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Morphological and Anatomical Features of Cosmopolitan Hornwort: *Phaeoceros carolinianus* (Michx.) Prosk.

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Abstract

The morphological and anatomical characteristics of *Phaeoceros carolinianus* (Michx.) Prosk. were investigated. Samples were collected from Chiang Mai, Thailand, during September - October 2013. The gametophyte and the sporophyte samples including spores and pseudoelaters were examined under a light and scanning electron microscope. The gametophyte exhibits a single large chloroplast with a pyrenoid in the center, the absence of a schizogenous cavity, both smooth and tuberculate rhizoids along the median region of the ventral side and the presence of *Nostoc* colonies located on the ventral side of the thallus. The sporophyte consists of 4 - 5 assimilative layers and one layer of tetrads that surround the 16-celled columella (4×4 rows of cell). The crescent-shaped foot can usually be seen. The sporogenesis with successive division begins at the base and moves forwards to the tip in an acropetal direction. The spores are spinose on the distal surface while on the proximal face ornamentation performed slight variations with medium papillae over vermiculated triradiate faces.

Keywords: Gametophyte, hornworts, *Phaeoceros*, sporophyte

Introduction

Hornworts (Division Anthocerotophyta), comprising 10 genera [1-3] with approximately 200 - 250 species worldwide, are a relatively small group of plants exhibiting a great diversity in the tropics and subtropics throughout the world [4,5]. Hornworts represent a key group in understanding the evolutionary trend of plant forms because they are considered a sister group of tracheophytes [5-7]. Their fragility and difficulty in identification has caused them to remain unexplored worldwide [7]. The scarcity of morphological and anatomical information has limited understanding of hornwort diversity [4]. However, generic boundaries and relationships among hornwort species is in question [8] because hornworts' systematic still lack critical evaluation of morphological and anatomical characters of many taxa [4]. The diagnostic characters of hornworts are horn-like sporophyte that lack seta, the presence of a basal meristem and dehiscence along one or 2 vertical lines [3]. The gametophyte is always thalloid, mostly uniplastidic containing pyrenoids [5]. Therefore, comprehensive morphological and anatomical studies are a stepping stone for understanding the typical features of individual hornwort and for resolving taxonomic controversy. Due to the fact that the anatomical and morphological features are significant and determinant keys for appropriate identification and classification, studies on morphology and anatomy of hornwort species might help confirming the features of a particular genus which can be considered later in defining taxonomic relationships. Furthermore, these features would then provide the necessary data to clarify the evolution of the characters within the hornworts and between primitive land plants as mentioned by Cargill *et al*. [8].

The genus *Phaeoceros* Prosk. was first described by Proskauer in 1951, with about 40 currently accepted species [5]. The genus is easily recognized by a solid and smooth thallus, antheridial chambers with usually (1-)2-6(-8) antheridia, capsule walls without a dehiscence line, yellow spores with spines

either only on the distal face or on both faces [5,9]. There are only 2 species of this genus reported from Thailand [9-12], probably due to the paucity of bryological studies in this country [13]. *Phaeoceros carolinianus* (Michx.) Prosk. is one of the most widespread species and considered a cosmopolitan species [26]. Despite its widespread distribution, detailed descriptions of the *P. carolinianus* are still lacking. Since both gametophyte and sporophyte of hornworts possess features important for delimitating taxa [7], more comprehensive morphological and anatomical studies of both gametophyte and sporophytes are needed. Therefore, the aim of this study was to provide more detailed gametophyte and sporophyte features of *P. carolinianus*.

Materials and methods

Plant materials and plant sources

Samples of *Phaeoceros carolinianus* were collected from Doi Suthep, Chiang Mai, Thailand in September - October 2013. Some plants were maintained in a growth chamber at 22 - 25 °C, relative humidity at 80 - 90 % and with a 16/8 h light/dark photoperiod. The gametophytes and the sporophytes were examined with a light microscope (LM) and scanning electron microscope (SEM). Samples for the LM study were prepared using the whole mount method and the standard method of paraffin [14]. Fresh samples were cleaned, dissected and fixed in FAA I and SEM fixatives for LM and SEM studies, respectively as described next. Three to 10 pieces (replicates) for each sample were prepared. The voucher specimen (*Penjor 02*) collected from Doi Suthep-Pui National Park was deposited in the PSU herbarium.

General characteristics of the gametophyte and the sporophyte

Shape, colour, length and width/diameter of the gametophytes and the sporophytes were measured using a digital vainer caliper (Insize, USA).

The number of chloroplasts in each cell and the presence of pyrenoid were detected from the gametophyte thallus. The number of antheridia per chamber, the colour of mature antheridium were observed. The orientation of the jacket cells and the number of cover cells around the archegonial neck were also noticed.

