The Charophytes (Charophyceae, Characeae) from Dagestan Aquatic Habitats, North Caucasus: Biogeographical and Barcoding Perspectives

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Article

The Charophytes (Charophyceae, Characeae) from Dagestan Aquatic Habitats, North Caucasus: Biogeographical and Barcoding Perspectives

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Abstract: To fill in the blanks for charophytes in the Caucasus to improve our knowledge of species distribution areas in Eurasia, a field survey and study of available specimens from Dagestan (North Caucasus, Russia) was conducted based on morphological observation and molecular genetic analysis. Seventeen new localities for six *Chara* species and one *Tolypella* species, with six new species and one new genus for the region studied, and one new species for the Caspian Sea region were found. Some species records changed the outline or filled in the gaps in the species distribution area. The presence of species with a distribution area mainly in the centre of Eurasia (*C. globata, C. neglecta*) with mainly Mediterranean – Middle East species (*C. gymnophylla*) is notable for this region, as well as for other studied regions of the Caucasus having a combination of species with different distribution areas. The frequent occurrence of *C. gymnophylla* points is similar to that in the Mediterranean region and the Middle East. Small brackish waterbodies at the coast of the Caspian Sea, freshwater mountain rivers, small waterbodies associated with them, and water reservoirs are the main habitats of charophytes in the regions. The habitat preference and distribution in the Caucasus allowed suggestion some species for protection. The absence of endemic species among charophytes from Dagestan and Caucasus contrasts with the flora of terrestrial magnoliophytes, which have a large number of endemic species.

Keywords: Characeae; *Chara*; *Tolypella*; Dagestan; Caucasus; Caspian Sea; DNA barcoding; protection

1. Introduction

Charophytes are a distinct group of macroscopic algae widely known mainly as ecosystem engineers and pioneer species [1]. Their life strategies and ecology are diverse [2–4] and still poorly outlined for many species, especially outside of Europe and Australia. Regional surveys of charophytes are an essential step towards better knowledge of their distribution and ecology. They allow further testing and improvement of species concepts and clarification of the bioindicator potential of charophytes. The barcoding of widely and narrowly distributed species fits the same targets, allowing testing of the presence of cryptic and undescribed taxa. Less-known and remote areas are especially important from this perspective.

Knowledge about the charophytes of Dagestan, a region east of the North Caucasus, is limited and almost unknown worldwide. All available data are basically limited to three old records of *Chara vulgaris* L. reported in two articles covering large areas [5,6], later summarized by Hollerbach [7]. A

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few recent records, mainly at the genus level (*Chara* L.) are available at iNaturalis.org [8]. A similar situation could be noted for the entire North Caucasus, based on nearly the same set of references.

As a first step towards better knowledge of this group of freshwater macrophytes, a keystone in some ecosystems, notable for their ecological role, we present here the main results of some recent field and herbarium investigations. In previous studies of Caucasian charophytes, species identification was carried out using only a morphological approach, which did not allow precise species identification in all cases. The aim of this study was to investigate the species compositions of the charophytes of Dagestan using a polyphasic approach, which included morphological observation and molecular genetic analysis of the DNA of the studied species.

2. Materials and Methods

2.1. Morphological Identification

The specimens were usually collected by hand during a careful survey of waterbodies. They were dried as herbarium specimens and stored in LE (acronyms according to [9]). Some specimens collected in the XIX and XX centuries were found in LE. Almost all of them had no identification before our study. Our efforts to search charophyte specimens from Dagestan stored elsewhere yielded no results. Few records of charophytes were available at iNaturalist.org [8], but only two of them can be identified at the species level (*Chara globata* Migula).

The morphological features of the specimens were studied using Olympus SZ61 stereomicroscope (Olympus Corporation, Shinjuku, Tokyo, Japan). Photomicrographs of diagnostic traits were taken using a digital camera. Oospores taken from some recent specimens for scanning electron microscopy (SEM) were treated according to a previously described method [10]. The cleaned oospores were coated with gold and studied using a Jeol JSM 6390LA scanning electron microscope (JEOL Ltd., Tokyo, Japan). Taxonomy followed the most recent reference [11]. The Ecoregions' mapping program was used to map the individual charophyte species distribution in the studied territory [12]. The BioDiversity Pro 2.0 program was used for the similarity calculation [13].

2.2. DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted as described previously by Echt et al. [14] with some modifications [15]. Part of the *rbc*L gene was amplified as described previously [16]. 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 analyzer (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 [17] and aligned manually in the SeaView program [18]. The *rbc*L sequences were deposited in GenBank (*C. connivens* OQ607406; *C. neglecta* OQ607411; *C. neglecta* OQ607412; *C. globata* OQ607413; *C. gymnophylla* OQ607410; *C. gymnophylla* OQ607409; *C. gymnophylla* OQ607408; *C. vulgaris* var. *longibracteata* OQ607407; Supplementary Table S1).

Before the phylogenetic analyses, the sequences of the *rbc*L gene were compared with those available at the National Center for Biotechnology Information (NCBI, Bethesda, USA) using a BLAST search [19] to estimate their taxonomic position.

2.3. Phylogenetic Analyses

The *rbc*L dataset was used to access the affinity of our *Chara* species with the genus representatives retrieved from the NCBI. The dataset was assembled as described by Romanov et al. [16,20]. Maximum likelihood (ML) analyses were carried out using PAUP 4.0b10 [21]. Bayesian inference (BI) was performed using MrBayes 3.1.2 [22]. To determine the most appropriate DNA substitution models for our datasets, we used the Akaike information criterion (AIC; [23]), which was applied with jModelTest 2.1.1 [24]. The GTR+I+G model was selected as the best fits for the *rbc*L dataset. ML analyses were carried out using heuristic searches with a branch-swapping algorithm (tree bisection-reconnection). Using BI, four parallel MCMC runs were carried out for 3 million

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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 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; [25]) 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/; [26]; accessed on 01 March 2023).

3. Results

3.1. Charophytes Diversity and Distribution

Seven species of charophytes, including six species of *Chara* L. and one species of *Tolypella* (A.Braun) A.Braun were found at 22 localities in Dagestan according to the all available data. The presence of a few taxa known before from this territory, i.e. *C. vulgaris* f. *vulgaris* and f. *longibracteata* (Kütz.) H.Groves [5,6], was confirmed with the studied specimens. Twenty localities of charophytes were found for the region, and eighteen of them are based on vouchers available for study.

