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'Unfinished' Morphogenesis Hides Different Speciation Pathways in Charophytes: Evidence from the 190-Year-Old Original Material of *Chara denudata* (Charales, Charophyceae)

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Abstract: Several *Chara* L. species have 'unfinished' morphogenesis that is recognizable because of their imperfect stem and branchlet cortication compared to the perfectly corticated species. *Chara denudata* A. Braun, described from South Africa, is one of these species, assumed for a long time to be conspecific with *C. dissoluta* A. Braun ex Leonhardi, as described from Central Europe. An attempt to resolve this long-lasting uncertainty in the framework of integrative taxonomy is implemented here. The restudy of the original material of both species showed similarities but did not identify a hiatus in their morphological traits, which represents evidence for their placement in the subsection *Chara* R.D. Wood according to morphology. Bifid adaxial bract cells, a trait rarely encountered among charophytes, were found for the first time in *C. dissoluta*. According to the *rbc*L and *mat*K sequences, *C. denudata* was unexpectedly placed within the section *Grovesia* R.D. Wood, far from the clusters of the section *Chara* with *C. dissoluta*. This is in obvious disagreement with the position of *C. denudata* according to morphology. Both species were distinct according to their biology, habitat preference, and distribution and were accepted as distinct species. Therefore, the 'unfinished' morphogenesis resulting in morphological similarity hides different speciation pathways in charophytes.

Keywords: *Chara denudata*; *Chara dissoluta*; section *Chara*; section *Grovesia*; morphology; *rbc*L; *mat*K; oospores; ecology; integrative taxonomy

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1. Introduction

Charophytes are easily recognizable plants because of their typical morphology and the general habit arising from repeating more or less similar modules. Many entities described as species are still debatable and not clearly outlined in individual clusters of macrospecies, according to the species concept in the last world monograph available [1,2]. The wide species concept implemented within it has been almost abandoned in regional treatments of charophytes, but uncertainties and enigmas remain among more than 400 extant species described to date. Some species have transitional, intermediate forms, leaving no space for a clear hiatus between extreme variants of the visible 'continuum'.

The simplified morphology of some *Chara* L. species due to them not being fully developed, or, in other words, the incomplete cortication of individual internodes of the stem and branchlets, resulted in the descriptions of several species differing in the degree of cortex development. The species *Chara brionica* Stapf, *C. denudata* A. Braun, *C. disjuncta* Nordstedt, *C. dissoluta* A. Braun ex Leonhardi, *C. howeana* (Wood) Ling, Xie et Qiu, *C. imperfecta* A. Braun in Durieu, and *C. scepusienis* Fil. are the best examples of

these cases. Some of them have been known for a long time from small areas or from only a few localities. Their morphology can be recognized as a result of 'unfinished' morphogenesis because of their imperfect stem and branchlet cortication compared to species with the complete cortication of the stem internodes and branchlet segments. It has been hypothesized that this morphology originates from the different growth rates of internodal and cortical cells during plant ontogenesis. All species with imperfect cortication have 'counterparts' with perfect cortication, e.g., *C. contraria* A. Braun ex Kützing for *C. denudata* and *C. dissoluta*, *C. tomentosa* L. for *C. disjuncta*, *C. vulgaris* L. for *C. brionica*, *C. lipkinii* R.E. Romanov et al. for *C. imperfecta*, and *C. contraria* or *C. vulgaris* for both *C. howeana,* and *C. scepusiensis*, which can be easily linked to these species with impoverished morphology but only from a morphological perspective. Both morphology and distribution are good bases for doubts about their species' rank, raising questions about the reliability of these taxonomic decisions based on a limited number of studied plants whose morphology can be environmentally related.

The uncertainty associated with *C. denudata* and *C. dissoluta* [3,4] needs to be resolved in the framework of the ongoing project 'The European Charophyte Monograph,' which aims to cover all species known in Europe [5]. An integrative taxonomy, i.e., the combination of the results of morphological (both light and electron microscopy) and genetic studies with ecological and distributional traits, is the most fruitful for charophytes [6–13], although it is not helpful for all cases [14–16]. Here, we implemented a delineation between *C. denudata,* described from the shallow water bodies in South Africa, and *C. dissoluta,* described from the deep stratifying lakes in the Alps (for a long time, it was assumed that these were conspecific (cf. [17–22]; they were treated separately as forms, varieties or species from the subsection *Chara* R.D. Wood in only a few cases [1,2,4]).

2. Materials and Methods

2.1. Morphological Study

Specimens stored in the collections of the Komarov Botanical Institute of the Russian Academy of Sciences (LE), the Botanic Garden and Botanical Museum of Berlin-Dahlem (B), the Natural History Museum in Vienna (W), and the Papanin Institute for Biology of Inland Waters of the Russian Academy of Sciences (IBIW) and the living plants were studied at different magnifications without any treatment with the help of a Carl Zeiss Stereo Discovery V12 stereo microscope equipped with an AxioCam MRs-5 digital camera (Carl Zeiss AG, Oberkochen, Germany), stereo microscope Olympus SZ61, and microscope Olympus BH2 (Olympus Corporation, Shinjuku, Tokyo, Japan) equipped with Canon EOS80D digital camera (Canon Inc. Operations, Ohta-ku, Tokyo, Japan) and a Zeiss Stemi 305 stereo microscope (Carl Zeiss AG, Oberkochen, Germany), as well as a Dino–Lite Digital Microscope Pro (AnMo Electronics Corporation, Taiwan). Oospores taken from the original material of *C. denudata* (specimens LE A0001555 and LE A0001560) for scanning electron microscopy (SEM) were treated according to the method described by Romanov et al. [10]. The cleaned oospores were coated with gold and studied using a Zeiss EVO 40 scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany).

Studied Specimens

Chara denudata

Lectotype [2]: hand-written inscription at the envelope: 8847 *Chara denudata* Alx. Br./Cap./no date/Drege. Label: *Chara denudata* Alex. Br./Cap. d. g. Hoff[nung]. Strombergen, in einem Fläche u[nd] Niederung/no date [XII 1832]/Drège (W 0207500).

Isolectotype: *Chara* 8847/[South Africa] Cap. b. sp. [Caput bonae spei; "Stormbergen, in einer Fläche und Niederung, 5000–6000 Fuss" [23]]/no date [XII 1832]/Drege (LE A0001555).—The plants are notably infected with disc-shaped colonies of *Coleochaete* sp.—The fragments were taken for SEM studies.

Isolectotype: Herb. Acad. Petrop. 8847 = *Chara denudata* Al. Braun.—Vidi Drege Append. Floram 1843. p. 172/Africa australis [South Africa, "Stormbergen, in einer Fläche und Niederung, 5000–6000 Fuss" [23]]/no date [XII 1832]/Drege (LE A0001560).—The fragments were taken for SEM and genetic studies.

Isolectotype: *Chara denudata* A. Braun/not indicated [South Africa]/no date [XII 1832]/not indicated [Drège] (LE A0001559).—This envelope seems to harbor a fragment of one of the samples listed above and fragments of female plants of triplostichous *Chara* (*C. kraussii* A. Braun ex Kützing?), but plants of *C. denudata* looks really similar to plants from LE A0001555 and have the same infestation pattern with *Coleochaete*. Therefore, it could be recognized as a part of specimen LE A0001555.

[South Africa] Cap. b. sp. [Caput bonae spei] / no date [XIX century]/Zeyher; distrib. Drege 1847 (Linnaea. XIX. XX) / original label: 4650 (LE A0001881).—This specimen consists of mostly *Chara kraussii* (?).

Plants from Oman/Oman: Oman, Dhofar: lower Wadi Hinna between the road Salalah-Mirbat and Atair, along the brook with running water, alt. 100–130 m, 17°02′ N, 54°36′ E. *Chara* is forming dense submerse mats, together with the fern *Ceratopteris thalictroides*./4 X 1998/Leg.: P. Hein & N. Kilian PH 5255/Krause: Algae 38653/*Chara* sp., det. H. Korsch, 18 X 2012; *C. denudata*, det. R. Romanov, 15 III 2017 (B 40 0040768).

The same label, Leg.: Hein 5255/*Chara polyacantha* A. Br., det. W. Krause, IV 1999; Seems to be *C. socotrensioides* (R.D. Wood) R.D. Wood, det. R. Romanov, 19 IV 2018 (W 2015-03597).—This specimen, a duplicate of B 40 0040768, contains upper fragments of plants with somewhat elongated upper stipulodes but no traceable cortex tubes.

Plants from Yemen. P. Hein, S. Achmed, S. Bahan, S. Ghoufaili, N. Kilian, S. Mohamed & S. Saad/Yemen, gov. Al Mahra, coastal mountains between Al Faydami and Hawf, track W of Jadib to the plateau with *Anogeissus* woodland, near the spring "Ain Ayn", alt. 460 m, 16°38′ N, 52°57′ E.—Growing in shallow flowing water, with *Potamogeton natans*, *Chara* spec., *Samolus valerandii* and *Ceratopteris thalictroides*/23 XI 1999/Hein 6791/Seems to be *C. socotrensioides* (R.D. Wood) R.D. Wood, det. R. Romanov, 19 IV 2018 (W2015-03598).—This is a new species record for Yemen.

