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Resolving relationships within the hornwort genus *Anthoceros*

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Abstract

The family Anthocerotaceae is amongst the last remaining hornwort families to be tested for their monophyly. The recent publication of genomes for *A. punctatus, A. agrestis* and *A. angustus* has emphasized the need to understand generic and species boundaries within the family. Twenty-eight taxa within the family and three chloroplast genes were used to reconstruct a phylogeny, which supports a monophyletic Anthocerotaceae. Within the family, four clades are recognised, comprising subgenus *Anthoceros*, subgenus *Indici*, *subg. nov*, subgenus *Australienses*, *subg. nov.* and subgenus *Sphaerosporoceros*. Species currently circumscribed as *Folioceros* also form a separate clade, but as most taxa within the genus, including the type species, are yet to be sequenced no formal decisions have been made for this group.

Keywords: Anthocerotaceae, *Folioceros*, hornworts, subgenus *Australienses,* subgenus *Indici*

Introduction

The use of molecular phylogenies as an independent assessment of morphological taxonomic concepts in bryophytes is commonplace nowadays. In the last 20 years, an increasing number of taxa, including genera, have been circumscribed using molecular data (e.g., Larraín *et al.* 2013), especially in hornworts. The family Anthocerotaceae is amongst the last remaining hornwort families to be tested for their monophyly. The recent publication of genomes for *A. punctatus, A. agrestis* and *A. angustus* (Dong *et al.* 2018, Li *et al.* 2020) has emphasized the need to understand generic and species boundaries within the family.

Hornwort molecular phylogenies have changed generic concepts considerably. Duff *et al.* (2007) provided evidence of the paraphyly of *Megaceros* Campbell (1907: 484) splitting it into two entities, comprising *Megaceros sensu stricto*, in Oceania and the Old World (Villarreal *et al.* 2010a, b; Cargill *et al.* 2013) and the mostly New World *Nothoceros* (Schuster 1987: 200) Hasegawa (1994: 32), (Villarreal *et al.* 2010a, b; Villarreal & Renner 2014). In this case, homoplasy in both gametophyte and spore characters between these two clades invites deep ultrastructural and chemical studies to find clear characters to circumscribe both lineages. Duff *et al.* (2007) also provided genetic evidence supporting the proposal of two new genera, *Phaeomegaceros* Duff *et al.* (2007: 241) and *Phymatoceros* Stotler *et al.* (2005: 114). After careful taxonomic and ultrastructural studies, all three genera have clear-cut synapomorphies in congruence with molecular analyses.

Currently, monophyly of most hornwort genera has been tested, except those in the family Anthocerotaceae (*Anthoceros* Linnaeus (1753: 1139)*, Folioceros* Bharadwaj (1971: 9)*,* and *Sphaerosporoceros* Hässel de Menéndez (1988: 79)). Recently, the publication of the genomes of *A. punctatus* Linnaeus (1753: 1139) and *A. agrestis* Paton (1979: 257) (Li *et al.* 2020), and *A. angustus* Stephani (1916: 1001) (Zhang *et al.* 2020) have emphasized the need to understand generic and species boundaries within the Anthocerotaceae. For example, the widespread *A. agrestis* appears to be composed of at least three clades. One of them includes the European *A. agrestis* (including plants from

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the type locality), two clades from North America, and one from China and Australia (Dawes *et al.* 2020). There is an obvious need to use molecular tools to further explore hornwort diversity, especially in the species-rich genus *Anthoceros*.

Anthoceros is the oldest published genus name of the phylum and has been a 'catchall' genus since Stephani's treatment (1916–1924). Eighty or so species of *Anthoceros* are recognized today, although a modern formal taxonomic treatment is still needed (Villarreal *et al.* 2010b; Söderström *et al.* 2016). Species within the genus share common characteristics of a thallus with internal mucilage-filled schizogenous cavities; antheridial jacket cells in tiers opening at the apex and darkly pigmented spores and pseudoelaters.

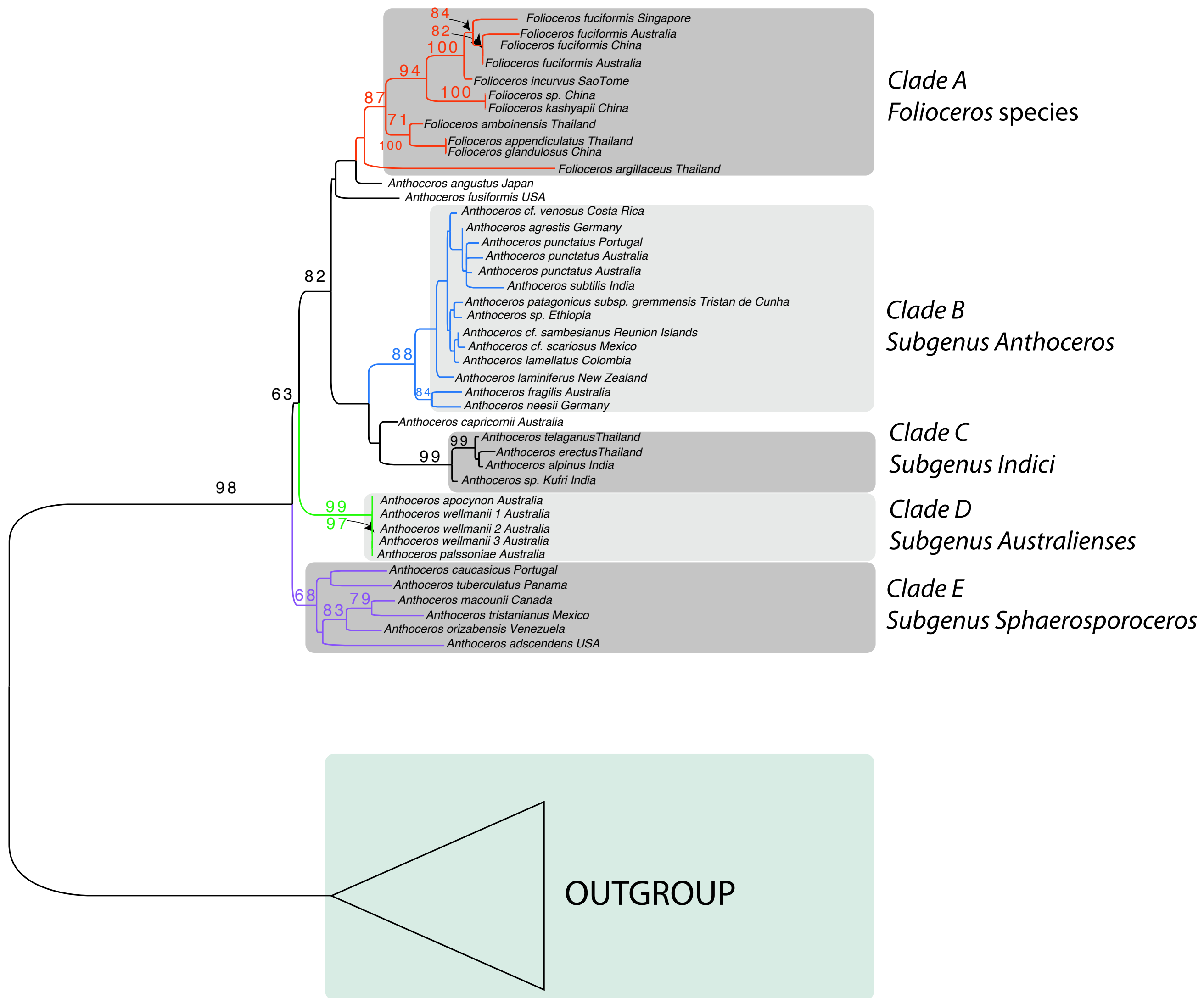

FIGURE 1. Maximum likelihood (ML) tree for 56 species of hornworts (from 3173 aligned nucleotides of plastid DNA) with ML bootstrap values above branches. A bootstrap value above 95% is considered very good, a value between 70% and 94% is reasonably good. Any value below 60-70% is poorly supported. Values lacking or below 60% on branches, mean no support. Outgroup from different hornwort families is presented as collapsed cartoon branches.

