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ORIGINAL ARTICLE

Discrimination of the Species and Toxic Heavy Metal Contents of *Alternanthera Sessilis* (Sessile Joyweed), *A. Philoxeroides* (Alligator Weed) and Other Related *Alternanthera* Species

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Abstract

Alternanthera sessilis [sessile joyweed (SJ)] is a staple leafy vegetable in Sri Lanka. However, *A. philoxeroides* [alligator weed (AW)] often gets mixed up with SJ due to the similar appearance. Alligator weed is an invasive species and also bio-accumulate toxic heavy metals. Genus *Alternanthera* has some other species that are occasionally being consumed in rural areas as leafy vegetables or grown as ornamental plants. It is essential to delimit AW from SJ and the rest of the *Alternanthera* species and study the heavy metal profiles of the shoot-top samples. In the present study, the species delimits and the phylogenetic relationships of the commonly grown *A. philoxeroides*, *A. sessilis*, *A. caracasana*, *A. paronychioides*, *A. ficoidea*, and *A. bettzickiana* were assessed using *rbcL*, *ITS* and *matK-trnT* DNA barcoding markers. The composition of the heavy metals in the market samples of the AW and SJ and field-grown samples of the other species were assessed using X-ray fluorescence (XRF) and Atomic Emission Spectroscopy (AES) methods. *A. philixeroides* and *A. sessilis* are positioned as sister taxa showing the close evolutionary relationship. The results showed that AW contains the highest amounts of the heavy metals, Pb and Sn. Red-SJ, a type of *A. sessilis*, contains the highest amount of As. The market samples of LS-1 and LS-2 did not contain significant levels of toxic heavy metals. The haplotypes of the markers *ITS*, and *matK-trnT* provide a clear basis to discriminate AW from the other *Alternanthera* spp.

Keywords: *Alternanthera phylogenetics*, *Bioaccumulation of heavy metals*, *dwarf*

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1. Introduction

Alternanthera sessilis of family Amaranthaceae, commonly known as sessile joyweed (SJ) or dwarf copperleaf, is one of the two most important leafy vegetables (LV) in Sri Lanka. Sessile joyweed is known as *Mukunuwenna* and *Ponnanganni-Keerai* in Sinhala and Tamil languages respectively. It is a staple leafy vegetable in Sri Lanka. The Sri Lankans including the immigrants living all over the world consume SJ as a unique recipe called '*Mellun'*. This recipe is prepared using finely chopped immature shoot-tops (*i.e*., young stems and leaves) of SJ mixed with grated coconut, chopped pieces of the other condiment herbs (onion, garlic, and green chili), black pepper powder and salt. The mixture is tempered for one to three minutes in low heat and mixed with some lime juice. Due to the heavy demand, SJ is a cash crop in Sri Lanka, and commercial cultivations exist in many locations throughout the country. It is a very popular LV among the Sri Lankan expatriates living in Australia too (Gunasekara and Bonila 2001; Jayasinghe 2008).

The members of the genus *Alternanthera* possess medicinal and nutritional properties. The plant extracts of *Alternanthera* spp. exhibit anti-viral, (Zhou et al. 1988), immunomodulatory (Guerra et al. 2003), antimalarial (Jacqueline et al. 1998), anti-tumor (Fang et al. 2007) and anti-diarrheic (Zavala et al. 1998) activities. In traditional medicine; leaves, stem, and shoots of SJ are used to treat stomach and gastric problems (Jubilee and Nath 2006), galactagogue (Jayaweera 1981), and night blindness (Mishra et al. 2008). The leaf extracts of the SJ is very effective in wound healing (Jalalpure et al. 2008), and as an antidote for snake bite (Gayathri et al. 2006). It has been reported that *Alternanthera* spp. contain the phytonutrients such as flavones, glycosides, steroids, betaines, and saponins (Sanoko et al. 1999). Also, *Alternanthera* spp. are rich in amino acids, lipids, and vitamins (Rudravarapu and Lakshminarayana 1993; Hosamani et al. 2004).

In addition to SJ, there are many other species in the genus *Alternanthera* (Iamonico and Pino 2016). Out of these species, at least eight *Alternanthera* spp. are commonly grown in Sri Lanka. They are grown for human consumption or ornamental purposes. There are three types of *A. sessilis* existing in Sri Lanka. Although the *Alternanthera* spp. have been characterized and named using conventional taxonomic methods, the modern DNA barcoding methods have not been employed to identify the species delimits and phylogenetic relationships.

Out of the many *Alternanthera* spp. in Sri Lanka, *A. philoxeroides*, commonly known as alligator weed (AW) is considered as one of the noxious and invasive weeds. AW can grow fast and overtake many other plants in the vicinity within a very short period and cover the entire habitat completely disturbing the ecosystem (Wu et al. 2017). Besides the ferocious invasiveness, AW is a plant that profoundly bio-accumulates toxic heavy metals (Gunasekara and Bonila 2001; Rane et al. 2015). The biggest threat of AW to the industry of SJ is often; growers and consumers get deceived by the similar appearance of AW to SJ. It has been reported that Sri Lankan expatriates in

Australia have introduced AW to Australia mistaking it to be SJ. Then the Australian legislators had to intervene with the awareness programs and eradication measures to control the distribution of AW within the country and introduce less invasive and safe *Alternanthera* species for the consumers (Gunasekara and Bonila 2001; Jayasinghe 2008). In Sri Lanka, because of the significant concerns in the diseases like Chronic Kidney Disease with unknown etiology (CKDu), the plants that are prone to the bioaccumulation of toxic heavy metals must be studied and identified to remove them from the food plate. Therefore, the present study was conducted to determine the species-delimits of commonly grown *Alternanthera* spp. in Sri Lanka, introduce a DNA based method to identify AW from the rest of the *Alternanthera* species and evaluate the presence of toxic heavy metals in market samples of AW in comparison to SJ and other species.

