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RESEARCH ARTICLE

Molecular and taxonomical study of the genus *Chara* (Linnaeus 1753) in Israel

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ABSTRACT
Israel's climate is characterized by hot and dry summers and cool, rainy winters in the northern and coastal regions, whereas the southern and eastern regions are arid. The aquatic macrophyte *Chara* is a common genus in Israel, and its distribution varies across Israel's climatic gradient. Although few studies described the abundance and taxonomy of the local *Chara* species in Israel, only little is known about the molecular taxonomy of this group in the region. We sampled *Chara* species along Israel's climatic gradient and identified specimens using the classical taxonomy key and molecular identification methods. We characterized the molecular taxonomy of four *Chara* species: *C. vulgaris, C. contraria, C. gymnophylla,* and *C. globata*. Notably, the latter species, which was found in a hyper-arid area, was last recorded in Israel ~50 years ago on the northern Mediterranean coast and Judaean Mountains. *Chara gymnophylla* was more abundant in northern Israel, while *C. vulgaris* and *C. contraria* were common in southern Israel. We developed Inter Simple Sequence Repeat markers to identify the four species and sequenced their chloroplast gene *rbc*L. The phylogenetic trees generated using the *rbc*L sequences and ISSR-based dendrogram were consistent with the classical taxonomy of *Chara*.

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Introduction

Chara is a macrophytic algae that grows submerged in fresh and brackish calcareous water (Kufel and Kufel, 2002). *Chara* species are distributed worldwide from the tropics to cold temperate zones. They play an essential role in studying the evolution of land plants due to their place between algae and terrestrial organisms (Karol *et al.*, 2001; Sanders *et al.,* 2003). Since the genus *Chara* exhibits high plasticity in response to environmental conditions, it is difficult to distinguish between phenotypic characters prompted by habitat conditions and those derived from a common ancestor (Urbaniak and Combik, 2013; Wood and Imahori, 1965). Consequently, the characterization of *Chara* has been controversial for a long time. Wood and Imahori (1965) discriminated between 18 polymorphic *Chara* species worldwide with a few forms and varieties, known as the macro-species concept. Krause (1997), on the other hand, recognized 45 species in Europe, suggesting that the genus includes many monomorphic species, known as the micro-species concept, which is traditionally used in Europe as a characterization key (Boegle *et al.*, 2007; Urbaniak, 2010). During the last few decades, the usage of molecular markers in studies related to charophyte taxonomy has substantially increased. These studies have particular significance because charophytes serve as a critical outgroup in phylogenetic studies on embryophytes (Sanders *et al.*, 2003).

Israel is characterized by a steep climatic gradient, owing to the combined effects of its geographical position (i.e. situated between Africa, Asia, and Europe) and climate variety (Goldreich, 2003; Jaffe, 1988). The aquatic macrophyte *Chara* is a common genus in Israel, and its distribution varies across the climatic gradient. Romanov and Barinova (2012) compared the historical

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and modern distribution and richness of charophytes in Israel, suggesting that the reduction in the natural charophyte habitats in this region is a consequence of urbanization and industrialization processes, which took place since the establishment of Israel ~70 years ago. On the other hand, artificial reservoirs have been created and occasionally occupied by charophytes (Romanov and Barinova, 2012). Notably, although previously investigated, little is known about the molecular taxonomy of this important group in Israel. To fill this empirical gap, we developed a set of ISSR (Inter simple sequence repeat) markers to identify *Chara* species in Israel. Fingerprinting with DNA-based molecular markers allows precise, objective, and rapid taxonomy classification (Khasdan *et al.*, 2005; Nagaoka and Ogihara, 1997; O'Reilly *et al.*, 2007). Arbitrarily amplified dominant (AAD) markers such as RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragment length polymorphism), and ISSR can be used to generate vast quantities of data addressing a diverse range of biological questions (Bussell *et al.*, 2005). The hundreds of studies that have utilized AAD markers in systematic and phylogenetic analysis may suggest a strong basis for such application (Bussell *et al.*, 2005). ISSR and SSR (Simple sequence repeats) are popular molecular markers in plant identification (Godwin *et al.*, 1997; Zhu *et al.*, 2011). ISSR markers detect polymorphism in inter-microsatellite loci using a single primer composed of SSR sequence anchored at the 3′ or 5′ end by 2–4 arbitrary nucleotides. ISSR markers have the advantages of relatively low cost, high polymorphism, and good reproducibility (Godwin *et al.*, 1997; Nagaoka and Ogihara, 1997; Patamsyté *et al.*, 2018; Tunaitiené *et al.*, 2017; Zhu *et al.*, 2011). Furthermore, this method does not require knowing the DNA sequence.

