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Chara lipkinii (Charales, Charophyceae): a new dioecious Mediterranean species under risk of extinction in the wild and some implications for the taxonomy of the genus *Chara*

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Abstract: A new species, *Chara lipkinii*, was described based on specimens from the Mediterranean, Israel. Dioecy, 10–13 branchlets in a whorl, complete tylacanthous to isostichous displostichous axial cortex with very short solitary spine cells, hardly visible diplostephanous bistipulate short obtuse stipulodes, frequently appearing as haplostephanous, in combination with completely ecorticate branchlets without cortex initials and with 1–3, mainly two nodes with unilateral bract cells distinguish it from species of *Chara* described to date. Its morphological traits, which are most important in the infrageneric taxonomy described by Wood, point towards its placement in the subsection *Chara*, rather than its distant clustering with both *Chara* and *Grovesia*. The oospore surface of *C. lipkinii* showed no features allowing identification based on oospore morphology only, but could be useful to distinguish it from some species. According to our analyses, the genetic affinity of species based on rbcL and ITS1 mostly does not support infrageneric division of the genus *Chara* based on morphological traits. *Chara lipkinii* is a freshwater and brackish water species, growing in small shallow inland water bodies and streams in the Eastern Mediterranean, i.e., in one of the most threatened water bodies of the region. The species faces a very high risk of extinction in the wild. It has disappeared from all previously discovered localities, which no longer support charophyte populations. The gradual decline and disappearance of living plants from one recently discovered locality of *C. lipkinii* were observed as a result of heavy sediment accumulation and eutrophication. Urgent action to restore the habitat needs to be taken.

Key words: Characeae, Israel, morphology, oospores, new species, phylogeny, species protection

Introduction

Charophytes are one of the most threatened groups of plants, as illustrated by the available regional Red Lists where the majority of species are recognized as threatened or even regionally extinct (cf. Becker 2016). The most severe threats to their survival can be found in arid and semiarid regions due to the almost hopeless combination of continuous human transformation of the environment and unfavourable climate changes. During our efforts to clarify the taxonomy of Israeli charophytes, their past and present species distribution, and conservation issues, a new species was found. The incorporation of a new species into the existing infrageneric division is essential for its evaluation. It could also be used as a tool to test the suitability of both the applied species concept and infrageneric division. The taxonomy by Wood (1965) is the most recent one covering the whole charophyte flora. The obvious incongruence of morphological and phylogenetic affinity within some species groups from the genus *Chara* L. has already been described (Meiers et al. 1999; Schneider et al. 2016; Trbojević et al. 2020), highlighting the importance of genetic studies for each new species. Here we describe a new species from Israel using a polyphasic approach and outline some implications for the taxonomy of the genus *Chara*.

Materials and Methods

Sample collection and studied specimens. During 2001–2021, we surveyed numerous water bodies in 29 river basins of Israel, with an emphasis on previously known localities. Our sampling efforts resulted in records from 46 recent localities based on 234 observations of 14 species. The specimens of the species studied were collected in Israel in different seasons with permission from the Israel Nature and National Parks Protection Authority. Water pH, conductivity (EC), and TDS were measured using HANNA HI 9813–0. The concentration of $N–NO_3$ was measured using HANNA HI 93728. The specimens were studied in living and pressed states. The only recently identified locality of the new species was surveyed during different seasons in 2012–2021. The vouchers are deposited in LE, TELA (acronyms according to Thiers 2021) and in the Herbarium of the Institute of Evolution of the Haifa University (IoE).

We also studied the TELA collection assembled by Dr. Y. Lipkin. This consisted of 623 herbarium sheets, with vouchers for 163 observations from 114 localities in 1924–1992 for 18 species. Some specimens collected in Israel were found in LE, B, BM, and NY. We expect that there will be a small number of specimens collected in Israel in other herbaria and hope to find them in the future. The 33 herbarium sheets containing specimens of the new species from 10 localities and stored in TELA and 30 new herbarium sheets storing more than 100 plants were studied. The 40 plants were measured for dimensions. The numerous specimens of other species from the Mediterranean and other regions were checked for comparison with this species.

Studied specimens. 1. Akko Plain, Qishon: [74 m a.s.l.], Nahalal, small stream 1 m wide, 09 September 1971, Y. Lipkin, as *C. aaronsohnii*, det. Y. Lipkin (TELA: 20538–20540).
2. Coastal Plain, central: 3 km south of Netanya, [34 m a.s.l.], seasonal Dora pool, 05 July 1967, Y. Lipkin as *Nitella translucens* (Pers.) C. Agardh, det. Y. Lipkin (TELA: 20063).
3. Coastal Plain, Hadera: Caesarea, [22 m a.s.l.], drainage canal near main road to Haifa, 22 February 1971, Y. Lipkin, as *C. aaronsohnii*, det. Y. Lipkin (TELA: 20400, 20402).
4. Coastal Plain, Hadera: entrance to Caesarea east of Haifa, [17 m a.s.l.], Khirbet Hudeidun drainage canal, ca. 50 cm deep, slow stream, 27 May 1970, Y. Lipkin, as *C. aaronsohnii*, det. V.W. Proctor (TELA: 20371, 20372).
5. Coastal Plain, Hadera: near Pardes Hanna, Binjamina road, [18 m a.s.l.], Nahal Ada, on muddy bottom of the stream 40–50 cm deep, rich population, 28 February 1967, Y. Lipkin, as *C. aaronsohnii*, det. V.W. Proctor (TELA: 20066–20068).
6. Coastal Plain, Hadera: source in Wadi Ara region, [21 m a.s.l.], Ein–El–Kasb [pool], 22 November 1966, Y. Lipkin, as *C. aaronsohnii*, det. V.W. Proctor (TELA: 10803–10805).
7. Coastal Plain, northern: near the Haifa–Tel Aviv highway, [26 m a.s.l.], Nahal Barqan [river], 26 April 1972, Y. Lipkin, as *C. aaronsohnii*, det. Y. Lipkin (TELA: 20658).
8. Upper Galilee, Dishon: [458 m a.s.l.], Dishon Reservoir, 23 May 1968, E. Cohen, as *C. aaronsohnii*, 2083 – det. Y. Lipkin, as *C. aaronsohnii*, 2083 – det. Y. Lipkin (TELA: 20139–20141, 20143, 20150, 20151, 20153–20155; x–135). A voucher of a cultivated plant for crossing experiments by V.W. Proctor originating from this site (x–135) is stored in BUT under the name *C. ecklonii* A. Braun ex Kützing (https://macroalgae.org/, catalog No. 501143). There are no obvious differences with other specimens of *C. lipkinii* collected by E. Cohen and studied by us.
9. Lower Jordan, Bet Shean valley: Neve Eitan, [234 m b.s.l.], artificial reservoir, 07 February 1970, E. Cohen, as *C. galilaea*, det. Y. Lipkin (TELA: 20413–20416; x–369).
10. Lower Jordan, Bet Shean valley: 2 km south of Bet–Shean (near Khavat Eden), [121 m b.s.l.], large artificial pool resembling fish pond, 07 May 1971, V. Proctor, Y. Lipkin, as *C. galilaea*, det. V. Proctor (TELA 20441–20444, 20446).
11. Paratypes: Strawberry Eye Pool – type locality (see below), 30 July 2015, S.S. Barinova (LE: A0000326, IoE: 2962); the same locality, 25 March 2017, S.S. Barinova (LE: A0000327, IoE: 2999, 3000); 22 April 2017, S.S. Barinova (LE: A0000328, IoE: 3002); 20 May 2017, S.S. Barinova (LE: A0000329, IoE: 3005).

