Contributions to the phylogeny of the haplolepideous mosses

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Chapter 2

Tackling relationships and species circumscriptions of *Octoblepharum*, an enigmatic genus of haplolepideous mosses (Dicranidae, Bryophyta)

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Introduction

Octoblepharum was the first genus that was described to accommodate mosses with a so-called leucobryoid morphology. Leucobryoid mosses are easily recognised by the whitish green colour of their gametophytes, which is due to their specific leaf anatomy. The leaves consist mainly of a very wide costa composed of two to several layers of large, hyaline cells (leucocysts or hyalocysts), surrounding one (or up to three) layer(s) of chlorophyllose cells (chlorocysts). Mosses with a leucobryoid morphology belong to the haplolepideous mosses (subclass Dicranidae), which constitute the second largest lineage of Bryophyta with about 4,000 species (Frey & Stech, 2009).

Octoblepharum comprises 18 accepted species (http://www.tropicos.org), including the pantropical *O. albidum* Hedw., the recently described *O. pocsii* Magill & B.H. Allen, recorded for Africa and Asia, and 16 species restricted to the Neotropics (10 species), tropical Africa (3), tropical Asia (2), and Australia (1), respectively (Eddy, 1990; He, 2014; Mägdefrau, 1983; Magill & Allen, 2013; Salazar Allen & Tan, 2010; Townsend, 1963; Yano, 1993). The genus was first included in the family Leucobryaceae (Schimper, 1856), which originally comprised all leucobryoid mosses. After Cardot (1899) separated *Octoblepharum* within the Leucobryaceae as tribe Octoblephareae Cardot, Fleischer (1904) described the family Leucophanaceae M. Fleisch. to include the former Leucobryaceae genera *Arthrocormus* Dozy & Molk., *Exodictyon* Cardot, *Leucophanes* Brid. and *Octoblepharum*, the latter in the subfamily Octoblepharoideae [‘Gruppe Octoblephareae’] (Cardot) M. Fleisch. Andrews (1947) considered the Leucophanaceae genera to belong to the Calymperaceae, based on similarities such as leaf structure, presence of a preperistome, and presence of leaf apex propagula. Finally, Eddy (1990) classified *Octoblepharum* in its own family, stating that ’the combination of gametophyte features, monoecious (autoecious) reproductive system and peculiar peristome structure appears to set *Octoblepharum* apart from Leucobryaceae on the one hand and Calymperaceae on the other’. Since Eddy did not follow the nomenclatural rules, Menzel (1991) validated the name Octoblepharaceae. This checkered taxonomic history is due to the fact that *Octoblepharum* differs from all other leucobryoid genera by its leaf cross section with

triangular chlorocysts in a single layer (Salazar Allen, 1991) as well as by a reduced peristome consisting of eight or 16 entire teeth, which made inferences about relationships difficult (Edwards, 1979).

Although authors such as Enroth (1989, 1990) acknowledged *Octoblepharum* as being anomalous among the leucobryoid Calymperaceae, the name Octoblepharaceae has not been widely used (e.g., Ellis, 2007), and the latest classifications of mosses (Frey & Stech, 2009; Goffinet et al., 2009) still treated *Octoblepharum* as part of the Calymperaceae. One reason may be that molecular phylogenetic data on *Octoblepharum* are still scarce (cf. Stech et al., 2012) and the monophyly of the genus has not yet been tested, since only *O. albidum* was included with one or two specimens in analyses of molecular evolution (Wall & Herbeck, 2003), moss relationships (Hedderson et al., 2004; La Farge et al., 2000; Tsubota et al., 2003, 2004) or the Calymperaceae relationships (as outgroup; Fisher et al., 2007). In these studies, *Octoblepharum albidum* was either resolved as sister to the remaining (well-supported) Calymperaceae with low support (Hedderson et al., 2004; La Farge et al., 2000; Tsubota et al., 2003) or separated from them (Tsubota et al., 2004).

If *Octoblepharum* and the remaining Calymperaceae are indeed sister groups, how they relate to other putatively close lineages, such as the Dicranaceae and the monogeneric Hypodontiaceae M. Stech (Cox et al., 2010; Stech et al., 2012; Tsubota et al., 2004), are still open questions. Only one phylogeny included *Octoblepharum*, the Calymperaceae and the Hypodontiaceae simultaneously (Tsubota et al., 2004), but yielded insufficient resolution for the relationships among these groups.