All localities were situated in two ecoregions: Pontic steppe and Caucasus mixed forests (Figure 1a). The first region is rich in both species and localities. All species except *C. contraria* were known from it. *Chara connivens*, *C. contraria*, *C. globata*, *C. gymnophylla* and *C. vulgaris* were found in a few water bodies in Caucasus mixed forests.

The distribution data, habitat and floristic novelty for the species found in the studied area are listed below.

Chara connivens Salzm. ex A.Braun (Figures 1b, 2a)

Studied specimens: 1. [Gergebilsky District], River Sulak, Chiryurt Reservoir, at shallow, in a pit, 08 VIII 1968, V.M. Katanskaya (LE A0002061). 2. [Babayurtovsky District] lower reach of the River Sulak, Lake Mekhteb [currently Mekhteb Reservoir], 1968, V.M. Katanskaya (LE).

Habitat: large mountain and coastal water reservoirs.

Floristic novelty: new species record for Dagestan.

Chara contraria A.Braun ex Kütz. (Figure 1b)

Studied specimen: *Dagestan, distr. Dargi. In paludosis ad fl. Akuscha inter pagos Urkhuwah et Urchaczi* [currently River Akusha or Akushinka, vicinity of the rural locality of Urkhuchimakhi (Urkhuchi)], 4000', 16 VII 1898, Th. Alexeenko. *Flora Caucasi* / *C. foetida* Al. Br., det. J. Vilhelm, 1927 (LE). – This locality was cited for *C. vulgaris* [5] because of specimen misidentification.

Habitat: wetland associated with mountain river.

Floristic novelty: new species record for Dagestan.

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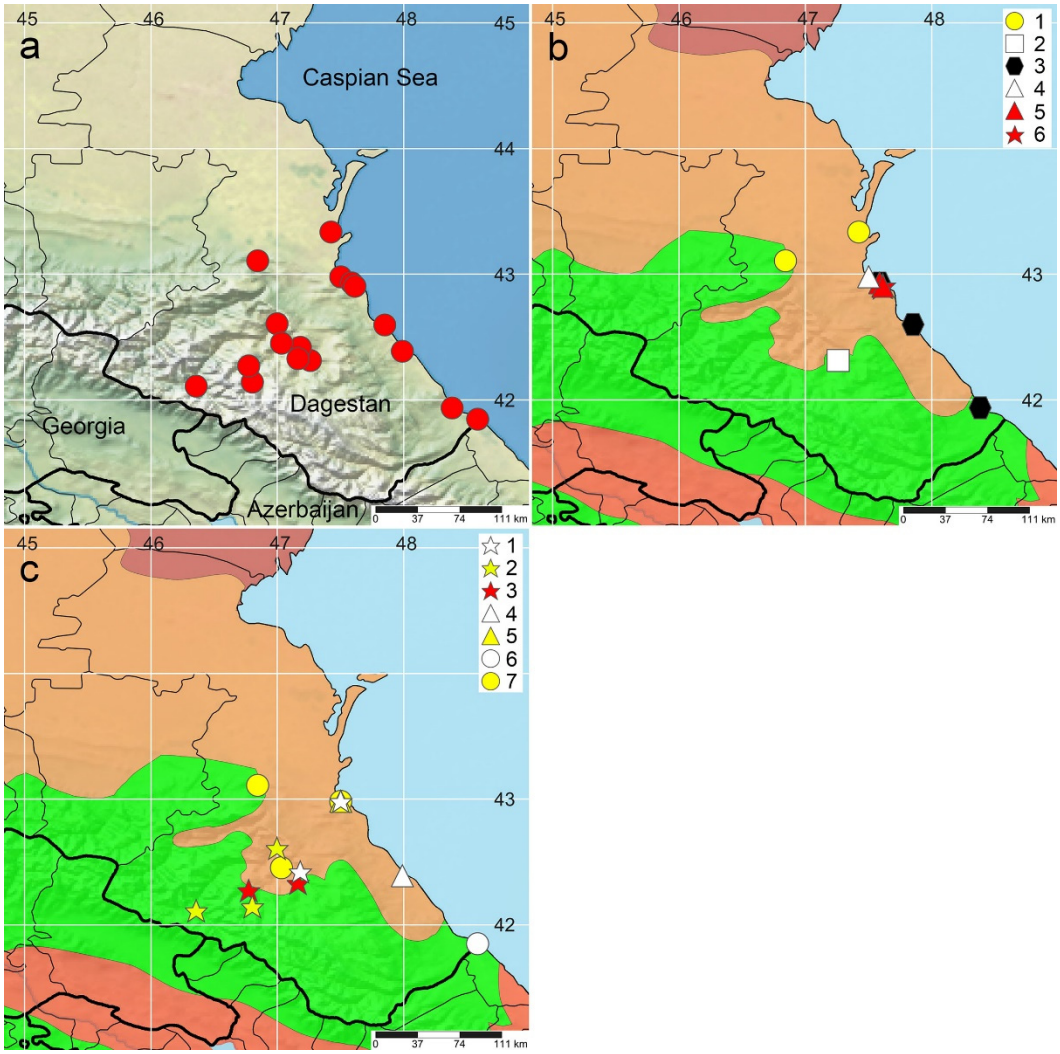

Figure 1. Species distribution in the context of elevation (**a**) and ecoregions (**b**,**c**) in the region studied at three sampling intervals: (**a**) – all species, (**b**): 1 – *Chara connivens*, XX century, 2 – *C. contraria*, XIX century, 3 – *C. globata*, XXI century, 4 – *C. neglecta*, XIX century, 5 – *C. neglecta*, XXI century, 6 – *Tolypella nidifica*, XXI century, (**c**): 1 – *C. gymnophylla*, XIX century, 2 – *C. gymnophylla*, XX century, 3 – *C. gymnophylla*, XXI century, 4 – *C. vulgaris* f. *vulgaris*, XIX century, 5 – *C. vulgaris* f. *vulgaris*, XX century, 6 – *C. vulgaris* f. *longibracteata*, XIX century, 7 – *C. vulgaris* f. *longibracteata*, XX century. Ecoregions [12]: dark brown – Caspian lowland desert, light brown – Pontic steppe, green – Caucasus mixed forests.

