Problems related to the taxonomic placement of incompletely preserved amber fossils: transfer of the Paleogene liverwort *Cylindrocolea dimorpha* (Cephaloziellaceae) to the extant *Odontoschisma* sect. *Iwatsukia* (Cephaloziaceae)

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Received: 2 March 2017 – Accepted: 20 March 2017 – Published: 12 April 2017

Abstract. A revision of the Baltic and Bitterfeld amber fossils assigned to *Cylindrocolea dimorpha* (Cephaloziellaceae) has yielded evidence of the presence of multicellular, bifid underleaves, which have not previously been reported for this species and conflict with the current circumscription of the family. We transfer the fossil species to *Odontoschisma* (sect. *Iwatsukia*) and propose the new combination *O. dimorpha* of the Cephaloziaceae. Characteristics of the fossil include an overall small size of the plant, entire-margined, bifid leaves and underleaves, more or less equally thickened leaf cell walls, ventral branching that includes stoloniform branches with reduced leaves, and the lack of a stem hyalodermis and gemmae. Placement of the fossil in Cephaloziaceae profoundly affects divergence time estimates for liverworts based on DNA sequence variation with integrated information from the fossil record. Our reclassification concurs with hypotheses on the divergence times of Cephaloziaceae derived from DNA sequence data that provide evidence of a late Early Cretaceous to early Eocene age of the *Odontoschisma* crown group and an origin of *O. sect. Iwatsukia* in the Late Cretaceous to Oligocene.

1 Introduction

Liverworts belong to the oldest lineages of plants on land and date back to the early Paleozoic (Taylor et al., 2009). They are characterized by a life cycle with a prominent leafy or thalloid gametophyte, an unbranched sporophyte, and the frequent presence of oil bodies and elaters (Renzaglia et al., 2007). Liverwort diversity today includes some 7000 species in ~400 genera; however, both species level and supraspecific classifications remain unstable despite considerable recent efforts to record the global diversity (Söderström et al., 2016). Accordingly, taxonomic studies still identify incongruences between morphology-based taxonomic hypotheses and DNA-based phylogenies and, consequently, genus and family concepts are frequently revised (e.g., Bechteler et al., 2016; Long et al., 2016; Patzak et al., 2016). Taking the considerable difficulties into account that hamper the classification of the present-day liverwort diversity (Renner et al., 2017), it comes as no surprise that fossils of liverworts often have an even complexer and more confusing taxonomic history (Grolle and Meister, 2004), especially if only fragments, rather than entire plants, are preserved (Heinrichs et
These fragments often do not display the whole complement of relevant taxonomic characters, and thus the classification of these forms often needs to be revised when additional, more completely preserved specimens become available.

*Jungermannia divaricata* Casp. was initially described by Caspary (1887) based on a single inclusion of an unbranched, male shoot enshrined in a piece of Baltic amber that is today kept in the Museum für Naturkunde Berlin. Baltic amber is considered Eocene in age (35 to 47 Myr old; Standke, 1998). The shoot lacks a hyalodermis and underleaves, and has two rows of bifid, entire-margined leaves consisting of relatively thin-walled cells lacking trigones, and an apical androecium with 5 pairs of shallowly bifid bracts (Grolle, 1980). Caspary and Klebs (1907) noted similarities of the fossil to the extant *Jungermannia divaricata* Sm. (= *Cephaloziella divaricata* (Sm.) Schiffn.; Söderström et al., 2016), and Grolle (1980) subsequently transferred the species to *Cephaloziella* (Spruce) Schiffn. (as *Cephaloziella dimorpha* (Casp.) Grolle) of the Cephaloziellaceae. Grolle and Meister (2004) described additional inclusions supposed to belong to this species from Baltic and Bitterfeld amber. However, no gemmae, which are a characteristic feature of most *Cephaloziella* species, were detected by these authors. As a result, they suggested that the fossils belong to the genus *Cylindrocolea* R.M.Schust., rather than *Cephaloziella* and, consequently, proposed the name *Cylindrocolea dimorpha* (Casp.) Grolle for the taxon.

