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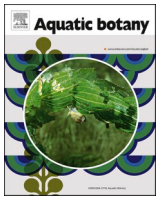

Species identification and pollination biology of an economically important true halophyte, *Salicornia brachiata* Roxb.

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ABSTRACT

Members of the genus *Salicornia* have gained a global attraction due to their ability to thrive under high saline conditions and as potential candidates in saline agriculture. However, it has been a taxonomically challenging genus for decades since the members show plastic responses to extreme environmental conditions and due to incongruences between morphological and molecular identification methods. While only a handful of commercially grown *Salicornia* species are fully described, most of the species including *S. brachiata*, a native species in the Indian sub-continent, Myanmar, and Sri Lanka are poorly described. With the potentials in adapting *S. brachiata* in saline agriculture, the aim of this study was to establish a morphology and DNA barcode-based species delineation system and to study pollination biology for future crop improvement projects. Tentatively identified *S. brachiata* plant samples were collected from two populations in Sri Lanka and completely described. GenBank lacked authenticated barcode data for *S. brachiata* except for one chloroplast genome to which the matK sequence obtained in the present study matched with 100 % identity. For the first time, well defined sequences of three barcode regions, ITS, ETS and matK, of *S. brachiata* were made available for accurate species identification. Reproductive dynamics in different parts of the inflorescence was studied. A facultative xenogamous mating system was recorded for the first time in the genus and while the lower florets in the cladode showed a preference towards outcrossing, the upper florets displayed adaptations for selfing. Data could be effectively utilized in future *Salicornia* breeding programs.

1. Introduction

Salicornia L. is a halophytic genus of the family Amaranthaceae (Chenopodiaceae) with specialized morphological and physiological adaptations to harsh saline environments and therefore, these plants serve as an excellent gene pool for salt-resistant genes (Ball and Tutin, 1959; Kadereit et al., 2007, 2012). With the soil salinization becoming a global issue, *Salicornia* spp. gained recognition as a potential crop in saline agriculture due to their ability to accumulate salt (Cárdenas-Pérez et al., 2021; Alfheeaid et al., 2022). Some of these species are rich in bioactivities and can accumulate up to 40–50 % NaCl from the dry weight serving as an excellent source of bio-salt and widely used in traditional medicine (Khare, 2007; Stanley, 2008; Jha et al., 2009; Alfheeaid et al., 2022). The genus is also well-reputed as an edible halophyte and secure a unique niche in the global food market as a functional food and as an organic seasoning (Khoshbakht and Hammer, 2008; Isca et al., 2014; Lopes et al., 2017; Rathore et al., 2019; Alfheeaid et al., 2022; Katel et al., 2023).

Regardless of numerous uses, *Salicornia* is one of the most taxonomically challenging genera (Kadereit et al., 2007, 2012). According to the Plants of the world online, (2023), 157 species are reported under the genus *Salicornia*, and 64 of them are accepted species names (https://powo.science.kew.org/). Historically, species delineation of *Salicornia* has been achieved by morphological characterization (Ball and Tutin, 1959; Tölken, 1967). However, unlike many other angiosperms, members of the genus *Salicornia* do not have prominent and discrete phenotypic traits for easy reference. For example, plants bear extremely reduced scale-like leaves, articulated or segmented succulent stems, and reduced flowers with fused tepals aggregated in dense terminal, cladode-like thyrsus (Kadereit et al., 2007; Murakeözy et al.,

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2007; Rathore et al., 2019).

Species determination of *Salicornia* based on morphology is often challenging, even for trained botanists, as the plants exhibit remarkable adaptations to frequent environmental fluctuations. These include high solar radiation and fluctuation in edaphic factors such as soil texture, pH, salinity, and nutrient supply caused by frequent inundations, resulting in a high degree of phenotypic plasticity (Ellison and Niklas, 1988; Kadereit et al., 2007; Rathore et al., 2019). In the reproductive stage, plants of the same *Salicornia* species in the same location can vary significantly in size, ranging from small individuals with only one or two segments to more robust, highly branched individuals that can reach heights around 20 cm (Piirainen, 2015). Rathore et al. (2019) found that the growth characteristics of *S. brachiata*, such as plant height, shoot and root development, branching patterns, and terminal cladode length, varied across six different sites. Numerical analysis of morphological variations, which is generally used to compare *Salicornia* species, failed to describe reliable features to distinguish *S. europaea* from *S. ramosissima* (Dalby, 1962). Teege et al. (2011) observed that *S. stricta* and *S. procumbens*, traditionally distinguished by their growth form, branching pattern, inflorescence length, and floral characteristics, are in fact intraspecific ecotypes resulted from high levels of inbreeding.

Therefore, in recent studies, traditional morphology-based species identification has been replaced with integrated morpho-molecular methods to solve the taxonomic dilemma of the genus *Salicornia* (Kadereit et al., 2006). DNA barcoding is becoming a widely applied tool for the quick and accurate identification of species with variable morphology. However, incongruences between morphological and molecular level identification have been reported (Kadereit et al., 2012; Piirainen et al., 2017). For example, plants with similar morphotypes were identified as different species based on molecular data and vice versa (Kadereit et al., 2012). Further, poorly or inadequately described taxonomy and difficulty to identify plants of the same genotype are few of the major constrains for accurate species delineation and use *Salicornia* sp. as a crop (Singh et al., 2014). Therefore, detailed morphological examination of any species is crucial and the use of morphological traits along with multiple barcoding regions for species identification of *Salicornia* has been proposed (Jamdade et al., 2022).

Among all the species of the genus, *S. europaea* is the most studied and well-documented species in terms of morphology, anatomy, and molecular identification since it is cultivated on a commercial scale (Dalby, 1962; Ellison and Niklas, 1988; Ellison et al., 1993; Davy et al., 2001). However, the names *Salicornia europaea* L., and *S. herbacea* L. are frequently used for several commercially grown species of this genus due to overlapping of phenotypic traits (Davy et al., 2001; Kadereit et al., 2012). Except for these studies, use of morpho-mlolecular traits in species identification have been conducted on *Salicornia* species found in Japan (Sagane et al., 2003), South Africa and Namibia (Slenzka et al., 2013), Egypt (Hussein et al., 2020), Iran (Chatrenoor and Akhani, 2021), United Arab Emirates (Jamdade et al., 2022), Tunisia (Hayder et al., 2022), and Italy (Sciuto et al., 2023) due to high economic potentials of the genus.

Salicornia brachiata, native to the Indian sub-continent, Myanmar, and Sri Lanka, has also been recognized as a promising species in saline agriculture (Pandya et al., 2006; Stanley, 2008; Alfheeaid et al., 2022). However, except for a brief description available in Flora of Ceylon (Boulos, 1995), and few ecological surveys published in Sri Lanka and India (Pemadasa et al., 1979; Rathore et al., 2019, 2021; Ahalya and Suresh, 2020), no detailed morphological characterization, DNA barcoding and descriptive reproductive studies are available on *S. brachiata,* the most common species in the Indian sub-continent (Katel et al., 2023). In spite of easy access to modern DNA sequencing technologies, a recent search in the NCBI GenBank resulted in only one curated chloroplast genome sequence of *S. brachiata* while three barcode sequences deposited under the name of *S. brachiata* were either not published or misidentified species highlighting the importance of filling this information gap.

Despite a dearth of information on the species identification, reproductive biology, floral biology, anthology, and pollination mechanisms are also not well understood for many species of the genus. Understanding reproductive biology is the initial step in identifying population subdivision, population heterogeneity, and is useful in breeding programs aiming to develop improved hybrids for saline agriculture.

Different *Salicornia* species are reported to exhibit different breeding systems. Self-pollination and cleistogamy are frequent among the species of *Salicornia* resulting in high levels of inbreeding allowing some level of local adaptations (Dalby, 1962; Ball, 1964). This results in the existence of different homozygous lineages, which might be responsible for the current taxonomic confusion (Davy et al., 2001; Kadereit et al., 2007). Though both dichogamy and cleistogamy have been reported in the genus, cleistogamy is a prominent phenomenon observed in species such as *S. ramosissima*, *S. europaea* (Jefferies et al., 1981; Jefferies and Gottlieb, 1982; Fernandez-Illescas et al., 2011) and *S. obscura* (Ball and Tutin, 1959). It is reported that some of these species are weakly protogynous as well (Dalby, 1962; Ferguson, 1964; Jefferies et al., 1981). Ferguson (1964) also reported that tetraploid species such as *S. dolichostachya* Moss. is weakly protogynous or homogamous. Protandrous flowers have been observed in *Salicornia pachystachya* (Tölken, 1967) and *S. ramosissima* (Fernandez-Illescas et al., 2011). However, no single study has attempted to selectively study the reproductive dynamics of florets in different regions of a spike of the genus.

Particularly *S. brachiata* is poorly studied in terms of fine-scale morphological characterization, molecular identification and reproductive biology despite its economic potential. The objectives of the present study are to fill the information gap in Flora of Ceylon by documenting fine-scale morphological traits of *S. brachiata*, to use morphological traits along with DNA barcodes for species identification, and to study floral biology and reproductive dynamics of different flowers of the same spike to understand the breeding system and pollination syndrome of the plant in its natural ecosystem. The study is particularly of significance since *S. brachiata* is a promising agent in saline agriculture in the tropics, where soil salinization is a major concern with the climate change.

