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## PRIMARY HEMIEPIPHYTISM IN *COLYSIS AMPLA* (POLYPODIACEAE) PROVIDES NEW INSIGHT INTO THE EVOLUTION OF GROWTH HABIT IN FERNS

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**Premise of research.** Epiphytes are prominent in many tropical floras; however, the evolution of epiphytism, especially among ferns, remains poorly understood. Transitions in habit have been proposed as evolutionary stepping stones in the radiation of ferns in the epiphytic niche, with hemiepiphytes playing an important role. However, few examples of hemiepiphytism have been conclusively documented in ferns, and evidence for this transition is limited. Because of this lack of information, the relationship between hemiepiphytism and epiphytism remains unclear. We fill this gap with new observations and analysis of habit evolution in the Polypodiaceae.

**Methodology.** We document primary hemiepiphytism in *Colysis ampla* from field observations of both gametophytes and sporophytes, and we examine its sporophyte anatomy in relation to its growth habit. Using cpDNA sequence data, we place the species within a phylogenetic context and perform ancestral state reconstruction of growth habit to infer the evolution of hemiepiphytism in *C. ampla*.

**Pivotal results.** Here we provide the first examination of gametophyte morphology and sporophyte growth habit in this species as well as the first conclusive documentation of primary hemiepiphytism in the Polypodiaceae. Phylogenetic analyses place *C. ampla* in a small clade of probable hemiepiphytes closely allied to the Old World ant-fern genus *Lecanopteris*. We infer a transition from epiphytism to hemiepiphytism in the clade consisting of *C. ampla* and closely related species, the first such transitional series reported for the polypod ferns. A hemiepiphyte syndrome consisting of gametophyte, rhizome, root, and leaf characteristics is presented to guide future investigation of this growth habit.

**Conclusions.** Hemiepiphytes are likely underreported among climber- and epiphyte-rich groups of ferns. Primary hemiepiphytes may be more frequently derived from holoepiphytic ancestors because epiphytic gametophytes and sporophytes are preadapted to hemiepiphytic growth. We propose that hemiepiphytism provides a mechanism for acquisition of stable nutrient and water supplies by tree-dwelling plants.

**Keywords:** hemiepiphyte, epiphyte, fern, microsoroid, growth habit.

### Introduction

Compared with most terrestrial habitats, the epiphytic niche is defined largely by stresses: nutrients in this environment are limited and patchily distributed, and the canopy is usually brighter, drier, and hotter than the forest floor (Wegner et al. 2003; Watkins et al. 2007c; Cardelús and Mack 2010). To successfully occupy these habitats, epiphytic plants must employ a suite of physiological and morphological adaptations. These are generally mechanisms to avoid and/or tolerate drought (desiccation tolerance, crassulacean acid metabolism photosynthesis), improve water and nutrient uptake (by absorptive trichomes or gross foliar uptake), or both (leaves arranged in tank-like rosettes and lined with absorptive trichomes in many species of tillandsioid bromeliads and some ferns and velamen radicum on the aerial roots of orchids and

some members of the Araceae; Benzing 1990; Zotz and Hietz 2001). In short, these and other adaptations of epiphytic species highlight both the unique constraints of the canopy habitat and the stark contrast between terrestrial and epiphytic species.

The ecophysiological challenges posed by the epiphytic habit are strongly reflected in the distribution and diversity of vascular epiphytes worldwide. Epiphytes are notably rare in dry regions and are by far most diverse and abundant in tropical cloud forests, which have a perhumid climate with limited seasonal fluctuation in temperature or precipitation (Still et al. 1999). Considering phylogenetic diversity, vascular epiphytes are almost entirely concentrated in just a few lineages: 80% of all vascular epiphyte species belonged to just four families (Orchidaceae, Bromeliaceae, Polypodiaceae, and Araceae) and 89% in eight families (Gentry and Dodson 1987). Considering just the leptosporangiate ferns, which include six of the 22 most epiphyte-rich vascular plant families (Gentry and Dodson 1987), the pattern is the same. We estimate that epiphytism has evolved a minimum of eight times in this group of at least 9000 species, on the basis of current phylogenies

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(Schuettpelez and Pryer 2007, 2009) and classifications (Smith et al. 2006; Rothfels et al. 2012). This attests to the remarkable success of groups that colonized and diversified within the epiphytic niche and suggests that there are strong barriers blocking the transition of plants from a terrestrial to epiphytic habit.

Among the major epiphyte clades, ferns seem unlikely candidates for success in abiotically stressful canopy habitats. Many aspects of fern ecology and physiology account for this apparent incompatibility with the epiphytic lifestyle: ferns have highly conservative water hydraulic systems (Watkins et al. 2010; Pittermann et al. 2011), passively controlled stomata (Brodribb and McAdam 2011; McAdam and Brodribb 2012), and an independent, morphologically simple gametophyte generation (Watkins et al. 2007b). However, recent studies have demonstrated that epiphytic ferns possess a suite of functional characteristics that are thought to have facilitated their radiation into the canopy, including changes in nutrient and water uptake mechanisms (Watkins et al. 2007a, 2010), shifts in water conductance strategies and stomatal function (Pittermann et al. 2011), and desiccation tolerance in both gametophyte and sporophyte generations (Watkins et al. 2007c; Testo and Watkins 2012). These adaptations represent a remarkable functional overhaul of nearly all aspects of the fern life cycle; these innovations and their ecological and evolutionary consequences have recently been reviewed by Watkins and Cardelús (2012).

The strong disparity between terrestrial and epiphytic habits and the limited number of successful epiphytic fern lineages pose an important question: how did ferns ascend into the canopy? Attention has been focused on the transitional stage as the critical step in the evolution of an epiphytic lifestyle. Transitions in habit have been proposed as an evolutionary stepping stone in the radiation of ferns into the epiphytic niche. Tsutsumi and Kato (2006) proposed one such transitional series in the epiphyte-rich davallioid-polygrammoid clade, with epiphytic taxa evolving from a secondary hemiepiphyte (a plant that establishes on the ground and loses physical connection with the soil at some point during its life), which in turn was derived from a terrestrial climber. In a study of the evolution of epiphytism in the bolbitidoid ferns (Dryopteridaceae), Lagomarsino et al. (2012) inferred a similar pattern: the climber-rich bolbitidoid clade gave rise to a lineage represented by the primary hemiepiphyte *Elaphoglossum amygdalifolium* (Mett.) H. Christ (a plant that establishes on trees and later gains connection with the ground), which in turn is sister to the rest of *Elaphoglossum*, a genus with ~600 spp. that comprises one of the largest clades of predominantly epiphytic ferns (Moran et al. 2010a). Studies of the evolutionary significance of hemiepiphytes are similarly sparse for angiosperms, but a similar pattern has been reported in the Gesneriaceae (Salinas et al. 2010).

