

RESEARCH ARTICLE

Molecular and Morphological Evidence Challenges the Records of the Extant Liverwort *Ptilidium pulcherrimum* in Eocene Baltic Amber

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Data Availability Statement: The amber fossil described as *Tetralophozia groehnii* sp. nov. is deposited in the collection of the Geological Palaeontological Institute Hamburg (GPIH 4575, Coll. Gröhn 5827). Information on the provenance of specimens used in divergence time estimates, vouchers, and herbarium where the voucher is deposited, as well as GenBank accession numbers are provided in [Table 1](#).

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Abstract

Preservation of liverworts in amber, a fossilized tree resin, is often exquisite. Twenty-three fossil species of liverworts have been described to date from Eocene (35–50 Ma) Baltic amber. In addition, two inclusions have been assigned to the extant species *Ptilidium pulcherrimum* (Ptilidiales or Porellales). However, the presence of the boreal *P. pulcherrimum* in the subtropical or warm-temperate Baltic amber forest challenges the phytogeographical interpretation of the Eocene flora. A re-investigation of one of the fossils believed to be *P. pulcherrimum* reveals that this specimen in fact represents the first fossil evidence of the genus *Tetralophozia*, and thus is re-described here as *Tetralophozia groehnii* sp. nov. A second fossil initially assigned to *P. pulcherrimum* is apparently lost, and can be reassessed only based on the original description and illustrations. This fossil is morphologically similar to the extant North Pacific endemic *Ptilidium californicum*, rather than *P. pulcherrimum*. Divergence time estimates based on chloroplast DNA sequences provide evidence of a Miocene origin of *P. pulcherrimum*, and thus also argue against the presence of this taxon in the Eocene. *Ptilidium californicum* originated 25–43 Ma ago. As a result, we cannot rule out that the Eocene fossil belongs to *P. californicum*. Alternatively, the fossil might represent a stem lineage element of *Ptilidium* or an early crown group species with morphological similarities to *P. californicum*.

Introduction

Liverworts belong to the oldest lineages of plants on land [1–2]; however, their exact position in the tree of life remains unclear [3–8]. The reconstruction of early land plant evolution is

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generally hampered by the incompleteness of the fossil record [9–10]. Moreover, widely differing hypotheses have been offered on the age of the land plant crown group [11] and their closest relatives [12]. In spite of these limitations, considerable progress has been made in recent years regarding the reconstruction of the evolutionary history of the liverworts. DNA-based divergence time estimates suggest a Paleozoic origin of several main liverwort lineages [13–14]. Moreover, the crown group diversification of most extant genera appears to have started during the Cretaceous or Cenozoic, concomitant with the diversification of angiosperms [15–18]. While the general pattern of liverwort diversification is well-supported by the available dating studies, age estimates fluctuate considerably, due in part to deviating node calibrations based on fossil evidence. This makes it all the more important to study the fossil record and determine the usability of fossils in constraining molecular dating analyses [19].

The Paleozoic and early Mesozoic fossil record of liverworts is meagre and most specimens are ill-preserved [20–21]. The situation changes for the better in the late Mesozoic [22–23]. Finally, Cenozoic strata have yielded numerous liverwort fossils in faithful cellular and ultra-structural preservation, most in the form of amber inclusions [24]. Amber, a solidified gymnosperm or angiosperm tree resin, is an excellent preservation medium that provides detailed insights into the diversity of soft-bodied organisms such as liverworts [25]. Approximately 200 amber deposits are known worldwide, the most widely known of which are located in the Baltic region [26]. Baltic amber has been dated as Eocene [27–28]. It was already mentioned by Pliny the Elder (*Naturalis Historia*, book 37), who considered it a type of spruce resin [29]. This “classic” hypothesis is largely accurate; however, the amber-producing tree was likely not a spruce, but rather a representative of the *Sciadopityaceae* [30] growing in a subtropical or warm-temperate mixed forest [31] together with several representatives of *Cupressaceae* and *Pinaceae*, as well as angiosperms, especially *Fagaceae* [28,32].

Baltic amber inclusions have been studied since the 18th century [33]; the first liverwort fossils in Baltic amber have been described in 1845 [34]. Several authors have since documented additional taxa or revised earlier classifications [35–38]. A comprehensive monograph [39] formally accepts 22 species of liverworts from Baltic amber, including *Ptilidium pulcherrimum* (Weber) Vain. *Ptilidium pulcherrimum* belongs to a small genus of terrestrial or epiphytic liverworts characterized by exclusively lateral branching and deeply lobed, incubous leaves with long uniseriate marginal cilia [40]. Three extant species are distinguished based on morphology: the North Pacific endemic *Ptilidium californicum* (Austin) Pearson; and the two widespread circumboreal *P. ciliare* (L.) Hampe and *P. pulcherrimum*. Leaves of *P. californicum* are sparsely ciliate or entire-margined, while the leaf lobes in *P. ciliare* and *P. pulcherrimum* are densely ciliate. Molecular phylogenies place *P. californicum* as sister to a clade containing the other two species [41]. Occurrence of the cold-temperate *P. pulcherrimum* in the warm Eocene [42] challenges the phytogeographical interpretation of the Baltic amber flora. Heinrichs et al. [13] questioned the conspecificity of the Eocene fossil and extant *P. pulcherrimum*; however, Frahm & Gröhn [43] identified a second inclusion as *P. pulcherrimum*, putting forth an adaptation to different climates during the evolutionary history of this species.

Here we present a reassessment of the fossils assigned to *Ptilidium pulcherrimum* based on morphological evidence and DNA sequence variation of extant *Ptilidium* specimens. We dismiss the fossils as evidence of the occurrence of *P. pulcherrimum* in the Eocene, and present an alternative taxonomic treatment for these amber inclusions.

Material and Methods

Amber fossils assigned to *Ptilidium*

The Eocene sediments that yield the majority of Baltic amber are 35–47 million years old, but some specimens are also found in strata up to 50 million-year-old [27–28]. The first piece of Baltic amber containing a liverwort inclusion assigned to *Ptilidium pulcherrimum* was published as specimen BB2379 of the Baltic amber collection of the Museum of Natural History Stuttgart (SMNS “coll. Velten”) [39]. We tried to find the specimen in the SMNS amber holdings, but were unsuccessful. Amber trader J. Velten informed us that he sold the specimen, but has no documents of who purchased it. As a result, the specimen is considered lost. We therefore base our revision of this liverwort inclusion on the description, images, and drawings in [39].