The surface structure of rhizoids from the ventral surface of thallus was observed. The location and type of tubers were also examined.

Capsule wall, spores and pseudoelaters

The presence of stomata in the capsule wall and the thickness of the epidermal wall were examined. The colour and size of spores and pseudoelaters were also determined.

Detailed anatomical features

Gametophyte: The number of epidermal cell layers and internal cells including their shapes and differentiation were observed.

Sporophyte: The features of the foot, the basal meristem, epidermal layer, the shape and number of assimilative cell layers, the sporogenous tissues and columella were examined.

LM observation

The dissected gametophyte thalli (5×5 mm^2) and the whole length of sporophytes (each with 3 or 4-cutting pieces) were prepared for transverse section and for both transverse and longitudinal sections, respectively. Each sporophyte, consisting of the meristematic region to mature capsule, was divided into 3 parts (tip, middle and foot). Transition of spore mother cells (SMCs) to mature spores and pseudoelater mother cells (PMCs) to pseudoelaters were also investigated. Samples were fixed in FAA I (formaldehyde: glacial acetic acid: 50 % ethyl alcohol; 5: 5: 90 v/v/v) for 24 h at room temperature [14]. After fixation, samples were dehydrated gradually through a graded ethanol-tertiary-butanol series, infiltrated and embedded in Histoplast (Histoplast PE; Richard-Allan Scientific, Michigan, USA) using Histo-embedding machine (Thermo Scientific; HistoStar Cold Plate). Serial sections 10 - 13 µm thick

were cut on a rotary microtome. Sections were affixed on slides and stained with safranin and fast green. They were observed using an Olympus BX 51 LM (Olympus America Inc., New York, USA) fitted with an Olympus DP72 digital camera (Olympus Latin America Inc., Florida, and USA).

SEM observation

The surface view of the gametophyte and the capsule as well as the pattern of spores and pseudoelater were examined. The samples were fixed in SEM fixative (10 % (v/v) formaldehyde, 5 % (v/v) acetic acid, 45 % (v/v) ethanol and 1 % (v/v) triton X-100) for 3 h at 4 °C. After rinsing 3 times with 0.1 M phosphate buffer (ph 7.2), samples were dehydrated in graded ethanol series and critical point dried using CO_2, affixed to stubs with sticky tape and coated with gold. These samples were photographed with a scanning electron microscope (FEI, Quanta 400) operated at 15 to 20 kV.

Results

Gametophyte characteristics

Thallus: *Phaeoceros carolinianus* (**Figure 1A**) grows in damp-shade areas. The rosette shaped thallus has a smooth margin, branched and it is green to dark green being 10 - 20 mm in length and 5 - 10 mm in width. No gemma is observed on the thallus. A transverse section illustrates that the thallus consists of the occasional enlarged cells without mucilage and schizogenous cavity. Dorsal epidermal cell exhibiting a rectangular-hexagonal shape is about 23 - 62 µm long and 20 - 32.5 µm wide and (**Figures 1B and 1C**) contains a single large chloroplast with pyrenoid in the center (**Figure 1D**). The chloroplasts of the epidermal layer and the interior cells are identical, but differ in size depending upon the size of the cell. The thallus is approximately 9 - 12 cells thick near the middle and it tapers gradually towards the margin (**Figure 1F**). The single-celled rhizoids are elongated. Two types of rhizoids, 1) smooth and transparent rhizoids and 2) pale brown tuberculate rhizoids (**Figure 1E**), are observed along the median thickened region on the ventral side of the thallus. The greenish blue spots of *Nostoc* colonies are irregularly scattered throughout the internal chambers at the ventral side of the thallus. (**Figure 1G**). Unfortunately, the tuber structure could not be observed.

Gametangia: *Phaeoceros carolinianus* is monoicous and produces both antheridia and archegonia on the dorsal side of the same thallus. However, the antheridia mature and release their spermatozoids before the archegonia are mature. Each antheridial chamber contains 1-8 antheridia (**Figure 1H**). The mature antheridium is yellow-orange, subglobose or rounded and approximately 75 - 142 µm in diameter. The antheridium with the irregular arrangement of the jacket cells can be shown. The solitary archegonium is located near the tip of the thallus.