We identified and characterized four *Chara* species along Israel's climatic gradient: *C. vulgaris* Linnaeus (1753), *C. contraria* Kütz (1839), *C. gymnophylla* Braun (1835), and *C. globate* Migula (1904). We illustrate that ISSR markers can be helpful for rapid and easy assay in identifying the species in this genus. The Consortium for the Barcode of Life (CBOL) approved the *rbc*L and *mat*K sequences as the official barcode for plants (CBOL Plant Working Group, 2009; http://www.barcoding.si.edu), showing that it is a valuable tool for taxonomy and conservation (Li *et al.*, 2011). Sequences for *rbc*L (the plastid-encoded large subunit of the enzyme ribulose-1,5-biphosphate carboxylase / oxygenase [Rubisco]) have been widely used in the phylogeny of extant genera in the Characeae species (McCourt *et al.*, 1996, 1999). Using DNA sequences of the *rbc*L, we constructed a phylogenetic tree. This tree was consistent with the ISSR-based dendrogram and classical taxonomy of these *Chara* species.

Materials and methods

Study area

The samples were collected from pounds and streams between 2013 and 2016 in (1) Northern Israel (Golan

Table 1. A list of *Chara* species by geographic zone/location as presented in Figure 1.

Population code	No of samples	Latitude N	Longitude E	Location	Species (%)	Ambient temperature[1]		Annual precipitation[1]
						max	min	(mm)
Nahal Dan	58	33 °13′47′	35 °38′12′	Upper Galilee	*Chara gymnophylla*	21 °C	9 °C	507
Nahal Dafna	63	33 °13′52′	35 °38′32′	Upper Galilee	*C. gymnophylla*	21 °C	9 °C	507
Ein Nevoria	1	33 °00′11′	35 °30′24′	Upper Galilee	*C. gymnophylla*	21 °C	13 °C	688
Ein Hemed	1	31 °47′45′	35 °07′27′	Judaean Mountains	*C. vulgaris*	22 °C	14 °C	522
Ein Mata	1	31 °42′56′	35 °03′03′	Judaean Mountains	*C. vulgaris*	23 °C	14 °C	550
Aminadav Pools	36	31 °45′19′	35 °07′56′	Judaean Mountains	*C. vulgaris*	22 °C	14 °C	522
Ein Avdat	49	30 °49′36′	34 °55′42′	Negev desert	*C. vulgaris* (~86%), *C. contraria* (~14%)	25 °C	12 °C	87
Ein Maarif	26	30 °49′23′	34 °45′49′	Negev desert	*C. vulgaris* (~85%), *C. contraria* (~15%)	25 °C	12 °C	87
Ein Aqev (upper)	6	30 °47′42′	34 °48′39′	Negev desert	*C. vulgaris*	25 °C	12 °C	87
Ein Aqev (lower)	43	30 °48′52′	34 °48′48′	Negev desert	*C. vulgaris* (~12%), *C. contraria* (~88%)	25 °C	12 °C	87
Nahal Shikma (kibbutz Ziqim)	12	31 °36′14′	34 °30′44′	Israeli coastal plain	*C. contraria*	26 °C	15 °C	500
Neot Ha-qikar Pond	132	30 °55′50′	35 °22′41′	Arava desert (near the Dead Sea)	*C. globata* (~68%), *C. contraria* (~24%), *C. vulgaris* (8%)	31 °C	23 °C	39

[1] Climate data were taken from the Israel Meteorological Service website: https://ims.gov.il/he/ClimateAtlas. Ambient temperatures represent measurements taken from 1995 to 2009, and annual precipitation data represent measurements taken from 1991 to 2020.