2. Materials and methods

2.1. Study site

The study materials were collected representing natural populations of *Salicornia* from two different geographical locations in the Puttalam lagoon located in Northwestern Province, Sri Lanka. The area usually experiences a semi-arid tropical climate with a mean annual temperature of about 27–28 °C (Pemadasa et al., 1979). The mean annual rainfall in the region is approximately 1500 mm, with most of the rainfall occurring during the northeast monsoon season from October to January (Department of Meteorology, Sri Lanka, 2023). From the lagoon, two sites Karative (80° 13′ 26.30″ N: 79° 47′ 42.22″ E) and Southwest of Seguwantive (80° 2′ 50.54″ N: 79° 48′ 48.99″ E) were selected based on the species abundance (Fig. 1) and easy accessibility for day and night data collection. Necessary approvals for the study were obtained from relevant authorities. Sampling was conducted from February to June of 2023 to study vegetative traits in non destructive manner.

The vegetation in the study area is often found on sandy and mudflats of the lagoon area that are periodically inundated with seawater. Vegetation in Seguwantive is dominated by *Suaeda maritima*, and *Salicornia brachiata* and the site selected in Karative is mainly dominated by *S. brachiata*, *Tecticornia indica* (*Halosacia indica*) and *Suaeda vermiculata* were also present. Based on our observation for several years, both the study sites are predominated with tentatively identified *S. brachiata* during the period of January to September. A minimal required amount of plant materials was collected from undisturbed sites of the areas following the standard guidelines.

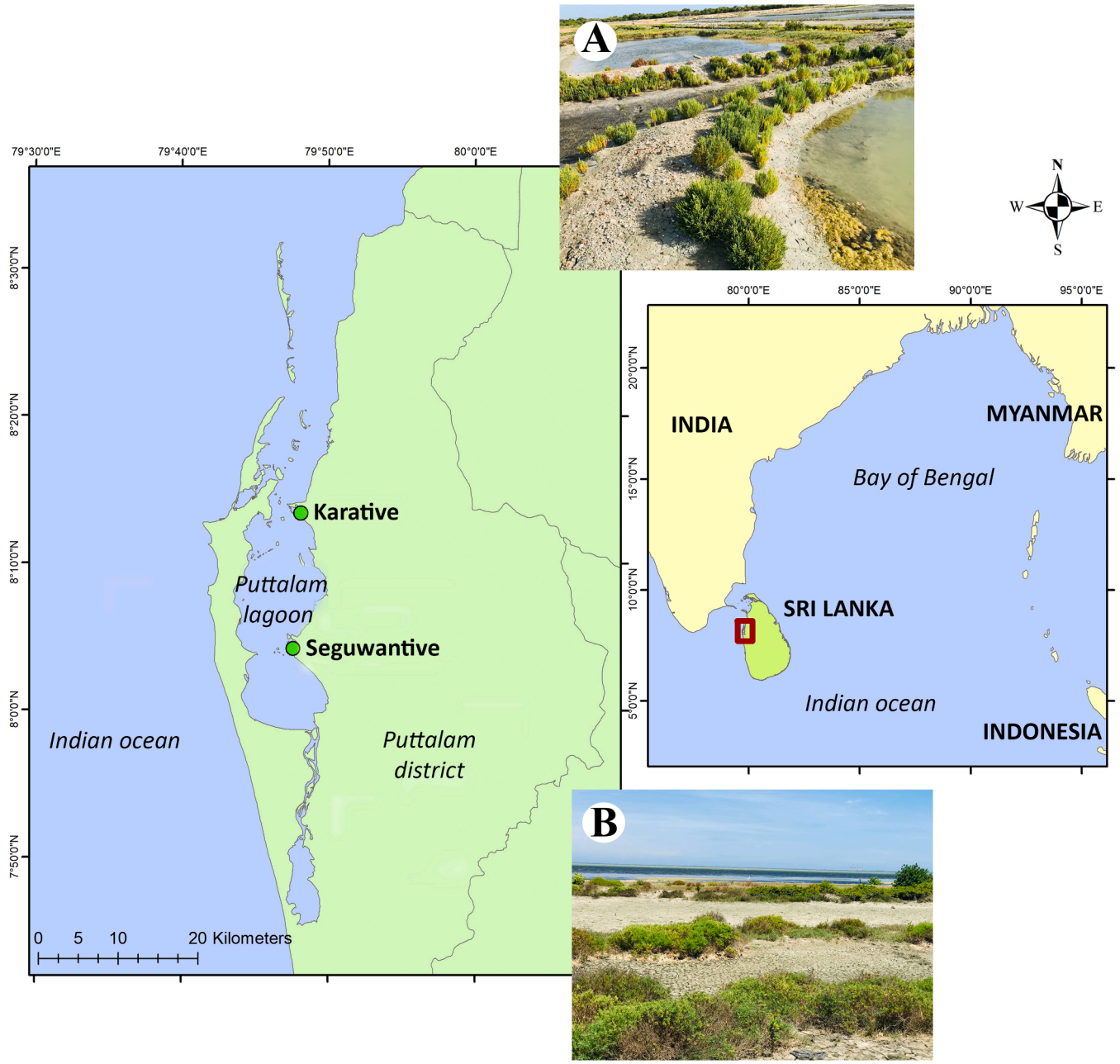

Fig. 1. Map of sampling sites in its natural habitats at Puttalam District in Sri Lanka. A: Karative B: Seguwantive.

Samples were collected excluding any damaged or apparently abnormal plants, for morphological, and anatomical characterization, and for DNA barcode-based species identification. Fresh samples collected for molecular studies were washed thoroughly, air dried for one day and stored at −80 °C.

2.2. Morphological characterization

The general morphology of vegetative parts (i.e. plant height, root length and canopy width.), and reproductive organs (i.e. fresh flowers/inflorescence, pollen grains, fruits, and seeds) of tentatively identified *S. brachiata* plants were studied in detail. Measurements were taken from randomly selected fresh plant materials (n = 20) as previously described (Kadereit et al., 2007; Ball, 1964; Davy et al., 2001; Shepherd et al., 2005; Chatrenoor and Akhani, 2021; Hayder et al., 2022). Qualitative/quantitative traits such as the angle of branching, height, and canopy diameter of the specimens were also recorded in the field. Herbarium specimens were prepared from samples showing typical traits and deposited in the National Herbarium, Peradeniya (PDA), Sri Lanka as well as in the herbarium of the Department of Plant and Molecular Biology, University of Kelaniya, and accession numbers were obtained.

2.3. Reproductive biology

2.3.1. Phenology and pollination biology

Plant samples at the reproductive stages were collected to observe apical and basal flowers during the peak flowering period, from August through September of 2023. Floral morphological traits were studied in the field as well as in the laboratory for several days around the clock. Periodic observation of marked flowers to determine the timing duration and variations in developmental stages, the classical method used in phenological studies in the field, was not feasible in *Salicornia* due to the presence of minute florets that are tightly embedded into cavities of the

main axis (Fernandez-Illescas et al., 2011) and therefore, microscopic dissection method was adopted as described below. Flowers with exerted and un-dehisced anthers were marked and observations were taken in hourly intervals at the field and in the laboratory for stigmatic receptivity and once in every two hours for pollen viability. Observations from unopened flowers were also obtained along with opened flowers at hourly intervals. Marked florets were dissected under a dissecting microscope in order to determine the position and functionality of stamens and stigma, their morphology and sex expression. General floral morphology and the changes in the morphological characters of flowers such as the orientation of the entire flower (erect versus pendent), dimensions of floral organs, relative position, shape, and color of tepals, timing and duration of stigmatic receptivity, anther dehiscence, changes in stamens, wilting and abscission of floral organs and floral visitors (if any) were recorded.

2.3.2. Pollen/ovule (P/O) ratio

The number of pollen grains of ten anthers in 10 flowers from randomly selected plants was determined as follows. A mature anther was crushed in 10 µL 0.5 % safranin dye on a cavity slide and pollen grains in the mixture were counted under the light microscope. As only one anther is in a flower, no further calculations were required. The number of ovules per flower was determined by dissecting ovaries on a glass slide and observing under a dissecting microscope.

Using the above-collected data, pollen/ovule ratios were calculated, and putative breeding systems were estimated by comparing the P/O ratios with the values given by Cruden (1977). As per Cruden (1977), the values of P/O ratio of 4.7 ± 0.7 indicates cleistogamy, 27.7 ± 3.1 indicates obligate autogamy, 168.5 ± 22.1 indicates facultative autogamy, 796.6 ± 87.7 indicates facultative xenogamy, and 5859.2 ± 936.5 indicates xenogamy.

2.3.3. Pollen dispersal mechanism

Microscope slides coated with Vaseline or double side self-adhesive transparent acrylic tapes attached to pieces of wooden holders were placed facing the wind direction at the level of canopy at different distances (1 m, 2 m, 3 m) in the vicinity of *Salicornia* plants in the field from 3.00 a.m. (before the beginning of anthesis) to 6.00 p.m. (Goodwillie, 1999). Five Vaseline-coated microscopic slides per each transect were placed about five-to ten-meter away from each other. Slides were observed under the light microscope for any attached pollen grains to identify the possibility of anemophily.

2.3.4. Pollen viability and germinability

The timing of the onset of the staminate phase, its duration, and pollen viability of un-opened and opened central and lateral flowers were assessed throughout the day at two-hour time intervals for several days in the field as well as in the laboratory. For laboratory observations, plants were uprooted with soil with intact root systems and brought to the laboratory so that plants would stay live for several days. Central and lateral flowers from apical and basal regions of the cladode from suitable plants were separately studied in two-hour time intervals for several days. This allowed us to observe more than 50 flowers of each type from each region from more than 40 plants. The number of viable pollen grains was assessed using 1 % acetocarmine stain. Pollen grains that took up the stain into deep red color were considered fertile, whilst those which remained colorless or pale, and usually mis-shapen or shrunken, were regarded as being sterile as described by Mosquera et al. (2021).