Sporophyte characteristics

The sporophytes consist of 2 parts as follows: 1) a spheroid foot which is embedded in the gametophyte tissue and 2) an elongated cylindrical capsule, having a length of approximately 40 - 60 mm, which is a spore-bearing region including epidermis, the assimilative layer, the sporogenous tissue, the columella, and the basal medium. This basal medium which intercalates between the capsule and the foot is encircled by conical-cylindrical involucres which reach to 4 - 5 mm long and 6 - 9 cells thick in the middle. The involucre remains as a cylinder that surrounds the base of the sporophyte. The details of the sporophyte parts presented herein are described next; starting from the foot and moving upward to the mature tip of the capsule.

Gametophyte-sporophyte junction

Phaeoceros carolinianus presents a spheroid foot which differentiates into 2 regions; a central parenchymatous region that subtends the basal meristem and the haustorial cells which are elongated or palisade-like cells (**Figure 2A**). These thin-walled parenchyma cells in the inner foot are 25 - 68.8 µm in diameter, compact and irregular in shape (**Figure 2B**) while the peripheral parenchyma cells differentiate into the haustorial cells which penetrate and interdigitate with the surrounding gametophyte cells. The placental region, the area of the sporophytic haustorial cells and the gametophytic transfer cells are

closely intermixed, exhibiting the presence and absence of wall ingrowth of the sporophyte cells and the gametophyte cells, respectively. These haustorial cells occasionally branch and infiltrate into the gametophytic tissues (**Figure 2C**). In addition, the placental cells of the gametophyte are smaller than those of the surrounding cells (**Figure 2C**).