2. Materials and Methods

2.1 Plant material

A total of nine *Alternanthera* spp. were assessed in the present study. The botanical, English, Sinhala and Tamil names, and the abbreviations used in the study are given Table 1. A. *philoxeroides* (AW), type-1 of *A. sessilis* (LS-1), *A. caracasana* (GS-1), A. *paronychioides* (GS-2), *A. ficoidea* (OS-1), *A. tenella* (OS-2), and type-2 *A. sessilis* (RS) were collected from *Gannoruwa,* Sri Lanka (GPS coordinates: N 7° 16' 59.30"; E 80° 35' 27.81"). The type-3 of *A. sessilis* (LS-2) was collected from *Nawalapitiya,* Sri Lanka (GPS coordinates: N 7° 2' 40.92"; E 80° 30' 57.82"). *A. bettzickiana* (WS) was collected from *Peradeniya*, Sri Lanka (GPS coordinates: N 7° 16' 11.51"; E 80° 35' 37.80"). The key morphological features of the shoot-tops (the edible part of sessile joyweed) were observed.

2.2 Assessment of the species delimits

DNA isolation, PCR and sequencing: The immature leaves were collected from each species/type (Table 1) and ground in liquid nitrogen. The genomic DNA was extracted from ground leaf material using a modified CTAB method (Porebski et al. 1997). The isolated DNA was PCR amplified using standard universal plant DNA barcoding markers *rbL* (Pf: ATG TCA CCA CAA ACA GAG ACT AAA GC; Pr: GTA AAA TCA AGT CCA CCR CG) (Levin et al. 2003; Kress et al. 2009; Hoot et al. 1995; Hoot and Taylor 2001), *matk-trnT* spacer (Pf: GCA TAA ATA TAY TCC YGA AAR ATA AGT GG, Pr: TGG GTT GCT AAC TCA ATG G) (Wicke and Quandt 2009), and *ITS1-4* (Pf: TCC GTA GGT GAA CCT TGC GG; Pr: TCC TCC GCT TAT TGA TAT GC) (White et al. 1990). The PCR amplification was carried out in a thermal cycler (Takara, Otsu Shiga, Japan) using the profile of initial denaturation 95 ºC for 3 min followed by 35 cycles of denaturation at 95 ºC for 1 min, primer annealing temperature (*rbcL* and *ITS1-4*: 55 ºC, and *matK-trnT*: 48 ºC), extension at 72 ºC for 2 min and final extension at 72 ºC for 10 min. A 15 µl PCR mixture was prepared by mixing 1× Go Taq® Green Master Mix (Promega Corporation, Madison, Wisconsin, USA) 1µl of each forward and reverse primers, 3.5 µl of spermidine and 2 µl of DNA template. The PCR products were purified

using QIAquick® PCR Purification Kit and sequenced (3×) using the Genetic Analyzer ABI 3500 (Applied Biosystems®). All the sequences generated during the present study were submitted to GenBank under the accession numbers of MK762528-MK762536, MK757182-MK757193, and MK744121- MK744121.

Phylogenetic analysis: The Geneious software (Version 9.17) (Kearse et al. 2012) was used to process the consensus sequence from the unedited sequence reads. A dataset was assembled by using the sequences of the Family Amaranthaceae reported in Xu et al. (2018) (Table 2), and the sequences generated in the present study. The separate multiple sequence alignments were carried out for the markers *ITS* and *rbcL*, and then the two alignments were concatenated using Geneious software. A partition homogeneity (ILD) test was conducted (Planet 2006) to check the phylogenetic congruence of *rbcL*, *ITS*, and *rbcL* + *ITS*. Due to the genomic positional differences and the differential evolutionary process of the markers used [a non-coding nuclear marker (*ITS*) and coding plastid marker (*rbcL*)], the best partition scheme search and accounting model selection was conducted in PartitionFinder 2 (Lanfear et al. 2016). The data matrix of the concatenated dataset was defined as coding and non-coding markers in PartitionFinder 2 platform in CIPRES Science Gateway (Miller et al. 2010). We implemented the algorithm which uses corrected Akaike information criteria (Cavanaugh 1997) (AICc), h cluster (Lanfear et al. 2014) method and K means (Frandsen et al. 2015). The best-fit partition scheme and the best fitting models were used for the downstream analysis. A phylogenetic tree search was conducted in Maximum Likelihood (ML) using rapid-bootstrap analysis (Stamatakis et al. 2008) for 1000 replications in RAxML-VI-HPC workflow (Stamatakis 2006) using CIPRES supercomputer. In this analysis, the best scoring tree of highest –log likelihood value was used to draw a single tree topology employing all the bootstrap sub-tree constructions. For higher statistical accuracy of the phylogenetic inferences, a Bayesian tree search was conducted in MrBays (Huelsenbeck and Ronquist 2001) on the CIPRES platform. The two hot and cold chains of Markov chain Monte Carlo (MCMC) for 60 million generations were used to probe trees in the virtual tree space. The 25% of the trees were discarded as burn-in, and the trees were examined after maximum chain convergence and used to draw the final 50% majority rule consensus tree. The MCMC chain performance was assessed by using Effective Sample Size (ESS) parameters for all the priors considered in TRACER v1. 4 (Rambaut and Drummond 2007). The edited phylogenetic trees were visualized in FigTree v1.4.3 (Rambaut 2014).

2.3 Assessment of the toxic heavy metals

XRF analysis: The bunches of LS-1 and LS-2 were purchased from the open market in Sri Lanka. The shoot-top samples of AW were picked from the LS bunches purchased in which AW shoot-tops can be frequently observed as mix-ups. All the other *Alternanthera* spp. were collected from the same sites that we collected samples for DNA extraction. The shoot-top material was thoroughly cleaned with the running tap water and then with the distilled

Table1: The shoot-top characteristics of the studied *Alternanthera* spp. studied.

