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Morphology, ultrastructure and phylogenetic affinities of the single-island endemic *Anthoceros cristatus* Steph. (Ascension Island)

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Oceanic islands, due to their geographical isolation, number, precisely defined boundaries and their geomorphological and climatic diversity, have provided enormous insights into speciation, dispersal, adaptive radiations and macroecological processes. One of the key components of these island studies is the role of single-island endemics (SIEs) as, in many instances, island biogeography models use the proportion of SIEs to infer evolutionary processes. It is, therefore, imperative to undertake critical taxonomic revisions to evaluate SIEs because changes in the number of SIEs have a key impact on downstream biogeographic analyses. We revise the special case of a putative SIE *Anthoceros cristatus* on Ascension Island using light and electron microscopy, as well phylogenomic tools. *A. cristatus* lies within the *A. agrestis*/*A. punctatus* complex but differs from the sister species *A. agrestis* and *A. punctatus* in spore morphology and gametophytic lamellae fringed with caducous marginal cells. The present confirmation, from both molecules and morphology, of the SIE status of *Anthoceros cristatus* and its restricted distribution on the Island makes the preservation of its habitat a conservation priority. Ascension Island is the tip of an undersea volcano that is thought to have emerged from the ocean 1 million years ago with an area of approximately 91 km^2, with Green Mountain as the highest elevation (~859 m a.s.l.). Ascension has a relative low bryophyte species diversity of 87 spp but this includes 12 endemics (~14%); a much higher level of endemism than on the far more speciose Macaronesian Islands.

Keywords: Oceanic islands, Single island endemics, Ultrastructure

Introduction

The importance of islands for exploring evolutionary questions dates back to Darwin's time (Losos & Ricklefs, 2009). Oceanic islands in particular, due to their geographical isolation, number, precisely defined boundaries and their geomorphological and climatic diversity, have provided enormous insights into speciation, dispersal, adaptive radiations and macroecological processes (Whittaker & Fernández-Palacios, 2007; Losos & Ricklefs, 2009). The complex interactions between migration, extinction and speciation within islands were first formally summarised by McArthur & Wilson (1967) and recently re-visited by an increasing number of more complex models of island biogeography including Pleistocene glacial fluctuations (e.g. Whittaker *et al.*, 2008; Fernández-Palacios *et al.*, 2016). One of the key components of these island studies is the role of single-island endemics (hereafter termed SIEs) as, in many instances, island biogeography models use the proportion of SIEs to infer evolutionary processes (Gray & Cavers, 2014). It is, therefore, imperative to undertake critical taxonomic revisions to evaluate SIEs because changes in the number of SIEs have a key impact on downstream biogeographic analyses (Gray & Cavers, 2014).

Recent studies on spore-producing plants (liverworts, mosses, lycophytes and ferns) (Patiño *et al.*, 2014, 2015) strongly suggest that the premises and predictions of the island biogeography theory (Whittaker *et al.*, 2008) do not entirely apply to these taxonomic groups. The most puzzling aspects of bryophyte island biology are the low levels of endemic species, with a very few confirmed SIEs (e.g. *Orthotrichum handiense* F.Lara, Garilleti & Mazimpaka endemic to Fuerteventura, Canaries; Patiño *et al.*, 2013). The high dispersal capabilities of bryophytes, as inferred

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from experimental, demographic and phylogeographic evidence are held to be responsible for the low levels of endemism in the group (Hutsemékers *et al.*, 2011; Patiño *et al.*, 2015). Many oceanic islands might be, therefore, insufficiently isolated to disrupt gene flow from continents and thus for speciation to occur (Rosindell & Phillimore, 2011). Conversely bryophyte diversity and, in particular, the proportion of SIEs might have been underestimated as a result of under-collecting, a preference by taxonomists for wide species definitions, and a lack of exhaustive taxonomic and floristic studies in a group that typically exhibits limited morphological complexity. The recent use of phylogenetic tools to uncover bryophyte diversity (Renner *et al.*, 2013; Hedenäs *et al.*, 2014) suggests that species concepts should now be revised by integrating both morphological and molecular approaches (Draper *et al.*, 2015). We revise the special case of a putative SIE *Anthoceros cristatus* Steph. on Ascension Island.

