Hemi-epiphytism in *Vandenboschia collariata* (Hymenophyllaceae)

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Abstract. Hemi-epiphytism represents an evolutionarily important yet poorly understood growth habit in ferns. Anatomy and ontogeny related to hemi-epiphytism in the fern *Vandenboschia collariata* (Hymenophyllaceae) were investigated at La Selva Biological Station, Costa Rica. Multiple specimens representing all stages of gametophyte and sporophyte growth were observed in the field, collected, and examined in the laboratory. Cross sections of sporophyte roots and stems were made by hand, and examined using light microscopy. Sporophytes were found to initiate growth on the base of trees up to ca. 25 cm above the ground. Two types of stems were produced: 1) a short stem that produces crowded roots, and 2) one or more climbing stems that branch from the short stem, climb up the trunk, and produce distant leaves but no roots. This study presents the first description of gametophytes in *V. collariata* and clarifies ontogeny and anatomy related to the hemi-epiphytic growth habit in ferns.

Key Words: Costa Rica, fern, gametophyte, hemi-epiphyte, Hymenophyllaceae, La Selva Biological Station, *Trichomanes*, *Vandenboschia*.

Although ferns are well known as an ancient lineage with a long fossil record, the majority of fern species evolved only recently, in the shadow of angiosperms (Schneider et al., 2004). The transition from a terrestrial to an epiphytic growth habit is thought to have been a critical step in fern diversification. About 30% of polypod ferns are epiphytes, completing their entire life cycle using another plant as a substrate (Schuettpelz, 2007). Besides terrestrial and epiphytic ferns, several species blur this distinction and grow as hemi-epiphytes. It is thought that hemi-epiphytism in these plants is a secondary evolution and does not represent the original transition from terrestrial to epiphytic growth (Schneider, 2000; Dubuisson et al., 2003; Hennequin et al., 2008); however, such ferns present a unique opportunity to investigate this evolutionarily important transitory growth form.

Due to the complexity of hemi-epiphytism, categorization of this growth habit is difficult. Benzing (1990) proposed two general types of hemi-epiphytism: primary hemi-epiphytism, in which growth is initiated on the host plant and adventitious roots descend to the soil secondarily, and secondary hemi-epiphytism, in which growth is initiated in the soil and a scandent stem subsequently climbs up the host plant. Dubuisson et al. (2003) segregated an additional type from these two categories: true lianescence, in which contact with the soil is mandatory, but a climbing stem may or may not be present (as opposed to a possible secondary loss of contact with the soil in secondary hemi-epiphytes).

Although Benzing's system provides a useful framework for describing hemi-epiphytes, it is difficult to assess without rigorous field observations. Herbarium specimens often feature only aboveground leaves and lack samples or descriptions of the stem or root. Routine field observations of only the adult plant are insufficient to determine how hemi-epiphytism was established, and omit the ecologically critical gametophytic phase of the life cycle (Farrar et al., 2008). Case studies including careful field

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observation of both gametophytes and sporophytes are needed to clarify the details of this complex growth habit. Such studies may shed light on the original transition from terrestrial to epiphytic growth in ferns, as well as reveal details of fern reproductive ecology that can aid conservation efforts.

The trichomanoid filmy fern *Vandenboschia collariata* (Bosch) Ebihara & Dubuisson (Hymenophyllaceae) was selected to investigate the anatomy and ontogeny of the hemiepiphytic growth habit in ferns. The mature sporophyte of *V. collariata* is dimorphic, with a long-climbing, rootless scandent stem that produces distant leaves and a short (<5 cm long) stem that produces approximate roots similar to those observed in fully terrestrial confamilial species (Fig. 1C). *Vandenboschia*