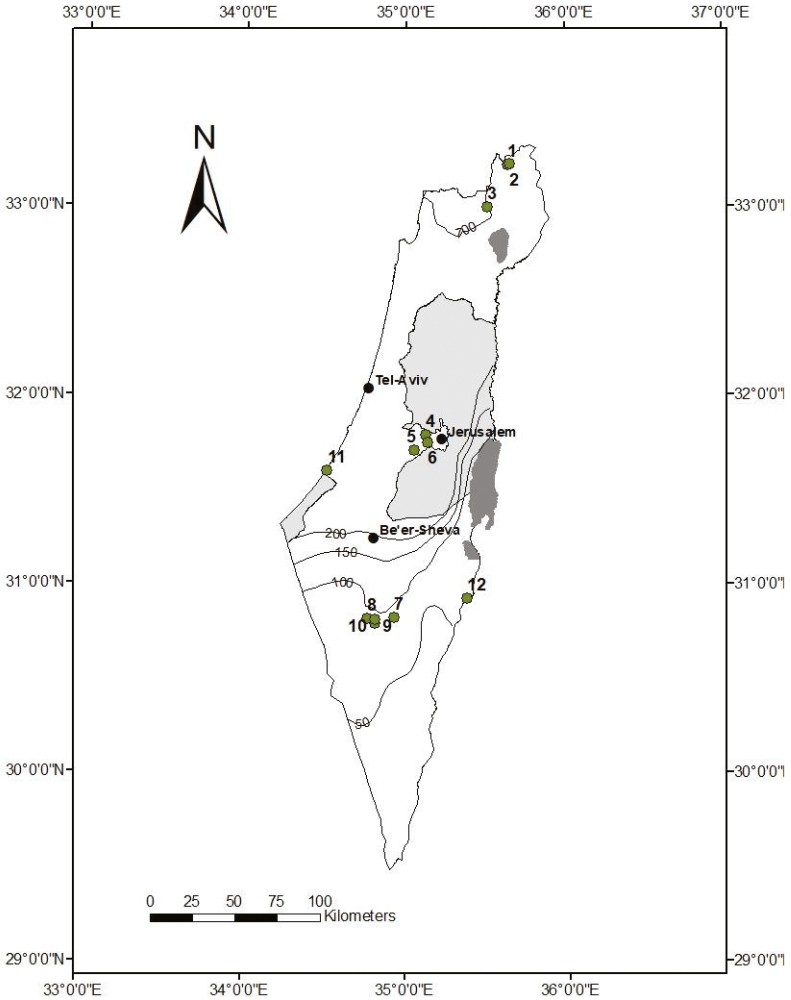

Figure 1. A map of Israel with the sampling locations: 1 – Nahal Dan; 2 – Nahal Dafna; 3 – Ein Nevoria; 4 – Ein Hemed; 5 – Ein Mata; 6 – Aminadav Pools; 7 – Ein Avdat (Sde Boker); 8 – Ein Maarif; 9 – Ein Aqev (upper); 10 – Ein Aqev (lower); 11– Nahal Shikma; 12 – Neot Ha-qikar Pond. Map contours represent annual precipitation in mm.

and Upper Galiley – Nahal Dan, Nahal Dafna, and Ein Nevoria); (2) Central Israel (Judaean Mountains – Ein Hemed, Ein Mata and Amidav pools); (3) Southern Israel (The Negev desert – Ein Avdat, Ein Maarif, and Ein Aqev); (4) Israeli coastal plain (Kibbutz Zikim – Nahal Shikma); and (5) Arava valley (Dead Sea area – Neot Ha-qikar Pond) (Table 1, Figure 1). The Northern and central parts of Israel are characterized by a Mediterranean climate, while an arid and hyper-arid climates characterize the southern part of Israel, and Arava valley, respectively (Goldreich, 2003).

Sampling procedure and study species

To obtain the samples, we collected from submersed *Chara* specimens. The material was placed in plastic zip bags with water from the pond or stream of origin and saved in a container with ice until getting to the laboratory. The *Chara* specimens were first examined under a Nikon SMZ800 dissecting microscope and placed in vials filled with a solution comprising DDW, ethanol, and formaldehyde (6:3:1, v/v/v).

Samples and laboratory procedures

Chara specimens were collected from 12 populations. Total DNA was extracted from frozen (-20 °C) samples using the DNeasy Plant Kit (Qiagen, Germany). DNA quantity and quality were measured electrophoretically and with a spectrophotometer.

ISSR PCR amplifications

PCR amplifications were carried out in 25 µl of reaction mixtures containing the following components: 1× PCR buffer A (*EURx*, Poland), 10 mM MgCl2, 5.0

mM dNTPs, 0.8 µM of the primer, 1.5 U of *Taq* DNA Polymerase (*EURx*, Poland) and 40 ng of total DNA. All amplifications were carried out in PTC-200 DNA Engine Cycler (*MJ Research*, Waltham, USA) programmed for 7 min at 94 °C initial DNA denaturation step, followed by 35 cycles of 30 s at 94 °C, 45 s at 52 °C and 120 s at 72 °C. The last cycle was followed by the final extension step for 7 min at 72 °C. The reaction mixture without DNA was used as a negative control. Six preselected 16- to 18-mers primers (UBC-818, -826, -847, -855, -856, and -857 (Nagaoka and Ogihara, 1997)) were used for the developing ISSR markers. Amplification products were fractionated in 1.2% agarose gel (1× TAE) and visualized with ethidium bromide. ISSR reproducibility was assessed by comparing at least two reactions. Primers generating ISSR profiles of low reproducibility were excluded from further analysis.

rbcL *gene amplification*

PCR amplifications were carried out in 25 µl of reaction mixtures containing the following components: 1× PCR buffer A, 40 mM MgCl2, 5.0 mM dNTPs, 1.0 U of *Taq* DNA Polymerase (*EURx*, Gdansk, Poland), 30 ng of total DNA and primers (RbcL_F_Chara: 5′–TTGTTTGATCGAGCAGACCTT–3′ and RbcL_R_Chara: 5′–TTGTTTGATCGAGCAGACCTT–3′). Primers were designed from the NCBI reference sequence *Chara vulgaris* chloroplast complete genome (accession DQ229107) using the Primer3 program. PCR conditions were as follows: 3 min at 94 °C initial DNA denaturation step, then 32 cycles of 30 s at 94 °C, 30 s at 65 °C, 120 s at 72 °C and a final step of 5 min at 72 °C. Produced from different *Chara* species samples, 1703 bp fragments were isolated from agarose gel, cleaned, cloned in pGEM-T Easy Vector Systems I (Promega, Madison, WI, USA), and sequenced.

Genetic data analysis

The ISSR bands (detected in the agarose gel) were first translated into a binary presence/absence table. Using the PRIMER v6 software (PRIMER-E Ltd, Plymouth, UK), we generated a similarity matrix comprising Jaccard's coefficient (Eq. 7.10 in Legendre and Legendre, 1998). Next, we used hierarchical agglomerative clustering (Legendre and Legendre, 1998), followed by a series of 'similarity profile' (SIMPROF) permutation tests, looking for statistically significant evidence for genuine clusters in the generated dendrogram.