The second Baltic amber specimen assigned to *P. pulcherrimum* originally comes from the private amber collection of Carsten Gröhn [43], but has recently been donated to the collection of the Geological Palaeontological Institute Hamburg, Germany (GPIH 4575, Coll. Gröhn 5827, Syninclusion: *Cylindrocolea dimorpha* (Casp.) Grolle). The surface of the amber piece was polished manually with a series of wet silicon carbide abrasive papers (grit from FEPA P 600–4000, 25.8 μm to 5 μm particle size, firm Struers) to minimize light scattering during analysis. The specimen was then placed on a glass microscope slide with a drop of water applied to the upper surface of the amber, and covered with a coverslip. Inclusions were studied using a Leica M50 dissection 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 illustrations accompanying our study represent digitally stacked photomicrographic composites obtained with the software package HeliconFocus 6.0. Drawings of the fossil were produced using a Leica DM1000 microscope equipped with a drawing tube. The liverwort was embedded in the resin in wet condition, and appears to have turned by a few degrees during fossilization. Resulting streaks in the amber exacerbate visual inspection and photography. Moreover, the inclusion has shrunken during fossilization and, as a result, is preserved in a cavity representing the original size of the plant. Refraction of light at the amber surface of the cavity obstructs the recognizability of the cell walls and surface structures, and further reduces digital image quality. However, the outlines and certain cellular details of the stem, leaf, and rhizoids in hydrated condition have been preserved as imprints on the surface of this cavity. Cell size measurements were taken from these imprints, as well as from plant fragments inside the cavity. Measurements of plant size and rhizoid diameter reflect the size of the cavity, rather than that of the shrunken plant.

The taxonomic treatment of the fossil is based on literature data on fossil and extant liverworts, as well as comparisons with herbarium specimens of liverworts housed at the Bavarian State Collection of Botany (M). Herbarium material of all three extant *Ptilidium* species was moistened and leaves were separated. Leaves were transferred into a drop of water on a microscope slide and covered with a coverslip. Images were captured digitally as described above for the fossil.

No permits were required for the described study, which complied with all relevant regulations.

Nomenclature

The electronic version of this article in a Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic

Table 1. Taxa used in divergence time estimates, including information on provenance of specimens, vouchers, and herbarium where the voucher is deposited, as well as GenBank accession numbers. Herbarium abbreviations: Göttingen University herbarium, Germany (GOET); East China Normal University herbarium (HSNU); National Museum of Nature and Science herbarium, Japan (TNS); University of British Columbia herbarium, Canada (UBC).

Taxon and locality	Voucher and herbarium	<i>trnL-trnF</i>	<i>atpB-rbcL</i>	<i>trnG</i>
<i>Neotrichocolea bissetii</i> (Mitt.) S. Hatt.				
Japan	Inoue, Br. Sel. Exs. 563 (GOET)	HQ329973	HQ330109	HQ330244
<i>Ptilidium californicum</i> (Austin) Pearson				
U.S.A.	Schofield 114357 (UBC)	HQ329991	HQ330126	HQ330261
<i>Ptilidium ciliare</i> (L.) Hampe				
China	Zhu 20080802–12 (HSNU)	HQ330016	HQ330151	HQ330286
Germany	Heinrichs & Schmidt 3741 (GOET)	HQ330023	HQ330158	HQ330292
Greenland	Long 13239 (JE)	HQ330026	HQ330161	HQ330295
Poland	Jedrezejko & Stebel, Hep. Pol. Exs. 204 (GOET)	HQ330030	HQ330165	HQ330299
U.S.A.	Schofield et al. 101305 (UBC)	HQ330036	HQ330171	HQ330305
U.S.A.	Schofield 118109 (UBC)	HQ330051	HQ330186	HQ330320
<i>Ptilidium pulcherrimum</i> (Weber) Vain.				
Canada	Schofield 124480 (UBC)	HQ330068	HQ330203	HQ330337
Japan	Higuchi 1197 (TNS)	HQ330077	HQ330212	HQ330345
Poland	Klama & Zarnowiec 206 (GOET)	HQ330080	HQ330215	HQ330348
U.S.A.	Schofield 116250 (UBC)	HQ330096	HQ330231	HQ330364

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Divergence time estimates

The evolutionary history of *Ptilidium* was reconstructed using a subset of the chloroplast DNA marker set of [41]. One accession per *Ptilidium*-haplotype was chosen and related sequences were downloaded from GenBank (Table 1). Only those haplotypes of which complete sequence stretches are available (*trnL* intron, *trnL* 3'-exon, *trnL-trnF* intergenic spacer; *atpB-rbcL*; *trnG*-intron) were considered. *Neotrichocolea bissetii* (Mitt.) S. Hatt. was chosen as outgroup based on phylogenetic hypotheses of [44]. Sequences were aligned manually using BioEdit version 5.0.9 [45] and ambiguously aligned positions were excluded. Divergence time estimates were conducted using the BEAST package v.1.8.2 [46] and the HKY substitution model with four rate categories. The tree prior was a pure-birth (Yule) tree with Markov chain Monte Carlo (MCMC) run for 40 million generations, sampling every 10,000 generations. The first 10% of trees were discarded as burn-in, and the remaining trees were combined using TreeAnnotator [47]. Following analyses of the extent of rate heterogeneity in Tracer 1.6 [48], we applied a strict clock model, calibrated with a plastid substitution rate of 5.0×10^{-4} substitutions/site/my from [49]. The ingroup was constrained to be monophyletic.

Results

Grolle & Meister fossil

According to the three published images and accompanying description, the fossil consists of a laterally branched gametophyte with deeply incised, (2–)3-lobed leaves and lobes with a few