Furthermore, species delimitations and relationships within *Octoblepharum* are still incompletely known. Half of the currently 18 accepted names represent little known species (Crosby et al., 1999), most of which are poorly described and/or never re-collected after their description. A single study investigated the genetic variation within and between three *Octoblepharum* species based on RAPD markers (Korpelainen & Salazar Allen, 1999), and the hypothesis that *Octoblepharum* is split into two evolutionary lines, one with eight and the other with 16 peristome teeth (Salazar Allen, 1991), is yet to be tested by molecular phylogenetic reconstructions.

Based on an extended dataset of Stech et al. (2012), including DNA sequence markers of all three genomes, the aim of this study was to (i) test the monophyly of *Octoblepharum*, (ii) infer its relationships with the Calymperaceae and *Hypodontium* (Hypodontiaceae) as putative close relatives, to conclude whether the genus should be placed in its own family or not, and (iii) provide a preliminary assessment of species circumscriptions and relationships within *Octoblepharum*.

Material and Methods

Plant material and sequence data sampling

For analyses at suprafamilial level within the haplolepideous mosses, the dataset from Stech et al. (2012), which comprised combined chloroplast *rps*4-*trn*T-*trn*L-*trn*F and *atp*B-*rbc*L sequences of 54 species of the Dicranidae as well as *Timmia austriaca* Hedw. (Timmiidae) and *Encalypta streptocarpa* Hedw. (Encalyptidae Ochyra et al.) as outgroup representatives, was extended by 21 specimens of *Octoblepharum*, and 12 samples of the (other) Calymperaceae. Material of seven species of *Octoblepharum* suitable for DNA sequencing was selected from herbaria L, MO, SING, and UB. The final sampling comprised four species, due to misidentifications or failure to obtain PCR products for some specimens.

Besides the extended taxon sampling, further markers were sequenced for the total dataset, namely mitochondrial *nad*5 as well as plastid *trn*S-*rps*4, to complete the *trn*S-*trn*F region (*trn*S-*rps*4-*trn*T-*trn*L-*trn*F; cf. Hernández-Maqueda et al., 2008). For analyses of relationships within *Octoblepharum*, a subset of the Dicranidae dataset including all *Octoblepharum* specimens as well as *Calymperes erosum* Müll.Hal., *Leucophanes angustifolium* Renauld & Cardot and *Syrrhopodon gardneri* (Hook.) Schwägr. as outgroup representatives was used, to which the nuclear ribosomal ITS1-5.8S-ITS2 region was added as an additional marker with high sequence variability.

Voucher information and GenBank accession numbers are listed in Appendix 1.

DNA extraction, amplification, and sequencing

DNA extractions of newly included specimens were performed with the NucleoSpin® Plant II Kit (Macherey-Nagel). Primers and PCR amplification protocols for all amplified regions are listed in Appendix 2. The PCR amplification mix was prepared with 14.3 µL MilliQ® water (Merck Millipore Corporation), 3 µL Q-solution® (Qiagen), 2.5 µL 10× CoralLoad® PCR buffer (Qiagen), 1 µL $MgCl_2$ (Qiagen), 0.9 µL dNTP, 1 µL of each primer (forward and reverse, ordered from Sigma-Aldrich Co.), 0.3 µL of Taq DNA polymerase (Qiagen), and 1 µL template DNA per sample for each marker except *nad*5, for which replacing Q-solution with MilliQ water yielded better results. PCR products were purified and sequenced at Macrogen Inc. (www.macrogen.com) and BaseClear B.V. (www.baseclear.com) using the amplification primers.

