

Anatomical features and SCoT profiles provide new insight into phenotypic plasticity in the halophyte *Suaeda maritima* in Thailand

KANOKPHORN RITTIRONGSAKUL¹, ANITTHAN SRINUAL^{1,*}, ONGKARN VANIJAJIVA²

¹Department of Biology, Faculty of Science, Srinakharinwirot University, 114 Sukhumvit 23, Bangkok 10110, Thailand.

Tel +66 2 649 5000, Fax +66 2 258 4007, *email: a.srinual.swu@gmail.com, vanijajiva@gmail.com

²Faculty of Science and Technology, Phranakhon Rajabhat University, 9 Chaeng Watthana Rd, Anusawari, Bang Khen, Bangkok 10220, Thailand

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Abstract. Rittirongsakul K, Srinual A, Vanijajiva O. 2020. Anatomical features and SCoT profiles provide new insight into phenotypic plasticity in the halophyte *Suaeda maritima* in Thailand. *Biodiversitas* 21: 1082-1090. Phenotypic plasticity is the variation in phenotypic traits of organisms to survive with fluctuating environments. Halophytes are plants that can naturally tolerate high concentrations of salt in the soil, and their tolerance to salt stress, which reflects the ability of the plants to produce different phenotypic traits in response to changing environmental conditions. *Suaeda maritima* (L.) Dumort is a polymorphic halophyte and prominent species complex from tropical to temperate regions due to diverse stressful saline habitats. Consequently, the identification of *S. maritima* based on morphological data is challenging due to high phenotypic plasticity. In Thailand, *S. maritima*, known as Seablite or Cha Khram, has only been reported for the genus *Suaeda* which frequently found in coastal salt marshes. The peculiarities of the prevalence of three distinctive color traits, green, green-reddish and reddish, were commonly observed in Thailand. This preliminary study aims to examine the morpho-anatomical characters and molecular analysis of *S. maritima* and to determine how their morphological and genetic characterizations are related to phenotypic traits, particularly in color variation within the population at Bang Khun Tien shoreline in Bangkok, Thailand. This combined investigation of anatomical characters and start codon targeted (SCoT) fingerprinting profiles provide first evidence of phenotypic variations and genetic characterizations of halophytic *S. maritima* in Thailand. Anatomical characters of leaf structures confirmed that the species is austrobassoid (C₃) types. Meanwhile, molecular investigation of thirty SCoT primers found that only two SCoT primers (SCoT10 and SCoT27) failed to amplify the *S. maritima* genomic DNA. Thoroughly, twenty-eight SCoT primers produced 162 unambiguous and reproducible banding profiles with 140-1200 bp product size. Despite, there was no genetic variation detectable within the population, SCoT technique was considered a suitable tool to produce adequate characterization for DNA fingerprinting of halophyte *S. maritima*. However, further studies involving additional samples of *S. maritima* are needed to obtain a more general view of the conspicuous variation and colonization history in Thailand.

Keywords: Anatomy, halophyte, SCoT, *Suaeda maritima*

INTRODUCTION

Phenotypic plasticity is the ability of organisms to modify phenotypic characters in response to changes in environmental selection pressures (Stearns 1989; Arnold et al. 2019). This important adaptive mechanism to respond to fluctuating environments plays a fundamental role in the evolutionary achievement of many organisms, particularly in plants whose no mobility lifestyle involves them to deal with surroundings environments in space and time (Schlichting 1986; Gratani 2014; Hiatt and Flory 2020). Halophytes are plants that can naturally tolerate high concentrations of salt in the soil, and their tolerance to salt stress growing in the heterogeneous salt marsh environment respond to abiotic and biotic stress influences through various mechanisms (Waisel 1972; Di Carlo et al. 2019). There is strong evidence among salt marsh plants of the existence of phenotypic plasticity, which reflects the ability of the plant to produce different phenotypic traits in response to changing environmental conditions (Flowers and Colmer 2008; Wetson et al. 2012).

Suaeda Forssk. ex J.F. Gmelin (Suaedoideae; Chenopodiaceae/Amaranthaceae) is a genus of halophytic plants that comprises approximately 80-110 species, mainly distributed in the coastal region from tropical to temperate areas of the world (Schenk and Ferren 2001; Kadereit et al. 2003; Munir et al. 2015). It comprises typically of halophytic leaf-succulents that grow in salty and basic wetlands and deserts. Based on recent morpho-molecular evidence, *Suaeda* is presently recognized in subfamily Suaedoideae of Chenopodiaceae (Fisher et al. 1997; Schütze et al. 2003; Brandt et al. 2015; Kim and Chung 2018; www.theplantlist.org). The subfamily is categorized by fleshy leaves, the occurrence of bracteoles, membranaceous. The difficulty lies in the fact that the genus includes wide-ranging polymorphic species, such as the coastal salt marsh plant *Suaeda maritima* (L.) Dumort., which has a worldwide distribution (Alghamdi 2012). The species, commonly known as Seablite or Cha Khram in Thailand (Pornpitakdamrong and Sudjaroen 2014), is an obligate halophyte that grows on both inland and coastal salt sites. It is an annual, typically autogamous species with small and inconspicuous flowers that have only been