For the pollen germinability test, pollen grains were randomly selected from fully exerted anthers before dehiscence. Pollen germination studies were conducted using four media with different concentrations in triplicates: i) sucrose solutions having a concentration gradient (0 %, 5 %, 10 %, 20 %, and 25 %), ii) sucrose solutions mixed with 0.01 M H_3BO_3 and 0.03 M $Ca(NO)_2$ (Dafni, 1992), iii) Brewbaker medium with the same sucrose concentration gradient (Brewbaker and Kwack, 1963) and, iv) NaCl solution with a concentration gradient (0 M, 0.001 M, 0.002 M, 0.003 M, 0.004 M, 0.005 M). Pollen grains were collected immediately after anther dehiscence and mixed with 10 µL of each of the above media in cavity glass slides. The slides were kept in a moist chamber of closed Petri dishes with damp filter paper at room temperature (28 ± 2 °C) for 24 hr. The pollen germination percentage was calculated using the following formula as given by Mosquera et al. (2021) at 2-hour intervals for 24 hrs.,

$$\text{Pollen viability\%} = \frac{\text{number of viable pollen grains}}{\text{total number of pollen grains on slide}} X100$$

where the rotal number of pollen grains = viable pollen grains + non-viable grains.

2.3.5. Stigmatic receptivity

As indicated previously, marked flowers with exerted and un-dehisced anthers were taken in hourly intervals for stigmatic receptivity. The stigma of each flower (lateral and central flowers from apical and basal regions of each cladode) was carefully excised, and receptivity was initially determined by immersing stigmas in a 3 % hydrogen peroxide (H_2O_2) drop and checking for bubble formation (Dafni, 1992). The intensity of bubble formation is proportional to the peroxidase enzyme activity of the stigma, which is an indirect indicator of stigmatic receptivity (Galen and Plowright, 1987). Stigmatic receptivity was studied for more than 100 flowers from each flower type (lateral and central flowers from both apical and basal regions) for several days.

2.4. SEM analysis

Scanning electron microscopy (SEM) was performed for pollen grains, seeds, and epidermis at the SEM facility of the Geology Department, University of Peradeniya (ZEISS-EVO LS15, Germany). Pollen grains obtained from mature anthers of newly collected samples were first acetolysed following standard procedures. Measurements were taken according to Dehghani et al. (2021) and the pollen morphology was described. Measurements of pore diameter and chord distance were taken using five SEM images.

Seeds were dehydrated in aqueous ethyl alcohol solutions of increasing concentration (from 50 % to 100 %), then in alcohol-acetone (1:1) solutions and pure acetone prior to the SEM analysis. Perianth was not chemically dehydrated. The epidermis of a vegetative internode was manually separated, cleaned and dehydrated as described above. Specimens were mounted on the stub using carbon tapes. The samples were sputtered with gold particles inside the ion sputter (SC7620 Mini Sputter Coater, UK) before being subjected to SEM.

2.5. Statistical analysis

Descriptive statistics were used to summarize the phenotypic data. Significant differences ($p<0.05$) in key phenotypic traits of central and lateral flowers, stomata and seeds was determined using Student's t-test or Mann-Whitney test (when assumptions are violated) implemented in Minitab 17. Assumptions of normality and equal variance were tested before the analysis.

2.6. Molecular analysis

2.6.1. Genomic DNA extraction and barcoding

DNA extraction from the young stems/branches (100 mg) of *Salicornia* samples was carried out using the DNeasy Plant Pro Kit (QIAGEN) following the manufacturer's instructions with minor changes. Briefly spin column was heated for 2 min at 60 °C before collecting DNA into collection tubes.

DNA barcoding was carried out targeting the core barcode consisting of one plastid coding region (matK) from the chloroplast genome (cpDNA), both internal transcribed spacer (ITS) and external transcribed

spacer (ETS) regions. Selected genes/regions were subjected to Polymerase chain reaction (PCR) (the Mastercycler® nexus PCR cycler, Germany) using universal/genus-specific primers, matK (Zeinalabedini et al., 2021), ITS (White et al., 1990) and ETS (Kadereit et al., 2007) (Table 1). For all markers, 25 μL reactions were prepared containing 1x Colorless GoTaq® Flexi Buffer, 1.7 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM forward and reverse primers, 1 U of GoTaq® DNA polymerase (Promega Inc., Madison, WI, USA), and 2 μL of template DNA. For matK, and ETS primers, 50 μg/mL of bovine serum–albumin (BSA) and DMSO (4 %) were added. The PCR reaction was carried out as initial denaturation for 5 min at 94 ℃ followed by 35 cycles of denaturation for 40 sec at 94 ℃, annealing at 42 ℃ or 52 ℃ for ETS and matK respectively for 30 sec, 1 min extension at 72 ℃ and final extension of 10 min at 72 ℃. Amplified PCR products were subjected to Sanger bidirectional sequencing at Genentech, Sri Lanka, with the same primers employed in the PCR reactions.

2.6.2. Sequence analysis

Bidirectional sequences were edited using BioEdit (Hall, 1999) and MEGA XI (Tamura et al., 2021). The edited sequences were subjected to The Basic Local Alignment Search Tool (BLAST) NCBI, GenBank for homology searches. The sequences were deposited in NCBI GenBank and accession numbers were obtained. Further. already published ITS, and ETS sequences were obtained from previously published records as indicated in Kadereit et al. (2007) and Piirainen et al. (2017) (Supplementary Table 1). Along with the individual barcode marker dataset, a concatenated dataset was prepared by combining the ETS and ITS sequences using MEGA XI.

The evolutionary history was inferred by using the maximum likelihood method implemented in MEGA XI. Multiple sequence alignments were generated with MUSCLE (Edgar, 2004), implemented in MEGA XI. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances.

The model that best fits the data was found with the "best DNA models" tool implemented in MEGA XI under the Bayesian information criterion (BIC) criterion. Tamura-3 model was selected for the ITS tree and Kimura-2 (Kimura, 1980) was selected for the ETS and concatenated trees and selected the topology with superior log likelihood value. The reliability of phylogenetic trees was assessed with the bootstrap support of 1000. All positions containing gaps and missing data were eliminated (complete deletion option). The out-group was selected according to Kadereit et al. (2007).

3. Results

3.1. Morphological characterization

The primary task of this study was to describe every possible morphological trait of tentatively identified *S. brachiata* since there is a severe dearth of taxonomic information in the current literature including the Flora of Ceylon (Boulos, 1995). The plant is an annual succulent, halophytic herb/subshrub. In general, seed germination starts with the northeast monsoon from December to February in the study site and other salt marsh vegetation of the country. Plants reach their maximum vegetative growth by the end of May. The glabrous stems are erect up to 12–31 cm (avg. 20.08 ± 4.72 cm) high, and woody at the base. They are typically much branched or have only primary branches or sometimes no branching at all depending on the environmental conditions. It was observed that when the environment is challenging and harsh, branching is less and the plant reach to the reproductive stage even without branching at all. The canopy diameter varied between 3 cm to 24 cm (avg. 10.73 ± 5.78 cm). The average length of the short, cylindrical internodes at the apical, middle, and basal regions were 0.73 ± 0.13 cm, 1.04 ± 0.20 cm, and 1.30 ± 0.20 cm respectively. The branches were straight or curved upwards, in opposite pairs with a decussate pattern. The uppermost primary branches make an acute angle with the main stem. In general, all these traits varied considerably depending on the sampling site, soil salinity, frequency of inundation etc. Highly reduced foliage was opposite, decussate, connate, forming minor collar-like scales at the upper part of each node, while the scarious margins, at the top and the fleshy lower parts, are fused and elongated around the stem and in agreement with Boulos (1995). The root system was superficial, often penetrating about 7.02 ± 1.76 cm into the sediment. In smaller individuals, the main root axis produces a few branches, while in larger plants, it develops several highly branched, woody roots.

During the reproductive stage, which lasts from June to August, primary, secondary, and even tertiary branches, with two branches at a node, were observed. Generally, the basal primary branches of a plant at the flowering stage was as long as the main stem. The young plants were dark green and by the end of the life cycle, both sterile segments and cladodes became yellowish green to yellow and turned into bright orange and lastly dark brown. Seed set begins by the end of August and can be collected until October or November. General morphology of the plant and typical morphological changes along different stages of the growth cycle are shown in Fig. 2. Vouchered specimens are available at the National Herbarium, Peradeniya, Sri Lanka for future reference under the Tag R2/RJ1_A.

The stomata type was anomocytic (Fig. 3A); the subsidiary cells were similar in size, shape, and arrangement to the epidermal cells (Keshavarzi and Zare, 2006) and the guard cells are usually accompanied by 2–4 subsidiary cells. The guard cells were located at about the same level as the epidermal and subsidiary cells (prominent stomata) (Fig. 3B). Stomata were abundantly scattered throughout the epidermis. In the vegetative internodes, they were arranged in their long axis at a right angle to the shoot axis. In the flowers, they were radially arranged.

The stomatal index of apical regions (14.08 ± 1.88 %) of a vegetative internode was higher than that of the basal region (12.35 ± 1.91 %) ($p=0.032$). The length and width of stomata (including guard cells) were 34.50 ± 3.31 μm and 23.12 ± 3.46 μm respectively. No difference was observed between stomata in the vegetative and fertile segments/cladodes.

3.2. Phenology

Terminal cladodes of *S. brachiata* were spicate with narrowly conical in shape (Fig. 4A). The length of terminal cladodes ranged from 1.10 to 8.70 cm long with an average of 3.86 ± 2.23 cm, and it makes an acute angle with the stem. On average, each branch had 11.45 ± 4.71 fertile

Table 1
Primers used in the study, sequence, annealing temperature, addition of BSA, and the reference.

