J.Natn.Sci.Foundation Sri Lanka 2023 51 (3): 491- 503
DOI: http://dx.doi.org/10.4038/jnsfsr.v51i3.11203

RESEARCH ARTICLE

Molecular Phylogeny

Rediscovery, identity, and conservation strategies of a critically endangered endemic plant, *Hedyotis quinquinervia* Thwaites (Rubiaceae) in Sri Lanka

A Gunarathne[1], HDRVL Harasgama[1], T Wijewickrama[2], A Attanayake[2], RN Attanayake[1*] and RMCS Ratnayake[1]

[1] *Department of Plant and Molecular Biology, Faculty of Science, University of Kelaniya, Kelaniya, Sri Lanka.*
[2] *Royal Botanic Gardens, Peradeniya, Sri Lanka.*

Submitted: 23 May 2022; Revised: 24 January 2023; Accepted: 24 March 2023

Abstract: Sri Lanka, a biodiversity hotspot in Asia, records 30 *Hedyotis* species of which 25 species and a variety are endemic. Among these 25 species, seven *Hedyotis* species were categorized as critically endangered (CR), and 13 as endangered (EN). During our field survey in 2014, an extremely attractive plant belonging to the genus *Hedyotis* was discovered from Mount Thotupola, Sri Lanka. The plant was tentatively identified as *H. quinquinervia*. For accurate species identification, morphological characters were compared with voucher specimens, and identification keys were also used. In addition, DNA barcoding using the sequence of the internal transcribed spacer region of the nuclear ribosomal DNA (rDNA-ITS) region was performed, followed by molecular phylogenetic analysis. A simple method to remove a thick cuticle layer on the leaves was employed to obtain a sufficient amount of DNA suitable for Polymerase Chain Reaction. A comparison with its protologue and type specimen along with molecular phylogenetic analysis confirmed that the unidentified plant was *H. quinquinervia* Thwaites. The National Red List of Sri Lanka (2020) had revised *H. quinquinervia* as a CR species upon the rediscovery confirmed after the lapse of a century. Habitat characteristics, *ex situ* and *in situ* conservation measures for *H. quinquinervia,* and general conservation strategies applicable to threatened heterostylous plants are also discussed.

Keywords: Critically endangered, *Hedyotis quinquinervia,* internal transcribed spacer (ITS), Rubiaceae.

INTRODUCTION

The genus *Hedyotis* L. is one of the largest genera in the family Rubiaceae, also known as the coffee family. Five hundred to 600 species belonging to the genus *Hedyotis* are found in tropical and subtropical zones (Wikström *et al*., 2013). Ridsdale (1998) has described 28 *Hedyotis* species found in Sri Lanka. Among them, the lesser-known endemic species *H. quinquinervia* was first described in 1859 from Mount Pedro, the tallest mountain in Sri Lanka, by Thwaites. The last available record of *H. quinquinervia* in Sri Lanka was the specimen collected and deposited in the National Herbarium, Peradeniya, Sri Lanka (PDA) by A. M. de Silva in 1906 from Mount Pedro, where four other previously collected specimens have been deposited as well. According to global red list categories and criteria (version 9) and the categories adapted at the national level (MOE, 2012), critically endangered, possibly extinct [CR(PE)] is defined as a species with no distribution records in the past 60 years. In the surveys conducted for the past 107 years, no *H. quinquinervia* plants have been found in the previously recorded location, Mount Pedro, or elsewhere. Hence, the National red list of Sri Lanka 2012 recognized *H. quinquinervia* as a critically endangered, possibly extinct [CR(PE)] species (MOE, 2012). However, based on the abstract publication of Harasgama *et al.* (2014) reporting the rediscovery of the plant in 2014, the status of CR(PE) was changed to CR in 2020 (MOE, 2020).

Species belonging to the genus *Hedyotis* show extensive variation in morphology and are often confused with the genus *Oldenlandia* (Guo *et al*., 2013). Morphological identification of species at times could be challenging due to the morphological plasticity and overlapping characteristics of the members. Therefore, for a controversial genus like *Hedyotis*, species identity based on molecular phylogenetic relationships is more suitable (Guo *et al*.,

* Corresponding author (renuka@kln.ac.lk; https://orcid.org/0000-0002-4875-777X)

This article is published under the Creative Commons CC-BY-ND License (http://creativecommons.org/licenses/by-nd/4.0/). This license permits use, distribution and reproduction, commercial and non-commercial, provided that the original work is properly cited and is not changed in anyway.

2013). A species identification method based on molecular characters, especially DNA barcoding is not affected by the age, environmental conditions, or handling and processing of the material (Kazi *et al*., 2013). However, DNA extraction from certain plant species presents great challenges at times due to the presence of various secondary metabolites and polysaccharides (Katterman & Shattuck, 1983). During DNA extraction from CR(PE) plant materials, extra care should be taken to use a minimal amount of plant materials. Therefore, molecular-based identification poses unexpected challenges at times especially when there is no or limited access to liquid N_2.

Heterostyly is a complex floral polymorphism controlled by its genetics and, is associated with self-incompatibility mechanisms (Barrett & Cruzen, 1994). Self-compatibility is rare among distylous Rubiaceae taxa (Mahadura & Saunders, 2021). Several species of the genus *Hedyotis* are heterostylous, such as *H. quinquinervia, H. brachiata* (Raju & Radhakrishna, 2018), *H. sithiravaraiensis*, *H. uncinella* (Muruganandam *et al*., 2018) and *H. caerulea* (Ornduff, 1980). *Hedyotis pulcherrima* is also a distylous species with heteromorphic self-incompatibility (Liu *et al*., 2021). Self-incompatible plant species in biodiversity hotspots of the world suffer from pollen limitation than the regions with lower biodiversity and therefore, subsequent population decline is evident. Hence, maintaining an adequate population for the long-term survival of the species is a must.

The present study was aimed at confirming the identity of *H. quinquinervia* rediscovered using morphological and molecular data and implementation of *in situ* and *ex situ* conservation strategies for this CR species. Such rediscoveries and accurate species identification have a tremendous impact on species conservation, especially on oceanic islands where geographic barriers hindered free pollen flow.