reported of the genus *Suaeda* for Thailand. However, its taxonomic identity is problematic since it displays variations due to diverse stressful saline habitats (Wetson et al. 2008; Polić et al. 2009; Wetson et al. 2012). Currently, very little study has been conducted on phenotypic variations within and between local populations of the salt-tolerant *S. maritima* in Thailand, even though the effects on the variation of the salt-marsh plant may be a key driver of reproduction, establishment, and existence in unpredictable and stressful environments. In addition, there is no evidence obtainable on the existing anatomical and genetic study in *S. maritima* in Thailand. Consequently, the classification of natural phenotypic and genetic data becomes essential for its efficient and sustainable management.

Distinguishing characters of the plants were initially based on morphology, anatomy and partly on micromorphology, these features are still valid today. Molecular markers effectively developed during the last few decades have largely overcome the problems that are associated with the phenotype-based classification. PCR-based start codon targeted (SCoT) marker, based on the short conserved nucleotide sequence that flanks the start codon ATG has been widely used to survey species identification, because their application simple, low cost, highly polymorphic, gene-targeted, and abundant in the genome (Collard and Mackill 2009; Amom and Nongdam 2017; Vanijajiva 2020). Because of the difficulties in the morphological determination of *Suaeda maritima* (Alghamdi 2012), this preliminary study purposes to examine the morpho-anatomical analysis and molecular investigation of *S. maritima* and to determine how their morphological and genetic characterizations are related to at Bang Khun Tien shoreline in Bangkok, Thailand.

MATERIALS AND METHODS

Study sites and sample collection

Thirty individuals of annual halophyte *Suaeda maritima* were used for genomic DNA extraction. All samples were collected representing a natural population of Bang Khun Tien shoreline, the only muddy shoreline located in the Bang Khun Tien, one of fifty districts of Bangkok in Thailand (Figure 1). The shoreline is located in the upper Gulf of Thailand where is distinguishing flat plain on the tropical delta. The length of this shoreline is about 5 km (Ekphisutsuntorn et al. 2010).

Anatomical studies

Among 30 *S. maritima* of used in this study, only 12 samples (four samples for each color morph) that representative as illustrations of qualitative anatomical study. The sections of the different plant organs were observed using a light microscope. In peeling approaches, the leaf bases and apexes were cut out, and then were peeled epidermis and mesophyll out by a razor blade. Subsequently, the sample was stained with 1 % (w/v) safranin O and dehydrated with series of ethanol (70%, 95%, and 100%) for at least 10 minutes in each grade. Following, the specimens were immersed in the mixed solution of xylene and absolute alcohol with a ratio of 1:1 (v/v) and pure xylene for 10 minutes in each solution. Finally, epidermal peels were mounted with DePeX. For the paraffin technique, transverse sections of the laminae and stems were dehydrated in graded series of TBA (tertiary butyl alcohol). The specimens were infiltrated by paraffin liquid and pure paraffin before embedding into molds. A thin section of 12-14 μm was made using phenotypic traits, particularly in color variation within population a sliding microtome and stained with safranin O and fast green, ahead mounting with DePeX (Johansen 1940; Feder and O'brien 1968; Ruzin 1999).