Morphological studies. Photos of living specimens were taken with a stereomicroscope Leica MZ 6 with Digital Camera OMAX A35180U3 and Dino–Lite Digital Microscope Pro. Oospores were prepared for scanning electron microscopy (SEM) as described by Romanov et al. (2015). The cleaned oospores were stored in 95% alcohol. They were coated with gold and studied using a ZEISS EVO 40 scanning electron microscope. The gametangia and oospore dimensions were measured in rewetted dried–pressed specimens. For practical convenience in the morphological description, we use "undifferentiated segment" for the node–less end part of the branchlet. The number of cells in a branchlet was counted irrespective of node presence. The isopolarity index for oospores was calculated as the largest polar axis length/largest equatorial diameter × 100. Map of records were made with SimpleMappr (http://www.simplemappr.net) according to the labels of studied specimens.

DNA extraction, amplification and sequencing. Total genomic DNA was extracted from the holotype, isotype, paratype (LE) and some old specimens (TELA) following Echt et al. (1992) with some modifications (Kiselev et al. 2015). Five attempts for sequencing of abundant old specimens in good condition were unsuccessful. The rbcL gene was amplified and sequenced in two fragments, using the following primer pairs for PCR: rbcL–RH1 (Zurawski & Clegg 1987) and rbcL–972R for the 5'–gene fragment; and rbcL–295F (Karol 2004) and rbcL–1379R (Pryer et al. 2001 with modifications) for the 3'–fragment in a T100 Thermal Cycler (Bio–Rad Laboratories, Inc., USA). The PCR cycling profile included an initial step of 3 min at 95 °C, followed by 38 cycles of denaturation at 95 °C for 20 s, 20 s of annealing at 49 °C, and 1 min at 72 °C, with a final extension at 72 °C for 5 min. The ITS1 region was amplified using newly designed Charales–specific primers: ChITS–1F (5'–CTGCGGAAGGATCATTGACAC–3') and ITS–IRm (5'–TTCTGCAAWTCRCATTGAGTRTCGC–3'; Hall et al. 2011 with modifications). The PCR cycling profile for this region included denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, elongation at 72 °C for 1 min and a final extension step at 72 °C for 5 min. The PCR products were purified by ExoSAP–IT PCR Product Cleanup Reagent (Affymetrix Inc., USA) and sequenced in both directions at the Instrumental Centre of Biotechnology and Gene Engineering of FSCEATB FEB RAS using an ABI 3500 genetic analyzer (Applied Biosystems, Maryland, USA) with a BigDye terminator v. 3.1 sequencing kit (Applied Biosystems, USA) and the same primers as used for PCR. Sequences were assembled with the Staden Package v.1.4 (Bonfield et al. 1995), and aligned manually in the SeaView program (Galtier et al. 1996). The sequences were submitted to GenBank under the accession numbers MW659838–MW659842 and MW659843–MW659847, for rbcL and ITS respectively (Supplement). Genotype identification was conducted with DnaSP 5.10 (Librado & Rozas 2009).

Phylogenetic analyses. Phylogenetic trees were inferred with ML–optimality criteria using PAUP 4.0b10 (Swofford 2002) and Bayesian inference (BI) with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). To determine the most appropriate model of nucleotide substitution, the Akaike information criterion (AIC) was applied with jModelTest 2.1.1 (Darriba et al. 2012). The GTR+I+Γ and HKY+Γ models were selected as the best fits for our rbcL and ITS1 data sets respectively. ML analysis was done using heuristic searches with a branch–swapping algorithm (tree bisection–reconnection).

In BI, two parallel MCMC runs were carried out for 3 million and 500 thousand generations, sampling every 100 generations for a total of 30000 and 5000 samples for the *rbc*L and ITS1 data sets respectively. Convergence of the two chains was assessed, stationarity was determined according to the 'sump' plot (the first 25% of samples were discarded as "burn–in"), and the posterior probabilities were calculated from the trees sampled during the stationary phase. The robustness of the trees was estimated by bootstrap percentages (BP; Stamatakis et al. 2008) in ML and posterior probabilities (PP) in BI. BP < 50% and PP < 0.95 were not considered. ML–based bootstrap analysis was inferred using the web service RAxML version 7.7.1 (http://embnet.vitalit.ch/raxml–bb/; Kozlov et al. 2019).

Results

Descriptions of species

***Chara lipkinii* R.E. Romanov, S.S. Barinova, V.Yu. Nikulin et A.A. Gontcharov sp. nov. (Figs 1–21)**

=*C. aaronsohnii* unedited name without description and comments, in herbarium

=*C. galilaea* Lipkin et Proctor unedited name without description and comments, in herbarium

Species description: Plants dioecious, without obvious sexual dimorphism, weakly to mostly moderately and strongly encrusted with lime, light green (Fig. 3) to light yellowish–brownish–green at well insolated tips (Figs 1, 4), scarcely branched, growing as individual shoots, having a decaying basal part without rhizoids and obvious nodal bulbils, up to 35 cm length. Sometimes plants unencrusted, becoming completely flat during pressing. Axis (295–)310–560 μm in diameter, appearing smooth to the naked eye. Axial cortex diplostichous, tylacanthous (Fig. 6), but usually tylacanthous to isostichous in the same plant and sometimes in parts erroneously recognized as isostichous triplostichous because of the narrower tubes due to more branchlets in a whorl in combination with inconspicuous spine cells (Figs 6, 13, 14). The secondary tubes sometimes override each other, producing short narrow longitudinal stripes of irregular diplo–triplostichous stem cortex (Fig. 14). Spine cells are solitary, short, several times shorter than the stem diameter, papillose, sometimes completely unrecognizable, very rarely slightly elongated and inclined, narrowing to the narrow–rounded ends (Figs 1–9, 13, 14).