Ascension Island is the tip of an undersea volcano that is thought to have emerged from the ocean 1 million years ago (Ashmole & Ashmole, 2000). It is located in the middle of the South Atlantic Ocean roughly 1505 kilometres from the coast of Africa, 2232 km from the coast of Brazil and 1500 km from St. Helena (~125 km^2). The island has an area of approximately 91 km^2, with Green Mountain as the highest elevation (~859 m a.s.l.). Both Ascension and St Helena have a relative low bryophyte species diversity (Ascension Island: 87 spp.; St. Helena: approximately 110 spp.), but a significant number of endemics (St. Helena: 24 spp. including one endemic genus (Wigginton, 2013); Ascension Island: 12 spp. (Pressel *et al.*, 2016)). Interestingly, the proportion of bryophyte SIEs is rather high on both islands (22% on St. Helena and 14% on Ascension Island) in stark contrast with islands with larger floras. For example, in Macaronesia: Azores (451 spp., 5 endemics), Madeira (463 spp., 10 endemics) and the Canary Islands (485 spp., 6 endemics); their low levels of SIEs can be explained by the proximity of these archipelagos to the potential continental species pools (Patiño *et al.*, 2013, 2015).

Anthoceros cristatus is now the sole hornwort possibly endemic to Ascension Island. A further possible endemic, *Anthoceros floribundus* Steph. which has highly diagnostic dorsal tubers, is now known to be conspecific with the Australian species *Phaeoceros evanidus* (Steph.) Cargill & Fuhrer (Pressel *et al.*, 2016). *Anthoceros cristatus* and *A. floribundus* were described, without illustrations, by Stephani (1916) based on specimens gathered by an unknown collector in 1900.

In the present study, we assess the affinities and evaluate the taxonomic status of the unique hornwort SIE to Ascension Island, *Anthoceros cristatus*, by using molecular and ultrastructural approaches. Using three molecular markers, two different genomic compartments, selected to fit in with existing datasets for the hornworts, were sequenced using recently collected samples from Ascension Island. Finally, we studied the anatomy and ultrastructure of the species to get complementary evidence about its phylogenetic affinities.

Materials and Methods

Taxon sampling, isolation of DNA, amplification and sequencing

The holotype specimen of *Anthoceros cristatus* (G) housed in Geneva (Villarreal *et al.*, 2015) (G-00045042!) and four recently collected samples were used for measurements and morphological studies. Three collections of *Anthoceros cristatus* from Ascension Island were studied and two of them were isolated for PCR using the protocols outlined by Villarreal & Renner (2013). Only one of these amplified successfully. Additionally we included a collection of *Anthoceros agrestis* Paton from Hessen, Germany. We used a dataset of 22 species of *Anthoceros* L. and 3 species of *Folioceros* D.C.Bhardwaj (Anthocerotaceae) and included 6 species of Notothyladaceae as the outgroup. To deduce phylogenetic relationships, we used the mitochondrial *nad*5-exon2 (excluding an intron of ~950 nucleotides that is unique to *Leiosporoceros* Hässel, *Anthoceros*, *Folioceros* and *Sphaerosporoceros* Hässel), the plastid gene *rbc*L and portions of the *trn*K intron and the *mat*K gene contained within it (Villarreal & Renner, 2013). Table S1 provides a list of the sampled species with taxonomic author names, geographic origin of material, herbarium vouchers and GenBank accession numbers for all sequences.

Phylogenetic analysis

Combined phylogenetic analyses were performed under likelihood (ML) optimisation and the GTR + G substitution model, using RAxML (Stamatakis *et al.*, 2008) with 100 bootstrap replicates. Bayesian analyses were conducted in MrBayes 3.2 (Ronquist *et al.*, 2012), using the default two runs and four chains (one cold and three heated), with default priors on most parameters. We partitioned by genome, applying the GTR + Γ + I. Model parameters for state frequencies, the rate matrix and gamma shape were unlinked, and posterior probabilities (PP) of tree topologies were estimated from both partitions. To assess burn-in and convergence we compared the bipartitions across the two runs. Convergence was usually achieved in MrBayes after 1×10^6 generations, with trees sampled every 5000th generation for a total length of 5×10^6 generations;