MATERIALS AND METHODS

Study area

The study site was Mount Thotupola, the third highest mountain (2,357 m) of Sri Lanka, located within the Horton Plains National Park, Central Province, Sri Lanka (summit, 6° 49.982' N - 80° 49.211' E and the bottom, 6° 50.121' N - 80° 48.634' E). Horton Plains is a gently undulating highland plateau at the southern end of the central mountain massif of the country. The weather of the sampling site is usually dominated by persistent cloud cover and strong winds, sometimes gale-force, during the southwest monsoon. The mean annual temperature is about 13 °C during the driest period of the year and night temperature drops below 5 °C. The mean total rainfall is about 2540 mm/annum (Premathilake & Risberg, 2003). The geological structure of this region is made up of highly crystalline, non-fossiliferous rocks of the Precambrian age, belonging to the Highland Series. Soil is characterized as a thick, black, organic layer at the surface, as indicated by Saroja and Gunatilake (2013). The bedrock consists of high-grade metamorphic rocks of charnockitic gneiss, quart feldspathic gneiss, hornblende biotite gneiss, quartzite, khondalite, and garnet biotite gneiss.

Vegetation sampling for the floristic structure of the study site

A sampling permit was obtained from the Department of Wildlife Conservation, Sri Lanka. To determine the floristic composition and plant species abundance on Mount Thotupola, a stratified random plot sampling method, as described by Abeywickrama (2014), was carried out in 2014. Overstorey vegetation was studied by using randomly selected 10 m × 10 m plots (Wright *et al*., 1997). To study understorey vegetation, two randomly selected 1 m × 1 m subplots were demarcated within each overstorey plot. Due to the extreme heterogeneity of the terrain, and dense scrub-type vegetation dominated by small bamboo, small square plot demarcation was relatively convenient. In transect selection for sampling plots, the direction of slope, physiognomy of the forest, and altitude were considered. Few plots were demarcated on the northern slope of the mountain, due to the steepness of the slope and the presence of a dense bamboo layer (Figure 1).

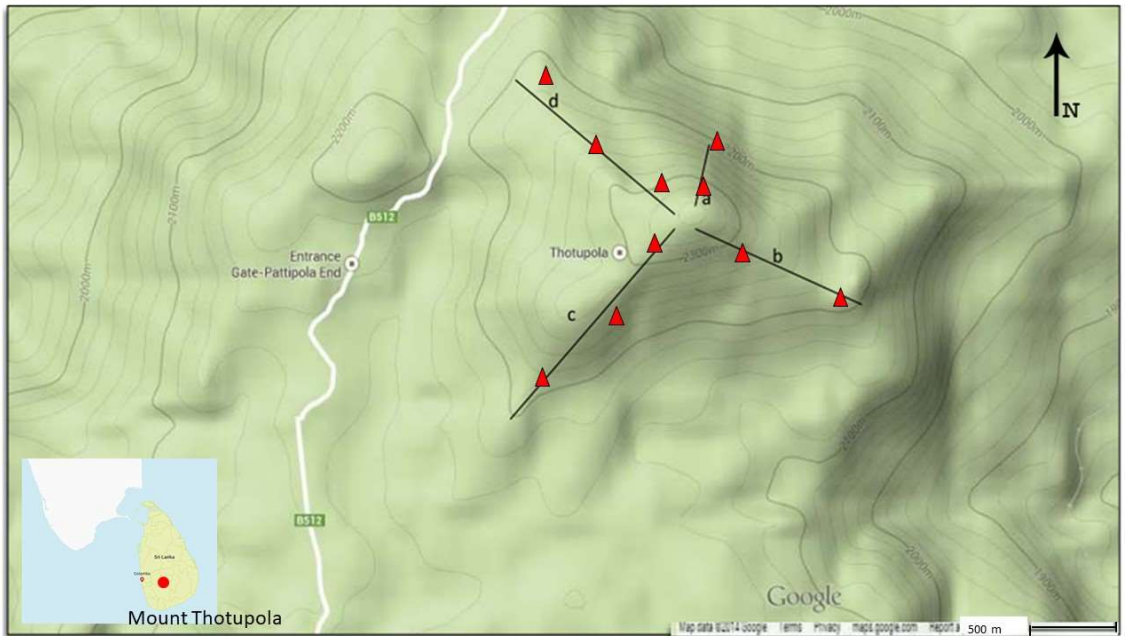

Figure 1: Distribution of overstorey and understory plots of the sampling sites in Mount Thotupola. (a) Northern slope; (b) Eastern slope; (c) Southern slope; (d) Western slope.

Field sampling covered three altitudinal ranges: lower elevation (2200 m – 2270 m), middle elevation (2270 m – 2340 m), and higher elevation (above 2340 m). The number of plots used for each elevation level is shown in Table 1. Data collection was done by both absolute (density, girth measurements, and basal area) and non-absolute (frequency) measures (Kent & Coker, 1996). Following Ratnayake *et al*. (1996) and Wright (1999), plants with girth at breast height (GBH) values ≥ 10 cm, and height > 1 m were considered as overstorey vegetation while plants below those limits were considered as understory vegetation. From each plant enumerated in each elevation level, specimens were collected and identified using identification keys and descriptions and comparison with the specimens deposited in the PDA.

Table 1: Number of over-story and understory plots sampled at each elevation level on Mount Thotupola.

Elevation (m)	No. of overstory plots	No. of understory plots
Lower (2200 - 2270)	15	30
Middle (2270 - 2340)	10	20
Higher (above 2340)	6	12
Total	31	62

Morphological identification of selected *Hedyotis* species

Among plant species found at mid-elevation, an extremely attractive plant of the genus *Hedyotis* was subjected to detailed morphological characterization following the protologue, keys, and information provided in the Revised Handbook to the Flora of Ceylon (Ridsdale, 1998). Specimens were compared with the type specimen and other specimens available at the National Herbarium, Peradeniya (PDA). Key morphological characters such as the presence of stipules and their morphology, position of the inflorescences, leaf arrangement, and leaf characteristics were examined. Herbarium specimens were prepared and deposited at the PDA (Figure 2).