Chara globata Migula (Figures 1b, 4a–f)

Studied specimen: Coast of the Caspian Sea, Makhachkala, the settlement of Turali, small lake, 42.9317 N, 47.5865 E, 25 m b.s.l., together with *C. neglecta*, 28 VI 2020, M.M. Mallaliev (LE).

Records at iNaturalist [8]: 1. Derbentskiy District, vicinity of the village of Arablyar, oxbow of the Rubas River near mouth at the coast of the Caspian Sea, 41.9381 N, 48.3816 E, 03 V 2023, https://www.inaturalist.org/observations/159590371. 2. Kayakentskiy District, northern of the town of Izberbash, small coastal water body, abundant, 42.6005 N 47.8506 E, 05 VI 2023, https://www.inaturalist.org/observations/166546785.

Habitat: small coastal brackish water bodies.

Floristic novelty: new species record for Dagestan.

Chara gymnophylla A.Braun (Figures 1c, 2b–e, 3a–c)

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Figure 2. *Chara connivens* and *C. gymnophylla* from Dagestan (LE): (**a**) – general habit of pressed *C. connivens* from the Mekhteb Reservoir showing elongated general appearance and long slender branchlets; (**b**–**e**) – *C. gymnophylla* from the stagnant water body on the bank of the Karalazurger River: (**b**) – general appearance of living plant; (**c**,**e**) – diplostichous aulacanthous stem cortex with tiny ((**c**),

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arrowhead) and slightly elongate solitary spine cells ((**e**), arrowhead); (**d**) – whorl of well-developed completely ecorticate branchlets with long adaxial bract cells and conjoined gametangia (arrowheads). Scale: (**a**) – 5 cm, (**c**,**e**) – 1 mm, (**d**) – 4 mm. Photos: (**a**,**c**–**e**) – by R.E Romanov, (**b**) – by M.M. Mallaliev.

Studied specimens: 1. *Caucasus orientalis. Dagestania borealis, pr. Chodshalmaihi* [currently Khadzhalmakhi] *ad calarzailem, 500-600 hex* [elevation], 22 VI 1861, Ruprecht (LE A0001459). 2. Dagestan. obl. [Dagestan Oblast], Aul Gunib, 17 V 1890, W. Lipsky. Flora Caucasica (LE A0001311). 3. Dagestan, Gunib Okrug, valley of the river Karasu-Koysu [currently Karakoisu, called Tleiseruch in its middle reach] between settlements of Yryb and Ylyb, lateral bay of river, ca. 1700 m a.s.l., 31 VIII 1929, A. Poretzky. *Plantae dagestanicae anno 1929 collectae*. Geobotanic Expedition of the Institute of Dagestanian Culture of 1929 (LE). 3. Untsukulsky District, near the Aul of Arakany [Arakani], road to pass, beyond the gorge, near waterfall, elevation ca. 400 m, 29 VIII 1953, Ya.I. Prokhanov, N.T. Cheldyshev; Dagestan Agricultural Institute. Ya.I. Prokhanov, Plants of North Dagestan, No. 386 / *Chara gymnophylla* A.Braun f. *subnudifolia* Mig., det. M.M. Hollerbach, 26 II 1955 (LE). 4. Caucasus, Dagestan, Tlyaratinsky District, village of Tlyarata, south-east part, in a gorge, in shallow flowing water, 25 VII 1960, Dzhafarov (LE). 5. Levashi District, vicinity of the village of Tsudakhar, Sana River, 42.3291 N, 47.1613 E, 1124 m a.s.l., 08 VII 2020, M.M. Mallaliev (LE). 6. Charodinsky District, vicinity of the village of Gochada, stagnant water body at the bank of the Karalazurger River, 42.2716 N, 46.7718 E, 1506 m a.s.l., 29 VII 2020, M.M. Mallaliev (LE).

Habitat: mostly water bodies associated with mountain rivers, slow flowing reaches of mountain rivers.

Floristic novelty: new species record for Dagestan.

Chara neglecta Hollerbach (Figures 1b, 3e,f, 5)

Studied specimens: 1. Dagestan, Petrovsk [Makhachkala], 02 VII 1891, W. Lipsky / *C. aspera* Willd., det. R. Romanov, 07 X 2015 (LE). 2. Coast of the Caspian Sea, the town of Kaspiysk, in small stagnant water body, 42.9048 N, 47.6117 E, 21 m b.s.l., together with *Tolypella nidifica* (O.F.Müll.) A.Braun, 07 VI 2020, M.M. Mallaliev (LE). 3. Coast of the Caspian Sea, Makhachkala, the settlement of Turali, small lake, 42.9317 N, 47.5865 E, 25 m b.s.l., together with *C. globata*, 28 VI 2020, M.M. Mallaliev (LE).

Habitat: small coastal brackish water bodies.

Floristic novelty: new species record for Dagestan, second species report for the Caucasus.

Chara vulgaris L. f. *vulgaris* (Figure 1c)

Studied specimens: 1. Dagestan, [no locality], *Flora Caucasica* No. 4428 (LE). 2. Makhachkala, bog, in a ditch, 25 V 1953, Prokhanov, No. 113 (LE).

Published records: 1. "Caucasus. Dagestan, distr. Dargi. In paludosis ad fl. Arkuscha inter pagos Urkhuwah et Urchaczi 4000′ (10. et 16. VII. 1898, leg. Th. Alexeenko)" (under C. foetida A.Braun; [5]). This specimen belongs to C. contraria (see above). 2. "Prov. Dagestan, distr. Kaitag – Tabassaran. Pr. st. Kajakent [currently Kajagent]. In fossis humidis (17/VII 1900, leg. Alekseenko)" (under C. foetida f. condensata A.Braun; Vilhelm, 1930).

Habitat: small lowland water bodies.

Floristic novelty: new, second one, population for Dagestan.