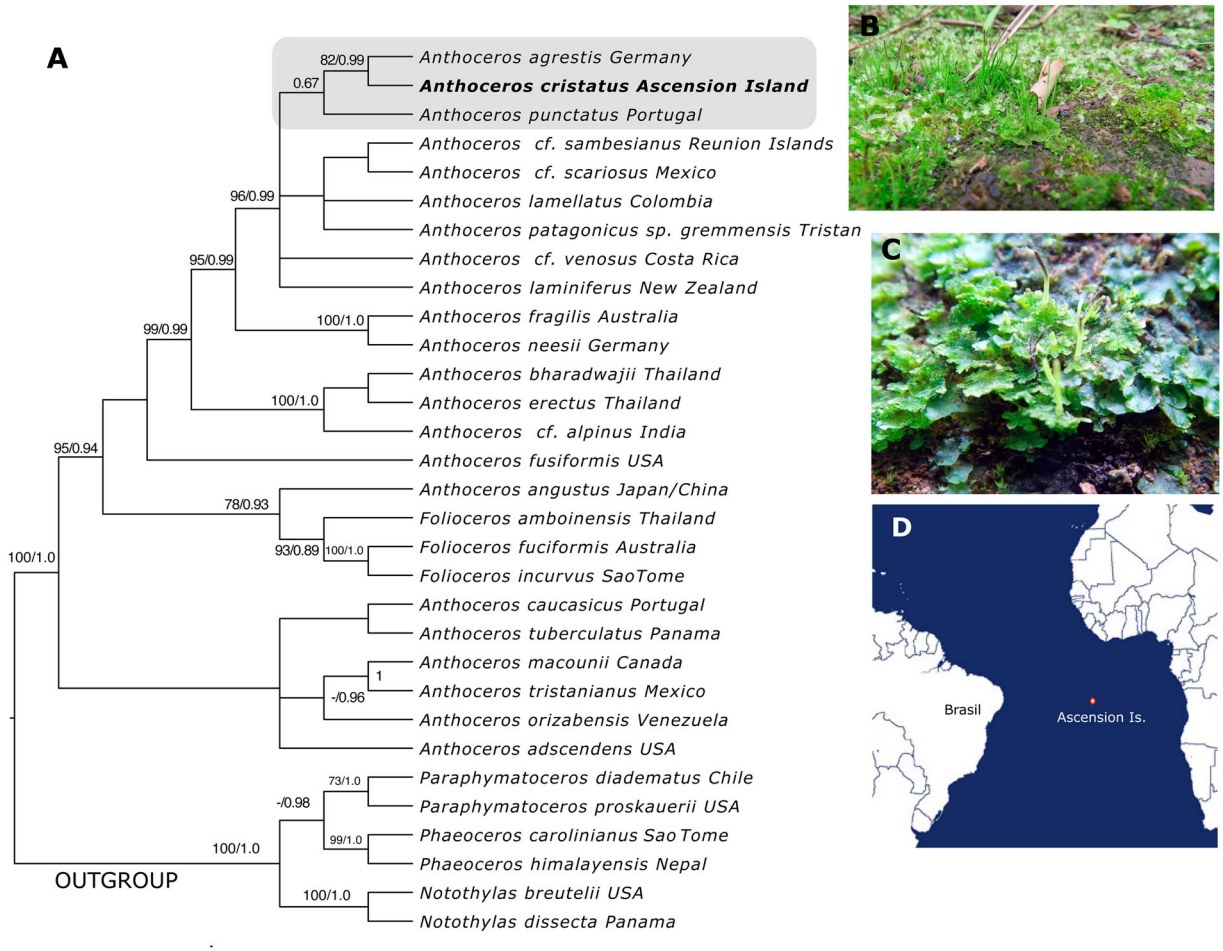

Figure 1 (A) Cladogram based on a maximum likelihood analysis of *Anthoceros* based on two loci of plastid, and mitochondrial DNA; ML bootstrap values above 50% and posterior probabilities are shown above branches. *Anthoceros cristatus* is related to a collection of *A. agrestis* from Hessen, Germany with high support. (B–C) Habitat pictures of *A. cristatus*. The species grows associated with *P. carolinianus* (C). (D) Ascension Island is located in the middle of the South Atlantic Ocean roughly 1505 kilometres from the coast of Africa, 2232 km from the coast of Brazil and 1500 km from St. Helena.

we discarded 25% of each run and then pooled runs. The final matrix is available at treebase, number = 19746.