Stipulodes diplostephanous (Fig. 15), short to mostly rudimentary and almost unrecognizable, ellipsoid, obtuse or frequently slight narrowing to narrow rounded ends to conical elliptic, usually hardly visible, especially lower row and in these frequent cases appearing haplostephanous or very rarely as having an incomplete lower row, rarely with slightly or somewhat elongated up to 443 μm length, pointed to very narrow rounded end (Figs. 1–9, 13, 15).

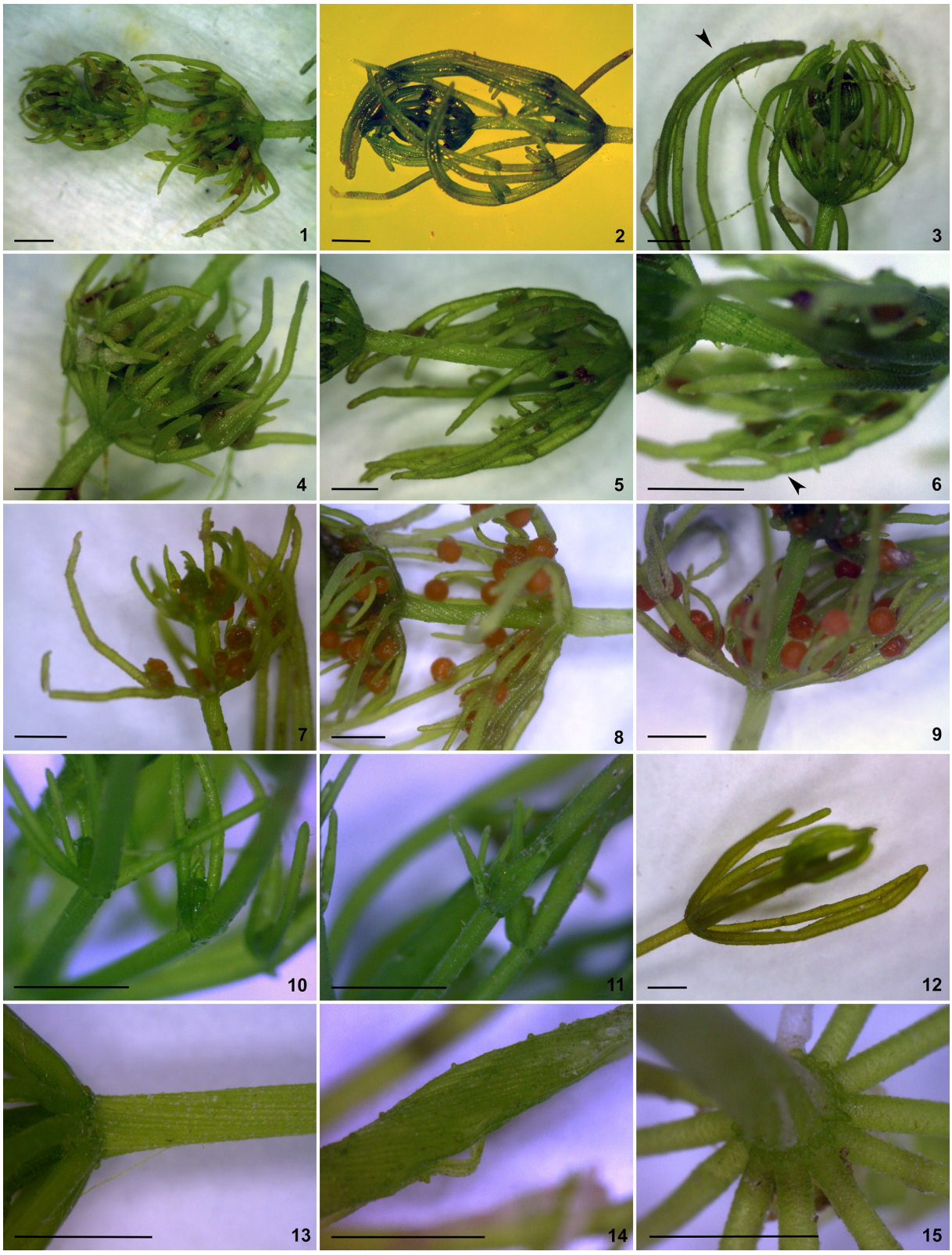
Branchlets 10–12(–13) in a whorl, 0.5–2.8 cm length, up to 5 cm in elongated plants but in these cases the longest branchlets are undifferentiated, 0.4–1.5–times longer than the internodes, always completely ecorticate, spread and more or less curved, but usually somewhat arcuate and sometimes even connivent above the apex (Figs 2, 3), consisting of one or mostly two nodes (Figs 1–9), very rarely of three nodes (Fig. 6), ecorticate cells between them and base of the whorl (segments) and undifferentiated end segment. The latter is 2–3–celled when there are two or three nodes and 3–4(–5)–celled in the case of a single node at the branchlet. Therefore, a branchlet consists of 4–6 but only five visible cells in most cases. The differentiated part of a branchlet is (0.22–)1/3–2/5–½–2/3 of its total length. The differentiated part is a negligible part of the elongated branchlets. The branchlets are rarely all undifferentiated in a whorl (Fig. 12) and undifferentiated branchlets are more frequent in the case of elongated plants with long branchlets.

The basal cell of the undifferentiated segment is the longest, almost equal to the last segment of the differentiated part or longer (Figs 1–7). End cell short, conical, narrow bluntly pointed, neither mucronate, nor obviously confluent with the base of the penultimate cell due to slight constriction between them and sometimes an obvious difference in the width of the end cell base with penultimate cell tip, not elongated, not curved (Figs 1–7). Gametangia on separate plants, at the first or mostly at the first and second nodes, solitary (Figs 1–9). Exceptionally the oogonia and antheridia can be geminate but they are found extremely rarely in separate populations.