Using the geological age of *Cylindrocolea dimorpha* as a minimum age constraint for *Cylindrocolea* in DNA-based divergence time estimates of liverworts results in estimates that indicate roughly 3 times older ages than analyses conducted without this fossil constraint (Feldberg et al., 2013, 2014; Laenen et al., 2014). This observation led us to reinvestigate the type material and additional fossils of *Cylindrocolea dimorpha*. We found that bifid underleaves occur in ascending shoots, while they are usually missing in prostrate shoots. The results from thorough re-analysis of the specimens, together with additional evidence from DNA-based divergence time estimates, are used in this study to transfer *Cylindrocolea dimorpha* to *Odontoschisma* sect. *Iwatsukia* (N.Kitag.) Gradst., S.C.A. Aranda & Vanderp. (Cephaloziaceae).

### 2 Materials and methods

#### 2.1 Investigation of amber inclusions

The amber inclusions (12 from Baltic and 6 from Bitterfeld amber) used in this study are housed at the Museum für Naturkunde at Berlin, the Georg August University of Göttingen (numbers preceded by GZG.BST), the SNSB-Bavarian State Collection for Palaeontology and Geology (numbers preceded by SNSB-BSPG), and the Carsten Gröhn amber collection. Specimens from the Museum für Naturkunde at Berlin were previously published under BHU-Palaeo collection numbers (e.g., Grolle and Meister, 2004). However, this acronym has recently been replaced by “MB.Pb”.

The surface of some of the amber pieces was polished manually with a series of wet silicon carbide abrasive papers (grit size from FEPA P 600–4000 (particle size: 25.8 to 5 µm), Struers) to minimize light scattering during analysis and photographic documentation. Specimens were then placed on a glass microscope slide with a drop of water added to the upper surface and covered with a coverslip. The amber inclusions were studied under a Leica M50 incident-light microscope and a Carl Zeiss Axioscope A1 compound microscope, the latter equipped with a Canon 60D digital camera. Incident and transmitted light were used simultaneously. The images compiled in Figs. 1 and 2 are digitally stacked photomicrographic composites of up to 145 individual focal planes obtained by using the software package HeliconFocus 5.0.

#### 2.2 Divergence time estimates

Divergence time estimates based on the DNA sequence variation obtained from extant representatives of cephalozioid liverworts were conducted to assess the level of congruence with our taxonomic placement of *Cylindrocolea/Odontoschisma dimorpha*. The DNA dataset that was used included 67 accessions of the family Cephaloziaceae and 2 outgroup species from Adelanthaceae (see Supplement). Sequences of the chloroplast *rbcL* gene and *trnL-F* region, as well as the nuclear *ITS1-5.8S-ITS2* region, were extracted from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), and were published previously in Feldberg et al. (2010, 2016) and Vilnet et al. (2010, 2012). Sequences were aligned manually in Bioedit version 7.0.5.2 (Hall, 1999); missing data were coded as missing.

*jModelTest* 2.1.7 (Guindon and Gascuel, 2003; Darriba et al., 2012) was employed to choose a nucleotide substitution model for both nuclear and plastid DNA datasets. With regard to the nuclear marker, the Bayesian information criterion (BIC) supported the TIM3+G+I model; regarding the combined chloroplast markers, BIC supported the TPM1uf+G+I model.