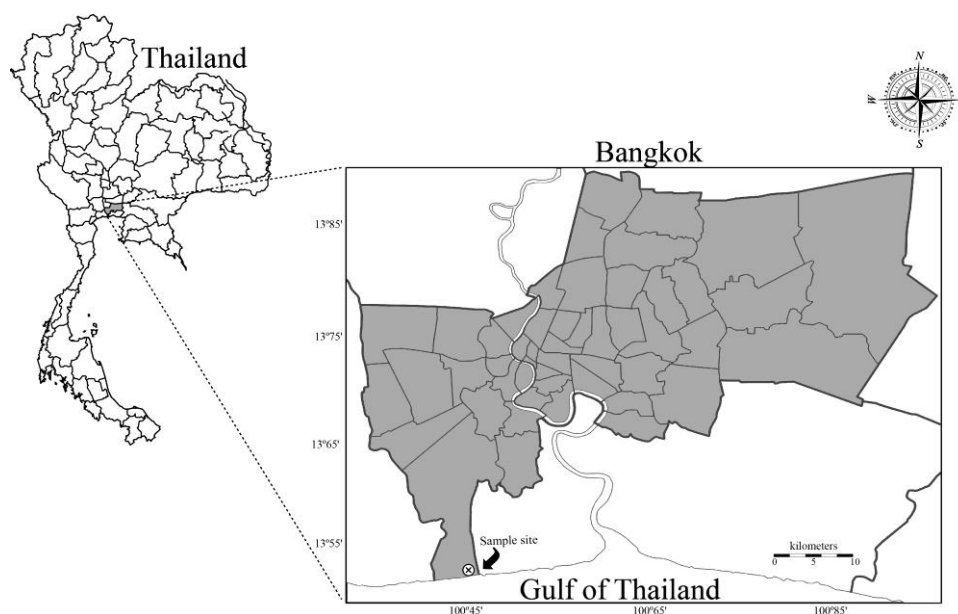


Figure 1. Sample site area (⊗) of *Suaeda maritima* population at Bang Khun Tien shoreline located in Bangkok province, Thailand

DNA extraction

Genomic DNA was extracted from the leaves of 30 *Suaeda maritima* accessions using the CTAB method with minor modification (Doyle and Doyle 1987; Doyle and Doyle 1990). The leaf material about 500 mg was ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris-HCl (pH 8), 0.7 M NaCl, 0.1% β -mercaptoethanol)] 500 μ l was added and the solution was incubated at 60 °C for 30 min. The homogenate was mixed with 25: 24: 1 phenol: chloroform: isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13,000 rpm for 15 min, the upper aqueous layer was transferred to a fresh tube. RNA was removed by treating with 2.5 μ l of the RNase (10 μ g/ μ l) for 30 min at 37°C. The extraction of DNA with phenol/chloroform/isoamyl alcohol was repeated one more time. DNA in the solution was precipitated with 0.6 volumes of ice-cold isopropanol and cleaned with 70% ethanol. Next, the DNA was extracted using CTAB DNA extraction procedure without RNase. The method was repeated until the DNA pellet was free of color (two to three times) and the final pellet was dissolved in sterile deionized water. The DNA quality was using Nanodrop Spectrophotometer (Thermo scientific Nanodrop 1000, USA) at the absorbance ratio of 260 and 280 nm providing a value of 1.7-1.8 which determines pure DNA preparation. Quality of DNA fragment was also electrophoretically analyzed through 0.8% agarose gel using 1X TAE buffer. The DNA was stored at -20 °C before use as templates for PCR amplification.

SCoT-PCR amplification

Thirty SCoT primers used in the present study were designed according to Collard and Mackill (2009) was initially screened for analysis by three repeating tests for each primer at different times (Table 1). PCR was performed using a Thermohybrid Px2 (Roche Molecular Systems, Inc., USA). PCR was optimized for testing the SCoT method. All PCR reactions were performed within a total volume of 20 μ L. PCR reaction mixtures contained PCR buffer (Promega; 20 mM Tris-HCl (pH 8.4), 50 mM

KCl), 2 mM $MgCl_2$, 0.24 mM of each deoxyribonucleotide triphosphates, 0.5 U of Taq polymerase (Promega), and 0.8 μ M of primer. Each reaction contained 50 ng of template DNA. A standard PCR cycle was used: an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; the final extension at 72°C was held for 5 min (Vanijajiva 2020).

Bands profile and analysis

The SCoT products were all analyzed by agarose (1.8% w/v) gel electrophoresis at 150 A for 30 minutes in 0.04 M TAE (Tris-acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with RedSafe™ Nucleic Acid Staining Solution and photographed under UV light by using gel documentation system alpha imager hp (Innotech, USA). To determine SCoT profiles, the size of each DNA band was inferred by comparison with a 100 bp DNA ladder (Promega), used as a molecular weight marker (M). Characterizations at all loci were confirmed by three repeating tests for each primer at different times.

RESULTS AND DISCUSSION

Phenotypic analysis

The plasticity phenotypic is particularly interesting in halophytes, where they can be related to the fact that, if a species that represent salt-tolerant species that thrive in the uncongenial environments of inland and coastal salt marshes, dunes, beaches, deserts, and salt flats. They are adapted to survive under extreme environments, represented by temperature, (freezing to very hot), salinity (hypo- to hyper-saline) and moisture (drought to water-logging). They must also cope with localized environmental variability (Waisel 1972; Alghamdi 2012; Di Carlo et al. 2019).