In 1971, *Folioceros* Bharadwaj (1971: 9) was proposed to accommodate species of *Anthoceros* with long, thick-walled pseudoelaters and baculate or spinose spores (Fig. 4F). Bharadwaj provided compelling statistical and morphological arguments to separate the genus from *Anthoceros* (Table 1). *Folioceros* comprises 18 species mostly from South Asia (India) and Africa with widespread species such as *Folioceros fuciformis* (Montagne 1844: 296) Bharadwaj (1975: 227) occurring in Reunion Island, Australia, Indonesia, China, Japan, Tanzania and islands of the South Pacific. *Folioceros* species possess an array of gametophyte body shapes (Fig. 2) and spore wall architecture (Fig. 3). The gametophyte may vary from an irregularly lobed thallus typically seen in the Indian species *Folioceros satpurensis* Bharadwaj & Srivastava (in Bharadawaj 1978: 112) to the irregularly pinnate, long, narrow strap-like male thallus of *F. physocladus* Bharadwaj (1978: 115) or the regularly pinnate thallus of *F. pinnilobus* Stephani (1907:

299) Bharadwaj (1975: 227) or *F. fuciformis* Bharadwaj (1978, Figs. 4A, B). The spore patterns are more diverse, with almost spherical spores varying in their patterning from verrucose to finely papillose to broad triangular spines to long narrow spines with or without a reduced trilete mark (Fig. 2). The genus *Sphaerosporoceros* was erected to accommodate species of *Anthoceros* from the Americas and Australia with large, almost spherical spores, indistinct trilete mark, connate-cristate ornamentation, hyaline sterile spores amongst fertile ones and short-ovoid pseudoelaters with thickening bands (Hässel de Menéndez 1988; Cargill & Scott 1997) (Table 1).

FIGURE 2. Habit photos of a number of *Anthoceros* and *Folioceros* species. A. *Folioceros amboinensis* (*S. Chantanaorrapint & C. Promma 549*); B. *Folioceros argillaceus* (*S. Chantanaorrapint & C. Promma 2850*); C. *F. fuciformis* (*S. Chantanaorrapint & C. Promma 506*); D-E. *F. appendiculatus* (*S. Chantanaorrapint & C. Promma 1884*). Image credits: S. Chantanaorrapint

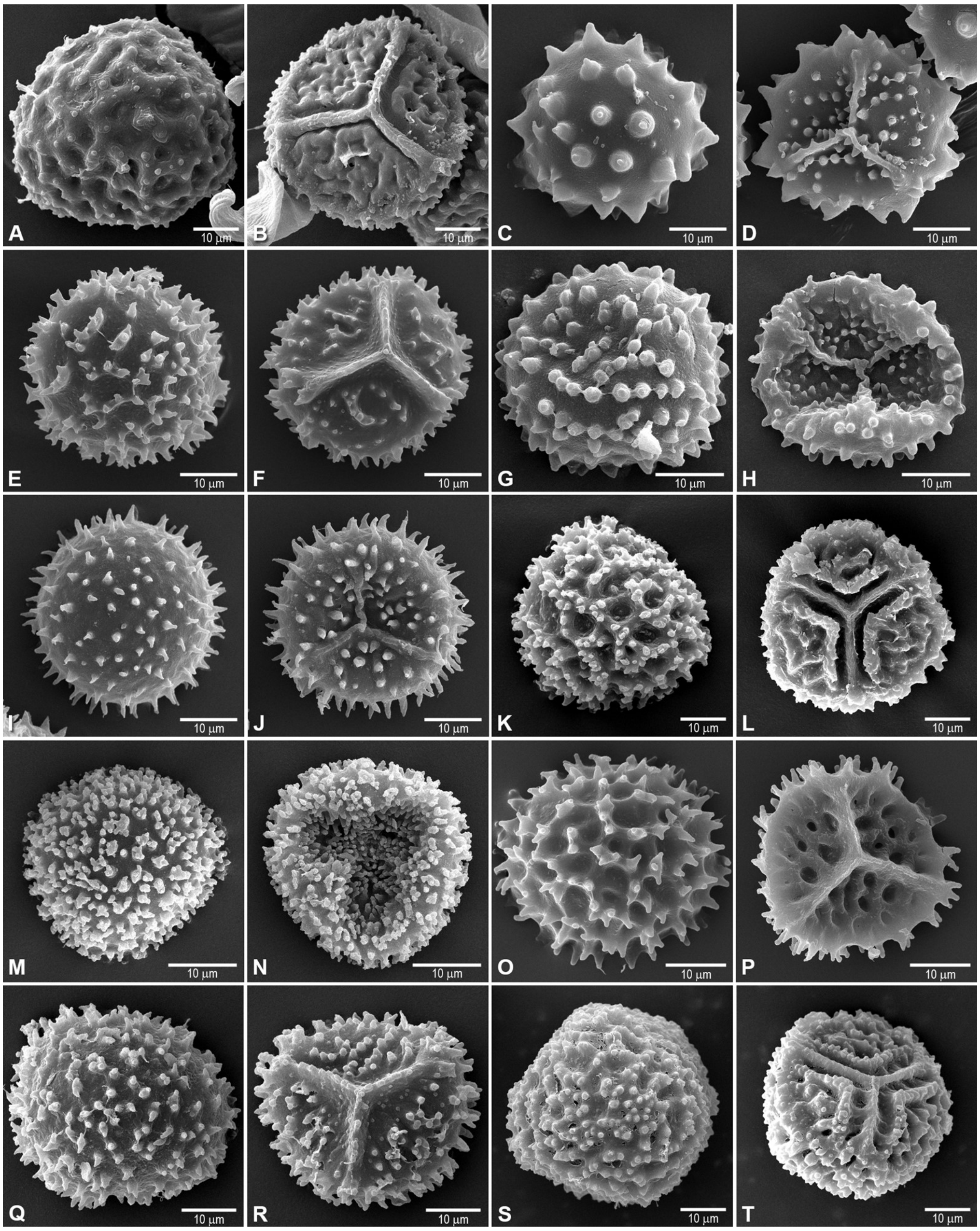

FIGURE 3. A-B. *Anthoceros alpinus* (*D.G. Long 18961*); C-D. *F. amboinensis* (S. Chantanaorrapint & C. Promma 549); E-F. *A. angustus* (*S.Chantanaorrapint & C.Promma 1599*); G-H. *Folioceros. appendiculatus* (*S.Chantanaorrapint & C.Promma 1884*); I-J. *F. argillaceus* (*S.Chantanaorrapint 2850*); K-L. *A. erectus* (*S. Chantanaorrapint & C. Promma 1701*); M-N. *F. fuciformis* (*S. Chantanaorrapint & C. Promma 506*); O-P. *A. punctatus* (*B.C. Ho 12-280*); Q-R. *A. subtilis* (*S. Chantanaorrapint & C. Promma 2816*); S-T. *A. telaganus* (*S. Chantanaorrapint & C.Promma 1730*). Image credits: S. Chantanaorrapint.