Light, transmission and scanning electron microscopy

Thalli were collected from the wild and were processed for transmission (TEM) and scanning electron microscopy (SEM) as described previously (Duckett *et al.*, 2006). For TEM, thalli were fixed in 3% glutaraldehyde, 1% fresh formaldehyde and 0.75% tannic acid in 0.05 M Na-cacodylate buffer, pH 7, for 3 h at room temperature. After several rinses in 0.1M buffer, the samples were post-fixed in buffered (0.1M, pH 6.8) 1% osmium tetroxide overnight at 4°C, dehydrated in an ethanol series and embedded in Spurr's resin via ethanol. 0.5–1 µm-thick sections were cut with a diamond histo-knife, stained with 0.5% toluidine blue and photographed with a Zeiss Axioskop light microscope fitted with a MRc Axiocam digital camera. Thin sections were cut with a diamond histo-knife, stained with methanolic uranyl acetate for 15 min and in Reynolds' lead citrate for 10 min, and observed with a Hitachi H-7100 transmission electron microscope at 100 kV. For SEM, thalli were fixed in 3% glutaraldehyde, dehydrated through an ethanol series, critical-point dried using CO_2 as the transfusion fluid, sputter coated with 390 nm palladium-gold and viewed using a FEI Quanta scanning electron microscope.

Results and Discussion

Taxonomic description and habitat preferences

Anthoceros cristatus Steph., Sp. Hepat. 5: 1916.

Typus: Ascension Island, *Unknown collector* (G!).

Figures 1–4

Thalli prostrate to ascending, with slightly dissected lobes at margins, up to 5 mm long and 1–2 mm wide; often overlapping to form crispate rosettes 0.5–2.0 cm in diameter; bright to pale green, older parts partially translucent. Thalli cavernous, cells with a single large discoid chloroplast 20–30 µm in diameter with a conspicuous pyrenoid. Thallus section 10–15 cells high, epidermal cells scarcely smaller than the