[Azerbaijan], Gusarsky Region [Qusar Region], settlement of Gaya-kend [Kayakend, Gayakend, ~41.407° N, 48.407° E], in water [it seems to be a river or small water body associated with river]/14 VI 1961/D.A. Aliev (LE).—This is a new species record for Azerbaijan and Caucasus.

Chara dissoluta

Lectotype [24]: [Switzerland] e lacu Neocomensi [Lake Neuchâtel, Neuenburgersee]/1854/Bulnheim (LE A0001500).—The plants are entangled with *Nitella syncarpa* (Thuill.) Chev.

[North] Makedonia, Lake Ohrid, north-western part, opposite of the town of Struga, depth 7 m/15 IX 2009/E. Chemeris/*Chara*, det. E. Chemeris (IBIW 54176).

[North Makedonia] Stenje, Prespasee, MK, N 40°56′13.5" O 20°56′35.0"/15 VII 2011/A.Ch. Mrkvicka/5086. Herbarium A. Ch. Mkrvicka. Rasterfeld Kart. Fl. MEur./*Chara* sp., det. C. Ch. Mrkvicka/*C. ohridana* (Kostić) Krause nom. inv., det. R. Romanov 19 IV 2018 (W 2011-03650).

Slovakia, Prešov District, Spišské Pohradie, together with *C. canescens* Loisel. (iNat ID: https://www.inaturalist.org/observations?place_id=any&taxon_id=486259&user_id=fero&verifiable=any; accessed on 2 January 2023) and *C. contraria* (iNat ID: https://www.inaturalist.org/observations?nelat=49.00694713768931&nelng=20.718710668085457&place_id=any&swlat=49.005975916243145&swlng=20.71712280034864&taxon_id=180685&user_id=fero&verifiable=any; accessed on 2 January 2023), 49.00636° N, 20.717796° E, 400 m a.s.l./27 X 2022/F. Bednár, iNat ID: https://www.inaturalist.org/observations/140705386; accessed on 20 December 2022. This is a shallow water depression in a meadow, where highly

calcified water runs from 4 active mineral springs forming travertine hill called Sivá Brada (Grey Beard) [25]. The travertine mound has a base diameter of about 500 m, a relative height of 25 m. This site was declared National Reserve in 1979. The location is managed, grazed, and mowed regularly.

Russia, Saint Petersburg, Kurortny District, vicinity of the settlement Molodyozhnoye, Gulf of Finland, at a spot with sparse vegetation sheltered from wind action with magnoliophytes, at sand, at the depth ca. 20 cm, together with *C. aspera* Willd. var. *subinermis* Kütz., rarely, 60.18919° N, 29.52842° E/27 VII 2020/R.E. Romanov (LE A0003221).—The fragments were taken for genetic studies. This is a new species record for Russia.

A map of distributional records was made with SimpleMappr (http://www.simplemappr.net; accessed on 20 December 2022) based on available records.

2.2. DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted as described previously by Echt et al. [26] with some modifications [27]. Part of the *rbc*L gene was amplified as described previously [12]. The PCR products were purified with ExoSAP-IT PCR Product Cleanup Reagent (Affymetrix Inc., Santa Clara, CA, USA) and sequenced in both directions using an ABI 3500 genetic analyser (Applied Biosystems, Waltham, MA, USA) with a BigDye terminator v. 3.1 sequencing kit (Applied Biosystems, Waltham, MA, USA). Sequences were assembled with Staden Package v.1.4 [28] and aligned manually in the SeaView program [29]. The *rbc*L and *mat*K sequences of *C. denudata* and *C. dissoluta* were deposited in GenBank under accession numbers ON502384 (*C. denudata*, LE A0001560, *rbc*L), OP919049 (*C. dissoluta*, LE A0003221, *rbc*L), OP946318 (*C. dissoluta*, LE A0003221, *mat*K), OP946319 (*C. denudata*, LE A0001560, *mat*K) (Supplementary Table S1).

2.3. Phylogenetic Analyses

The *rbc*L and *mat*K datasets were assembled as described by Romanov et al. [12,13]. Maximum likelihood (ML) analyses were carried out using PAUP 4.0b10 [30]. Bayesian inference (BI) was performed using MrBayes 3.1.2 [31]. To determine the most appropriate DNA substitution models for our datasets, we used the Akaike information criterion (AIC; [32]) was applied with jModelTest 2.1.1 [33]. The GTR+I+G and TVM+G models were selected as the best fits for the *rbc*L and *mat*K datasets, respectively. ML analyses were carried out using heuristic searches with a branch-swapping algorithm (tree bisection-reconnection). In BI, four parallel MCMC runs were carried out for 3 million generations. Sampling was carried out every 100 generations for a total of 30,000 samples. The convergence of the two chains was assessed, and stationarity was determined according to the 'sump' plot (the first 25% of the samples were discarded as 'burn-in'). The posterior probabilities were calculated from the trees sampled during the stationary phase. The robustness of the trees was estimated by bootstrap percentages (BP; [34]) in ML and posterior probabilities (PP) in BI. A BP < 50% and PP < 0.95 were not considered. An ML-based bootstrap analysis was inferred using the web service RAxML version 7.7.1 (http://embnet.vitalit.ch/raxml-bb/; [35]; accessed on 1 October 2022).

3. Results

3.1. Description of the Specimens of Chara denudata

The plants are evenly and moderately encrusted with lime and grow in clumps or mats, not as solitary shoots. The older ecorticate parts are infected with numerous discoid thalli of *Coleochaete* sp. Ring-shaped bulbils are sometimes formed at the lowermost nodes (Figure 1). They consist of two dense rows of more or less isodiametric rounded cells of (70)90–200 µm in length with starch inside. The stem cortex is nearly absent to incomplete. It consists of only narrow primary tubes, with wide empty spaces (furrows) between them. Their length varies from almost invisible short initials to more or less elongated tubes. The ends of the short tubes can be slightly peeled off the stem (plants from Oman). The most developed cortex, which is not a frequent case, always consists

of narrow primary tubes meeting in the center of the internode and having remarkably wide empty spaces between parallel tubes. The spine cells are solitary, small, and papillate and are barely recognizable. The stipulodes are bistipulate, diplostephanous, short, and obtuse, adpressed to the branchlets, and are up to 150 μm but are mostly much shorter than this. The cells in the upper rows can be slightly elongated, whereas, in the low rows, they can be hardly recognizable to almost invisible. The branchlets are ca. 10 in a whorl, somewhat or evidently longer than the internodes at the upper parts or from the near base of the plant up to the tip. They are sometimes several times longer, resulting, in this case, in the clasping of the branchlets from several apical whorls above the apex (plants from Oman). The branchlets consist of one–three ecorticate segments delineated with nodes and long, undifferentiated (or, in other words, nodeless) ecorticate three-celled end segments, comparable in length to differentiated part or longer. This can be broken at lower branchlets. Each or both segments can be abbreviated, constituting a negligible part of branchlet length (plants from Oman). Very narrow elongated initials of the branchlet cortex can be traced below the branchlet nodes in rare cases. The end cells of the branchlets are short, narrowly pointed, and neither acute nor mucronate. The adaxial (posterior) bract cells are short, bluntly conical, and mostly hardly recognizable. The abaxial (anterior) bract cells are cylindrical, narrowing mostly near their conical, shortly pointed tips, and are variable in length from two times shorter oogonia to several times longer than oogonia, slightly to evidently exceeding the length of adjacent branchlet cells (up to 2–2.3 times). The bracteoles are mostly and remarkably thinner and shorter than the adaxial bract cells. The plants are richly fertile, with solitary conjoined gametangia at one or two basal nodes of the branchlets. Oogonia can vertically geminate as an exception. The oospores are dark brown, with 11 or 12 low narrow ridges, together with (or without) a basal cage, having no empty spaces between the cage ribs (Figure 2). The oospore surface sculpture has two variants—granulated with prominent granules or roughened. The cage ribs can be irregularly flanged. Table 1 provides an overview of the measured morphometric data.

3.2. Description of the Specimens of Chara dissoluta

The plants are evenly and moderately encrusted with lime or completely incrusted, sparsely branching and growing in groups, being mostly lax to infrequently compact. The stem cortex is nearly absent to incomplete (Figures 3–5). It mainly consists of only narrow primary tubes, with wide empty spaces (furrows) between them. Their length varies from almost invisible short initials to more or less elongated tubes that are visible above and below the branchlet whorl base (plants from the Gulf of Finland—GF and Slovakia). The ends of short tubes can be peeled off the stem (GF and Slovakia). The short solitary spine cells and the round initials of the secondary tubes are recognizable in the case of the elongated cortex tubes (GF and Slovakia). The secondary tubes can be evidently elongated, but the empty spaces between the cortex tubes are typical in these cases too. Both the primary and secondary tubes are not regularly associated with stem (Slovakia). The most developed cortex consists of narrow primary tubes meeting in the center of the internode and having evident, empty spaces between the parallel tubes (typical of the lectotype (Figure 3) and found in plants from Slovakia). The spine cells are solitary, small, and papillate and are hardly recognizable. The stipulodes are bistipulate, diplostephanous, short, obtuse, and adpressed to the branchlets. The cells in the upper rows can be slightly elongated, whereas, in the low rows, they can be hardly recognizable to almost invisible.