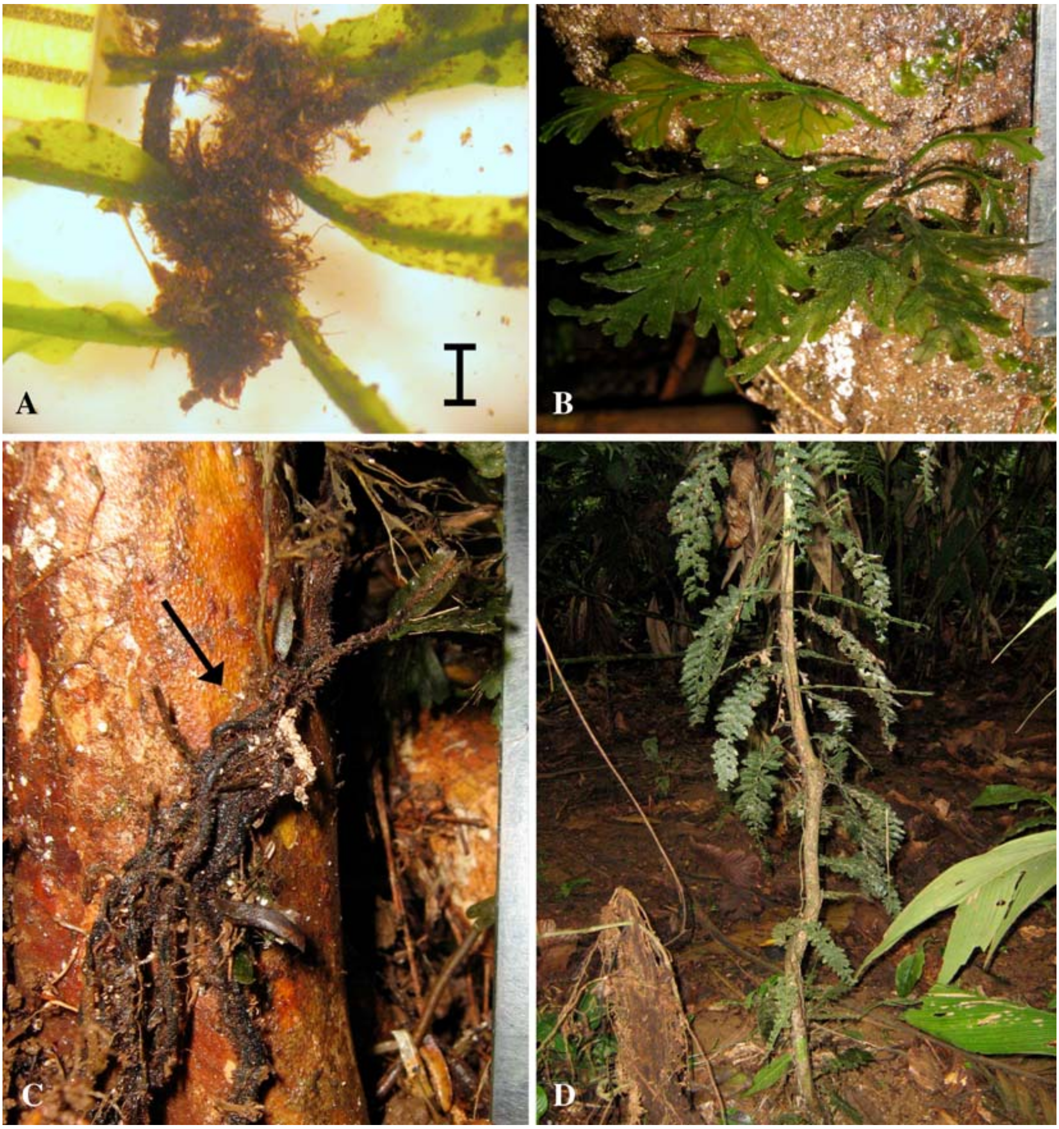

Fig. 1. *Vandenboschia collariata*, sporophyte habit and morphology. **A.** Juvenile sporophyte, showing short stem with leaves on short internodes. Scale bar = 1 mm (*Nitta 179*). **B**. Juvenile sporophyte on base of trunk (*Nitta 173*). **C**. Mature sporophyte, showing short stem (arrow) and roots extending to ground (*Nitta 179*). **D**. Mature sporophyte, habit (*Nitta 172*).

collariata was previously categorized by Dubuisson et al. (2003) as a secondary hemi-epiphyte; however, this conclusion was based on literature descriptions and herbarium samples rather than field observation. Field observation and anatomical examination of gametophytes and sporophytes at a variety of growth stages were conducted in order to clarify the ontogeny and anatomy related to the hemi-epiphytic growth habit in this fern and to provide a framework for further evolutionary studies investigating other hemi-epiphytic fern species.