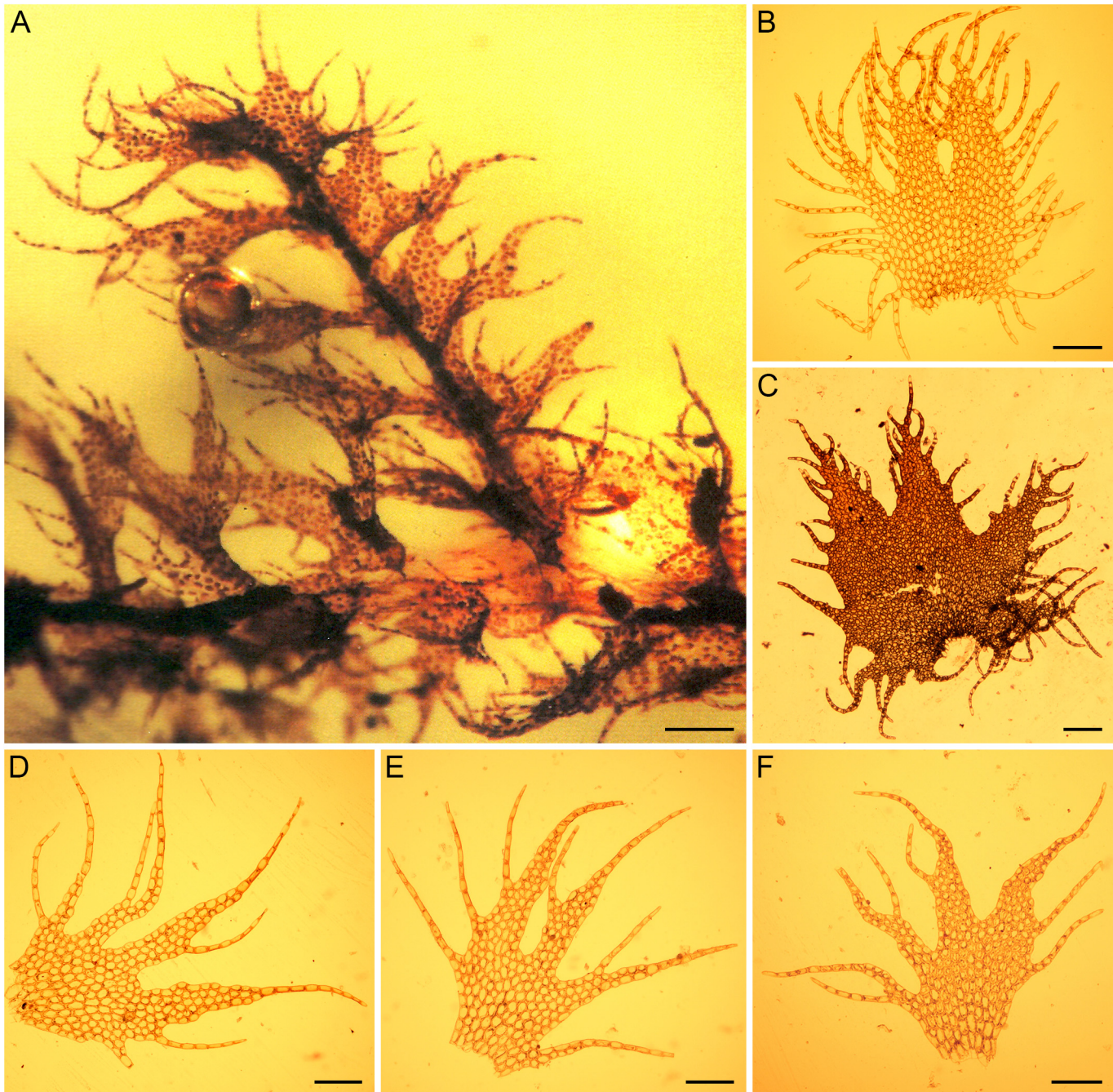


Fig 1. The Eocene liverwort fossil *Ptilidium* sp. (A) and leaves of extant representatives of *Ptilidium* (B–F) (scale bars 200 μ m). (A) Baltic amber fossil. (B) *Ptilidium pulcherrimum*. (C) *Ptilidium ciliare*. (D–F) *Ptilidium californicum*.

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loosely arranged, long, uniseriate cilia (Fig 1A). Fasciculate rhizoids are present at the base of ciliate underleaves. Available evidence does not argue against assignment of the fossil to *Ptilidium*. However, the fossil differs from the extant *Ptilidium pulcherrimum* (Fig 1B) in the low number of leaf cilia and their loose spacing.

Frahm & Gröhn fossil

Amber piece GPIH 4575 (Gröhn 5827) includes gametophyte fragments of two different leafy liverworts, along with stellate hairs of Fagales. The fragment identified as *Ptilidium* has quadrifid, succubously-transverse leaves with canaliculate lobes and marginal teeth up to 4 cells wide and cilia (Figs 2 and 3), and thus cannot be assigned to *Ptilidium*. The specimen is re-described below as an extinct species of the extant genus *Tetralophozia* (R.M. Schust.) Schljakov:

Tetralophozia groehnii Heinrichs, Váňa and Schäf.-Verw., sp. nov.

Holotype: Plant fragment with quadrifid leaves in Baltic amber piece GPIH 4575 (= Coll. Gröhn 5827) of the Geological Palaeontological Institute Hamburg, Germany (Fig 2).

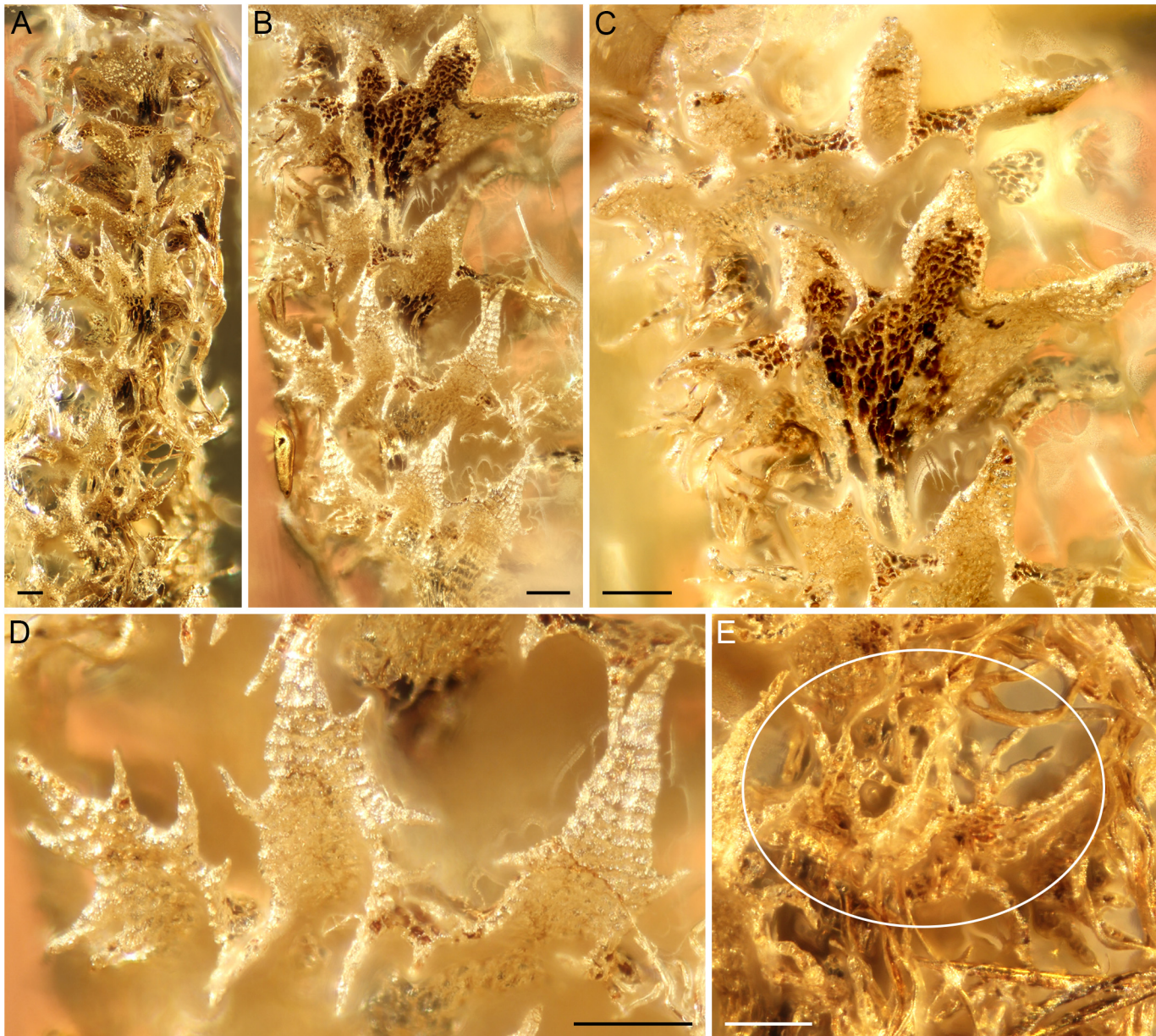


Fig 2. Holotype of *Tetralophozia groehnii* sp. nov. (GPIH 4575) from Baltic amber. The fossil was initially assigned to *Ptilidium* [43] but morphology is inconsistent with this genus (scale bars 100 μ m). (A) Upper portion of gametophyte in lateral view. (B, C) Deeply quadrifid leaves, teeth at lobe base pointing towards the plant base. (D) Impressions of leaf lobes showing leaf cells with papillose cuticle in ventral view. (E) Densely ciliate, bifid underleaf (circle).