Chara vulgaris f. *longibracteata* (Kütz.) H.Groves (Figure 1c)

Studied specimens: 1. Makhachkala, bog, in a ditch, 25 V 1953, Prokhanov, No. 595 (LE). 2. [Kizilyurtovsky District] Chiryurt Reservoir at the Sulak River, in the bay at right coast, at the depth of 0.3 m, at oozy-sandy bottom, 14 VIII 1958, V.M. Katanskaya (LE). 3. [Gergebilsky District], Gergebil Reservoir, at shallow, in water from spring, 07 VIII 1968, V.M. Katanskaya (LE A0002061).

Published record: "Prov. Dagestan, distr. Kurink. In littore ad ostium fl. Jaloma (11/VIII 1899, leg. Alekseenko)" (under C. foetida f. longibracteata Mig.; [6]).

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Habitat: small lowland water body, bay and shallows of mountain water reservoirs, mouth of lowland river.

Floristic novelty: new populations for Dagestan.

Tolypella nidifica (O.F.Müll.) A.Braun (Figures 1b, 3d, 4g,h)

Studied specimen: Coast of the Caspian Sea, the town of Kaspiysk, in small stagnant water body, 42.9048 N, 47.6117 E, 21 m b.s.l., together with *C. neglecta*, 07 VI 2020, M.M. Mallaliev (LE).

Habitat: small coastal brackish water bodies.

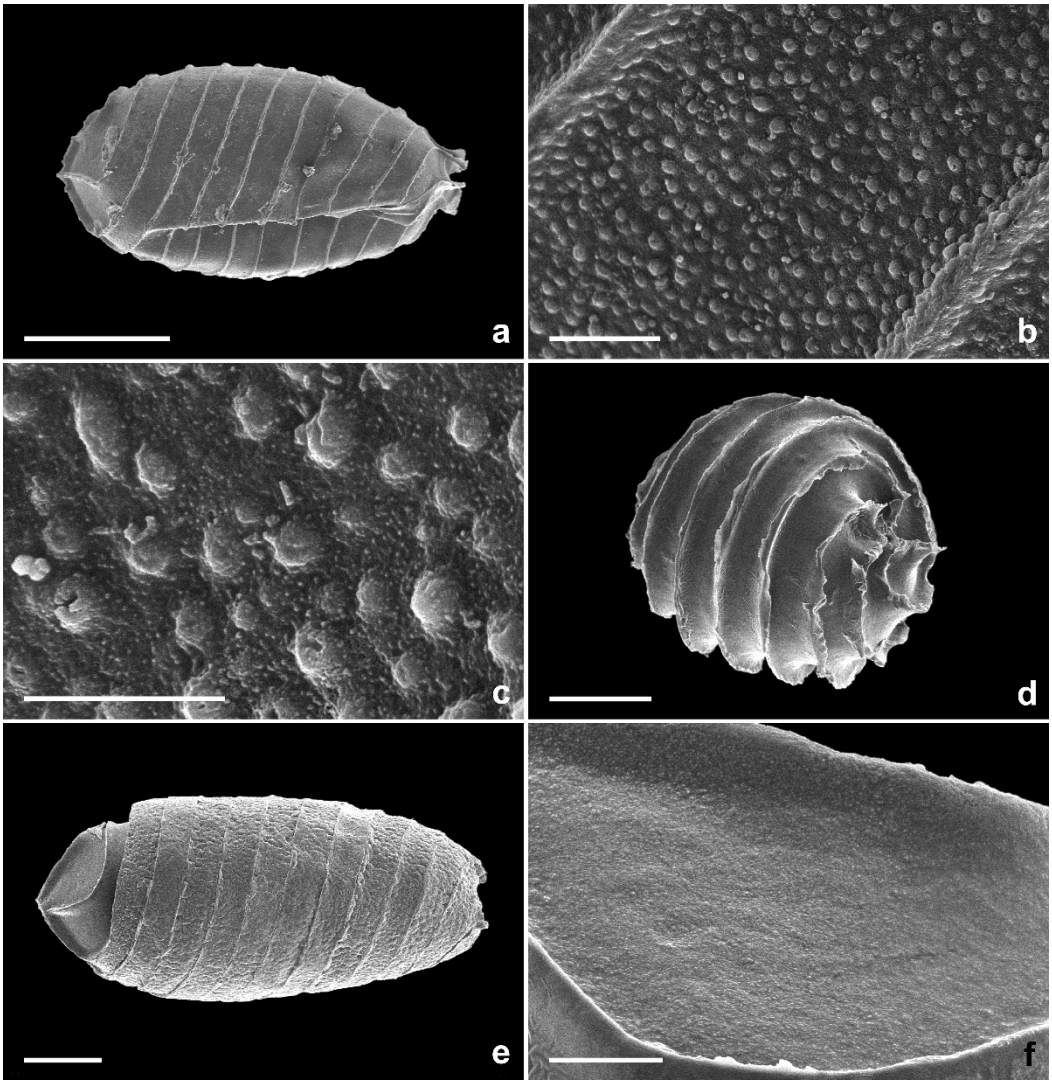

Figure 3. Oospores and gyrogonites of *Chara gymnophylla*, *C. neglecta* and *Tolypella nidifica* (LE), SEM: (**a**–**c**) – oospore of *C. gymnophylla* from the stagnant water body at the bank of the Karalazurger River: (**a**) – later view (ruptured during preparation), showing low ridges and absence of basal cage; (**b**,**c**) – surface of fossae, pustulate and densely minutely granulate between pustulae; (**d**) – tangential view of oospore of *T. nidifica* from small stagnant water body near the town of Kaspiysk, showing smooth surface and prominent ridges; (**e**,**f**) – gyrogonite and oospore surface of *C. neglecta* from small stagnant water body near the town of Kaspiysk: (**e**) – lateral view of gyrogonites missing apical part of lime shell, an apex of oospore with low ridges is visible; (**f**) – roughened surface of oospore fossa. Scale: (**a**) – 200 μm, (**b**) – 10 μm, (**c**) – 5 μm, (**d**,**e**) – 100 μm, (**f**) – 20 μm. Photos by R. Romanov.