Phylogenetic reconstructions

Sequences were manually aligned in Geneious® v8.0.5 (Biomatters Ltd). Phylogenetic reconstructions were performed under maximum parsimony (MP), maximum likelihood (ML),

and Bayesian inference (BI). In each analysis, gaps were either treated as missing data or coded as informative by simple indel coding (SIC) (Simmons & Ochoterena, 2000) using SeqState (K. Müller, 2004). Evolutionary model testing for ML and BI was performed for each of the two combined datasets, Dicranidae and *Octoblepharum*, in jModelTest 2 (Darriba et al., 2012; Guindon & Gascuel, 2003). According to the Akaike Information Criterion (AIC), the selected model for the Dicranidae dataset was GTR+Γ+I, followed closely by GTR+Γ, and for the *Octoblepharum* dataset it was GTR+Γ. Yang (2006) and other authors (as Jia et al., 2014 and references therein) recommended the use of the model GTR+Γ instead of GTR+Γ+I, with the support of mathematical and biological arguments, and we followed this recommendation in the analyses performed in this study. Since model testing for each partition (ITS, *nad*5, *trn*S-*trn*F, and *atp*B-*rbc*L) separately resulted in the selection of a GTR model (GTR, GTR+Γ, GTR+I, GTR+Γ+I), and maximum parsimony analyses of each partition did not reveal incongruent topologies, all model-based analyses were performed with the combined datasets under the GTR+Γ model.

Maximum parsimony analyses were performed in PAUP® 4.0b10 (Swofford, 2002). Heuristic searches were implemented using random sequence addition with 1,000 replicates and tree bisection-reconnection branch-swapping. Heuristic bootstrap searches were performed with 1,000 replicates and 10 random addition cycles per bootstrap pseudoreplicate with the same options in effect. Maximum likelihood searches and thorough bootstrap analyses were performed with RAxML v8 (Stamatakis, 2014) employing raxmlGUI v1.3.1 (Silvestro & Michalak, 2012). Ten independent ML searches and 1,000 bootstrap replicates were performed within each analysis. Bayesian inference analyses were performed in MrBayes v3.2.6 (Ronquist et al., 2012), on the CIPRES Science Gateway v3.3 (M. A. Miller et al., 2010). Four runs with four chains (5×10^6 or 10^7 generations each) were run simultaneously, with the temperature of the single heated chain set to 0.4. Chains were sampled every 1,000 generations and the respective trees were written to a tree file. After verifying the convergence of runs in Tracer v1.6 (Rambaut et al., 2014), 50% majority rule consensus trees and PP of clades were calculated after the chains converged.

Results

The Dicranidae alignment of combined mitochondrial and chloroplast markers comprised 5,096 positions, of which 2,126 were variable, and 1,513 of the variable positions were parsimony-informative (*nad*5 276, *trn*S-*trn*F 934, and *atp*B-*rbc*L 303 parsimony-informative positions). Simple indel coding added 745 (*nad*5 45, *trn*S-*trn*F 495, and *atp*B-*rbc*L 205) parsimony-informative indels.

The *Octoblepharum* alignment of combined nuclear, mitochondrial, and chloroplast markers comprised 5,374 positions, of which 1,156 were variable, and 684 of the variable positions

were parsimony-informative (ITS 152, *nad*5 74, *trn*S-*trn*F 347, and *atp*B-*rbc*L 111 parsimony informative positions). Simple indel coding added 295 (ITS 147, *nad*5 7, *trn*S-*trn*F 104, and *atp*B-*rbc*L 37) parsimony-informative indels.

Figure 6 shows the consensus tree from Bayesian inference of the Dicranidae dataset with indel coding, with indication of branch support for Bayesian inference, maximum likelihood, and maximum parsimony. Tree topologies for the suprageneric relationships between *Hypodontium* (Hypodontiaceae), the Calymperaceae, and *Octoblepharum* did not differ between phylogenetic analysis methods or datasets with and without indel coding (trees not shown). *Octoblepharum* was resolved as monophyletic with maximum support (Bayesian posterior probability [PP] 1.00, bootstrap support from maximum likelihood [ML BS] and maximum parsimony [MP BS] 100%). The clade of the Calymperaceae sister to *Octoblepharum* received maximum support in all analyses. *Hypodontium* sister to this clade received high support with indels (PP 1.00, ML BS 95%, MP BS 98%), but lower support without indels (PP 0.94, ML BS 77%, MP BS 89%). The Dicranaceae appeared as sister to this clade, also with high support (PP 1.00, ML BS 100%, MP BS 96–98%).