Bayesian divergence time estimates were generated in BEAST 1.8.4 (Drummond et al., 2012). The DNA dataset was split into a nuclear and a chloroplast partition, with unlinked substitution and clock models, and linked trees. An uncorrelated relaxed (lognormal) clock was employed for both partitions and the substitution models were implemented according to the results of the *jModelTest* analyses. A birth–death model for incomplete sampling was employed. The root of the tree was calibrated at 202.01 Ma based on estimates in Laenen et al. (2014) for the split between the Adelanthaceae and Cephaloziaceae in an analysis not factoring *Cylindrocolea dimorpha* as an age constraint. The prior had...
Figure 1. The Paleogene amber fossil *Odontoschisma* (sect. *Iwatsukia*) *dimorpha*. (a) Male shoot in ventral view; (b) androecium in dorsal view; (c) male shoot in dorsal view; (d) portion of shoot in dorsal view; (e) portion of shoot in dorsal view; leaf-free cell strip discernible; (f) shoot pp. in lateral and pp. in dorsal view, with asterisk indicating ventral branch; and (g) close-up from (f) (a–d from holotype; e–g from Gröhn 2082).
Figure 2. *Odontoschisma dimorpha*. (a) Two ascending shoots in top view – note physical connection to small bark fragments; (b) ascending shoots in ventral view – arrow points to underleaf; (c) deeply bifid underleaf; (d) leaf; (e) dense mat of creeping and ascending shoots on bark fragment; (f) portion of ascending shoot, arrow points to underleaf; and (g) portion of shoot – arrows point to underleaves on ventral side of shoot (a–d from Hoffeins 930-3; e, f from Gröhn 2038; g from Grolle M 12-8).
a normal distribution with a mean of 202.01 and standard deviation of 20 to account for the unknown confidence interval. The analysis was run for 500,000,000 generations and by sampling every 50,000th tree. Consequently, the final tree file contained 10,000 trees. After a burn-in of 25% a maximum credibility tree was compiled in TreeAnnotator 1.8.4, which is part of the BEAST package. Effective sample size was analyzed in TRACER v1.6 (Rambaut et al., 2014).

3 Results

3.1 Systematic paleontology

Odontoschisma (sect. Iwatsukia (N.Kitag.) Gradst., S.C. Aranda & Vanderp.) dimorpha (Casp.) Heinrichs, K. Feldberg, Váňa & Schäff.-Verw., comb. nov.


Holotype: MB.Pb.1979/687 (Künow amber collection 144a) (Fig. 1a–d).

Description

Plants small, prostrate or ascending, brown or reddish brown (sometimes appearing whitish-green or yellowish as a result of shrinking subsequent to embedding), creeping or forming dense mats; leafy shoots 1–14 mm long, 0.10–0.56 mm wide, sparingly ventral-intercalary branched (gyrothecal), leafy, flagelliform or stoloniform; leafy shoots often tapering into a long flagella or sectors with reduced, scaly leaves alternating with sectors producing well-developed leaves. Rhizoids diffusely distributed along ventral side of stem. Stems rigid, 0.05–0.11(–0.14) mm in diameter, 3–6(–ca. 8) cells high, epidermal cells surrounding slightly smaller or similar-sized inner cells (discernible in two broken edges of stems), epidermal cells short rectangular to rectangular, 15–25 × 18–30(–40) µm, walls moderately and evenly thickened, without trigones. Leaves bilobed to about 30–50(–60)% of length, more or less concave, distant to densely imbricate, successively inserted, standing upwards to spreading or leaning on the stem, insertion line oblique to subtransverse, usually not extending to dorsal midline, dorsal leaf-free strip narrow, (0–)1–2 cells wide; leaves variable in size and shape, sometimes reduced, scaly, if well-developed ovate to ovate-oblong to rectangular, ca. 0.12–0.28 mm long (including lobes), 0.10–0.2 mm wide, as long as wide to somewhat longer than wide, widest in or slightly below the middle and narrowing towards base and apex, subsymmetrical, margins entire, lobes broadly triangular to triangular, (2–)3–6(–8) cells wide at base, apex acute, usually ending in a (bluntly) triangular cell, sinus acute, narrowly to widely V-shaped. Leaf cells more or less isodiametrical, some slightly elongate, 14–20(–25) µm in midleaf, slightly smaller along margins, not becoming larger toward base, walls evenly thickened or becoming slightly thicker towards corners, sometimes thin-walled, cuticle verruculose, asperate or smooth. Underleaves absent or well developed, especially on ascending but also on some of the creeping shoots, up to ca. 0.12 mm long, elongate triangular or elongate-ovate to almost rectangular, undivided to deeply bifid, lobes 1–2 cells wide. Gemmae absent. Dioicous (?; only sterile or male plants known). Androecia with 3–8 pairs of bracts; bracts less deeply bifid and somewhat larger than leaves, terminal on elongate branches or becoming intercalary by continued vegetative growth of the branch; antheridia not observed. Gynoecia and sporophyte unknown.