The generic status of both *Folioceros* and *Sphaerosporoceros* has been questioned on morphological grounds by Hasegawa (1984, 1988), Schuster (1992) and Cargill & Scott (1997). Villarreal & Cargill in Söderström *et al.* (2016) no longer recognised *Sphaerosporoceros* as a distinct genus, with all species going back to *Anthoceros*. In this paper we have built a molecular phylogeny based on two of the three former species of *Sphaerosporoceros* and six of the 18 species of *Folioceros*. The aim of this paper is to test the monophyly of both genera and their placement within Anthocerotaceae. A morphological assessment and re-circumscription of the family Anthocerotaceae are provided.

TABLE 1. Comparison of morphological characters amongst the three described genera within the family Anthocerotaceae: *Anthoceros, Folioceros* and *Sphaerosporoceros.*

Morphological characters	*Anthoceros*	*Folioceros*	*Sphaerosporoceros*
Gametophyte:			
- thallus shape	rosettes or fans or straps	straps or rosettes or fans	rosettes or fans
- thallus margin	irregularly dissected	pinnate or irregularly dissected to broadly crenate	irregularly dissected to broadly crenate
- dorsal lamellae	abundant or absent	absent or few	Absent
- involucre length	0.5–6 mm	up to 8mm	up to 5 mm
Sporophyte:			
- capsule description and length	Thin or short and stout; 5–100 mm long	thin; up to 75 mm long	short and stout; 2–24 mm long
- columella size	16 cells (but see Campbell 1904 on *A. fusiformis*)	16 cells (4 × 4 rows)	16 cells (4 × 4 rows)
- assimilative tissue	?3–6 layers	4–5 layers	3–4 layers
- pseudoelater description	short to long; thin-walled; 1–3(–5)-celled; bands of thickening absent/present??	dark, continuous lumen, long, narrow and thick-walled; 2–5-celled; bands of thickening sometimes present	reduced, 1–3 (-5)-celled; thin-walled with irregular bands of thickening
Spores:			
- shape and diameter	tetrahedral; small to medium: 29–69 µm	hemispherical to almost spherical; small: 26-50 µm	hemispherical to almost spherical to tetrahedral(?); large: 56 -105 µm
- distal face	punctate or protuberances: either spines or cristate	protuberances: papillate, tuberculate, baculate or spines	protuberances: tuberculate, connate or papillate
- proximal face	foveolate or cristate	spiny or papillate	tuberculate, cristate or spiny
- triradiate mark	present and distinct; some species with smooth strips on either side of mark	absent or present and reduced; smooth strips absent	present and reduced or distinct; smooth strips absent

Materials and Methods

Taxon sampling, Light and Scanning Electron Microscopy (SEM) for Morphology

Light micrograph images of Australian plants of *Folioceros fuciformis* were taken using a Nikon Coolpix 5000 camera through a Lietz and Wild compound and dissecting microscopes respectively. Spores of the Australian *F. fuciformis* were air dried, attached to double-sided sticky tape and attached to aluminium stubs, gold coated and viewed with a Joel JSM 6400 SEM.

SEM photos of the spores of four *Folioceros* species were obtained from Thailand and the Malay Peninsula. Mature spores of *F. amboinensis* (Schiffner 1890: 45) Piippo 1993: 36), *F. appendiculatus* (Stephani 1896: 315) Hasegawa 1986: 382, *F. argillaceus* (Stephani 1916: 970) Villarreal & Cargill (in Villarreal *et al.* 2015: 93) and *F. fuciformis* were air dried and removed from sporangia and mounted on double-sided cellophane adhesive tape affixed on stubs. Spores were then plated with a thin layer of gold and examined with a FEI Quanta 400 SEM.

Taxon Sampling, Isolation of DNA, Amplification and Sequencing

Seven species of *Folioceros* and 28 species of *Anthoceros* covering its global distribution, were included in our molecular dataset. Additionally, several species of Notothyladaceae, Dendrocerotaceae and Phymatocerotaceae were included as outgroup. Unfortunately, the type species of *Folioceros, F. assamicus* Bharadwaj (1971: 10), was not available for the study. We included four accessions of *F. fuciformis* from China, Singapore and Australia. Table S1 provides a list of the sampled species with geographic origin, herbarium vouchers, and GenBank accession numbers for all sequences were used to deduce phylogenetic relationships. We used the plastid genes *rbc*L, portion of the *trn*K intron and the *mat*K gene contained within and the ribosomal gene *rps*4 (Villarreal *et al.* 2015) (Table 2).

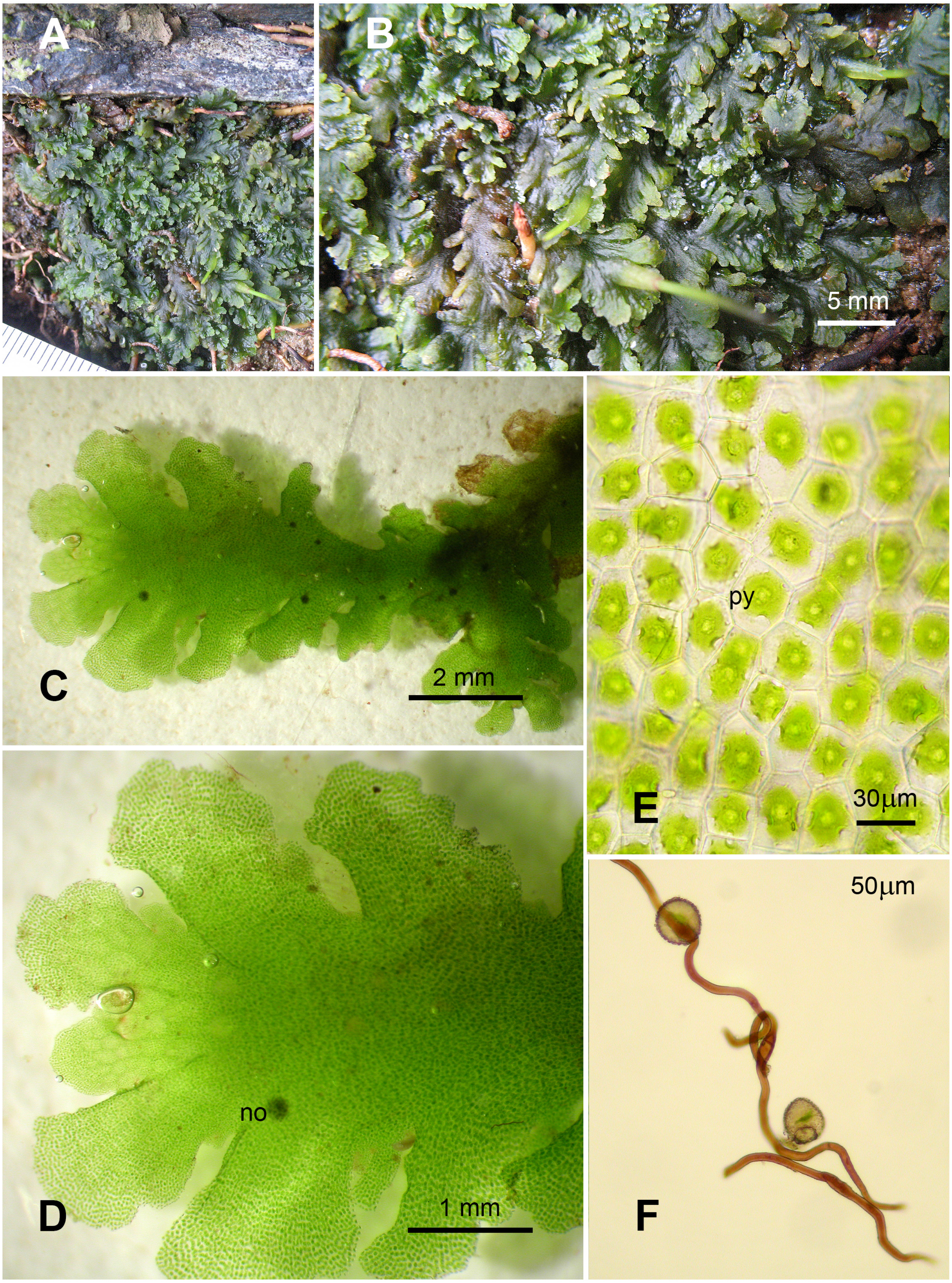

FIGURE 4. *Folioceros fuciformis*. A-B. Plants *in situ* (*P. Wellman* 485). C-D. Individual plant showing pinnate patterning of lobes and an *Nostoc* colony (no) (*P. Wellman 484b*). E. Dorsal epidermal cells with a single large chloroplast with associated pyrenoid (py) (*P. Wellman 484b*). F. Long thick-walled pseudoelaters with two spores (*P. Wellman 484b*). Image credits: A-B P. Wellman; C-F D.C. Cargill.