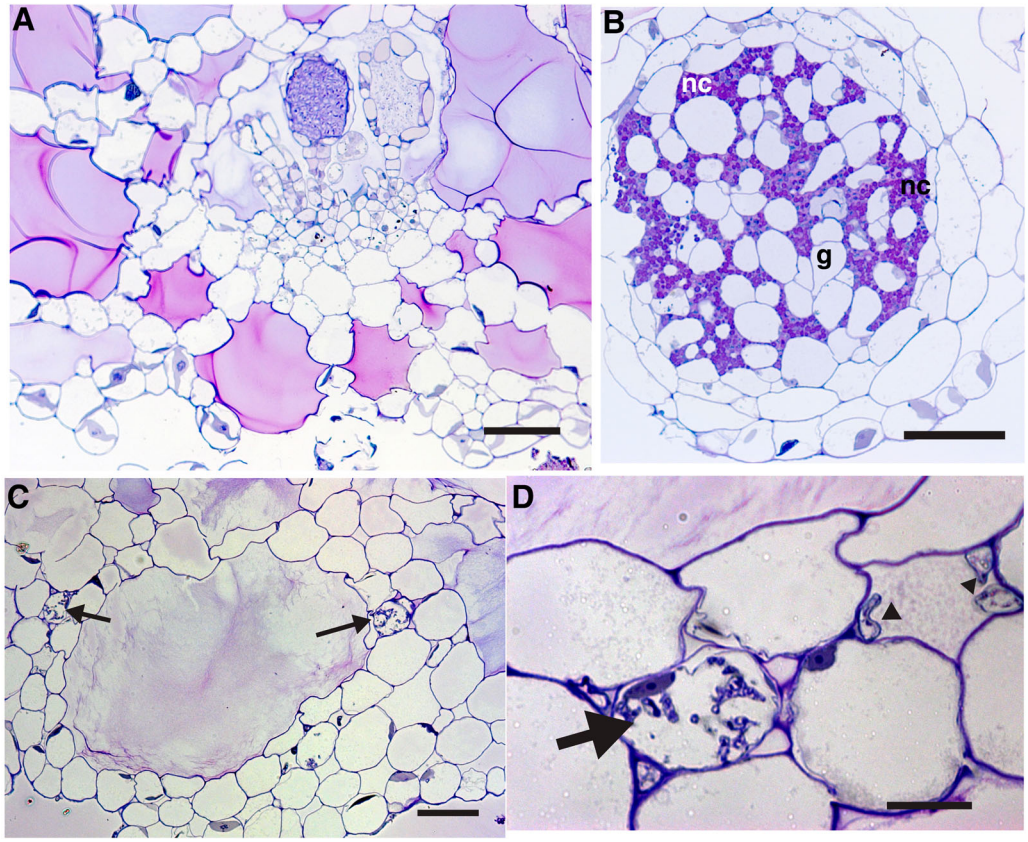

Figure 2 Gametophyte anatomy of *Anthoceros cristatus* Steph. 1 μm, toluidine blue, resin-embedded sections. (A) Transverse section of the thallus showing multiple mucilage-filled schizogenous cavities (purple). One antheridial cavity shows two entire antheridia (with their stalks) and several antheridia budding from its base. *Anthoceros cristatus* can present up to 20 antheridia per chamber. Bar = 20 μm. (B) A section of a *Nostoc* colony (nc) with numerous gametophytic cells (g) intertwined with the cyanobacterial filaments. Bar = 50 μm. (C) Transverse section of a large schizogenous cavity with a few cells infected with Mucoromycotina fungal hyphae around its periphery. Bar = 40 μm. (D) A subepidermal cell packed with hyphae (arrowed) and intercellular hyphae in a small mucilage cavity (arrowheads) Bar = 10 μm.

internal cells, 30–50 μm wide. Dorsal surface of thalli covered with highly conspicuous lamellae tipped by enlarged, swollen and often caducous cells 45–55 μm in diameter. Ventral region of thallus containing inter- and intracellular mucoromycote fungus (Rimington, Bidartondo, Pressel & Duckett, unpublished data). *Nostoc* colonies usually 1 per thallus lobe, up to 600 μm in diameter and bulging below the lower thallus surface.

Monoicous, most likely highly self-fertile and mostly protandrous. Antheridial chambers with up to 20 antheridia. Antheridial body 60–80 × 100–140 μm with 3–4-tiered stalk 15–25 × 50–80 μm. Involucre cylindrical, smooth, erect, usually less than 0.5 mm high, occasionally up to 2 mm.

Sporophytes very common, erect, bivalved, up to 3.5 cm long, 325–380 μm wide, with a well-developed columella. Epidermal cells of capsule walls rectangular to narrowly rectangular, 165–210 × 10–15 μm, thick-walled; stomata scattered, 52–69 × 29–36μm, with two reniform guard cells. *Spores* black, 40–60 (66) μm in diameter, distal surface with13–17 spinulate projections, proximal surfaces finely foveolate and with a distinct triradiate mark. Pseudoelaters light brown, usually 4 cells long, cells narrowly rectangular, thin-walled and sometimes branched.

Morphologically *A.cristatus* clearly fits within the '*Anthoceros punctatus*/*A. agrestis* complex' (Table 1) characterised by massively cavernous, lamellate monoicous thalli and distally spinose black spores with foveate proximal faces (Proskauer, 1958; Paton, 1999). The distal face of *A. cristatus* lacks the strong reticulate ornamentation found in *A. agrestis* (Polevova *et al.*, 2012). Although under the light microscope the proximal face appears smooth and similar to the Neotropical *A. lamellatus* Steph., SEM reveals the presence of regular shallow pits (Figure 4).

The very short involucres, swollen, caducous marginal lamellar cells and massive *Nostoc* colonies (Figure 2B) are the gametophytic features that clearly separate *A. cristatus* from *Anthoceros punctatus* and *A. agrestis* (Table 1). It should be noted that, although the *A. punctatus*/*A. agrestis* complex has recently received attention at both the ultrastructural (*A. agrestis*, Polevova *et al.*, 2012) and genomic levels (Li *et al.*, 2014; Szövényi, 2016) the genetic diversity and precise