In contrast, other authors have suggested that hemiepiphytism in ferns represents a secondary reversion from epiphytic ancestors; this appears to be the case in some members of the Hymenophyllaceae (Schneider 2000; Dubuisson et al. 2003; Nitta and Epps 2009). These conflicting results raise the question: do hemiepiphytes act as a stepping stone to epiphytism? Or is it a habit derived from epiphytism? Certainly, both are possible. Answering this question would provide important

insight into the evolution of epiphytic ferns; however, the lack of primary observations documenting the life histories of plants has prevented progress. If hemiepiphytes do play an important role in the transition from a terrestrial to epiphytic lifestyle, we would expect to find them in other large fern families with large numbers of holoepiphytes, such as the Polypodiaceae. Although several species from the family have been reported as hemiepiphytes, these designations either are erroneous (Pessin 1925) or require further substantiation (Lellingner 1988; León 1990; Tsutsumi and Kato 2006); to our knowledge, no hemiepiphytes have been described from the family to date.

Here, we report primary hemiepiphytism in *Colysis ampla* (F. Muell. ex Benth.) Copel. (Polypodiaceae) from Australia. We present evidence of this growth habit from in situ observations of gametophytes and sporophytes of various stages of maturity and consider the ecology and morphology of *C. ampla* in relation to previous reports of primary hemiepiphytism in the ferns as well as current hypotheses on the evolution of holoepiphytism. To better understand the phylogenetic position of *C. ampla* within the microsoroid clade to which it belongs and to evaluate the evolution of growth habit in this group, we compared chloroplast DNA sequence data from two coding regions (*rbcL* and *rps4*) and two intergenic spacers (*trnL-trnF* and *rps4-trnS*) obtained from *C. ampla* to data from previously published phylogenies of the microsoroids and obtained growth habit data from personal observations and regional floras. This report includes both the first description of the gametophytes of *C. ampla* and the first conclusive documentation of hemiepiphytism in the Polypodiaceae.

## Methods

### Plants

Plants of *Colysis ampla* at all stages of maturity were observed growing in the Daintree Rainforest, Queensland, Australia, in August 2011. Individuals were photographed to document growth habit of gametophytes and all sporophyte size classes. Gametophytes and young sporophytes were fixed in 70% ethanol, and large individuals were pressed and mounted. Voucher specimens (M. Kessler 14300, 14358) were collected under a permit issued by the Department of Environment and Resource Management Queensland (to M. Kessler, permit WISP09438311) and are deposited at VT.

Field-collected gametophytes and young sporophytes were examined in the lab with a Leica MZ8 stereoscope and an Olympus BX60 light microscope. Images were recorded with a SPOT Insight Firewire 2.0 camera (Spot Imaging Solutions, Sterling Heights, MI). Images were edited using Adobe Photoshop CS2 software. Editing options included cropping, scaling, and adjustment of contrast, brightness, and saturation. Mature sporophyte morphology was studied from herbarium material. Rhizomes and roots of mature plants were obtained from herbarium specimens, rehydrated in distilled water for 48 h, and sectioned by hand.

### DNA Extraction and Sequencing

Total genomic DNA was extracted from herbarium specimens of *C. ampla* using a modified cetyltrimethylammonium

bromide extraction protocol (Doyle and Doyle 1987). One chloroplast coding region (*rbcL*) and two chloroplast intergenic spacers (*trnL-trnF-rps4-trnS*) were amplified using the polymerase chain reaction (PCR). PCR amplifications were performed using 1  $\mu\text{L}$  of genomic DNA, 2.5  $\mu\text{L}$  of  $10\times$  *Taq* buffer with 15  $\mu\text{M}$  of  $\text{MgCl}_2$  added, 2.5  $\mu\text{L}$  of deoxyribonucleotide triphosphates, 2.5  $\mu\text{L}$  of 2.5  $\text{mg mL}^{-1}$  bovine serum albumin solution, 1.25  $\mu\text{L}$  of each primer at 10  $\mu\text{M}$ , 0.125  $\mu\text{L}$  of *Taq* DNA polymerase, and 17.857  $\mu\text{L}$  of purified water. PCR conditions for the amplification of *rbcL* consisted of an initial denaturation cycle of 5 min at 94°C and then 40 cycles of 60 s at 94°C, 30 s at 55°C, 90 s at 72°C, and a final extension period of 7 min at 72°C. Amplification protocols for both spacers were as follows: initial denaturation cycle of 5 min at 94°C and then 35 cycles of 60 s at 94°C, 30 s at 55°C, 90 s at 72°C, and a final extension period of 7 min at 72°C. The *rbcL* gene was amplified using the primers ESRBCL1F and ESRBCL1361R (Schuettpelz and Pryer 2007); for sequencing, the primers and ESRBCL628F and ESRBCL654R were used with the amplification primers. We used the universal primers e and f (Taberlet et al. 1991) for the *trnL-trnF* spacer and the primers *rps4-3r.f* (Skog et al. 2004) and *trnSr* for the *rps4-trnS* spacer (Souza-Chies et al. 1997). For both intergenic spacers, the same primers were used for amplification and sequencing. PCR products were electrophoresed and visualized on 1% agarose gel with ethidium bromide. PCR products were purified using ExoSAP-IT (USB, Cleveland, OH), following manufacturer protocols. Automated sequencing was performed using an ABI Prism 3130x1 sequencer at the Vermont Cancer Center (Burlington, VT).

#### Taxon Sampling

To place *C. ampla* in a phylogenetic context within the microsoroid ferns, we obtained sequence data from an additional 69 taxa in 17 genera published in prior phylogenetic studies of the group by Haufler et al. (2003), Schneider et al. (2006) and Kreier et al. (2008) and included representatives from all major genera within the core microsoroids. Accession numbers for sequences used are shown in the appendix. Of the 70 taxa sampled, 43 were represented by all three markers sequenced, 24 were represented by *rbcL* and *trnL-trnF*, and three were represented by *rbcL* alone.

#### Nomenclature

Generic circumscription of microsoroid ferns is in transition following the results of recent phylogenetic studies (Haufler et al. 2003; Schneider et al. 2006; Kreier et al. 2008). We apply names used in the most recent floristic treatments available (Brownlie 1969; Rödl-Linder 1990; Bosman 1991; Hovenkamp 1998; Crouch et al. 2011; Hovenkamp 2013; Zhang et al. 2013).

#### Sequence Alignment and Phylogenetic Analyses

Sequences were edited in Geneious (ver. 6.16; Biomatters) and aligned using the MAFFT plugin. We produced alignments for each gene region (*rbcL*, *rps4*, and *rps4-trnS*, *trnL-F*) and then chose substitution models using BIC in Partition Finder (Lanfear et al. 2012). The substitution models and partition

scheme was used in a maximum likelihood (ML) phylogenetic analysis in RAxML (Stamatakis 2006). We also analyzed our data using maximum parsimony (MP) using TNT v1.1 (Goloboff et al. 2008), performing a driven search strategy employing sectorial search, parsimony ratchet (up and down weights set to 4%), and tree fusing, with an initial level of 60 and set to find minimal length 10 times. Support for nodes for the combined data set analysis was calculated by bootstrap (BS) analyses, with the same settings.