The *rbc*L sequences were first aligned and analyzed using Sequencher 4.8 and Clustal X. A Maximum Likelihood phylogenetic tree (Kimura 2-parameter model) based on the aligned sequences was created using MEGA version 6.06 (Tamura *et al.*, 2011). The analysis included 8 *Chara* samples from Israel, 3 genera as out groups, and 49 sequences downloaded from NCBI (Supplementary Table S1). Specifically, our samples included: 4 *C. globata* (Neot Ha-qikar) (the following isolates: 101 (accession number MN273738), 105 (MN817123), 110 (MN817124) and 116 (MN817125); 2 *C. gymnopylla* (Nahal Dan) (the following isolates: 66 (accession number MN793053) and 74 (MN793052); 1 *C. contraria* (Neot Ha-qikar) (isolate 63 accession number MN793054) and 1 *C. vulgaris* (Ein Avdat) (isolate 13 accession number MN793055) (Table 1). The list of *rbc*L 47 sequences and 3 genera as an out group, downloaded from NCBI, is shown in Supplementary Table S1.

Results

Chara *species detected in Israel*

We have surveyed ~160 water bodies across Israel. Our sampling effort resulted in the detection of four *Chara* species: *C. vulgaris*, *C. contraria, C. gymnophylla,* and *C. globata*. In the upper Galilee (Nahal Dan, Nahal Dafna, and Ein Nevoria), we detected *C. gymnophylla*. In the Judaean Mountains (Aminadav pools, Ein Hemed, and Ein Mata), we found *C. vulgaris.* In the Arava Valley (Neot Ha-qikar pond), we detected *C. globata*, *C. contraria,* and *C. vulgaris*. In the coastal plain (Shikma stream), we found *C. contraria*. In the Negev Desert (Ein Maarif, Ein Avdat, and Ein Aqev), we detected *C. vulgaris* and *C. contraria*. Both Nahal Shikma (Israeli coastal plain) and Aminadav pools (Judaean Mountains) have been used for morphology classification only.

Morphological identification of Chara

The four Israeli species included *C. vulgaris*, *C. contraria, C. gymnophylla,* and *C. globata*, all of which were characterized based on the updated *Chara* keys, with orientation to the Mediterranean region. We characterized these *Chara* species using the essential morphology features described in the Supplementary Table S2.

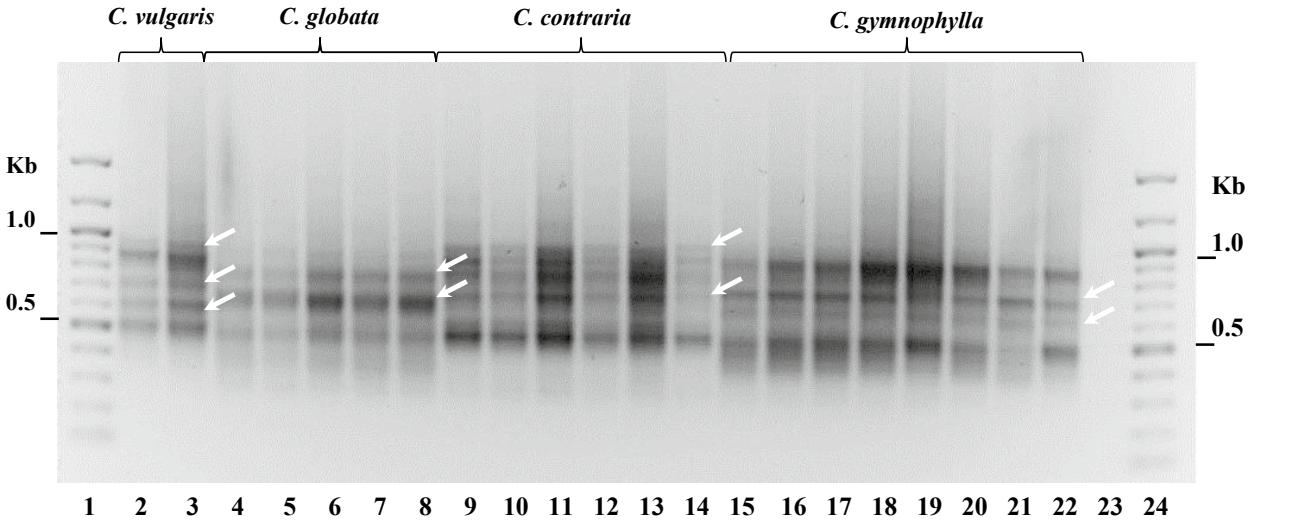

Figure 2. PCR-ISSR analysis of *Chara* species DNA from different populations with primer UBC-856. Lanes1 and 24, 100 bp DNA Ladder (EURx, Poland); Lanes 2 and 3, samples of *C. vulgaris* obtained from Ein Avdat; lanes 4–8, *C. globata* from the pond Neot Ha-qikar; lanes 9–14, *C. contraria* from Ein Avdat, Ein Maarif, and Neot Haqikar; lanes 15–22, *C. gymnophylla* from Nahal Dan, Nahal Dafna, and Ein Maarif; lane 23, a control without DNA. Arrows mark the position of species-specific bands.