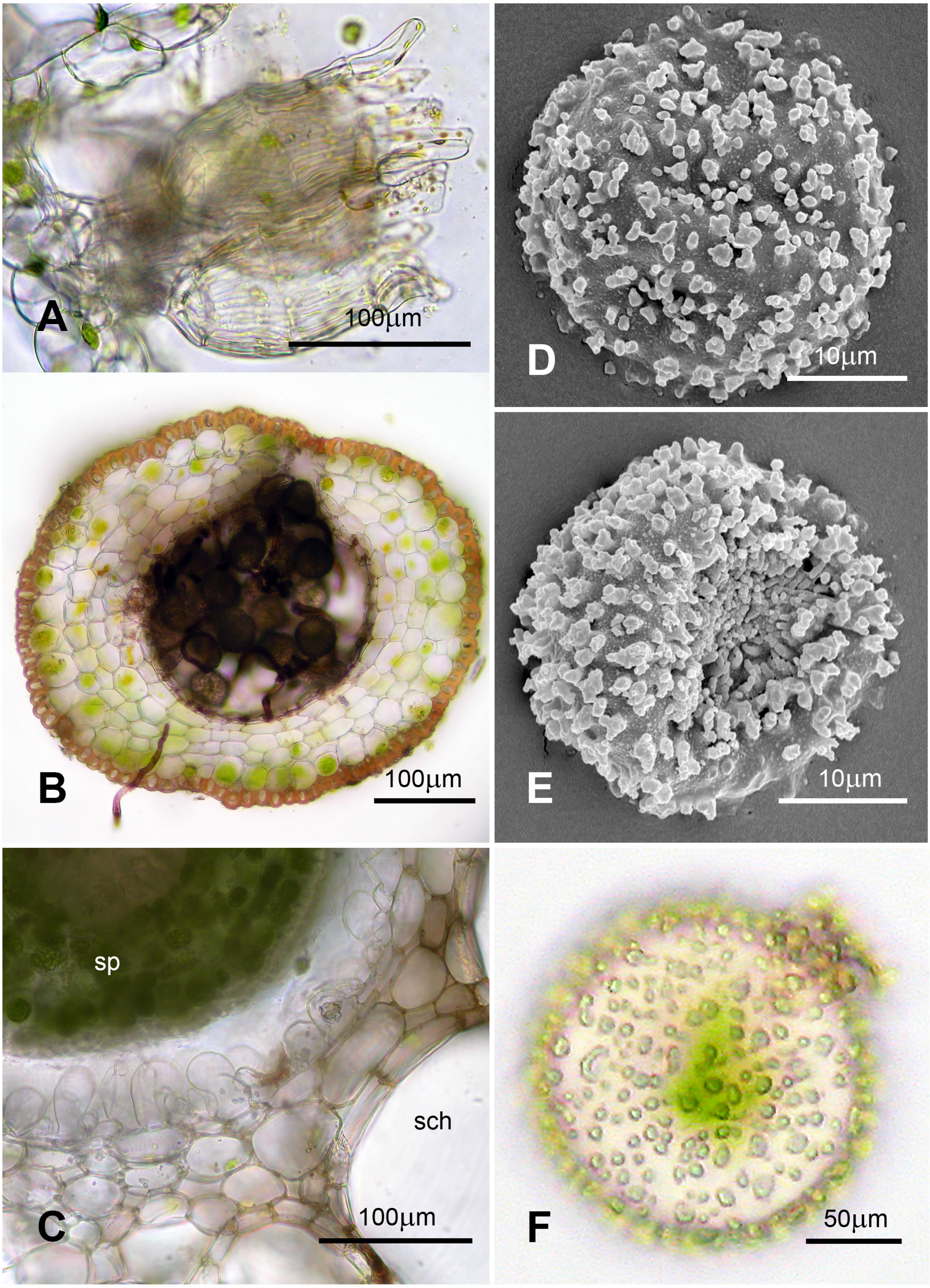

FIGURE 5. *Folioceros fuciformis*. A. Antheridia (*P.Wellman 483*). B. Transverse section through sporophyte with sporogenous layer in middle, 4-layered assimilative section and outer epidermal layer (*P.Wellman 483*). C. Partial transverse section through involucre showing schizogenous cavities (sch) and inner layer of endodermal cells (*P.Wellman 483*). SEM micrographs of spores (*D.C. Cargill 62*). D. Distal face of spore. E. proximal face of spore. F. Light micrograph of distal face of spore (*P.Wellman 484b*). Image credits: D.C. Cargill.

TABLE 2. Primers used for the molecular markers and publication sources.

Region	Primer	Direction	Sequence	Publication
*rbc*L	*rbcLF*	Forward	GTCACCACAAACGGARACTAAAGC	Duff *et al.* (2004)
*rbc*L	*rbcLHR*	Reverse	CTTTCCATACTTCRCAAGCAGC	Duff *et al.* (2004)
*rbc*L	*rbcL660F*	Forward	CAAGGTCCACCTCATGGTA	Duff *et al.* (2004)
*rbc*L	*rbcL660R*	Reverse	AACGATCTCTCCAACGCA	Duff *et al.* (2004)
*rbc*L	*rbcL946F*	Forward	ACACGAAAGTGAATACCATG	Duff *et al.* (2004)
*rbc*L	*rbcL946R*	Reverse	ACACGAAAGTGAATACCATG	Duff *et al.* (2004)
*rps*4	rps40F	Forward	TCGTCTGGGGACTCTACCAG	Villarreal *et al.* (2013)
*rps*4	rps540R	Reverse	AACCAATCCAGTCACGATCT	Villarreal *et al.* (2013)
*mat*K	*matk-horn1F*	Forward	TTC GAA TTT TTC GTA GAC GAG T	Villarreal & Renner (2013)
*mat*K	*horn_R2_trnk*	Reverse	TAA TGT AAA AGT TTC TTC CG	Villarreal & Renner (2013)

TABLE 3. PCR conditions for each of the primer sets.

Primer	PCR conditions
*rbc*L	95ºC for 5 min; 95ºC for1min; 42ºC for 1min, 68ºC (or 72ºC depending on the taq) for 1 min (34 or 40 cycles from 95ºC for 1min); 68ºC (or 72ºC) for 7 min.
*trn*K intron+*mat*K	95ºC for 5 min; 95ºC for 1min; 51ºC for 1min, 68ºC (or 72ºC depending on the taq) for 1.5 min (34 cycles from 95ºC for1min); 68ºC (or 72ºC) for 7 min.
*rps*4	95ºC for 5 min; 95ºC for 1min; 50ºC for 1min, 68ºC (or 72ºC depending on the taq) 1 for min (34 cycles from 95ºC for 1min); 68ºC (or 72ºC) for 7 min.