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Figure 4. *Chara globata* and *Tolypella nidifica* from water bodies on the coast of the Caspian Sea (LE): (**a**,**c**) – upper parts of first morphotype of *C. globata* lacking evident lime incrustation and having long abaxial bract cells (arrowhead); (**b**) – upper part of the second morphotype of *C. globata* showing evident lime incrustation and abaxial bract cells of moderate length; it is really similar to *C. baltica* (Hartm.) Bruzelius and differs with strong lime incrustation only; (**c**) – whorl of branchlets of second morphotype of *C. globata* showing acute stipulodes of moderate length (arrowhead), abaxial bract

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cells of moderate length (double arrowhead) and mainly solitary aculeate spine cells of moderate length; (**e**) – nodes of branchlet of first morphotype of *C. globata* showing corticated segments, long verticillate bract cells and solitary conjoined gametangia (oogonium – arrowhead, antheridium – double arrowhead); (**f**) – starchless nodal bulbils of first morphotype of *C. globata*; (**g**) – apical part of rewetted specimen of *T. nidifica* showing obtuse end cells of branchlets (arrowhead); (**h**) – fertile head of rewetted specimen of *T. nidifica* showing presence of oogonia (arrowheads) and at least one antheridium (double arrowhead). Both morphotypes of *C. globata* were found in the same lake near the settlement of Turali. Scale: (**c**,**d**) – 5 mm, (**e**,**f**) – 1 mm. Photos: (**a**,**b**) – by M.M. Mallaliev, (**c**–**h**) – by R.E Romanov.

Floristic novelty: new species record for Dagestan, first species and genus record for the Caspian Sea region.

Few other records of *Chara* are known from Dagestan [8], but they cannot be identified at the species level according to available photos.

3.2. Species Identification and Phylogenetic Analysis

The identification of some *Chara* samples was based on a combination of morphological and molecular genetic approaches. DNA was isolated from most species, but our efforts were successful for only eight samples (five species: *C. connivens*, *C. globata*, *C. gymnophylla*, *C. neglecta*, *C. vulgaris* var. *longibracteata*). 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). Samples of *C. connivens, C. vulgaris* var. *longibracteata* and *C. globata* were placed within species clades (60/0.96, –/– and 65/– respectively), being part of the species haplotypes (Table S1). Samples of *C. neglecta* fell in shared haplotype with sequences of *C. galioides* and *C. aspera*. Three samples of *C. gymnophylla* represented a single haplotype that differed from the nearest (*C. gymnophylla* MN793052) by one substitution.

4. Discussion

Seven species of charophytes from two genera, *Chara* and *Tolypella* are reliably known from Dagestan. Six species are reported here for the first time for the area studied. The investigation of the biodiversity of charophytes of Dagestan using a polyphasic approach allowed us to conduct precise taxa identification of *Chara* species. Morphological traits of oospores studied with SEM were considered. They are in good agreement with species variability [11,29]. The image of gyrogonites and surface of oospore of *C. neglecta* was taken for the first time.

The overall topology of our *rbc*L tree (Figure 6) was similar to that presented in the previous studies of the genus *Chara* [16,20,30–32]. The sequences for *C. neglecta* were obtained for the first time. According to the molecular data, all samples (except *C. gymnophylla)* were part of the species haplotypes. Neither cryptic nor new species were found. The subsection *Hartmania* R.D.Wood of the genus *Chara* is widely known as "*crux et scandalum botanicorum*". In other words, it represents a group of species with still debatable and uncertain boundaries [11]. Molecular genetics failed to be helpful several times in this case [30,31,33,34] except for *C. globata*, a species delineable with *rbc*L sequences [10,35]. This was confirmed with this study too.

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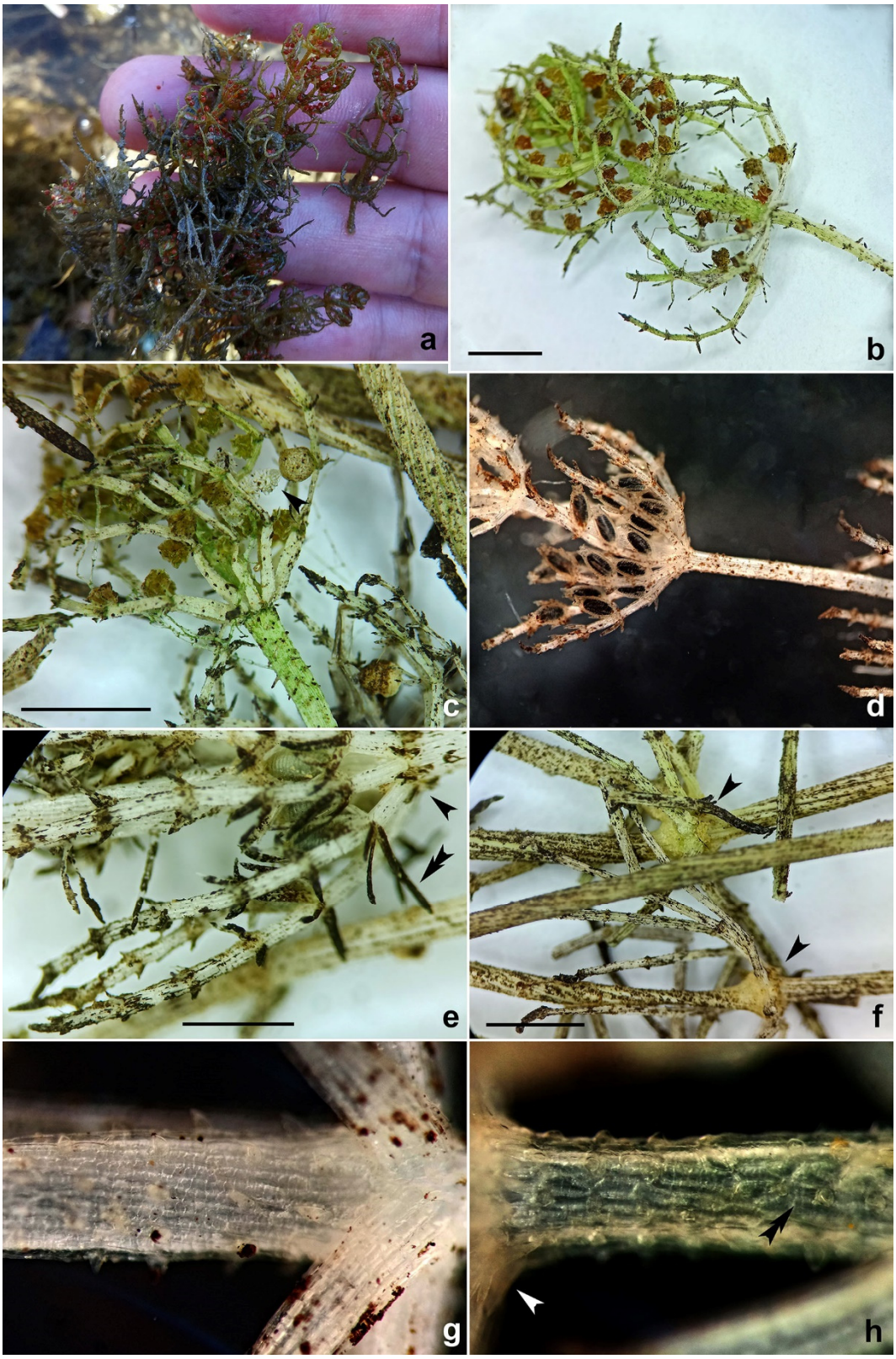