Botanical name	English name	Sinhala name	Tamil name	Acronym	Leaf					Stem	
					Color	Shape	Arrangement	Petiole	Margin	Color	Other
A. philixeroides (Mart.) Griseb.	Alligator weed	*Kimbulwenna*	*Atalari*	AW	Shiny dark green	Elongated/ obovate	Simple and opposite	Sessile	Entire margin with prominent rib	Green with purple coloration	Hallow, branched stems ascending or creeping, stolen: 50-100 cm long
A. caracasana Kunth	Giant sessile joyweed-1	Giant (*Yodha*) *mukunuwenna*-1	*Peru Ponnanganni keerai – 1*	GS-1	Shiny dark green	Obovate or ovate	Opposite	Sessile	Entire and slightly undulate	Dark green	Prostrate-branched ,10-120 cm long branches; hairs present
A. paronychioides A. St.-Hil.	Giant sessile joyweed-2	Giant (*Yodha*) *mukunuwenna*-2	*Peru Ponnanganni keerai – 2*	GS-2	Green	Ovate-Rhombic	Simple and opposite	Sessile	Entire	Green	Prostrate-branched stems sparsely covered with hairs
A. sessilis (L.) R. Br. ex. DC.	Lowland sessile joyweed Type-1	Field (*Vel*) *mukunuwenna*-1 / *mukunuwenna*	*Ponnanganni keerai – 1*	LS-1	Green	Obovate to lanceolate-eliptic	Simple and opposite	Sessile	Entire and narrow and pointed leaf base	Green	Branched, 10-100 cm long branches, prostrate or ascending stems prominent rooting at the nodes
	Lowland sessile joyweed Type-2	Field (*Vel*) *mukunuwenna*-2 / mukunuwenna	*Ponnanganni keerai – 2*	LS-2	Green	Obovate to lanceolate-eliptic	Simple and opposite	Sessile	Entire and straight	Green	
A. ficoidea (L.) P. Beauv.	Ornamental sessile joyweed-1	Ornamental (*Wisithuru*) *mukunuwenna*-1	*Alangaara Ponnangaani keerai – 1*	OS-1	Bright green with blotches (orange, brown, yellow, purple, and red)	Eliptic to broad ovate	Simple and opposite	Sessile	Undulated	Green to brown	Erect to procumbent, branched stems 15-30 cm long
	Ornamental sessile joyweed-2	Ornamental (*Wisithuru*) *mukunuwenna*-2	*Alangaara Ponnangaani keerai – 2*	OS-2	Brightly colors with blotches (orange, red, brown, yellow, and purple)	Obolng - lanceolate	Simple and opposite	Sessile	Undulated	Green to brown	
A. sessilis (L.) R. Br. ex. DC.	Red sessile joyweed	Red (*Rathu*) *mukunuwenna*-1	*Sivappu Ponnangaani keerai*	RS	Red	Elongated	Simple and opposite	Sessile	Entire	Red maroon	Smooth, branched, prostrate to erect stems, prominent rooting at the nodes
A. bettzickiana (Regel) Voss	Wild sessile joyweed	Wild (*Wal*) *mukunuwenna*	*Kaatu Ponnangani keerai*	WS	Shiny dark green	Obovate or ovate	Opposite	Sessile	Entire	Dark green	Prostrate branched 10-120 cm long stem, hairs present

Table 2: The metadata of the sequences used in the phylogenetic analysis.

Species	Variety	Voucher No.	Geographic origin	GenBank Accessions		
				ITS	*rbcL*	*matK-trnT*
Alternanthera bettzickiana	Wild sessile joyweed (SJ)	DMB77	Sri Lanka	MK757182	MK744121	MK762528
Alternanthera caracasana	Giant SJ 1	DMB78	Sri Lanka	MK757183	MK744122	MK762529
Alternanthera ficoidea	Ornamental SJ 1	DMB79	Sri Lanka	MK757184	MK744123	MK762530
Alternanthera paronychioides	Ornamental SJ 1	DMB80	Sri Lanka	MK757185	MK744124	MK762531
Alternanthera philixeroides	Alligator weed	DMB81	Sri Lanka	MK757186	MK744125	MK762532
Alternanthera sessilis	Red SJ	DMB82	Sri Lanka	MK757187	MK744126	MK762533
Alternanthera sessilis	Lowland SJ Ecotype 1	DMB83	Sri Lanka	MK757188	MK744127	MK762534
Alternanthera sessilis	Lowland SJ Ecotype 2	DMB84	Sri Lanka	MK757189	MK744128	MK762535
Alternanthera ficoidea	Ornamental SJ -2	DMB85	Sri Lanka	MK757190	MK744129	MK762536
Alternanthera philixeroides	Alligator weed	DMB86	Sri Lanka	MK757191	MK744130	-
Alternanthera philixeroides	Alligator weed	DMB87	Sri Lanka	MK757192	MK744131	-
Alternanthera philixeroides	Alligator weed	DMB88	Sri Lanka	MK757193	MK744132	-
Amaranthus cruentus	-	201509205	Hunan, Zhuzhou	KY968887	MF135386	-
Alternanthera philoxeroides	-	201509153	Hunan, Changsha	KY968872	MF135371	-
Alternanthera philoxeroides	-	X12	Hunan,	KY968963	MF135473	-
Alternanthera philoxeroides	-	201509177	Hunan, Changsha	KY968879	MF135378	-
Amaranthus blitum	-	201509155	Hunan, Changsha	KY968874	MF135373	-
Amaranthus blitum	-	2015054	Liaoning, Dalian	KY968859	MF135357	-
Amaranthus hybridus	-	13369	Jiangsu, Zhenjiang	KY968907	MF135408	-
Amaranthus hybridus	-	201509228	Hunan, Leiyang	KY968895	MF135396	-
Amaranthus hybridus	-	13489	Jiangsu, Jiangyin	KY968931	MF135436	-
Amaranthus palmeri	-	201509229	Hunan, Leiyang	KY968896	MF135397	-
Amaranthus spinosus	-	X13	Jiangsu	KY968964	MF135474	-
Amaranthus spinosus	-	201509140	Hunan, Changsha	KY968864	MF135362	-
Amaranthus tricolor	-	201509212	Hunan, Zhuzhou	KY968890	MF135390	-
Amaranthus tricolor	-	201509213	Hunan, Zhuzhou	KY968891	MF135391	-
Amaranthus tuberculatus	-	13461	Jiangsu,	KY968925	MF135430	-
Amaranthus tuberculatus	-	13495	Jiangsu, Jiangyin	KY968934	MF135440	-
Amaranthus tuberculatus	-	2015052	Liaoning, Dalian	KY968858	MF135356	-
Amaranthus viridis	-	201509139	Hunan, Changsha	KY968863	MF135361	-
Aster subulatus	-	201509142	Hunan, Changsha	KY968866	MF135364	-
Basella alba	-	201509233	Hunan, Leiyang	KY968898	MF135399	-
Celosia argentea	-	13482	Jiangsu	KY968928	MF135433	-
Celosia argentea	-	13398	Jiangsu, Taizhou	KY968912	MF135413	-
Celosia cristata	-	13442	Jiangsu, Taizhou	KY968920	MF135424	-
Celosia cristata	-	13519	Beijing, Haidian	KY968942	MF135449	-
Chenopodium ficifolium	-	13505	Jiangsu, Jiangyin	KY968936	MF135442	-
Chenopodium glaucum	-	13506	Jiangsu, Jiangyin	KY968937	MF135443	-
Dysphania ambrosioides	-	13493	Jiangsu, Jiangyin	KY968933	MF135439	-
Dysphania ambrosioides	-	201509136	Hunan, Changsha	KY968860	MF135358	-
Dysphania ambrosioides	-	201509137	Hunan, Changsha	KY968861	MF135359	-
Dysphania ambrosioides	-	201509237	Hunan, Leiyang	KY968902	MF135403	-
Dysphania ambrosioides	-	201509236	Hunan, Leiyang	KY968901	MF135402	-
Dysphania pumilio	-	13316	Jiangsu, Jiangyin	KY968827	MF135321	-
Oxybaphus nyctagineus	-	13524	Beijing, Haidian	KY968946	MF135453	-
Phytolacca octandra	-	13305	Jiangsu, Jiangyin	KY968823	MF135317	-
Phytolacca octandra	-	13311	Jiangsu, Jiangyin	KY968825	MF135319	-
Phytolacca thyrsiflora	-	201507058	Yunnan, Kunming	KY968845	MF135339	-
Portulaca pilosa	-	13359	Guangdong	KY968836	MF135330	-
Solanum pseudocapsicum	-	201507061	Yunnan, Kunming	KY968847	MF135342	-