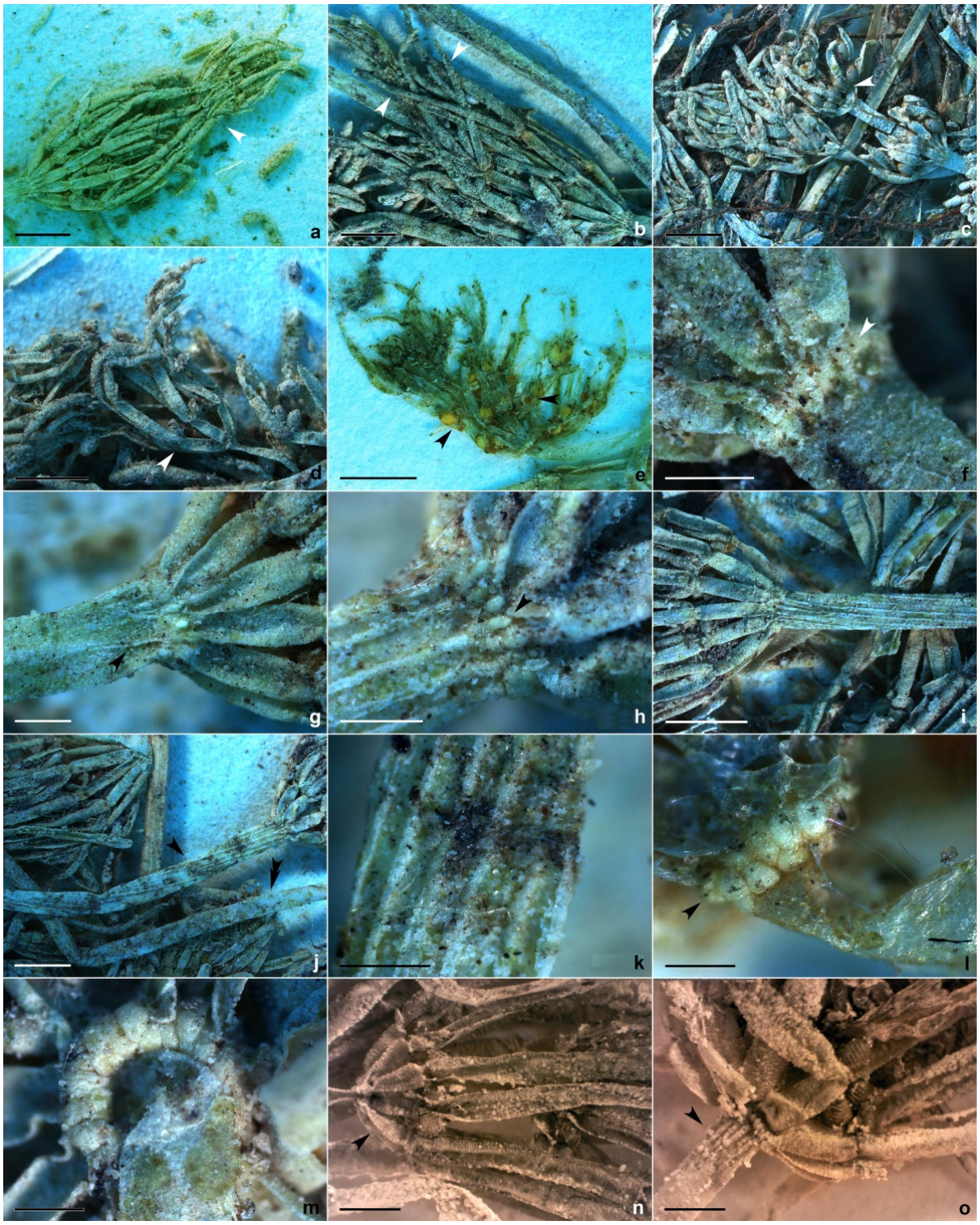

Figure 1. *Chara denudata*: (**a–m**)—the isolectotype from South Africa (LE); (**n**,**o**)—a specimen from Oman (B); (**a**)—apical part with branchlets having long adaxial bract cells, the arrowhead indicates the unbroken long undifferentiated ecorticate end of a branchlet; (**b**)—differentiated part of the branchlet, arrowheads indicate long adaxial bract cells; (**c**,**d**)—apical part with short (**c**) and very short (**d**) adaxial bract cells, arrowheads indicate fertile branchlet node; (**e**)—upper part of plant after decalcification with acid and subsequent pressing, probably implemented by Hollerbach, arrowheads indicate conjoined gametangia; (**f**)—base of branchlet whorl, arrowhead indicates hardly recognizable

short stipulodes; (**g**)—base of branchlet whorl and stem section below it with narrow elongated incompletely developed primary tubes of the stem cortex (arrowhead); (**h**)—base of branchlet whorl and stem section below it with narrow elongated primary tubes of the stem cortex with wide empty spaces between them, the arrowhead indicates slightly elongated diplostephanous stipulodes; (**i**)—base of branchlet whorl and stem section below it with narrow and long primary tubes of the stem cortex with wide empty spaces between them; (**j**)—stem sections showing narrow and long primary tubes of the stem cortex with wide empty spaces between them (arrowhead) and ecorticate internode (double arrowhead); (**k**)—stem sections showing narrow and long primary tubes of stem cortex with wide empty spaces between them; (**l**,**m**)—base of branchlet whorl with a ring-shaped nodal bulbil consisting of cells with starch grains (arrowhead at l); (**n**)—base of branchlet whorl with abbreviated ecorticate basal segments (arrowhead) below single fructifying branchlet nodes and long undifferentiated ecorticate branchlet segments; (**o**)—base of branchlet whorl and stem section below it with narrow elongated primary tubes of the stem cortex slightly peeling of the stem (arrowhead) with empty spaces between them; (**a**,**b**,**f**–**k**)—LE A0001560; (**c**–**e**,**l**,**m**)—LE A0001555; (**n**,**o**)—B 40 0040768. Scale: (**a**–**e**,**i**,**j**)—2 mm, (**f**–**h**)—0.5 mm, (**k**,**m**)—0.4 mm, (**l**)—0.2 mm, (**n**,**o**)—1 mm. Photos by R.E. Romanov.

There are six–eight branchlets in a whorl (6(7?) in case of a lectotype), which are somewhat longer than the internodes at the upper parts of the plant and are 1.2–2.5 times shorter than the internodes in most parts of the plant. The branchlets consist of one–three ecorticate or corticated segments that are delineated with nodes and long, undifferentiated (or, in other words, nodeless) ecorticate two–four-celled end segments, which are longer than in the differentiated part. It can be broken at lower branchlets if it is incrusted. The number of cells in the undifferentiated segment is inversely correlated with the number of segments. All segments are usually abbreviated (lectotype and GF), constituting a small part of the branchlet length. The branchlet segments of a lectotype are corticated, with the cortex unclearly appearing in a dry state having narrow, empty longitudinal spaces between the tubes. The tubes from the upper and lower nodes meet each other at the center of the segment or reach the base of the branchlet at the basal segments. The narrow and elongated or usually rounded initials of the branchlet cortex can be traced below the branchlet nodes in some cases but not at all nodes (GF). The specimens from Slovakia have an intermediate expression of this trait (Figure 4). They have round initials or long tubes of branchlet cortex with empty spaces between them, which neither meet each other at the center of the segment nor reach the branchlet base. The end cells of the branchlets are short or somewhat elongated, narrowly pointed, and not clearly mucronate, yet the base is usually clearly narrower than the tip of the penultimate cell. The adaxial (posterior) bract cells are short and bluntly conical to conical. The abaxial (anterior) bract cells are more or less conical, with bluntly pointed tips, and are variable in length: from 0.6 of the oogonium length to mostly slightly shorter to slightly up to two times longer, and are evidently shorter (lectotype) to mostly slightly shorter and nearly equal to the length of the adjacent branchlet cells (GF; Figure 5). The bracteoles are similar in appearance to the adaxial bract cells. The plants are poorly fertile with unripe gametangia (lectotype) to richly fertile with unripe gametangia and clear protandry (Slovakia), with ripe oospores (GF), with solitary conjoined gametangia at one–three branchlet nodes. The oogonia can be laterally geminate in rare cases (GF). The oospores are black. Table 1 provides an overview of the measured morphometric data.