Methods

Field observations were made of multiple *Vandenboschia collariata* individuals representing a variety of growth stages (gametophyte, juvenile sporophyte, mature sporophyte) at La Selva Biological Station, Costa Rica (10.4310 N, −84.0036 W). Field observations for gametophytes included substrate type, location on substrate, and area of gametophyte mat. Field observations for juvenile sporophytes included substrate type and location on substrate. Field observations for mature sporophytes included the following: substrate type, number of roots, length of short stem, diameter of short stem, location of short stem on substrate, number of climbing stems, leaf internode length, and height of first fertile leaf. Voucher specimens (*Sundue 1479* and Nitta *171–179*) were deposited at UC, CR, INB, and TI.

To investigate anatomy, specimens were fixed in 70% EtOH. Cross-sections were made by hand and stained with cresyl violet acetate. Sections were mounted with 40% calcium chloride and examined under an Olympus CH30 compound microscope. Photographs were taken with a Canon PowerShot S5 camera.

To confirm species identification, representative specimens of both gametophyte and sporophyte growth stages were selected for DNA sequencing of chloroplast *rbcL*. This gene is a reliable species-level marker in the trichomanoid lineage (Nitta, 2008). Total genomic DNA was extracted from silica-dried samples using the Plant Dneasy Mini kit (Qiagen, Valencia, California) following the manufacturer's protocol. Chloroplast *rbcL* was amplified in 20 μL reactions containing 2X Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 10 μM primer 26F, 10 μM primer 1379R, 1 μL of total genomic DNA, and de-ionized water to volume following the protocol of Pryer et al. (2001). PCR products were checked for successful amplification on a 1% agarose gel in TAE buffer. PCR products were cleaned using Montage PCR Centrifugal Filter Devices (Millipore, Billerica, Massachusetts) following the manufacturer's protocol. Automated sequencing of purified PCR products was performed on a CEQ 2000 Genetic Analysis System (Beckmen Coulter, Fullerton, California) following the manufacturer's protocol. In addition to the primers used for PCR amplification, internal primers H1R1, TKT-F2N-2—TKT-R3N-2, and TKT-F1—TKT-2PRN were also utilized for sequencing (Ebihara et al., 2003, 2007). The resulting chromatograms were assembled into sequences using ATGC multialignment software (Genetyx, Tokyo, Japan), and edited manually. Sequences are available on GenBank (http://www.ncbi.nlm.nih.gov/).

Results

Results of field observations are summarized in Table I. Gametophytes were observed to form mats on the base of tree trunks from 6–50 cm above ground (Fig. 2A). The mats varied in size, ranging from 0.5×1 cm to 5×

TABLE I
RESULTS OF FIELD OBSERVATIONS OF *VANDENBOSCHIA COLLARIATA.*

Gametophyte		Juvenile Sporophyte	Mature Sporophyte - Short Stem					Mature Sporophyte - Climbing Stem	
Height (cm)	Mat Area (cm^2)	Height (cm)	Height (cm)	Diameter (cm)	Length (cm)	# Roots	# Shoots	Average Internode Distance (cm)	Height First Fertile Frond (cm)
22.9±14.2 (*n*=9)	27.6±39.8 (*n*=9)	34.6±17.7 (*n*=5)	14.9±10.9 (*n*=11)	0.7±0.5 (*n*=8)	2.1±0.9 (*n*=8)	19.2±16.1 (*n*=9)	5.3±4.2 (*n*=8)	4.8±2.6 (*n*=12)	124±38.2 (*n*=6)

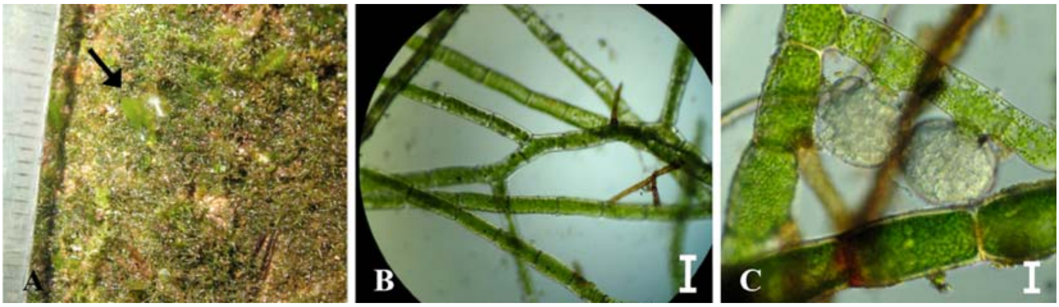

FIG. 2. Gametophytes of *Vandenboschia collariata*. **A.** Gametophyte mat on base of tree (young sporophyte marked by arrow; *Nitta 179*). **B.** Gametophyte structure under magnification. Scale bar = 100 μm (*Nitta 171*). **C.** Gametophyte bearing spherical antheridia. Scale bar = 25 μm (*Nitta 171*).