3.2 Additional specimens examined

3.2.1 Baltic amber

Bavarian State Collection for Palaeontology and Geology, Munich, Germany:
SNSB-BSPG 1958 VIII 44 (Bachoven-Echt amber collection P44); SNSB-BSPG 1958 VIII 95 (Bachoven-Echt amber collection P95)

Geoscientific collections, Georg August University Göttingen, Germany:
GZG.BST.21957 (Hoffeins amber collection 5-43); GZG.BST.21959 (K7.319)

Gröhn amber collection, Glinde, Germany:
2015, 2038, 2082

Museum für Naturkunde Berlin, Germany:
MB.Pb.1979/654 (Künow amber collection 95); MB.Pb.1979/688 (Künow amber collection 145); MB.Pb.1979/689 (Künow amber collection 146); MB.Pb.1979/708 (Künow amber collection 165a)

3.2.2 Bitterfeld amber

Geoscientific collections, Georg August University Göttingen, Germany:
GZG.BST.21958 (Hoffeins amber collection 930-3)

Museum für Naturkunde Berlin, Germany:
MB.Pb.1997/2 (Kutscher amber collection H006); MB.Pb.1997/16 (Kutscher amber collection M 8/6); MB.Pb.1997/24 (Grolle amber collection M 10/5);
3.3 Divergence time estimates

The DNA-based divergence time estimates (Fig. 3) support a late Early Cretaceous to early Eocene age of the Odontoschisma crown group (53.5–102.2 Myr). Odontoschisma sect. Iwatsukia originated sometime between the Late Cretaceous and Oligocene (28.5–66.1 Myr).

4 Discussion

4.1 Morphological evidence and ecology

Grolle and Meister (2004) transferred the Eocene amber fossil Cephaloziella dimorpha to Cylindroclea (Cephaloziozaceae) because they respected the complement of morphological features displayed by the fossil (i.e., small size of the plant, the presence of entire-marginated, bifid leaves with uniformly thickened leaf cell walls, ventral branching, and the absence of a stem hyalodermis, underleaves and gemmae) as more congruent with features seen in the latter genus than the former. Our evaluation of the taxon based on series of amber inclusions (Figs. 1, 2) corroborates the species circumscriptions in Grolle (1980) and Grolle and Meister (2004). However, multicellular, deeply bifid underleaves may sometimes occur, e.g., on the arcuately ascending shoots of specimens Gröhn 2038 and Hoffeins 930-3, as well as on a prostrate species. Underleaves are not entirely unknown in Cephaloziozaceae but typically are unlobed and therefore propose the new combination Odontoschisma dimorpha for the taxon. It is impossible to determine whether wax crystals were present in the fossil; however, a few leaves display a striately papilllose cuticle that might constitute of surface wax. Moreover, no plasmodesmata were detected in the fossil leaf cells, but this might as well be a preservation artifact. Finally, it is interesting to note that most O. dimorpha fossils are brownish to reddish-brown in color because this coloration is unknown in extant representatives of sect. Iwatsukia, but is characteristic of several other extant Odontoschisma species.

Based on the preceding considerations and comparison, we believe that O. dimorpha should be retained as a separate species, rather than regarded as fossil O. jishibae. Caspary (1887), in his initial report on the holotype specimen, selected the epithet “dimorpha” for the name because he failed to correctly identify the androecia. Rather, this author assumed that the plant produced two different types of leaves (see Grolle, 1980). Although originally based on a simple misinterpretation, the epithet is actually quite fitting for the species. Leaves of ascending O. dimorpha shoots may be more robust than those of prostrate shoots, which often are somewhat scaly and more loosely arranged.