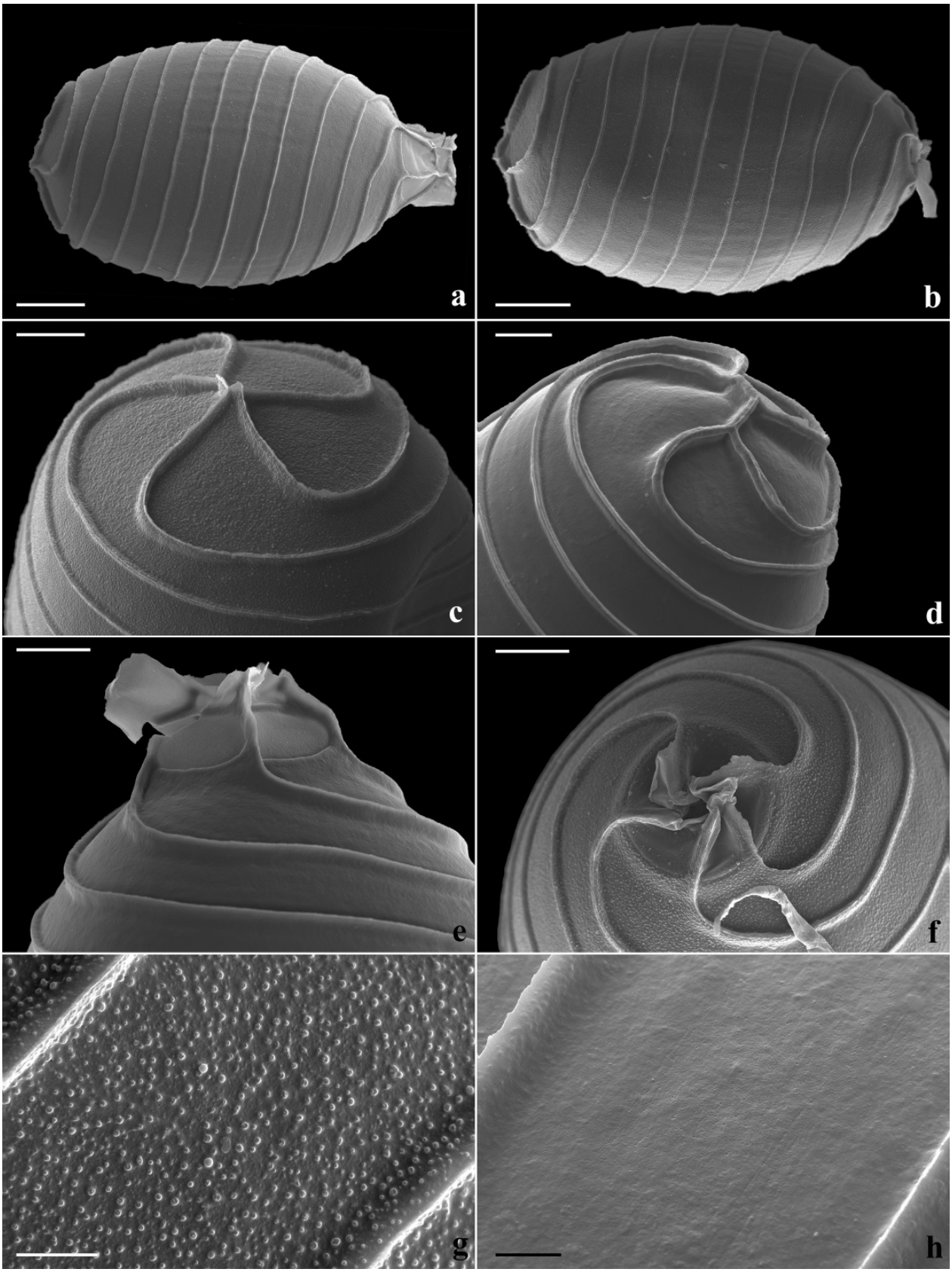

Figure 2. Oospores of the isolectotype of *Chara denudata* (LE), SEM: (**a**,**b**)—general appearance: (**a**)—oospore with basal cage having no open spaces between partly flanged ridges; (**b**)—oospore

without basal cage; (**c**,**d**)—apical parts of oospores with different expression of surface sculpture; (**e**,**f**)—basal parts of oospores: (**e**)—basal cage without open spaces between ribs; (**f**)—basal part without evident basal cage, without open spaces between basal claws; (**g**)—granulated surface of oospore fossa and ribs; (**h**)—roughened surface of oospore fossa with indistinct granulations at oospore ribs; (**a**–**c**,**f**,**g**)—LE A0001555; (**d**,**e**,**h**)—LE A0001560. Scale: (**a**,**b**)—100 µm, (**c**–**f**)—50 µm, (**g**,**h**)—10 µm. Photos by R.E. Romanov.

Table 1. Morphometry of the studied specimens of *Chara denudata* and *C. dissoluta*. All measurements were taken in a dry state from the pressed specimens of *C. denudata* (isolectotypes) and from the pressed (lectotype)/living plants of *C. dissoluta*.

Morphological Traits	Values	
	C. denudata	***C. dissoluta***
Length of plant, cm	7–8, 13–14	>20/10–11
Diameter of stem, µm	(486)513–945	453–757/243–392, 653–720
Diameter of nodal bulbil, mm	0.9–1.1	–/–
Length of branchlet, mm	7–13	12–33/6–14
Length of adaxial bract cell, µm	420–6380	(233)286–960/(91)455–887
Length of oogonium without coronula, µm	(456)651–810	(427)495–785/455–721
Width of oogonium, µm	419–513	(214) 256–486/(232)405–450
Length of oogonium coronula, µm	–/–	–/130–170
Width of oogonium coronula, µm	–/–	–/208–263
Length of oospore without basal cage, µm	538–723	–/486–587
Width of oospore, µm	350–423	–/233–328
Length of basal cage, µm	69–79	–/–
Diameter of antheridium, µm	(214)228–288(317)	209–428 / 243–500(645)

3.3. Phylogenetic Positions

The topologies of the ML and BI trees based on the *rbc*L dataset were similar to that of the BI tree, except for some differences in clade support (Figure 6). A total of 85 Characeae sequences were distributed between the 12 main clades. A sample of *C. denudata* placed with *C. tenuispina* A. Braun, with weak support (63 BP) in the subsection *Grovesia* R.D. Wood. This clade was part of a larger clade (composed of *C. tomentosa* L., *C. contraria*/*C. filiformis* A. Braun in Hertzsch, *C. globata* Mig., *C. canescens*/*C. altaica* A. Braun in A. Braun et Nordst., *C. lipkinii* Romanov et al., *C. aspera* Willd./*C. galioides* DC., *C. vulgaris* L./*C. gymnophylla* A. Braun, and complex clade (877/–), which included six identical sequences of six different species (*C. polyacantha* A. Braun in A. Braun, Rabenh. & Stizenb. nom. inv., *C. hispida* L., *C. intermedia* A. Braun nom. ill., *C. rudis* (A. Braun) Leonh. nom. ill., *C. baltica* (Hartm.) Bruzelius, *C. horrida* Wallman ex Wahlst.). The clades *Hartmania* R.D. Wood and *Chara* R.D. Wood were resolved as sister lineages (63/0.97).

Despite incongruent taxon sampling and marker length, the topology of the *mat*K tree was similar to that obtained with *rbc*L, and it resolved the same subsections and section clades with a comparable significance (Figure 7). *Chara denudata,* again, was a sister to *C. tenuispin*a, while *C. dissoluta* was placed distantly from it to *C. contraria*/*C. filiformis* subclade (82/–). Our *C. denudata* specimen had an identical *mat*K sequence to that of four European "*C. denudata*" accessions: *C. contraria*, *C. contraria* var. *hispidula* A. Braun, and *C. filiformis*.