Figure 2: Herbarium specimen of *Hedyotis quinquinervia* plant (Reproduced with the permission of the Director General of the Department of National Botanic Gardens, Sri Lanka). Magnification 1 x 0.5

DNA barcoding and molecular phylogeny

DNA barcoding work was done in 2019. The uprooted plant was established in the Hakgala Botanic Garden (described under conservation) and leaves were used for DNA extraction. Young leaves (n = 5) from the specimen were collected in a plastic zip-lock bag and transported to the University of Kelaniya for further studies. Leaves were surface sterilized by washing with tap water and dipping in 70% ethanol for 10 s, followed by three serial washings in sterilized distilled water. The excess water was removed using tissue papers. Leaf samples with cuticles were initially used, but it did not work well, so cuticle removal was attempted. Half of the samples were subjected to cuticle removal and the other half was used with the cuticle. Cuticle removal was done by placing a piece of clear tape on the adaxial surface of the leaf and quickly removing it so that the epidermis with the cuticle was separated from the leaf. Cuticle removal was confirmed by observing it under the microscope. Samples, with or without the cuticle, were stored at -80 °C and in silica gel at room temperature (25 °C). DNA extraction was performed using three protocols, Inglis *et al*. (2018), Guo *et al*. (2011), and modified Doyle & Doyle (1987), with varying β–mercaptoethanol concentrations, with a minimal amount of plant materials. Due to the limited amount

of material available from the desired specimen, optimization of DNA extraction was conducted with another commonly found species of the genus, *H. auricularia* L. which was recently synonymized to *Exallage auricularia* (L.) Bremek. Samples of *H. auricularia* stored in silica gel and at -80 °C were used for DNA extraction using the above three methods. The quality of DNA was determined using spectroscopic methods. Genomic DNA was subjected to polymerase chain reaction (PCR) amplification of the internal transcribed spacer (*ITS*) *region* of the nuclear *ribosomal DNA* (rDNA) using either the primer pairs, ITS1/4 (Hadi *et al*., 2016) or P17 and 26S-82R (Popp & Oxelman, 2001). P17 and 26S-82R primers (Popp & Oxelman 2001) were specific for the ITS region of the genus *Hedyotis* (Table 2). Once the protocol was optimized, leaves of *H. quinquinervia* were used to extract DNA, which was subjected to PCR.

Table 2: Primer name, sequence, and reference used in PCR amplification of selected *Hedyotis* sp.

No.	Primer Name	Primer Sequence (5' – 3')	Reference
1	ITS 1	AGGAGAAGTCGTAACAAGGT	Hadi *et al*. (2016)
2	ITS 4	TCCTCCGCTTATTGATATGC	
3	P17	CTACCGATTGAATGGTCCGGTGAA	Popp & Oxelman (2001)
4	26S-82R	CCCGGTTCGCTCGCCGTTACTA	

The PCR reaction mixture consisted of 1X buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM forward and reverse primers, and 0.25 U/µL of GoTaq® DNA polymerase (Promega, USA). The PCR reaction regime was carried out as initial denaturation for 5 min at 94 ℃ followed by 40 cycles of denaturation for 30 s at 94 ℃ followed by annealing at 55 ℃ for 30 s, or as described in Popp & Oxelman (2001), 1 min extension at 72 ℃ and final extension of 10 min at 72 ℃. Amplicons were separated in 1% agarose gel using agarose gel electrophoresis (Bio-Rad, USA). Successfully amplified PCR products were subjected to Sanger bidirectional dideoxy chain termination reaction at Gen-Tech., Colombo. Sequences were manually edited using Bioedit (version 7.2.5) and compared with the authenticated or vouched for sequences available in the GenBank using the BLASTn search tool. Good sequences were deposited in the GenBank and accession numbers were obtained.

Molecular phylogenetic analysis was performed using MEGA ver. 6 software (Kumar *et al.*, 2018) to determine the phylogenetic relatedness of putatively identified *H. quinquinervia* with the other *Hedyotis* spp. available in the GenBank using ITS sequence data. Twenty-two sequences of *Hedyotis* sp. mentioned in Guo *et al*. (2011) were obtained from the NCBI database and used in the phylogenetic analyses (Table 3). *Paraknoxia parviflora* (Stapf ex Verdc.) Verdc. ex Bremek. was selected as the outgroup according to Wikström *et al*. (2013). Sequences were aligned using ClustalW (Thompson *et al*., 1994) and the maximum likelihood method based on the Kimura-2 parameter model (Kimura, 1980) was performed as implemented in MEGA. Branch lengths were measured in the number of substitutions per site and the tree was drawn to scale. Every position that contained missing data and/or gaps were eliminated.

In situ and *ex situ* conservation of *Hedyotis quinquinervia*

In situ conservation of *H. quinquinervia* has been ensured as the location is situated within a conserved montane forest, Mount Thotupola located within Horton Plains, Sri Lanka. Officers of the Wildlife Conservation and Forest Departments of Sri Lanka, university academia, and policy decision-making bodies have been made aware of the discovery (Harasgama *et al*., 2014). As *ex situ* conservation measures, a few individual specimens of *H. quinquinervia* were carefully root balled by trained staff of the Botanical garden, immediately transported, and transplanted in the Botanical Gardens, Hakgala, Sri Lanka in 2014, where similar climatic and weather conditions prevailed (tropical wet montane forests). The plant has been established and maintained following general plant establishment and maintenance procedures by the Hakgala Botanical Garden, Sri Lanka. In 2019, leaves from the established plant were used for molecular work described earlier. Common conservation strategies for critically endangered heterostylous plant species were also suggested.

RESULTS AND DISCUSSION

Floristic structure of the study site

Plant heights of Mount Thotupola were variable depending on the elevation of the sampling site. The maximum plant height recorded in the lower elevation was about 12 m, while the minimum height was about 1.5 m in the higher elevation. Most of the species at lower elevations had a height of 2 - 8 m. Clearly indistinguishable, two different strata were arbitrarily identified as canopy and sub-canopy. The canopy height was about 6 - 12 m in height and mostly dominated by plant species such as *Michelia nilagirica* (Zenker) Figlar, *Calophyllum walkeri* Wight, *Litsea ovalifolia* (Wight) Trimen, *Vaccinium symplocifolium* (D.Don ex G.Don) Alston, *Actinodaphne ambigua* Hook. f. and *Symplocos* spp. The sub-canopy layer was mostly dominated by the species such as *Rhododendron arboreum* Sm., *Syzygium* spp., *Hedyotis* spp., *Rhodomyrtus tomentosa* var. *parviflora* (Alston) A.J. Scott. Hook. f., *Lasianthus gardneri* (Thwaites) Hook. f. and *Osbeckia* spp.

The middle elevation was occupied by a fairly open area dominated by pigmy forests, a preliminary version of tropical upper montane forests. The vegetation at this elevation was only 1 – 5 m in height. Species such as *Elaeocarpus montanus* Thwaites, *Vaccinium symplocifolium* (D.Don ex G.Don) Alston, and *Litsea ovalifolia* (Wight) Trimen were the dominant plants, and species like *Ochlandra stridula* Thwaites, *Psychotria gardneri* Hook. f., and *Lycianthes bigeminata* (Nees) Bitter were the dominant shrubs.