Figure 7 shows the consensus tree from Bayesian inference of the *Octoblepharum* dataset with indel coding, with indication of branch support for Bayesian inference, maximum likelihood, and maximum parsimony. For the *Octoblepharum* infrageneric relationships, the clades for each of the included species had maximum support in all analyses and datasets, as well as the sister group relationship of *O. cocuiense* Mitt. and *O. pulvinatum* (Dozy & Molk.) Mitt. (PP 1.00, ML and MP BS 100%). However, a difference between analyses was observed in the placement of *O. arthrocormoides* N. Salazar Allen & B.C. Tan, which appeared as sister to the clade formed by *O. cocuiense* and *O. pulvinatum* with high support in the BI and ML analyses (PP 1.00, ML BS 94–100%), whereas in the MP analyses the species was resolved with maximum bootstrap support as sister to a weakly supported clade (MP BS 60–74%) formed by all other *Octoblepharum* samples (trees not shown).


Timmia austriaca
Encalypta streptocarpa
Catoscopium nigritum
Ditrichum flexicaule
Drummondia prorepens
Dicranoweisia crispula
Bryoxiphium norvegicum
Blindia acuta
Ptychomitrium polyphyllum
Schistidium apocarpum
Racomitrium canescens
Eustichia longirostris
Archidium alternifolium
Ochrobryum gardneri
Leucobryum juniperoideum
Campylopus flexuosus
Pilopogon africanus
Atractylocarpus alticaulis
Campylopodiella flagellacea
Brothera leana
Dicranodontium denudatum
Amphidium mougeotii
Amphidium lapponicum
Rhabdoweisia crenulata
Oncophorus wahlenbergii
Oncophorus virens
Cynodontium polycarpum
Oreoweisia bogotensis
Erpodium biseriatum
Aulacopilum cf. abbreviatum
Ceratodon purpureus
Trichodon cylindricus
Pleuridium acuminatum
Syntrichia ruralis
Tortula muralis
Cinclidotus riparius
Diobelonella palustris
Aongstroemia longipes
Cheilothela chloropus
Dicranella heteromalla
Dicranella cerviculata
Microcampylopus khasianus
Leptotrichella flaccidula
Campylopodium medium
Fissidens fontanus
Fissidens bryoides
Leucoloma procerum
Dicranoloma plurisetum
Chorisodontium wallisii
Paraleucobryum longifolium
Dicranum polysetum
DICRANACEAE
Hypodontium dregei
Hypodontium pomiforme
HYPODONTIACEAE
Syrrhopodon incompletus Sint Eustatius 93
Calymperes afzelii Sint Eustatius 95
Calymperes erosum MSCe
Calymperes palisotii Sint Eustatius 94
Calymperes motleyi MS1023
Syrrhopodon gardneri MSSyg
Mitthyridium fasciculatum Malaysia 99
Mitthyridium flavum Malaysia 100
Syrrhopodon prolifer Brazil 96
Syrrhopodon trachyphyllus Australia 98
Arthrocormus schimperi Malaysia 86
Leucophanes molleri Gabon 90
Leucophanes molleri Guinea 92
Leucophanes sp. Netherlands 80b
Leucophanes angustifolium Gabon 89
Leucophanes octoblepharioides China 91
CALYMPERACEAE
Octoblepharum albidum Brazil 32
Octoblepharum albidum Thailand 107
Octoblepharum albidum Java 83
Octoblepharum albidum Australia 77
Octoblepharum albidum Australia 81
Octoblepharum albidum Nicaragua 50
Octoblepharum albidum Netherlands 80a
Octoblepharum albidum Honduras MSOal
Octoblepharum albidum Brazil 20
Octoblepharum cocuiense Ecuador 72
Octoblepharum cocuiense Brazil 109
Octoblepharum cocuiense Brazil 111
Octoblepharum cocuiense Brazil 108
Octoblepharum cocuiense Bolivia 51
Octoblepharum cocuiense Brazil 110
Octoblepharum cocuiense Bolivia 49
Octoblepharum pulvinatum Brazil 112
Octoblepharum pulvinatum French Guiana 75
Octoblepharum pulvinatum Nicaragua 52
Octoblepharum arthrocormoides Malaysia 84
Octoblepharum arthrocormoides Malaysia 85
OCTOBLEPHARACEAE
-/100
100/-
87/-
95/75
89/-
70/-
100/-
-/-
79/-
-/-
71/-
85/-
79/-
98/83
89/96
87/74
86/-
72/-
82/78
81/73
81/81
95/-
0.03