ISSR markers

The ISSR bands appeared on the gel (Figure 2) in a range between 500 to 1000 bp, representing four *Chara* species. Each species was distinguished by a unique pattern of bands on the gel, although there was an overlap in some bands. Similar band patterns were found in *C. contraria* from two different areas in Israel: (1) Ein Avdat in the Negev desert and Neot Ha-qikar in the Arava Valley, and (2) in *C. gymnopylla* sampled from two different rivers in the upper Galilee of Israel, Dan and Dafna. We generated a Jaccard similarity matrix using these results and created a dendrogram (Figure 3).

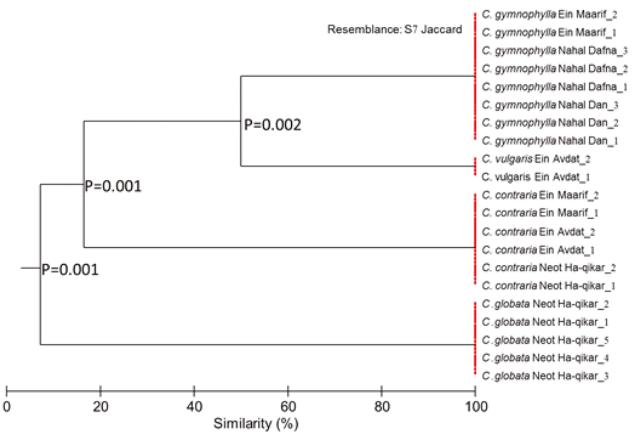

Figure 3. A dendrogram based on a Jaccard similarity matrix created using the presence/absence of ISSR bands in the different Chara specimens. Significant differences ($P < 0.05$) between clusters are presented in the dendrogram with the respective *P*-values.

Analysis of rbcL *sequences*

Obtained Rubisco large subunit gene (*rbc*L) sequences were analyzed using MEGA 6.06. The local *Chara* species from Israel were clustered with their relative species worldwide. *C. vulgaris* from Israel was clustered with *C. vulgaris* from Canada, the USA, Taiwan, Japan, Germany, Egypt, and South Africa Coastline. *C. contraria* from Israel was clustered with *C. contraria* from Egypt and Germany. *C. globata* from Israel was clustered with *C. globata* from Russia and Israel sampled in 1970 in Ma'agan Michael Quarry. The *C. globata* species located relatively close to the *C. hispida* complex included *C. hispida, C. rudis, C. baltica, C. horrida,* and *C. polyacantha. C. gymnophylla* was clustered close to *C. vulgaris*. We found no *rbc*L *C. gymnophylla* sequences available in NCBI, and our specimen was not distinguishable from *C. vulgaris*. The remaining *Chara* species in the phylogenetic tree were clustered according to their taxon position. *Nitella* and *Nitellopsis* were used as an outgroup (Figure 4). The local Israeli *Chara* species were clustered according to their genetic distance. *C. vulgaris* (Canada) was used as a control cluster with *C. vulgaris (*Israel), and it was located close to *C. gymnophylla*. Four *C. globata* (Israel) clusters were located on the same branch, not far from *C. contraria*. *C. braunii* (Japan) was found relatively far from most of the group, and *Nitella* (USA) was used as an outgroup (Figure 5).