Primer ID	Sequence	Annealing temp.	BSA (50 ug/mL) and/or 4 % DMSO	References
matK_For	TGTAGCACAGGAAAGTCGAAGT	52 ℃	BSA and DMSO	Zeinalabedini et al. (2021)
matK_Rev	CGATCTATTCATTCAATATTTC			
ETS_P708_F	CTCTAACTGATTTAATGAGCCATTCGCA	42 ℃	BSA and DMSO	Kadereit et al. (2007)
ETS_P707_R	GTCCCTATTGTGTAGATTTCAT			
ITS4	TCCTCCGCTTATTGATATGC	56 ℃	No	White et al. (1990)
ITS1	TCCGTAGGTGAACCTGCGG			

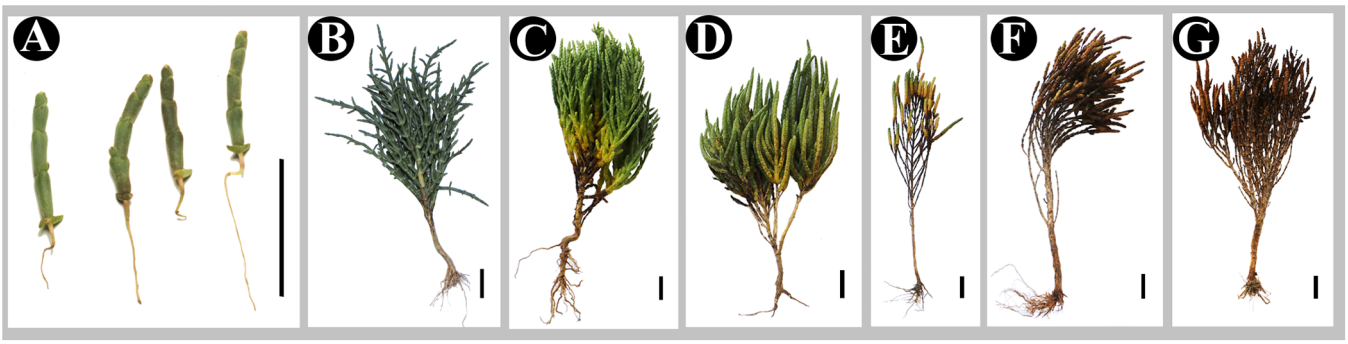

Fig. 2. *Salicornia brachiata* plants at different developmental stages. (A, B) Vegetative stage, with primary branch; (C) Early reproductive stage; (D, E) Reproductive stage; (F,G) Late seed shedding stage. Scale bars: (A-G) = 2.5 cm.

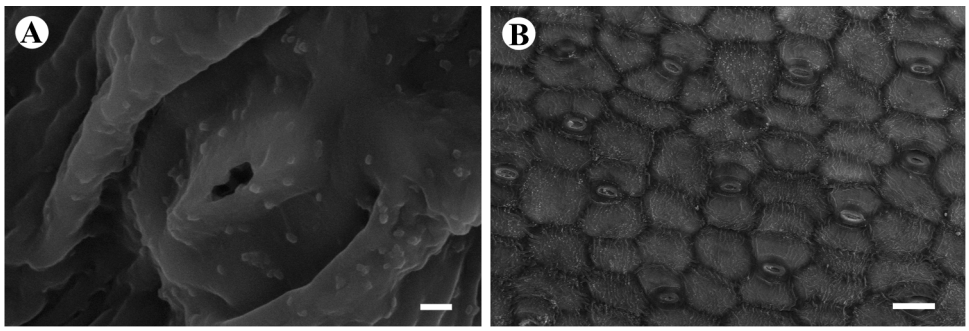

Fig. 3. (A) SEM image of epidermis with stomata; (B) SEM image of a stomata on a vegetative segment. Scale bars: (A) = 40 μm (B) = 4 μm.

segments and 1–10 sterile segments. The average height and width of fertile segments were 2.99 ± 1.07 mm and 3.18 ± 1.27 mm. The height and width (at the center) of the basal vegetative segment of the cladode were 4.92 ± 1.62 mm and 2.72 ± 0.34 mm, respectively.

Each fertile segment had two convex triangular three-flowered cymes in the cavities of the stems on opposite sides and alternating with cymes on the adjacent segments in a cladode (Fig. 4A,C). On some rare occasions, abnormal fertile segments bearing a single flower, instead of three flowers were also observed. The florets were sessile in the axils of bracts and were subtended by bracts. The free part of the bract was 0.73 ± 0.1 mm tall. The inconspicuous scarious border was 0.24 ± 0.06 mm wide on the sides.

The height and width of the central flower, were bigger (2.14 ± 0.38 mm×1.98 ± 0.25 mm) than the lateral flowers (1.27 ± 0.37 mm × 1.40 ± 0.16 mm) and the difference is significant (p = 0.00). The upper edge of the central flower was round with a dome shape and about 0.15 ± 0.20 mm away from the top of the segment and this distance gradually reduced at the apical flowers. The fleshy, cup shaped perianth of *Salicornia* species consisted of one whorl of united sepal-like tepals similar to those described in Beer et al. (2010). The perianth lobes were green, similar to the fleshy shoot. The overlapping, small free part of the tepals, which are transparent and colorless, covered the narrow opening of the floral tube enabling the excretion of anthers, pollen, and stigma during reproductively active stages. The central flower harbored four tepals and lateral flowers consisted of three tepals. However, the number of tepals often varies. Central flowers with three and two tepals (often at the apical region of the terminal cladode) and lateral flowers with two tepals were often observed. The basal flowers develop before apical flowers and hence, the flower opening pattern is acropetal.

Florets were mostly bisexual, usually consisting of a single stamen (Fig. 4C,D), always inserted in an adaxial position. However, in some rare cases, two anthers were observed on a few flowers (both central and lateral flowers) at the apical region. In such cases, the abaxial anther was not well developed and the pollens were sterile. The average length and width of anthers were 0.82–0.60 (avg. 0.70 ± 0.03) mm and 00.4–0.21 (avg. 0.29 ± 0.04) mm respectively. There was no statistically significant difference between the anther length (p = 0.229), anther width (p = 0.126) or filament length (p=0.212) of central and lateral flowers. Filaments could be straight or curved and the length varied from 1.07 to 0.06 mm (avg. 0.45 ± 0.34 mm). Filaments were short in cleistogamous and unopened flowers (0.12 ± 0.03 mm). The average length of the style was 0.24 ± 0.08 mm (no significant difference was detected between the style lengths of central and lateral flowers (p = 0.097). The stigma consisted of plumose stigmatic lobes which were about 0.11 ±0.03 mm long (Fig. 4-F). The average length of a stigma in both central and lateral flowers was 0.39 ± 0.11 long. Though there was no significant difference in the length of stigma in central and lateral flowers (p=0.507) mostly trifid, and sometimes bifid stigma were observed in central flowers, while in lateral flowers they were mostly bifid, sometimes trifid (Fig. 4-B). However, in rare cases, stigmas with four lobes were also observed in both types of flowers. The deeply embedded ovary was ovoid and unilocular with a solitary basal ovule with an average length of 0.22 ± 0.03 mm (Fig. 4E).

3.3. Anther dehiscence, stigmatic receptivity, and inferences about pollination

3.3.1. Anther dehiscence

Anther exertion started around 4.00 a.m., and anther dehiscence started around 6.00 a.m. and continued until around 8.00 a.m. (Fig. 5). Generally, anthers were entirely exerted before dehiscence. However, in some instances, anther dehiscence occurred before exertion displaying cleistogamy. Cladodes consisted of 0–2 cleistogamous flowers mostly at the apical segments and sometimes among basal flowers. In general, 2–3 % cleiostogamous flowers per cladode was observed. Anthers dehisced longitudinally, dispersing yellow-colored pollen grains. The mature anthers were whitish-yellow, and the color became slightly orangish-light brown with time. The pollen sacs were empty by 12.00 p.m., and the