water. The plant material was then cut into small pieces and oven-dried at 80 ºC for five days in crucibles. The oven-dried samples were crushed into a fine powder and subjected to X-ray fluorescence (XRF) analysis using X-Ray Xan instrument (BW/526/00/Ro-Type: XAN). The device was first calibrated using standard elements [S, Ti, Cr, Ni, Cu, Zn, Zr, Mo, Ag, Sn, W, Au, and Pb standard metal plate (CALSS Pure Elements Analysis)]. The spectra generated were examined to identify the peaks for heavy metal elements.

Atomic Emission Spectroscopic (AES) analysis: The same set of plant samples used in XRF were subjected to AES analysis to quantify the trace metals. Approximately 0.2 g of the powdered oven-dried sample was mixed with 6 ml of conc. HNO_3 and 2 ml of H_2O_2. The sample digestion was conducted at 180 ºC for 30 mins in Mars-6 Microwave Digester (CEM; Matthews, NC). After that, the element composition of digested samples was measured using the AES system, Agilent technologies 4200 MPAES (5301 Stevens Creek Blvd, Santa Clara, CA 95051, United States). The elemental concentrations were calculated using the following equation (Van de Wiel 2003).

$$W\ (mgkg^{-1}) = (C_1 - C_0)D_fV/m$$

W: Weight of the element ($mgkg^{-1}$)

C_1: Concentration of the element

C_0: Concentration of the element in the standard sample

D_f: Dilution factor

V: Sample volume

m: Sample weight

3. Results

3.1 Morphological appearance of shoot-tops

The studied *Alternanthera* spp. mainly contain the sessile leaves and flowers. The flowers form in the leaf pedicel-stem junctions as clusters. However, AW flowers have an extended peduncle. The size of the leaves and the plants are depending on the environmental and soil conditions. However, GS-1, GS-2 possess relatively larger shoots and leaves. The ornamental types; LS-1, LS-2, and RS possess variegated or purple colored shoots. The images of the adaxial and abaxial sides of the leaves, harvestable shoots-tops for consumption, matured branch with flowers and the close-up view of flowers and sections of the field-grown plants are shown in Plate 1. The key morphological features of the studied species/types are summarized in Table 1.

3.2 Species delimits and phylogenetic positions

The final combined sequence alignment was separated into four partitions, and four models of evolutions were assigned (Subset 1: TVM+I+G, subset 2: K81+I+G, subset 3: K81UF+G and subset 4: GTR+I+G) to each portion in the data partition analysis. The MCMC chains converged maximally at initial 500,000 runs, and all the priors had ESS value over 200. The phylogenies constructed in Bayesian and ML frameworks had similar branching patterns. However, the Bayesian tree had higher resolution at basal nodes with higher support values; thus, the inferences were made with respect to the Bayesian majority rule consensus tree (Fig. 1).