#### Reconstruction of Ancestral Habit

We scored categorical habit traits for all taxa employing the states terrestrial (including epipetric), climber, hemiepiphyte, and holoepiphytic. *Colysis ampla* was scored as hemiepiphytic on the basis of our observations presented here. All other taxa were scored from descriptions provided in the literature (Brownlie 1969; Rödl-Linder 1990; Bosman 1991; Tryon and Stolze 1993; Hovenkamp 1998; Crouch et al. 2011; Zhang et al. 2013). Plants that exhibit more than one growth habit were scored for the state they exhibit most commonly, as described in the literature. Character matrices were assembled and optimizations performed using Winclada v1.7 (Nixon 1999–2002) and Mesquite v2.75 (Maddison and Maddison 2011). Ancestral character state reconstructions were performed under both MP and ML. Parsimony reconstructions were performed using Winclada and explored using acctran and deltran, and states were considered unordered. ML optimizations were performed using Mesquite on the best tree resulting from our RAxML search under the Mk1 model (Lewis 2001).

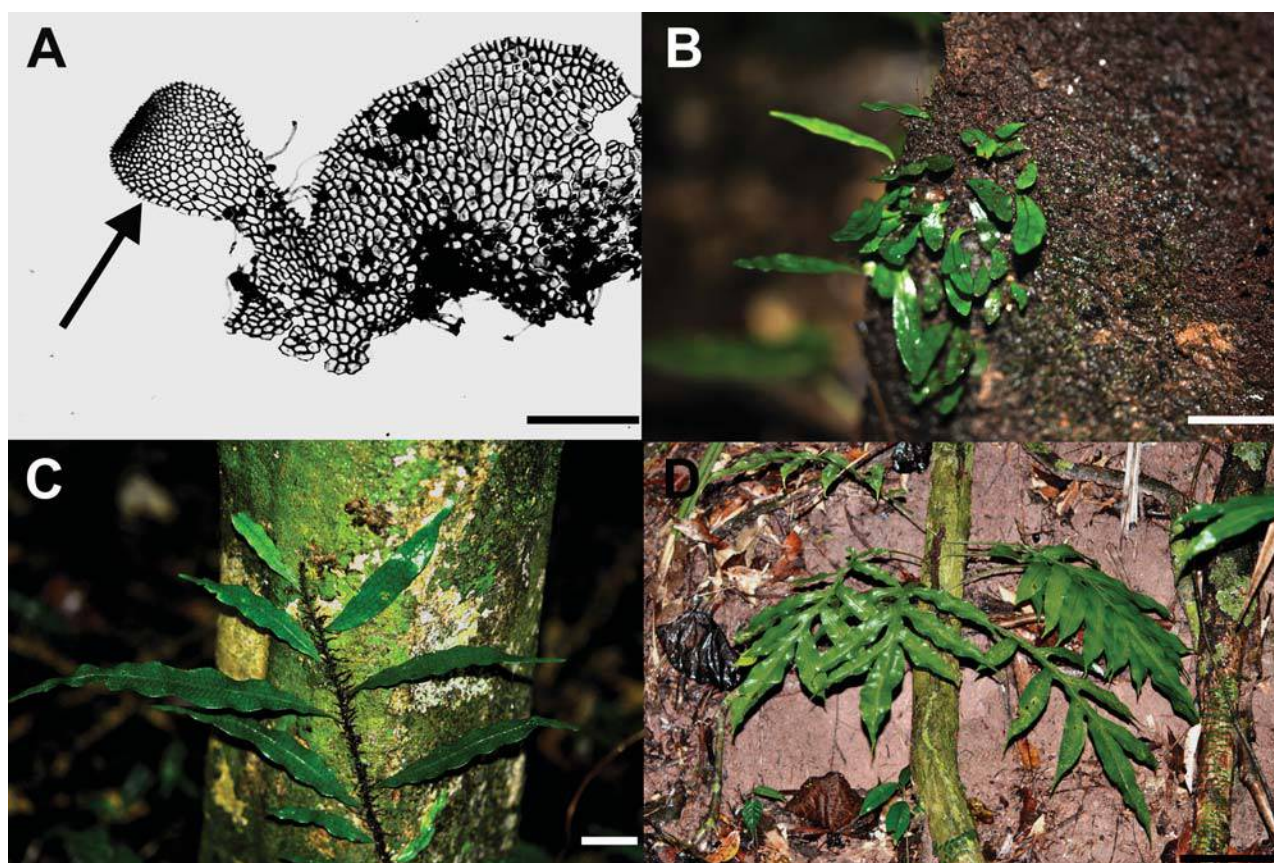
## Results

#### Gametophyte Morphology

Gametophytes were found growing on both large boulders and the trunks of small- and medium-diameter trees, from just above ground level to ~1 m. In some cases, gametophytes were found attached to the base of young sporophytes, helping to confirm their identity. All gametophytes observed were 2.2–4.6 mm broad, strap shaped, and sparsely branched (fig. 1A). Multiple marginal meristems were observed on each gametophyte. Rhizoids were short and generally restricted to the posterior margin, where gametophytes attached to the host plant. Several of the gametophytes observed had produced elongate to cordiform marginal proliferations (fig. 1A). These proliferations possessed apical pluricellular meristems and rhizoids restricted to their ventral side. Unicellular, chlorophyllous, nonglandular papillate hairs were observed on the margins of all gametophytes observed. Numerous antheridia were present on the dorsal side of one gametophyte and were restricted to branched segments of the thallus; no archegonia were observed. Antheridia consisted of basal, ring, and cap cells.

#### Sporophyte Morphology

Mature sporophytes of *Colysis ampla* were climbing on tree trunks and attained a maximum height of 1.5–2 m above the ground. All sporophytes possessed long-creeping, dorsiventral rhizomes; distichous leaves; and roots that were inserted both



**Fig. 1** Growth habit and gametophyte and sporophyte morphology of *Colysis ampla*. *A*, Mature gametophyte of *C. ampla* with spatulate asexual outgrowth (arrow) with marginal meristem and rhizoids. Scale = 1 mm. *B*, Gametophytes and young, simple-leaved sporophytes of *C. ampla* on tree trunk. Scale = 2.5 cm. *C*, Growth habit of young *C. ampla* sporophyte climbing by rhizome and clasp roots prior to root-soil contact. Scale = 2.5 cm. *D*, Mature *C. ampla* sporophyte after root-soil contact. Scale = 10 cm.

laterally and ventrally. Leaves of immature plants were lanceolate, simple, and entire, whereas those of mature plants were deeply lobed, with five to 10 pairs of pinnae (fig. 1*B–D*).