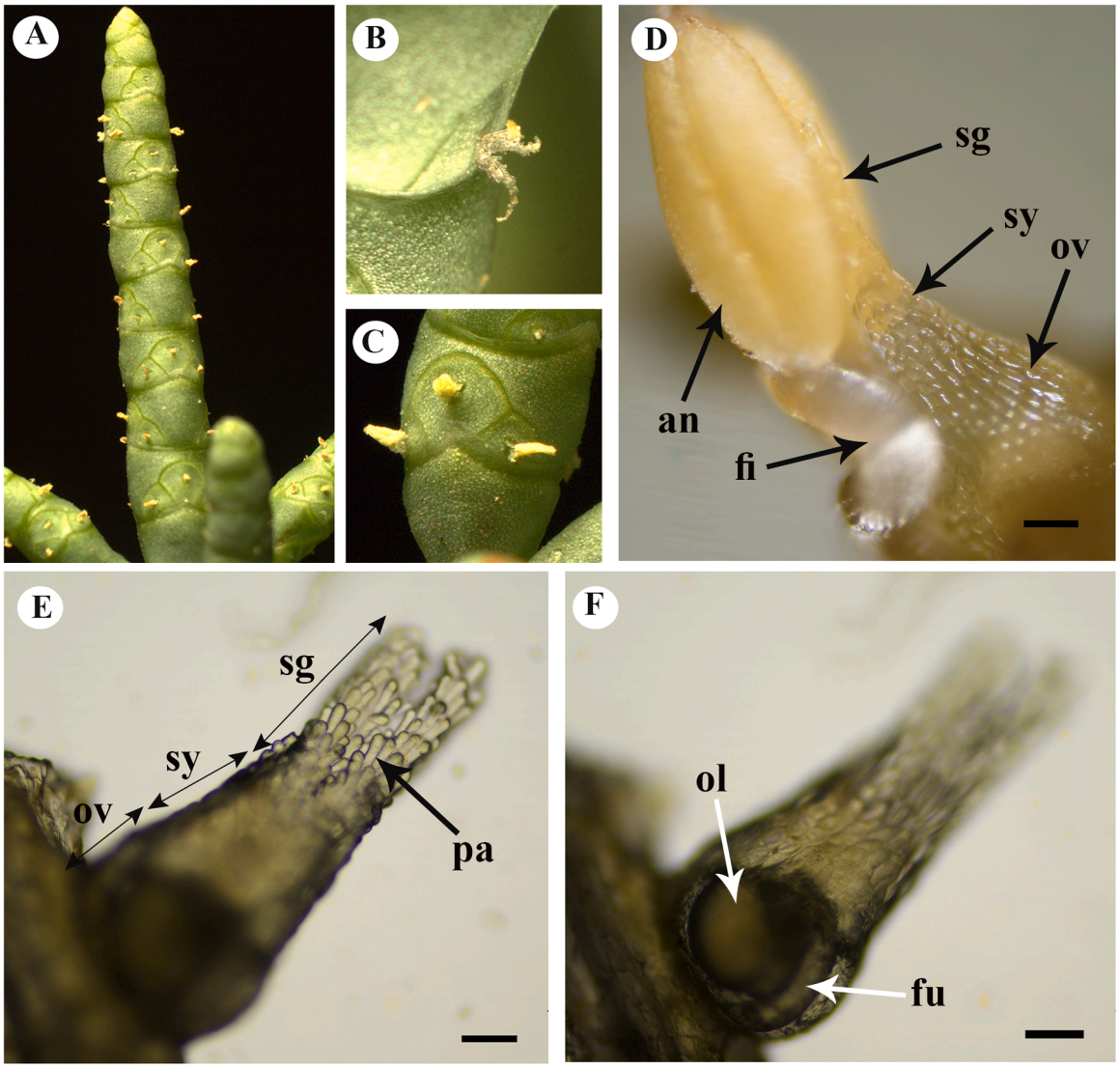

Fig. 4. Morphological anatomical features of *S. brachiata* flower. (A) The terminal cladode at the flowering stage; (B) Lateral flower with three stigmatic lobes; (C) A cyme with bisexual flowers (after anther dehiscence); (D) closely attached Stamen and pistil; (E, F) Morphology of pistil an = anther; fi = filament; fu = funicle; ol = ovule; ov = ovum; pa = papillae; sg = stigma; sy = style. Scale bars: (D, E, F) = 100 μm.

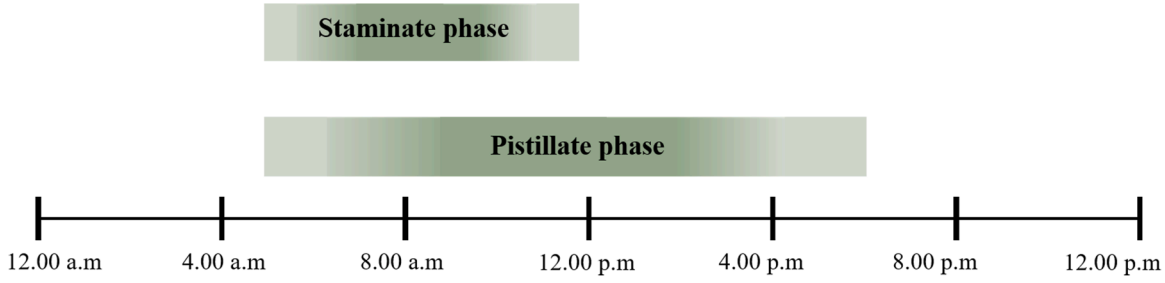

Fig. 5. Duration of staminate and pistillate phases of *Salicornia brachiata*.

anther was easily detached from the filament after that. No noticeable difference was observed in the timing and duration of anther dehiscence of central and lateral flowers at apical and basal segments.

3.3.2. *Stigmatic receptivity*

The pistillate phase started simultaneously with the staminate phase around 4:00 a.m. and lasted until around 6:00 p.m. (Fig. 5). The intensity of the stigmatic receptivity was higher in the afternoon than in the morning hours as indicated by the amount of bubble formation in the presence of H_2O_2 and in the morning hours before the anther exertion, no stigmatic receptivity was observed in most of the flowers. Stigmatic receptivity began simultaneously with the anther dehiscence. The pistillate phase remained longer than the staminate phase. The stigma receptivity of open central flowers was slightly stronger than opened lateral flowers based on the amount of bubble formation observed. No difference was observed in pistillate and staminate phases between basal

and apical flowers of a cladode and also no difference was observed in timing and duration of stigma receptivity of central and lateral flowers.

3.4. Pollen morphology, viability and germinability

There was no major noticeable difference between the mature pollen morphology of central and lateral flowers. Yellow color pollens (non-acetolysed) were globular in shape with a diameter ranging from 36 to 22 (avg. 26.79 ± 3.66) μm (Fig. 6C). Exine was 1.53 ± 0.18 μm thick. The average pore diameter and chord distance (the distance between two pores) were 1.59 ± 0.2 μm, and 2.18 ± 0.2 μm respectively in acetolysed pollen grains (Fig. 6A,B).

The tectum and operculum were covered with minute spinules (Fig. 6B). Average pollen counts and the pollen: ovule ratios in central flowers was 1470 ± 86.61 whereas in the lateral flowers it was 1374 ± 72.06 (since the flower has only one ovule) indicating facultative xenogamous breeding system according to Cruden (1977). There was no significant difference in the pollen count and pollen:ovule ratios (p=0.667) between central and lateral flowers.

Pollen grains did not germinate in any of the four media used and pollen germination rate was 0 % in all media. However, in Acetocarmine staining assay, the highest percentage of viable pollens (from exerted and un-dehisced anthers) were recorded as 90 % in the flowers collected around 8.00 a.m. to 8.30 a.m. indicating the presence of viable pollen grains and pollen viability remained for 3–4 hours after anthesis. The glass slides with sticky tapes captured pollen clumps of *Salicornia* indicating dispersal of pollen by wind.

3.5. Fruit and seed micromorphology

After fertilization, the papillae in the stigma become dry, and the withered stigma curls up towards the flower opening. Later, the dried tepals seal the opening of the floral tube. Enlargement of the ovule, reduction of the transparency, and the formation of a small projection indicative of a successful fertilization and the development of seed (Fig. 7A). Fruit development in flowers was observed three to four days after anther dehiscence in including cleistogamous flowers. *Salicornia brachiata* fruit consisted of one seed. The young embryo is green in color and turned golden brown in mature dried seeds (Fig. 7F,G).

Horseshoe-shaped, embryo of *S. brachiata* was relatively large occupying almost the entire seed, and lacked a perisperm. The fleshy perianth became spongy at maturity and it does not fully enclose the pericarp and the seed (Fig. 7B,C). The pericarp also does not completely enclose the seed (Fig. 7D). The central perianth was larger than the lateral ones. Mature dry perianth does not split centrally and falls releasing the seed. The membranous pericarp fussed with the perianth and loosely attached to the seed hairs (Fig. 7G). The pericarp was easily detached from the seed coat. In cross sections, air cavities (about 66.27 ± 24.94 μm in length) were observed between the inner layer of the pericarp and the outer layer of the seed testa. In the same cyme, the seeds of the central flowers were larger than the seeds in lateral flowers with an average length x width of 1.34 ± 0.17 mm × 0.66 ± 0.10 mm for central and 1.2 ± 0.19 mm × 0.67 ± 0.11 mm lateral flowers respectively. However, the difference was not statistically significant (n=20, p = 0.129, and for width p = 0.89). Seeds were horizontal in orientation relative to the inflorescence axis, and vertical to the axis of the ovary. The seeds had coriaceous and transparent seed coats. The mature seeds were light brown/golden brown in color. It appeared that the seed color was determined by the embryo as described in Shepherd et al. (2005). The outer exotesta epidermal cells exhibited uneven elongation to form short, hooked hairs (Fig. 7E,H).

3.6. DNA barcoding

3.6.1. PCR amplification and sequence analysis

Both samples collected from Karative and Seguwantive populations successfully amplified all three barcode regions, ETS, ITS and matK. Almost all markers showed successful PCR amplification and produced good sequencing results. In some instances, a supplement of 50 μg/mL of bovine serum–albumin BSA) and/or DMSO (4 %) enhanced PCR amplification. ITS sequences of both samples were identical to each other, and BLAST results showed no single match to vouchered ITS sequences of *S. brachiata* in GenBank. When 679 nucleotides of the ITS region was used, 98.8 % sequence similarity was found with *Salicornia ramosissima* (ON685420 -unpublished and OX596238 deposited under the BioProject: PRJEB62065, from Darwin Tree of Life) and with *Salicornia europaea* (MT923358 - unpublished) with 100 % query coverage.

Similarly, both ETS sequences obtained from the samples of Karative and Seguwantive were identical. BLAST searches showed multi-species assignment (with identity values ranging from 97 % to 100 %) and there were no published/vouchered *S. brachiata* sequences in the GenBank. The highest similarity of 97.7 % was found for *Salicornia ramosissima* (BioProject: PRJEB62065, Darwin Tree of Life Project) with 99 % query coverage. The next best match showed 97.5 % similarity to several sequences of *S. persica* (Jamdade et al., 2022) with 97 % query coverage.

Most importantly, 881 nucleotide fragment of matK gene with 100 % query coverage showed 100 % similarity to *S. brachiata* chloroplast sequence deposited under chloroplast genome project PRJNA927338. The next best match showed 99.89 % similarity to *S. ramosissima* (OX596242, NC066032), and *S. europaea* (NC027225). Since the lack of barcode sequences of *S. brachiata* in the GenBank severely affected accurate species identification, molecular phylogenetic analysis was conducted.

