SEM and light microscopic studies in seeds of *Hibiscus surattensis* L. and phylogenetic attributes in Puducherry region, India

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**ABSTRACT**

The micro-morphological and scanning electron microscopic (SEM) studies in seeds of *Hibiscus surattensis* L. has brought out five interesting as well as taxonomically and phylogenetic important traits for the first time. *Hibiscus surattensis* L. belongs to the family Malvaceae, located at Puducherry region, India. 1. There is a change in the number of locules from pentacarpellary in the ovary during seed maturation and tetra-carpellary after seed maturation in the capsule; 2. Capsules contain both aborted and fully matured seeds recording an average of 4 aborted in 10 capsules; 3. Trichome on seeds consisted of bundle of unicellular finger-like structures, non-glandular and appressed; more importantly hilum is lined with trichomes; 4. Trichomes originate from the outer seed coat as extension/elongation of reticulate-foveate-type seed surface. 5. Embryo was folded occupying both poles and represented approximately the one-third of embryo. Size of the seed, colour, and shape of the seed, surface patterns of seed were examined using SEM. Factors responsible for the production of abortive seeds, functional attributes of non-glandular trichomes and other features are discussed in detail with relevant SEM and microscopic images. Further, phylogenetic importance in elucidating evolutionary clade is also explained. The partial sequence of chloroplast DNA of *Hibiscus surattensis* L. and neighbourhood joining trees are done for the first time in the study plant growing in India.

**Introduction**

The seeds of angiosperms vary greatly in their features and because of their reliable consistency these characters can be used significantly in the taxonomic range at different levels. The seeds of macro- and microstructure are very significant in the data classification of angiosperm taxa. Seed morphology is the important taxonomic delimitation tool of various families and genera (Ather & Qaiser, 2013; Corner & Corner, 1976; Fawzi, 2018; Hufford, 1995; Kanwal, Abid, & Qaiser, 2016; Karez, Engelbert, & Sommer, 2000; McClure, 1957). The report (Duke, 1961) mentioned that the seed structure provides a rather critical indication of the systematic position of species. The report (Boesewinkel & Bouman, 1984) stated that the study of seed structure may be helpful at lower taxonomic level. Further, comparative anatomical and micro morphological studies in combinations with palynology, systematics-related disciplines bring out many evolutionary relationships. To be precise, such studies provide the possibility of identifying specimens when other information is lacking or contradictory for assessing relationships of various populations (Judd & Manchester, 1997). Most of the traits of the family Malvaceae shares advanced and primitive traits. Similarly, morphology of seed enlightens primitive or advancement in their features. Most of the seed coats exhibit valuable taxonomic clues because of their complex and diverse morphological and anatomical features (Barthlott, 1981). Fruit or seed surface texture has been used in a variety of ways to solve taxonomic problems, illustration of the evolutionary relationship, and illumination of adaptive characters of fruit or seed surface (Karaismailoglu, 2015, 2019). The Brassicaceae, one of the largest angiosperm families include Ca. 340 genera and 3350 species. The northern hemisphere of temperate regions is distributed worldwide (Karaismailoglu, 2017).

During the last two decades, the use of scanning electron microscope has greatly increased our knowledge of the microstructures of seeds. Data relating to micro-morphological traits in seeds are used for a variety of purpose in solving systematic problems, identification of hybrids from segregating progenies, understanding the dormancy and associated physiological phenomenon, testing seed quality and understanding structural variations associated with pest and disease resistance.

In that pursuit, tribe Hibisceae (Family Malvaceae) has attracted great interest from biologists because of its potential as a source of fibre and its ornamental attraction. This tribe includes Gossypium and Hibiscus. Hibiscus species are utilised as food and as a source of cordage by many indigenous peoples in Africa. The wide variability of most species has offered annoying difficulties to
botanists when trying to delineate these species or intraspecific taxa resulting in an alarming accumulation of names (Van Borssum Waalkes, 1966).