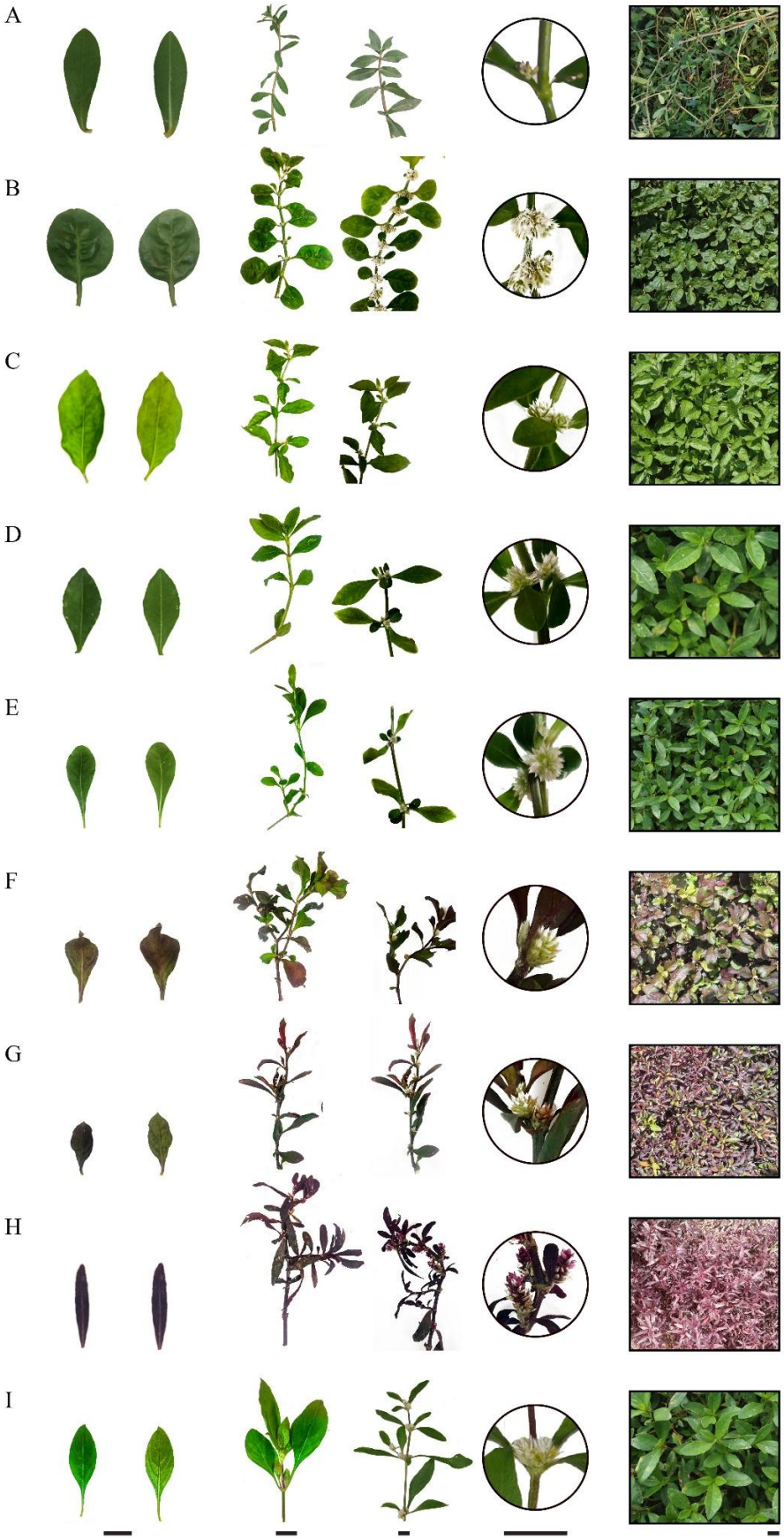

Plate 1: Morphological diversity of *Alternanthera* spp. A: *A. philoxeroides* [alligator weed (AW)]; B: *A. caracasana* [giant sessile Joyweed-1 (GS-1)]; C: *A. paronychioides* [giant sessile Joyweed-1 (GS-1)]; D: *A. sessilis* [lowland sessile joyweed-1(LS-1)]; E: *A. sessilis* [lowland sessile joyweed-2(LS-2)]; F: *A. ficoidea* [ornamental sessile joyweed-1 (OS-2)]; G: *A. tenella* [ornamental sessile joyweed-1 (OS-2)]; H: *A. sessilis* [red sessile joyweed (RS)]; I: *A. bettzickiana* [wild sessile joyweed (WS)]. The adaxial and abaxial surfaces of the leaves, shoot-tops with and without flowers, close-up view of the flower in a node, and a section of the field grown plants are shown. The scale bars represent one cm.

In the phylogeny, all the *Alternanthera* spp. showed a monophyletic origin. The highly supported *Alternanthera* clade (PP=100 and bs=100) got separated into two clades; clade A (PP=100 and bs=100), and clade B (PP=100 and bs=100) (Fig. 1). The clade B was separated into two main clusters in the phylogeny. The clade BI (PP=100 and bs=92) exclusively contained the three types (LS-1, LS-2, and RS) of *A. sessilis*. Similarly, all the *A. philoxeridea* (AW) samples were clustered into the clade BII (PP=100 and bs=100). *A. philoxeridea* found in Sri Lanka was nested separately (clade C; PP=100 and bs=100) compared to the *A. philoxeridea* plants sequenced in Xu et al. (2018).