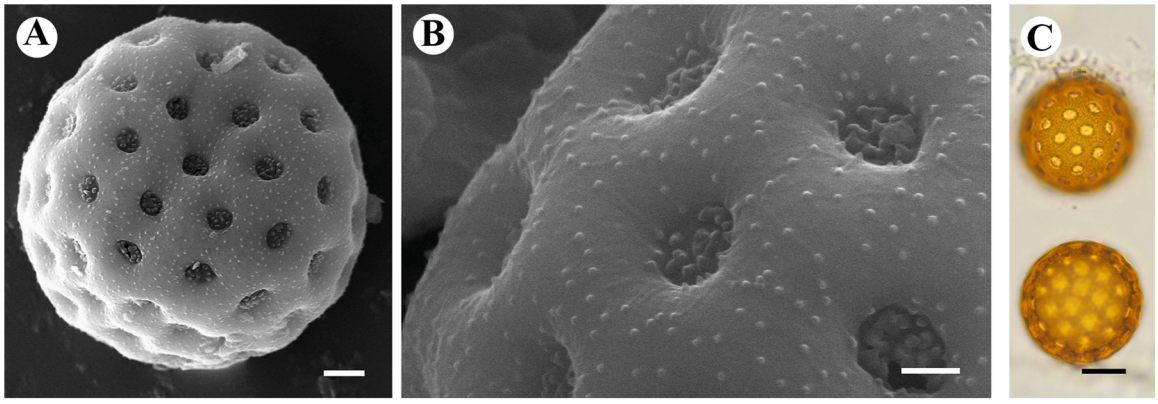

Fig. 6. *Salicornia brachiata* pollen morphology. (A), SEM image of pollen surface morphology; (B), SEM image of acetolysed pollen; (C) Acetolysed pollens under light microscope. Scale bars: (A) = 1 μm; (B) = 2 μm; (C) = 10 μm;.

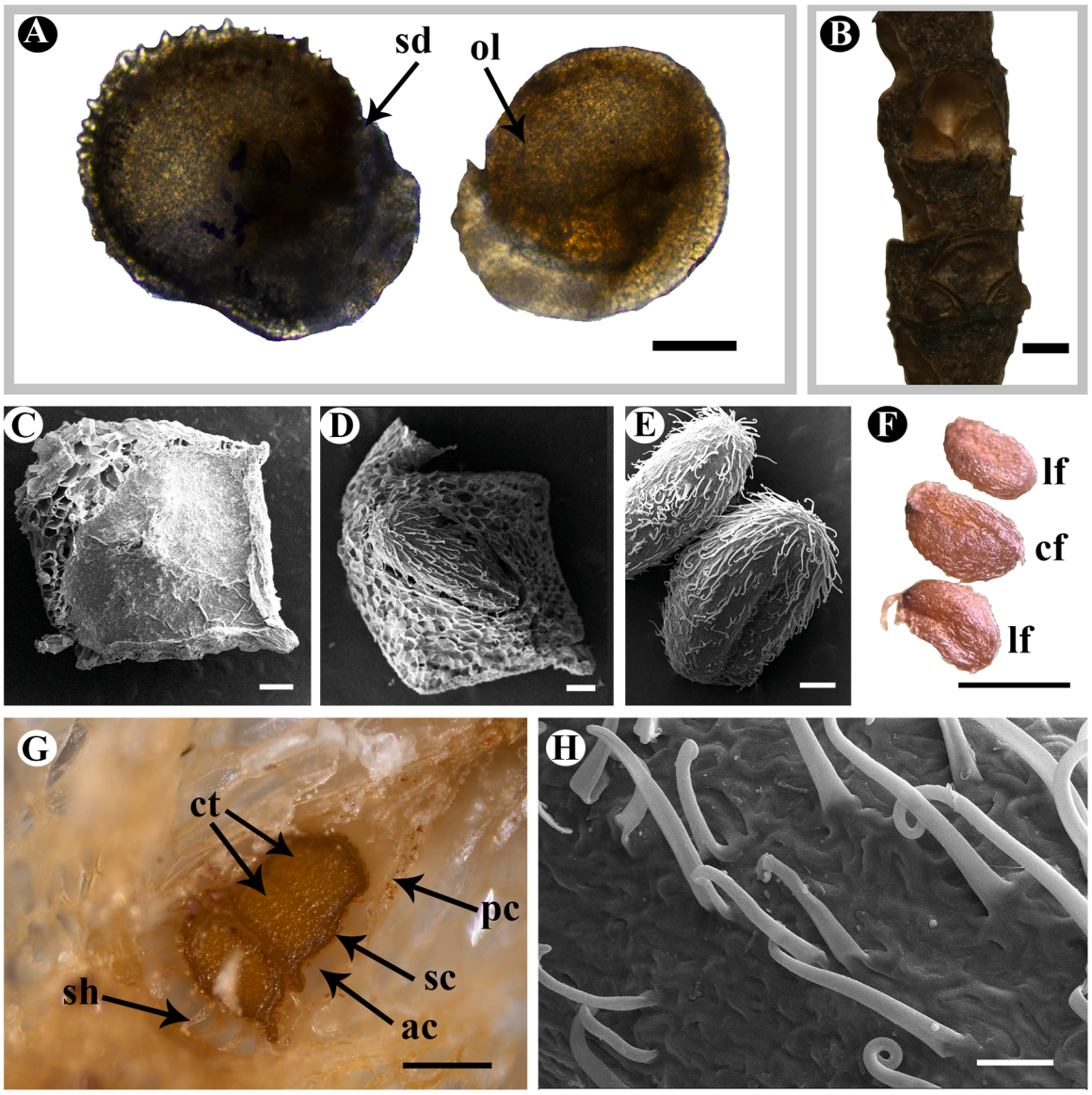

Fig. 7. Morphological and anatomical features of *S. brachiata* seeds. (A) ovule (ol) (in an un-opened flower) and an 3–4 days old seed (sd); (B) seed bearing dry cladode; (C, D) fruit bearing dry perianth; (E, F) seeds; lf=lateral flower, cf=central flower; (G) anatomical aspects of seeds; (H) Seed coat ornamentation and hooked hairs; ac = air cavities; ct =cotyledons; pc = pericarp, sc = seed coat, sh = seed hair. Scale bars: (A, G) 100 μm; (B, F) = 1 mm; (C,D, E) = 200 μm; (H) = 40 μm.

3.6.2. Phylogenetic analysis

Phylogenetic analysis was conducted for each gene region separately as well as for concatenated data set of ETS and ITS sequences. The phylogenetic tree constructed using ITS sequences was shown in Fig. 8A. The analysis involved 14 published and vouched nucleotide sequences. There were a total of 507 positions in the final dataset and *S. brachiata* separated from the other species with 88 % bootstrap support as indicated in red color.

Phylogenetic tree obtained with ETS sequences was shown in Fig. 8B. This analysis also involved 15 nucleotide sequences obtained from published and vouched specimens. There were a total of 406 positions in the final dataset and *S. brachiata* separated from the other species with 97 % bootstrap support as indicated in red color.

In the case of ETS and ITS sequences and when concatenated, *S. brachiata* used in the present study clustered separately with strong bootstrap support (Fig. 8C). This analysis also involved 14 nucleotide sequences. After eliminating all positions containing gaps and missing data, 913 positions yielded in the final dataset. The matK phylogenetic analysis performed using few vouchered sequences suffered mostly from the lack of resolution in delineating *S. brachiata* (tree not shown). However, when ITS, ETS and concatenated sequences were used, it was clear that other species were well separated from *S. brachiata* samples used in the present study with 99 % bootstrap support (indicated in red color in Fig. 8C). In addition, neighbor-joining trees were also drawn for each case mentioned above and tree topologies were identical to the ML trees (data not shown). All the sequences obtained in this study were deposited in the GenBank under accession numbers OR838462, OR826158, OR838460, OR838463, OR826159, and OR838461.

4. Discussion

A true halophyte, *S. brachiata* is of interest in the present study because of its tolerance to salinity, and its recognition as a potential crop in saline agriculture since soil salinisation is an increasing problem across the globe (Jha et al., 2009; Alfheeaid et al., 2022). The first attempt in such an endeavour is to accurately identify the species.