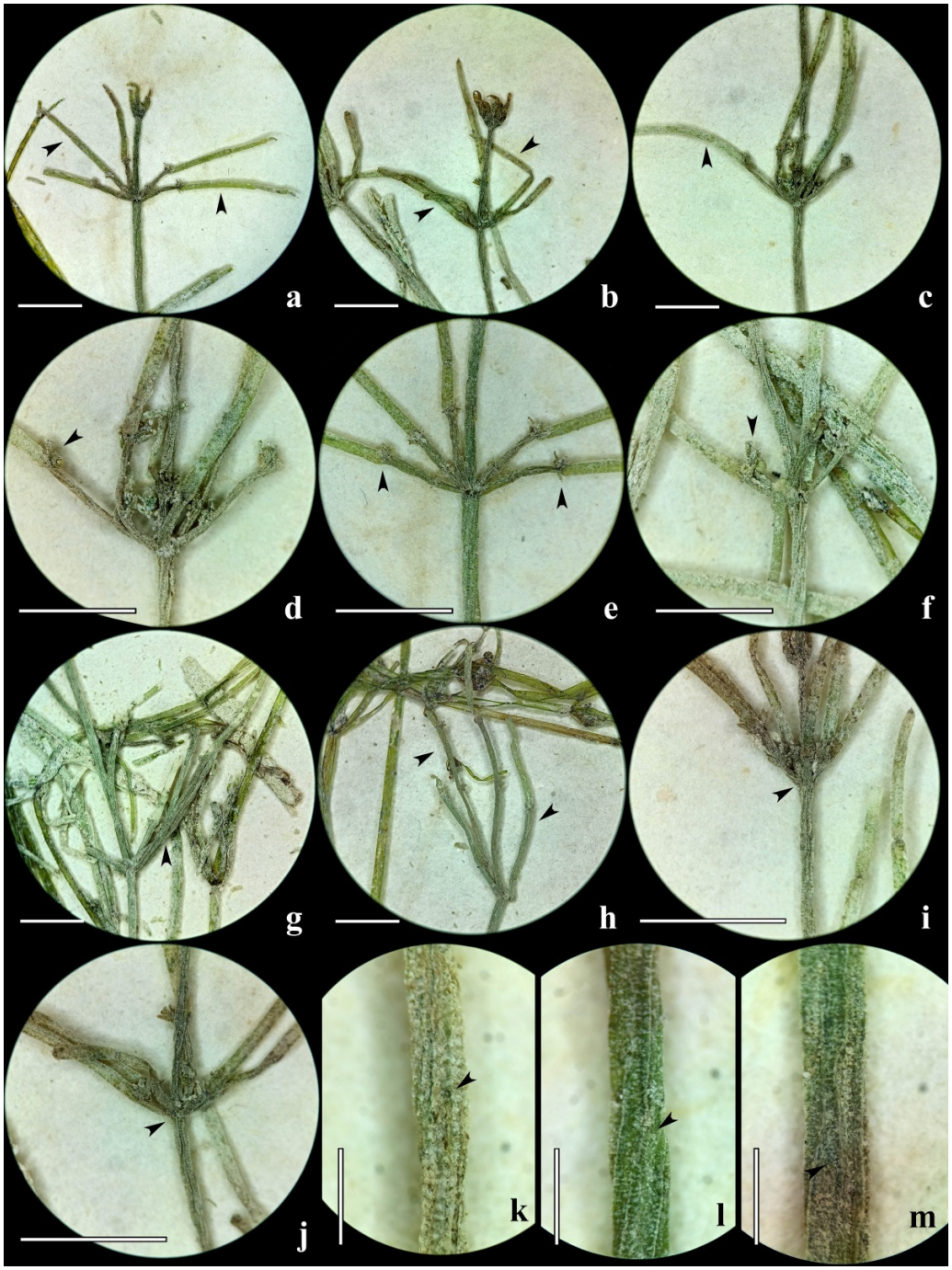

Figure 3. Lectotype of *Chara dissoluta* (LE A0001500): (**a**,**b**)—lax apical parts showing long ecorticate undifferentiated segments of branchlets (arrowheads); (**c**)—base of branchlet whorl, arrowhead indicates long ecorticate undifferentiated segment of branchlet; (**d**–**f**)—bases of branchlet whorls showing short corticated basal segments, short bracts and conjoined gametangia (arrowheads); (**g**,**h**)—bases of branchlet whorls showing long corticated basal segments (arrowheads); (**i**,**j**)—bases of branchlet whorls showing short hardly recognizable stipulodes (arrowheads); (**k**–**m**)—stem cortex, arrowheads indicate short barely visible spine cells (**k**) or empty spaces between primary cortical tubes (**l**,**m**). Scale: (**a**–**j**)—5 mm, (**k**–**m**)—1 mm. Photos by R.E. Romanov.

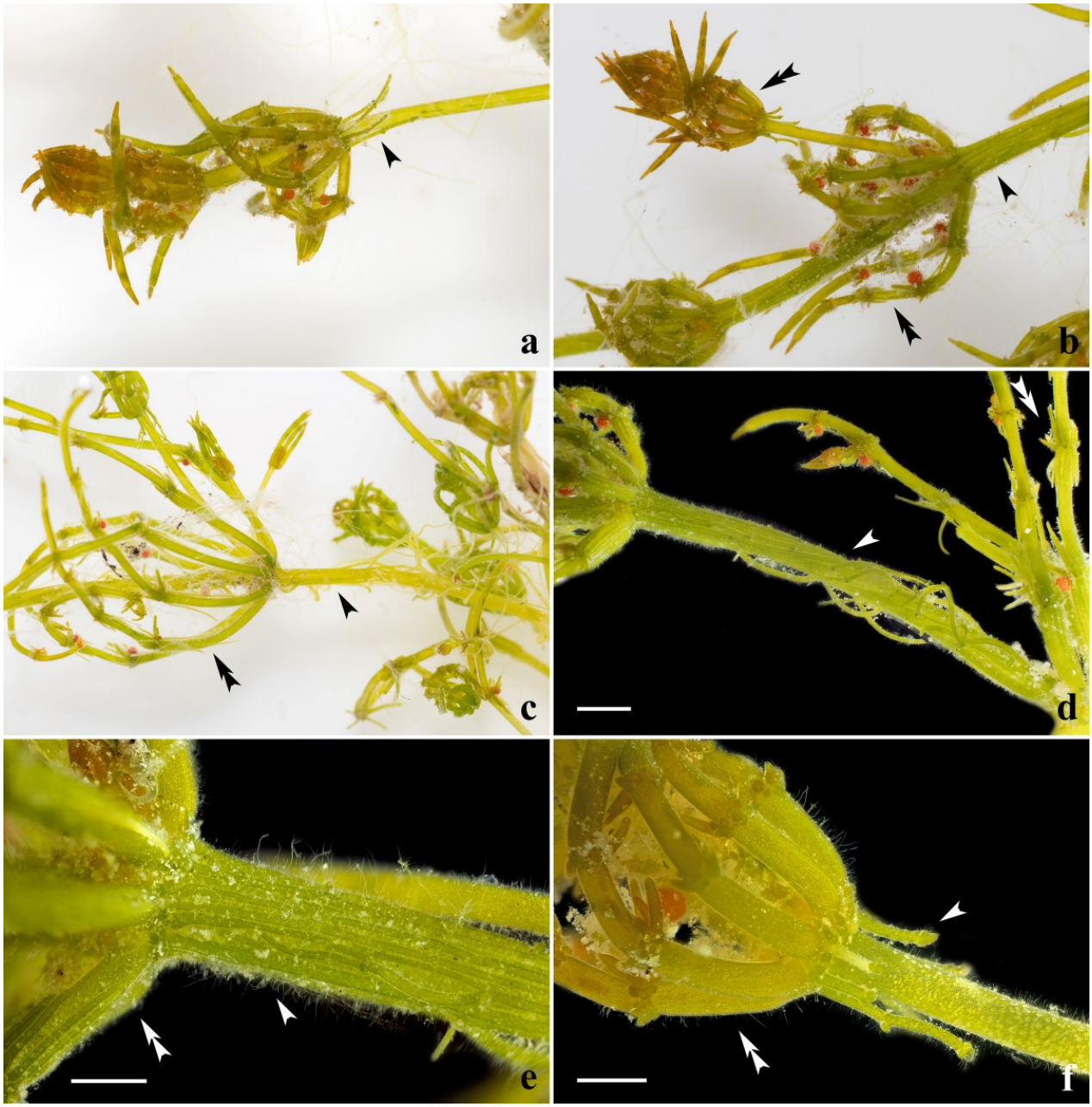

Figure 4. *Chara dissoluta* from minerotrophic wetland in Slovakia: (**a**,**b**)—apical and subapical parts showing short ecorticate undifferentiated segments of branchlets; (**c**)—middle part with arcuate branchlets and stem cortex peeling of the stem; (**d**)—stem cortex consisting of long primary tubes and short secondary tubes with empty spaces between them; (**e**)—base of branchlet whorl showing short stipulodes and cortication pattern of basal branchlet segment and stem; (**f**)—base of branchlet whorl with initials of branchlet cortex and short primary tubes of stem cortex peeling of the stem. Different length of tubes of stem cortex (arrowhead) and branchlet cortex (double arrowheads) is notable. Scale: (**d**,**f**)—1 mm, (**e**)—0.5 mm. Photos by F. Bednár.