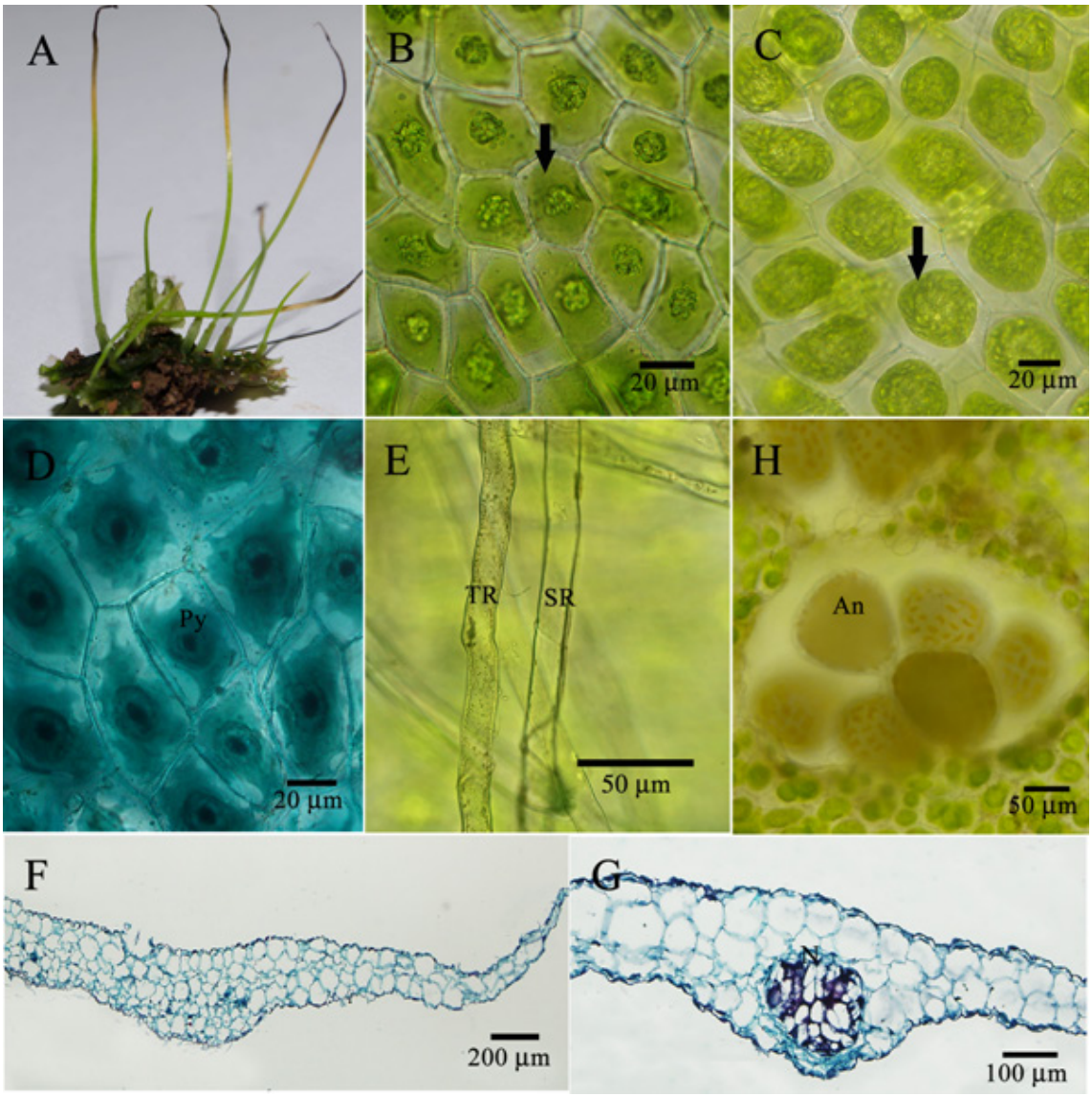

Figure 1 *Phaeoceros carolinianus* plant. A. Gametophyte with mature sporophytes. B. Dorsal epidermal cell with chloroplast (arrow) of fresh plant. C. Dorsal epidermal cell with chloroplast (arrow) of plant grown in a growth chamber. D. Gametophyte showing pyrenoid (Py) stained with Amido-black; Black dot in the centre of chloroplast. E. Tuberculate rhizoid (TR) and smooth rhizoid (SR) in the ventral surface of the gametophyte. F, G. Transverse section of thallus showing a *Nostoc* colony (N). H. Antheridia (An) in a chamber. All photographs from *Penjor 02* (PSU).

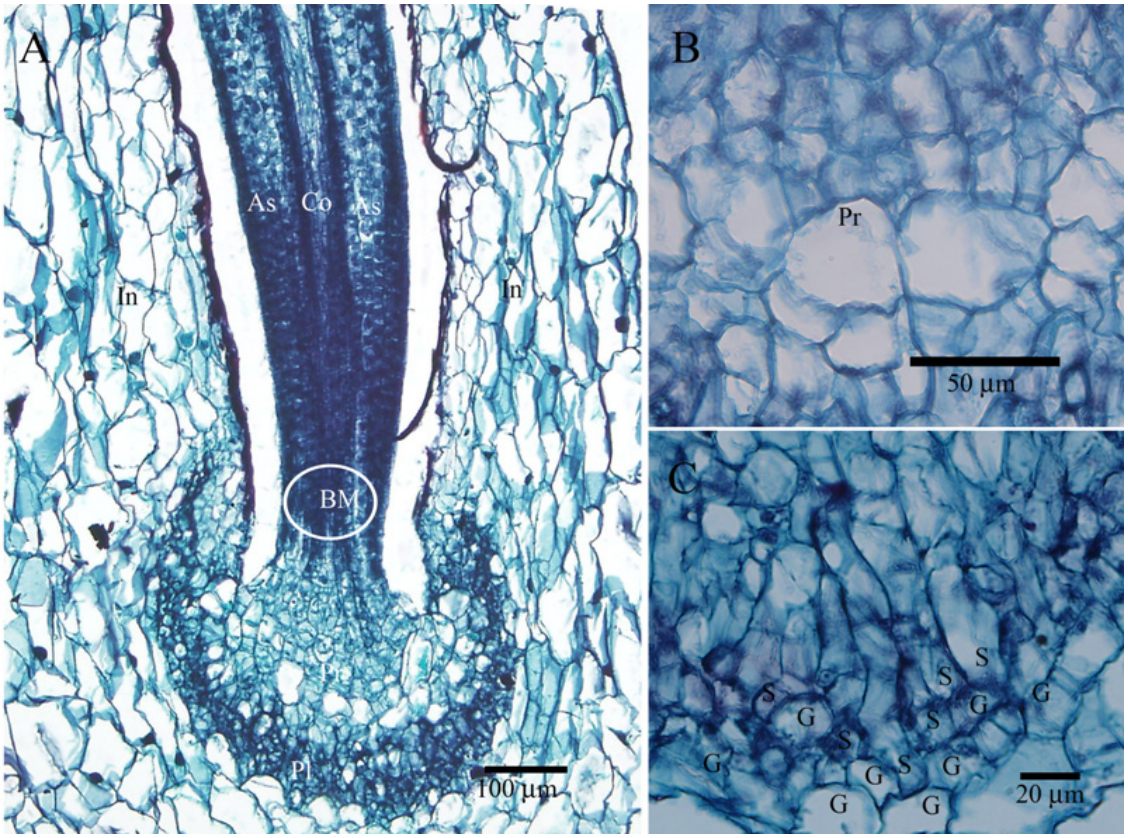

Figure 2 A. Longitudinal sections of basal part and foot of the sporophyte exhibiting involucre (In), placental region (Pl), parenchymatous region (Pr), basal meristem (BM) and other sporophytic tissues above intercalary meristem. B. High magnification of parenchymatous region. C. High magnification of the peripheral part of foot in placental region showing sporophyte haustrorial cells (S) and gametophyte cells (G). All photographs from *Penjor 02* (PSU).