The extant Odontoschisma jishibae occurs in tropical and warm-temperate regions of South Siberia, Japan, South Korea, Nepal, Malaysia and Papua New Guinea, East Africa, Guinea and Costa Rica, from the lowlands to ca. 2700 m alti-
Figure 3. Phylogenetic chronogram of Cephaloziaceae based on DNA sequence variation of extant species, with secondary calibration from Laenen et al. (2014). Confidence age estimate intervals shown as horizontal bars. Vertical bar indicates age interval of Baltic amber. Transfer of the fossil Jungermannia dimorpha to Odontoschisma sect. Ivatsukia concurs with presented age estimates.
tude, and typically grows on decaying wood, trunk bases and soil (Gradstein and Ikliu-Borges, 2015). Several specimens of O. dimorpha are enshrined together with small bark fragments, suggesting that they grew on trunk bases of the resin-exuding trees, i.e., conifers in the families Pinaceae or Sciadopityaceae (Wolfe et al., 2016). Other fossils of O. dimorpha occur in the amber without providing insights into the substrate on which they grew; however, the presence of these liverworts in amber indicates that they must have grown in close proximity to a resin-exuding tree. Odontoschisma sect. Iwatsukia today does not occur in the Baltic region; however, the Baltic amber forest grew in a distinctly warmer climate (Zachos et al., 2001). Most Baltic amber-bearing strata have been dated as Priabonian, but a few likely extend into the Lutetian. Baltic amber therefore ranges in absolute age from 47 to 35 Myr (Standke, 2008). Although there is some evidence to suggest that most Baltic amber fossils originate from the youngest strata, the exact provenance and geologic age of the O. dimorpha fossils remain unknown. Liverwort fossils similar to O. dimorpha have also been reported in Bitterfeld amber, which is usually interpreted as late Oligocene in age (24–25 Myr; Knuth et al., 2002; Blumenstengel, 2004; Führmann, 2004; Standke, 2008). If the age estimates for both amber are correct, then morphological stasis occurred in O. dimorpha over a period of at least 10 million years. However, the incomplete preservation of the fossils has to be taken into account; female structures, sporophytes and oil bodies remain unknown.

Odontoschisma dimorpha appears to be a relatively common element in both the Baltic and Bitterfeld amber liverwort floras. Originally based on a single individual (Caspary, 1887), additional specimens have been reported over the years (Caspary and Klebs, 1907). Grolle and Meister (2004) list some 12 specimens from Baltic and 9 from Bitterfeld amber. Additional specimens were reported by Frahm and Gröhn (2013a, b). Odontoschisma dimorpha is not the first fossil representative of the family Cephaloziaceae. Katagiri (2015) recently described the Baltic amber fossil Cephalozia veltenii T. Katag. and separated this form from O. dimorpha based on the alleged presence of a hyalodermis. However, the images of C. veltenii in Katagiri’s study suggest that the fossil shrank subsequent to the curing of the resin and, as a result, is preserved in a cavity which depicts the original size of the plant. We therefore submit that what appear as brownish stem portions in this specimen represent the plant material, while the whitish stem layers surrounding the brownish stem represent the amber cavity with imprints of the outer plant surface on the cavity wall. Cephalozia veltenii thus may be synonymous with O. dimorpha.

4.2 DNA-based divergence time estimates

Deviations from DNA standard substitution rates are commonplace and have been documented for seed plants (Bromham et al., 2013), ferns (Rothfels and Schuettpelz, 2014; Zhong et al., 2014; Crusz et al., 2016), and liverworts (Villarreal et al., 2016). As a result, age estimates for Cylindrocolea that turn out roughly 3 times older by using the Baltic amber fossil as a minimum age constraint than estimates based solely on DNA sequence variation (Feldberg et al., 2013) come as no surprise. However, with regard to the amber fossils detailed in this study these differences are based on a misinterpretation of morphological evidence (Grolle and Meister, 2004). Interpretation of Jungermannia dimorpha as a member of Odontoschisma, rather than Cylindrocolea, favors estimates indicating a Miocene age (Laenen et al., 2014) over others that suggest an Eocene age (Feldberg et al., 2014) of Cylindrocolea.

