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A preliminary systematic analysis of leaf epidermal characters for six *Smilax* species in Thailand

Bruce Roger Moore[a,*], **Theeraporn Moore**[a], **Nual-Anong Narkkong**[b]

[a] Palaeontological Research and Education Centre, Faculty of Science, Mahasarakham University, Mahasarakham, 44150, Thailand

[b] Instrumentation Lab, Faculty of Science, Mahasarakham University, Mahasarakham, 44150, Thailand

*Corresponding author, e-mail: br-moore@hotmail.com

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ABSTRACT: Accurate identification of *Smilax* (Smilacaceae) species in Thailand is mostly dependent on plants of both sexes being collected in the right reproductive phase. To explore an alternative method, abaxial and adaxial leaf surfaces of six *Smilax* species were examined for delimiting architecture. Five taxa have individually unique abaxial reticulums and all species presented different abaxial ornamentation such as papillae, granulae, and bullae. Likewise, stomatal ultra-structure varied remarkably between each species. These results strongly suggest that at least some Thai *Smilax* possess taxonomically useful cuticular features.

KEYWORDS: taxonomy, stomata, cuticle, Smilacaceae

INTRODUCTION

The genus *Smilax* L. (Smilacaceae Vent) is cosmopolitan with about 350 species[1] primarily distributed throughout the sub-tropics and tropics. In Thailand, 21 species and 7 sub-species occur in five sections; *Macranthae* Kunth, *China* T. Koyama, *Vaginatae* T. Koyama, *Coilanthus* A. DC, and *Cospromanthus* Torrey[2,3]. Easily recognizable at the genus level, Thai *Smilax* have leaves with acrodromous or campylodromous primary venation on stems bearing mixed combinations of vegetative characters such as tendrils, prickles, and petiolar prophylls. In contrast, identifying and/or delimiting species, especially with sterile specimens, is rigorous. Although botanists widely hold to the use of floral characters in taxonomic work, *Smilax* is dioecious and the species in Thailand have small, morphologically similar flowers. Consequently, to identify specimens correctly requires examination of flowers of both sexes, plus leaves, stems, and sometimes fruits[2]. Unfortunately, locating populations of mature individuals in all reproductive phases is often impractical. Therefore, a simplified taxonomic aid to distinguish individual species accurately is appealing.

A number of alternative methods are available to the taxonomist. Pollen morphology is a well recognized tool. Chen et al[4] evaluated *Smilax* pollen over a wide range of species. Unfortunately, their results showed the taxonomic limits of pollen to be at the genus level. In contrast to pollen, numerous workers have shown that morpho-histology of *Smilax* taxa provides significant taxonomic characters from flowers[5] and vegetation[6,7]. A large number of investigations have examined the diagnostic value of leaves. Leaves are probably the most varied anatomical organ of the angiosperms[8]. In spite of this, the taxonomic value of features such as stomata structure, surface ornamentation, and epidermal cell wall configuration has been repeatedly demonstrated[9–14].

When Ferguson[15] evaluated the leaf epidermis of Florida *S. laurifolia* L., he determined that a braid-like reticulum remained constant as a diagnostic character. Contemporaneous to Ferguson's study, Yates and Duncan[16] recognized the difficulties to correctly identify sterile *Smilax* specimens of southeast North America. Assessing quantitative and qualitative leaf cuticular features, epidermal and mesophyllous cells and vascular systems, they circumscribed characters in a supplemental taxonomic key. It should be noted that care must be taken when assessing the taxonomic reliability of stomatal and epidermal cell numbers and anticlinal cell wall patterns. Variations in ecological and climate conditions during leaf development may affect epidermal cell configuration and numbers[17–21]. In consideration of this, Moore[22] and Moore et al[3] used modified approaches similar to Yates and Duncan to provide evidence that leaf epidermal surfaces and stomata characters varied between a number of *Smilax* species in Thailand. Here, we emphasize and describe the nature of leaf cuticular character and stomatal ultra-structure as a simplified taxonomic aid for diagnosing six *Smilax* species from Thailand.

MATERIALS AND METHODS

Materials

Field and herbarium leaves ($n = 45$) from six arbitrarily selected *Smilax* species from northern and northeast Thailand were examined. Materials cited in the study bear the first author's field collection voucher acronym PS (personal specimen) and number. Specimens from the Royal Forest Herbarium, Bangkok are referred to by using the collection acronym (BKF) and specimen number. The species and sample sizes are as follows: Section *Macranthae* - *S. verticalis* Gagnep., PS4 (3 leaves, female with mature fruits); *S. megacarpa* A. DC., BKF47680 (3 leaves, female with fruits) and BKF4238 (3 leaves, male); *S. bracteata* Presl ssp. *verruculosa* (Merrill) T. Koyama, PS12 (3 leaves, female with flowers and developing fruits); Section *China* - *S. micro-china* T. Koyama, PS8 (3 leaves, male with flowers); Section *Coilanthus* - *S. corbularia* Kunth ssp. *corbularia*, T. Koyama, PS16, BKF4251, BKF4807, BKF34075, BKF50936, BKF096058, BKF115942, BKF136158, BKF49783 (3 leaves each, $n = 27$, genders not noted); Section *Coprosmanthus* - *S. pottingeri* Prain, PS25 (3 leaves, gender unknown). Specimens sampled are located in the Royal Forest Herbarium collection at Bangkok.