Basal meristem

The thin-walled cells of the basal meristem located just above the foot are completely enclosed within the involucre. The basal meristematic cell contains a deeply stained nucleus in a dense cytoplasm (**Figures 3A** and **3D**). These compact cells have no intercellular space.

Immature capsule

Capsules are erect and cylindrical. Immature capsules are green and constitute all capsule tissues: the epidermal layer, the assimilative cell layers, the spore mother cells (SMC), the pseudoelater mother cells (PMC) and the 16-cells columella (**Figures 3B** and **3E**). The epidermal cells lacking stoma are uniformly thickened wall along the outer surface. The thin-walled assimilative cell has a single chloroplast. The innermost assimilative layer is elongated and has thicker wall towards the sporogenous space. SMC and PMC are found in meiotic and mitotic phases, respectively (**Figure 3E**). Tetrad of microspores which are inside the SMC wall are found at 7 - 10 mm above the involucre.

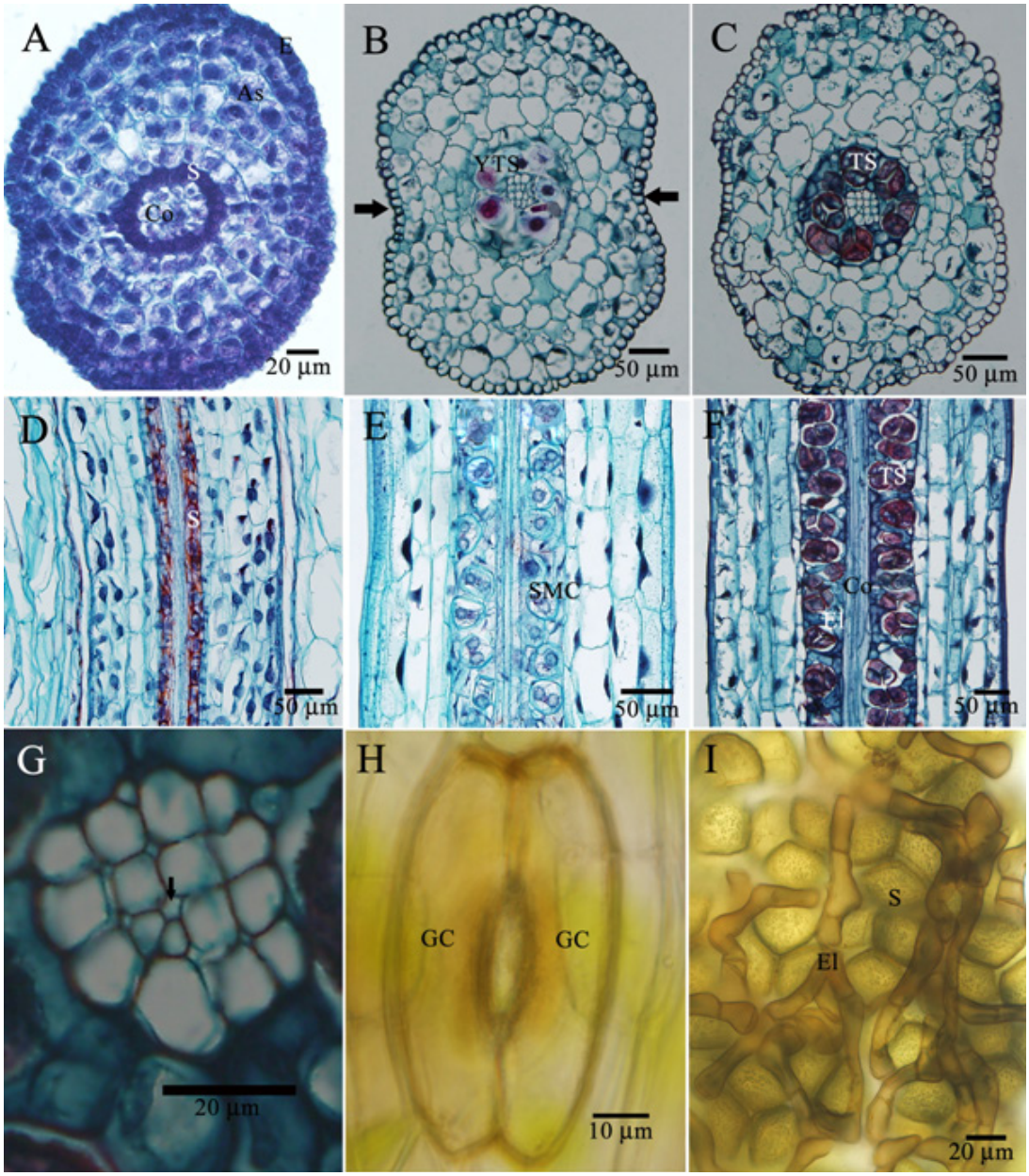