Total DNA from fresh, silica-dried, and herbarium material was extracted using a Qiagen Dneasy Plant Mini kit and a Stratec Invisorb Spin Plant Mini kit, according to the manufacturer's instructions. PCR protocols are listed in Table 3.

PCR products for Australian species *A. apocynon* Cargill & Palsson (2021: 327), *A. capricornii* Cargill & Scott (1997: 55), *A. palssoniae* Cargill (2021: 334), *A. punctatus* and *A. wellmanii* Cargill (2021: 340) were sent to Macrogen (Seoul, Korea), all other samples were sequenced at the Genomic Platform of the Institute of Biological Systems (Laval University).

All taxa included in the analysis are listed in Table 4.

Phylogenetic Analyses

We used PARTITIONFINDER (Lanfear *et al.* 2012) to obtain the optimal data partition scheme (by locus) and the associated nucleotide substitution models, resulting in three partitions. The dataset was analyzed under the maximum likelihood (ML) criterion using RAxML black box (Stamatakis *et al.* 2008) with 500 bootstrap replicates (MLP) using the Cipres Portal (Miller *et al.* 2010).

Results and Discussion

Molecular topology

ML analyses of the Anthocerotaceae data matrix resulted in a well-supported phylogeny, with major ingroup nodes receiving ML bootstrap values >80% (Fig. 1). There are five main clades within Anthocerotaceae with various levels of support. Clade A includes nearly all *Folioceros* species (except *F. argillaceus*) with good support (87%) in a polytomy with the Paleotropical *Anthoceros angustus* a species known for abundant gemmae and long thin-walled pseudoelaters, *F. argillaceus* and *A. fusiformis* Austin (1875: 28). Clade B (88% MLP) includes species with spinose spores and thin-walled pseudoelaters comprising *Anthoceros neesii* Proskauer (1958: 1312), *A. subtilis* Stephani (1916: 1003) and *A. fragilis* Stephani (1916: 1006) sister to a clade that includes species with similar morphology to *Anthoceros punctatus* and *A. agrestis*. Clade C comprises three Indian species: *A. alpinus* Stephani (1923: 425), *A. erectus* Kashyap (1915: 9), *Anthoceros* sp. (from Kufri, India) and a species from Thailand, *A. telaganus* Stephani (1916: 1005) with high support

(MLP 99%). Almost all species are characterized by having a smooth strip along either side of the trilete mark on the proximal face of the spores. The endemic Australian species *A. capricornii* (Cargill & Scott 1997: 55) is sister to all three within this clade with no support. The Australian clade (Clade D, MLP 99%) comprises three recently described Australian endemic taxa (Cargill & Palsson 2021), including *A. palssoniae*, *A. apocynon* and *A. wellmanii.* All taxa with disparate spore and thallus morphology. (Cargill & Scott 1997: 55) The clade E has low to moderate support (MLP 68%) and includes *A. adscendens* Lehmann & Lindenberg (1832: 24), *A. tuberculatus* Lehmann & Lindenberg (1832: 25), *A. macounii* Howe (1898: 19)*, A. tristanianus* Villarreal, Engel & Vaìn̆a (2013: 33)*, A. orizabensis* (Stephani 1916: 965) Hässel (1990: 211) and *A. caucasicus* Stephani (1923: 427).

The nested nature of both *A. adscendens* and *A. capricornii* supports their inclusion within *Anthoceros*. Similarly, most *Folioceros* species form a strongly supported clade but nested within *Anthoceros*. The relatively well-sampled and well-supported clades B and E and the morphological synapomorphies of these two clades support a subgeneric rank to accommodate these species.

Morphological and ultrastructural diversity

Taxonomic implications

Based on these molecular phylogenetic results we propose to recognise four subgenera within *Anthoceros*: subgenus *Anthoceros* to accommodate *Anthoceros punctatus* and allied species (Clade B), and two new subgenera to accommodate a subset of Australian species (subgenus *Australienses* Clade D), a subset of Indian species (subgenus *Indici* Clade C) and a group of species with a number of synapomorphies which are not shared by all taxa in subgenus *Sphaerosporoceros* (Hassel 1988: 79) Cargill & Scott (1997: 58) (Clade E). Clade A comprises species with long-thick walled pseudoelaters, and could be recognised as subgenus *Folioceros*, but at present, molecular data for this clade lacks sequences from the type species, *F. assamicus* and the remaining 12 species from the genus. Consequently, no taxonomic decisions have been made at this point in time, but it appears clear with the taxa that have been sequenced that they are a monophyletic group with relatively good bootstrap support. We would predict that the type species, *F. assamicus* and the 12 remaining species will also fall within the *Folioceros* clade.

The relationship of Clade A to other clades is not resolved and lacks robust support. Previously, a relationship between *A. angustus* and *Folioceros* had been suggested by Hasegawa (as *A. formosae* Stephani (1916: 1002)) based on morphological data (Hasegawa 1984). *Anthoceros angustus* has long pseudoelaters (up to 400 µm), typical of *Folioceros* species (Table 2) and abundant gemmae on the thallus margin. However, this relationship is not supported by the molecular data as evidenced by the lack of bootstrap support. The positions of both *A. angustus* and *A. fusiformis* currently remains unresolved and therefore *incertae sedis*. With the addition of more genetic markers and taxa, their positions may be clarified.

Subgenus *Anthoceros* accommodates all species with short, thin-walled pseudoelaters, and dark, spiny spores with distally reticulate ornamentation and the absence of a smooth stripe along the trilete mark of the proximal face. This subgenus contains the following species *A. agrestis*, *A. laminifer* Stephani (1892: 266), *A. scariosus* Austin (1869: 230), *A. lamellatus* Stephani (1916: 1000), *A. neesii, A. venosus* Lindenberg & Gottsche (Gottsche *et al.* 1846: 584), *A. patagonicus* Hässel de Menéndez (1990: 207)*, A. patagonicus* subsp. *gremmensis* Villarreal, Engel & Vaňa (2013: 32), *A. sambesianus* Stephani (1916: 996) and *A. muscoides* Colenso (1884: 361). *Anthoceros fragilis* spores are the only exception to this particular spore pattern synapomorphy, lacking the distal reticulate patterning and pitting of the proximal face.

Subgenus *Australienses* is characterised by its unique and distinctive spores and comprises four Australian endemic taxa. All have a smooth strip either side of the trilete mark which falls short of the equatorial girdle and a flange running along either side of the trilete mark within each triangular proximal facet (Figs. 6 A-B, G-K). All three species, *A. apocynon, A. palssoniae* and *A. wellmanii* are ephemeral and grow in the semi-arid regions of western New South Wales in eastern Australia (Figs. 7 A, C, E-F). It is possible that the cluster of these three new endemic Australian taxa together could be a product of a selective sweep in plastid genes. Or it may be due to a recent divergence with clear morphological shared traits, but without the yet simultaneous accumulation of molecular *cp*DNA synapomorphies.