← *Figure 6. Bayesian inference consensus tree of 88 representatives of haplolepideous mosses (Dicranidae) based on mitochondrial and chloroplast DNA sequences (nad5, trnS-trnF region, and atpB-rbcL), with indel coding.* Timmia austriaca *(Timmiidae) and* Encalypta streptocarpa *(Encalyptidae) were used as outgroup representatives. Branch support is indicated for Bayesian inference (BI), maximum likelihood (ML), and maximum parsimony (MP) analyses of the same dataset. Bold branches represent posterior probabilities (PP) ≥ 0.95 for BI and bootstrap (BS) values ≥ 90% for ML and MP. Actual BS values are shown if in the range of < 90% and ≥ 70% for ML and/or MP. BS values below 70% are not shown ("-"). Branch lengths are to scale, except the ones indicated by the symbol "//" (shortened four times).*

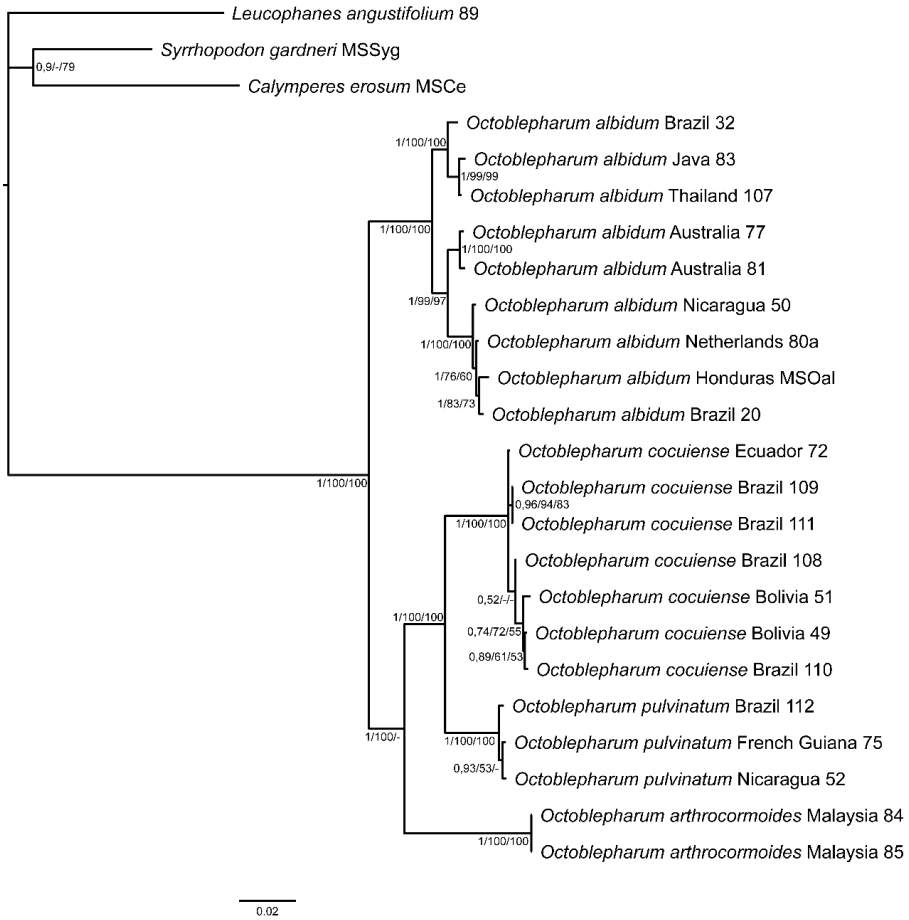

Figure 7. Bayesian inference consensus tree of 21 representatives of Octoblepharum *(Octoblepharaceae) based on nuclear, mitochondrial, and chloroplast DNA sequences (ITS,* nad*5,* trn*S*-trn*F region, and* atp*B*-rbc*L), with indel coding.* Calymperes erosum, Leucophanes angustifolium, *and* Syrrhopodon gardneri *(Calymperaceae) were used as outgroup representatives. Support values are shown for Bayesian inference, maximum likelihood, and maximum parsimony analyses (BI PP/ML BS/MP BS) of the same dataset.*