Figure 3 Details of sporophyte and spore development of *P. carolinianus.* A-C. Transverse sections at different regions of the sporophyte with different developmental stages; A. Meristem with a layer of sporogenous tissue (S) surrounded columella, single layer epidermal cells (E) and 4 layers assimilative cells (As), B. Section above involucre with young tetrad spores (YTS). C. Section from mature capsule with tetrad spores (TS). D-F. Longitudinal sections of sporophyte: D. Meristem region with sporogenous tissue, E. immature region containing spore mother cells (SMC) in meiotic phase, F. mature capsule with TS. G. High magnification of columella with intercellular space (arrow). H. Stoma with a pair of guard cells (GC) on the capsule wall. I. Bright yellow spores (S) and brown pseudoelaters (El). All photographs from *Penjor 02* (PSU).

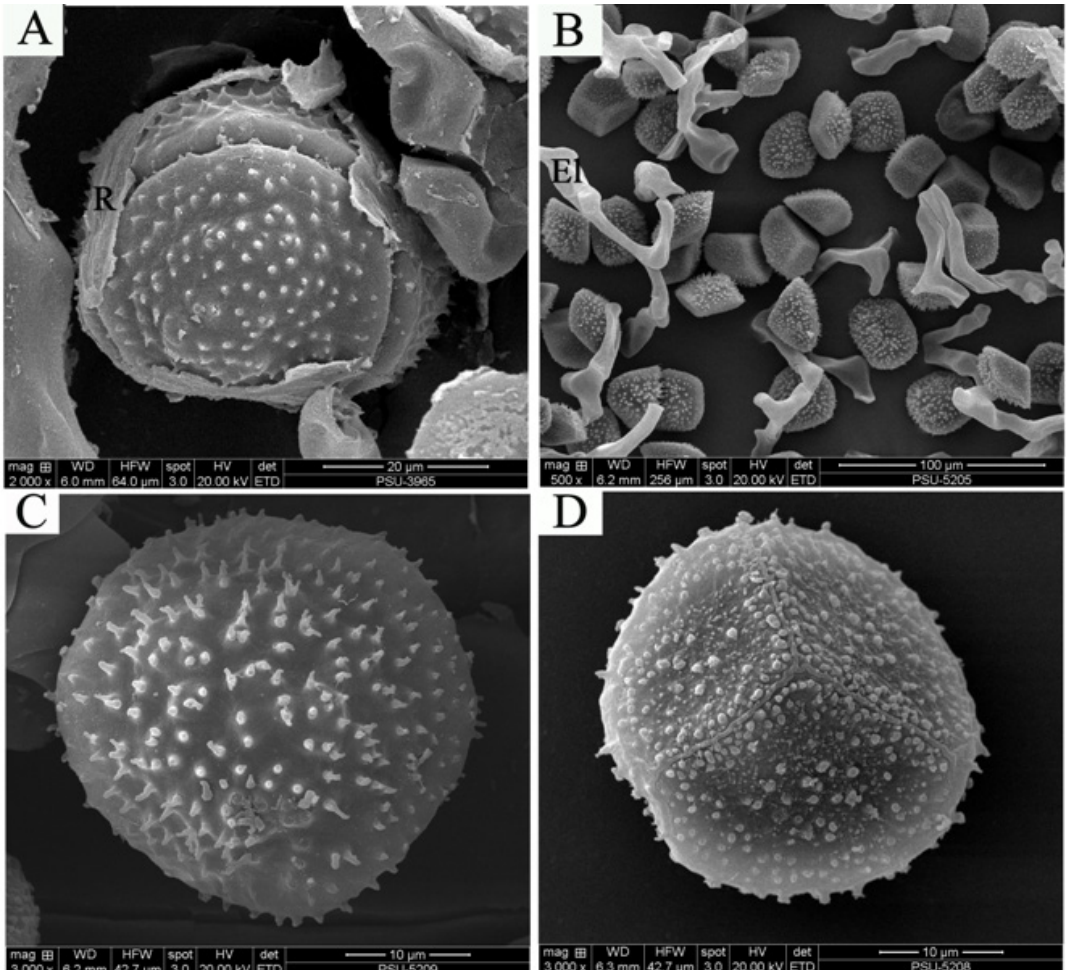

Figure 4 SEM micrographs of *P. carolinianus.* A. Tetrad of spores inside the remnant (R) of spore mother cell wall. B. Spores and pseudoelaters (El). C. Distal face covered with spines. D. Proximal face with medium-minute papillae. All photographs from *Penjor 02* (PSU).