Table1: SCoT primers used in this study

SCoT	Sequence (5'-3')	SCoT	Sequence (5'-3')
SCoT1	CAACAATGGCTACCACCA	SCoT16	ACCATGGCTACCACCGAC
SCoT2	CAACAATGGCTACCACCC	SCoT17	ACCATGGCTACCACCGAG
SCoT3	CAACAATGGCTACCACCG	SCoT18	ACCATGGCTACCACCGCC
SCoT4	CAACAATGGCTACCACCT	SCoT19	ACCATGGCTACCACCGGC
SCoT5	CAACAATGGCTACCACGA	SCoT20	ACCATGGCTACCACCGCG
SCoT6	CAACAATGGCTACCACGC	SCoT21	ACGACATGGCGACCCACA
SCoT7	CAACAATGGCTACCACGG	SCoT22	AACCATGGCTACCACCAC
SCoT8	CAACAATGGCTACCACGT	SCoT23	CACCATGGCTACCACCAG
SCoT9	CAACAATGGCTACCAGCA	SCoT24	CACCATGGCTACCACCAT
SCoT10	CAACAATGGCTACCAGCC	SCoT25	ACCATGGCTACCACCGGG
SCoT11	AAGCAATGGCTACCACCA	SCoT26	ACCATGGCTACCACCGTC
SCoT12	ACGACATGGCGACCAACG	SCoT27	ACCATGGCTACCACCGTG
SCoT13	ACGACATGGCGACCATCG	SCoT28	CCATGGCTACCACCGCCA
SCoT14	ACGACATGGCGACCAACG	SCoT29	CCATGGCTACCACCGCC
SCoT15	ACGACATGGCGACCGCA	SCoT30	CCATGGCTACCACCGCG

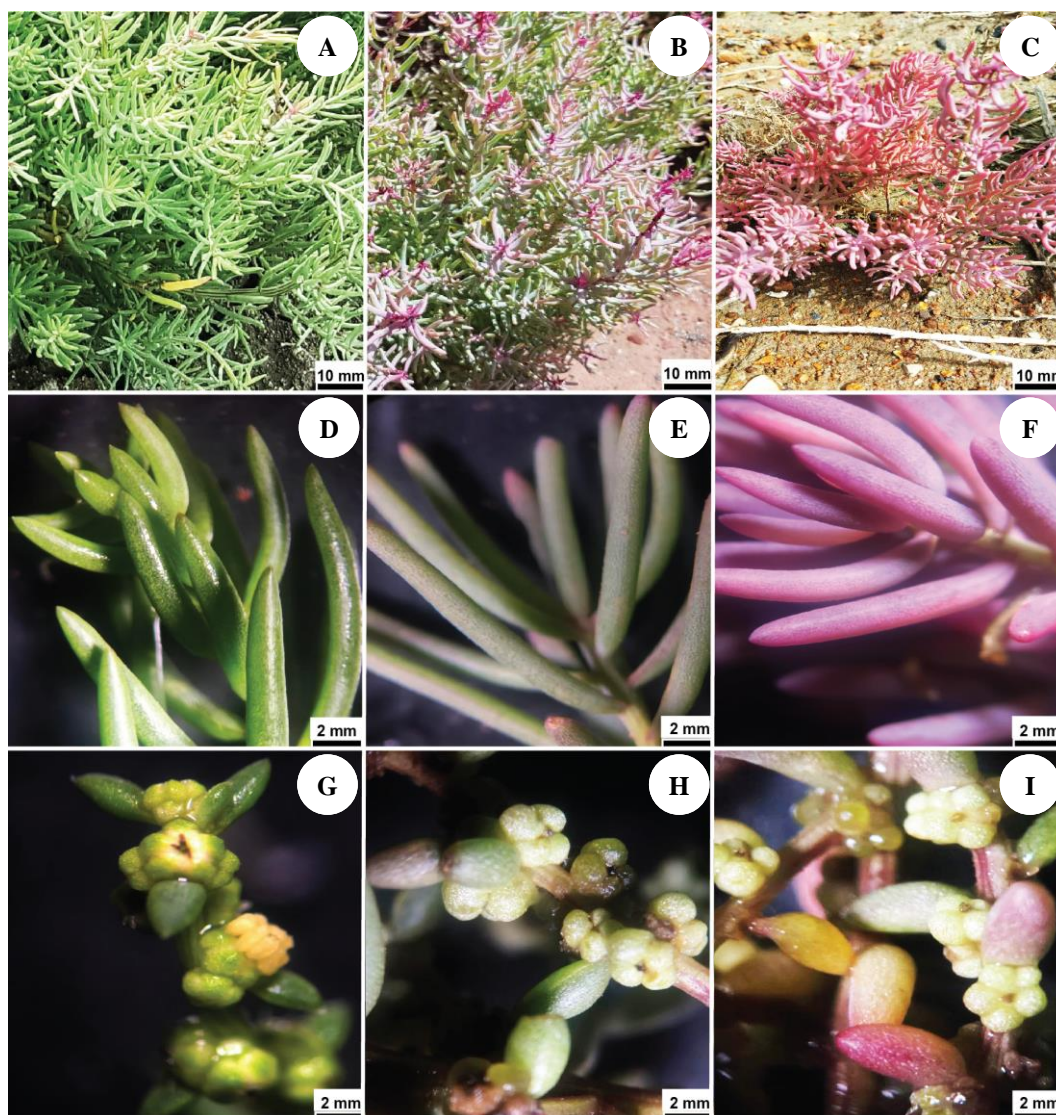


Figure 2. Photo plate illustrating different color morphs of *Suaeda maritima*: green, green-reddish and reddish color morphs by left to right column. Habitats (A-C); Leaves (D-F); Infructescences (G-I)