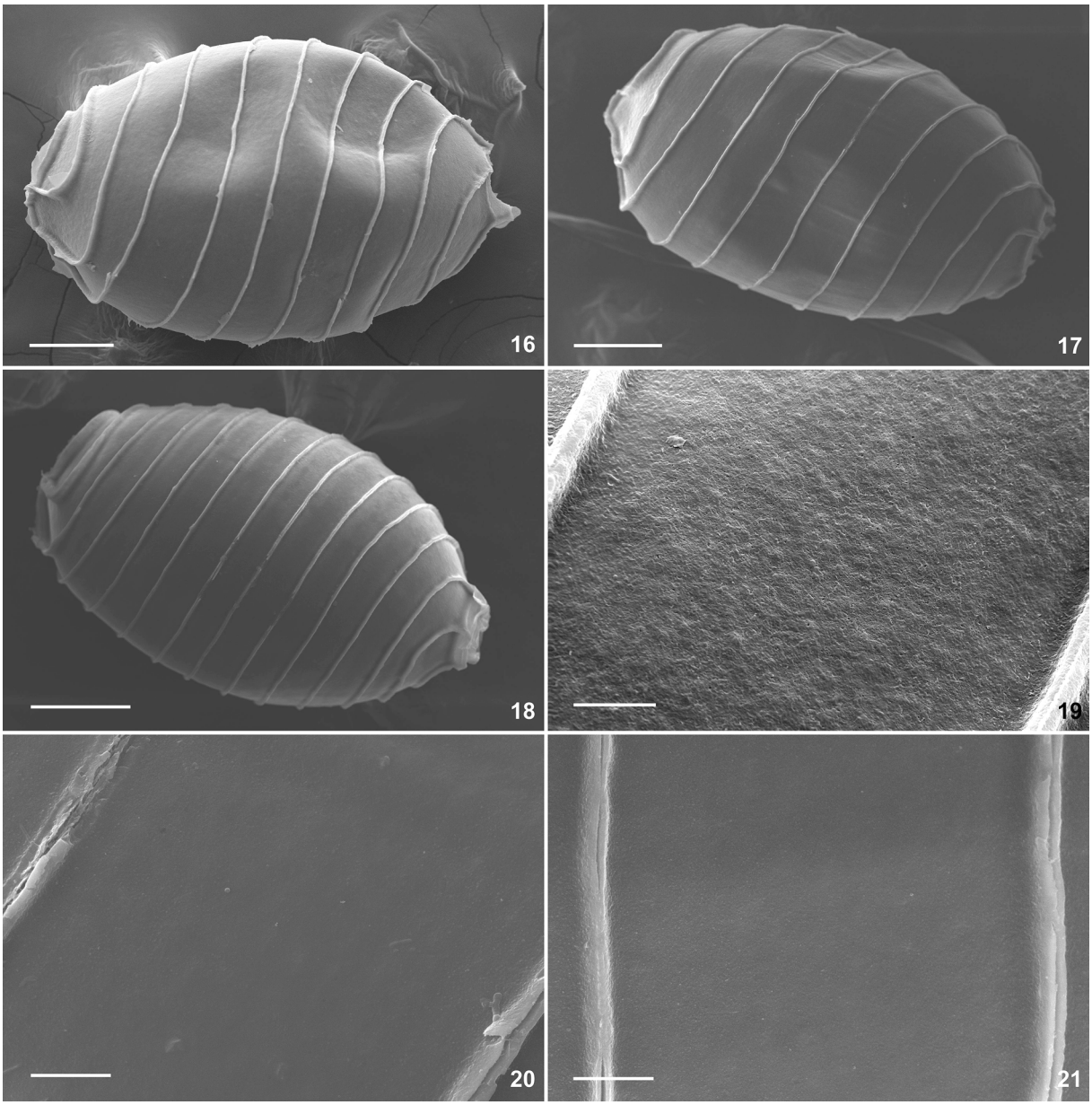
Bract cells always obviously unilateral; posterior bract cells rudimentary or very rarely short conical; anterior ones very gradually narrowing or almost cylindrical ending with narrow obtuse tips, frequently unequal in length, somewhat developed, 0.3–0.8 of the length of the conjoining cell of the branchlet to almost equal in length (Figs 1–11).

Male plants have two anterior bract cells only, no bracteoles could be traced (Figs 7–9). The branchlets of male plants look more "empty" in comparison with female ones because of the absence of bracteoles and usually shorter bract cells. Both anterior bract cells are more or less elongated, usually unequally, rarely with only one developed and its counterpart appearing as missing, 1/3–3/5 of neighbouring cell of branchlet, and 2–6–times, but frequently up to several times, longer or rarely obviously shorter than the antheridium diameter. Female plants have two unequal anterior bract cells, 1.4–6 to many times longer than the oogonium, 0.3–0.8 to 1.3–times longer than the neighbouring cell of a branchlet, up to 5 mm long in elongated plants, or shorter, sometimes poorly developed to the extent that they appear absent at lover magnification (Figs 1–6, 10, 11). The bracteoles present in the case of oogonium development are from almost equal to more or less shorter than the anterior bract cells. The bractlet is short or 2–2.8–4 to many times longer than the oogonium.

Oogonia ellipsoid, light green to light orange and brownish green if unripe (Figs 1–6, 10, 11), blackish if ripe due to translucent spiral cells, (479–)514–608(–626) μm length, 365–443 μm width, with 11 or 12 convolutions,



Figs 1–15. *Chara lipkinii* sp. nov. (holotype and isotype, LE): (1) shoot apex of female plants; (2, 3) shoot apices of female plants with connivent branchlets, arrowhead – elongated hook–shaped branchlets; (4) somewhat condensed whorl of branchlets, female plant; (5) less condensed whorl of branchlets, female plants; (6) tylacanthous axial cortex and branchlet with three nodes, arrowhead – third node; (7) shoot apex of male plant; (8, 9) lower parts of fertile male branchlets with different lengths of anterior bract cells; (10, 11) nodes of fertile female branchlets with different lengths of anterior bract cells and bracteoles; (12) whorl of undifferentiated branchlets; (13) base of the whorl with almost rudimentary stipulodes and part of stem with isostichous cortex with rudimentary spine cells; (14) slightly spiral almost isostichous stem cortex with papillate spine cells; (15) base of the whorl with short stipulodes. Scale bars 1 mm. All photos by S.S. Barinova.



Figs 16–21. The oospores of *Chara lipkinii* sp. nov., SEM: (16–18) general view of oospores, illustrating their variability; (19–21) different degree of surface ornamentation, from nearly smooth (20), slightly (21) to obviously roughened (19, isotype, LE); (16) type locality (isotype, LE); (17, 20, 21) Dishon Reservoir (TELA); (18) drainage channel near entrance to Caesarea (TELA). Scale bars 100 µm (16–18), 10 µm (19–21). All photos by R.E. Romanov.

coronula compact, not spreading, 61–78(–82) µm length, 365–436 µm width.

Oospores mostly black, rarely dark brown (unripe?), with 8–11(–12) low but distinct spiral ridges, (393–)407–560 µm length, (286–)300–350 µm width, isopolarity index (119–)126–163, without basal cage, with roughened surface in SEM (Figs 16–21). Gyrogonites not found.