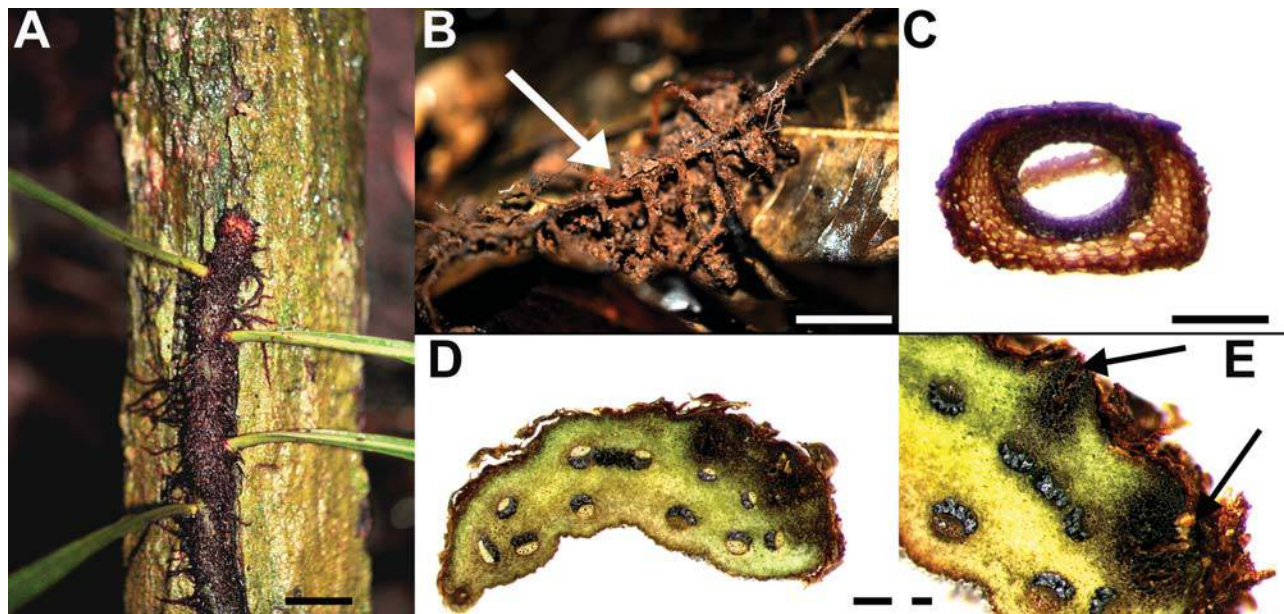
Young sporophytes climbed upward from their point of establishment on the trunks with the aid of short lateral clasp roots produced on the lateral surface. Their rhizomes also produced long ventrally inserted feeding roots that grow downward along the tree trunk, eventually making contact with the ground (fig. 2*A, B*). (The terminology used here of “clasp” and “feeding” roots follows that used for *Elaphoglossum amygdalifolium* by Lagomarsino et al. [2012].) The anatomy of clasp and feeding roots was similar but differed in size and symmetry. Both consisted of an epidermal layer with short root hairs, a cortex comprised of a three or four layers of parenchyma cells subtended by two or three layers of sclerenchyma, and a haplostelic vascular cylinder (fig. 2*C*). Clasp roots were 0.1–0.3 mm in diameter, terete, and rarely longer than 5 cm. They do not penetrate the bark of the host tree but appear to secrete a glue that attaches them to the substrate. In contrast, feeding roots were 0.4–2 mm across at their widest axis, dorsiventrally flattened, and frequently exceeded 1 m in length. These feeding roots penetrate the soil and become

highly branched (fig. 2*B*). The establishment of root-soil contact also coincided with a change in leaf morphology, with plants developing their first lobed leaves shortly after making contact with the soil. In one case, we were able to count 23 simple leaves on a plant before the first lobed leaf.

Rhizomes of mature plants were 6–14 mm wide, greenish, the older portions becoming brown, and densely covered with dark brown, lanceolate scales. In all specimens examined, rhizomes were moderately to strongly dorsiventrally flattened and often arched on their ventral face (fig. 2*D*). The rhizome stele consisted of a dictyostele comprised of eight to 15 scattered vascular bundles, each partially sheathed by a strand of sclerenchyma (fig. 2*D, E*). In some cross sections, small cavities within the cortex of the rhizome were observed; these were surrounded by regions of sclerified tissue and typically found near the dorsal surface. These cavities occasionally perforated the epidermis, forming small external openings on the dorsal surface of the rhizome (fig. 2*E*).

#### Phylogenetic Analysis

MP parsimony search returned 20 trees (length, 2468; confidence index, 51; retention index, 70). These trees were largely



**Fig. 2** Rhizome and root anatomy of *Colysis ampla*. *A*, Climbing rhizome of mature sporophyte showing distichous leaf arrangement and lateral short clasping roots. Scale = 1 cm. *B*, Long feeding root branching extensively upon making contact with soil (arrow). Scale = 1 cm. *C*, Cross section of long feeding root showing epidermis, cortical cells, and sclerotized inner cortex. Scale = 0.5 mm. *D*, Cross section of rhizome showing arched ventral face, perforated dictyostele with sclerenchyma bundles, and sclerotized internal cavities. Scale = 0.5 mm. *E*, Close-up of rhizome cross section showing sclerotized internal cavities with external openings (arrow). Scale = 0.5 mm.

congruent with those derived from ML search (fig. 3) and recovered the same main clades. Microsoroids were recovered as sister to *Goniophlebium* (100% ML MP BS), and monophyletic, albeit with weak support. Our topology was similar to that of Kreier et al. (2008), with Microsoroids s.s and Lepisoroids forming a clade (87% ML BS) and in turn sister to the clade comprising the Lecanopteroid and Membranaceoid ferns. *Colysis ampla* was found to be sister to *Phymatosorus scandens* (G. Forst.) Pic. Serm. (ML 92%, MP 84%), followed by *Microsorium varians* (Mett.) Henning & Hett. These taxa were resolved as sister to *Microsorium linguiforme* (Mett.) Copel. (ML 99%, MP 89%) and these together sister to *Lecanopteris* (ML 71%, MP 51%). A clade of austral ferns including *Phymatosorus novae-zealandii* (Baker) Pic. Serm., *Phymatosorus pustulatus* (G. Forst.) Large, Braggins, & P.S. Green, and *Microsorium vieillardii* (Mett.) Copel., followed by the sister pair *Microsorium membranaceum* (D. Don) Ching and *Microsorium lastii* (Baker) Tardieu, were in turn sister to the preceding groups.