Suaeda maritima is a polymorphic annual halophytic species that in Thailand is frequently found in the landward margin of mangroves and also in the area bordering the estuarine water and land. This species is usually associated with dry areas and grows on dry, clay and brackish to saline soils (Pornpitakdamrong and Sudjaroen 2014). Several studies reported that *S. maritima* revealed strong evidence of the existence of phenotypic plasticity, which reflects the ability of the species to produce different physiological and morphological phenotypes in response to changing environmental conditions (Ihm et al. 2004; Prinz et al. 2009a; Alghamdi 2012). The species is highly plastic within certain parameters, and this plasticity is demonstrated in varying growth rates, sizes at maturity, reproductive characteristics and rates, and tissue chemistry (Alghamdi 2012; Wetson et al. 2012).

This preliminary research is focused on the analysis of phenotypic variation, particularly in the coloration of *S. maritima* within Bang Khun Tien shoreline population. The

plants observed in this study show obviously variations in terms of color morph of *S. maritima* in three distinctive color traits: green, green-reddish, and reddish (Figure 2). These three color variations more or less display similar growth responses depending on the locality, high or low salt marshes. The growth forms could be notable, the one which grows in low marsh locality is smaller and less branched than the other one which grows in high marsh zone. Differing color appearances in vegetative parts such as stems, leaves, and shoot, reflecting this heteromorphism have been reported in *Suaeda* species (Alghamdi 2012; Raju and Kumar 2016). The variations in color morph characters could be situational depending on the nutrient and water levels, temperature, age, and other ecological factors such as the spectral quality of shade treatments (Ihm et al. 2004; Polić et al. 2009; Alghamdi 2012; Aluri et al. 2016; Raju and Kumar 2016).

The widespread occurrence of phenotypic plasticity has significant implications for taxonomy and plant

identification, since similar responses of different species to environmental conditions may cause them to converge phenotypically. If a character is highly plastic, it is less useful for taxonomic purposes. Meanwhile, phenotypic traits that are plastic under some environmental conditions may be constant under others and later taxonomy is based on a separation of all possible characters, the resulting plant identifications may be influenced by the place and time that collections were made (Sultan 1995; Parsons et al. 2020). In the *Suaeda*, leaf blade surface morphology and internal anatomy are traditional taxonomic characters that have been particularly useful at the subgeneric affiliations and species levels. The peculiarities of leaf morphology and anatomy may be important as a source of characters at the species level when reproductive material has few definitive characters or is not available (Fisher et al. 1997; Wetson et al 2012).

Preliminary observations on the muddy coastline of *Suaeda maritima* with apparently glabrous leaves found that the same plants' leaf subacute to acuminate, flat above, convex beneath; the lower leaves held horizontally, the upper erect. Among 30 samples of *S. maritima* used in this study, only 12 samples (four samples for each color morph) that representative as illustrations of qualitative anatomical study. All samples examined have relatively centric leaves. The epidermal cells contain few chloroplasts and have relatively thin cuticles. Stomata are positioned on both sides of the leaves and are associated with the other epidermal cells (Figure 3). The leaf peeling showed paracytic stomata dispersed over the whole adaxial and abaxial surfaces. Epidermal cells are relatively round shape, moderately thin cuticle with straight, and consist of papillae on both sides (Figure 3.A-3.F).