Antheridia are round, brick orange, solitary, sessile, octoscutate MIN 322 µm, mostly 350–536 µm, AVG 453 µm, MAX 579 µm (n=131) in diameter (Figs 7–9).

Taxonomic remarks: *Chara lipkinii* differs from *C. conimbrigensis* Cunha, *C. contraria* A. Braun ex Kütz., *C. denudata* A. Braun, *C. dissoluta* A. Braun ex Leonh., *C. grovesii* Pal, *C. gymnophylla* A. Braun, *C. papillosa* Kütz., *C. subspinosa* Rupr., and *C. vulgaris* L. by its dioecy, from *C. canescentiformis* Hollerb. in Hollerb. et Krassavina, *C. denudata*, *C. dissoluta*, and *C. imperfecta* A. Braun in Durieu – with perfect cortication of the stem, from *C. aspera* Willd., *C. connivens* Salzm. ex A. Braun, and *C. galioides* DC. – with diplostichous stem cortex and ecorticated branchlets, and from *C. hereroensis* Nordst. – with diplostichous stem cortex, much smaller gametangia and oospores.

Holotype (designated here): Accession number LE A0000323, herbarium sheet deposited in the Herbarium of

the Komarov Botanical Institute of the Russian Academy of Sciences, Saint Petersburg.
Isotypes (designated here): Accession numbers LE A0000324, A0000325, herbarium sheets, Herbarium of the Komarov Botanical Institute of the Russian Academy of Sciences, Saint Petersburg, LE. Accession numbers 3007, 3008, Herbarium of the Institute of Evolution of the Haifa University. Collection numbers 3007, 3008, Herbarium of the Tel Aviv University, TELA.
Type locality: Israel, southern part of Carmel Mount region, Hagit Customer Forest Natural Reserve, south of Ein Tut interchange between highway 6 and road 70, Strawberry Eye Pool, elevation 121 m a.s.l., 32°36'40"N, 35°02'15"E, collected 10 June 2017 by S.S. Barinova.
Habitat: freshwater small permanent flowing pool.
Etymology: named in honour of Dr. Yaacov Lipkin (1935–2019), highlighting his outstanding efforts in the collection and preparation of charophyte specimens from Israel, stored in TELA.

Phylogenetic analysis
To assess the phylogenetic affinity of the new species, its rbcL and ITS1 rDNA sequences were obtained and compared these with all *Chara* sequences available in GenBank (February 5, 2020). Of the total of 348 rbcL sequences retrieved, only 207 sequences were longer than 900 bp and were retained in the dataset. These were further screened to eliminate redundant (>1 substitution) sequences. Identical sequences differing in length were also regarded as redundant. Different taxa having identical sequences were retained in the data set. The final rbcL alignment included 85 *Chara* sequences (39 species) representing 69 haplotypes (Supplement). Eight haplotypes of these were common for two or more species. The alignment was 1349 nt long, of which 87 characters were parsimony–informative.

In the unrooted tree *Chara* sequences were distributed between 13 major clades (Fig. 22). Nine of these were resolved in ITS1 sequence comparisons (I–IX; see below). Five identical *C. lipkinii* accessions formed a well–supported species clade (98/0.99) that was part of a larger clade (composed by *C. tomentosa* L. (VI), *C. tenuispina* A. Braun, *C. contraria* (I) / C. *filiformis* A. Braun in Hertzsch, *C. globata* Mig., *C. canescens* Loisel./ C. *altaica* A. Braun in A. Braun et Nordst. (V), *C. aspera* (VII) / C. *galioides*, *C. vulgaris* / *C. gymnophylla* (IV), and clade II (84/–), which included six identical sequences of six different species. *C. lipkinii* showed no affinity to any of these lineages despite generally supported relationships in their assemblage.

Chara was represented by only 19 ITS1 sequences in GenBank. Two of them had multiple sequencing errors (indents and substitutions) and were not included in the analysis. The final ITS1 data set consisted of 22 sequences (15 species). It was 960 nt long, of which 139 characters were parsimony–informative. Due to the limited taxon sampling, ITS analyses were less informative but we were able to establish the same nine clades (I–IX) described above (Fig. 23). Clade VIII, which was well supported by rbcL, was resolved as paraphyletic, and clade II, which comprised six species having identical rbcL sequences, was split into two clades (Fig. 23). *C. lipkinii* showed strong affinity (100/1.00) to clade II, comprised by identical *C. papillosa* (as *C. intermedia* A. Braun ex A. Braun, Rabenh. et Stizenb.) and *C. subspinosa* (as *C. rudis* (A. Braun) Leonh.) ITS1 sequences. The robust *C. contraria* / *C. filiformis* clade (I) was resolved as a sister to this lineage (94/0.99).

Species ecology and associated species
The type locality Strawberry Eye Pool is a small flowing pond created on a small stream in a natural area protected by the Israeli Ministry of Environmental Protection. Charophytes were growing in this locality during March to July 2015, 2017 and 2018 when its depth was up to 1 m. Their abundance was variable in different seasons, ranging from dense stands almost completely covering the bottom to sparse plants. Charophytes have not been recorded since August 2019, when the maximal depth of the pond decreased to 0.4 m and signs of eutrophication were apparent. During charophyte growth the water temperature was 18.6–24.9 °C, pH – 7.2–8.4, electrical conductivity – 0.66–1.02 $mSm.cm^{-1}$, total dissolved solids – 478–739 $mg.l^{-1}$, nitrate nitrogen content – (0.0–)0.3–1.7 $mg.l^{-1}$. In the type locality *Chara lipkinii* was growing together with *C. vulgaris*, Zygnemataceae, *Cladophora glomerata* (L.) Kütz., *Ranunculus* sp., and *Zannichellia* sp. In another 10 localities, i.e. small streams, drainage channels, artificial reservoirs and seasonal pool, it was found during February and November, mostly at the end of winter and during spring, but except at the type locality it was never sampled again in the same place. It was associated with *C. braunii* C.C. Gmelin, *C. contraria*, *C. globularis* Thuill., *C. vulgaris* and *Nitella mucronata* (A. Braun) Miq., but frequently seemed to be the only species of Characeae growing in water bodies surveyed by Y. Lipkin.