With reference to genus Hibiscus, it is known from intensive literature collection on seed morphology that detailed studies on ultrastructural features of seeds of *Hibiscus surattensis* L. are very lacking. The report (Thakor, 2009) on the use of morphology of seed in 51 species of genus Hibiscus growing in Gujarat, India in which *Hibiscus surattensis* L. was not included. The recent report (Abid, Ather, & Qaiser, 2016) on the use of studies in 75 taxa belonging to 6 subfamilies in which only four species of genus Hibiscus viz. *Hibiscus caesium*, *Hibiscus lobatus*, *Hibiscus aristivalvis*, and *Hibiscus micranthus* were included but not *Hibiscus surattensis* L. Moreover, reports of Pfeil (2009) explained the phylogenetic status of many species of Hibiscus but not on micro-morphological- and SEM-based study. Therefore, it is reported that the present contribution is considered as first of its kind with reference to *Hibiscus surattensis* L. Hence, the aim of the study was (1) to determine the changes in locules of ovary during seed maturation, structure of trichome, ultrastructure of indumentum, hilum zone, and nature of embryo. (2) To examine the seed size, weight, colour, shape, and surface pattern of the seed. (3) To identify the species by morphological traits as well as molecular sequencing.

### Materials and methods

The study plant, *Hibiscus surattensis* L. [Figure 1(a), Figure 2(b)], is growing abundantly in dry lands of Puducherry region located at 12.0219°N and 79.8575°E along the Coromandel Coast of India. The *Hibiscus surattensis* L. is an indigenous scrambling annual commonly known as Wild Sour distributed throughout Africa and Asia. Leaves and stems are densely pubescent, with silvery hairs visible to the naked eye. It is a scrambling, annual herb that produces stems up to 10 feet (3 m) long. All parts of the plant, including the leaf stalks and weak stems are covered with small downward-pointing, soft prickles, and naked hairs. Leaf margins are serrated and often appear reddish, suggesting the presence of anthocyanin compounds. Flowers are bisexual, and pentamerous. The ability of *Hibiscus surattensis* L. to self-pollinate is considered as an evolutionarily advanced trait. Fresh and healthy

![Figure 1.](image1.png)  
(a) *Hibiscus surattensis* L.  
(b) *Hibiscus surattensis* L. in Habitat

![Figure 2.](image2.png)  
(a) Dry fruit  
(b) C.S. of dry fruit with seed
plants were collected and identified by the experts at Pondicherry French institute and Pondicherry University, Puducherry. Identification done based on morphological traits is also further confirmed by molecular sequencing and submitted to NCBI.

**DNA extraction and molecular sequencing**

The chloroplast DNA was extracted by the modified method of Makimura, Murayama, & Yamaguchi (1994). The purified plant DNA was resolved by electrophoresis in a 1% agarose gel in 1× TAE buffer. **PCR Amplification:** a PCR was performed in a total volume of 20 μl containing 10 μl master mixture, 1 μl of μM each of plant ITS FP (5’ ATGCGATACTTGGTGTGAAT3’) and plant ITS RP (5’ GACGCTTCTCCAGACTACAAT3’) and 40 ng of template DNA. Sequencing reactions were carried out in both directions using same forward and reverse primers used for amplification with Big Dye Version 3.1 kit (Applied Bio-systems) on an ABI-PRISM 3730 DNA Sequencer (Applied Biosystems). The sequences were assembled with BioEdit (Version 7.0.9.0). BLAST programme (NCBI) was used to found out the homology of potential sequences. The sequences were submitted to Gen Bank to display in public domain and received accession number from NCBI. The phylogenetic trees depicted the evolutionary relationship between taxonomic groups. The tree was generated with neighbour Joining tree of various species (Figure 10). Since the study plant showed phylogenetically as well as taxonomically important variations, molecular sequencing was done for authentication of the taxonomic position of the study plant *Hibiscus surattensis* L. neighbour joining tree of various species of Hibiscus based on plant ITS gene sequence were constructed and presented (Figure 10).