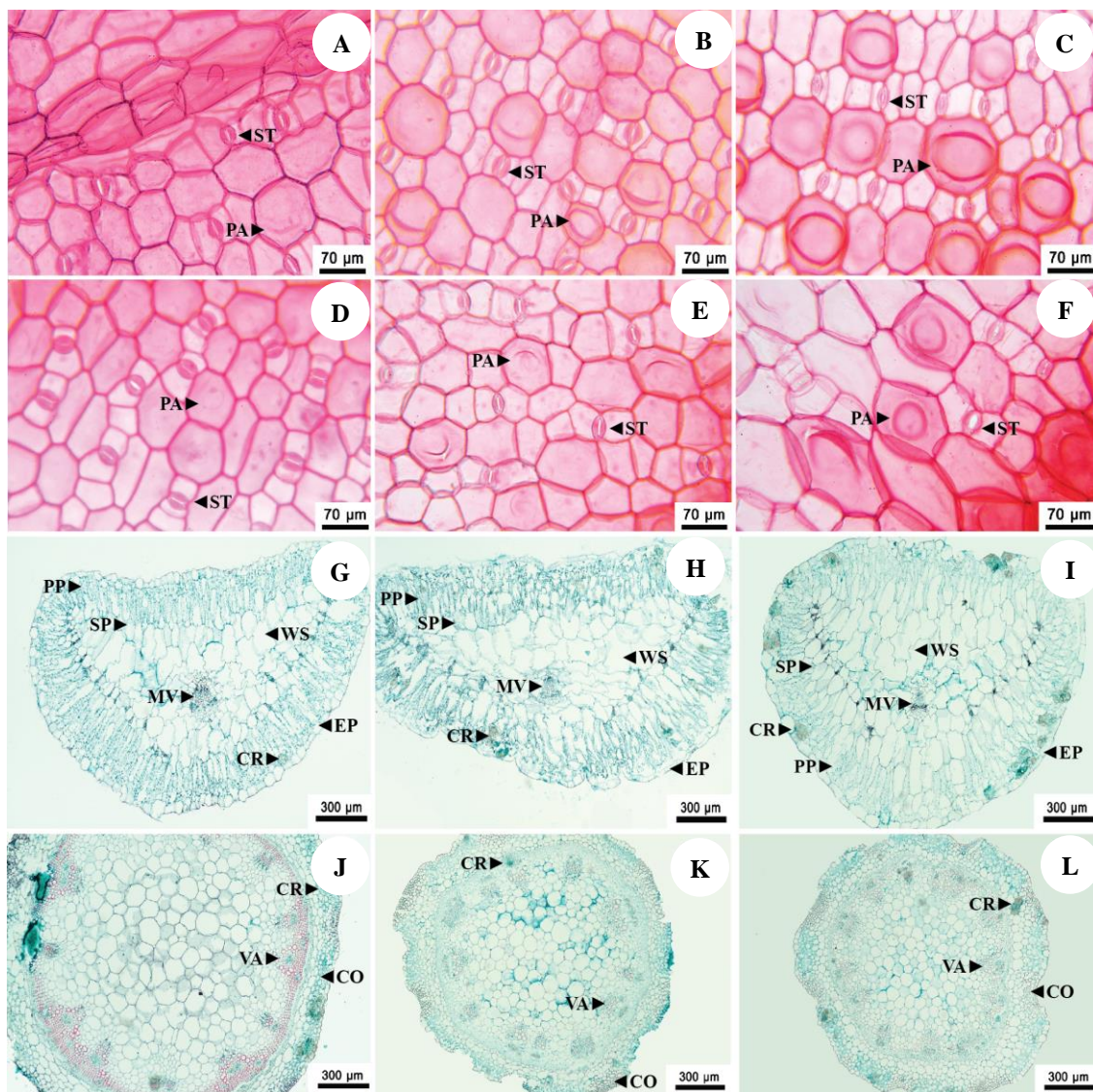


Figure 3. Anatomical features of leaf and stem of *Suaeda maritima*: green, green-reddish and reddish color morphs by left to right column. Adaxial surface (A-C); Abaxial surface (D-F); Cross-sectional leaf (G-I); Cross-sectional stem (J-L). Black arrows indicate distinctive of *S. maritima* anatomy. Abbreviations: CO, collenchyma; CR, crystalloid; EP, epidermis; MV, main vascular bundle; PA, papillae; PP, palisade parenchyma; SP, spongy parenchyma; WS, water-storage tissue; VA, vascular bundle

In leaves transverse section, *S. maritima* has isolateral leaf arrangements. It showed circular-shaped leaf with uniseriate epidermis and slightly sunken stomata. The mesophyll is differentiated into two distinct layers: a layer of palisade parenchyma below the epidermis, and central water-storage tissue, containing main vascular bundles. The main vascular bundle is in the center of the leaf, surrounded by water storage parenchyma, while other bundles gradually became smaller towards the tip part of the leaf. Remarkably, leaves from *S. maritima* species contain small amounts of spongy parenchyma, which primarily consists of elongate cells that resemble palisade cells. This anatomy, termed austrobassoid (Carolin et al. 1975; Fisher et al. 1997; Schütze et al. 2003), is typical for most C_3 plants of the genus *Suaeda* (Figure 3G-3I). Furthermore, sections of stems from all coloration morphs of *S. maritima* reveal round epidermal cells. The cortical tissue with water storage parenchyma of the species generally contains angular collenchyma under epidermis. Stele is eustele with collateral vascular bundle and center of pith were large parenchyma cells (Figure 3J-3L). Druse crystals or crystalloids were found in stems and leaves of all types of color phenotype.