Discussion

Phylogenetic analysis and taxonomic implications for genus *Chara*
The combination of phenotypic traits of *Chara lipkinii*, i.e. displostephanous stipulodes and diplostichous stem cortex with solitary spine cells, suggests that the species should be classified in the subsection *Chara* as defined by Wood (1965). However, according to the molecular data this species showed no affinity to representatives of either the subsection *Chara* or the subsection *Grovesia*. It was actually placed between even higher infrageneric taxa, i.e. the section *Chara* (subsection *Chara*) and the section *Grovesia* (subsection *Grovesia*). In rbcL analyses *C. lipkinii* was part of the large supported assemblage that comprised the section *Chara* with the traditional subsections *Chara*, *Hartmania*, *Desvauxia*, and part

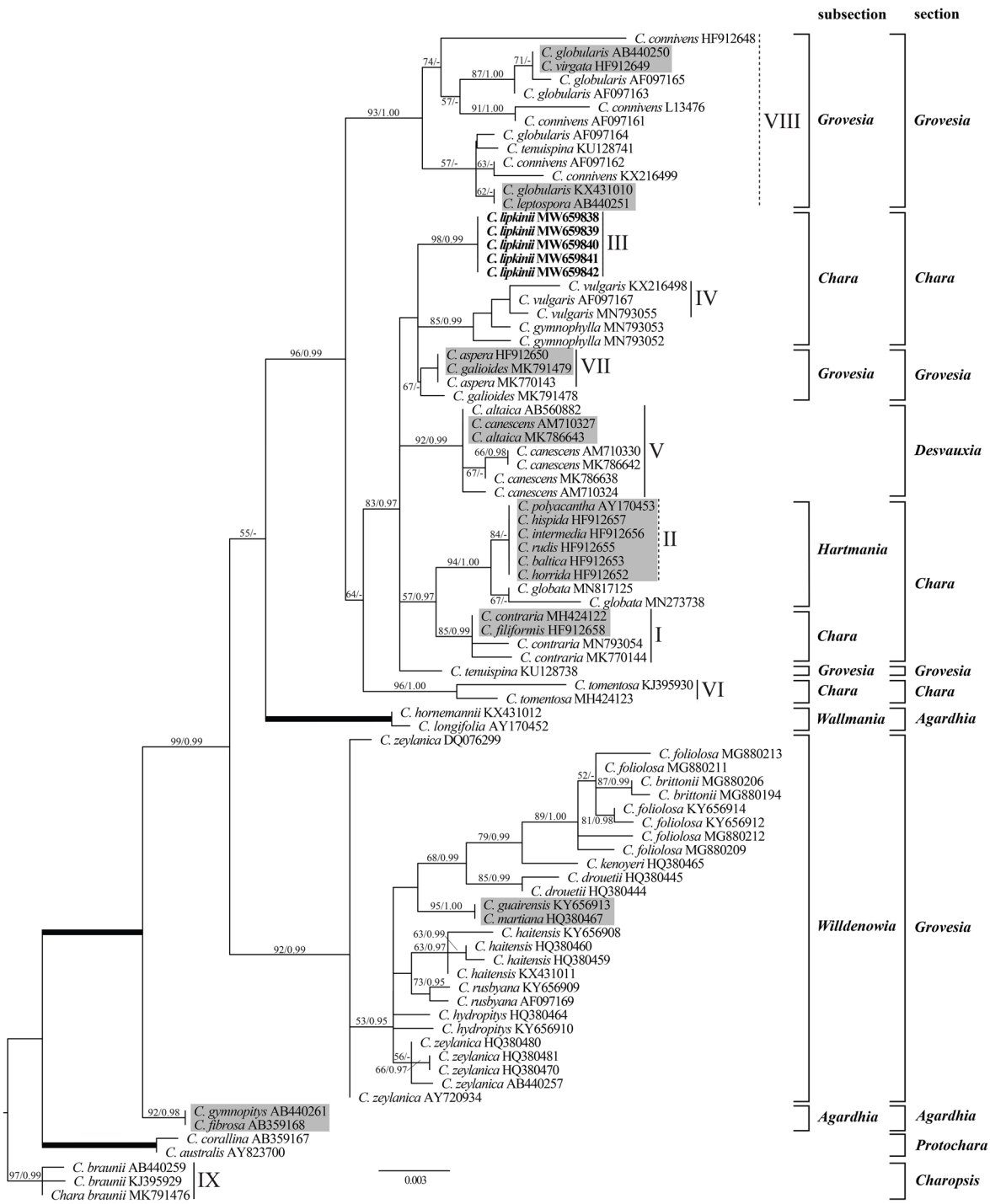


Fig. 22. ML–phylogenetic tree inferred in PAUP with GTR+I+Γ nucleotide substitution model from 85 rbcL sequences of *Chara*. ML BP (> 50%) and BI PP (> 0.95) are shown. Branches received 100% BP and 1.00 PP support and the newly obtained sequence of *C. lipkinii* sp. nov. is shown in bold. Sequences of different species carrying one genotype are marked with grey. *Chara* sections and subsections are according to Wood (1965) with changes (Casanova & Karol 2014). Roman numerals (I–VIII) denote clades resolved with rbcL and ITS1, the dotted line shows the clades not supported in the ITS analysis.

of the section *Grovesia,* i.e. members of the subsection *Grovesia* only, having rather unresolved relationships. Although our taxon sampling included only a small number of *Chara* species, we were unable to resolve the respective section and subsection clades. Moreover, nuclear (ITS1) and chloroplast (rbcL) markers were generally consistent in resolving only a distant relationship between representatives of the same sections (e.g. lack of affinity between the subsection *Chara* members *C. contraria*/*C. filiformis* (clade I) and *C. vulgaris* (clade IV) and the same pattern in the subsection *Grovesia* (Figs 22, 23)). To date no phylogenetic analyses have

supported morphology–based intrageneric *Chara* classifications (cf. Wood 1962; Wood 1965; and Meiers et al. 1999; Schneider et al. 2016); therefore, this result was not unexpected. The worldwide coverage of most species could allow testing and improvement of the morphology–based infrageneric classification of *Chara*. *Chara lipkinii* differs from other similar species with a tylacanthous cortex, in particular by showing no affinity to *C. contraria* according to the rbcL sequence, i.e. one of the most conservative molecular markers for charophytes. *Chara contraria* and *C. arcadiensis* [U. Raabe in] Schneider et al. nom. nud. are indistinguishable from each other based on matK sequences (Schneider et al. 2016), i.e. a more variable molecular marker. Therefore, it can be concluded that *Chara lipkinii* is not conspecific with the still undescribed *C. arcadiensis*. Interestingly, in ITS1 analyses *C. lipkinii* showed close affinity to clade II (subsection *Hartmania*) and clade I (subsection *Chara*) but these relationships could not be established with rbcL even topologically, although affinity with clades I and II was weakly supported (Fig. 22). The same sister relation between the *C. hispida* cluster (clade I here) and the *C. contraria* cluster (clade II) was supported in an analysis based on another chloroplast marker matK (Schneider et al. 2016).