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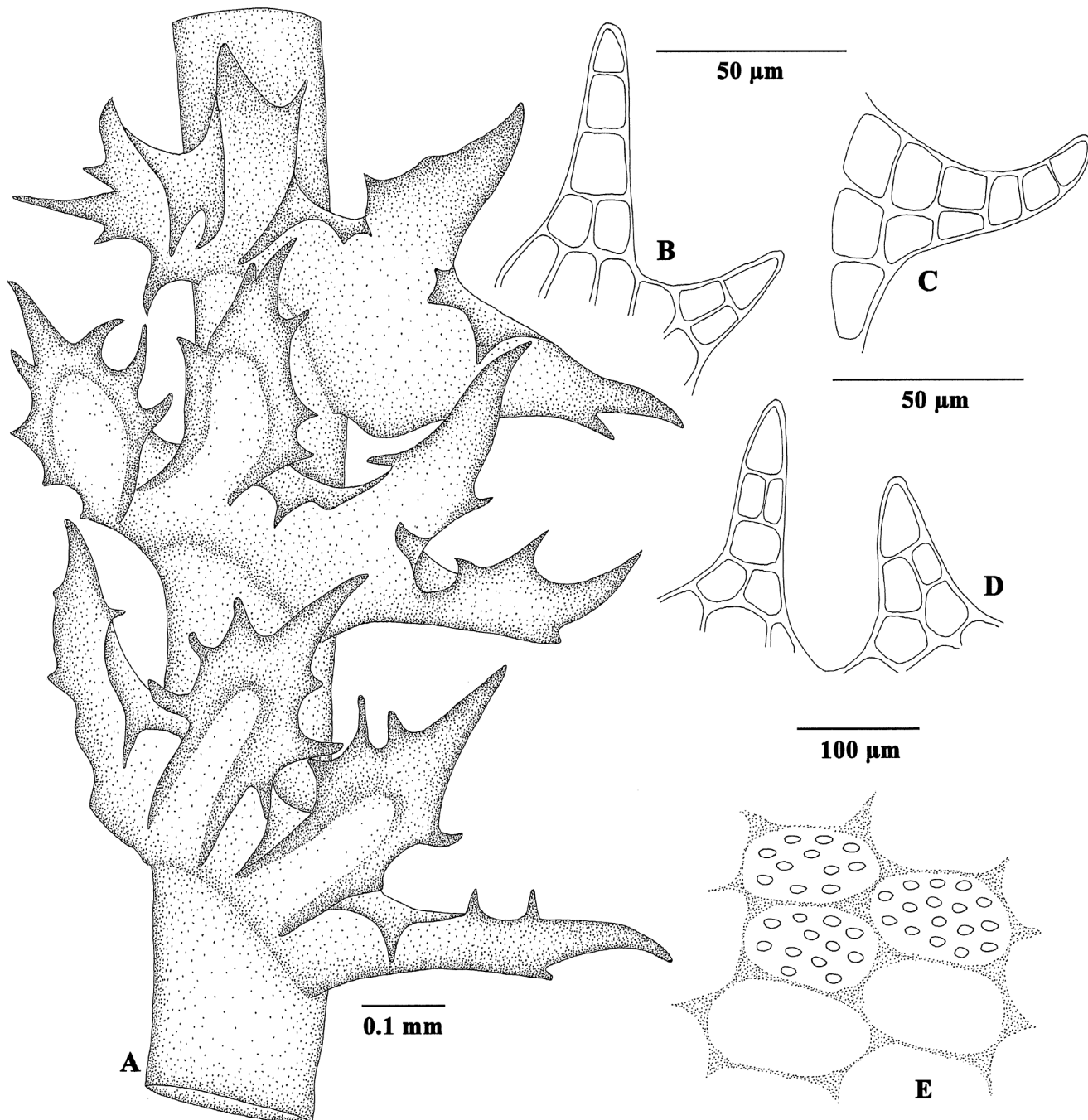


Fig 3. Reconstruction of *Tetralophozia groehnii* based on the holotype. (A) Portion of shoot with 3 quadrifid, succubous-subtransversely arranged leaves and partly canaliculate lobes. (B–D) Triangular to elongate-triangular, partly biseriolate teeth extending from lobe margins. (E) Leaf cell pattern; papillae indicated in upper cells.

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Diagnosis: Succubously-transverse foliated gametophyte with bifid, ciliate toothed underleaves and 2 rows of deeply quadrifid leaves with canaliculate, dentate-ciliate lobes ending in uniseriate tip 1–3(–5) cells long.

Description: Sterile shoot fragment, 4.8 mm long and up to 1.2 mm wide, brown to reddish brown. Stem prostrate, round, ca. 170–250 μm in diameter, cortical cells rectangular, (25–)40–60

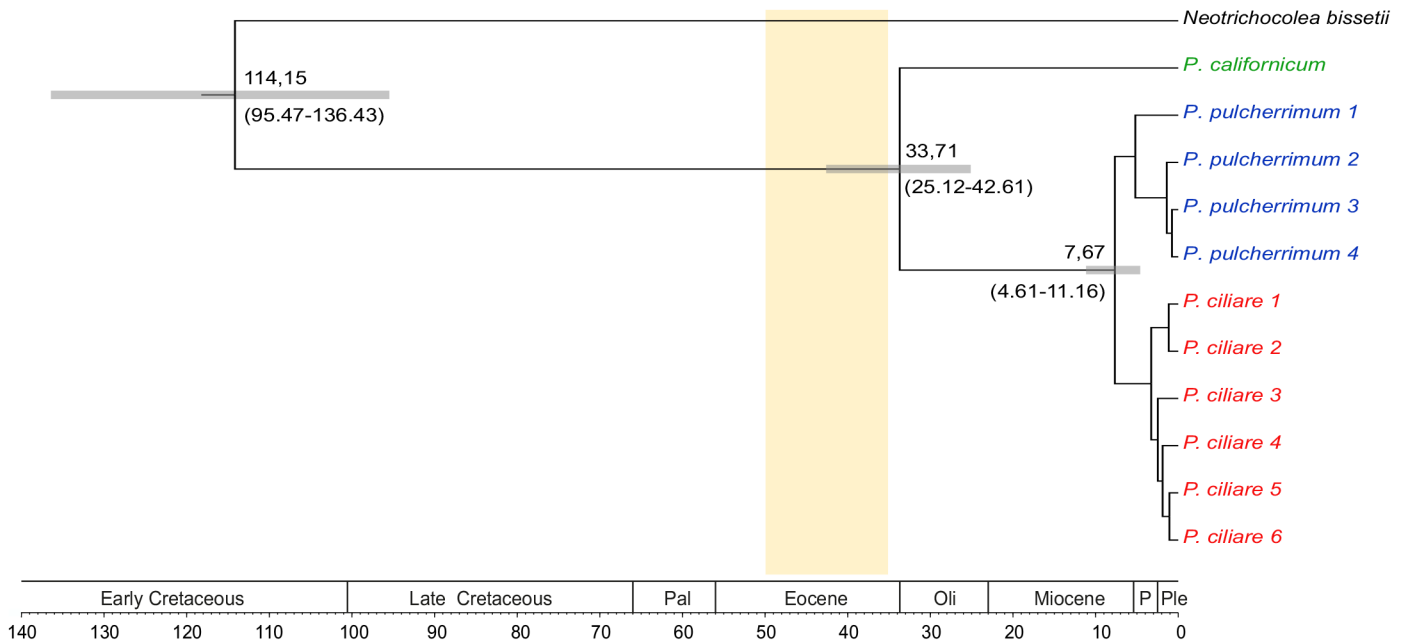


Fig 4. Phylogenetic chronogram for *Ptilidium* plotted against the geological time scale (Pal = Paleocene, Oli = Oligocene, P = Pliocene; Ple = Pleistocene/Holocene). Time scale shown in million years to present. Confidence age estimate intervals (split *Ptilidium*-*Neotrichocolea*, *Ptilidium* crown group, split *Ptilidium ciliare*/*P. pulcherrimum*) shown as grey horizontal bars. Vertical bar indicates time interval for Baltic amber [27–28].