Discussion

Circumscription and relationships of Octoblepharum

The present study allows the most comprehensive phylogenetic inference of *Octoblepharum* available so far, based on 21 specimens from the Americas, Asia, and Australia, representing four species. Our data strongly support the monophyly of *Octoblepharum*, which could not be assessed in previous studies that included only samples of *O. albidum* (Hedderson et al., 2004; La Farge et al., 2000; Tsubota et al., 2003, 2004; Wall & Herbeck, 2003). The sister group relationship of *Octoblepharum* with the remainder of the Calymperaceae sensu Frey & Stech (2009), which was already resolved with moderate support in La Farge et al. (2000), receives maximal support in all present analyses. *Hypodontium* is resolved sister to this clade with significant support at least in the analyses with indels included. Previous phylogenetic reconstructions either lacked resolution for those relationships, or did not include representatives of all three clades (*Octoblepharum*, other Calymperaceae, and *Hypodontium*; Cox et al., 2010; La Farge et al., 2000; Stech et al., 2012; Tsubota et al., 2004).

Based on the molecular data, we followed Eddy (1990) and Menzel (1991) in classifying *Octoblepharum* in its own family Octoblepharaceae. *Octoblepharum* and the remaining Calymperaceae are sister groups, but high genetic divergence between them is evident. Morphological synapomorphies for *Octoblepharum* are the peculiar leaf shape and leaf anatomy. Leaf shape is a character not commonly highlighted as distinctive in the genus description (e.g., Eddy, 1990; Salazar Allen, 1994). Leaves are ligulate, the entire strap-shaped portion composed exclusively by costa, with a cuspidate to mucronate leaf apex and a distinct sheathing basal hyaline lamina. The alternate position of the hyalocysts of the different layers in costa cross-section can as well be considered a synapomorphy for *Octoblepharum* (see below). Eddy (1990) described *Octoblepharum* (and the Octoblepharaceae) as being monoecious, but his description was based mainly on *O. albidum* (the single species reported in his study for Malesia), and there are Neotropical species of the genus which are dioecious (Salazar Allen, 1991).

Octoblepharum has been retained in the Calymperaceae based on questionable arguments. First, there was a common notion that the leucobryoid Calymperaceae (genera *Arthrocormus*, *Exodictyon*, *Exostratum* L.T.Ellis, and *Leucophanes*) and *Octoblepharum* should be classified together, as in Fleischer (1904) and Andrews (1947), due to the shared leucobryoid morphology. However, the leaf structures of the leucobryoid Calymperaceae genera and *Octoblepharum* have little in common, and Ellis (1985) states that *Octoblepharum* leaves resemble more the ones of the leucobryoid Dicranales (e.g., *Leucobryum*). As is evident from Cardot's (1899) illustrations of the leaf sections, *Octoblepharum* can be distinguished by the above mentioned triangular chlorocysts, while the leucobryoid Calymperaceae show diamond-shaped chlorocysts. In addition, the leucobryoid Calymperaceae genera *Arthrocormus*, *Exodictyon*, and *Exostratum* exhibit additional layers of chlorocysts, not found in any other

leucobryoid genus, and *Leucophanes* has a distinctive bundle of stereids, also absent in all other leucobryoid genera. The close relationship between the leucobryoid Calymperaceae genera is well supported by molecular data (Fisher et al., 2007; present study). They compose a derived clade within the family Calymperaceae and thus are only distantly related to *Octoblepharum*.

Second, some species of *Octoblepharum* may occasionally present gemmae at the leaf tips, very similar in shape to those found among the Calymperaceae (e.g., Andrews, 1947; Harrington & Egunyomi, 1976; Maciel-Silva et al., 2013). However, elongate gemmae at leaf tips are also found in other unrelated moss families, e.g., in the Orthotrichaceae Arn. of subclass Bryidae (Vitt, 2014). For that reason, we do not consider this specific character adequate to support, alone, the circumscription of a family.