Figure 5. *Chara neglecta* from a small water body on the coast of the Caspian Sea near Kaspiysk (LE): (**a**) – upper parts of living robust male plants showing arcuate upper branchlets; (**b**,**c**) – upper part of pressed male plants showing arcuate completely corticated branchlets with solitary antheridia and verticillate bract cells, sparsely short spiny stems, aculeate diplostephanous stipulodes of moderate length (arrowhead indicates triangular shields of antheridium); (**d**) – well-developed whorl of branchlets of female plant (rewetted specimen) having smaller and slender appearance in comparison with male plants (sexual dimorphism), showing completely corticated branchlets with verticillate bract cells of moderate length and solitary oogonia with ripe black oospores, short aculeate stipulodes,

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short spine cells; (**e**) – branchlet and base of branchlet whorl of pressed female plant showing aculeate diplostephanous stipulodes of moderate length, completely corticated branchlets with short end cells, well-developed verticillate bract cells (double arrowhead), whose length gradually decrease from the base to the tip of branchlet; (**f**) – nodal starchless bulbils (arrowheads) of pressed plants; (**g**) – isostichous triplostichous stem cortex with short solitary acute spine cells (rewetted plant); (**h**) – short aculeate diplostephanous stipulodes (arrowhead) of rewetted plant and irregular diplo-triplostichous stem cortex, slightly aulacanthous (double arrowhead). Scale: (**b**,**c**,**f**) – 2 mm, (**e**,**g**) – 1 mm. Photos: (**a**) – by M.M. Mallaliev, (**b**–**h**) – by R.E Romanov.

Some further perspectives could be outlined. A detailed study of *C. neglecta* is required and will be conducted by the authors in the future for more populations for evaluation of its delineation or merger with *C. galioides*, because these species seem to be undistinguishable using *rbc*L sequences, although they can be separated with stem cortex arrangement, being weakly tylacanthous to isostichous triplostichous in case of *C. galioides* and weakly aulacanthous diplo-triplostichous to isostichous triplostichous in case of *C. neglecta* [11].

There are 25 species of charophytes known in the Caucasus (Table 1), but some species records require confirmation. Other Russian regions of the Caucasus as a whole and Azerbaijan have the richest charophyte floras among the regions compared. The species composition of the regions compared largely overlaps but is not identical to each other. Seven species known from Dagestan represent less than one third of the species richness of the Caucasus. This number seems to be low and new species records are expected according to the species distribution in the Caucasus and neighboring regions (Table 1). The species composition of charophytes from Dagestan is similar to those from Caucasian regions, whereas all other regions compared are closer to each other than to Dagestan if only reliable data are considered (Figure 7).

A few old species records [7] and specimens could not be georeferenced, even up to administrative regions or to the whole territory of the Caucasus, but they could be evidence of the possible occurrence of some species in the area studied. The collection by F.A. Marschall von Bieberstein (1768-1826) stored in LE (LE 01157109-01157113) and checked by the first author contained *Chara* cf. *globularis* Thuill., *C. vulgaris*, *Nitella opaca* (C.Agardh ex Bruzelius) C.Agardh and *Tolypella glomerata* (Desv. in Loisel.) Leonh. Some of them were collected in "*deserto Cumanum*" [36] i.e. in arid region of drainage basin of the Kuma River that belongs to Dagestan, Kalmykia and Stavropol Territory of Russia. The exact georeferencing of these specimens is not possible. Old records of *C. aspera* Willd. and *C. tomentosa* L. from the lake Atu-Kol of the Terek Oblast of the Russian Empire [37] could belong to the current territory of the Republic of Kalmykia. The occurrence of some species of *Nitella* C.Agardh, *Sphaerochara* Mädler, *Nitellopsis obtusa* (Desvaux) J.Groves and *Lamprothamnium papulosum* (Wallr.) J.Groves seems to be possible in Dagestan, although they are rarely found in West Asia [38–40].

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Table 1. Species of charophytes from different regions of the Caucasus.

Species	Dagestan[1]	Others[2]	Georgia[3]	Armenia[4]	Azerbaijan[5]
Chara baltica (Hartm.) Bruzelius	–	+	–	–	(+)
C. braunii C.C.Gmel.	–	–	+	–	+
C. canescens Desv. & Loisel. in Loisel.	–	+	+	+	+
C. connivens Salzm. ex A.Braun	+	+	+	–	+
C. contraria A.Braun ex Kütz.	+	+	+	+	+
C. denudata A.Braun	–	–	–	–	+
C. globata Migula	+	+	+	+	–
C. globularis Thuill.	–	+	+	+	+
C. gymnophylla A.Braun	+	+	+	+	+
C. hispida L.	–	–	+	–	(+)
C. neglecta Hollerbach	+	–	–	–	+
C. papillosa Kütz.	–	–	+	(+)	+
C. squamosa Desf.	–	+	–	–	–
C. strigosa A.Braun	–	–	–	–	(+)
C. tomentosa L.	–	(+)	–	–	–
C. vulgaris L.	+	+	+	+	+
Lamprothamnium papulosum (Wallr.) J.Groves	–	+	+	–	+
Nitella capillaris Krock.	–	–	+	–	+
N. flexilis (L.) C.Agardh	–	+	–	–	–
N. mucronata (A.Braun) Miq.	–	+	–	–	–
N. opaca (C.Agardh ex Bruzelius) C.Agardh	–	–	+	–	–
Nitellopsis obtusa (Desvaux) J.Groves	–	–	+	–	–
Sphaerochara prolifera (Ziz ex A.Braun) Soulié-Märsche	–	–	+	–	–
Tolypella glomerata (Desv. in Loisel.) Leonh.	–	–	+	–	+
T. nidifica (O.F.Müll.) A.Braun	+	+	+	–	–
Number of species	7	13(14)	17	6(7)	13(16)