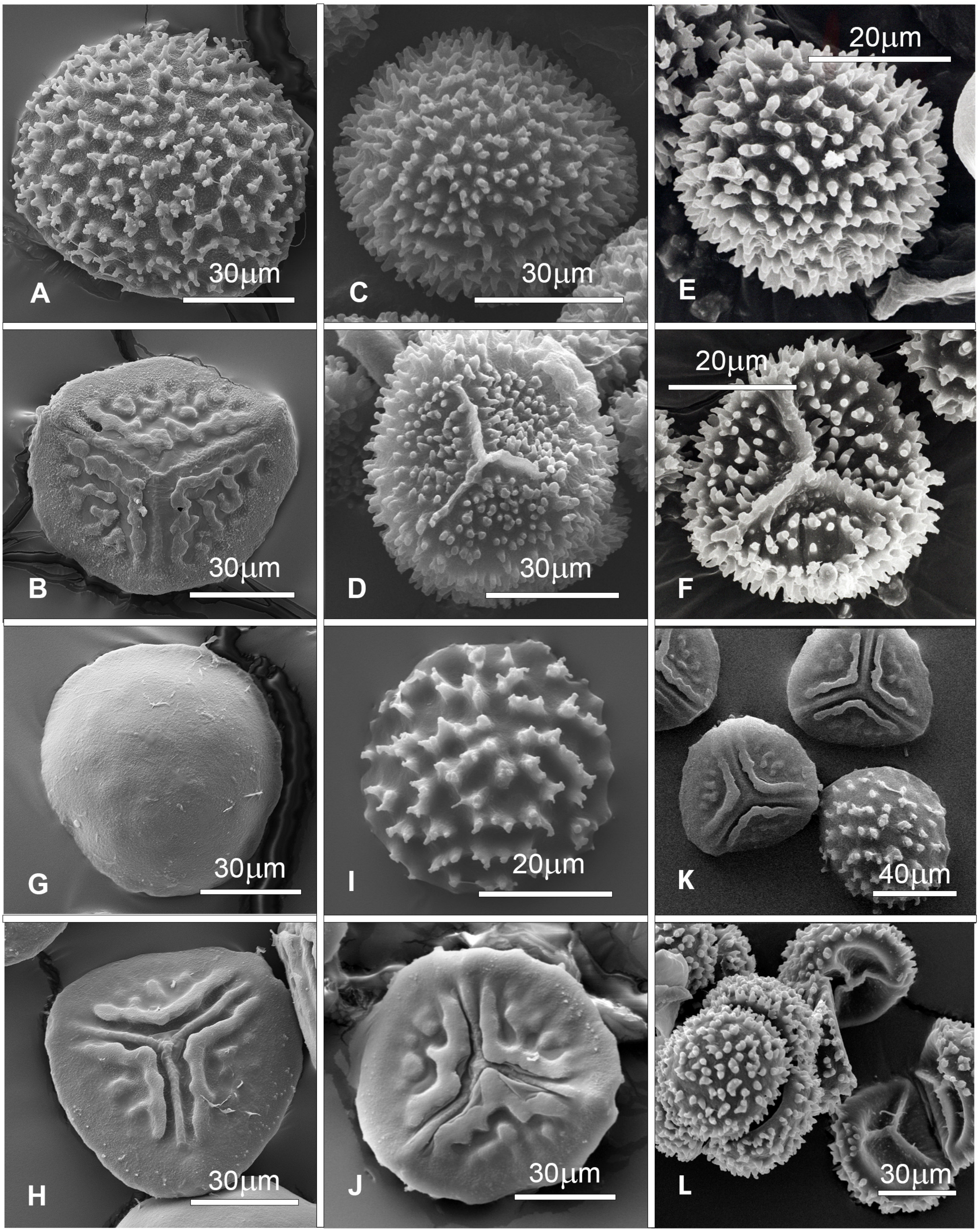

FIGURE 6. SEM images of spores of the Australian clade and one Indian taxon of the new Indian clade. A-B. *Anthoceros apocynon* distal and proximal views (*R.L.Palsson 398(3)*). C-D. *A. capricornii* distal and proximal views (*Z.Madycki ZM023*). E-F. *A. fragilis* distal and proximal views (*A.Dietrich s.n.*). G-H. *A. palssoniae* distal and proximal views (*R.L.Palsson 398(2)*). I-J. *A. wellmanii* distal and proximal views (*PW488A.1*). K. *A. wellmanii* distal and two proximal views. (*R.L.Palsson 473*). L. *A. sp_Kufri India* distal and proximal views (*J.Duckett IW57*). Image credits; A-K. D.C. Cargill, L. J.C. Villarreal.

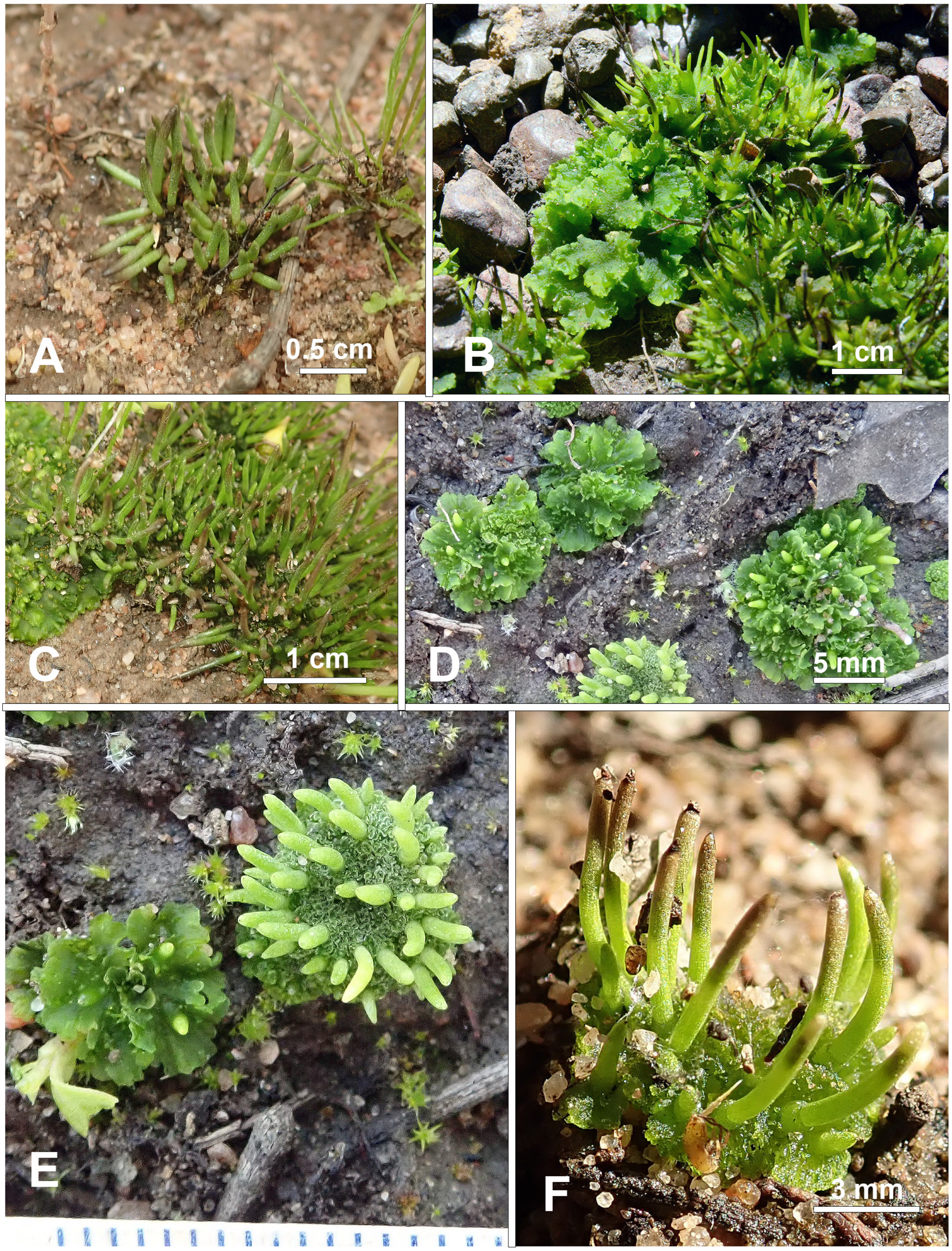

FIGURE 7. Habit photos of species in the Australian clade. A. *Anthoceros apocynon* (*R.L.Palsson 398(3)*); B. *A. capricornii* (*Z. Madycki ZM023*); C. *A. palssoniae* (*R.L.Palsson 398(2)*); D. *A. punctatus* (*D.C.Cargill 1670*); E. *A. wellmanii* (*D.C.Cargill 1670*); F. *A. wellmanii* (*R.L.Palsson 465A*). Image credits: A, C-E D.C. Cargill; B. Z. Madycki and F. R.L. Palsson.