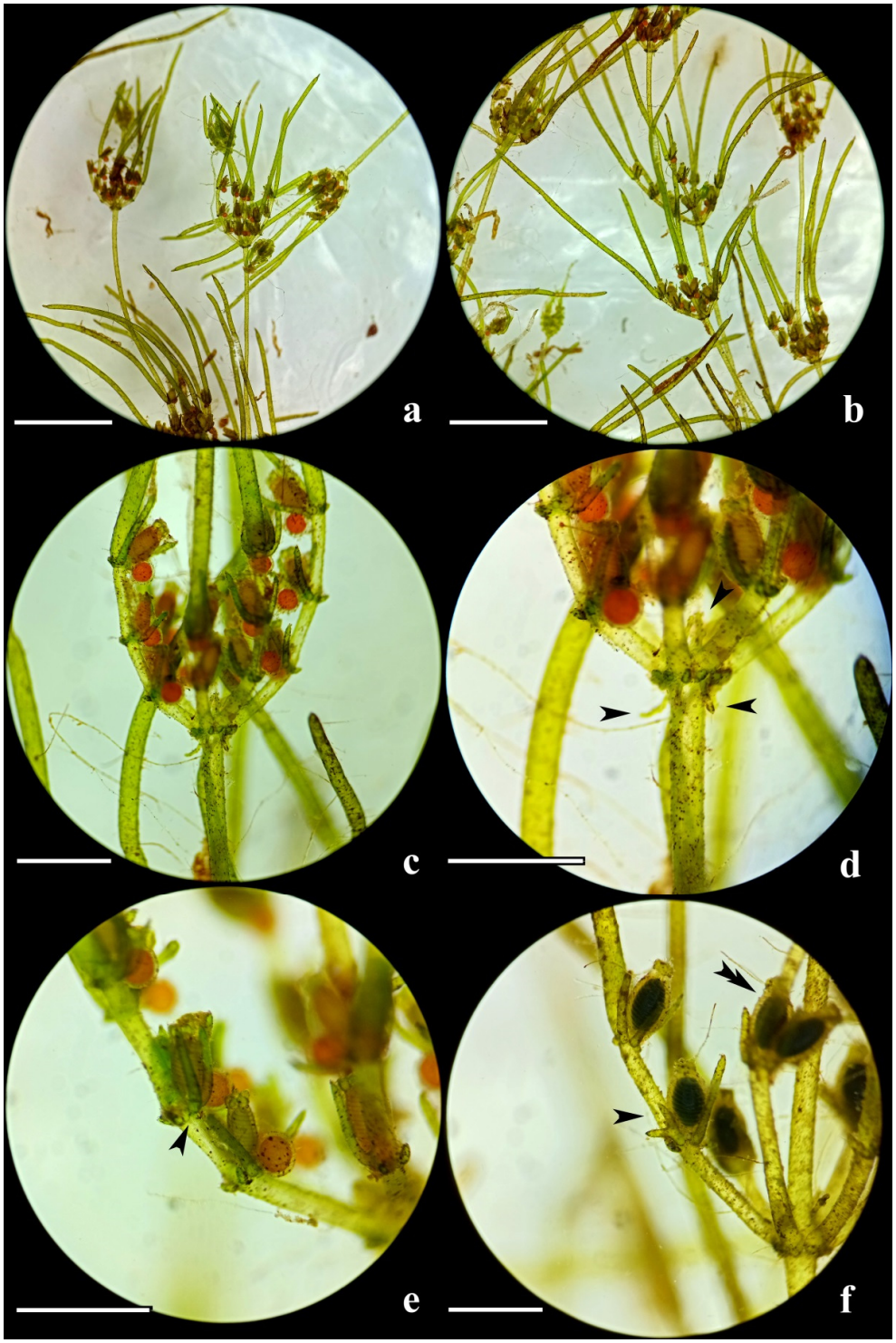

Figure 5. *Chara dissoluta* from the Gulf of Finland (LE A0003221): (**a**)—apical parts showing long ecorticate undifferentiated segments and abbreviated basal segments of branchlets; (**b**)—subapical part base of branchlet whorl, showing long ecorticate undifferentiated segments and abbreviated basal segments of branchlets; (**c**)—base of branchlet whorl showing short ecorticate basal segments, short bracts, and conjoined gametangia; (**d**)—base of branchlet whorl showing slightly elongated upper stipulodes, short primary tubes of stem cortex above and below of stem node peeling of the stem (arrowheads); (**e**)—branchlet nodes with conjoined gametangia, short bracts and short initial of branchlet cortex (arrowhead); (**f**)—branchlet nodes with ripe oogonia containing ripe black oospores, arrowhead indicates bifid bract cell. Scale: (**a**,**b**)—5 mm, (**c**–**f**)—1 mm. Photos by R. Romanov.

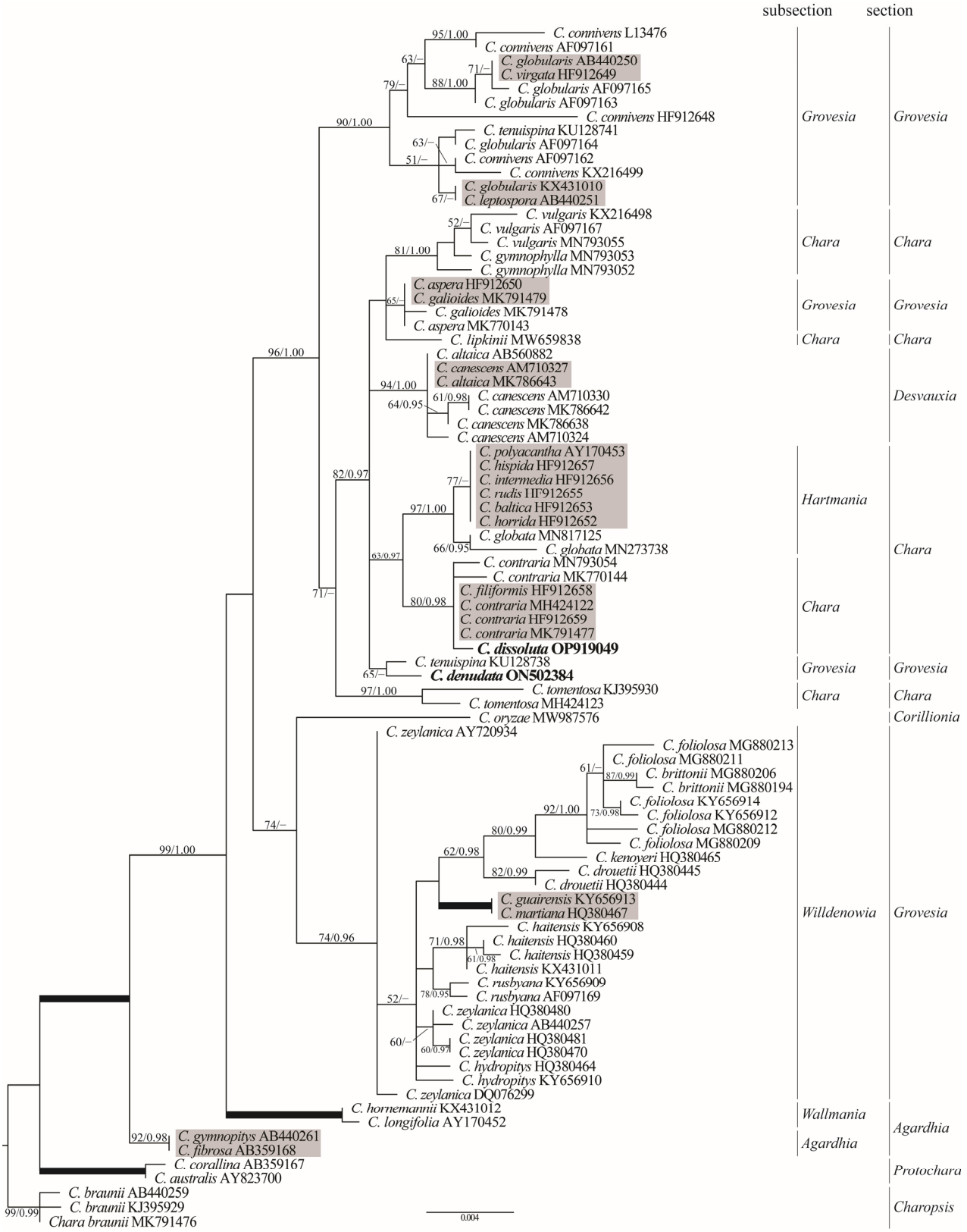

Figure 6. Maximum likelihood phylogenetic tree inferred in PAUP with the GTR+I+G nucleotide substitution model from 86 *rbc*L sequences of *Chara*. ML BP (>50%) and BI PP (>0.95). Branches received 100% BP and 1.00 PP support, and the newly obtained sequences are shown in bold. Sequences of the different species carrying one genotype are marked with grey. *Chara* sections and subsections are based on [2] with changes from [13,36].