**Micro-morphological and scanning electron microscopic studies**

To examine ovary structure in terms of number of locules, matured and aborted seeds and carpals, 10 capsules from 10 plants were collected and average of each parameter was considered for discussion. Micro-morphological studies on cross-sections of ovary seed shape and colour were carried out using UBS linked Digital Microscope (AMCAP linked to computer). For SEM images of seed surface configuration, ornamentation, indumentum, C.S. of embryo and trichomes SEM available at Central Instrumentation Facility, Pondicherry University was used.

**Results**

Seeds of *Hibiscus surattensis* L. were collected freshly from the matured capsule. The ovary was pentacarpellary and the whole ovary was with soft hairy covering [Figure 2(a,b)] and it was ovate with pointed beak-like structure. There were 7–8 seeds in each capsule and exhibited axial placentation. Though the ovary was pentacarpellary, in the matured fruit/capsule the seeds were present in four locules with axial placentation [Figure 3(a–c)]. The shape of seed was reniform and centrally depressed. The colour of the seed was light brown to dark brown. The length of the seeds was in the range of 331–345 mm and estimated breadth was 270–279 mm. Weight of each seed ranged 302–309 mg in weight. The surface was reticulate-foveate with discontinuous concentric rings that increase in density towards hilum. Trichomes were present along the ridges discontinuously [Figure 4(a–c)]. SEM images of seed showing strophiole and hilum [Figure 5(a,c)]. The wider view of hilum lined with trichomes [Figure 5(c)]. Base attachment of seed with funicle (Hilum) (Figure 6). These trichomes were whith, unicellular, appressed, and non-glandular. Trichomes were fan like due to the fused appearance of 20–25 finger-like structure each measuring approximately 80–120 μm. A single bunch fan of trichomes looks like tentacles of octopus [Figure 7(a–d)]. Embryo was folded occupying both poles and represented approximately the one-third of embryo [Figure 8(a–c)]. The seed coat was double layered; the outer layer with trichomes on the outer side of the seed and inner covering endosperm (Figure 9). Since the study plant showed phylogenetically as well as taxonomically important variations, molecular sequencing was done for authentication of the taxonomic position of the study plant *Hibiscus surattensis* L. neighbour joining tree of various species of Hibiscus based on plant ITS gene sequence were constructed and presented (Figure 10).

**Discussion**

The most useful clues for recognition of seeds are usually the seed shape, colouring, hilum shape, surface architecture, and seed weight. The present study on these lines in *Hibiscus surattensis* L. has brought out many interesting as well as taxonomically and phylogenetic important traits in seeds based on SEM images for the first time in *Hibiscus surattensis* L.

Cross-sections of freshly collected ovaries of *Hibiscus surattensis* L. showed ovate-globase and spherical-ovoid with acute apex covered by tiny hooked spines [Figure 2(a)]. One such interesting aspect observed in ovary and capsule/dry fruit was the variation in the number of locules. Ovary was pentacarpellary with 5 locules and 12–13 developing seeds with axial placentation [Figure 3(a–c)], but it was tetra-locular in the
capsule stage with only 7–8 matured seeds with axial placentation [Figure 3(c)]. Such condition was noticed in 4 capsules out of 10 observed in 10 plants growing in different locations. Moreover, these variations were also found in capsules from the same plant. From these observations, it is learned that during the maturation of seeds in the ovary few of them gets aborted. Generally, seed maturation is controlled/regulated both by external environmental factors and internal hormonal regulations. In the case of Hibiscus surattensis L., these aborted seeds were found in the same plant as well as plants growing in different localities. Such occurrence indirectly implies that apart from the external factors, genetic factor in scripted in the genes operate the programmed cell death in the maturing seeds at one point of time during the seed maturation and/or hormonal regulations controlled by specific genes (Matilla, 2019; Ozga, Kaur, Savada, & Reinecke, 2016; Wiens, King, Nickrent, Calvin, & Vivrette, 1989). In such cases, it could reasonably be presumed that abortive seed production might also be a genetic character and this character can be considered as species specific in Hibiscus surattensis L. However, to elucidate such presumption, further study is required to trace out at what stage and what genetic cue is responsible for such abortion during seed maturation. Therefore, elucidation of important events that take place in the plant is undoubtedly one of the interesting areas of seed maturation.