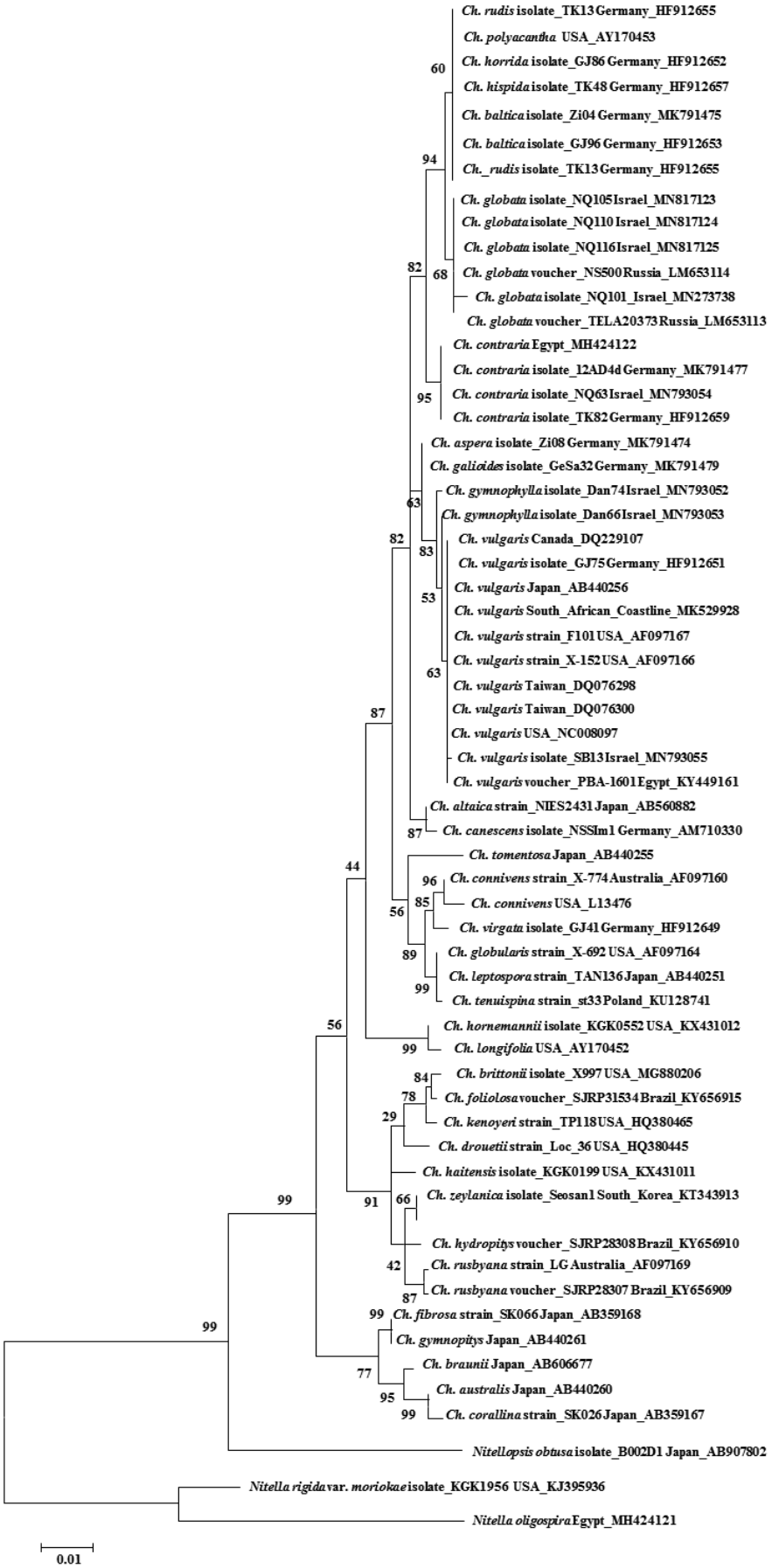

Figure 4. Maximum likelihood phylogenetic tree (Kimura 2-parameter model) of *Chara* specimens from Israel and abroad based on rbcL sequences. The species *Nitella rigidia* var. *moriokae* KJ395936 (USA) serves as outgroup, NCBI GenBank.

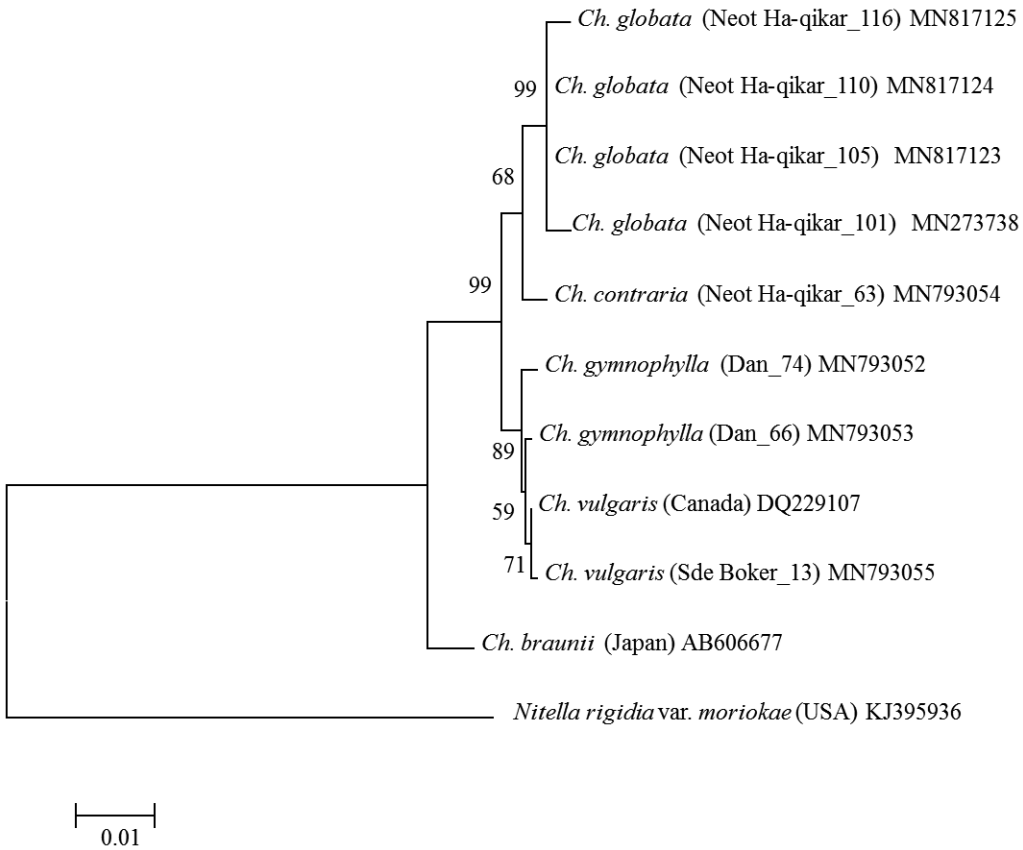

Figure 5. Maximum likelihood phylogenetic tree (Kimura 2-parameter model) of the local Israeli *Chara* species based on rbcL sequences. The species C. *vulgaris* DQ229107 (Canada) and *C. braunii* AB606677 (Japan) serve as control, and *Nitella rigidia* var. *moriokae* KJ395936 (USA) outgroup, NCBI GenBank.