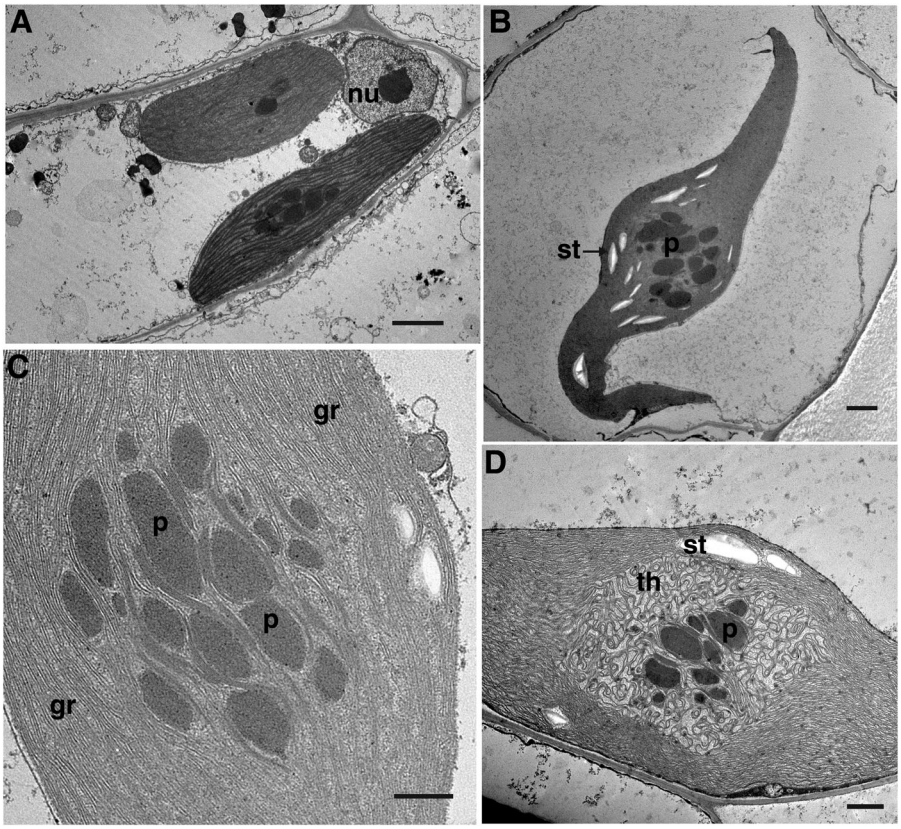

Figure 3 Transmission electron micrographs of gametophyte cells of ***Anthoceros cristatus*** Steph. **(A)** Internal cell showing two lobes of the plastid on either side of the nucleus (nu). Bar = 10 μm. **(B)** Near median transverse section of an epidermal plastid showing the central pyrenoid (p) flanked by numerous starch grains (st). The multiple pyrenoid units (p) are flanked by several starch grains (St). Bar = 5 μm. **(C)** Details of the multiple pyrenoid and grana (gr) lacking end membranes. Bar = 2 μm. **(D)** Many plastids have highly pleomorphic channel thylakoids (th) around the pyrenoid. Bar = 5 μm.

affinities of the species within the complex have yet to be resolved. This is likely to change in the near future with the adoption of *A. punctatus*/*A. agrestis* as the next bryophyte model 'species' because of the small genome size (~85 Mb, data.kew.org/cvalues/vienna05_report.pdf) and increasing available genomic resources (Li *et al*., 2014; Szövenyi, 2016). In the molecular context, it is also noteworthy that *Anthoceros cristatus* is the only hornwort to date that seems to associate exclusively with Mucoromycotina fungi; all other taxa examined to date harbour both Glomeromycota and Mucoromycotina fungi, sometimes simultaneously (see Dèsiro *et al*., 2013, Table 1). In common with all other hornworts, fungal colonisation is both inter and intracellular (Figure 2C, D) and there is no evidence of fungal entry via the rhizoids.

Ecology: Frequent on bare ground along paths (Elliot's, Bishop's, Dew Pond) and on friable banks between 600 and 700 m (Pressel *et al*., 2016). Lowest localities: bank by The Residency (560 m) and on soil in a shaded south-facing ravine in Cricket Valley (420 m). Highest locality; Dew pond track (780 m). The associates are the hornworts *Phaeoceros carolinianus* (Michx.) Prosk. and *P. evanidus*, the liverworts *Fossombronia husnotii* Corb. and *F. indica* Steph. and the mosses, *Bryum dichotomum* Hedw., *B. sauteri* Bruch & Schimp., *Dicranella goughii* O'Shea, *Hyophila ascensionis* Cardot and *Leptophascum leptophyllum* (Müll.Hal.) J.Guerra & M.J.Cano. In general terms, the *A. cristatus* habitats and many of its associates are similar to those of many temperate hornworts that also colonise arable fields