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(–90) x 12–20(–30) μm, ventrally, especially in lower sectors, with scattered colourless to pale-brownish rhizoids 12–15 μm thick; near base with a single, densely foliated young branch emerging from between rhizoids, and thus likely of ventral or ventro-lateral origin. Leaves vertically oriented, succubously-subtransverse, hand-like, deeply quadrifid, with spreading bases but upwardly bent to suberect, finger-like, abaxially canaliculate lobes, lobe margins and disc with triangular to elongate-triangular teeth 1–3(–4) cells wide and 2–8 cells long and cilia, those at lobe base often pointing towards the plant base. Teeth oriented perpendicularly to leaf margin, pointed forward, some pointing backwards, (1–)2–7 per lobe margin, straight or curved. Leaf cells ca. 8–15 x 12–25 μm, near leaf base mostly irregularly rectangular, towards apex subisodiametric or a mixture of subisodiametric and rectangular ones, walls thickened, trigones present. Cuticle with relatively large, low, globose to elliptic papillae. Underleaves conspicuous, bifid, densely ciliate, to more than 250 μm long (including cilia). Asexual reproduction not observed.

Divergence time estimates

Ptilidium separated from *Neotrichocolea* S. Hatt. during the Cretaceous (Fig 4). Crown group diversification of *Ptilidium* started in the Eocene or Oligocene (25–43 Ma). *Ptilidium californicum* is placed sister to a clade with accessions of *P. pulcherrimum* and *P. ciliare*. The separation of *P. pulcherrimum* and *P. ciliare* occurred in the Pliocene or late Miocene (4.6–11.2 Ma).

Discussion

Fossil record of *Ptilidium*

Only one of the two Eocene amber fossils initially assigned to *Ptilidium pulcherrimum* can actually be assigned to *Ptilidium* with some degree of confidence, whereas the second represents a different genus. While the Grolle & Meister fossil in fact appears to belong to *Ptilidium*

(Fig 1A), affinities to *P. pulcherrimum* (Fig 1B) are dismissed. *Ptilidium pulcherrimum* is characterized by densely ciliate leaves [40,50], but the leaves of the fossil possess only a few loosely arranged cilia [39]. This arrangement of cilia resembles the North Pacific endemic *P. californicum* (Fig 1D–1F), which is characterized by sparsely ciliate to entire-margined leaves [41,51–52]. The fossil seems to have a slightly wider leaf sinus than extant *P. californicum*; however, the variation in leaf shape is difficult to determine from the images. *Ptilidium californicum* is an epiphyte that grows on the bark of gymnosperms, especially at the base of large trees or fallen logs. The species occurs southwards to northern California where it is restricted to middle-elevation forests, in a more mediterranean climate than the other two extant *Ptilidium* species [52–53]. The ecological amplitude of the present-day *P. californicum* does not contradict the presence of this taxon in the Baltic amber forest; however, assignment of incompletely preserved Paleogene fossils to extant species is problematic [24], especially in light of the extensive morphological homoplasy among extant taxa [54]. On the other hand, the morphological data gathered from the fossil do not suffice to reject affiliation of the fossil with *P. californicum*.

DNA sequence variation of extant species provides information on evolutionary history, and thus can be used to review the morphology-based classification of fossils [13,55–56]. So far, only *P. ciliare* and *P. pulcherrimum* have been included in divergence time estimates of liverworts [3,15,18]. These large-scale studies all indicate a Miocene origin for the two species, and thus cast doubt on the assignment of Eocene fossils to *P. pulcherrimum*. Our divergence time estimates (Fig 4) also indicate a Miocene age of *P. pulcherrimum*, and thus provide a further argument against the identification of the fossil by Grolle & Meister [39]. According to our reconstruction, *Ptilidium* separated from *Neotrichocolea* some 114 Ma ago, in the Early Cretaceous. This estimate corresponds well with the estimates provided by [15] (118 Ma ago), [18] (103 Ma ago) and [16] (94 Ma ago). Our divergence time estimates are the first that include *P. californicum*, indicating a late Eocene origin of this species (33.7 Ma ago). However, the age estimate confidence interval (25.1–42.6 Ma) largely overlaps with the age of Baltic amber of 35–50 Ma [27–28]. As a result, we feel confident to hypothesize that the *P. californicum* lineage may have already existed at the time of the Baltic amber forest. We cannot rule out that the Grolle & Meister fossil in fact belongs to this lineage. It is also possible, however, that this fossil represents a stem lineage element of *Ptilidium* or an early crown group species with morphological similarities to *P. californicum*.

Although little is known about the evolutionary age of bryophyte species and their morphological changes through time, an Eocene origin has previously been reconstructed for the leafy liverwort *Bryopteris diffusa* (Sw.) Nees [57,58]. Separation of the liverwort *Haplomitrium gibbsiae* (Steph.) R.M. Schust. from the rest of the genus possibly occurred at some point during the Permian [15]. On the other hand, speciation and extinction events are not necessarily visible in the fossil record or in chronograms based on DNA sequence variation of extant species. Such speciation events imply younger species ages than seen in the chronograms, but can only be reconstructed if a dense fossil record is available [59] or if fossil DNA is still accessible [60]. With regard to *Bryopteris diffusa*, the Miocene amber fossil *Bryopteris bispinosa* Grolle is of special interest [61]. Both taxa share a toothed lobule, and thus may belong to the same lineage. However, the fossil can be interpreted as a Miocene *B. diffusa* only if fundamental changes in the morphology of this species have occurred during its evolutionary history. Accepting the fossil as an extinct sister lineage of *B. diffusa* might imply a younger age of this species than seen in chronograms considering only the extant diversity.

In the absence of additional *Ptilidium* fossils we are unable to dismiss an Eocene origin of *P. californicum*, yet we acknowledge the uncertainties connected with this assumption. All other published Baltic amber fossils of liverworts have been assigned to extinct species. Conspecificity of the extinct *Nipponolejeunea europaea* Grolle with the extant *N. subalpina* (Horik.) S.

Hatt., as suggested by [39], has subsequently been rejected based on divergence time estimates indicating a late Miocene or Pliocene origin of *N. subalpina* [13]. As a consequence, the missing *Ptilidium* fossil of Grolle & Meister [39] represents the only Baltic amber inclusion of a liverwort with a possible affiliation to an extant species.