Subgenus *Indici* is defined by unique and distinct spores characterised by their smooth, unpatterned strips either side of the proximal trilete mark, a prominent flange bordering the smooth strips and an elaborate ornamentation of smooth or knobbly vermiculae and papillae (this is in contrast with the proximal ornamentation seen in the Australian subgenus, see Figs. 6B, H, J. K)) (Figs. 3B, L, N) and comprises the following species: *A. alpinus, A. erectus*, *A. telaganus* and an unpublished species *Anthoceros* sp. Kufri, also from India (Fig. 6 L). *Anthoceros capricornii* is a northern Australian species from the monsoon tropics and is characterised by its relatively large (55-85 µm), almost spherical spores, densely papillate on both faces (Figs. 6B-C). This species had been placed in the subgenus *Sphaerosporoceros* because of its morphological affinities to *A. adscendens* (Cargill & Scott, 1997). At present it only has an unsupported sister relationship to subgenus *Indici* and does not fall within any of the current subgenera and is therefore of uncertain placement.

Lastly, subgenus *Sphaerosporoceros* comprises a group of species which share a single morphological synapomorphy – non-alveolate spores. *Sphaerosporoceros* was erected by Hässel de Menéndez to accommodate two species, *Anthoceros adscendens* Lehmann & Lindenberg (1832: 24) and *A. granulatus* Gottsche (1863: 275). The genus was characterised by spherical spores with reduced trilete mark, patterning more or less the same on both sides of the spore, sterile spores amongst mature and pseudoelaters broken up into short ovoid cells. Schuster also recognised the distinctiveness of *A. adscendens* but placed this species with the North American *A. macounii* in section *Brachyanthoceros* Schuster (1992: 815). He defined the section on non-alveolate spores; reduced pseudoelaters and short sporophytes (Schuster, 1992). Cargill and Scott, recognised *Sphaerosporoceros* but treated it at subgeneric level, and included an Australian endemic species, *A. capricornii* (Cargill & Scott, 1997). Clade E contains two of the four taxa originally placed in subgenus *Sphaerosporoceros* by Cargill & Scott (1997): *A. adscendens* and *A. macounii*. It also contains *A. caucasicus, A. orizabensis, A. tristanianus* and *A. tuberculatus*. As mentioned above, all taxa share the character of non-alveolate spores, but three of the five taxa also share relatively short sporophytes (3-24 mm); two of the species share a smooth strip either side of the trilete mark on the proximal face of their spores; two species have short pseudoelaters (19-42 x 17-45 µm) sometimes with vestigial thickenings and two species share a spore pattern of a 'wart' in the centre of each of the proximal facets. Other characters may come to the fore with further studies and more careful and detailed descriptions of species.

Recognition at the level of subgenus rather than genus level for each of the clades was deliberately chosen based on an incomplete dataset – not all taxa have been sequenced. This is particularly true of the taxa within the *Folioceros* species clade. By recognizing a single genus within the family Anthocerotaceae and therefore making it monotypic, a position which is not unusual within bryophyte classification, avoids instability and from a practical viewpoint, also avoids creating new combinations which may revert with the introduction of new information.

Further taxon and gene sampling within *Anthoceros* is required to help resolve species circumscription, as well as assessment of *Folioceros* species to test the morphological concepts proposed by Bharadwaj and later authors. Nuclear markers would also be useful to further test the clades proposed here, and whether the Australian endemic species form a clade, or their current cluster is a product of a selective sweep or a very recent divergence.

Taxonomic Treatment

Anthoceros Linnaeus, Sp. Pl. 1: 1139 (1753)

Type: *Anthoceros punctatus* Linnaeus

Subgenus *Anthoceros*

Included species: *Anthoceros agrestis*, *A. fragilis*, A. *lamellatus*, *A. laminiferus*, *A. neesii*, *A. patagonicus subsp. gremmensis*, *A. punctatus*, *A. subtilis*

Subgenus *Sphaerosporoceros* (Hässel de Menéndez 1988: 79) Cargill & G.A.M.Scott, J. Hattori Bot. Lab. 82: 58: (1997)

Basionym: *Sphaerosporoceros* Hässel, J. Hattori Bot. Lab. 64: 79 (1988)

Type: *Anthoceros adscendens* Lehm. & Lindenb.

Synonym: Section *Brachyanthoceros* R.M.Schust. The Hepaticae and Anthocerotae of North America. Vol. VI: 815 (1992)

Type: *Aspiromitus adscendens* (Lehm & Lindb.) Schust.

Included species: *Anthoceros adscendens*, *A. caucasicus*, *A. macounii*, *A. orizabensis*, *A. tristanianus*, *A. tuberculatus*

Subgenus *Indici* Cargill, Chantanaorr., R.L.Zhu, A.K.Asthana, Renzaglia & J.C.Villarreal *subgen. nov.*

Type: *Anthoceros alpinus* Steph.

Diagnosis: plants with spores similar to those of subg. *Australienses* but differing by the ornamentation of the flange with papillae and tubercles which continue into each of the three proximal facets or the proximal face may be completely smooth apart from the trilete mark.

Included species: *Anthoceros alpinus*, *A. erectus*, *A. telaganus*

Subgenus *Australienses* Cargill & J.C. Villarreal, *subgen. nov.*

Type: *Anthoceros wellmanii* Cargill

Diagnosis: plants with spores similar to those of subg. *Indici* but differing in the smooth flattened form of the flange and the reduced ornamentation in each of the three proximal facets, or facets filled with papillae and verrucae.

Included species: *Anthoceros apocynon*, *A. palssoniae*, *A. wellmanii*

This paper is dedicated to our colleague and friend Dr Jeff Duckett who has contributed enormously to the field of bryology.

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TABLE S1. List of all taxa used in the phylogeny with collection and herbarium details, country of origin and Genbank accession numbers. New sequences are in bold red font.