#### Reconstruction of Ancestral Habit

The ancestral habit of microsoroid ferns was reconstructed as holoepiphytic in all reconstructions (fig. 3). A terrestrial habit is derived six times. Most of these occur in single species or in sister species; however, terrestrial habit also characterizes the clade of nine species comprising *Leptochilus* + *M. pteropus*, with a single reversal back to epiphytism. Climbing ferns evolve from epiphytic ancestors three times, in *Tricholepidium normale* (D. Don) Ching, *M. vieillardii*, and for both *M. varians* and *P. scandens*. *Colysis ampla* is nested among these

latter two taxa; thus, following this coding, hemiepiphytism in *C. ampla* is derived from a climbing habit.

## Discussion

### Gametophyte Morphology

In ferns, gametophyte form is tightly linked to habitat, and the development of a long-lived, noncordiform gametophyte has been hypothesized to be an important step in the evolution of holoepiphytism in ferns (Dassler and Farrar 1997, 2001; Watkins and Cardelús 2012). The short-lived, cordiform gametophyte typical of most terrestrial ferns appears to be poorly suited for survival in relatively stable and competitive epiphytic habitats; most holoepiphytic ferns possess filamentous, strap- or ribbon-shaped gametophytes that are capable of persisting for long periods of time via indeterminate growth and frequently asexual reproduction. This morphology is thought to promote longevity in these relatively stable habitats, which may facilitate outcrossing, which is thought to predominate in epiphytic ferns (Dassler and Farrar 2001). Thus, gametophytes of primary hemiepiphytic species should be either intermediate in form relative to terrestrial and epiphytic gametophytes or similar to those of epiphytic species. In cases where the gametophyte morphology of primary hemiepiphytes has been studied, gametophytes are generally reported as strap shaped (Ebihara et al. 2013) or filamentous (Nitta and Epps 2009), though cordate to cordate-elongate gametophytes were reported for the primary hemiepiphyte *Elaphoglossum amygdalifolium* (Mett.) H. Christ (Lagomarsino et al. 2012). The strap-shaped gametophytes of *Colysis ampla* fit well within

this range of morphology, though the presence of thallus branching and marginal proliferation as described here has not been reported for any other eupolypod hemiepiphytes. As demonstrated for many epiphytic taxa (Chiou and Farrar 1997; Dassler and Farrar 2001), gametophytic proliferation in *C. ampla* likely allows for the establishment of clones and promotes reproductive success; however, more extensive study of gametophyte populations is necessary to confirm this hypothesis. Interestingly, the gametophytes of *C. ampla* more closely resemble those of epiphytic relatives than terrestrial ones, whereas the converse is true for *E. amygdalifolium*. It is possible that gametophyte form in hemiepiphytic ferns may provide insight into the habit of closely related species and indicate the directionality of possible transitions in growth habit.

Gametophyte morphology has been relatively well studied in the microsoroid ferns (Nayar 1962, 1963; Nayar and Kaur 1971; Chiou and Farrar 1997; Takahashi et al. 2009), providing a useful context in which to consider that of *C. ampla*. Both cordate and ribbon-/strap-shaped gametophytes are known from the microsoroids and allied groups, with cordate gametophytes reported for *Microsorium*, *Aglaomorpha*, and *Drynaria*, whereas ribbon- and strap-shaped gametophytes are found in *Colysis*, *Leptochilus*, *Paraleptochilus*, *Selliguea*, and *Dendroglossa* (Nayar and Kaur 1971). Although relationships between gametophyte form and habitat are difficult to interpret in this group because of limited information on growth habit for many species, the generalized terrestrial/epiphyte split in gametophyte morphology does not seem to describe diversity in gametophyte form exhibited in this group, since wholly epiphytic genera (*Drynaria*, *Aglaomorpha*) possess cordate gametophytes (Nayar 1965) and gametophytes of some terrestrial and epilithic species in other genera (e.g., *Colysis decurrens*; Takahashi et al. 2009) are strap or ribbon shaped. The prevalence of noncordiform gametophytes in both epiphytic and terrestrial species of microsoroids is unusual and may be linked to the high proportion of species that have been reported to grow both terrestrially and epiphytically in the group (Tsumi and Kato 2006; Dong et al. 2008; Kreier et al. 2008). Future investigations of the microsoroids should include careful investigation of growth habit to improve understanding of the evolution of gametophyte form in this group.

#### *Sporophyte Morphology*

Nearly all studies documenting hemiepiphytism in ferns have reported a shared sporophyte body plan that includes a dorsiventrally flattened, long-creeping rhizome, often distichously arranged leaves, and the presence of both laterally inserted clasping roots and ventrally inserted feeding roots. We found that *C. ampla* conforms well to this body plan, providing additional support to the hypotheses proposed by earlier authors who suggested that these characters are crucial components of the hemiepiphytic habit in *C. ampla* and several other primary hemiepiphyte species (e.g., *Lomariopsis* spp., *Vandenboschia collariata* [Bosch] Ebihara & K. Iwats.). Establishment of physical contact with the ground induces a sudden change in leaf morphology, with plants attaining their mature leaf morphology after root-soil contact is made. We suspect that juvenile plants are more desiccation tolerant than mature ones and that