[1] A comprehensive bibliography is impossible to report within the scope of this article so the cited references are those that include all known species from the territory. [1] this study, [2] Russian regions of the Caucasus excl. the Republic of Dagestan: Chechen Republic, Kabardino-Balkarian Republic, Karachayevo-Circassian Republic, Krasnodar Territory, Republic of Adygeya, Republic of Ingushetia, Republic of North Ossetia – Alania, Stavropol Territory [7,40–46], [3] [7,37–49], [4] [7,11,37,50–53], [5] [6,7,32,37,40,54–57]. (+) –: Other Russian regions of the Caucasus – a record needs to be confirmed with specimen study, existence of voucher is unknown; Armenia – a record from Lake Sevan needs in confirmation, because it could be actually based on misidentification of *C. globata*, Azerbaijan – recent record of *C. baltica* needs to be confirmed with independent study of specimen, the old record of *C. hispida* needs in confirmation because it could be based on another species.

Despite a long interval of collecting, 1861–2020, only 22 localities are still known from this region, which could be explained by the fact that the charophytes were a neglected group in this region for a long time. The new species records from Dagestan improve species distribution ranges, filling in gaps and even changing their outlines in several cases. New localities of *C. connivens* filled the gap between a few localities in the South Caucasus and the Lower Volga region [7,11,40,58].

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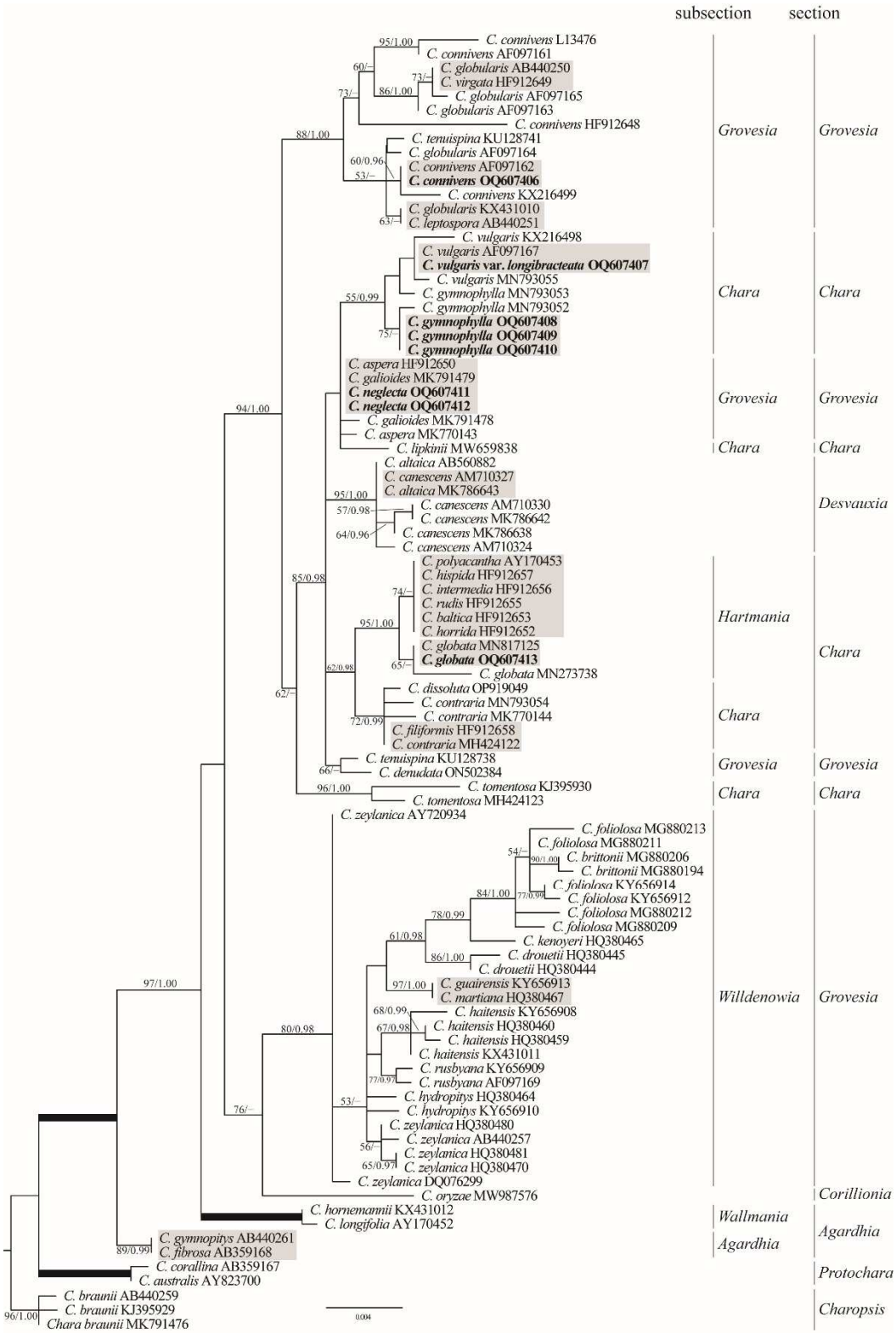

Figure 6. Maximum likelihood phylogenetic tree inferred in PAUP with the GTR+I+G nucleotide substitution model from 92 *rbc*L sequences of *Chara*. ML BP (>50%) and BI PP (>0.95). Branches received 100% BP and 1.00 PP support, and the newly obtained sequences are shown in bold. Sequences carrying one genotype are marked with grey. *Chara* sections and subsections are based on [27] with changes from [20,28].