Mature capsule

The mature capsule is yellow and reaches a length of 60 mm and 0.3 - 0.5 mm in diameter. The mature capsule is comprised of a layer of epidermal cells, 4 - 5 layers of assimilative cells with the intercellular space, a single row of spores and pseudoelaters, and the 16-celled columella (4×4 rows of cells) in the center (**Figures 3C** and **3F**). The thin-walled columella cells are small (4.5 - 15.5 µm in diameter and 1 µm thick wall) and maintain small intercellular spaces (**Figure 3G**). The epidermal cells are 95 - 195 µm long, 10 - 12.5 µm wide with uniformly thickened outer tangential walls. Stomata each with a pair of narrow guard cells are dispersed among the epidermal cells (**Figure 3H**). The guard cell is approximately 70 - 76 µm long and 12 - 16 µm wide. The thin-walled and undifferentiated assimilative cells are 30 - 55 µm in diameter and possess small intercellular space. These cells are larger in size than those of the epidermal cells. The sporogenous tissue is located between the assimilative region and the columella.

A tetrad of microspores comes out from the remnant of the SMC wall to get separated into individual spores. Concurrently, most of the long multicellular pseudoelaters break themselves into a

unicellular pseudoelater (**Figure 3I**). The dehiscence region of the capsule is slightly depressed inward (**Figure 3B**) due to the comparatively smaller and lesser layers of the assimilative cells. This dehiscence begins from just below the tip with 2 valves. Spores are released through the valves which are held together at the tip and enlarge progressively downward. The part of capsule that had released spores gets dried and valves twist and curl outwards.

Spores and pseudoelaters

Sporogenesis and spore maturation appear from the base to the apex of the sporophyte capsule. The spores in the capsule develop asynchronously. The early developmental stage of sporophyte development including the sporogenesis and the elaterogenesis begins at the young sporophyte still remained in the involucres. At the base of sporophyte, the basal meristem is composed of a ring of a single cell row of archesporium (single layered sporogenous tissues) encircling the columella (**Figure 3A**). Further division appears in the archesporium to produce a row of spore mother cells (SMC) which are alternatively with a row of the elaterocytes (PMC) embedding in the mucilaginous space surrounds the columella (**Figure 3D**). SMC is round with conspicuous large nucleus while PMC is small and slightly elongated. These elaterogenous cells are continue to divide by repeated mitotic division and then elongate to form the short-elongated pseudoelaters. SMC undergoes meiosis and the complete meiotic division can be observed at 4 - 5 mm above the involucre (**Figure 3E**). Young tetrad is enclosed in the remnant of the SMC wall until it is fully matured (**Figure 4A**). The mature spores are released and dispersed at 15 - 20 mm above the involucre (**Figure 3F**). Mature spores are bright yellow to pale brown, rounded-tetrahedral with conspicuous proximal and distal surfaces (**Figures 3I** and **4B**). The spore size is approximately 32.5 - 42.3 μm in diameter (**Figure 4C**). Distal surface of the spore is covered with numerous spines (**Figure 4C**). Spore proximal surface exhibits the triangular faces which are covered with the minute-medium papillae on vermiculate ornamentation (**Figure 4D**).

PMCs undergo repeated mitotic division to form the multicellular pseudoelaters. Mature pseudoelaters are pale brown, smooth and usually branched (**Figures 3I** and **4D**). A pseudoelater cell is approximately 37 - 80 μm long with a 1.25 - 1.5 μm thick wall.

Discussion

Phaeoceros carolinianus is basically identical to those of other hornworts, for instance, having a foot embedded in gametophyte and a cylindrical, upright sporangium. This species is a monoicous plant and exhibits the simple dorsoventral thallus lacking differentiation. The dorsal epidermal cells containing a single large chloroplast are rectangular-hexagonal in shape. The internal thallus cells are isodiametric and smaller than the cells in the ventral side. The conspicuous globose *Nostoc* colonies invade the thallus through the ventral mucilage clefts, which is a common feature of Anthocerotophyta [15]. The *Nostoc* colonies are seen residing in the intercellular space.