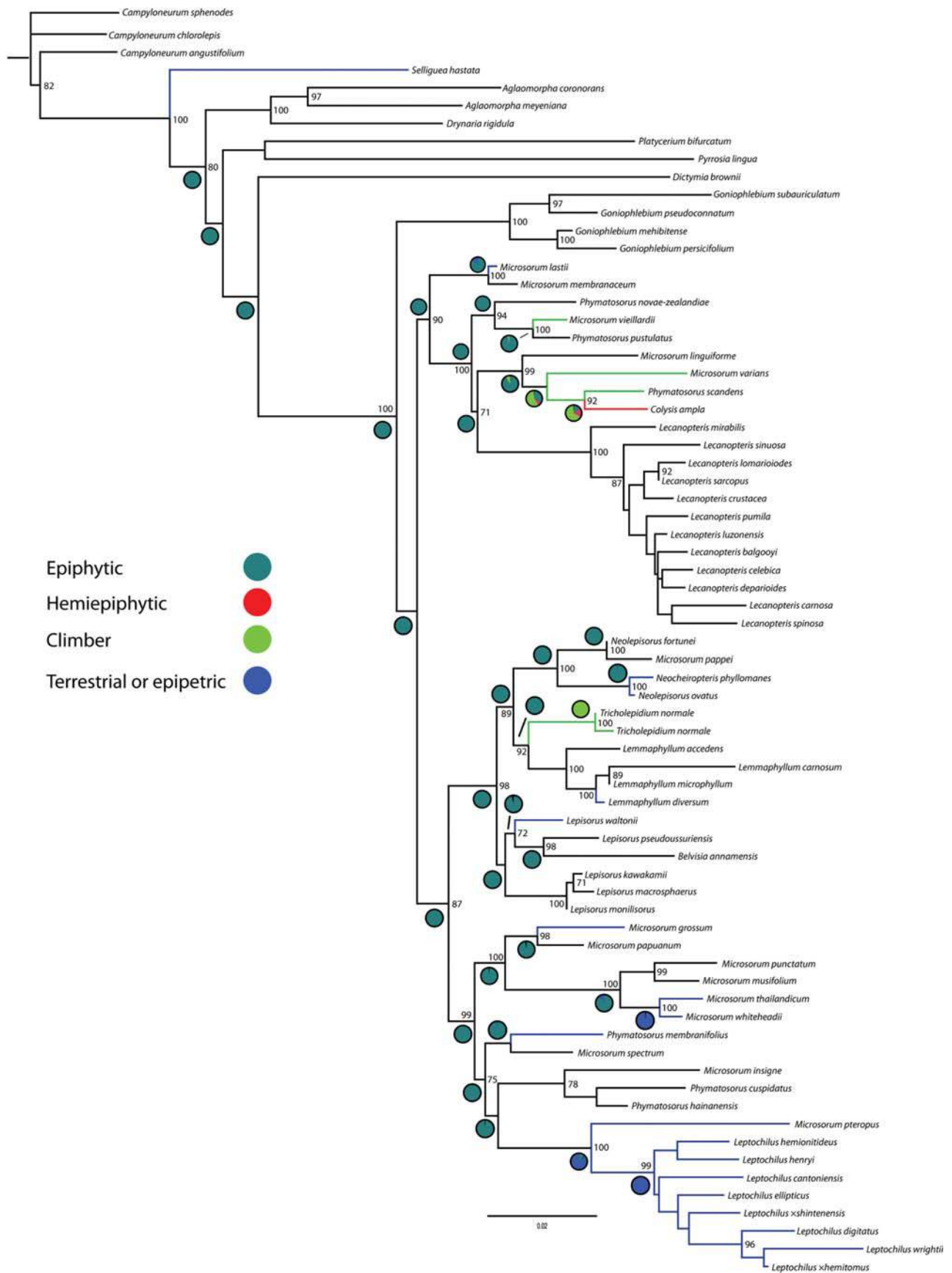
physiological change coincides with the morphological transition.

Recent work by Watkins et al. (2010) suggests that the transition from a terrestrial to an epiphytic lifestyle in ferns was accompanied by a dramatic shift in vascular system structure and function and that hemiepiphytes are intermediate to these life forms in nearly all anatomical and ecophysiological traits. While intermediacy in these traits supports the habit transition hypothesis, the same pattern would be expected in cases in which hemiepiphytes are derived from holoepiphytic taxa. This, coupled with the epiphyte-like sporophyte body plan of most hemiepiphytic ferns, suggests that primary hemiepiphytes may more commonly represent a transition from epiphytic to terrestrial growth than the converse. This possibility has not been considered for most groups and highlights the importance of including phylogenetic information in the study of hemiepiphytic fern taxa.

The rhizome morphology of *C. ampla* is noteworthy, given the taxon's placement in a clade sister to *Lecanopteris*. In the Malesian genus *Lecanopteris*, ants live within large cavities in the plants' rhizomes in a symbiotic association (Gay 1993a; Haufler et al. 2003). The evolution of these cavities has remained enigmatic in part because of uncertainty regarding the genus's closest outgroups as well as the lack of internal rhizome cavities in the earliest-divergent species in the genus, *Lecanopteris mirabilis* (C. Chr.) Copel., which instead hosts ants underneath its dorsiventrally arched rhizome. Several species in the closely allied *Microsorium linguiforme* group have been shown to possess small cavities in their rhizomes; however, the homology of these structures to the ant domatia has been doubted because *L. mirabilis* lacks internal cavities (Haufler et al. 2003). In *C. ampla*, both internal cavities and an arched rhizome structure were observed, a combination of characters not previously reported for this group. While the internal cavities observed in this species were not large enough to host ants, they did possess external openings that have been hypothesized to be a critical character in the evolution of the lecanopterid rhizome (Haufler et al. 2003). No ants were observed in the space formed under the rhizome in *C. ampla*; however, such associations were not looked for during the initial collection. Future work should examine the rhizome morphology of species in this group (including *L. mirabilis*) in situ to identify a potential transitional series in rhizome morphology and possible ant associations.

#### *Evolution of Hemiepiphytism in Colysis ampla*

Though epiphytes are an important ecological component of many tropical floras, epiphytism has rarely evolved among vascular plants, as evidenced by the small number of epiphytic lineages (Gentry and Dodson 1987). Ferns have been remarkably successful in radiating into the epiphytic niche; however, the mechanisms by which they have accomplished this ecologically drastic transition remain poorly understood. Climatically and edaphically, terrestrial and epiphytic habitats are fundamentally different, and the shifts in physiology and morphology associated with the evolution of epiphytism in ferns are drastic. Seeking to bridge the disparity between terrestrial and epiphytic life forms, some authors have hypothesized that hemiepiphytes play an important role in the transition from





terrestrial to epiphytic life in both ferns (Tsutsumi and Kato 2006) and angiosperms (Salinas et al. 2010). Among ferns, several examples conforming to this pattern have been described recently in the Davalliaceae and Dryopteridaceae (Tsutsumi and Kato 2006; Lagomarsino et al. 2012). Evidence from the Hymenophyllaceae (Schneider 2000; Dubuisson et al. 2003; Nitta and Epps 2009), however, present an alternative scenario, with hemiepiphytes representing a reversion from epiphytism. With the discovery of both transitional and reversionary primary hemiepiphytic taxa, the evolution of this growth form is now known to go both ways, down from the trees or up from the soil; as such, it is important that each case of primary hemiepiphytism be evaluated in relation to its outgroups to determine the direction of this transition.

Our phylogenetic analyses place the primary hemiepiphyte *C. ampla* sister to the climbing species *Phymatosorus scandens* and both taxa sister to another reported climber, *Microsorium varians*. In addition to our phylogenetic results, our confidence that *C. ampla* is closely related to these species is based on their similar morphology, including leaf venation and aspects of rhizome anatomy that are not found in other microsoroids. Geography also supports this close relationship; all three are austral species nested within the otherwise epiphytic lecanopterid clade (sensu Kreier et al. 2008) comprised of species of primarily Malesian and Austral distributions. Optimization of growth habit using data from floristic descriptions indicates an epiphyte → climber → primary hemiepiphyte transition in growth habit within this group; however, careful observation of the two climbing species suggests that they are most likely primary hemiepiphytes as well. Similar to *C. ampla*, they have been mischaracterized as climbers in floristic accounts. We base this assessment primarily on observation of several photographs of both species that show multiple young sporophytes establishing on tree trunks well above the ground and a similar heteroblastic leaf series to that observed in *C. ampla*. We believe that the most likely scenario is a direct transition from epiphytism in the lecanopterid ferns to primary hemiepiphytism in the clade comprised of *M. varians*, *P. scandens*, and *C. ampla*. Our analysis recovered eight transitions from holoepiphytic to terrestrial growth within the microsoroid clade without hemiepiphytic intermediates. This pattern may be obscured by a lack of careful observations of growth habit among these species; indeed, most truly hemiepiphytic ferns have only recently been discovered. Future work should carefully investigate the growth habit of species in clades with these transitions to identify possible primary hemiepiphytes.