It is interesting to note an incongruence in the structuring of the subsection *Hartmania* between rbcL and ITS1 (Figs 22, 23). Its species (clade II in rbcL analyses), which are among the most difficult to distinguish, were placed in two distinct clades by ITS1 – brackish water *Chara baltica* (Hartman) Bruzelius and *C. horrida* Wahlst. and freshwater *C. papillosa* (as *C. intermedia*) and *C. subspinosa* (as *C. rudis*). In contrast, in the rbcL analyses this subsection was monophyletic with only *C. globata* having no sequences identical to other species (Fig. 22). Non–monophyly of the subsection in ITS1 analyses is somewhat unexpected because earlier attempts to delineate its species with different molecular markers were not successful (Urbaniak & Combic 2013; Schneider et al. 2015, 2016; Nowak et al. 2016; Urbaniak & Sakayama 2017; Urbaniak & Kwiatkowski 2019). Therefore, the vouchers available for *C. baltica, C. horrida, C. intermedia,* and *C. rudis* should be checked to rule out possible misidentifications based on ITS1 and rbcL sequences.

Problems of delineation and identification

The combination of morphological traits distinguish *C. lipkinii* from all other species of *Chara* described to date (Table 1). Some morphological traits of *C. lipkinii* are more common in the subsection *Grovesia*, but they are not useful in the infrageneric taxonomy of *Chara* implemented by Wood (1965). The large number of branchlets in a whorl (10–13) typical for *C. lipkinii* is not a usual trait for species of the subsection *Chara*, although the number overlaps with the maximum numbers for some species (10, 11). This trait is more common for the subsections *Grovesia* and *Willdenowia*. The small number of nodes in a branchlet (1, 2, rarely 3) in our species is a relatively common trait for gymnophyllous

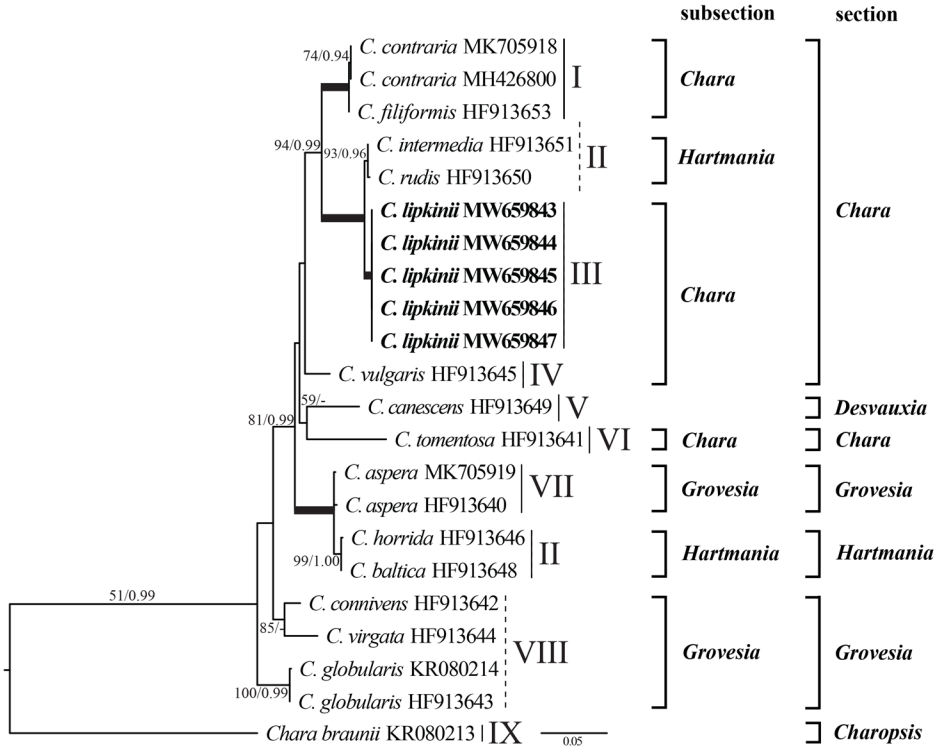


Fig. 23. ML–phylogenetic tree inferred in PAUP with HKY+Γ nucleotide substitution model from 22 ITS1 sequences of *Chara*. See the Figure 22 legend for details.

and subgymnophyllous species and forms from the subsection *Chara*. The hardly visible to almost unrecognizable stipulodes, with a frequently inconspicuous lower row, is typical for *C. lipkinii* and mostly known for some species from the subsection *Grovesia* and typical for the section *Protochara*. The length of the bract cells and proportions between differentiated and undifferentiated parts of the branchlet in combination with the axial cortex arrangement seem to be the most variable traits of this species. The size of the antheridia overlaps with values known for monoecious species from the subsection *Chara* but can be obviously bigger. This trait seems to agree with the general pattern known for dioecious species of *Chara*, which usually have bigger antheridia in comparison with monoecious species (Groves & Bullock–Webster 1920; Maszewski 1994).

In the sterile state dioecious *Chara lipkinii* could be expected to be indistinguishable from monoecious *C. conimbrigensis*, which has conjoined and sometimes partly sejoined gametangia and also occurs in the Mediterranean (Romanov et al. 2019). However, the sterile state of both species could only be observed in suppressed or juvenile plants at the beginning of their growth. Plants with a small number of gametangia growing in very shallow water should be studied very carefully to avoid misidentification. The general appearance of *Chara lipkinii* is similar to that of species of the subsection *Chara*, e.g. *C. gymnophylla*, *C. vulgaris*, *C. conimbrigensis* and *C. contraria* but showed little affinity to them in the molecular data analyses.

Plants from the artificial reservoir in Bet Shean valley (x–396, TELA) and from another locality, Dishon Reservoir (x–146, not found in TELA and at the Macroalgal Herbarium Consortium Portal, https://macroalgae.org/), were assigned by Proctor (1971) to dioecious species from the subsection *Grovesia* and were used for breeding experiments to test the biological species concept for charophytes. The plants from Dishon Reservoir (x–146) were described as large plants with both rows of stipulodes well developed (Proctor 1971), which does not fit the description of *C. lipkinii*. The specimens were interfertile but could not be crossed with plants from other populations of species from the subsection *Grovesia*. They have a chromosome number of 14, which could be accepted as suitable for *C. lipkinii*.