The vegetation at a higher elevation was only 1 – 5 m in height, the same as at the middle elevation. The tree layer was composed of species such as *Symplocos bractealis* Thwaites and *Symplocos cochinchinensis* (Lour.) S. Moore. The shrub layer was mainly dominated by *Rhodomyrtus tomentosa* var. *parviflora* (Alston) A.J. Scott. Hook. f. and *Psychotria gardneri* var. *gardneri* (Thwaites) Hook. f. An extremely attractive plant belonging to the genus *Hedyotis* was found in the open, marshy, or wet grasslands on rocky substrates of small valleys on mountain slopes. Seasonal or non-seasonal streams associated with these valleys maintain a small seepage on the rocky substrate providing semi-aquatic conditions. Accordingly, herbaceous species, *Eriocaulon* spp. and *Neanotis nummularia* (Arn.) W.H. Lewis which prefer semi-aquatic conditions, were commonly found in the habitat. In addition, some herb or shrub species such as *Strobilanthes calycina* Nees, *Knoxia platycarpa* var. *hirsuta* (Arn.) Thwaites, *Hedyotis ceylanica* N. Wikstr. & Neupane, *Anaphalis subdecurrens* Gamble, *Emilia speeseae* Fosberg, *Osbeckia parvifolia* Arn., *Chrysopogon nodulibarbis* (Hochst. ex Steud.) Henrard, *Impatiens leptopoda* Arn., are also commonly inhabited in this herb-dominated vegetation.

Table 3: Gene sequences used in phylogenetic analyses (species, GenBank accession number, and reference).

Species	GenBank accession number	Reference
Hedyotis assimilis Tutcher	JF699903.1	Guo *et al*. (2011)
Hedyotis auricularia L.	JF699904.1	Guo *et al*. (2011)
Hedyotis biflora (L.) Lam.	JF699908.1	Guo *et al*. (2011)
Hedyotis bodinieri H. Lév.	JF699909.1	Guo *et al*. (2011)
Hedyotis cantoniensis F.C. How ex W.C. Ko	JF699912.1	Guo *et al*. (2011)
Hedyotis diffusa Hance	JF699933.1	Guo *et al*. (2011)
*Hedyotis mellii*Tutcher	JF699936.1	Guo *et al*. (2011)
Hedyotis ovatifolia Cav.	JF699940.1	Guo *et al*. (2011)
Hedyotis pulcherrima Dunn	JF699946.1	Guo *et al*. (2011)
Hedyotis quinquinervia Thwaites	AM939458.1	Karehed *et al*. (2008)
Hedyotis shenzhenensis Tao Chen	JF699951.1	Guo *et al*. (2011)
Hedyotis tenuipes Hemsl.	JF699960.1	Guo *et al*. (2011)
Hedyotis trichoclada Merr. & L.M. Perry	HE657714.1	Wikström *et al*. (2013)
Hedyotis trimenii Seb & Ratna Dutta	HE657716.1	Wikström *et al*. (2013)
Hedyotis uncinella Hook. & Arn.	JF699964.1	Guo *et al*. (2011)
Hedyotis verticillata Blume	JF699969.1	Guo *et al*. (2011)
Hedyotis yangchunensis W.C. Ko & Zhang	JF699972.1	Guo *et al*. (2011)
Paraknoxia parviflora (Stapf ex Verdc.) Verdc. ex Bremek.	AM267020.1	Kårehed and Bremer (2007)

Morphological identification of *Hedyotis* species

Among plant species identified in the mid-elevation of the forest, which was an open grass and rocky marsh area, an extremely attractive plant specimen (Figure 3) belonging to the genus *Hedyotis* was collected, subjected to further studies, and identified as *H. quinquinervia*. The plant is a perennial woody shrub with a height of 0.5-1 m and leaves oppositely and compactly arranged. The leaves are small (1-1.5 cm), curled, waxy, and glabrous on both surfaces. They are simple and ovate-orbicular or broadly obovate-shaped with two to three pairs of lateral nerves with five nerves appearing at the base. Leaves have entire margins and the apex is acute (Figure 3b and 3d). *Hedyotis quinquinervia* was not found at the higher and lower elevations of the forest.

Morphology of flower in relation to pollination

The flowers are arranged as terminal inflorescences with a short (0.5-1 cm) axis, which stands out prominently above the foliage (Figure 3a). The corolla is white and funnel-shaped (2 mm long). Pubescent hairs arranged as a ring are found at the throat of the corolla tube. Small, actinomorphic, bisexual sub-sessile, four-petaled flowers are heterostylous. Due to its heterostylous nature, natural self-pollination would not happen. The style of the flower is 1 - 3 mm or 4.6 mm long. Filaments of the stamens were short in pin-flowers (0.3 - 0.5 mm) and long in thrum-flowers (1-2 mm). Hence both pin and thrum floral morphs were evident. Filaments were shorter than the style favouring cross-pollination. The stigma is bi-lobed. The ovary is bi-locular. The fruits are brownish, ellipsoid small (2 - 3 × 1.5 - 2 mm) capsules with four to six brownish seeds per placenta.

Figure 3: Rediscovered *Hedyotis quinquinervia* plant from Mount Thotupola, Sri Lanka. (a) Terminal inflorescence with each floret having four white petals; (b) and (d) Compactly arranged, curved leaves on a woody shrub; (c) Brownish ellipsoidal capsules arranged in fruit.

Conspicuous terminal inflorescence above the foliage with white flowers facilitates the attraction of pollinators as a visual cue. To prevent the entrance of floral larcenists a ring of pubescent hairs at the throat of the corolla tube is important. Heterostyly enhances the possibility of cross-pollination to increase genetic variations within a population. Heterostyly promotes outbreeding (Barrett, 1992; Barrett & Shore, 2008). The bi-lobed stigma of *H. quinquinervia* may facilitate efficient pollen attachment. Accordingly, the pollination syndrome would be

entomophilous with the help of small insects. Most of the heterostylous flowers are pollinated by insects, particularly bees, flies, moths, butterflies, and beetles (Ganders, 1979), bombyliid flies (Ornduff, 1980), and *Apis cerana*, the Asian honeybee (Mahadura, 2020). Several findings indicated that *Hedyotis* species are mainly meiophilous and pollinated by small bees (Mahadura & Saunders, 2021) as reported for *H. shenzhenensis* Tao Chen (syn. *H. shiuyingiae*), *H. acutangula* Champ. ex Benth., *H. loganioides* Benth. (also syn. *H. bodinieri* H. Lév.), and *H. vachellii* Hook. & Arn. species. Another dehiscence and stigmatic receptivity in the morning also coincide with the timing of bee foraging.