Cytology of the thalli of *A. cristatus*: (Figures 2 and 3) The massive nature of the mucilage cavities in *A. cristatus* is clearly apparent in semi-thin sections (Figure 2A, C). The antheridial cavities contain multiple antheridia at different stages of development, ensuring continuous and staggered sperm production in each gametophyte (Figure 2A). The large, ventrally bulging *Nostoc* colonies show extensive proliferation of gametophyte cells within each colony (Figure 2B). These gametophytic projections within the cyanobacterial colonies most likely enhance the transfer of metabolites between partners. However, in *A. cristatus,* as in other hornworts, these comprise highly vacuolated cells and lack the wall ingrowths found in the liverwort *Blasia* L. (Duckett *et al*., 1977). The endophytic Mucoromycotina fungus is located in the ventral parts of the thalli and around the mucilage cavities (Figure 2C, D). The fungus is both inter- and intracellular.

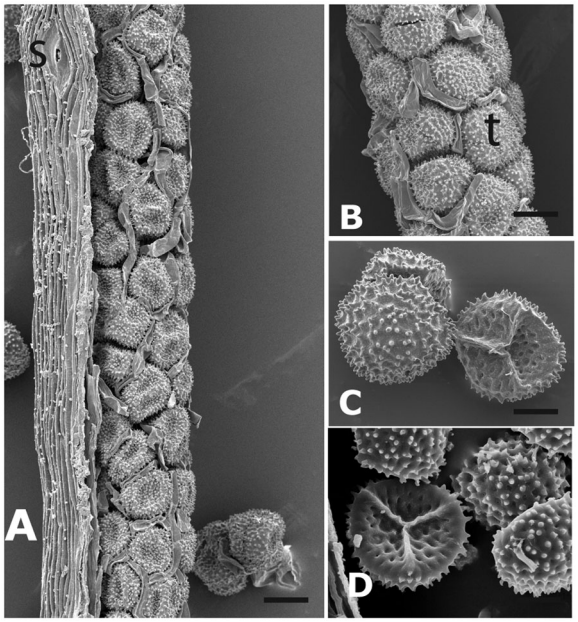

Figure 4 Scanning electron micrographs of the sporophyte and spore morphology of *Anthoceros cristatus* Steph. (A) A longitudinal view of the sporophyte immediately above the involucre showing a shrunken open stoma (s) and the spore mass tightly intertwined with the pseudoelaters (ps). Bar = 50 μm. (B) A close-up of the mass the spore tetrads. The tetrads (t) are ready to separate and be dispersed with the aid of the pseudoelaters. Bar = 20 μm. (C) Individual spores showing a distal surface with 13–17 spinulate projections, finely foveolate proximal surfaces and with a distinct triradiate mark. Bar = 15 μm (D) Image of the type material of *A. cristatus* (G–00045042!). In the type material the distal foveoles are slightly more pronounced, probably due to the antiquity of the material (1900). Bar = 15 μm.

Each epidermal cell contains a single discoid plastid with a multiple pyrenoid (up to 12 subunits in section). Each pyrenoid unit is traversed by single thylakoids or grana (Figure 3C) containing 3–6 thylakoids and lacking end membranes (Vaughn *et al.*, 1992). Many of the plastids have a network of highly convoluted channel thylakoids around the pyrenoid (Figure 3D) typical of most hornworts (Vaughn *et al.*, 1992).

Phylogenetic affinities

Our dataset includes 3591 total characters with 624 variable characters that are parsimony informative (Figure 1). Phylogenetic analyses using plastid and mitochondrial data support the placement of *Anthoceros cristatus* within the genus *Anthoceros* and excluded from *Folioceros*. The data cluster *Anthoceros cristatus* in a highly supported clade (95% bootstrap value) with all species of *Anthoceros* with spiny distal spore faces that lack a smooth stripe along the trilete mark. The clade includes the cosmopolitan species *A. punctatus*, New Zealand *A. laminifer* Steph., American *A. scariosus* Austin, Neotropical *A. lamellatus* and *A. venosus* Lindenb. & Gottsche, Tristan de Cunha endemic *A. patagonicus* subsp. *gremmenii* J.C.Villarreal, J.J.Engel & Váňa and African *A. sambesianus* Steph. (Figure 1). In our tree, the closest relative of *Anthoceros cristatus* is a collection of *Anthoceros agrestis* from Germany with good support (84%). Both collections differ by less than 1% of their included nucleotide sequences. This nucleotide similarity is amazing considering the large geographic distance between Ascension Island and Germany. Such similarity may be also an indication that the slow evolving molecular markers are not suitable for a species-level resolution. *Anthoceros agrestis* is an annual species of temperate zones, commonly and almost exclusively found in arable fields (Bisang, 1995). Its small genome size (85Mb) and relatively low abundance of repetitive elements (Li *et al.*, 2014; Szövényi, 2016) in comparison to all other *Anthoceros* species (Bainard & Villarreal, 2013) may have been due to a species shortened life history and self-compatibility as a late adaptation to arable fields since the outset of agriculture in Europe near 6–12,000 years ago. It should also be noted that the distribution of another of the four hornworts on Ascension, *Phaeoceros evanidus* otherwise only known from Australia (Cargill & Fuhrer, 2008), is equally remarkable.