Thallus of *P. carolinianus* contains occasional enlarged cells without schizogenous cavity which is a primary character of *Phaeoceros* [16]. The species reported herein has both types of rhizoids, smooth and tuberculate, exhibiting along the median region of the ventral side of the thallus. The occurrence of these rhizoid types might depend on the developmental stages of the thallus. The smooth rhizoids were regularly noticed on the young thalli or near the thallus apices while the tuberculate rhizoids were observed on the mature or the old thalli. However, the intermixing between the smooth rhizoids and the tuberculate rhizoids were rarely noticed. According to Crandall-Stotler *et al*. [17], the appearance of the tuberculate rhizoid is due to the deposition of wall material in the inner surface of the cell wall, but the author did not specify the actual components of the deposits.

Although the hornwort thallus usually develop many tubers in the ventral side to protect its survival from the unfavorable environmental conditions particularly in winter [18,19], no tuber formation can be observed in the *P. carolinianus* presented here. It is possible that the habitat at Doi Suthep, Chiang Mai, Thailand could be most well-suited for *P. carolinianus.* The young antheridium is green but turns yellow-orange at maturity. This change is classic and common due to the loss of green chlorophyll resulting from the conversion of chloroplast to chromoplast within the antheridial cells [20,21. In addition, a less regular

arrangement of the antheridial jacket cells of *Phaeoceros* is common appearing when the antheridium grows up [16]. Renzaglia [20] noted that the genus *Anthoceros, Dendroceros* and *Phaeoceros* exhibit several archegonia in a single gametophyte resulting in the formation of one or more sporophytes after fertilization. However, this *P. carolinianus* has shown that the antheridium may form before the archegonium which resemble those of archegonia and begin to develop when the mature antheridia are noticed [20,22]. More research of the reproductive crisis, such as on the fertility of gametes, of this species are required.

Although the sporophyte foot of *P. carolinianus* reported herein is a spheroid, the sporophyte foot shape of hornworts generally exhibit a great variation and usually presents a globose [20,21]. In addition, the anatomical organization of the foot is highly variable among species. Renzaglia [20] revealed that a more rounded, less massive foot can be found in *Anthoceros* while a large massive foot is presented in *Phaeoceros*.

The members of *Phaeoceros* usually exhibit a difference in the thickness of the placental region and cellular organization due to the sporophytic haustorial cells are straight and runs directly from the parenchyma region towards the surrounding gametophyte tissues. The features of the gametophytic transfer cells are similar to other hornworts with smaller in size than those of the surrounding gametophyte cells [15]. Besides, the intermixing of sporophyte and gametophyte cells in the placental region is to increase the surface area for up-take nourishment by the sporophyte [20-22].

The basal meristem produces specified cells above the foot that differentiate upwardly. The meristem undergoes repeated periclinal division to form the epidermal tissue layer, the assimilative tissue layers, the sporogenous tissues and the columella [15,21,22]. These tissues add spontaneously the new cells resulting in the elongation and the expansion of the sporophyte tissues which can continue to produce the spores throughout the growing season [15,21].

The capsule of *Phaeoceros* contains 4 - 5 layers of the assimilative cells. The outer layer cells are irregular in size with large chloroplast and intercellular spaces. According to Villarreal and Renzaglia [15], photosynthesis occurs in these outer layers of assimilative cells and the intercellular spaces facilitate gas exchange among cells. *Phaeoceros carolinianus* also contains a single layer of microspore tetrad, product of archesporial cell layer, which is similar to the genus *Phaeoceros* [16,20]. In addition, this species presents epidermal cells and the assimilative cells of the dehiscence region are smaller and fewer than the surrounding cells, respectively.

The sporogenesis begins at the early stage of the sporophyte development which is similar to those of other hornworts [23]. The immature spores of *P. carolinianus* are yellow and they remain yellow even at maturity. The spore color is related to the spore longevity, for instance, yellow and brown spores are long-lived because of thicker wall filled with oils. Pseudoelaters show great range of variation within the hornwort group [20]. For instance, elongated, branched, unspiralled multicellular pseudoelaters are found in *Phaeoceros* while elongated, unbranched, unispiralled pseudoelaters occur in *Dendoceros* and *Megaceros.*

In the present study, *P. carolinianus* bears the spores with spines in distal face which looks identical to the spores described by previous authors [19,24,25]. In addition, the species presented here has the spore exhibiting slight variation which possesses minute-medium papillae architecture throughout the vermiculated triangular faces of proximal face. However, the previous studies reported that this species hold the spore with papillae concentrated in the center of the triangular faces of proximal face [4,19,24]. Spore shape, wall ornamentation and pseudoelater architecture are variable across taxa and are widely used in taxonomy.

Conclusions

The features of *P. carolinianus* are upholding the taxonomic status. However, 2 apparent variations, lack of tubers in the ventral side of the thallus and architecture of the spore throughout the vermiculated triangular faces of proximal face, could be noticed.

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