Third, some authors included *Octoblepharum* in the Calymperaceae based on peristome characters (Salazar Allen, 1994, and references therein). However, according to Ellis (1985), the presence of a preperistome is the only shared peristome trait between *Octoblepharum* and the remaining Calymperaceae. The *Octoblepharum* peristome cannot be considered as alike that of *Syrrhopodon* Schwägr., i.e., formed by 16 undivided teeth with equally thick inner and outer layers, usually papillose and without trabeculae, without basal membrane and often with a preperistome (Frey & Stech, 2009), since it is not papillose and may have trabeculae (Salazar Allen, 1994). Neither can it be considered as dicranoid, i.e., formed by 16 teeth divided to half, dorsally trabeculate and vertically striate, without a developed basal membrane (Frey & Stech, 2009), since it bears a preperistome and has undivided (even reduced in number) teeth (Salazar Allen, 1994).

Edwards (1979) has briefly described the peristome of *O. albidum* as reduced, with eight simple teeth, the peristome formula 2(−3):2, deviating from the typical haplolepideous formula, and no trabeculae, with dorsal and ventral peristome plates slightly convex. He claimed this reduction would make it difficult to make inferences on *Octoblepharum* relationships. However, the non-reduced *Octoblepharum* peristomes with 16 teeth, not as well described in the literature, show a different combination of characters, and may be more informative regarding relationships. On preliminary examination, the *O. pulvinatum* teeth (specimen French Guiana 75 in Figures 6, 7), for example, are well-developed, with strong trabeculae, vertical striae, no papillae, without the ventral zig-zag line usually seen in other haplolepideous peristomes (absent also in the eight teeth species, but due to their reduction), and differ from the eight teeth pattern as well as from the syrrhopodontoid or dicranoid types. Thus, to compare the Octoblepharaceae and the Calymperaceae based on peristome characters requires further studies on the variability of this structure among the species of *Octoblepharum*. Ellis (1985) may be correct, and the *Octoblepharum* peristome may even represent a fifth main expression of the haplolepideous peristome, diverging from the

dicranoid, seligerioid, syrrhopodontoid, and pottioid ones already named in the literature (Frey & Stech, 2009).

Delimitation and relationships of Octoblepharum *species*

The four included *Octoblepharum* species (*O. albidum*, *O. arthrocormoides*, *O. cocuiense*, and *O. pulvinatum*) are molecularly well-defined according to the present study (Figure 7). The molecular data support the present morphological circumscriptions of these *Octoblepharum* species, and our morphological studies indicate that the available literature allows the correct identification of the specimens. The closely related *O. cocuiense* and *O. pulvinatum* (Figure 7) both possess a peristome with 16 teeth, whereas the peristome of *O. albidum* consists of eight teeth. The sporophyte (and thus peristome) of *O. arthrocormoides* is still unknown. Salazar Allen (1992) suggested the state of eight peristome teeth found in *O. albidum* would be a derived condition, while 16 peristome teeth, the most common number among the Dicranidae, also present in the closest relatives of *Octoblepharum* (*Hypodontium* and most of the Calymperaceae), would most likely be plesiomorphic for the genus. However, a larger sampling of *Octoblepharum* species is necessary to test this hypothesis as the current topology does not provide sufficient evidence. In addition, the placement of *O. arthrocormoides* diverges between BI/ML and MP analyses (cf. Results), either sister to the two included species with 16 peristome teeth (Figure 7) or sister to all other included *Octoblepharum* species in MP trees (trees not shown). Since in the trees for BI and ML the two samples of *O. arthrocormoides* form a very long branch, the topology in MP may be a result of long-branch attraction.