Although, the genus *Suaeda* has both C_3 and C_4 photosynthetic pathways (Fisher et al. 1997; Jacobs 2001; Schütze et al. 2003), anatomical structure of *Suaeda* in Thailand is still poorly studying. Features found in this study is initial report anatomical structure of *Suaeda maritima* in Thailand showed that the species is austrobassoid (C_3) types of leaf anatomy which the leaf is distinctly succulent, flattened to more or less semiterete, on adaxial side usually concave, at base somewhat attenuated; vascular system in a curved more or less central horizontal with the 3-4 mesophyll layers on each side strongly increasing in size towards the center and with decreasing numbers of chloroplasts, the innermost 1 or 2 layers as aqueous tissue, usually lacking of chloroplasts, without air spaces (Schütze et al. 2003). A similar statement was drawn for populations of many regions such as North American, European and Asian coastal areas in which a considerable proportion of the variation observed in field-collected material was attributed to phenotypic plasticity (Yeo and Flowers 1980; Fisher et al. 1997; Lomonosova et al. 2008; Wetson et al. 2012; Prinz et al. 2013; Raju and Kumar 2016; Kim and Chung 2018; Voronin et al. 2019).

Molecular analysis

Extraction of high-quality DNA from *Suaeda maritima* is notoriously difficult due to the high contents of polyphenols, tannins and other secondary metabolites, which is agreeable for downstream analyses (Schütze et al. 2003; Gurudeeban et al. 2011). The protocol of high-quality DNA extraction was optimized by re-extracting the DNA using CTAB DNA isolation procedure with minor modification by using phenol: chloroform: isoamyl alcohol extraction instead of chloroform: isoamyl alcohol extraction. The polyphenolics with the DNA were basically removed and decent SCoT electrophoretograms were obtained with all samples. DNA extracted from Seablite leaf using an above modified gave a good and adequate

quality DNA for PCR reaction. DNA isolated by minor modification method yielded strong and reliable amplification products and the amount of DNA extracted from the accessions ranged from 180 to 245 $\mu\text{g/g}$ fresh weight leaf material. The ratios of A260/A280 varied from 1.78 to 1.85. The quality of DNA was also tested by PCR, which confirmed that the DNAs were suitable for PCR reaction. The parameters for the SCoT protocol from samples were also studied. Several parameters affected banding patterns and reproducibility such as concentration of dNTPs, magnesium chloride concentration, concentration of enzyme, concentration of primer and concentration of template DNA, but the concentration of template DNA and magnesium chloride were most important. Similar to Gurudeeban et al. (2011), effectively employed CTAB DNA isolation protocol to increase the quality and quantity of genomic DNA which the result clearly revealed that at 50 ng template DNA and MgCl_2 2 mM concentration were suitable for further PCR analysis.

The significant mechanism in response to the heterogeneity of salt marshes is the presence of genetic differentiation within halophyte species. Indeed, there is evidence that much of the morphological variation evident within halophytes is genetically based, and that this genetic differentiation is the driver in maintaining the variation in phenotypes, both within and between populations. The present study is first reported to have shown significant genetic characterization of *Suaeda maritima* using SCoT markers (Ihm et al. 2004; Prinz et al. 2009a; Prinz et al. 2009b; Wetson et al. 2012; Prinz et al. 2013). This examination, a set of 30 SCoT primers were preliminary verified, out of total, 30 SCoT primers produced an unambiguous and reproducible banding profile with 140-1200 bp product size but only two SCoT primers, SCoT10 and SCoT27, failed to amplify the Seablite genomic DNA. A total of 162 scorable bands were identified through the amplification of 28 SCoT primers in 30 samples (Figure 4). The amplification ranged from two bands (SCoT 7) to ten bands (SCoT 18 and SCoT 19). However, there was no genetic variation detectable within populations of the same region. As SCoT technology has proved to be a reliable method to find residual genetic characterizations in many studies (Guo et al. 2012; Agarwal et al. 2019; Vanijajiva 2020), this study may provide that populations within the same regions in the Bang Khun Tien shoreline of Bangkok consist of only one single genotype.

Maintenance of the evolutionary perspective is the primary objective of natural conservation by maintaining higher levels of genetic diversity. Hence, first-hand information on the genetic characterizations at intra-specific levels helps to formulate effective conservation policies (Hoffmann et al. 2015; Frankham et al. 2019). The SCoT method is sensitive to low levels of genetic variations and thus provides a very useful tool for analyzing population genetics on a wide range of plants as well as identifying species or populations of the same species (Collard and Mackill 2009; Amom and Nongdam 2017; Vanijajiva 2020). In the present study, the representatives of the *S. maritima* collected from Bang Khun Tien shoreline, where the plant can still be found in its natural habitat, revealed

that no differentiated by the DNA fingerprinting patterns using SCoT primers. Similar results were reported by Prinz et al. (2009b) that using amplified fragment length polymorphism (AFLP) markers examined 120 samples from 40 *S. maritima* populations indicated an unexpectedly low level of diversity among the investigated *S. maritima* plants. Moreover, extremely low levels of genetic variation among *S. maritima* samples from coastal and inland sites in Germany were also informed by Weising and Freitag (2007). An overall reduced genetic variation may also be caused by selfing or mixed mating system (Hamrick and Godt 1996; Nybom and Bartish 2000). Despite the lack of genetic diversity, the natural population of *S. maritima* exhibits large population sizes and does not appear threatened by extinction. This may be attributable to large phenotypic plasticity, enabling the production of numerous seeds under a wide range of environmental conditions. Although phenotypic plasticity may favor the viability of *S. maritima* in the short term by allowing the production of numerous seeds under a wide range of environmental situations, the species survival may be compromised in the long term, as phenotypic plasticity without genetic variation is not sufficient to face large environmental changes (Alghamdi 2012; Wetson et al. 2012).