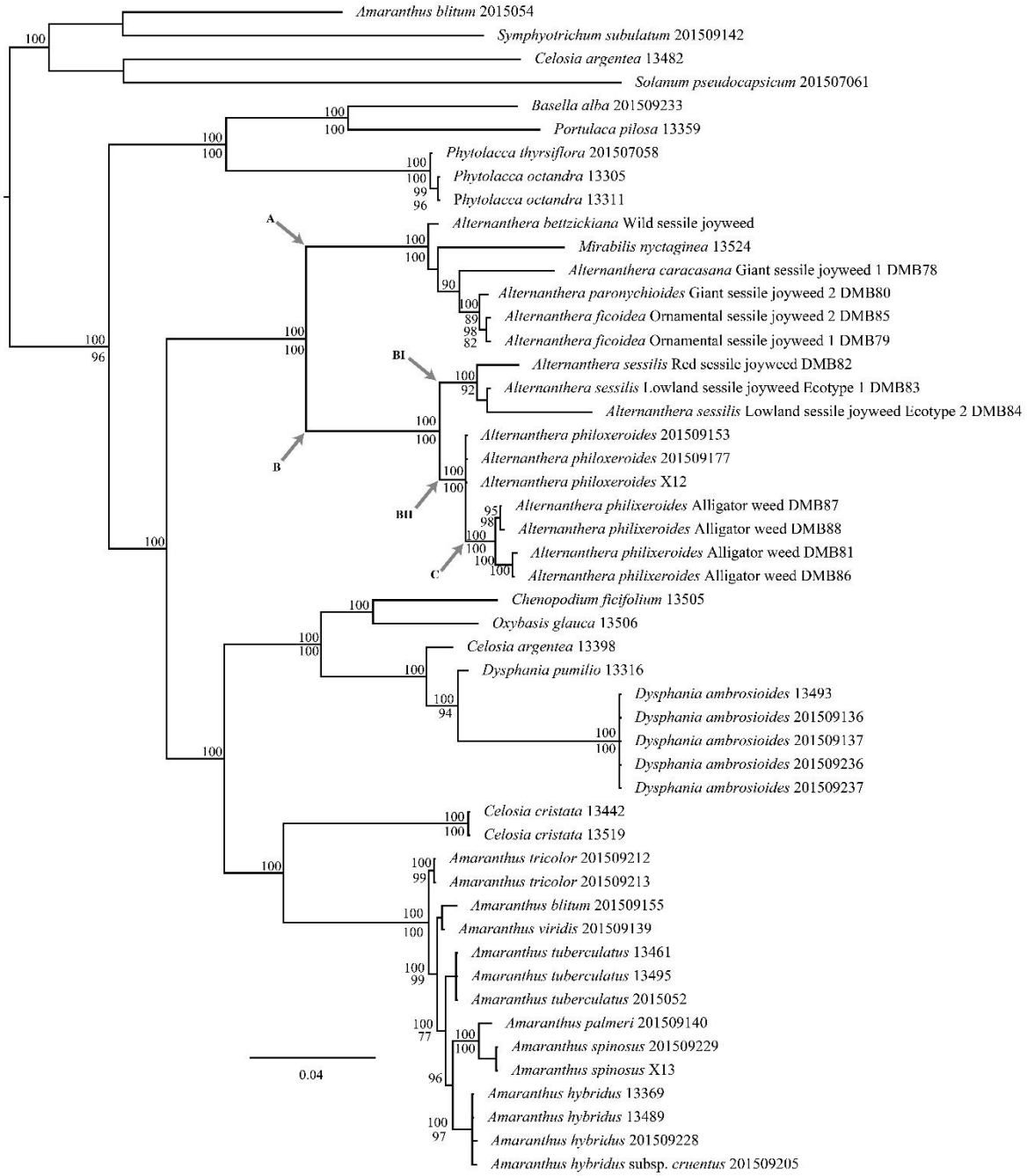

Figure 1: The Bayesian majority rule consensus tree which exhibits the phylogenetic relationships among *Alternanthera* spp. with respect to the other closely related taxa. Arrows show the major clades within genus *Alternanthera.* The bootstrap values (>70) are given below the nodes, and the posterior probability values (>90) are provided above the nodes.

3.3 Variation of the elemental contents

The significantly highest contents of toxic elements, Pb and Sn, were found in AW indicating its ability to bio-accumulate toxic elements. The Pb and Sn contents were approximately three times higher in AW than in LS-2. The As content was significantly highest in RS (2.306 mg/kg) indicating that RS (*A. sessilis* with red shoots and leaves) can bio-accumulate As. Ni and Cd were not detected in AW whereas, Cd was detected in minimum amounts in LS-1 and LS-2. The Cr content was not significantly different among the *Alternanthera* spp. studied. The micronutrient, Cu content was significantly highest in AW, and Fe and Mn contents were significantly highest in LS-2 and LS-1 respectively indicating their high nutritional value (Table 3).

4. Discussion

The phylogeny constructed for *rbcL+ITS* revealed that the studied *Alternanthera* spp. are separated into two main lineages (*i.e.*, clades). The Clade A is represented by WS, GS-1, GS-2, OS-1, and OS-2. WS was the most distantly related taxa of the Clade A, suggesting possible evolution or agronomic selection of other taxa from the diverse array of the WS germplasm. OS-1 and OS-2 are grouped into one cluster indicating that they are differently colored morphotypes of *A. ficoidea*. AW and three types of SJ represent clade B. The Clade BII which includes AW that was placed sister to the Clade BI which includes LS-2 and LS-2, the two most commonly consumed types of *Alternanthera* spp. RS (characterized with red shoots and foliage) is within the species limit of *A. sesslis.* However, RS is occasionally indicated as *A. polygonoides* in Sri Lanka. Therefore, it must be corrected as *A. sessilis*. The nucleotide divergence between LS-1 and LS-2 is 0.0067 (*matK-trnT*), 0.00 (*rbcL*), and 0.00 (*ITS*) indicating that they are belonged to the same species. For the first time, we introduce the evolutionary relatedness (nucleotide similarity of %) between *A. philixeroides* (AW) and *A. sessilis* (SJ) based on *ITS* and *rbcL* phylogeny, which favors the possibility of misidentification. The markers with less resolving power can also mislead the discrimination of AW from SJ. Thus, we introduce a set of species-specific haplotypes that are found in *ITS* and *matK-trnT* spacer to distinguish AW from SJ and rest of the *Alternanthera* spp. (Table 4). The haplotypes shown in Table 4 can be used to identify AW from SJ. Also, the seven studied *Alternanthera* spp. are characterized by the unique haplotypes present in *ITS* and *rbcL* loci indicating their power in defining species delimits within genus *Alternanthera*.

In the present study, we focused on five toxic elements with respect to the macronutrient K and six other elements including essential micronutrients. It has been reported that the elemental detection techniques such as AES and XRF cannot detect the entire range of elements with the required accuracy (Dulski 2017). The present elemental analysis demonstrated that *A. phylozeroids* (AW) is bioaccumulating Pb and Zn. However, As content is found to be significantly higher in OS-2 (*A. ficoides*) and RS (*A. sesslis*) implying that AW is not the only bioaccumulating species within the genus *Alternenthera*. Because of the sample

Table 3: Variation of the contents of a selected set of elements in *Alternanthera* species.

Species	Detection method and elemental concentrations measured in ppm											
	XRF			AES						XRF	AES	XRF
	K	Fe	Cu	Mn	Al	Cr	Ba	Ni	Cd	Sn	As	Pb
*A. philixeroides** (AW)	6.880b	1.610b	**0.700a**	0.062e	0.375d	**0.008a**	0.040e	-	-	**0.340a**	0.152c	**0.350a**
A. caracasana (GS-1)	**8.900a**	0.590c	0.280b	0.040f	0.653c	**0.003a**	0.065b	0.007c	0.009b	0.050b	0.071c	0.110c
A. Paronychioides (GS-2)	**9.17a**	0.260c	0.340b	0.093c	0.128f	**0.002a**	0.027f	-	**0.012a**	0.050b	0.129c	0.120c
*A. sessilis*** (LS-1)	**8.86a**	0.470c	0.390b	**0.345a**	0.335d	**0.002a**	0.029f	**0.010a**	0.002e	0.070b	0.047c	0.140c
*A. sessilis*** (LS-2)	6.780b	**2.480a**	0.350b	0.188b	0.989b	**0.007a**	0.052c	-	0.004d	0.140b	0.0360c	0.190b
A. Ficoidea (OS-1)	**9.380a**	0.240c	0.200b	0.0400f	0.989b	**0.003a**	0.017g	-	0.007c	0.050b	0.053c	0.070c
A. Tenella (OS-2)	**9.500a**	0.230c	0.140b	0.024g	**1.070b**	**0.002a**	0.047d	0.007c	-	0.070b	1.323b	0.050c
A. Polygonoides (RS)	**9.100a**	0.220c	0.330b	0.078d	1.639a	**0.002a**	**0.081a**	0.008b	-	0.110b	**2.306a**	0.160c
A. bettzickiana (WS)	**9.350a**	0.230c	0.230b	0.071d	0.240e	**0.001a**	0.0280f	-	-	0.030b	-	0.080c