20 cm. The gametophytes are branched and filamentous, as in several other *Trichomanes* s.l. species (Stokey, 1940; Fig. 2B). Filaments consist of cylindrical cells, 100–200 μm long and 50–80 μm wide. The rhizoids of the gametophyte are brown and occasionally branched. They are separated from the parent cell by a single wall. Sites where rhizoids have been lost are often marked by small circular brown scars. Antheridia are stalked and spherical, with multiple ring cells (Fig. 2C). No archegonia were observed. Gemmifers were common and may bear brown, circular scars resulting from gemma dehiscence. These scars are larger than those left by lost rhizoids. No intact gemmae were seen. Gametophyte and sporophyte *rbcL* sequences (GenBank accession numbers FJ460462 and FJ460461 respectively) were found to be identical, confirming the specific identification of putative gametophytes.

Juvenile sporophytes (Fig. 1A, B) were found growing on the base of tree trunks (from ca. 5–50 cm above ground) and often

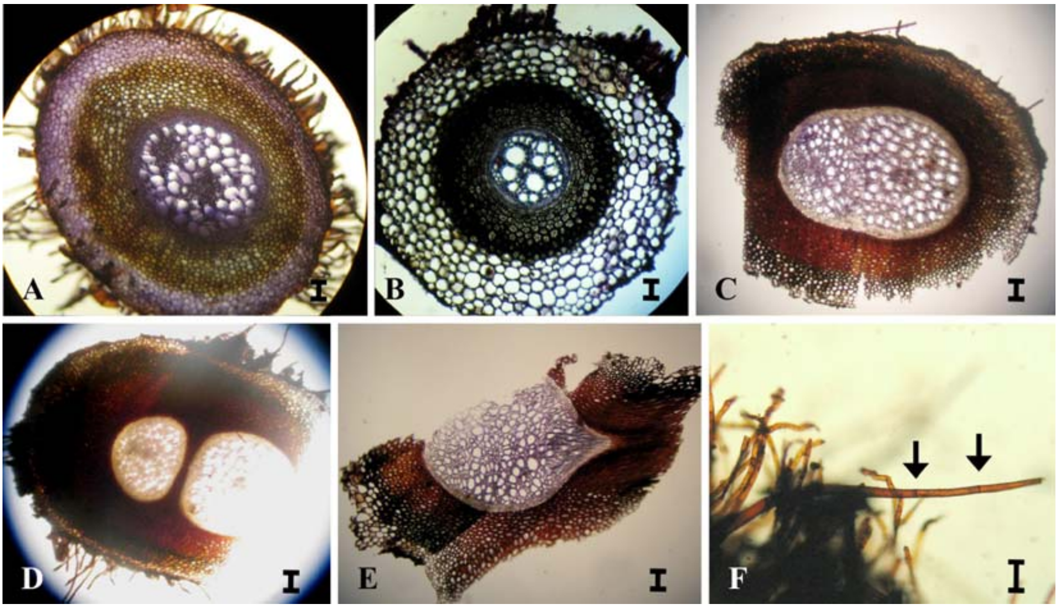

FIG. 3. Anatomy of *Vandenboschia collariata.* **A**. Climbing stem section. Scale bar = 100 μm (*Nitta 179*). **B**. Root section. Scale bar = 50 μm (*Nitta 172*). **C**, **D**. Sequential cross-sections at branch point of short stem, showing initial bifurcation of stele and fully divided stele. Scale bar = 100 μm (*Nitta 179*). **E**. Short stem cross-section, showing endogenous development of root. Scale bar = 100 μm (*Nitta 179*). **F**. Detail of hairs from climbing stem, showing septae (indicated by arrows). Scale bar = 25 μm (*Nitta 179*).

associated with gametophyte mats (Fig. 2A). Juvenile sporophytes have a short stem (up to 1.5 cm long) with short internodes (ca. 0.5–1 mm), from which they produce up to ten leaves and one to several roots that grow down the trunk. Juvenile sporophytes at more advanced stages of growth produce one or more creeping stems that climb vertically up the trunk.