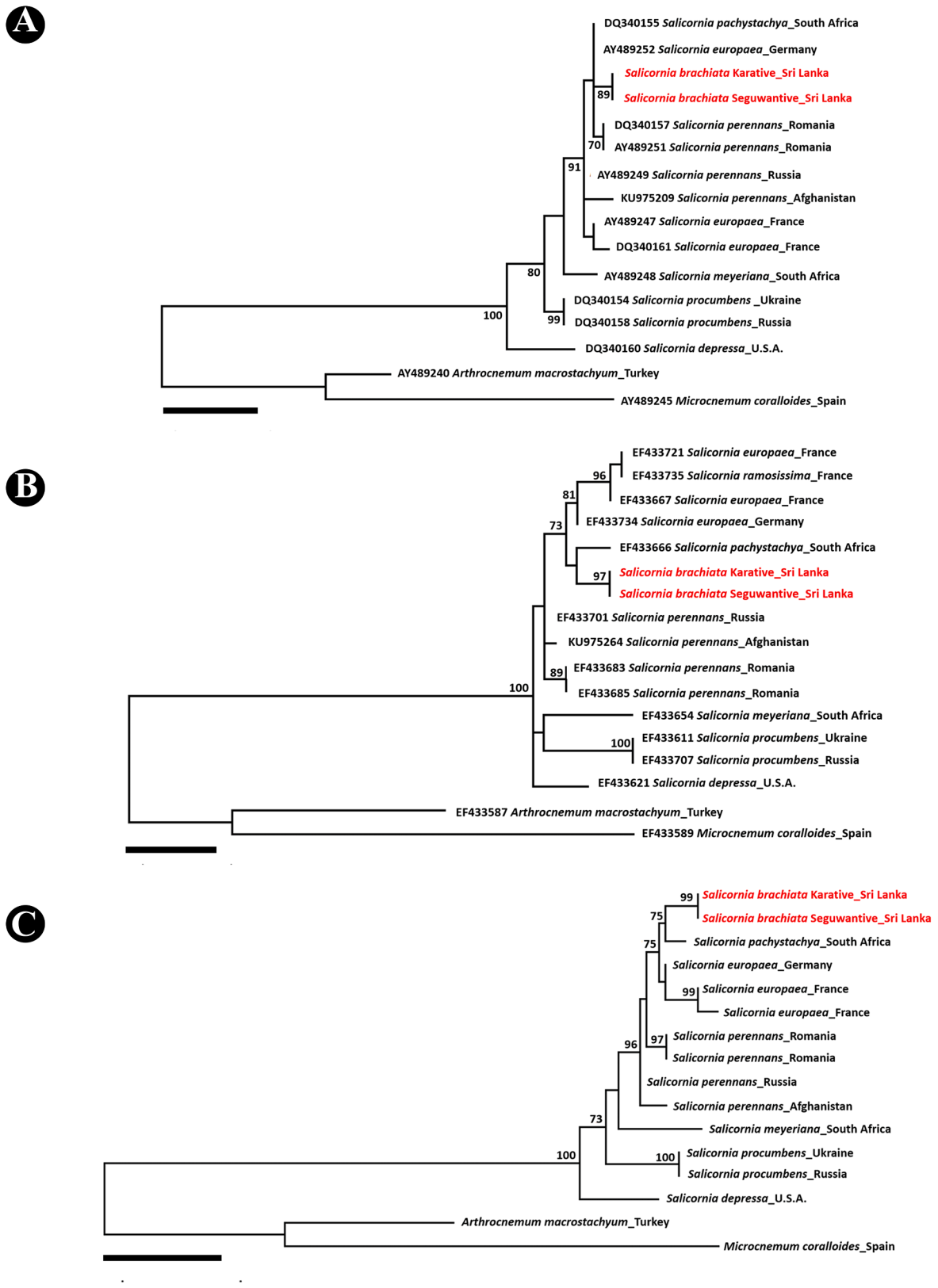

Fig. 8. Maximum Likelihood tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches (above 70 %). The trees were drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). (A) The highest log likelihood tree of −1181.96 for ITS sequences. Sri Lankan samples were separated with 88 % bootstrap support indicated in red color (B) The tree with the highest log likelihood value of −1206.66 for ETS sequences. Sri Lankan samples were separated with 97 % bootstrap support indicated in red color (C) The tree with the highest log likelihood value of −2687.96 for the concatenated data set of both ITS and ETS. Sri Lankan samples were separated with 99 % bootstrap support indicated in red color. Scale bar = 0.02.

However, most of the members of the genus *Salicornia* have been particularly known for their taxonomic dilemma for decades due to reduced plant structure with weak morphological differentiation, overlapping phenotypic traits or morphological parallelism, high phenotypic plasticity and lack of detailed and descriptive studies. This makes it challenging to identify these species even for botanists also. To our surprise, the GenBank database was also poor in authenticated records and most of the studies on morpho-molecular characterization have been conducted in Europe, the Mediterranean region, and Japan for other *Salicornia* species while no information is available for *S. brachiata,* the prominent species in Southeast Asia. Further, understanding the reproductive biology is the key in variety development and it is interesting to note that the reproductive biology of *Salicornia* sp. also has received little or no attention in the literature. To facilitate such attempts, it is crucial to have a deep understanding of biology, taxonomy and reproductive ecology of any plant species before introducing it as a crop.

Here we fill this information gap by providing a descriptive report on morphology, molecular characterization and reproductive biology revealing the cryptic reproductive behavior of *S. brachiata* for the first time. Firstly, this article elaborated on fine scale phenotypic traits of *S. brachiata*. Like any other halophyte, *S. brachiata* also shows drastic variations in vegetative traits depending on the habitat and the substrate composition. Mature plants, at reproductive stage, with heavy branching as well as no branching at all were observed in the field indicating the unreliability of exclusively morphology-based species identification (Rathore et al., 2019, 2022). Up until the present study, the colors of the fleshy segments were considered to be a useful field character in separating species in Salicorniadea family (Tölken, 1967). It has been accepted that *S. brachiata* produces no red coloration at any stage of development and used to differentiate it from other *Salicornia* species (Davy et al., 2001). However, during our field surveys, light pink or pinkish-orange coloration was observed in some cases in the field depending on the developmental stage of the plant and when the substrate quality was poor (soil analysis data not shown). Further, such coloration was most pronounced in the Karative site where the soil quality was poor (general observation and soil analysis was not shown). Much information on *Salicornia brachiata* seed anatomy is also reported in this study.

Secondly, the study elaborates on the reproductive traits, which are often used in species delineation in any angiosperm, yet poorly described in *S. brachiata* and in the genus in general. Reproductive traits such as color of the terminal of the cladode, number of flowers per cyme, number of sessile flowers per cyme and number of anthers and shape of central flower are often used in differentiating between species in the genus *Salicornia* with some reservations mainly because of phenotypic parallelism and overlapping features among closely related species (Shepherd et al., 2005; Beer, 2009; Beer et al., 2010; Hayder et al., 2022). For an instance, unique pentagonal central flower of *S. persica* could be used to separate it from *S. brachiata* and other *Salicornia* species (Akhani, 2003). Even though Tölken (1967) mentioned that there were no clear morphological characteristics that can separate *S. pachystachya* from *S. brachiata*, the number of anthers per flower is useful in separating them. While *S. pachystachya* has two fertile anthers per flower (Tölken, 1967), only one anther was reported in *S. brachiata* (present study and Boulos, 1995) and can be used in separating them. However, identifying these microscopic traits is not trivial even for a trained taxonomist. By comparing the morphological features of *S. brachiata* with those of other *Salicornia* species from published literature, we identified the dome shape of the upper edge of the central flower, frequent occurrence of single anther, and color changes of the plant at different growth stages as key distinguishing characteristics. A combination of seed characteristics such as transparent seed coat, golden-brown seed colour and presence of seed hair also could be used to separate *S. brachiata* from multiple *Salicornia* species (Shepherd et al., 2005).

Thirdly, the study focuses on multilocus DNA barcoding approach. Even though genomic data are freely available with sequencing becoming cheap in this omics era, to our surprise, DNA barcode data for *S. brachiata* was scarce or the available sequences were from apparently misidentified species. For instance, there were only three ITS sequences for *S. brachiata* in the GenBank (KJ784580.1, KF848296.1 and JQ341058.1), but those were neither published nor detailed morphological characterization was available. Interestingly BLAST searchers of these three sequences, KJ784580.1, KF848296.1 and JQ341058.1, did not match any *Salicornia* species even within the first 100 matches and instead resulted in *Arthrocaulon macrostachyum* (Moric.); synonym *Arthrocnemum macrostachyum*, indicating the importance of careful interpretation of data and the need for a descriptive study of *S. brachiata*. However, in other species of the genus *Salicornia*, ITS, ETS sequences and chloroplast genome have been often used in species delineation (Kadereit et al., 2006, 2007; Murakeözy et al., 2007; Jamdade et al., 2022). The ETS and ITS sequences of the members of the genus *Salicornia* published in GenBank are well-defined by previous studies (Piirainen et al., 2017). Even though morphometric data are often used to distinguish *Salicornia* species, high phenotypic plasticity makes it less desirable in phylogenetic analysis. Even though embryogenic data have been used in species identity, in the case of *Salicornia* it is not easy to detect. On the other hand, even though phylogenetic analysis based on multiple DNA barcoding regions have been effective in distinguishing species for some extent, morphologically identical accessions with different genotypes as well as morphologically variable accessions with identical genotypes have been reported (Kadereit et al., 2007, 2012). Therefore, along with the morphological description, molecular analysis is a must to properly define a *Salicornia* species.

Based on our analysis, the concatenated phylogenetic tree showed a clear separation of *S. brachiata* from other species. Even though matK sequences were not used for phylogenetic analysis due to a lack of sufficient accessions in the GenBank, it matched with the available single chloroplast genome of *S. brachiata* confirming the accurate species identity of the species used in present study.

Understanding the breeding systems of any crop plant is of great importance when it comes to variety development, maintenance of generations and production of seedlings and understanding population genetic structure. However, the breeding system has not been studied many *Salicornia* spp. including *S. brachiata*, hence fourthly, we presented novel information on the reproductive biology of the species. The uniqueness of this study is that different types of reproductive dynamics of flowers on the same stalk are presented for the first time in the genus *Salicornia*. As *S. brachiata* bear high economic significance mainly in food industry, reproductive biological information is of utmost impotence for its productivity enhancement via crop improvement strategies.

However, studying the minute reproductive organs of *Salicornia*, especially observing stigmatic receptivity was challenging. Damage to tissues and pollen grains on the stigma is known to induce a reaction with hydrogen peroxide, independent of the stigmatic receptivity (Dafni, 1992) and so does in the present study on *S. brachiata* as well. Potential damage which can occur during the separation of the delicate gynoecium from the flower might have affected the bubble formation. However, careful handling of the flowers/gynoceium, and continuous monitoring and studying of hundreds of lateral and central flowers throughout the day over several days, consistently showed the same pattern confirming that the observations are not merely due to the tissue damage. Stigmatic receptivity was considered positive if bubbles reached the tips of all two or three stigmas in the gynoecium. Samples were discarded if bubbles formed on only one stigma or on only one side of a stigma. Any stigmas that came into contact with pollen during gynoecium separation were also discarded. Peroxidase activity was assessed immediately after separating the gynoecium to prevent drying. Further, we determined peroxidase activity throughout reproductive active period and determined the stigmatic receptivity comparing intensity of bubble formation in whole stage and therefore, peroxidase

activity is unlikely to be an artifact.