Means denoted by same letters within columns are not significantly different at P<0.05. Significantly highest means are shown in bold case letters.

limitations and the impossibility to conduct trials by adding toxic heavy metals to the environment, the degree of elemental bioaccumulation by each species cannot be precisely documented under field or greenhouse conditions. However, the assessment of the elemental contents of the market samples provides the profiles of toxic heavy metals in bunches that are available for the consumers.

Sri Lanka has a total human population of 21.44 million and almost all of them eat SJ as a LV in daily basis. Although migrated and settled down in foreign countries, the dietary habits of Sri Lankan expatriates have not been changed dramatically. Sri Lankan expatriates prepare meals according to the Sri Lanka style and SJ *mellun* is a popular dish in any kind of menu. In Australia a total of 75136 Sri Lankans is living in which 22,945 is Sinhalese and 19426 is Tamils (Australian Bureau of Statistics 2011) and all of them consume SJ. The consumption of AW in place of SJ is problematic as indicated in previous studies (Ramachandra et al. 2018; Lin et al. 2018; Suthari et al. 2017). In the present study, we also proved that higher Pb concentrations can be found in AW however, other *Alternenthera* spp. such as RS may also contain toxic heavy metals. The accurate methods such as DNA barcoding is essential to identify the food adulterants as reported in meat (Cavin et al. 2018), flour (Barcaccia et al. 2016), and spice (Osman et al. 2019) industries. Therefore, the present study is important as it provides the molecular basis to differentiate AW from edible and safe LS thus the harmless consumption of SJ can be safeguarded.

5. Conclusions

The phylogenetic analysis of the commonly grown *Alternanthera* spp. in Sri Lanka based on the *ITS* and *rbcL* markers revealed the existence of two major clades. *A. philixeroides*, and *A. sessilis* are sister taxa implying the basis for misidentification of AW as SJ. However, the haplotypes of *ITS*, and *matK-trnT* can be used to discriminate AW from SJ and other *Alternanthera* spp. accurately. The elemental

analysis of the market/available samples shows that AW contains the highest amounts of Pb and Sn. However; RS, a type of *A. sessilis* contains the highest amount of As. The market samples of LS-1 and LS-2 did not contain higher amounts of toxic heavy metals proving the level of safety for the consumption. Moreover, LS contain the highest amounts of micronutrients Fe and Mn indicating its nutritional value.

Table 4: The unique haplotypes that can distinguish AW (*A. philixeroides*) from *A. sessilis* and other *Alternanthera* spp. studied.

Marker	Position in the alignment (bp)	*A. philixeroides*	*A. sessilis* spp.	Other *Alternanthera spp.*
ITS	117	G	T	C/T
	159	CACAG	CATAG	ATAGC
	175	TTG	TCG	ATT
	195	GAG	GGG	ATT
	200	CGA	TGA	CTT
	383	A	G	G
	447	CCTG	ATTG/ACTG	CGGG
	456	TTG	TTA	TTG/AGG/ATG
	527	Deletion	ATGCAGGGG	AGCATCGCG/AGCCTCGCG
	575	TA	TG	AA
matK-trnT	68	A	C	C

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7. References

Australian Bureau of Statistics (ABS) (2011) Fact sheet. https://www.abs.gov.au/websitedbs/censushome.nsf/home/factsheetsancsri?opendocument&navpos=450. Accessed on 12th April 2019.

Barcaccia G, Lucchin M, Cassandro M (2016) DNA barcoding as a molecular tool to track down mislabeling and food piracy. Diversity 8:2.

Cavanaugh J E (1997) Unifying the derivations for the Akaike and corrected Akaike information criteria. Stat Probabil Lett 33:201-208.

Cavin C, Cottenet G, Cooper K M, Zbinden P (2018) Meat vulnerabilities to economic food adulteration require new analytical solutions. CHIMIA International journal for chemistry 72:697-703.

Dulski T (2017) Trace elemental analysis of metals: methods and techniques. CRC press, Tayler & Francis Group, London, New York.

Fang J B, Duan H Q, Zhang Y W, Yoshihisa T (2007) Antitumor constituent from *Alternanthera philoxeroides*. J Asian Nat Prod Res 9:511-515.

Frandsen P B, Calcott B, Mayer C, Lanfear R (2015) Automatic selection of partitioning schemes for phylogenetic analyses using iterative k-means clustering of site rates. BMC Evol Biol 15(1):13.

Gayathri B M, Balasuriya K, Gunawardena G S, Rajapakse R P V, Dharmaratne H R W (2006) Toxicological studies of the water extract of green leafy vegetable Sessile joy weed (*Alternanthera sessilis*). Curr. Sci 91:1517-1520.

Guerra M J M, Barreiro M L, Rodríguez Z M, Rubalcaba Y (2003) Actividad antimicrobiana de um extracto fluido al 80% de *Schinus terebinthifolius* Raddi (Copal). Rev Cub Salud Publica 5:23–25.

Gunasekara L, Bonila J (2001) Alligator Weed: Tasty Vegetable in Australian Backyards. J Aquat Plant Manag 39:17-20.

Hoot S B, Culham A, Crane P R (1995) The utility of *atpB* gene sequences in resolving phylogenetic relationships: comparison with *rbcL* and 18S ribosomal DNA sequences in the Lardizabalaceae. Ann Mo Bot Gard:194-207.

Hoot S B, Taylor W C (2001) The utility of nuclear *ITS*, a LEAFY homolog intron, and chloroplast *atpB-rbcL* spacer region data in phylogenetic analyses and species delimitation in Isoetes. Am Fern J 91: 166-177.