Method

One $\sim$ 0.5 cm $\times$ 0.5 cm tissue was removed from the mid-lamina region of each mature dried leaf. After gold sputtering, digital images were made with the aid of a scanning electron microscope at 10 kV. We observed the stomata ultra-structure and epidermal surface architecture from three fields of view per tissue. The descriptive terminology in this report follows Harris and Harris [23]. The term stomatal ultra-structure here is used to describe the exo-skeletal features on the cuticular surface. The term cuticular membrane refers to the complex covering of waxes, cutin, cellulose, and pectin associated with the external surface of leaf cell walls [24].

RESULTS

Section *Macranthae*:

Smilax verticalis (Fig. 1a–b)

Leaves amphistomatic. The abaxial and adaxial stomatal polar orientation is generally towards the leaf midvein. Each stomate is over-arched by the cuticular membrane that begins with an encircling crease and terminates in a distinctly elevated-elliptical pore rim (Fig. 1a). The abaxial surface is bullate and subdivided by a sinuous reticulum (Fig. 1b). The adaxial surface is bullate with a sinuous reticulum.

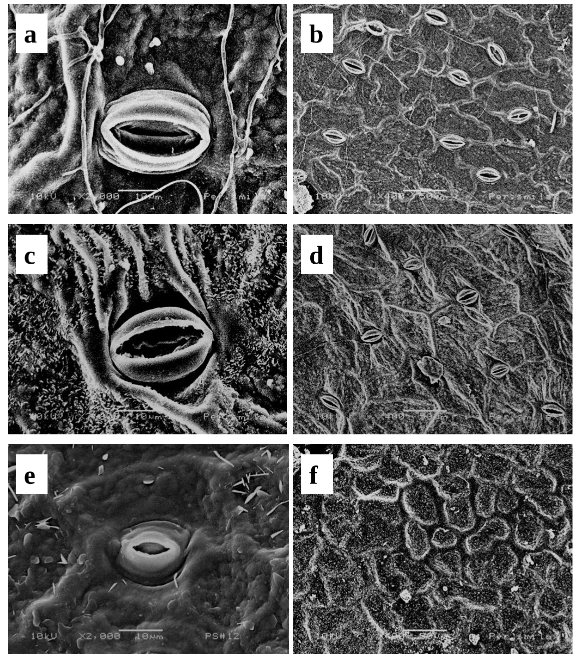

Fig. 1 *Smilax verticalis* (a–b): (a) SEM ($\times$ 200) abaxial stomatal architecture and bullate surface (scale bar = 10 µm). (b) ($\times$ 40) stomata orientation and abaxial sinuous reticulum (scale bar = 50 µm). *S. megacarpa* (c–d): (c) SEM ($\times$ 200) abaxial recessed stomate, perpendicular striae and minute furfuraceous ornamentation (scale bar = 10 µm). (d) SEM ($\times$ 40) abaxial stomata orientation, zig-zag shaped reticulum (scale bar = 50 µm). *S. bracteata* ssp. *verruculosa* (e–f): (e) SEM ($\times$ 200) recessed stomata and architecture, bullate ornamentation and reticulum (scale bar = 10 µm). (f) SEM ($\times$ 40) adaxial reticulum formed by convex periclinal epidermal cell walls and corresponding surface ornamentation (scale bar = 50 µm).

Smilax megacarpa (Fig. 1c–d)

Leaves amphistomatic. The abaxial and adaxial stomatal polar orientation is generally towards the leaf midvein. The stomata ultra-structure is elevated-oviform, recessed into the epidermal surface and has an elliptic pore. Striae radiate outward perpendicular to the stomatal polar axis (Fig. 1c). The abaxial surface has a zig-zag reticulum (Fig. 1d). The adaxial reticulum is similar the abaxial side.

***Smilax bracteata* ssp. *veruculosa* (Fig. 1e–f)**

Leaves are hypostomatic. The stomatal polar orientation is random. Stomata are recessed and over-arched by the cuticular membrane beginning with an encircling tangential perimeter that terminates centrally in a slightly elevated tangentially flattened pore rim with an elliptical pore. The abaxial surface is furfuraceous and has a distinctly sinuous reticulum (Fig. 1e). The adaxial surface has periclinal epidermal cell walls that are convex with 4–7 straight-sides and collectively form the reticulum (Fig. 1f).