According to previous studies, *Salicornia* sp. displays adaptations to both self-pollination as well as cross-pollination since dichogamy, cleistogamy, and homogamy have been reported in the genus (Ball and Tutin,1959; Tölken, 1967; Fernandez-Illescas et al., 2011; Ferguson, 1964; Jefferies and Gottlieb, 1982). Here we report key morphological adaptations that promote self-pollination as well as cross-pollination in *S. brachiata* in its natural environment. First, we highlight the following floral morphological characters of *S. brachiata* favor self-pollination; 1. The simultaneous occurrence of anther dehiscence and stigmatic receptivity of the same flower revealed homogamy in *S. brachiata*. 2. The presence of developing embryos and viable seeds in cleistogamous flowers provided evidence for self-compatibility. 3. The stigma and the anther were always in close contact with each other in minute florets of *S. brachiata*. 4. Style of the pistil of *S. brachiata* is always shorter than the stamen and therefore, the anthers always remain above the stigmas which promotes autogamy with downward movements of pollen grains with gravity. 5. In some flowers long filaments were curved to keep the anther inside the flower, which also helps autogamy. 6. Anthers of some flowers bent inwards the flower without exerting outside regardless of having a longer and straight filament (flexistyly). 7. The anthers in *S. brachiata* emerge slightly earlier than the stigma, which also ensures selfing when the stigma is receptive. 8. Anthers do not exert fully before the dehiscence and remain at the same level as the receptive stigma, facilitating autogamy. It was identified that flowers occur in shorter cladodes. Full exertion of anther and the stigma was blocked by bracts of the flowers in apical positions of the cladode preventing the exposure of the stigma to the atmosphere for anemophily. This is also an adaptation to autogamy. Within a plant, flowers in two to three subsequent segments per cladode opened at the same time and receptive stigmas of multiple flowers exerted simultaneously inferring self-pollination by geitonogamy. Hence these evidence support self-pollination, specifically autogenous self-pollination. The flexistyly is identified in several families in the angiosperms by previous studies i.e. Zingiberaceae. However, based on the mismatching in the time periods of highest pollen release and highest stigmatic receptivity, it can be inferred that this plant can functionally behave as a protogynous plant. Since the homogamy is also possible during the early morning due to simultaneous occurrence of anther dehiscence ad stigmatic receptivity, *S. brachiata* could be considered as weakly protogynous. Some *Salicornia* plants have also been reported to be weakly protogynous with persistent stigmas (Dalby, 1962).

Even in strongly self-compatible populations, outcrossing is unavoidable through wind pollination due to strong wind currents in the natural habitats of *Salicornia*. Adaptations for wind pollination was observed in *S. brachiata* in nature, in the present study, as point out below; 1. Stamens produced a higher amount of pollen grains, which is a characteristic of anemophily. 2. Diameter of pollen grains were in between 20 and 40 μm, which fits with the size of the anemophilous pollen. 3. As continuous tectum was observed; pollen grains were eutectate. The operculum protected the pollen grains from desiccation until the appropriate time of fertilization as an adaptation to survive in harsh, and arid habitat of *S. brachiata*. 4. Most of the stamens consisted of long straight filaments and anthers are exerted out before dehiscence in most of the flowers and are well-exposed. 5. Wind-pollinated plants usually consist of very few ovules and in the present study, *S. brachiata* had only one and consisted of well-exposed stigmas with papillae to increase the surface area of the stigmas. 6. The reduced morphological features such as leafless nature of the plant also facilitated pollen deposition on stigma without any hindrance from leaves, thereby reducing pollen loss due to plant architecture (Dafni, 1992). 7. When plants grow in close proximity to each other in the population, the chances of deposition of pollen grains of the nearby stigma is possible due to the short travel distance (Dafni and Firmage, 2000). This phenomenon is valid for *S. brachiata* as a small plant growing in contact with other individuals in the field. *Salicornia* in vegetation is also fairly dense and open in structure, which is also an important feature of wind-pollinated plants (Whitehead, 1969). 8. Further to facilitate anemophily, in the habitat, it was observed that *Salicornia* plants were the tallest plants in the salt marsh vegetation facilitating wind-blown pollen grains.

Adaptations of *S. brachiata* plant to wind pollination were also evaluated as per Dafni (1992). Floral level phenology and floral morphology were also in agreement with chasmogamous breeding system as well(Dalby, 1962; Ferguson, 1964). All previous studies on *Salicornia* assumed the presence of anemophily in these species based on floral characteristics (Fernandez-Illescas et al., 2011) and Dalby (1962) proved that pollen is carried by the wind in a population of *S. europaea*. Minute, extremely reduced, green colore flowers of *S. brachiata* were similar to the plant. These extremely reduced, transparent and colorless tepal extensions are unattractive to insects and covers the floral tube before exertion of anther and stigma. In opened flowers, this extremely narrow opening of the flower is completely blocked by the emerging anther and stigma that prevents entry of floral visitors or insects to the floral chamber. Other than these traits, features that indicate entomophily such as nectar glands were not found in *S. brachiata* flowers.

The extended pistillate phase, along with floral adaptations of *S. brachiata* also suggest wind pollination promoting out-crossing. Most flowers at the apical region of the cladode, which open at the last stages of the reproductive cycle, exhibited more adaptations to autogamy indicating late-acting selfing, compared to flowers at the early stages (basal flowers), which promote both outcrossing and self-pollination. These floral morphological features also fit with selfing and anemophily. Therefore, considering floral morphological traits in relation to pollination syndrome and pollen:ovule ratio results (compared to the values given by Cruden (1977), the facultative xenogamy is concluded to be the breeding system of *S. brachiata* for the first time.

In general, different populations of the same species can exhibit different reproductive mechanisms, and it is reported for *S. ramosissima* as well (Fernandez-Illescas et al., 2011). However, in the present study, flowers from the two populations of *S. brachiata* did not show a significant difference, but further studies in diverse geographic regions should be included to conclude this.

Collection and storage of pollen until pollination is one of the practical aspects in artificial pollination. During hand pollination, mature, viable pollen grains should be placed in a receptive stigma. Therefore, pollen viability and germinability studies are of great significance. Pollen germination study was conducted in commonly used media such as sucrose medium, Brewbaker medium and a medium containing only NaCl, with negative results. The medium suggested for sea-shore plants by Dafni (1992) was also unable to produce positive results. It has been reported that freshly shed pollens of some species need to be exposed to special conditions before germination or they exhibit low germinability (Dafni, 1992). The low germinability of the pollen grains of *S. brachiata* also could be due to the fact that they are adapted to germinate under particular environmental conditions. Since all the media are generally used for terrestrial angiosperms, further studies in pollen germination studies are needed for *Salicornia* species.

The palynology of *S. brachiata* also revealed some interesting findings. Even though differences between pollen sizes and pollen grain volumes between diploid and tetraploid species were reported (Ball and Brown, 1970; Dalby, 1962), Dehghani et al. (2021) revealed that the pollen characteristics of different taxa do not show significant differences, making it impossible to distinguish any pollen type or group in the genus *Salicornia.* Therefore, pollen characteristics are not useful for species delineation. In the family Amaranthaceae (Chenopodiaceae), Boutin et al. (1987) and Dufay et al. (2008) reported non-viable pollen grains being smaller than viable pollen grains, which was also observed in the microscopic studies of *S. brachiata.* The pollen diameter of *S. brachiata* ranges from 36 to 22 μm and they were viable. They were found in fully exerted, undehised anthers. These viable larger pollen grains were only observed in the early morning hours and were not observed in the evening and not found in developing or unexerted

anthers. Non-viable pollens observed were always smaller with a diameter less than 22 μm.

The information collected by this study could be valuable for breeding (usually high salt tolerance) of domesticated *S. brachiata*. It should be noted that since the flowers at the middle/basal regions of the cladode has a higher chance to be fertilized by out-crossing, seeds in these regions could have higher genetic variation than those in the apical areas. From the plant breeding point of view, pollen grains should be collected before 8:30 a.m. to get maximum benefit from artificial pollination and the central flowers are better adapted for out-crossing than lateral flowers based on the stigma receptivity data because central flowers had strong stigmatic receptivity based on peroxidase activity as inferred by H_2O_2 bubble formation. Further, when hybridization studies are performed, a better outcome would be obtained if researchers focus on the middle and lower flowers of the cladode.

In summary, the current study reports several important aspects in relation to the species identity and reproductive biology of *S. brachiata*. It provides a comprehensive report of morphological, anatomical, and molecular traits of *S. brachiata* for the first time. Barcode sequences of ITS and ETS regions were identified as preferred regions for species delineation purposes. Facultative xenogamous mating system was reported for the first time in the genus. Finally, it identified the best pollen collection time, pollination time, and preferred flower types for out-crossing and the information can be used as a foundation for future crop improvement programmes of *S. brachiata*.

5. Conclusions

The combination of molecular and morphological analysis was used to describe *S. brachiata* species in Sri Lanka for the first time which helps differentiating the species from other species. ETS, ITS, and matK sequences of authenticated, vouchered specimens of *S. brachiata* were submitted to NCBI GenBank for future reference. The mating system of the plant was finally resolved describing the cryptic nature of facultative xenogamy. Overall, the paper provides useful information for plant biologists, and reproductive biologists and provides a strong foundation for future *Salicornia* breeding programs.

CRediT authorship contribution statement

Dinum Perera: Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Renuka Attanayake:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Kalmi Siridewa:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Wasana De Silva:** Writing – review & editing, Investigation. **R M C S. Ratnayake:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Siril Wijesundara:** Writing – review & editing, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2024.103827.

Data availability

Data will be made available on request.

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