Hosamani K M, Ganjihal S S, Chavadi D V (2004) *Alternanthera triandra* seed oil: A moderate source of ricinoleic acid and its possible industrial utilization. Ind Crops Prod 19: 133-136.

Huelsenbeck J P, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.

Iamonico D, Sánchez-del Pino I (2016) Taxonomic revision of the genus *Alternanthera* (Amaranthaceae) in Italy. Plant Biosyst 150:33-342.

Jacqueline M, Sylvia B, Jairo S, Adriana C, Carmona J (1998) In vitro antimalarial evaluation of the plants *Alternanthera lanceolata* and *Pollalesta discolor*. Anales de Quimica 94: 67-70.

Jalalpure S S, Agrawal N, Patil M B, Chimkode R, Tripathi A (2008) Antimicrobial and wound healing activities of leaves of *Alternanthera sessilis* Linn. Int J Green Pharm 2:141-144.

Jayasinghe H (2008) Please don't eat mallung leaf lookalike Sunday Times, Sri Lanka. http://www.sundaytimes.lk/080504/News/news0013.html. Accessed on 14th April 2019.

Jayaweera D (1981) Medicinal Plants (Indigenous and Exotic) used in Ceylon. The National Science Council of Sri Lanka, 47/5, Maitland Place, Colombo 07.

Jubilee P, Nath S (2006) Biological activities of ethnomedicinal claims of some plant species of Assam. Indian J Tradit Know 5: 229-236.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

Kress W J, Erickson D L, Jones F A, Swenson N G, Perez R (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. Proc Natl Acad Sci U S A 106:18621–18626.

Lanfear R, Calcott B, Kainer D, Mayer C, Stamatakis A (2014) Selecting optimal partitioning schemes for phylogenomic datasets. BMC Evol Biol 14: 82.

Lanfear R, Frandsen P B, Wright A M, Senfeld T, Calcott B (2016) PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Mol Biol Evol 34: 772-773.

Levin R A, Wagner W L, Hoch P C (2003) Family-level relationships of Onagraceae based on chloroplast rbcl and ndhf data. Am J Bot 90:107-115 (modified from Soltis, P. (1992) Proc Natl Acad Sci U S A 89: 449-451).

Lin H, Liu J, Dong Y, Ren K, Zhang Y (2018) Absorption characteristics of compound heavy metals vanadium, chromium, and cadmium in water by emergent macrophytes and its combinations. Environ Sci Pollut Res Int 25:17820-17829.

Miller M A, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees, Gateway Computing Environments Workshop (GCE). 1–8. http://www.ieeexplore.ieee.org/abstract document/5676129 Accessed on 16th April 2019.

Mishra S B, Dwivedi S, Shashi S, Prajapati K (2008) Ethnomedicinal uses of some plant species by ethnic and rural peoples of the Salem District of Tamilnadu with special reference to the conservation of vanishing species. Ethnobotanical Leaflets 12:873-887.

Osman A G, Raman V, Haider S, Ali Z, Chittiboyina A G, Khan I A (2019) Overview of analytical tools for the identification of adulterants in commonly traded herbs and spices. J AOAC Int 102: 376-385.

Planet P J (2006) Tree disagreement: measuring and testing incongruence in phylogenies. J Biomed Inform 39: 86-102.

Porebski S, Bailey L G, Baum B R (1997) Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol Biol Report 15(1):8-15.

Ramachandra T V, Sudarshan P B, Mahesh M K, Vinay S (2018) Spatial patterns of heavy metal accumulation in sediments and macrophytes of Bellandur wetland, Bangalore. J Environ Manage 206: 1204-1210.

Rambaut A (2014) FigTree, a graphical viewer of aphylogenetic trees. http://tree. bio. ed.ac. uk/software/figtree. Accessed on 20th August 2017.

Rambaut A, Drummond A (2007) Tracer v1. 4. http://beast. bio. ed. ac. uk/Tracer. Accessed on 15th August 2017.

Rane N R, Chandanshive V V, Watharkar A D, Khandare R V, Patil T S, Pawar P K, Govindwar S P (2015) Phytoremediation of sulfonated Remazol Red dye and textile effluents by *Alternanthera philoxeroides*: an anatomical, enzymatic and pilot scale study. Water Res 83:271-281.

Rudravarapu S, Lakshminarayana G (1993) Lipid classes, Fatty acids, and Tocopherols of leaves of six edible plant species. J Agric Food Chem 41: 61-63.

Sanoko R, Speranza G, Pizza C, De Tommasi N (1999) Triterpene saponins from *Alternanthera repens*. Phytochemistry 51: 1043-1047.

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web servers. Syst Biol 57:758-771.

Suthari S, Kiran B R, Prasad M N V (2017) Health risks of leafy vegetable *Alternanthera philoxeroides* (Alligator weed) rich in phytochemicals and minerals. The EuroBiotech Journal 1: 293-302.

van de Wiel H J (2003) Determination of elements by ICP-AES and ICP-MS. National Institute of Public Health and the Environment (RIVM), Bilthoven, Netherlands.

White T J, Bruns T, Lee S, Taylor J (1990) in Innis A, Gelfand D H, Sninsky J J (Eds.) PCR Protocols, Academic Press, San Diego, USA. 315-322.

Wicke S, Quandt D (2009) Universal primers for the amplification of the plastid *trnK/matK* region in land plants. Anales del Jardín Botánico de Madrid 66: 285-288.

Wu H, Carrillo J, Ding J (2017) Species diversity and environmental determinants of aquatic and terrestrial communities invaded by *Alternanthera philoxeroides*. Sci Total Environ 581:666-675.

Xu S Z, Li Z Y, Jin X H (2018) DNA barcoding of invasive plants in China: A resource for identifying invasive plants. Mol Ecol Resour 18:28-136.

Zavala M A, Perez S, Perez C, Vargas R, Perez R M (1998) Antidiarrhoeal activity of *Waltheria americana*, *Commelina coelestis* and *Alternanthera repens.* J Ethnopharmacol 61: 41-47.

Zhou B N, Blaskò G, Cordell G A (1988) Alternanthin, a C-glycosylated flavonoid from *Alternanthera philoxeroides*. Phytochemistry 27:3633-3636.