Compared with the terrestrial → hemiepiphyte → epiphyte transition that has been inferred for the bolbitidoid (Lagomarsino et al. 2012) and davallioid (Tsutsumi and Kato 2006) ferns, the epiphyte → hemiepiphyte transition may be common in ferns for several reasons. First, epiphytic ferns possess numerous preadaptations amenable for hemiepiphytic growth that are rare or absent in terrestrial species, including (usually)

noncordiform, long-lived gametophytes; dorsiventral rhizomes; and ventrally and/or laterally inserted roots (Holttum 1978; Dassler and Farrar 2001; Watkins et al. 2007b). If this is the case, it is possible that other intermediate growth habits, such as root climbers (sensu Darwin 1875), may play a more important role in terrestrial → epiphyte transitions, since the life histories of these plants are more similar to terrestrial species.

Though hemiepiphytic growth clearly played an important role in the evolution of epiphytism in some fern groups, the growth habit is certainly not restricted to such transitional scenarios. Conversely, the evolution of primary hemiepiphytism in ferns may more often represent a novel and favorable ecological approach for accessing reliable stores of water and nutrients from mineral soil while occupying a relatively stable and noncompetitive low-trunk habitat. We propose that a transition from epiphytism to primary hemiepiphytism may allow species to avoid the physiological constraints associated with holoepiphytism while maintaining a competitively favorable position above the forest floor. In this scenario, primary hemiepiphytism arises when low-trunk holoepiphytes develop root dimorphy, with both short, laterally inserted clasping roots and longer, likely ventrally inserted feeding roots. These long roots connect with the forest floor, providing the sporophyte with a steady supply of water and nutrients from the forest floor while allowing the plant to persist in the relatively noncompetitive tree trunk habitat in which it established. This view of hemiepiphytes as a novel functional form rather than an evolutionary intermediary is more consistent with the treatment of this group in several angiosperm lineages, notably the Araceae (López-Portillo et al. 2000; Lorenzo et al. 2010), Clusiaceae (Zotz and Winter 1993; Zotz et al. 1994; Wanek et al. 2002), and Moraceae (Holbrook and Putz 1996). This possibility should be considered in future studies of hemiepiphytic ferns. Again, synthesis of careful ecological and morphological observations with phylogenetic analyses is necessary to better understand the evolutionary and ecological significance of hemiepiphytism among ferns and other groups.

With these factors considered, we propose a hemiepiphyte syndrome that should inform much-needed searches for additional primary hemiepiphytic fern species. Potential primary hemiepiphytes should be searched for among epiphyte-rich groups found growing low on the trunks of trees in tropical forests with seasonally dry climates, where connection with a more stable soil water supply would be particularly favorable. Such species are expected to possess noncordiform (i.e., filamentous, ribbon, or strap-shaped) gametophytes, dorsiventrally flattened rhizomes, dimorphic (short-clasping and long-feeding) laterally and/or ventrally inserted roots, and a leaf developmental series that exhibits change associated with root-soil contact.

Since its inception, the term “hemiepiphyte” has been a source of considerable confusion, evidenced by its inconsistent

**Fig. 3** Phylogenetic relationships of *Colysis ampla* inferred from maximum likelihood (ML) analysis of *rbcl*, *rps4* and *rps4-trnS*, and *trnL-F* and reconstruction of ancestral habit. Numbers at nodes are ML bootstrap values. Branch colors depict maximum parsimony character state reconstruction, and pie charts depict ML reconstruction employing the MK1 model (dark blue, epiphytic; light blue, terrestrial or epipetric; green, climbing; red, hemiepiphytic).

and incorrect application across ferns and angiosperms, recently reviewed in detail by Zotz (2013). Characterization of this growth habit in ferns has proven especially difficult because of the challenges in locating and identifying gametophytes and young sporophytes, and misapplication of the term has contributed to the confusion. Most of the ferns that have been described as hemiepiphytes—notably members of *Cyclocladum* (Smith 1986), *Lomagramma* (Gay 1993b), *Mickelia* (Watkins et al. 2007c; Moran et al. 2010), *Maxonia* (Mehlreter and Palacios-Rios 2003), *Olfersia* (Watkins and Cardelus 2009), and *Polybotrya* (Moran 1987; Young and León 1991)—are in fact terrestrial climbers that never lose root-soil contact (Canestraro et al. 2014), and other reports from the Davalliaceae (Tsutsumi and Kato 2006), Dryopteridaceae (Holtum 1978; Moran et al. 2010b), Hymenophyllaceae (Dubuisson et al. 2013), Oleandraceae (Tsutsumi and Kato 2006), and Polypodiaceae (Lellinger 1988; León 1990; Tsutsumi and Kato 2006) lack sufficient documentation to distinguish hemiepiphytism from other growth habits. In order to distinguish hemiepiphytes from climbers or low-trunk holoepiphytes, it is critical to document all life stages, including gametophytes,

since sporophytes of primary hemiepiphytes frequently establish root-soil contact early in development. To date, primary hemiepiphytism has been conclusively documented (including observation of gametophytes) for only a handful of fern species, and no hemiepiphytic species have been described for many primarily epiphytic or climbing groups. Additional detailed fieldwork is necessary to document this life form and to better understand the significance of hemiepiphytic ferns in both an ecological and evolutionary context.

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### Appendix

#### Voucher Information and GenBank Accession Numbers

Voucher information and GenBank accession numbers for sequences used in this study. Taxa are listed in alphabetical order. Species (in bold), voucher (herbarium), GenBank accessions, *rbcL*, *rps4-trnS*, *trnL-F*. A dash indicates that the sequence was not available; *unk.* indicates that the voucher specimen is unknown.