DNA barcoding and molecular phylogeny

In a recent floristic survey conducted at Mount Thotupola, *H. quinquinervia* was rediscovered. The plant was identified using morphological catalogues, comparison with authentic specimens deposited in the National Herbarium, Peradeniya, Sri Lanka (PDA), following taxonomic keys, and DNA barcoding. A field study by Prabhukumar *et al*. (2018) confirmed the identity and rediscovery of *Hedyotis beddomei* Hook. f. from the Western Ghats, India, only with morphological comparison. However, in the present study, we confirmed the identity of the species with the currently most accepted DNA technology too. Except for the limited availability of plant materials, the biggest challenge in this study was to isolate DNA without liquid N_2. DNA isolation was successful by the modified Doyle & Doyle (1987) procedure and PCR was successful with the DNA isolated. The removal of the cuticle from the leaves was critical, and the novel modified method could be applied to other plant species with a thick cuticle layer.

After several attempts and modifications of DNA extraction methods, high-quality DNA of *H. quinquinervia* was isolated by the modified method described in Doyle & Doyle (1987). The modifications were: removal of the leaf cuticle layer by the clear tape method, followed by storage at -80 °C. In addition, the reduction of β-mercaptoethanol from 4% to 2%, and using 0.2 g of leaf material was found to be effective in obtaining a relatively high-quality DNA. OD value at 260/280 nm was 1.69 whereas all the other methods yielded below 1.4. The simple method of cuticle removal was successful, and it was evident when the stained clear tapes were observed using a light microscope. All the samples stored in silica gel gave very poor-quality DNA and were not suitable for PCR. Good ITS sequences were obtained from the above sample in both directions. The rDNA-ITS sequence was deposited at the GenBank under accession number MT373691. BLASTn analysis showed our sample was 99% similar to the sequence of the *H. quinquinervia* (AM939458.1) deposited by Karehed *et al*. (2008), from a voucher specimen that was also from Sri Lanka. The sequences have been available since 2008, from the DNA that was extracted most likely from an old herbarium specimen.

Guo *et al*. (2011) reported that in the genus *Hedyotis*, the success rate of PCR amplification of the ITS region was the greatest, whereas all the other barcoding regions (*matK, trnH–psbA, petD, rbcL* of plastid DNA) yielded the lowest amplification. Therefore, only the ITS region of *H. quinquinervia* was used in the phylogenetic analyses. The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (-1893.67) is shown (Figure 4). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 25 sequences. There was a total of 321 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

The *H. quinquinervia* sequence (MT373691) was clustered with the *H. quinquinervia* (AM939458.1) DNA barcode (Karehed *et al*. 2008) obtained from a Sri Lankan voucher specimen (Figure 3). In addition, the ML approach, NJ and maximum parsimony analyses produced the same tree topology and hence are not included. For a complete picture of the evolutionary history of our sample, a multigene approach should be applied (Guo *et al*., 2011; Wikström *et al*., 2013). However, this study was able to clarify the phylogenetic placement of *H. quinquinervia* from Sri Lanka in the genus *Hedyotis* (Salmaki *et al*., 2019).

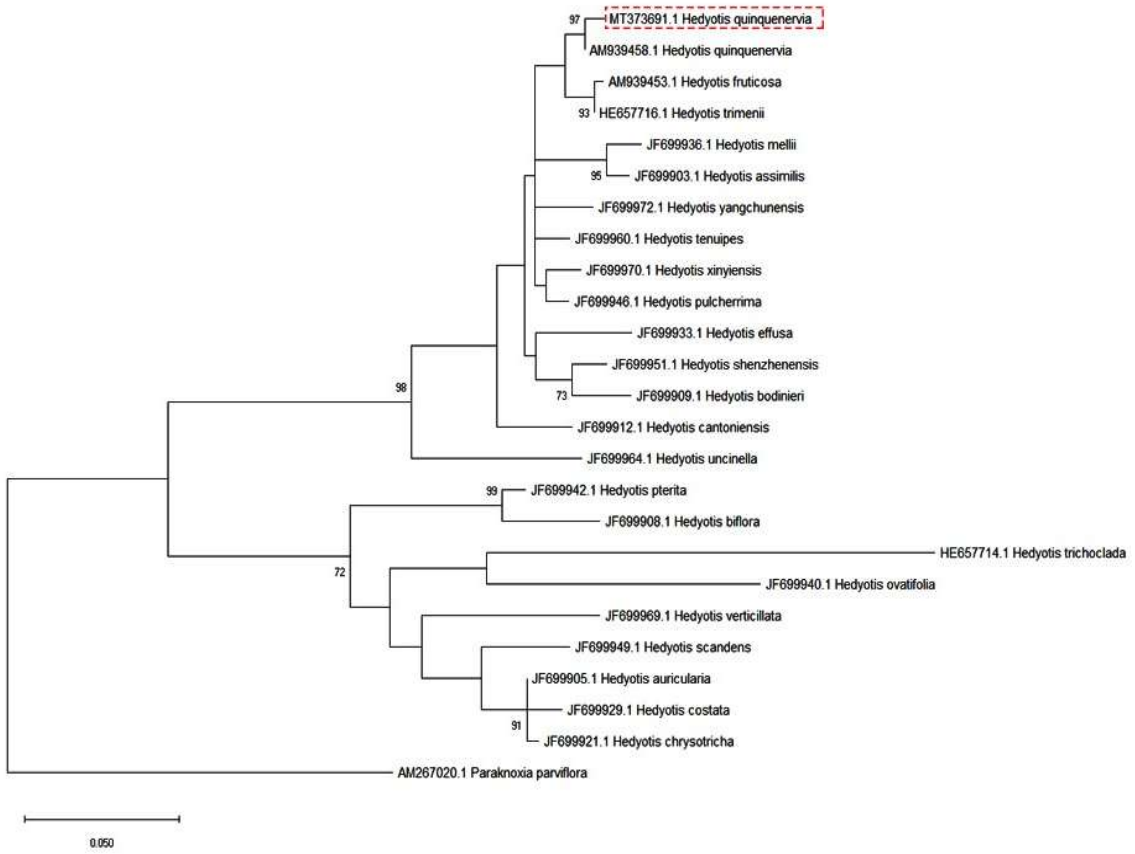

Figure 4: Phylogenetic relationships of *Hedyotis quinquinervia* with selected *Hedyotis* sp. The tree was constructed using maximum-likelihood method. The tree is drawn to scale (branch lengths proportional to evolutionary distances) and has been rooted with *Paraknoxia parviflora* (AM267020.1). Bootstrap support values are shown above branches (values > 70 are shown). Sample of the current study is in a box with a dotted line.