Tetralophozia groehnii

Ptilidium is an isolated genus that has been interpreted as an early diverging lineage of the liverwort order Porellales [62] or a member of the Ptilidiales [63]. Porellales/Ptilidiales are characterized by a complement of several structural features, including incubous foliation, exclusively lateral branches, and fasciculate rhizoids. Specimen GPIH 4575 [43] cannot be attributed to *Ptilidium*; rather, the succubous leaves and diffusely distributed rhizoids are suggestive of affinities to Jungermanniales, a generalistic main lineage of liverworts that originated in the Paleozoic [15,16]. Ten fossil representatives of Jungermanniales have been recorded for Baltic amber to date [39,64–66]; however, GPIH 4575 is not related to any one of these fossils. The rigid stem, brown colour, succubously transverse quadrid leaves (Fig 2A and 2B), canalliculate leaf lobes (Fig 2D) and bifid underleaves (Fig 2E) correspond to the extant genus *Tetralophozia* of the Scapaniaceae sensu [62] and [67], or Anastrophyllaceae sensu [68]. Divergence time estimates provide some evidence in support of an Eocene origin of *Tetralophozia* [16,67], and thus do not contradict the morphology-based classification.

Tetralophozia includes four extant species that thrive in temperate and tropical Asia, Africa, and the Holarctics [50,69–71]. Molecular phylogenies [67] suggest that *Tetralophozia* and the morphologically closely related genus *Plicanthus* R.M. Schust. may be synonymous; however, current sampling does not suffice to resolve the actual relationship between the two taxa. *Plicanthus* is the younger taxon, and is separated from *Tetralophozia* by 3- rather than 4-lobed leaves and freely ciliate leaf margins. *Plicanthus* currently includes four or five species with a Palearctic distribution [72].

Tetralophozia groehnii (Figs 2 and 3) represents the first fossil evidence of the genus *Tetralophozia*. The fossil differs from the extant species by the relatively regular dentition of the leaf lobes and teeth with a 1–4(5) celled uniseriate tip.

Conclusions

The two Baltic amber inclusions initially assigned to the boreal species *Ptilidium pulcherrimum* in fact do not represent this species, and thus do not challenge the phytogeographical interpretation of the subtropical or warm-temperate Baltic amber flora. The specimen published by Frahm & Gröhn [43] is a jungermannialean liverwort, representing the first fossil record of *Tetralophozia*. The second inclusion, described in Grolle & Meister [39], may represent an extinct crown group element of *Ptilidium* or a representative of its stem lineage. Alternatively, this fossil might belong to the extant *P. californicum*. If the latter is correct, then this fossil represents the only Baltic amber fossil of a liverwort with a possible direct relationship to an extant species.

In spite of the dedicated work by Grolle [39], the inventory of liverworts in Baltic amber remains incomplete. Only three species have been added to this inventory since the publication of the monograph by Grolle & Meister [39], i.e. *Cephalozia veltenii* T. Katag. [66], *Notoscyphus balticus* Heinrichs, A.R. Schmidt, Schäf.-Verw., Gröhn et M.A.M. Renner [65], and *Tetralophozia groehnii* (this paper). Liverworts preserved in amber likely were epiphytes on the resin-exuding gymnosperms, or lived in the immediate vicinity and became embedded in resin trickling or dripping from trunks and branches. Epiphytes that prefer angiosperm bark, as well as terrestrial species growing in some distance to the resiniferous gymnosperms are rarely

preserved in Baltic amber. Wind (e.g. during storms) and water (e.g., during floods) transport of liverwort fragments into resin flows may occur, but is exceedingly rare. Based on the fact that the diversity of epiphytic liverworts in present-day gymnosperm forests is usually lower than that in angiosperm-dominated forests [73–76], it is reasonable to conclude that only a minor proportion of the liverworts of the Baltic amber forest has been documented to date.

Supporting Information

S1 File. Agreement for use of the previously copyrighted image of the *Ptilidium* fossil for Fig 1A.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: JH AS MK ARS. Performed the experiments: JH AS. Analyzed the data: JH AS GEL JV ASV MK ARS. Wrote the paper: JH AS MK ARS.

References

1. Kenrick P, Crane PR. The origin and early evolution of plants on land. *Nature* 1997; 389: 33–39.
2. Wellman CH, Osterloff PL, Mohiuddin U. Fragments of the earliest land plants. *Nature* 2003; 425: 282–285. PMID: [13679913](#)
3. Nishiyama T, Wolf PG, Kugita M, Sinclair RB, Sugita M, Sugiura C, et al. Chloroplast phylogeny indicates that bryophytes are monophyletic. *Molec Biol Evol* 2004; 21: 1813–1819. PMID: [15240838](#)
4. Goremykin VV, Hellwig FH. Evidence for the most basal split in land plants dividing bryophyte and tracheophyte lineages. *PI Syst Evol* 2005; 254: 93–103.
5. Groth-Malonek M, Pruchner M, Grewe F, Knoop V. Ancestors of trans-splicing mitochondrial introns support serial sister group relationships of hornworts and mosses with vascular plants. *Molec Biol Evol* 2006; 22: 117–125.
6. Qiu YL, Li L, Wang B, Chen Z, Knoop V, Groth-Malonek M, et al. The deepest divergences in land plants inferred from phylogenomic evidence. *Proc Natl Acad Sci USA* 2006; 103: 15511–15516. PMID: [17030812](#)
7. Cox CJ, Li B, Foster PG, Embley TM, Civiá P. Conflicting phylogenies for early land plants are caused by composition biases among synonymous substitutions. *Syst Biol* 2014; 63: 272–279. doi: [10.1093/sysbio/syt109](#) PMID: [24399481](#)
8. Wickett NJ, Mirarab S, Nguyen N, Warnow T, Carpenter E, Matasci N, et al. Phylotranscriptomic analysis of the origin and early evolution of land plants. *Proc Natl Acad Sci USA* 2014; 111: E4859–E4868. doi: [10.1073/pnas.1323926111](#) PMID: [25355905](#)
9. Taylor TN, Taylor E, Krings M. *Paleobotany. The Biology and Evolution of Fossil Plants*. Burlington: Academic Press; 2009.
10. Kenrick P, Wellman CA, Schneider H, Edgecombe GD. A timeline for terrestrialization: consequences for the Carbon cycle in the Palaeozoic. *Phil Trans Royal Soc B, Biol Sci* 2012; 367, 519–536.
11. Clarke J, Warnock RCM, Donoghue PCJ. Establishing a time-scale for plant evolution. *New Phytol* 2011; 192, 266–301. doi: [10.1111/j.1469-8137.2011.03794.x](#) PMID: [21729086](#)