Another taxonomically important seed trait is the morphometric characters. The length of the seeds examined was 331–345 mm and breadth was estimated in the range of 270–279 mm [Figure 4(b)] where the seed size range of Malvacean members is 1.8–4.8 × 1.2–5 mm. Weight of each seed ranged from 302 to 310 mg. Judd and Manchester (1997) found that the use of smaller seeds reflects herbaceous nature of the plant and larger seeds indicate primitive shrub or tree habit. Thus, the seed weight proves the herbaceous nature of the plant. The shape of seeds was reniform and centrally depressed [Figure 3(c)]. Fawzi (2018) found that the use of five species belongs to the genus Corchorus and family Malvaceae, the shape of the seed was characterized as ellipsoid in C. trilocularis, angular oblong in C. tridens and
C. depressus, triangular-cuneiform in C. capsularis, rhomboidal in C. olitorius. Present studies showed, in general, shape of the seed is directly related to the insertion are more or less reniform, as similar as the work by Chuang & Heckard (1983) and Juan, Pastor, & Fernandez, (2000). Abid et al. (2016) found that uses of reniform types of seeds are the characteristic trait of genus Hibiscus.

Seed colour was light to dark brown. Seed colour is another good reliable constant character and are widely used for delimitation of various taxa (Ahmed & Qaiser, 1989; Duke, 1961).

Indumentum on the seed surface of Hibiscus surattensis L. was interesting as well as taxonomically important trait because seed surface pattern is also considered an effective diagnostic parameter to delimit the various taxa (Jones & Safa, 1982; Juan et al., 2000; Kanwal, Abid, & Qaiser, 2012). Under light microscope, discontinuous concentric rings around basally located hilum were seen [Figure 4(a)]. SEM images further demonstrate distinct surface architecture of the seed. The outer surface layers of seeds were reticulate-foveate from which trichomes were originating in bunches. Trichomes are whitish structures that were randomly present along the concentric rings. Each trichome was unicellular hair-like units variously fused along their length to form fan-like structure as seen in Figure 7(a–c) and was appressed [Figure 7(d)]. Each fused fan-shaped trichome consisted of about 24–30 finger-like structures [Figure 7(b)]. The length of each finger-like unit approximately measured between 60 and 140 µm. It was understood from SEM images that trichomes were non-glandular as there was no secretary cells either outside or inside the unicellular finger-like structures or at the base of trichome. The defensive functions of non-glandular trichomes are less characterized; however, it serves as physical barriers that impede herbivore feeding in Solanum spp. both pre- and post-ingestion defences against caterpillars (Kariyat, Smith, Stephenson, De Moraes, & Mescher, 2017; Tattini et al., 2007). Trichomes are also considered as important theoretical and phylogenetic significance and useful in phylogenetic verification (Xiao et al., 2017). From the Figure 7(b,c), it is understood that trichomes seemed to be an extension/elongation of the reticulate-foveate seed surface. In this study, the surface of the seed ornamentations, which are reticulate-foveate may be

Figure 4. (a) Microscopic view showing seed shape, surface morphology, and position of hilum (red arrow indicates hilum) (b) SEM image of seed surface and seed length (c) Magnified hilum lined with trichomes (red arrow indicates hilum).
Figure 5. (a) SEM image of seed showing strophiole (b) Hilum (c) Hilum lined with trichome and wider view of hilum.