All mature sporophytes were observed growing as hemi-epiphytes on trees (Fig. 1D; a single epipetric individual was also observed). A single short, thick stem (ca. 2.1× 0.7 cm) is present on the base of the tree trunk (usually 5–25 cm above ground). The short stem produces multiple long-creeping stems that climb up the tree and bear distant leaves (internode length ca. 4.8 cm) but no roots. These climbing stems occasionally produce axillary branches and bear dense brown, septate hairs (Fig. 3F). Fertile leaves are first produced after the climbing stem reaches ca. 120 cm in length. The short stem produces roots that grow vertically down the tree and branch several times after penetrating the soil. The roots have hairs similar to those on the climbing stem (brown and septate).

The root, short stem, and climbing stem all have similar anatomy (Fig. 3A, B, C), and appear to differ only in cell density (Table II). The cortex is heterogeneous, consisting of an inner cortex of cells with thickly sclerified walls and an outer cortex of cells with thinner, non-sclerified walls. This anatomy matches previous descriptions of filmy fern root (Schneider, 2000) and stem (Ebihara et al., 2007) cortices. The walls of the cells in the inner cortex become increasingly thick towards the outside. Cells of the cortex often contain starch granules. The stele consists of a central core of xylem with an outer ring of phloem. Protoxylem may be present as discrete strands or distributed throughout the xylem. This corresponds to a reduced protostele sensu Ebihara et al. (2007). The root and climbing stem differ markedly in development. Roots are generated endogenously (Fig. 3E), originating in the stele and pushing outwards through the cortex. New stems are produced through bifurcation of the stele (Fig. 3C, D).

Discussion

Although it was not possible (nor the goal of the current study) to observe continuous growth of a single individual from gametophyte to mature sporophyte, observations of multiple individuals at a variety of growth stages allow inference of the ontogeny of hemi-epiphytic growth in *Vandenboschia collariata.*

Young sporophytes begin growth from gametophyte patches located on the bases of trees. A short, oblique stem is first produced. This produces leaves on short internodes and also sends roots down the tree. Some time after roots have been initiated, a climbing stem is produced by the short stem. It is unknown whether climbing stems are produced before or after roots contact the soil. Although gametophytes and short stems of young sporophytes were observed on tree trunks up to ca. 50 cm above the ground, short stems of mature sporophytes were not observed at heights greater than ca. 25 cm. Although additional quantitative study is needed, this result implies that the height of gametophytes on the substrate may be a limiting factor in determining sporophyte recruitment.

As the sporophyte matures, the climbing stem grows up the tree and produces distant leaves. The leaves initially produced by the young sporophyte on the short stem senesce and are not observed on older specimens. The short stem continues to produce additional roots and climbing stems; although the short stem itself does not increase in diameter, the stem becomes surrounded by root and climbing stem bases, and may appear quite thick (up to 1.5 cm observed). The cortices of the roots and climbing stems appear continuous with the cortex of the short stem in cross section,

TABLE II

ANATOMICAL COMPARISON OF ROOT, SHORT STEM, AND CLIMBING STEM IN MATURE SPOROPHYTE OF *VANDENBOSCHIA COLLARIATA.*

	Root	Short Stem	Climbing Stem
No. cell layers in outer cortex	4–6	6–7	4–6
No. cell layers in inner cortex	6–7	10–12	9–10
No. xylem cells	9–15	>40	20–40

and may be responsible for the greater number of cell layers in the short stem cortex relative to the climbing stem cortex and root cortex.

This study clarifies several aspects of the previously uninvestigated hemi-epiphytic habit of *Vandenboschia collariata*. Observations of gametophytes and juvenile sporophytes on tree trunks support categorization of this species as a primary hemi-epiphyte sensu Benzing, as it begins growth on the tree and subsequently sends roots to the soil. Observations of individuals at a variety of growth stages show that the leafless short stem of mature individuals is developmentally homologous to the leafed short stem of juveniles. Gametophyte morphology is similar to congeners (branched, filamentous, with mat-like growth habit). Height of gametophytes above the ground is a possible limiting factor in sporophyte recruitment and bears further quantitative investigation. Similar field-based studies of other hemi-epiphytic climbers in Hymenophyllaceae (e.g. *Vandenboschia radicans* (Sw.) Copel., *Trichomanes tuerckheimii* H. Christ.) as well as other ferns (*Lomariopsis, Bolbitis, Polybotrya*), are necessary to construct evolutionary hypotheses concerning the hemi-epiphytic growth habit and the transition from terrestrial to epiphytic growth in ferns.

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