12. Wodniok S, Brinkmann H, Glöckner G, Heidel AJ, Philippe H, Melkonian M, et al. Origin of land plants. Do conjugating green algae hold the key? *BMC Evol Biol* 2011; 11: 104. doi: [10.1186/1471-2148-11-104](https://doi.org/10.1186/1471-2148-11-104) PMID: [21501468](https://pubmed.ncbi.nlm.nih.gov/21501468/)
13. Heinrichs J, Hentschel J, Wilson R, Feldberg K, Schneider H. Evolution of leafy liverworts (Jungermanniidae, Marchantiophyta): estimating divergence times from chloroplast DNA sequences using penalized likelihood with integrated fossil evidence. *Taxon* 2007; 56: 31–44.
14. Fiz-Palacios O, Schneider H, Heinrichs J, Savolainen V. Diversification of land plants: insights from a family-level phylogenetic analysis. *BMC Evol Biol* 2011; 11: 341. doi: [10.1186/1471-2148-11-341](https://doi.org/10.1186/1471-2148-11-341) PMID: [22103931](https://pubmed.ncbi.nlm.nih.gov/22103931/)
15. Cooper ED, Henwood MJ, Brown EA. Are the liverworts really that old? Cretaceous origins and Cenozoic diversifications in Lepidoziaceae reflect a recurrent theme in liverwort evolution. *Biol J Linn Soc* 2012; 107: 425–441.
16. Feldberg K, Schneider H, Stadler T, Schäfer-Verwimp A, Schmidt AR, Heinrichs J. Epiphytic leafy liverworts diversified in angiosperm-dominated forests. *Sci Rep* 2014; 4: 5974. doi: [10.1038/srep05974](https://doi.org/10.1038/srep05974) PMID: [25099137](https://pubmed.ncbi.nlm.nih.gov/25099137/)
17. Laenen B, Shaw B, Schneider H, Goffinet B, Paradis E, Désamoré A, et al. Extant diversity of bryophytes emerged from successive post-Mesozoic diversification bursts. *Nature Com* 2014; 5: 6134.
18. Sun Y, He X, Glenn D. Transantarctic disjunctions in Schistochilaceae (Marchantiophyta) explained by early extinction events, post-Gondwanan radiations and palaeoclimatic changes. *Mol Phylogenet Evol* 2014; 76: 189–201. doi: [10.1016/j.ympev.2014.03.018](https://doi.org/10.1016/j.ympev.2014.03.018) PMID: [24680916](https://pubmed.ncbi.nlm.nih.gov/24680916/)
19. Parham JF, Donoghue PC, Bell CJ, Calway TD, Head JJ, Holroyd PA, et al. Best practices for justifying fossil calibrations. *Syst Biol* 2012; 61: 346–359. doi: [10.1093/sysbio/syr107](https://doi.org/10.1093/sysbio/syr107) PMID: [22105867](https://pubmed.ncbi.nlm.nih.gov/22105867/)
20. Krassilov VA, Schuster RM. Paleozoic and Mesozoic fossils. In: Schuster RM, editor. *New Manual of Bryology*. Nichinan: The Hattori Botanical Laboratory; 1984. pp. 1172–1193.
21. Oostendorp C. The bryophytes of the Paleozoic and Mesozoic. *Bryophyt Bibl* 1987; 34: 5–112, Plates I–XLIX.
22. Schuster RM, Janssens JA. On *Diettertia*, an isolated Mesozoic member of the Jungermanniales. *Rev Palaeobot Palynol* 1989; 57: 277–287.
23. Heinrichs J, Schäfer-Verwimp A, Feldberg K, Schmidt AR. The extant liverwort *Gackstroemia* (Lepido-laenaceae, Porellales) in Cretaceous amber from Myanmar. *Rev Palaeobot Palynol* 2014; 203: 48–52.
24. Heinrichs J, Kettunen E, Lee GE, Marzaro G, Pócs T, Ragazzi E, et al. Lejeuneaceae (Marchantiophyta) from a species-rich taphocoenosis in Miocene Mexican amber, with a review of liverworts fossilised in amber. *Rev Palaeobot Palynol* 2015; 221: 59–70.
25. Penney D. (editor) *Biodiversity of fossils in amber from the major world deposits*. Manchester: Siri Scientific Press; 2010.
26. Weitschat W, Wichard W. Baltic amber. In: Penney D, editor. *Biodiversity of fossils in amber from the major world deposits*. Manchester: Siri Scientific Press; 2010. pp. 80–115.
27. Standke G. Die Tertiärprofile der Samländischen Bernsteinküste bei Rauschen. *Schriftenreihe für Geowissenschaften* 1998; 7: 93–133.
28. Standke G. Bitterfelder Bernstein gleich Baltischer Bernstein?—Eine geologische Raum-Zeit-Betrachtung und genetische Schlußfolgerungen. *Exkurs.f. und Veröfftl. DGG* 2008; 236: 11–33.
29. Garrett RM. *Precious stones in old English literature*. Naumburg a. S.: Lippert & Co.; 1909.
30. Wolfe AP, Tappert R, Muehlenbachs K, Boudreau M, McKellar RC, Basinger JF, et al. A new proposal concerning the botanical origin of Baltic amber. *Proc Royal Soc B: Biol Sci* 2009; 276: 3403–3412.
31. Frahm JP. *Die Laubmoosflora des Baltischen Bernsteinwaldes*. Jena, Weissdorn; 2010.
32. Conwentz H. *Monographie der Baltischen Bernsteinbäume. Vergleichende Untersuchungen über die Vegetationsorgane und Blüten, sowie über das Harz und die Krankheiten der baltischen Bernsteinbäume*. Leipzig, Engelmann; 1890.
33. Sendel N. *Historia succinorum corpora aliena involventium et nature opere pictorum et caelatorum ex Augustorum I et II cimeliis Dresdae conditis aeri insculptorum*. Leipzig: Gleditsch; 1742.
34. Goeppert HR, Berendt GC. *Der Bernstein und die in ihm befindlichen Pflanzenreste der Vorwelt*. Berlin: Nicolai; 1845.
35. Caspary R. Einige neue Pflanzenreste aus dem samländischen Bernstein. *Schr Physik-Ökonom Ges (Abh)* 1887; 27: 1–8.
36. Caspary R, Klebs R. Die Flora des Bernsteins. *Abh Preuss Geol Landesanst NF* 1907; 4: 11–182.
37. Mägdefrau K. Flechten und Moose im Baltischen Bernstein. *Ber Dt Bot Ges* 1957; 79: 433–435.
38. Grolle R. Lebermoose im Bernstein 1. *Feddes Repert* 1980; 91: 183–190.