Section *China*:

***Smilax micro-china* (Fig. 2a–b)**

Leaves hypostomatic. The stomata polar orientation is random. Stomata are over-arched by the cuticular membrane and densely ornamented with granulae. The stomata have a narrowly asymmetrical pore (Fig. 2a). The abaxial surface is heavily granular (Fig. 2b). The adaxial surface is sparsely granular and has a faint sinuous reticulum formed by the epidermal anticlinal cell walls.

Section *Coilanthus*:

***Smilax corbularia* ssp. *corbularia* (Fig. 2c–d)**

Leaves hypostomatic. The stomata polar orientation is random. Stomata are over-arched by the cuticular membrane and ornamented with granular stacks (Fig. 2c). The abaxial surface is papillate. Papillae form loose rows. Granular stacks ornament lamina surface (Fig. 2d). The adaxial surface is unremarkable.

Section *Coprosmanthus*:

***Smilax pottingeri* (Fig. 2e–f)**

Leaves hypostomatic. The polar axis of stomata is generally oriented towards the leaf mid-vein. Stomata are over-arched by the cuticular membrane and have furfuraceous ornamentation (Fig. 2e). The abaxial surface is ornamented by a reticulum of semi-circular to arcuate elevated rims and dense furfuraceous covering (Fig. 2f). The adaxial surface is sparsely furfuraceous.

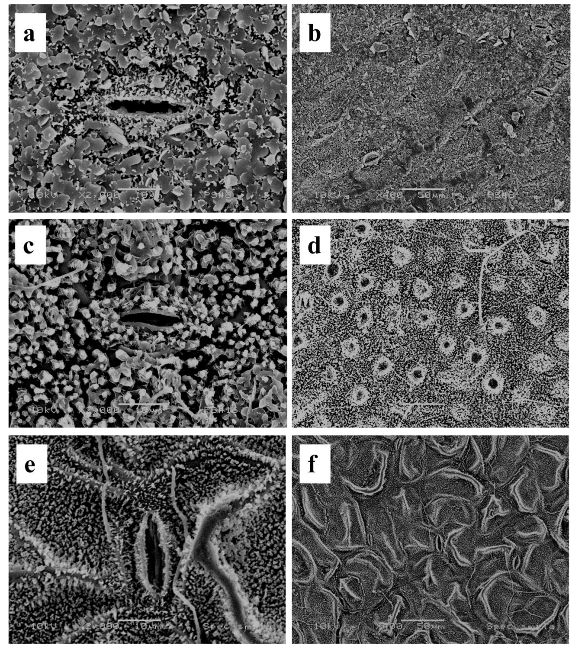

Fig. 2 *S. micro-china* (a–b): (a) SEM (× 200) stomatal architecture and granular abaxial surface (scale bar = 50 µm). (b) SEM (× 40) adaxial surface finely granular (scale bar = 50 µm). *S. corbularia* ssp. *corbularia* (c–d): (c) SEM (× 200) abaxial stomatal architecture and granular stacks (scale bar = 10 µm). (d) SEM (× 40) abaxial papillae (scale bar = 50 µm). *S. pottingeri* (e–f): (e) SEM (× 200) abaxial stomatal architecture and furfuraceous ornamentation (scale bar = 10 µm). (f) SEM (× 40) abaxial arcuate reticulum (scale bar = 50 µm).

DISCUSSION

Review of the summary of characters is given in the key of epidermal characters in Table 1. Of the six species studied *S. verticalis* and *S. megacarpa* have amphistomatic leaves.

Four species bear a surface reticulum on one or both leaf surfaces. All species show one or a combination of individually unique ornaments. Each taxon also exhibits a unique stomatal ultra-structure. One feature absent in section *Macranthae* species studied is the lack of ornamentation on stomatal ultra-structures. Presently, the importance of this is subjective. For the other three species, all displayed some form of ornamentation on the stomata.

Striking differences occur between cuticular architecture such as the densely granular abaxial surface of *S. micro-china*, the abaxial papillae and granular stacks of *S. corbularia* ssp. *corbularia* leaves, and the elevated arcuate-semi circular rims on the abaxial surface of *S. pottingeri*. The amphistomatic *S. verticalis* has an abaxial sinuous reticulum and bullate surface while *S. megacarpa* has a zig-zag reticulum.

The larger sample size ($n = 9$ plants) for *S. corbularia* ssp. *corbularia* represents a broader phytogeographic range and greater level of confidence for using abaxial papillae as a defining tool. It is the only *Smilax* species in Thailand with abaxial papillae[22].