In-situ and *ex-situ* conservation of *Hedyotis quinquinervia*

According to the National Red List of Sri Lanka 2012, *H. quinquinervia* was a critically endangered, possibly extinct [CR(PE)] species (MOE, 2012). After the abstract publication of the same study, the status had been revised to critically endangered (CR) in 2020 (MOE, 2020). The discovery of *H. quinquinervia* at Mount Thotupola is important because it had not been found during recent floristic surveys conducted on Mount Pedro where the species had been reported more than 100 years ago (Attanayake, unpublished data). Therefore, at present, the plant is found only on Mount Thotupola. Mount Thotupola, in Horton Plains, is a conserved forest under the Department of Wildlife Conservation, which is the responsible institute for the *in situ* conservation of the species on Mount Thotupola. Due to the limited number of individuals found on Mount Thotupola, only a single plant was removed for *ex situ* conservation keeping more individuals intact in the forest for natural seedling establishment.

As per the *ex situ* conservation measures, one individual of *H. quinquinervia* was successfully transplanted to the Hakgala Botanic Garden, Sri Lanka (6.9266° N, 80.8215° E). However, as no studies are available on the reproductive biology of the species, its breeding system is not yet defined. Experimental pollination revealed that both pin and thrum flowers of *Oldenlandia salzmannii* (DC.) Benth. & Hook.f. ex B.D.Jacks. (syn. *Hedyotis salzmannii* (DC.) Steud.) are self-compatible. Karunarathne *et al.* (2005) found that three other *Hedyotis* species, *Oldenlandia corymbosa* L. (syn. *Hedyotis corymbosa* (L.) Lam.), *Hedyotis fruticosa* L., and *Hedyotis trimenii* Deb & Ratna Dutta, are pollinated by a diverse array of hymenopterans. Based on the floral phenology, there is a high probability that *H. quinquinervia* is also entomophilous. If that is the case, the low abundance of individuals in the original site limits the probabilities of cross-pollination. That might have been one of the reasons for its

rarity and its being categorized as critically endangered (CR). Under such circumstances, the possibility of colonization by a single plant is uncertain under *ex situ* conservation, since the fecundity of the next generations after self-pollination is uncertain due to its heterostyly and the presence of two floral morphs. Therefore, the future addition of other floral morphs for *ex situ* conservation would facilitate cross-pollination and can be used in the expansion of the populations. Therefore, detailed studies of the reproductive biology and pollination biology of *H. quinquinervia* is essential. Evidence proposes that pollen limitation for reproduction is severe in plants in biodiversity hotspots than those in less diverse areas, mainly due to the response of self-incompatible species (Alonso *et al*., 2010). The given IUCN category of CR is also due to the self-incompatibility of heterostylous species in which only pin flowers can fertilize thrum flowers, and vice versa. That prevents further expansion by selfing. Most of the Rubiaceae individual plants can only have pin flowers or thrum flowers, but not both and therefore, one heterostylous rubiaceous plant in the Hakgala botanical garden is not sufficient to address *ex situ* conservation fully. Therefore, addition of a minimum of two heterostylous individuals, one pin and one thrum, are essential for effective *ex situ* conservation. According to the replanting of the plant successfully *ex situ*, future addition of another floral morph will complete the *ex situ* conservation attempt. However, the available plant can be used for attempts of vegetative propagation experiments like layering and tissue culture. There is a potential to use a derived mechanism like pre-anthesis cleistogamy which ensures reproductive assurance of a single individual conserved *ex situ,* despite potential mate and pollinator limitations for *H. quinquinervia,* as it was successfully used for *H. bodinieri* in Hong Kong by Mahadura (2020). Mahadura & Saunders (2021) found that the breakdown of self-incompatibility facilitates interspecific hybridization in *Hedyotis*. With the advancement of technology, even with a single plant, colonization may be possible.

The genus *Hedyotis* has 30 species in Sri Lanka and out of that, 25 species and a variety are endemic to the island (MOE, 2020). Seven of them are critically endangered (CR), 13 are endangered (EN), 1 each vulnerable (VU) and near threatened (NT) while only 2 are of least concern (LC) (MOE, 2020). The rediscovery of this CR species is of great interest from the conservation point of view because Sri Lanka is extraordinarily rich in biodiversity with 3087 indigenous plant species of which 863 are endemic (MOE, 2020). However, the plant is, at present, restricted to the area of Mount Thotupola. Even in the past, *H. quinquinervia* was only recorded from the natural montane forest in the central highland of Sri Lanka. For the conservation of threatened species protection of the whole ecosystem is essential. Species in biodiversity hotspots may thus be more at risk due to limited reproduction success and subsequent population decline (Alonso *et al*., 2010). Watanabe & Sugawara (2015) stated that in some oceanic islands, a shift from heterostyly to other sexual systems may occur and that contributes to the rarity of heterostyly and the difficulty in colonization of heterostylous species on oceanic islands.

The current state of its low abundance might be due to several facts, such as anthropogenic activities, global warming, alien invasive species, plant diseases, and wildfires. Rapidly spreading alien invasive species such as *Ageratina riparia* (Regel) R.M. King & H. Rob., *Ageratina adenophora* (Spreng.) R.M. King & H. Rob., *Aristea ecklonii* Baker, *Austroeupatorium* spp., and *Pennisetum* Rich. spp. (Ranwala *et al*., 2012) are a threat to the native flora, including *H. quinquinervia*. Additionally, grasslands are more prone to fire than tree vegetation. Therefore, the implementation of appropriate conservation measures is essential, not only for the protection of *H. quinquinervia* but also to conserve other endemic and native flora and fauna in the area. In distylous plants, the supergene controls a diallelic sporophytic self-incompatibility system according to Ganders (1979). This could be another reason for the resulting lack of pollen for cross-fertilization leading to the decline of the population of *H. quinquinervia*.