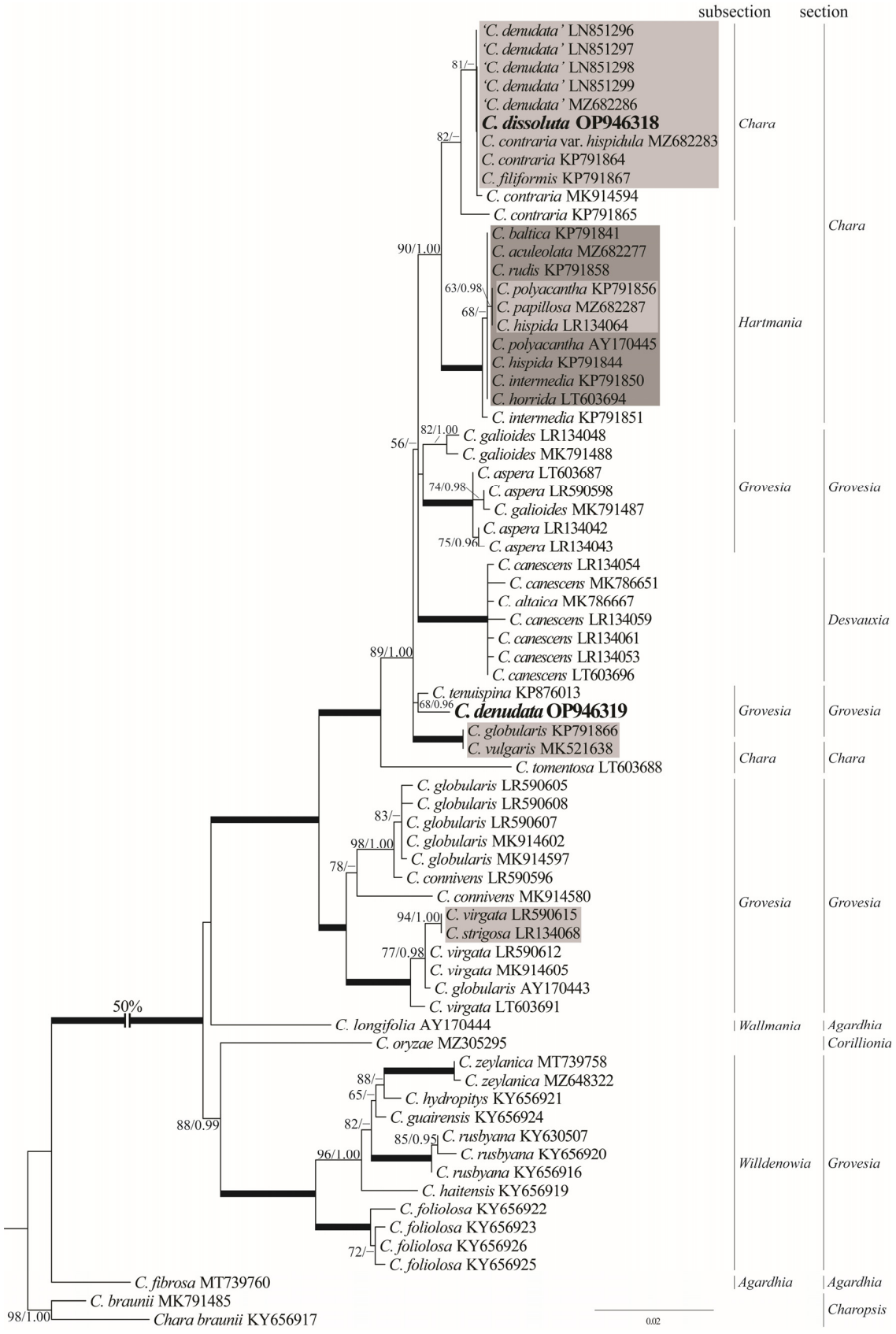

Figure 7. Maximum likelihood phylogenetic tree inferred in PAUP with the TVM+G nucleotide substitution model from 71 *mat*K sequences of *Chara*. One branch was reduced by 50% in length. See the Figure 3 legend for details.

4. Discussion

4.1. Morphological Perspective

The restudy of the original material of the charophyte species described many years ago allows for the testing and improvement of existing species concepts (e.g., [1,2,37–40]) and may serve as an essential keystone for checking and confirming species' distributional records. Integrative taxonomy is a fruitful approach, but its great age and sometimes a low amount of original material can limit the opportunities for adding new important data, e.g., nucleotide sequences and the scanning electron microscopy images of oospores. The storage of abundant original material in several collections can overcome these limitations, as was possible during this study.

The original material of *Chara denudata* was stored in at least three herbaria (B, LE, W). The part stored in B seemed to have been lost during the Second World War. The lectotype was selected by Wood [2] based on a specimen stored in W. Morphological descriptions and illustrations of plants from the original material, which has been published by several authors [1,2,4,17,41,42].

We believe that the three envelopes stored in LE and studied by us, namely LE 0001555, LE 0001559, and LE 0001560, belong to the original material of *C. denudata* and should be recognized as isolectotypes. They were collected by Drège in South Africa in December 1832 [23]. This material is not fragmented and is evidently more abundant and in better condition in comparison to the lectotype stored in W [2,4]. It revealed variability in plant arrangement, e.g., the length of the stem cortex tubes and the adaxial bract cells. Both parts were variable, from really short to invisible in the case of the stem cortex tubes, to very long within the same population.

The differences between the clumps of plants stored in LE 0001555 and LE 0001559 in comparison to ones from LE 0001560 were outlined, as the former consisted of more elongated, noncondensed plants with more elongated internodes, stem cortex tubes, and somewhat longer leaves with longer adaxial bract cells, which resulted in differences in the plant's general appearance. It may be recognized as variability within the same population, reflecting the differences in the microenvironment in the same locality.

The morphological studies of the original material of *C. denudata* [1,2,4,17,41,42] described less variability than we found during this study. Few descriptions of this species based on materials other than the original material are known from Oman [43].

The morphology of the European "counterpart," *C. dissoluta*, is well described and illustrated, relying on these references for comparison to *C. denudata*. Moreover, we implemented a restudy of a part of the original material of both species and some other specimens (LE, IBIW, W, and [24], this study). The great similarity in morphology between the European and South African plants is remarkable, and all differences that could be suggested in comparison to the original material for both species become insignificant if all ranges of variability that are described for the European plants are considered ([3,4,18–22,41,42,44–46]; see the discussion in [24]).

The compared species have different combinations of variable expressions of the same traits, resulting in their appearance as largely intergrading morphotypes. One morphological trait is remarkable. The adaxial bract cells of *C. denudata* were significantly longer than those of *C. dissoluta*. They were shorter, equal to, or somewhat longer than the oogonium length but were up to 2.5 times longer than the oogonium length in southeast-European populations of *C. dissoluta* [3,4,18–22,24,41,42,44–46]. The lower number of branchlets in the whorl is also notable, according to the original studied material (6(7?) vs. ca. 10), agreeing with some (e.g., [3,20,42]) but not all descriptions of the European plants. The size of the gametangia has overlapping values for the compared species, but *C. dissoluta* can produce smaller oospores and larger antheridia. The reliability of species delineation with the vertically geminating oogonia of *C. denudata* vs. the laterally geminating oogonia of *C. dissoluta* is unclear because of the rare occurrence of this trait in both cases.

A single case of bifid adaxial bract cell found in *C. dissoluta* from the Gulf of Finland is a rare morphological trait unknown for this species. A few examples of bifid cell occurrence

have been reported for charophytes. The bifid cells found among the bracteoles, bract cells, and spine cells of *C. aspera* Willd. [47,48] and *C. baltica* (Hartm.) Bruzelius [2], the bract cells of *Lychnothamnus barbatus* (Meyen) Leonh. [2,41] and *C. flaccida* A. Braun [49], and the branchlet end cells of *Nitellopsis obtusa* (Desv. in Loisel.) J.Groves ([49], the identification of *N. obtusa* needs confirmation) are exceptional cases. This trait can be recognized as a rare charophyte abnormality, at least for a few species, but we still do not know the true scale and causes of this phenomenon. The bi-tripartite or bipartite branchlet end cells of *Nitella bicornuta* F.S. Han et W.Q. Chen, *N. guangxiensis* Y.J. Ling, C.Z. Deng et Z. Li, *N. inaequipartita* L.B. Liang [50], *N. partita* Nordst. [2,50], the bifurcate bracteoles, and the adaxial bract cells of *C. kieneri* E.F.K. Daily [51] have been suggested as key traits for only these species.

The oospores of the plants from the original material of *C. denudata* have two types of oospore surface: granulated and roughened (Figure 2). The granulated variant is similar to the pattern described for the specimen of this species from Tanganyika Plateau, Tanzania, i.e., granulated and roughened on the same surface [52,53]. The oospores of *C. dissoluta* var. *ohridana* Kostić have been described as smooth using SEM [52], which coincides with our observations [24]. The morphologically similar dioecious *C. imperfecta* has a roughened and minutely perforate oospore surface [52,53], i.e., overlapping with the variant found by us for *C. denudata*. However, even two of the patterns found in this study seemed to be not unique and sufficient for delineation with other species based on this trait if only species of *Chara* studied with SEM are considered (e.g., [52–62]).

The plants of *C. dissoluta* are usually poorly fertile or sterile [3,4,18,20,22,41,42,44–46], in contrast to the richly fertile plants of *C. denudata*, although this delineation seems to be not so crucial in the case of the plants of *C. dissoluta* growing in the shallows of large lakes and water bodies that are associated with them in the Balkans [24] or in the shallows of the Gulf of Finland and the shallow water depressions formed with the discharge of active mineral springs in Slovakia (this study).

4.2. Phylogenetic Perspective

The successful amplification of partial *rbc*L and *mat*K sequences from a 190-year-old herbarium isolectotype specimen allowed us to assess the phylogenetic relationship of *C. denudata* with *C. dissoluta* and the rest of the genus. The overall topologies of our *rbc*L and *mat*K trees (Figures 3 and 4) were similar to those presented in the previous studies of the genus *Chara* [12–14] and generally congruently resolved the relationship between the species. According to the molecular data, *C. denudata* was placed in a large, supported assemblage that comprised sections *Grovesia* R.D. Wood, *Chara* R.D. Wood, and *Desvauxia* R.D. Wood and the traditional subsections of *Chara* R.D. Wood, *Hartmania* R.D. Wood, and *Desvauxia* R.D. Wood. *Chara denudata* was significantly positioned outside this lineage and showed close relationships with *C. tenuispina* in the subsection *Grovesia*. These species differed from each other in only one substitution in the *rbc*L sequence and eight substitutions in *mat*K. Thus, our molecular data fully confirmed the distinctness of *C. denudata* and its distant relationship with other species of the subsection *Chara*. Therefore, *C. contraria* belongs to another group only distantly related to the group of the species associated with *C. contraria*, including the European populations earlier referred to as *C. denudata*.

Our results conflict with the phylogeny presented by Schneider et al. [14]. These authors analyzed the phylogenetic relationships between 327 charophyte accessions (including four accessions of European plants of *C. dissoluta* placed in GenBank under the name of '*C. denudata*') using partial (518 bp) *mat*K gene sequences. According to their results, European *C. dissoluta* (under the name '*C. denudata*') was placed in the *C. contraria* cluster (*C. imperfecta*, *C. filiformis*, *C. ohridana* (Kostić) Krause nom. inv., *C. contraria*, *C. arcadiensis* [U. Raabe in] Schneider et al. nom. nud., *C. gymnophylla*) of the section *Chara*. This placement is in good agreement with [63] and mirrors the position of *C. dissoluta* in our tree. However, in the dataset studied by Schneider et al., '*C. denudata*' was repre-

sented in European plants only [14] (i.e., under the name of species not reliably known from Europe [24]), whereas our specimens (isolectotypes of *C. denudata*) were collected in South Africa. The *mat*K sequences of the *C. contraria* cluster also revealed limited genetic variation (identical for 17 individuals of three different species: *C. contraria*, *C. filiformis* and *C. dissoluta* placed in GenBank under '*C. denudata*').