Discussion

Chara *distribution in Israel*

In the Upper Galilee region (North Israel), we have found patches of *C. gymnophylla* over tens of meters along the Dan and Dafna rivers during the entire study period, i.e. 3 years. In addition, *C. gymnophylla* was found at other sites in Galilee, such as Ein Nevoria (Barinova and Romanov, 2014). In the Negev desert region, during the last decade of sampling and monitoring, we have found both well-adapted species, *C. contraria* and *C. vulgaris*, which were successfully established in Ein Maarif, Ein Avdat and Ein Aqev alkaline streams (Yehuda *et al.*, 2013). *C. vulgaris* was recently found in the Judaean Mountains, i.e. Aminadav Pools, and in the Southern Negev Desert in Neot Smadar pools (Barinova and Romanov, 2015b). *C. contraria* was found in the Shikma stream, near the Mediterranean Sea. Indeed, we have found *C. contraria* in Neot Ha-qikar pond located in the Arava desert, like Barinova (2015a), who sampled this pond in 2012. The unexpected event was the occurrence of the rare species *C. globata* in Neot Ha-qikar pond. It is a small artificial freshwater pond in the arid Arava desert, located in southeastern Israel, near the Dead Sea. The Israeli water authority 'Mekorot' regulates this pond; since 2016, the pond has dried out.

The last record of *C. globata* in Israel and Egypt was collected by E. Cohen, Y. Lipkin, and W. Proctor in 1969 and 1970. This collection was stored in Tel Aviv University Herbarium and was published by Romanov and Barinova (2012). Around the world, *C. globata* was found in China (Ling *et al.*, 2000; Migula, 1904), southern Kazakhstan (Kostin and Shoyakubov, 1974), and recently in Oxbow Lake of the Yeya River, Krasnodar Russian Federation (Romanov *et al.*, 2015), and Targhrud Lake in Iran (Noedoost *et al.*, 2015). The *C. globata* species was found mainly in arid and semi-arid regions of Asia, Europe, and the Sahara-Arabian Desert, with a disjunctive distribution (Romanov *et al.*, 2015). The habitat of the Neot Ha-qikar pond is like those mentioned above, which can explain its preference and establishment in the unique Arava desert conditions. One of the limitations in this area is the

appearance of mines that prevent entry into regional riparian zones, which reduces the monitoring of *C. globata* in this region.

Morphological identification

The *Chara* species delineation has long been controversial, mainly because of the limited morphology features, intermediate forms, and phenotypic plasticity influenced by habitat conditions (O'Reilly *et al.*, 2007). Our study focused on characterizing the local *Chara* species in the main areas of Israel. We have used the relevant *Chara* keys to accurately delineate Chara species based on the morphology features. Using the illustrations and morphology description found in Wood and Imahori (1965) and Krause (1997), we characterized four Israeli species: *C. vulgaris, C. contraria, C. gymnophylla*, and *C. globata*. We have used the 'micro-species concept', suggesting that the *Chara* genus is composed of many separated species as suggested by Krause (1997) and Cirujano *et al.* (2009), and has been supported by molecular studies (Mannschreck *et al.*, 2002; O'Reilly *et al.*, 2007; Schneider *et al.*, 2015). The *Chara* morphology characters are summarized in Supplementary Table S2. The gametangia of the four *Chara* species are monoecious and conjoined. They have two tiers of stipulodes, which are slightly different in size. *C. gymnophylla* branchlet segments are ecorticated (Supplementary Figure S1A,B), whereas the rest have cortex in the branchlet segments. Wood and Imahori (1964) characterized *C. gymnophylla* (A. Braun) as a variety of *C. vulgaris* based on their similar morphology. However, Krause (1997) and Gollerbach and Krasavina (1983) characterized *C. gymnophylla* as a distinct species that is currently accepted. We have distinguished between aulacanthous/isostichous *C. vulgaris* and tylacanthous *C. contraria* (Supplementary Figure S1C,D) due to the spine cells' morphology and position on the cortex. The bract cells' length, position, and number have been characterized. We found that *C. vulgaris* is a Longibracteate variety characterized by its long bract cells (Supplementary Figure S1G,H); likewise, the bract cells in *C. globata* are long, acute, and almost verticillate that may use one of the hallmarks of this species (Supplementary Figure S1 E,F). Oospores in *C. vulgaris* and *C. contraria* have shown variety in colors (mainly orange and dark) and sizes, although not measured directly, only general observation by the binocular (Supplementary Figure S1I,J). The morphology of *C. globata* and the Mediterranean *C. baltica* type have shown some features in common that may confuse the delineation; however, the irregular triplo- to diplo-stichous cortex and the conjoined gametangia are unique characters found in *C. globata* and not in Mediterranean *C. baltica*, determined our samples to *C. globata*. The oospore morphology of our *Chara* sample is suitable for *C. globata*, although we have been measuring a small sample (750-850 µm in length, 440-550 µm in width, 11-13 number of striae) (Supplementary Figure S1E,F). Blume *et al.* (2009) found that the 'French Baltica' oospores had a higher number of striae and a higher length-to-width ratio than *C. baltica* from the Baltic Sea. The 'French Baltica'' is associated with the Mediterranean *C. baltica* and has more striae than *C. globata*, supporting our delamination.