Lineages of plants usually are somewhat older than their oldest indisputable fossil representatives. Heinrichs et al. (2015a) and Schneider et al. (2016) therefore proposed to involve age hypotheses from independently generated molecular chronograms in the taxonomic treatment of fossils. These integrative approaches, which focus on the integration of evidence from different origins (Dayrat, 2005; Will et al., 2005), may be misleading if the molecular clocks greatly vary; however, extreme rate variations (Rothfels and Schuettpelz, 2014) have rarely been reconstructed for seed-free land plants, and approaches involving secondary calibrations and standard substitution rates have therefore been advocated (Villarreal and Renner, 2014). We present divergence time estimates of Cephaloziaceae based on a secondary calibration obtained from the most comprehensive chronogram of liverworts generated without using the fossil Jungermannia dimorpha as an age constraint (Laenen et al., 2014). Our divergence time estimates support a late Early Cretaceous to early Eocene age of the Odontoschisma crown group, and suggest that O. sect. Iwatsukia originated sometime between the Late Cretaceous and Oligocene. Assignment of Jungermannia dimorpha to Odontoschisma sect. Iwatsukia does not conflict with current hypotheses relative to the evolution of Cephaloziaceae and lends further support to assumptions of a minimum age of 35 Ma of O. sect. Iwatsukia based on the age reconstruction of Baltic amber (Standke, 1998).

The chronogram for Cephaloziaceae shown in Fig. 3 is the most comprehensive assessment to date with regard to taxon sampling. The results are congruent with the divergence time estimates provided in Feldberg et al. (2013, 2014) and Laenen (2014), and support a Cretaceous to Paleogene age of most generic crown groups, a recurrent pattern in the evolution of leafy liverworts (Cooper et al., 2012). Similar hypotheses have been derived from amber fossils of liverworts that usually match the morphology of extant genera (Heinrichs et al., 2015b). Taxonomic conclusions drawn on the basis of the gross morphology of incompletely preserved amber fossils are problematic, and hence additional evidence is always intensively sought after and highly welcome. Integrative approaches using a combination of morphological evidence and evidence generated from the DNA variation of extant species (Heinrichs et al., 2007, 2015a) have dis-
missed hypotheses on affinities of certain Eocene amber fossils to the extant species Nipponolejeunea subalpina (Hori.) S.Hatt. and Ptilidium pulcherrimum (Weber) Vaino (Grolle and Meister, 2004). Cylindrocolea dimorpha is only the third example of a fossil liverwort in which molecular data were used to substantiate or revise a taxonomic decision that was based on morphological evidence.

5 Conclusions

An integrative taxonomic approach using morphological and independent, DNA-based evidence suggests that the fossil liverwort Cylindrocolea dimorpha needs to be transferred from Cephaloziaceae to Cephaloziaceae, and supports affinities of the fossil to the extant genus Odontoschisma. This study underlines the importance of correctly identified fossils for our understanding of evolutionary patterns in liverworts, an early diverging lineage of land plants with a poor fossil record (Taylor et al., 2009), and advocates the assessment and integration of different lines of evidence in taxonomic studies of incompletely preserved fossils. We anticipate that such integrative studies will lead to a better understanding of the liverwort fossil record and will provide more precise insights into the evolution of this early land plant lineage than studies considering only a single line of evidence.

Data availability. All necessary data are available in the Supplement.

Information about the Supplement

Taxa used in the divergence time estimates, including information about the origin of the studied material, voucher information, and GenBank accession numbers.

The Supplement related to this article is available online at doi:10.5194/fr-20-147-2017-supplement.

Competing interests. The authors declare that they have no conflict of interest.

Acknowledgements. We thank Christel and Hans Werner Hoffeins (Hamburg) for providing their liverwort fossils to the Geoscientific Museum Göttingen, and Alexander Gehler (Göttingen), Christian Neumann (Berlin) and Martin Nose (Munich) for making the amber collections of the Geoscientific Museum Göttingen, the Museum für Naturkunde at Berlin and the Bavarian State Collection for Palaeontology and Geology available for study.

Edited by: C. Bickelmann
Reviewed by: A. Hagborg and one anonymous referee

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