The oospore surface under scanning electron microscopy is a trait of primary importance for the taxonomy of *Nitella* because of its typical patterns for most species (Leitch et al. 1990; Sakayama 2008; Casanova 2009). It seems to be much less variable and sometimes overlapping between species of *Chara* or differing among populations of the same species (Leitch et al. 1990; Casanova 2005; Urbaniak 2011; Urbaniak & Blaženčić 2012; Urbaniak & Sakayama 2017; Romanov, unpubl. data), and, finally, it looks somewhat less useful for the taxonomy of this genus. *Chara lipkinii* has a roughened oospore surface that distinguishes it from *C. conimbrigensis*, *C. gymnophylla* and *C. vulgaris*, which have a pustulate to granulate oospore surface ((Ray et al. 2001; Urbaniak 2011; Romanov, unpubl. data). The oospores of some populations of *C. aspera, C. contraria, C. denudata, C. imperfecta* and even *C. vulgaris* were described as having the same pattern (roughened surface; Leitch et al. 1990; Romanov, unpubl. data), similar to that of *C. lipkinii*. Therefore, it can be concluded that the oospore surface of *C. lipkinii* cannot be used for identification based on oospores only, but can be useful to differentiate it from certain other species.

Fig. 24. The distribution range of *Chara lipkinii* sp. nov. according to the specimens studied (LE, TELA, IoE). The red / grey dots – previously known populations that have now disappeared, dark blue / black dot – recently discovered population.

Distribution

All currently known localities of *C. lipkinii* are in northern Israel, including only one recently discovered locality (Fig. 24). The species has been found in several regions of the country, namely Mount Carmel, Akko Plain, Coastal Plain, Upper Galilee and Lower Jordan. This pattern is consistent with the differentiation of the Israeli territory between the northern and southern areas according to species occurrence (Romanov & Barinova 2012; Barinova & Romanov, unpubl. data), reflecting sharp differences in the environment. *Chara lipkinii* is known from water bodies situated at 234 m b.s.l. up to 458 m a.s.l. i.e. strictly within the Thermo–Mediterranean and Meso–Mediterranean vegetation belts (Vargas 2020). Therefore, the occurrence of this species in the regions north of Israel seems possible. In addition, the distribution range of dioecious *C. lipkinii* could be restricted compared to most similar monoecious *C. conimbrigensis* and C. *gymnophylla*, which occur in different regions of the Mediterranean, according to the general pattern for dioecious and monoecious species of *Chara* (Proctor 1980).

Species protection issues

The continuous efforts made by S.S. Barinova have identified some new localities of charophytes in Israel and confirmed the loss of many of the previously

identified localities. We suggest that the variable distribution of charophytes in space and time is the result of the absence of large stable water bodies in this region (with the exception of Lake Kinneret, which has been unable to support them at all for a long time), the strong variability of the natural environment and long–term, persistent, severe human transformation of the environment and management of water bodies in a way that is inappropriate for charophyte protection, in combination with climate–driven aridification, which is more pronounced in southern Israel. This combination could be recognized as a somewhat hopeless situation for charophyte conservation in the region.

The localities of *Chara lipkinii* known previously have mostly disappeared according to surveys conducted by S.S. Barinova between 2012 and 2021. The Dishon Reservoir, Nahalal stream, Khirbet Hudeidun drainage channel do not exist anymore. The revealed but not studied yet Ein Ada pool in Samaria as well as Ada stream itself can be suitable for charophytes and will be surveyed in future. The drainage channel near the main road to Haifa could not be located. The fish pools in Neve Eitan and near Khavat Eden are undergoing regular reconstruction and charophytes cannot survive in them. The Nahal Barqan river, Ein–El–Kasb pool and seasonal Dora pool still exist but no longer harbour any charophytes according to numerous surveys, nor has its type locality since 2019. Any oospores buried in the bottom sediments and covered with rapidly accumulating thick new layers are the last opportunity for recovery of its population at the latter site. This might be possible because charophyte oospores can survive for a long time even after disappearance of the water body (Beilby & Casanova 2014; Stobbe et al. 2014; Alderton et al. 2017), but the number of viable oospores declines over time (Beilby & Casanova 2014). Therefore, according

Table 1. Differences with species similar in morphology or distantly related in rbcL analyses (clade VII; fig. 22) (a – Imahori 1964; Wood 1965; b – Hollerbach & Krassavina 1983; c – Hussain et al. 2003; d – Krause 1997; e – Romanov et al. 2019; f – own observations).

Similar species	Key difference with *C. lipkinii*	Distribution range	Reference
C. aspera	triplo– to diplo–triplostichous stem cortex, rhizoidal bulbils, aculeate stipulodes, spine cells and bracts, nearly verticillate bract cells, corticated branchlets, more nodes within a branchlet	Holarctic	a, b, d, f
C. canescentiformis	corticated branchlets, haplo–diplostephanous stem cortex and sometimes geminate (and even triplicate) spine cells	Central Asia	b, f
C. conimbrigensis	monoecy	Mediterranean	a, b, e, f
C. connivens	perfect triplostichous isostichous stem cortex, completely corticated branchlets, very short bract cells, more nodes within a branchlet	Palearctic	a, b, d, f
C. contraria	monoecy, corticated branchlets	subcosmopolite	a, b, d, f
C. denudata	monoecy, imperfect cortication of the stem	South Africa, Oman	a, c, f
C. dissoluta	the same	Europe	a, f
C. galioides	triplo– to diplo–triplostichous stem cortex, aculeate stipulodes, spine cells and bracts, nearly verticillate bract cells, corticated branchlets, more nodes within a branchlet	Mediterranean	a, b, d, f
C. grovesii	monoecy		a
C. gymnophylla	monoecy, aulacanthous stem cortex	typical of the Mediterranean	a, b, f
C. hereroensis	irregular, mostly triplostichous, stem cortex, acute stipulodes, short bract cells, bracteoles longer than the anterior bract cells, much bigger oogonia, oospores and antheridia	Namibia	a
C. imperfecta	imperfect cortication of the stem, peeling of the stem, geminate gametangia	West Mediterranean	a, d, f
C. vulgaris	monoecy, corticated branchlets, aulacanthous stem cortex	cosmopolite	a, b, d, f

to the IUCN criteria (IUCN 2012) *Chara lipkinii* can be recognized as critically endangered. Habitat destruction and eutrophication are the main threats for the species.

The protection of a single recently identified locality and urgent actions for the reestablishment of populations are essential for the survival of *Chara lipkinii* in a rapidly changing world. The control of eutrophication as well as accurate removal of the surface layer of sediments accumulated in its type locality is essential for the recovery of its population. The study of oospore distribution in sediments is the first step required for subsequent reasonable restoration of the water body as a habitat for the species. This may help avoid the destruction of the type locality as occurred in the case of *Chara hellenica* Langangen (Langangen 2017).

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Supplementary material

The following supplementary material is available for this article:

Species and strains used for the present rbcL and ITS1 gene phylogeny.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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