In conclusion, this is the first study on the anatomical and genetic characterizations using SCoT markers as a tool for determining genetic variation of *S. maritima* within population at Bang Khun Tien shoreline in Bangkok, Thailand. Our research indicated that the species is austrobassoid (C_3) types and SCoT is a simple, efficient and inexpensive DNA marker technique that is useful for assessing the genetic diversity of halophyte *S. maritima*.

Lack of genetic variability displayed by this halophyte could indicate that selfing or apomixis has an important influence on the genetic structure of *S. maritima*. Therefore, the results provided much more useful anatomical and genetic information about *S. maritima*, which are beneficial for the maintenance approach of the species preservation of planning conservation strategies. In our future work, more halophyte *S. maritima* will be included as well as reciprocal transplantation of seedlings to verify whether the findings hold when even more population in Thailand are included. In addition, although no genetic variation of the species in Thailand is revealed here, comparison of genetic diversity of *S. maritima* from different parts of the world will be needed in order to understand the overall genetic structure of this halophyte species.

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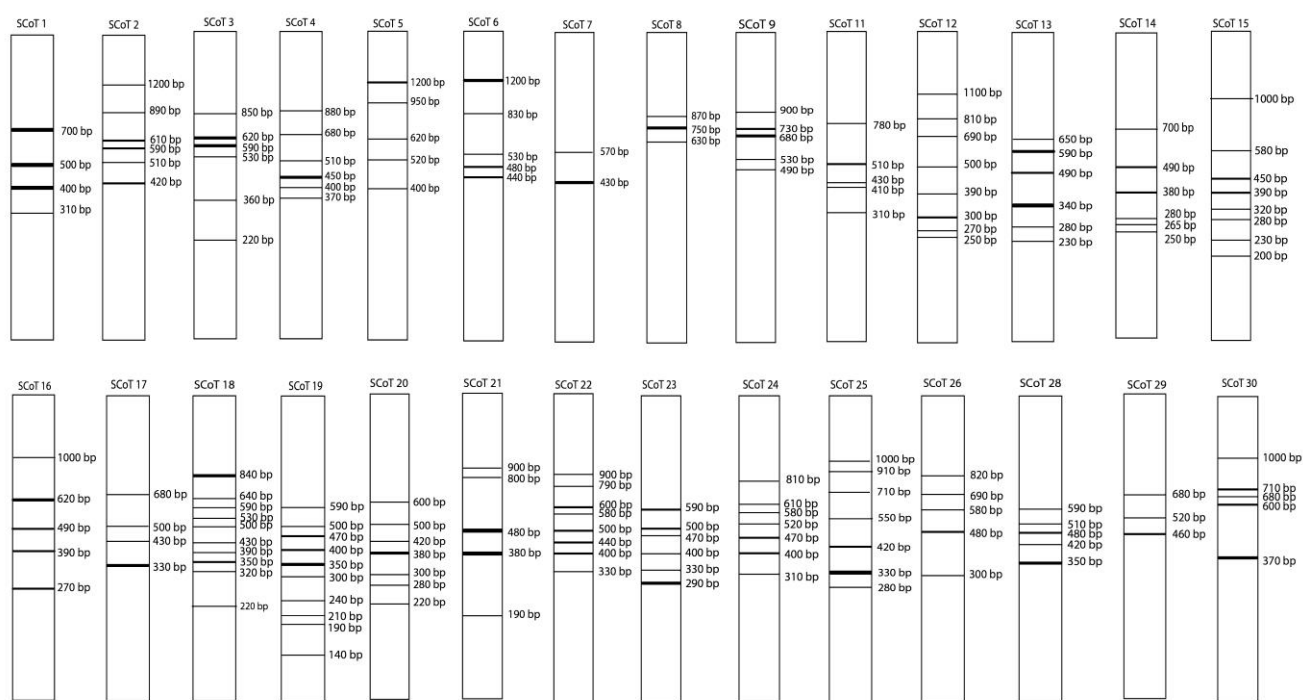


Figure 4. The representative SCoT profiles in this study. The electrophoretograms are employed as representative of clear, distinguished, stable profiles from 28 primers of samples

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