Perhaps the simplest scenario for the origin of *Anthoceros cristatus* is that it evolved anagetically from an ancestor in the *A. agrestis*/*punctatus* group that initially colonised Ascension Island. All the species in the aggregate share several similarities: monoicous sexual system, spore size and small thallus. The demonstration in axenic cultures that the swollen

Table 1 Comparisons with other members of the *Anthoceros agrestis*/*punctatus* agg.

Gametophyte:

	Nostoc colonies	Fungal endophyte	Thallus section	Dorsal lamellae	Marginal cells	Antheridial dimensions (μm)	Involucre length (mm)
A. cristatus	Usually 1/thallus up to 600 μm	Mucoromycotina	10–15 cells	Abundant	Rounded, caducous	60–80 × 100–140	Up to 0.5 (2.0)
A. punctatus	Usually > 1/thallus 200–450 μm	Mucoromycotina and/or Glomeromycota	10–20(30) cells	Few or none	Not as above	60–85 × 104–150	0.7–3.0
A. agrestis	Usually > 1/thallus 200–450 μm	Mucoromycotina and/or Glomeromycota	6–12(18) cells	Frequent	Not as above	45–56 × 56–88	2.0–5.0

Sporophyte:

	Sporophyte dimensions (mm)	Spores diameter (μm)	Spore ornamentation distal face	Spore ornamentation proximal face
A. cristatus	0.3–0.4 × 35	40–60 (66)	13–17 spinulate projections	Finely foveolate
A. punctatus	0.4–0.8 × 20–100	42–62	15–18 spines and tubercles united at their base to form incomplete alveoli	Finely foveolate
A. agrestis	0.25–0.5 × 8–20(30)	46–60	8–12 spines with irregularly curved tips, coarsely reticulate	Finely foveolate to coarsely reticulate

caducous marginal lamellar cells produce new thalli is a phenomenon in line with the tenet of increased asexual reproduction in island taxa. A second of the four hornworts on Ascension, *Phaeoceros evanidus*, also reproduces asexually via prominent dorsal tubers.

Conservation considerations

The present confirmation, from both molecules and morphology, of the SIE status of *Anthoceros cristatus* demands an examination of its conservation status. Like most oceanic islands Ascension has been changed out of all recognition by human influences. Because of the barren nature of the original treeless landscape Joseph Hooker, following his visit in 1843, suggested a major 'improvement programme'; today the Island flora contains over 200 introduced vascular plants and the only man-made cloud forest in the world (Wilkinson, 2004). Although over 150 years of diverse plant introductions many of the habitats previously dominated by bryophytes and pteridophytes have become severely overgrown, there is little or no evidence for wholesale extinctions. Indeed, of the 86 species confirmed for the Island only one is most likely extinct (*Marchantia berteroana* Lehm. & Lindenb.) and very few are critically endangered. Many of the indigenous bryophytes have now spread onto the introduced trees. Today *Anthoceros cristatus* and the other Island hornworts almost certainly occupy the same kind of open banks that must have been present before human activities. The only serious threat to hornwort abundance on the Island, aside from their highly restricted altitudinal range and the small size of the Island, is the spread of herbaceous plants over bare areas on the tracks and banks around Green Mountain. As a conservation measure, to augment the hornwort populations, areas are now being cleared on a regular basis (Pressel *et al.*, 2014).

Online supplementary material

Supplementary material is available at [10.1080/03736687.2017.1302153].

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