We concluded that South African and European plants usually refer to *C. denudata* (before our study) actually belong to different sections and, therefore, should be treated as a separate species despite their morphological similarity. This decision about their difference was implemented in the monograph covering charophytes in Germany [4] based on a morphological study of a lectotype of *C. denudata*, a small, fragmented specimen. The same decision was accepted in the monograph about European charophytes [24], and this study provides thorough evidence for it according to an integrative taxonomy.

4.3. Ecological Perspective

The short description of the type of locality for *C. denudata* [23] allows only a tentative outline of the environment [64–66]. It was situated in the Stormberg Mountains in Eastern Cape, an arid region with very hot summers and very cold winters. It seems to be a temporary, seasonal, or permanent wetland fed by both subsurface and surface water, only subsurface water inputs, or surface flow only. It could be due to an ephemeral, seasonally flowing stream, spring, seep, or the rock pools associated with them or the temporary pools in the depressions (the latter is locally called vleis or pans). The water in this habitat seems to be rich in total dissolved solids because of its high evaporation. The other habitats of *C. denudata* known from other regions are streams, rivers, wadis, and stagnant water bodies with alkaline waters ([43,67,68], studied specimens from B, W). The ecology of *C. denudata* looks strongly dissimilar to that of *C. dissoluta*. The latter is exclusively freshwater and mainly deep-water species of strongly alkaline stable standing water bodies, mainly deep transparent lakes on marl and limestone in temporal regions [69], and, rarely, the smaller water bodies associated with them [24,70]. New localities in Slovakia and Russia are remarkable because they belong to new types of species habitats, a minerotrophic wetland in Central Europe, and the shallows of the easternmost freshwater part of the Baltic Sea. However, when the species are compared, they seem to be ecologically distinct. *Chara denudata* can be recognized as a shallow water species of arid regions; it is usually richly fertile. *Chara dissoluta* is mostly a deep-water species of deep, stratified, transparent, temperate lakes; it has mostly low fertility.

4.4. Distribution

All distributional records from Africa and Eurasia are clearly or tentatively attributable to *C. denudata* ([2,17,23,41,43,52,67,68,71,72], studied specimens from LE, B, and W) and *C. dissoluta* ([24], this study) are summarized on the map (Figure 8). This allowed for a preliminary outline of the possible distribution range of *C. denudata*. One record from Tanzania, Tanganyika Plateau [52], could not be located exactly without specimen checking because not enough label data have been published. Moreover, it could actually be another species, and the study of its voucher is desirable. Another locality from the Middle East, cited as Kurdistan [73], also seems to belong to this species but cannot be depicted on the map because no exact locality is known.

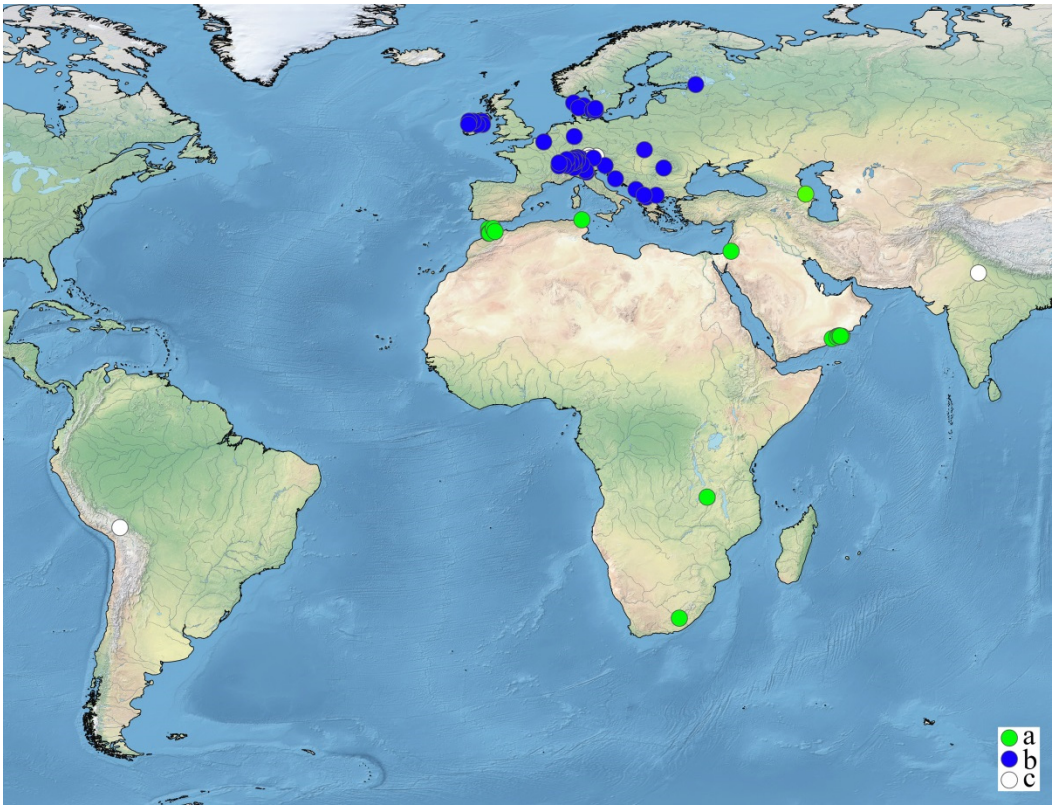

Figure 8. Distribution area of the records tentatively referring to *Chara denudata* in Africa and Asia ([2,17,23,41–43,52,67,68,71,72], LE, B, W, studied specimens), distribution area of *C. dissoluta* in Europe ([24], this study), and published records associated with these species [24,74–76]: a—*C. denudata*; b—*C. dissoluta*; c—published records associated with these species, restudy of their vouchers is required.

The records from Lake Titikaka, South America, are based on deep water plants [75,76] and could belong to another species. The record from India seems to also be based on another species because its specimens were described as having remarkably long spine cells [74] that are different from the minute, hardly recognizable spine cells of *C. denudata* and *C. dissoluta*.

The sterile specimens fitting the description of monoecious *C. denudata* are actually impossible to delineate from dioecious *C. imperfecta*. At least one specimen illustrating this case was studied by the first author: Pflanzen der Türkey/C5 Nigde. Bolkar Daglari, ca. 100 m östl. Kara Göl. Bachlauf, ca. 20 cm Wassertiefe; 2630 m, 37°25′ N 034°37′ E/05 VIII [19]92/E. Raab-Straube Nr. KG 2-7/*C. gymnophylla*, det. W. Krause (B, Algae 38518).

Therefore, nearly all distributional records referring to *C. denudata* are situated in the tropical and subtropical arid regions of Africa and Asia (Figure 8). This is evidently dissimilar to *C. dissoluta*, which is mostly known from the temperate regions of Europe [4,20,24,41]. The lack of successful hybridization between the distant populations of the same morphospecies of *Chara* from the subsections *Chara* and *Grovesia* [77–79] could be an additional argument to attribute the morphologically similar, but geographically far distant taxa *C. denudata* and *C. dissoluta* to different species.

5. Conclusions

The study of the original material of *Chara denudata* in the framework of integrative taxonomy allowed for the clarification of its position and found its unexpected placement in the infrageneric division of the genus *Chara* according to *rbc*L sequences, which is in contrast to its position according to morphology. This proved the suggestions expressing that *C. dissoluta* and *C. denudata* are different entities [1,2,4]. The similarity and large

overlap in the expression of the morphological traits of both species, as a result of the unfinished morphogenesis "stopped" at different stages, are making their clear separation impossible according to morphology only. It actually masks the different origins of *C. denudata* and *C. dissoluta*, as is evident from their phylogenetic affinity. This case indicates that the same incompletely developed or simplified morphology of charophytes could have been derived from different ancestors. There seems to be further evidence of a disagreement and the partial reflection of morphological infrageneric taxonomy of *Chara* with phylogenetic pattern [12]. Therefore, morphological similarity can lead to incorrect suggestions and taxonomic decisions, which can be avoided or at least softened in the framework of integrative taxonomy.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d15020249/s1, Table S1: Species name, GenBank accession number, and the haplotypes for the taxa used in our analyses. The sequences obtained in this study are in bold. Shared haplotypes are highlighted in yellow.

Author Contributions: Conceptualization, R.E.R.; methodology, R.E.R., S.S.B., V.Y.N. and A.A.G.; software, V.Y.N.; validation R.E.R., S.S.B., V.Y.N. and A.A.G.; formal analysis, R.E.R., S.S.B., V.Y.N. and A.A.G.; investigation, R.E.R., S.S.B., V.Y.N. and A.A.G.; data curation, R.E.R., S.S.B., V.Y.N. and A.A.G.; writing—original draft preparation, R.E.R., V.Y.N.; writing—review and editing, R.E.R., S.S.B., V.Y.N. and A.A.G.; visualization, R.E.R. and V.Y.N.; funding acquisition, R.E.R., S.S.B., V.Y.N. and A.A.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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