Grass species *Cenchrus clandestinus* (Hochst. ex Chiov.) Morrone (syn. *Pennisetum clandestinum* Hochst. ex Chiov.) and *Cenchrus geniculatus* Thunb. (syn. *P. glabarum* Steud.) in the Horton Plains National Park have been reported to be infected by the fungus *Laetisaria fuciformis* (McAlpine) Burds. causing the red thread disease (Adikaram *et al*., 2001). Such diseases might also pose a threat to the natural habitat of *H. quinquinervia.* In addition, it has been reported that a high level of Pb in soils on the slopes leads to forest die-back in the montane forests (Fernando *et al*., 2009). In addition to the global climatic changes and other reasons discussed above, wildfires might have affected the disappearance of the species. It is recommended to establish wildfire protection procedures, such as maintaining a fire belt, removing combustible materials, and frequent monitoring. As Ashton & Zhu (2020) suggested possible catastrophic restrictions on forests, and the reproduction, dispersal, and survival of species should be predicted and appropriate conservation strategies should be implemented.

CONCLUSION

During a field survey in 2014, a tentative morphology-based identification of *H. quinquinervia* was done, and it was recognized that the plant is CR(PE). The finding of this study led to the revision of the status to CR in 2020. By 2019, both *in situ* and *ex situ* conservation strategies were addressed for the species. DNA extraction was also attempted in 2019 using the conserved plant from Hakgala Botanic gardens. This extremely attractive plant was accurately identified as *H. quinquinervia* using a DNA barcoding approach. An efficient DNA extraction protocol for *H. quinquinervia* and possibly for both *Hedyotis* and *Oldenlandia* genera was developed by removing the thick cuticle, which facilitated efficient DNA extraction and PCR amplification. Pre-treatment of leaves by storing at -80 ℃ prior to DNA extraction was more suitable for the leaf samples than silica gel drying. This method is suitable for resource-limited settings of developing countries with no regular access to liquid N_2. The floral trials of *H. quinquinervia* are indicative of entomophilous pollination syndrome. The presence of heterostylous flowers suggests a requirement of cross-pollination between individuals. These facts need to be considered when taking conservation measures for the species.

Acknowledgement

The authors would like to acknowledge the Department of Wildlife conservation, Sri Lanka for research permit, Royal Botanic Gardens, Peradeniya for their support in obtaining photographs of herbarium specimens from the rare plant section, the Biodiversity Secretariat of the Ministry of Environment and Renewable Energy, Department of National Botanic Gardens (for sampling and fieldwork), and University of Kelaniya for molecular identification (Research Grant RP/03/02/01/01/2014).

REFERENCES

Alonso C., Vamosi J.C., Knight T.M. & Steets J.A. (2010). Is reproduction of endemic plant species particularly pollen limited in biodiversity hotspots? *Oikos* **119**: 1192–1200.
DOI: https://doi.org/10.1111/j.1600-0706.2009.18026.x

Ashton P. & Zhu H. (2020). The tropical-subtropical evergreen forest transition in East Asia: an exploration. *Plant Diversity* **42**: 255–280.
DOI: https://doi.org/10.1016/j.pld.2020.04.001

Barrett S.C.H. (1992). Heterostylous genetic polymorphisms: model systems for evolutionary analysis. In: *Evolution and Function of Heterostyly* (ed. S.C.H. Barrett), pp. 1–29. Springer, New York, USA.
DOI: https://doi.org/10.1007/978-3-642-86656-2_1

Barrett S.C.H. & Cruzan M.B. (1994). Incompatibility in heterostylous plants. In: *Genetic Control of Self-incompatibility and Reproductive Development in Flowering Plants* (eds. E.G. Williams, A.E. Clarke & R.B. Knox), pp. 189-219. Springer, Dordrecht, Netherlands.
DOI: https://doi.org/10.1007/978-94-017-1669-7_10

Barrett S.C.H. & Shore J.S. (2008). New insights on heterostyly: comparative biology, ecology, and genetics. In : *Self-incompatibility in Flowering Plants – Evolution, Diversity, and mechanisms* (Ed. V.E. Franklin-Tong), pp. 3–32. Springer, Berlin, Germany.
DOI: https://doi.org/10.1007/978-3-540-68486-2_1

Doyle J.J. & Doyle J.L.A. (1987). Rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Fernando G.W.A.R., Ranasinghe P., Wimalasena M.D.N.R. & Ekanayake S.P. (2009). Dieback in tropical montane forests of Sri Lanka: anthropogenic or natural phenomenon? *Journal of the Geological Society of Sri Lanka* **13**: 27–52.

Ganders F. (1979). The biology of heterostyly. *New Zealand Journal of Botany* **17**(4): 607–635.
DOI: https://doi.org/10.1080/0028825X.1979.10432574

Guo X., Simmons M., But P., Shaw P. & Wang R. (2011). Application of DNA barcodes in *Hedyotis* L. (Spermacoceae, Rubiaceae). *Journal of Systematics and Evolution* **49**(3**)**: 203–212.
DOI: https://doi.org/10.1111/j.1759-6831.2011.00130.x

Guo X., Wang R., Simmons M., But P. & Yu J. (2013). Phylogeny of the Asian Hedyotis–Oldenlandia complex (Spermacoceae, Rubiaceae): Evidence for high levels of polyphyly and the parallel evolution of diplophragmous capsules. *Molecular Phylogenetics and Evolution* **67**(1): 110–122.
DOI: https://doi.org/10.1016/j.ympev.2013.01.006

Hadi S., Santana H., Brunale P., Gomes T., Oliveira M., Matthiensen A., Oliveira M., Silva F. & Brasil B. (2016). DNA barcoding green microalgae isolated from Neotropical Inland Waters. *PLOS ONE* **11**(2).
DOI: https://doi.org/10.1371/journal.pone.0149284

Harasgama H.D.R.V.L., Wijewickrama T., Ratnayake R.M.C.S. & Attanayake A. (2014). Rediscovery of *Hedyotis quinquinervia* Thwaites (Rubiaceae): a critically endangered shrub from Horton Plains, Sri Lanka. *Proceedings of the 19th International Forestry and Environment Symposium,* 24- 25 October. University of Sri Jayewardenpura, Sri Lanka, pp. 24.