The two specimens of *O. arthrocormoides* studied here are probably the first report after its original description. Their identity was revealed by molecular analyses, since they were originally labelled as *O. albidum*. *Octoblepharum arthrocormoides* is very similar to *O. albidum* in gametophytic features such as colour, size of the leaves and leaf shape. The only literature which compares the two species is the original description of *O. arthrocormoides* by Salazar Allen & Tan (2010). *Octoblepharum arthrocormoides* differs from *O. albidum* in its broken leaf apices, shorter lamina hyalocysts, more hyalocyst layers, and lack of inflated and porate marginal hyalocysts (Salazar Allen & Tan, 2010). According to our observations, the main distinctive feature between these species is the general appearance of the gametophytes, slenderer and with broken leaf apices in *O. arthrocormoides*, and more compact with entire leaves in *O. albidum*. The central lamina hyalocysts are indeed shorter and mostly quadrate in both specimens of *O. arthrocormoides* studied here, as opposed to longer and mostly short-rectangular to rectangular hyalocysts in *O. albidum*. Number of hyalocyst layers is almost overlapping in the specimens included in this study, ranging from 5–7 layers on each side of the chlorocyst layer in *O. arthrocormoides* versus 2–5 in *O. albidum*. In addition, we observed

that the pores between lamina hyalocysts are clearly visible in *O. albidum* but only visible as small dots in *O. arthrocormoides*.

Although all specimens in the well-supported *O. cocuiense* clade exhibit fragile leaves with the pseudocosta characteristic of the species, they display considerable morphological variability. Two of these specimens were previously misidentified as *O. erectifolium* Mitt. ex R.S. Williams. Gametophytically, the two species may be mistaken due to similarities in the fragility of leaves, shape of lamina cells, dentation of upper lamina margins, and in some specimens of *O. cocuiense*, unusually long leaves for the genus (according to descriptions in Salazar Allen, 1994). However, they differ in that *O. erectifolium* shows leaves even longer than the range of variation of *O. cocuiense* (from 2 mm long), lacks a pseudocosta and never shows a purple tone in its leaves. The misidentified specimens in this study were indeed in the larger range of leaf sizes for *O. cocuiense*, but in all other characters they fit well the species description. They did not bear sporophytes, otherwise they would have been easily identified correctly as *O. cocuiense*, since *O. erectifolium* has eight peristome teeth. Two other *O. cocuiense* specimens were mistaken for *O. pulvinatum*. These had a pale green tone, hexagonal basal lamina cells similar to those of some *O. pulvinatum* specimens, and small plant and leaf sizes corresponding to smaller specimens of *O. cocuiense*. These characteristics resemble those of *O. pulvinatum*, which has its name in reference to its also fragile leaves. The main trait which allowed their correct identification as *O. cocuiense* was the presence of a pseudocosta, although in specimens such as these, which lack the strong pink pigmentation, this structure can be quite inconspicuous and is only visible at low magnifications.

Octoblepharum pulvinatum also shows vegetative similarities to species with eight peristome teeth. It resembles *O. albidum*, the Neotropical *O. cylindricum* Schimp. ex Mont., and the recently described Paleotropical *O. pocsii* (He, 2014; Magill & Allen, 2013) in the green colour and lack of a pseudocostal area. It further resembles *O. albidum* and *O. cylindricum* in occasionally showing a pink tone at its leaf bases, and differs from those species in its fragile leaves and quadrate to short hexagonal basal lamina cells (Salazar Allen, 1991). On the other hand, it further resembles *O. pocsii* in its fragile, long leaves, and short basal lamina cells, differing in having shorter leaves, and in occasionally showing a pink tone at its leaf bases (Magill & Allen, 2013).

Octoblepharum albidum, although being well-supported based on the molecular data (Figures 6, 7), displays a considerable intraspecific molecular variability. Furthermore, the three well-supported clades resolved within *O. albidum* based on our preliminary data indicate the presence of geographical structure. One lineage is formed by Neotropical samples (Brazil, Honduras, and Nicaragua), plus a specimen from a glasshouse in the Hortus Botanicus Leiden, which thus is likely of Neotropical origin. The second lineage, sister to the first, includes only Australian samples, and the third lineage, sister to the clade formed by the other two, comprises one Brazilian and two Asian samples, indicating that *O. albidum* populations in Brazil

belong to two different lineages. The three *O. albidum* clades fit the general morphological description of the species. However, as *O. albidum* is a reportedly variable species (Florschütz, 1955; Magill & Allen, 2013; Salazar Allen, 1991, 1994), further studies are necessary to verify if these clades correspond to morphologically distinguishable groups within a species complex.