**Aglaomorpha coronans**, *unk.* (UC), AF470349, —, AY083652. **Aglaomorpha meyeniana**, *Giauque s.n.* (UC), AF470338, —, AY083641. **Belvisia annamensis**, *Hovenkamp 05-277* (L), EU482931, EU482976, EU483025. **Campyloneurum angustifolium**, *Chisaki & Carter 1004* (UC), AF470344, —, AY083647. **Campyloneurum chlorolepis**, *A.R. Smith 1158* (UC), AF470345, —, AY083648. **Campyloneurum wercklei**, *Horich s.n.* (UC), AF470346, —, AY083649. **Colysis ampla**, *Kessler 14358* (VT), KF570108, KF570110, KF570110. **Dictymia browni**, *Hodel s.n.* (UC), AF470348, —, AY083651. **Drynaria rigidula**, *Skottsberg 156* (UC), AF470339, —, AY083642. **Goniophlebium mehibitense**, *Hovenkamp 05-278* (L), EU482932, EU482977, EU483026. **Goniophlebium persicifolium**, *Cult. BGB; 239-12-90-33* (B), EU482933, EU482978, EU483028. **Goniophlebium pseudoconnatum**, *Cult. BGB 239-36-90-30* (B), EU482934, EU482979, EU483029. **Goniophlebium subauriculatum**, *Cult. BGB A.R. Smith s.n.* (UC), AF470342, —, AY083645. **Lecanopteris balgooyi**, *Hennipman s.n.* (L), AF470328, —, AY083631. **Lecanopteris carnosa**, *Cranfill 153* (UC), AF470322, EU482980, AY083625. **Lecanopteris celebica**, *Schneider s.n.* (GOET), AF470323, EU482981, AY083626. **Lecanopteris crustacea**, *A.R. Smith s.n.* (UC), AF470329, EU482982, AY083632. **Lecanopteris deparioides**, *Hennipman 7865* (U), AF470324, —, AY083627. **Lecanopteris lomarioides**, *Hennipman s.n.* (U), AF470326, —, AY083629. **Lecanopteris luzonensis**, *Schneider s.n.* (GOET), AF470325, EU482983, AY083628. **Lecanopteris mirabilis**, *Hennipman s.n.* (U), AF470330, EU482984, AY083633. **Lecanopteris pumila**, *Hennipman s.n.* (UC), AF470331, —, AY083634. **Lecanopteris sarcopus**, *Ridl 171* (E), EU482935, EU482985, EU483030. **Lecanopteris sinuosa**, *Hennipman 7821* (L), AF470321, —, AY083624. **Lecanopteris spinosa**, *Hennipman s.n.* (U), AF470327, —, AY083630. **Lemmaphyllum accedens**, *Hovenkamp 05-298* (L), EU482936, EU482986, EU483031. **Lemmaphyllum carnosum**, *A.R. Smith s.n.* (UC), AF470332, —, AY083635. **Lemmaphyllum diversum**, *Ranker 2079* (COLO), EU482937, EU482987, EU483032. **Lemmaphyllum microphyllum**, *Schneider s.n.* (GOET), EU482938, EU482988, EU483033. **Lepisorus kawakamii**, *Ranker 2051* (COLO), EU482940, EU482990, EU483035. **Lepisorus macrosphaerus**, *Ranker TW018* (UC), EU482941, EU482991, EU483036. **Lepisorus monilisorus**, *Ranker TW012* (UC), EU482942, EU482992, EU483037. **Lepisorus pseudoushuriensis**, *Ranker TW093*, EU482943, EU482993, EU483038. **Lepisorus waltonii**, *Cranfill 94-266-29* (UC), EU482944, EU482994, EU483039. **Leptochilus cantoniensis**, *Dong 172* (PE), EU482946, EU482996, EU483042. **Leptochilus digitatus**, *Smith 00-036* (UC), EU482948, EU482998, EU483044. **Leptochilus ellipticus**, *Zhang 1923* (PE), EU482949, EU482999, EU483045. **Leptochilus hemionitideus**, *Moran s.n.* (NY), EU482950, EU483000, EU483046. **Leptochilus henryi**, *Zhang 2541* (PE), EU482952, EU483002, EU483048. **Leptochilus shintenensis**, *Zhang 3800* (PE), EU482953, EU483003, EU483049. **Leptochilus wrightii**, *Craig s.n.* (UC), AF470340, EU483004, AY083643. **Leptochilus x hemitomus**, *Zhang 3302*, EU482951, EU483001, EU483047. **Microsorium grossum**, *Lorence 9155* (DL), EU482956, EU483007, EU483053. **Microsorium insigne**, *Liu 204* (PE), EU482957, EU483008, EU483054. **Microsorium lastii**, *Perier 7937* (P), EU482961, EU483012, EU483058. **Microsorium linguiforme**, *Ranker 1776* (UC), AF470334, —, AY083637. **Microsorium membranaceum**, *Li 95* (PE), EU482962, EU483013, EU483059. **Microsorium musifolium**, *unk.* (UC), AF470333, —, AY083636. **Microsorium pappi**, *Cult. BGL* (L), AF470336, —, AY083639. **Microsorium papuanum**, *Cult. BGB*

*Schuettpelz 603* (GOET), DQ642162, EU483015, DQ642246. *Microsorium pteropus*, *Kreier s.n.* (GOET), EU482965, EU483016, EU483061. *Microsorium punctatum*, *Ridsdale s.n.* (UC), AF470337, EU483017, AY083640. *Microsorium spectrum*, *Wood 10936* (LOA), EU482967, EU483018, EU483064. *Microsorium thailandicum*, *Schwertfeger s.n.* (GOET), EU482969, EU483020, EU483066. *Microsorium varians*, *Cult. BGG, Schneider s.n.* (GOET), AY362566, AY362638, DQ179643. *Microsorium vieillardii*, *Cult. ABG Smith s.n.* (UC), DQ179634, DQ179637, DQ179644. *Microsorium whiteheadii*, *Whitehead s.n.* (UC), EU482970, EU483021, EU483067. *Neochiropteris phyllomanes*, *Nicholson s.n.* (E), EU482973, EU483024, EU483069. *Neolepisorus fortunei*, *Zhang 3446* (PE), EU482955, EU483006, EU483052. *Neolepisorus ovatus*, *Zhang 728/1* (PE), EU482972, EU483023, EU483068. *Phymatosorus cuspidatus*, *unk.*, AF470335, —, AY083638. *Phymatosorus hainanensis*, *Wang 1348* (PE), EU482960, EU483011, EU483057. *Phymatosorus membranifolius*, *Dunn 458* (LOA), EU482964, EU483014, EU483060. *Phymatosorus novae-zealandiae*, *Perrie s.n.* (WELT), DQ401120, DQ401126, DQ401124. *Phymatosorus pustulatus*, *Perrie s.n.* (WELT), DQ401117, DQ401127, DQ401122. *Phymatosorus scandens*, *Perrie s.n.* (WELT), DQ401118, DQ401128, DQ401123. *Platynerium bifurcatum*, *Ornduff 9618* (UC), AF470341, —, AY083644. *Pyrrosia lingua*, *Bartholomew 566* (UC), AF470343, —, AY083646. *Selliguea hastata*, *Craig s.n.*, AF470347, —, AY083650. *Tricholepidium maculosum*, *Zhang 3100* (PE), EU482974, —, EU483070. *Tricholepidium normale*, *Shen S4-1* (PE), EU482975, —, EU483071.

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