Inglis P., Pappas M., Resende L. & Grattapaglia D. (2018). Fast and inexpensive protocols for consistent extraction of high-quality DNA and RNA from challenging plant and fungal samples for high-throughput SNP genotyping and sequencing applications. *PLOS ONE* **13**(10): e0206085.
DOI: https://doi.org/10.1371/journal.pone.0206085

Kårehed J. & Bremer B. (2007). The systematics of Knoxieae (Rubiaceae)-molecular data and their taxonomic consequences. *Taxon* **56**(4): 1051–1076.
DOI: https://doi.org/10.2307/25065904

Karehed J., Groeninckx I., Dessein S., Motley T. & Bremer B. (2008). The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoceae. *Molecular Phylogenetics and Evolution* **49**(3): 843–866.
DOI: https://doi.org/10.1016/j.ympev.2008.09.025

Karunarathne I., Edirisinghe J. & Gunatilleke C.V.S. (2005). Floral relationships of bees in selected areas of Sri Lanka. *Ceylon Journal of Science* **34**: 27–45.

Katterman F.R.H. & Shattuck V.I. (1983). An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. *Preparative Biochemistry and Biotechnology* **13**: 347–359.
DOI: https://doi.org/10.1080/00327488308068177

Kazi T., Hussain N., Bremner P., Slater A. & Howard C. (2013). The application of a DNA-based identification technique to over-the-counter herbal medicines. *Fitoterapia* **87**: 27–30.
DOI: https://doi.org/10.1016/j.fitote.2013.03.001

Kent M. & Coker P. (1996). *Vegetation Description and Analysis: A Practical Approach*, pp. 363. John Wiley, New York, USA.

Kumar S., Stecher G., Li M., Knyaz C. & Tamura K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35(6)**: 1547–1549.
DOI: https://doi.org/10.1093/molbev/msy096

Liu X., Wu X. & Zhang D. (2021). Distyly and heteromorphic self-incompatibility of *Hedyotis pulcherrima* (Rubiaceae). *Biodiversity Science* **20**(3): 337–347.
DOI: https://doi.org/10.3724/SP.J.1003.2012.11243

Mahadura A.D. (2020). Mating systems in sympatric *Hedyotis* species (Rubiaceae) in Hong Kong: the breakdown of distyly and its impact on hybridization and self-fertilization. *M. Phil thesis*, University of Hong Kong, China.

Mahadura A.D. & Saunders R.M.K. (2021). Correlation of self- and interspecific incompatibility among sympatric *Hedyotis* species (Rubiaceae) and consequences for hybridization. *Journal of Systematics and Evolution*: **60**: 998–101.

MOE (2012). *The National Red List 2012 of Sri Lanka- Conservation Status of the Fauna and Flora,* pp. 324. Ministry of Environment, Colombo, Sri Lanka.

MOE (2020). *The National Red List 2020- Conservation Status of the Flora of Sri Lanka*, pp. 153. Ministry of Environment, Colombo, Sri Lanka.

Muruganandam S., Devanathan K., Ravikumar S. & Narasimhan D. (2020). *Hedyotis sithiravaraiensis* (Rubiaceae): a new species from Southern India. *Journal of Asia-Pacific Biodiversity* **13**(4): 749–754.
DOI: https://doi.org/10.1016/j.japb.2020.08.003

Ornduff R. (1980). Heterostyly, population composition, and pollen flow in *Hedyotis caerulea. American Journal of Botany* **67**(1): 95–103.
DOI: https://doi.org/10.1002/j.1537-2197.1980.tb07627.x

Prabhukumar K., Jagadeesan R., Aiswarya P., Sunil C., Thomas V., Prasad K. & Balachandran I. (2018). On the identity and rediscovery of *Hedyotis beddomei* Hook. f. (Rubiaceae): a lesser known endemic species of Western Ghats, India. *Phytotaxa* **375**(3): 229–234.
DOI: https://doi.org/10.11646/phytotaxa.375.3.5

Popp M. & Oxelman B. (2001). Inferring the history of the polyploid *Silene aegaea* (Caryophyllaceae) using plastid and homoeologous nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **20**(3): 474–481.
DOI: https://doi.org/10.1006/mpev.2001.0977

Premathilake R. & Risberg J. (2003). Late quaternary climate history of the Horton Plains, central Sri Lanka. *Quaternary Science Reviews* **22**(14**)**: 1525–1541.
DOI: https://doi.org/10.1016/S0277-3791(03)00128-8

Raju A.J.S. & Radhakrishna J. (2018). Pollination ecology of the annual herb, *Hedyotis brachiata* (Rubiaceae). *Annali di Botanica* **8**: 9–16.

Ranwala S., Marambe B., Wijesundara D. & Silva P. (2012). Post-entry risk assessment of invasive alien flora of Sri Lanka - present status, gap analysis, and the most troublesome alien invaders. *Pakistan Journal of Weed Science* **18**: 863–871.

Ratnayake R.M.W., Jayasekara L.R. & Solanaarachchi S.M. (1996). A quantitative study of overstory vegetation of an upper montane rain forest. *The Sri Lanka forester* **22**(3,4): 43–49.

Ridsdale C.E. (1998). Hedyotis. In: *A revised handbook to the flora of Ceylon- volume 12* (Ed. M.D. Dassanayake) pp. 238–268. A.A. Balkema, Rotterdam, Netherlands.

Salmaki Y. & Müller J. (2019). Rediscovery of the enigmatic *Scutellaria xylorrhiza* (Scutellarioideae; Lamiaceae) - a rare endemic species from Iran. *Phytotaxa* **394**(4): 267–275.
DOI: https://doi.org/10.11646/phytotaxa.394.4.4

Saroja K.G.N. & Gunatilake J. (2013). Compilation of detailed geological map and soil geochemical maps for Horton Plains National Park. *Proceedings of 29th Technical Sessions of Geological Society of Sri Lanka.* Colombo, Sri Lanka, pp. 79–82.

Thompson J., Higgins D. & Gibson T. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**(22): 4673–4680.
DOI: https://doi.org/10.1093/nar/22.22.4673

Thwaites G.H.K. (1859). *Enumeratio Plantarum Zeylaniae.* William Pamplin, Soho Square, London, UK.

Watanabe K. & Sugawara T. (2015). Is heterostyly rare on oceanic islands? *Annals of Botany* **7**: 1–16.
DOI: https://doi.org/10.1093/aobpla/plv087

Wikström N., Neupane S., Kårehed J. & Motley T.J. (2013). Phylogeny of *Hedyotis* L. (Rubiaceae: Spermacoceae): redefining a complex Asian Pacific assemblage. *Taxon* **62**(2): 357–374.

Wright S.J. (1999). Plant diversity in tropical forests. In: *Handbook of Functional Plant Ecology* (Eds F.I. Pugnaire & F. Valladares), pp. 449–472. Dekker, New York, USA.
DOI: https://doi.org/10.12705/622.2