Figure 6. Base attachment of seed with funicle (Hilum).
given as good diagnostic characters at the specific condition. The seed surface ornamentation is expressed as reticulate-verrucate (Pinar, Adiguzel, & Geven, 2007) and verrucate (Atceken, Dural, & Citak, 2016) reticulate foveate, reticulate, verruculate, reticulate-ocellate, ocellate, ruminate (Karaismailoglu, 2019).

The density, length, and distribution pattern of trichomes on the seed coat play corresponding physiological roles in resisting natural stresses because of their specific structure (Xiao et al., 2017). Trichomes have an extensive role in plant–environment interactions (Figueiredo, Resende, Morales, Gonçalves, & Da Silva, 2013; Mershon, Becker, & Bickford, 2015; Wei et al., 2013). The trichome types have been successfully used in the classification of genera and even of species in certain families and in the recognition of interspecific hybrids (King & Robinson, 1969, 1970; Metcalfe & Chalk, 1950; Ramayya, 1972; Sporne, 1956). Ramayya (1962) used trichome types to determine the genetic limits among Compositae also.

Another phylogenetically and physiologically important trait observed in the seeds of *Hibiscus surattensis* L. was hilum – the scar or mark left on a seed coat indicating the point of attachment of seed (ovule) to funicle on ovary wall during developmental stages and it can be used as distinguishing characteristics of seeds. Type and nature of hilum are considered as fingerprint of the attachment of ovules to the ovary during the ovary maturation period. In *Hibiscus surattensis* L. hilum located at the depressed part of the reniform
seed was oval-round shaped [Figure 4(c), Figure 5(a–c)]. It was lined with trichomes and these trichomes might be involved in either protecting the hilum and/or regulating moisture/heat around hilum [Figure 5(c)]. After dehiscence, seeds fall on the soil and become vulnerable to atmospheric and soil temperature and moisture. The trichomes protect the seeds against environmental conditions till the seeds start germinating. According to the dendrogram evolved by Abid et al., (2016), strophiolate seed is one of the salient features of sub family Malvoideae under which five genera, Sida, Abutilon, Malva, Hibiscus, Malvastram, and Althaea fall in first subgroup by having reniform, broad reniform, transversally cuneate seeds with strophiole. Hibiscus surattensis L. was having reniform seed with strophiole. It was clearly seen in Figure 5(a,b) that strophiole was present as an outgrowth of hilum adjacent to hilum. Cross-section of embryo demonstrated
folded nature of embryo occupying both poles and lying as bent loop embedded in endosperm. Endosperm represented approximately one-third of the embryo [Figure 8(a–c)] and it was surrounded by inner layer of seed coat and trichomes were present in the outer surface of the seed coat (Figure 9). Due to some technical reasons, the thicknesses of these two layers were not possible to measure (Figure 9).

Since the study plant showed phylogenetically as well as taxonomically important variations, molecular sequencing was done for authentication of the taxonomic position of the study plant *Hibiscus surattensis* L. neighbourhood Joining tree of various species of Hibiscus based on Plant ITS gene sequence were constructed and presented (Figure 10). After submission to NCBI, the accession number for the study plant was obtained as MN 337237. The tree also throws light on the interspecies variation both in clad and genetic distance. It is also understood from the accession numbers and the countries that this is the first time where *Hibiscus surattensis* L. from India is sequenced.

**Conclusions**

Based on the microscopic and SEM studies in the seeds of *Hibiscus surattensis* L., it is reported that the present study is the first to provide a detailed micro-morphological features relating to the production of abortive seeds, changes in number of locules and seeds during seed maturation and structure of trichomes in the seeds of *Hibiscus surattensis* L.; second, all these information are taxonomically and phylogenetically significant and these can be utilized as an additional tool to strengthen the taxonomic delimitation and tracing the phylogenetic relationship of various taxa within the family Malvaceae.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

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