39. Grolle R, Meister K. The liverworts in Baltic and Bitterfeld amber. Jena, Weissdorn; 2004.
40. Schuster RM. The Hepaticae and Anthocerotae of North America. Vol. 1. New York: Columbia University Press; 1966.
41. Kreier HP, Feldberg K, Mahr F, Bombosch A, Schmidt AR, et al. Phylogeny of the leafy liverwort *Ptilidium*: cryptic speciation and shared haplotypes between the Northern and Southern Hemispheres. Mol Phylogenet Evol 2010; 57: 1260–1267. doi: [10.1016/j.ympev.2010.10.002](https://doi.org/10.1016/j.ympev.2010.10.002) PMID: [20950690](https://pubmed.ncbi.nlm.nih.gov/20950690/)
42. Pearson PN, van Dongen BE, Nicholas CJ, Pancost RD, Schouten S, Singano JM, et al. Stable warm tropical climate through the Eocene epoch. Geology 2007; 35: 211–214.
43. Frahm JP, Gröhn C. Neue Nachweise von Moosen aus baltischem Bernstein. Arch Bryol. 2013; 175: 1–8.
44. Liu Y, Jia Y, Wang W, Chen Z-D, Davis EC, Qiu Y-J. Phylogenetic relationships of two endemic genera from East Asia. *Trichocoleopsis* and *Neotrichocolea* (Hepaticae). Ann Missouri Bot Gard 2008; 95: 459–470.
45. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1998; 41: 95–98.
46. Drummond AJ, Suchard MA, Xie D, Rambaut A. Bayesian phylogenetics with BEAUTi and the BEAST 1.7. Molec Biol Evol 2012; 29: 1969–1973. doi: [10.1093/molbev/mss075](https://doi.org/10.1093/molbev/mss075) PMID: [22367748](https://pubmed.ncbi.nlm.nih.gov/22367748/)
47. Rambaut A. FigTree v. 1.4.2. 2014; Available: <http://tree.bio.ed.ac.uk>.
48. Rambaut A, Suchard MA, Xie D, Drummond AJ. Tracer v1.6. 2014; Available: <http://beast.bio.ed.ac.uk/Tracer>.
49. Villarreal JC, Renner SS. 2014. A review of molecular-clock calibrations and substitution rates in liverworts, mosses, and hornworts, and a timeframe for a taxonomically cleaned-up genus *Nothoceros*. Mol Phylogenet Evol 2014; 78: 25–35. doi: [10.1016/j.ympev.2014.04.014](https://doi.org/10.1016/j.ympev.2014.04.014) PMID: [24792087](https://pubmed.ncbi.nlm.nih.gov/24792087/)
50. Paton JA. The liverwort flora of the British Isles. Colchester, Harley Books; 1999.
51. Schofield WB. Field guide to liverwort genera of Pacific North America. Seattle, University of Washington Press; 2002.
52. Doyle WT, Stotler RE. Contributions toward a bryoflora of California III: Keys and annotated species catalogue for liverworts and hornworts. Madroño 2006; 53, 89–197.
53. Botting RS, Fredeen AL. Contrasting terrestrial lichen, liverwort, and moss diversity between old-growth and young second-growth forest on two soil textures in central British Columbia. Canad J Bot 2006; 84: 124–129.
54. Renner MAM. Lobule shape evolution in *Radula* (Jungermanniopsida): one rate fits all? Bot J Linn Soc 2015; 178: 222–242.
55. Lóriga J, Schmidt AR, Moran RC, Feldberg K, Schneider H, Heinrichs J. 2014. The first fossil of a bolbitidoid fern belongs to the early divergent lineages of *Elaphoglossum* (Dryopteridaceae). Am J Bot 2014; 101: 1466–1475. doi: [10.3732/ajb.1400262](https://doi.org/10.3732/ajb.1400262) PMID: [25253707](https://pubmed.ncbi.nlm.nih.gov/25253707/)
56. Schneider H, Schmidt AR, Nascimbene PC, Heinrichs J. A new Dominican amber fossil of the derived fern genus *Pleopeltis* confirms generic stasis in the epiphytic fern diversity of the West Indies. Org Divers Evol, 2015; 15: 277–283.
57. Hartmann FA, Wilson R, Gradstein SR, Schneider H, Heinrichs J. Testing hypotheses on species delimitations and disjunctions in the liverwort Bryopteris (Jungermanniopsida: Lejeuneaceae). Int J Pl Sci 2006; 167: 1205–1214.
58. Wilson R, Heinrichs J, Hentschel J, Gradstein SR, Schneider H. Steady diversification of derived liverworts under Tertiary climatic fluctuations. Biol Lett 2007; 3: 566–569. PMID: [17686755](https://pubmed.ncbi.nlm.nih.gov/17686755/)
59. Willmann R. 1985. Die Art in Raum und Zeit. Berlin, Parey; 1985.
60. Willerslev E, Anders HJ, Binladen J, Brand TB, Gilbert MTB, Shapiro B, et al. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science, 2003; 300:791–795. PMID: [12702808](https://pubmed.ncbi.nlm.nih.gov/12702808/)
61. Grolle R. *Bryopteris bispinosa* spec. nov. (Lejeuneaceae), ein weiteres Lebermoos in Dominikanischem Bernstein. J Hattori Bot Lab 1993; 74, 71–76.
62. Heinrichs J, Gradstein SR, Wilson R, Scheider H. Towards a natural classification of liverworts (Marchantiophyta) based on the chloroplast gene *rbcL*. Cryptog Bryol 2005; 26: 131–150.
63. Crandall-Stotler B, Stotler RE, Long DG. 2009. Phylogeny and classification of the Marchantiophyta. Edinb J Bot 2009; 66: 155–198.
64. Frahm J-P. Neue Moosfunde aus Baltischem Bernstein. Limprichtia 2006; 29: 119–129.

65. Heinrichs J, Schmidt AR, Schäfer-Verwimp A, Gröhn C, Renner MAM. The leafy liverwort *Notoscyphus balticus* spec. nov. (Jungermanniales) in Eocene Baltic amber. *Rev Palaeobot Palynol* 2015; 217: 39–44.
66. Katagiri T. First fossil record of the liverwort family Cephaloziaceae (Jungermanniales, Marchantiophyta) from Baltic amber. *Nova Hedwigia* 2015, doi: [10.1127/nova_Hedwigia/2015/0276](https://doi.org/10.1127/nova_Hedwigia/2015/0276)
67. Feldberg K, Heinrichs J, Schmidt AR, Vá a J, Schneider H. Exploring the impact of fossil constraints on the divergence time estimates of derived liverworts. *Pl Syst Evol* 2013; 299: 585–601.
68. Söderström L, De Roo R, Hedderson T. Taxonomic novelties resulting from recent reclassification of the Lophoziaceae/Scapaniaceae clade. *Phytotaxa* 2010; 3: 47–53.
69. Urmi E. *Tetralophozia filiformis* (Steph.) comb. nov. in Europa. *J Bryol* 1983; 12: 393–401.
70. Vá a J. Taxonomic results of the BRYOTROP expedition to Zaire and Rwanda 11. Cephaloziaceae, Cephaloziellaceae, Gymnomitriaceae, Jungermanniaceae, Lophoziaceae. *Trop Bryol* 1993; 8: 99–103.
71. Schuster RM. Austral Hepaticae Part II. *Nova Hedwigia Beih* 119; 2002: 1–606.
72. Schuster RM. Revisionary studies of the Chandonanthoideae (Jungermanniales, Jungermanniaceae). *Nova Hedwigia* 2002; 74: 465–496.
73. Gustafsson L, Eriksson I. Factors of importance for the epiphytic vegetation of aspen *Populus tremula* with special emphasis on bark chemistry and soil chemistry. *J Appl Ecol* 1995; 32: 412–424.
74. Kuusinen M, Penttinen A. Spatial pattern of the threatened epiphytic bryophyte *Neckera pennata* at two scales in a fragmented boreal forest. *Ecography* 1999; 22: 729–735.
75. Cleavitt NI, Dibble AC, Werrier DA. Influence of tree species on epiphytic macrolichens in temperate mixed forests of northern Italy. *Can J For Res* 2009; 39: 785–791.
76. Király I, Nascimbene J, Tinya F, Ódor P. Factors influencing epiphytic bryophyte and lichen species richness at different spatial scales in managed temperate forests. *Biodiv Conserv* 2013; 22: 209–223.