Species	Collector & #	Country	rbcL	trnL-matK	rps4
Anthoceros adscendens Lehm. & Lindenb.	Hays 4201-3 (M)	U.S.A. (Florida)	KF482293	---	KP238761
Anthoceros agrestis Paton	Villarreal 1353 (M)	Germany	KP238681	KP238675	KP238684
Anthoceros alpinus Steph.	Duckett IW110 (M)	India	KF482268	KF482215	KP238685
Anthoceros angustus Steph.	Zhang 7704 (SZG)	China	AB086179.1	AB086179.1	AB086179.1
Anthoceros apocynon Cargill & Palsson	Palsson 398(3) (CANB)	Australia	---	---	OP921816
Anthoceros capricornii Cargill & Scott	Madycki ZM023 (CANB)	Australia	---	---	OP921809
Anthoceros caucasicus Steph.	Garcia s.n. (M)	Portugal	JX872419	KF482294	KP238687
Anthoceros cf. sambesianus Steph.	Serousiaux s.n. (M)	Reunion Islands	JX872425	KF482295	---
Anthoceros fragilis Steph.	Lovatt & Holland TH 9821 (CANB)	Australia	KF482270	KF482298	---
Anthoceros cf. scariosus Aust.	Villarreal 1217 (M)	Mexico	JX872420	KF482296	---
Anthoceros erectus Kash.	Chantanaorrapint 212 550 (PSU)	Thailand	KF482269	KF482216	KP238689
Anthoceros fusiformis Aust.	Doyle 11347 (ABSH, M)	U.S.A. (California)	DQ845677	KF482217	---
Anthoceros lamellatus Steph.	Duckett s.n. (ABSH), Venezuela, Rincon s.n. (M)	Colombia	DQ845679	KF482299	KP238690
Anthoceros laminiferus Steph.	Duckett s.n. (ABSH), Slack 211056 (M)	New Zealand	KF482271	KF482300	KP23869
Anthoceros macounii M.A.Howe	Faubert s.n. (M, QFA)	Canada	JX872421	KF482301	KP238692
Anthoceros neesii Prosk.	Manzke s.n. (M)	Germany	JX872422	KF482302	KP238693
Anthoceros orizabensis (Steph.) Hässel	Villarreal 770 (M)	Venezuela	JX872423	KF482303	KP238694
Anthoceros palssoniae Cargill	Palsson 398(1) (CANB)	Australia	OP921805	---	OP921810
Anthoceros patagonicus subsp. gremmenii J.C.Villarreal et al.	Gremmen 2005-T028 (F)	Tristan de Cunha	JX872424	KF482304	KP238695
Anthoceros punctatus L.	Sergio s.n. (LISU, M) Portugal)	Portugal	KF482272	KF482305	KP238696
Anthoceros punctatus	Cargill 1670 (CANB)	Australia	OP921804	---	OP921812
Anthoceros punctatus	Palsson 457	Australia			
Anthoceros sp.1. Ethiopia	Hylander 4504 (M)	Ethiopia	KF482273	KF482306	KP238697
Anthoceros sp.2_Kufri India	Duckett IW57 (BM)	India	KF482274	KF482218	KP238698

...*Continued on the next page*

TABLE S1. (Continued)

Species	Collector & #	Country	rbcL	trnL-matK	rps4
Anthoceros subtilis Steph.	Villarreal 1236A (M)	India	---	---	KP238699
Anthoceros telaganus Udar & Asthana	Chantanaorrapint 229 (PSU)	Thailand	KF482267	KF482214	KP238686
Anthoceros tristanianus J.C.Villarreal et al.	Villarreal 1032 (M)	Tristan de Cunha	JX872426	KF482219	KP238700
Anthoceros tuberculatus Lehm. & Lindenb.	Villarreal & Rodríguez 857 (CONN)	Panama	JX872427	KF482307	---
Anthoceros cf. venosus Lindenb. & Gottsche	Salazar & al. 20654 (PMA)	Costa Rica	JX872428	KF482297	KP238688
Anthoceros wellmanii Cargill 3	Wellman 588A.1 (CANB)	Australia	OP921807	---	OP921815
Anthoceros wellmanii Cargill 2	Palsson 457pp (CANB)	Australia	OP921808	---	OP921811
Anthoceros wellmanii Cargill 1	Cargill 1670pp (CANB)	Australia	OP921806	---	OP921814
Dendroceros cichoraceus (Mont.) Gottsche	Larraín 31162 (CONC)	Chile	JX872430	KF482310	KP238701
Dendroceros crispatus (Hook.) Nees	Cargill 28 (CANB), Paterson s.n. B570 (CANB)	Australia	AY463048	KF482311	
Dendroceros crispus (Sw.) Nees	Villarreal 1296 (M)	Panama	JX885633	KF482312	KP238702
Dendroceros validus Steph.	Duckett D27 (M)	Malaysia	JX885634	KP238673	---
Folioceros amboinensis (Schiffn.) Steph.	Chantanaorrapint 2493 (PSU)	Thailand	KF482280	KF482315	KP238706
Folioceros appendiculatus (Steph.) J.Haseg.	Chantanaorrapint 1884 (PSU)	Thailand	KP238683	KP238677	---
Folioceros argillaceus (Steph.) Verdoorn	Chantanaorrapint 2737A PSU)	Thailand	KP238682	KP238676	KP238707
Folioceros_fuciformis (Mont) D.C.Bharadwaj	Tan s.n.	Singapore	AY463050	---	---
Folioceros_fuciformis (Mont) D.C.Bharadwaj	Cairns s.n. (CANB)	Australia	AY463051	---	---
Folioceros fuciformis (Mont) D.C.Bharadwaj.	Gradstein 12350 (M), Cairns s.n. (CANB)	Australia	KF482281	KF482316	KP238708
Folioceros_fuciformis (Mont) D.C.Bharadwaj.	Zhang 4465 (SZG)	China	JF815572	---	---
Folioceros glandulosus (Lehm. & Lindenb.) D.C.Bharadwaj	Zhang 5622 (SZG)	China	JF815573.1	---	---
Folioceros incurvus (Steph.) D.C.Bharadwaj	Shevock 39742 (M)	Sao Tomé	KF482282	KF482225	KP238709
Folioceros kashyapii Udar & Srivastava	Peng 20111015-85 (HSNU)	China	KF482283	KF482226	KP238710

...Continued on the next page

TABLE S1. (Continued)

Species	Collector & #	Country	rbcL	trnL-matK	rps4
Folioceros sp.	Peng 20111016-3 (HSNU)	China	KF482284	KF482227	KP238711
Megaceros flagellaris (Mitt.) Steph.	Cargill 885 (CANB)	Australia	GQ845371	JN559929	KP238713
Megaceros tjibodensis Campb.	Duckett IE52 (M)	India	KF482286	KF482231	---
Nothoceros fuegiensis (Steph.) J.C.Villarreal	Goffinet 9527 (CONN)	Chile	HM056156	JN559934	KP238718
Nothoceros minarum (Nees) J.C.Villarreal	Cargill & Prieto 2625 (CANB)	Uruguay	JX872433	KF612916	KP238720
Nothoceros renzagliensis J.C.Villarreal et al.	Villarreal & al. 1080 (COL)	Colombia	HM056162.1	JN559940	KP238721
Notothylas himalayensis Udar & Singh	Duckett IW56 (M)	India	KF482287	KF482232	KP238727
Notothylas pandei Udar & Chandra	Chantanaorrapint 1666 (PSU)	Thailand	KF482289	KF482235	---
Paraphymatoceros diadematus Hässel	Larraín 34069 (CONC)	Chile	JX872438	KF482321	KP238732
Phaeoceros proskauerii Stotler et al.	Doyle 11339 (ABSH)	U.S.A. (California)	EU283415.1	KF482323	KP238735
Phaeoceros himalayensis (Kash.) Prosk.	Long 30423 (E)	Nepal	JX872444	KF482239	KP238741
Phaeoceros laevis (L). Prosk.	Sergio s.n. (LISU)	Portugal	DQ845673	KF482240	KP238743
Phaeoceros perpusillus S.Chantanaorrapint	Chantanaorrapint 1551 (PSU)	Thailand	KF482292	KF482333	KP238747
Phaeomegaceros fimbriatus (Gottsche) Duff et al.	Villarreal 779 (ABSH), Villarreal & al. 881 (CONN)	Panama	HM056149	JN559928	KP238752
Phaeomegaceros hirticalyx (Steph.) Duff et al.	Duckett s.n. (ABSH, M)	New Zealand	AY463043	KF482336	KP23875
Phymatoceros bulbiculosus (Brot.) Stotler et al.	Sergio s.n. (LISU)	Portugal	DQ268978.1	KF482241	KP238759
Phymatoceros phymatodes (M.A.Howe) Duff et al.	Doyle s.n, Doyle 11480 (ABSH, M)	U.S.A. (California)	DQ845660.1	KF482342	KP238760