Molecular identification

In this study, we have used two different molecular methods that confirmed our morphological separation of the *Chara* species. It was shown previously that ISSR markers are suitable tools for plant molecular taxonomy at the level of related species (Butkuvienė *et al.*, 2017; Fajardo *et al.*, 2014; Zhu *et al.*, 2011), and Rubisco large subunit gene (*rbc*L) was approved as the official DNA barcode for land plants (Hollingsworth *et al.*, 2011; Li *et al.*, 2011), and *Chara* taxonomy studies. The *Chara* molecular taxonomy studies carried out in recent years primarily focused on revealing the incompatibility of *Chara* species delimitation, mainly those defined according to Wood and Imahori (1965), which was not occurring in our study. The fact that both analyses focused on different genomes (nuclear and chloroplast DNAs) in *Chara* cells, while ISSR focused mainly on nucleus DNA, and the *rbcL* included the chloroplast DNA, considerably validates the species separation. In addition, a previous study based on AFLP (Yehuda *et al.*, 2013) supported the current study results. Using the ISSR method, we found that each *Chara* species has a unique fingerprint, which is the basis of the species separation. So far, we have not found any publications that used this technique in *Chara* studies. Fingerprinting with arbitrarily amplified dominant molecular markers allows precise, objective, and rapid taxonomy classification of closely related species (Archibald *et al.*, 2006; Bussell *et al.*, 2005; Khasdan *et al.*, 2005). ISSR markers have the advantages of relatively low cost, high polymorphism, and good reproducibility (Butkuvienė *et*

al., 2017; Zhu *et al.*, 2011). Furthermore, this method does not require knowing the DNA sequence for ISSR primer design. The potential of this method is the ability to assess intraspecific variability using relatively simple procedures and to create species-specific markers by isolating and sequencing a unique band, which may be a helpful tool for certain *Chara* species identification. However, in this study, the species identity was confirmed using *the rbc*L marker, widely applied in plant barcoding (Hollingsworth *et al.*, 2011; Li *et al.*, 2011; Sheth and Thaker, 2017).

We constructed two *rbc*L phylogenetic trees; the first includes our specimens and an additional 53 sequences (NCBI) of *Chara* species worldwide (Figure 4). The second tree includes mainly the local *Chara* species of Israel (Figure 5). According to the comprehensive *rbcL* phylogenetic tree, we can indicate that the *Chara* species from Israel matched their types in the world. The species *C. vulgaris* from Israel clustered with *C. vulgaris* from seven countries worldwide. Similarly, *C. contraria* was clustered with *C. contraria* from Germany and Egypt. There is no available sequence of *C. gymnophylla* in NCBI; thus, we could not compare our data with other *C. gymnophylla* specimens worldwide. Like the local tree, the species *C. gymnophylla* was clustered near *C. vulgaris*. The species *C. globata* from Israel (Arava desert) was clustered with *C. globata* from Russia and Israel, which was sampled in 1970 in Ma'agan Michael Quarry (located along the northern Mediterranean coast of Israel) by Y. Lypkin and kept in Tel Aviv University Herbarium (TELA), and recently was sequenced (Romanov *et al.*, 2015). In addition, the Hispida complex, which includes *C. baltica, C. horrida, C. hispida, C. intermedia,* and *C. polyacantha,* was clustered close to *C. globata* as a relative species that was found similarly in Romanov *et al.* (2015) study. The local *Chara* species clustered according to their genetic distance. The species *C. gymnophylla* and *C. vulgaris* clustered nearby in the *rbc*L phylogenetic tree as accepted from relative species (Grant and Proctor, 1972). Similarly, *C. contraria* and *C. globata* were clustered close to each other, as was found in the Romanov *et al.* (2015) study.

In summary, this study presented a partial distribution of *Chara* throughout Israel in recent years. It indicated that the distribution of this genus changes as a function of geographic and climatic gradients. The water characteristics of most sampling sites have not yet been studied. Future studies should examine the water characteristics of all *Chara* sampling sites to investigate the relationships between the distribution of different *Chara* species and the respective water characteristics.

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Conflict of interest

The authors have declared no conflict of interest.

Supplementary material

Supplementary material can be found online at https://doi.org/10.6084/m9.figshare.27968598

Figure S1. The *Chara* morphology characters.
Table S1. The NCBI *Chara* and 3 out group samples used to construct a phylogenetic tree, based on *rbc*L sequences, together with the eight *Chara* samples from Israel.
Table S2. *Chara* morphology characters of the four species found in Israel.

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