Table 1 Key to taxa based leaf cuticular characters.

1. Leaves amphistomatic 2
    2. Leaves with abaxial and adaxial sinuous reticulum; stomata ultra-structure elevated, steeply-elliptic pore rim, abaxial and adaxial surfaces bullate . . . . *S. verticalis*
    2. Leaves with abaxial reticulum; stomatal ultra-structure oviform, elevated in recessed cuticular depression, striae radiate outward perpendicular to stoma polar axis, abaxial and adaxial reticulum zig-zag . . . . *S. megacarpa*
1. Leaves hypostomatic 3
    3. Leaves with abaxial and adaxial reticulum 4
        4. Abaxial reticulum sinuous, surface bullate, adaxial reticulum polygonal, stomatal ultra-structure recessed, encircled with non-ornamented tangential perimeter terminating centrally into an elevated tangentially flat pore rim with narrow elliptic pore . . . . *S. bracteata* ssp. *verruculosa*
    3. Leaves with abaxial reticulum 5
        5. Reticulum of individually-elevated semi-circular to arcuate rims, surface furfuraceous, stomata ultra-structure slightly elevated, stomatal ornamentation furfaceous . . . . *S. pottingeri*
    3. Leaves with adaxial reticulum 6
        6. Adaxial reticulum faint-slightly sinuous, abaxial surface heavily granular, stomatal ultra-structure heavily ornamented with granulae . . . . *S. micro-china*
    3. Leaves lacking reticulum 7
        7. Abaxial lamina papillate, papillae collectively forming loose rows, surface ornamented with minute granular stacks, stomatal ultra-structure ornamented with granular stacks, pore rim un-ornamented . . . *S. corbularia* ssp. *corbularia*

Although clear differences were seen in stomatal ultra-structure and cuticular characters for *S. micro-china* and *S. bracteata* ssp. *veruculosa* our confidence in these features is tentative for their use as an alternative taxonomic tool due to the small sampling pool, and an additional study using a larger sample size is desirable. These two species have only been reported from Phu Kradueng mountain, a remote location in Loei province. The former inhabits the mixed grass-pine forest on the mesa-like pinnacle in full sun; the coriaceous nature of leaves reflected by the heavy layering of abaxial granulae implies a utilitarian character for moisture conservation. For the latter, our study specimen grew under the closed canopy seasonally-wet forest nearly 1100 m above sea level. A cuticular membrane over-arching a stoma may be hypothesized as a moisture conservation feature. In this matter, all species of our study exhibited a thickened stomatal covering intimately associated with the leaf cuticle membrane which may be a common response character to extended seasonal dry periods.

Because male flowers are unknown for *S. pottingeri* and the single specimen available to assess cuticular morphology, our results should be considered preliminary. A search should be made to acquire additional specimens for further analysis. Similarly, analysis of *S. verticalis* should be applied to a larger sample of leaves to test the taxonomic significance of characters shown here. For *S. megacarpa*, a significant amount of herbarium material is available and further examination can be made on leaves from both genders. If it is shown that our results hold true for male and female leaves through the entire phytogeographic range, the diagnostic dependency on fruits or flowers would be less critical. Flowers of *S. micro-china* have not been described and although we collected males with flowers, two field-trips failed to produce flowering female plants. *Smilax verticalis* is one of the most difficult species to identify because of the large range in leaf polymorphism and verification of characters here will be very helpful to assess the extent of leaf variability. The geographic range of *S. bracteata* ssp. *veruculosa* and *S. micro-china* are presently understood to be limited to the same remote mountain location and it is interesting that Moore et al[3] described another locally-restricted taxon (*S. petiolatumidus*) from this isolated region.

Fossil *Smilax* remains have been reported from numerous Cenozoic localities in Europe[15,25] and North America[26–28]. For the US, the palaeodiversity appears similar to present day and a systematic investigation of North American Eocene species is presently underway (Moore, personal data).

Asia arguably may be a centre of diversity for the genus. The Flora of China (http://www.efloras.org/florapage.aspx?floraid=2) reports 79 *Smilax* taxa, ten of which also occur in Thailand where there are 28 species and subspecies[2,3]. These two floras thus represent about 25% of 350 species worldwide[1]. *Smilax* primarily inhabits sub-tropical and tropical regions but extends to temperate latitudes. Because the diversity is larger at low latitudes where there are proportionately fewer collections and as there is widespread difficulty in identifying taxa correctly, it is very likely that other taxa have not been recognized. Our preliminary data provides a basis for further investigation of leaf surface architecture to diagnose *Smilax* species. Because of the difficulty in collecting specimens in optimum reproductive condition, our results show that cuticular features in Thai *Smilax* taxa have the potential for the correct identification of incomplete or sterile specimens.

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