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New localities of *C. contraria*, *C. vulgaris* and, especially, *C. gymnophylla* add essential details to their distribution in the Caucasus. *Chara contraria* and *C. vulgaris* are widely distributed species. They are generalists in many temporal regions [59]. *Chara gymnophylla* is the most frequently found species in the area studied. It has a wide distribution area in Eurasia, but most records are concentrated in the Mediterranean region and the Middle East [11,60–62]. It is the second most common species in Iran [61] and northern Israel [62]. Its records are unknown north of the area studied [11,40], i.e. in the south-eastern margin of Eastern Europe, and its northern distribution range can be tentatively outlined with the piedmont area of the North Caucasus.

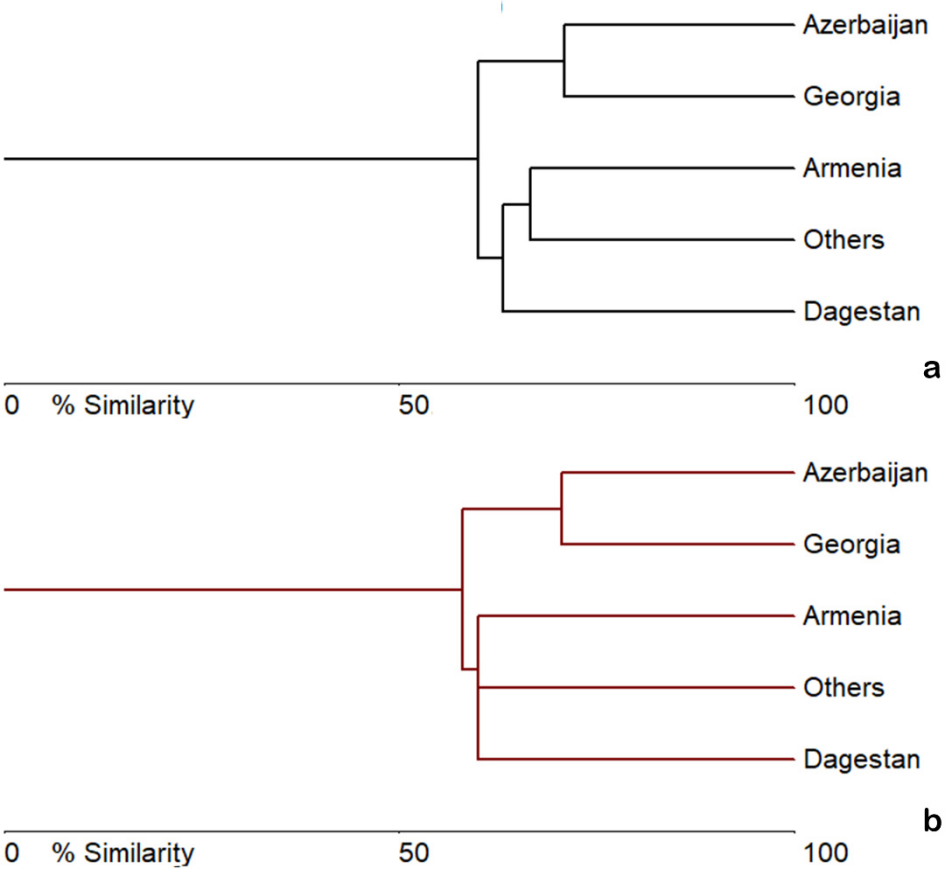

Figure 7. The similarity of charophytes species composition of different regions of the Caucasus according to Bray-Curtis cluster analysis (single link): (**a**) – based on reliable records, (**b**) – based on all records; others: Russian regions of the Caucasus excl. the Republic of Dagestan: Chechen Republic, Kabardino-Balkarian Republic, Karachayevo-Circassian Republic, Krasnodar Territory, Republic of Adygeya, Republic of Ingushetia, Republic of North Ossetia – Alania, Stavropol Territory.

Chara globata is mainly a Central Asian species with several localities in Southeast Europe, North Africa, Middle East and China [16,35,61,63,64]. Its distribution area extends north of the Caucasus to arid regions of Volga and Don interfluve [11,40]. It is known from few localities in the Caucasus, although its remarkable habit and size nearly excludes its undersampling during targeted searches for charophytes and allows it to be used as a flagship species for biogeographical studies [10]. The records from small water bodies on the coast of the Caspian Sea add a new type of habitat known for this species mostly associated with much bigger permanent lakes [11,62].

Chara neglecta is a species mostly known from the south of Eastern Europe and Central Asia [11]. In the Caucasus, it was formerly known only in Azerbaijan [47,54,55]. New records in Dagestan point towards its wider distribution in coastal regions of the Caspian Sea.

Tolypella nidifica is a species occurring mostly in coastal regions of Europe, where it is mostly known from north and west Europe as well as west Mediterranean [11]. Only two reliable small areas

are known from the North Black Sea region [11,40,43,65,66] and one recent record from inland Georgia by V.S. Vishnyakov [8,49], https://www.inaturalist.org/observations/156074494. The record from the inland of Crimea [66,67] belongs to *Sphaerochara prolifera*, according to published images. Lake Issyk Kul is the sole locality for this species in Central Asia [47]. The unexpected new record from Dagestan is the first for the Caspian Sea region and fills the gap in the eastern part of the species distribution area in Eurasia.

The combination of species with distribution areas mainly in the centre of Eurasia, such as *C. globata* and *C. neglecta* with mainly Mediterranean – Middle East *C. gymnophylla* is a notable trait of charophyte flora of the area studied. It seems to be a trait common to other Caucasian regions.

The habitat preference of species recorded from the area studied allows tentative suggestion of two main groups of habitats having dissimilar species compositions. *Chara contraria*, *C. gymnophylla* and *C. vulgaris* have been found mostly in freshwater small water bodies usually associated with rivers. *Chara globata*, *C. neglecta* and *Tolypella nidifica* are known only from small brackish water bodies at the coast of the Caspian Sea. They grew together here with *Ruppia maritima* L. during our survey, which evidently indicates a brackish environment. *Chara connivens* falls outside this scheme because it is known only from two large artificial mountain and coastal water reservoirs. Surveys of the region, especially of oligotrophic mountain, brackish coastal and lowland temporal spring water bodies, seem to be the most fruitful in further clarification of biogeography of charophytes in the North Caucasus.

The suggestion of some species for protection is possible based on the habitat preference and distribution in the Caucasus. Coastal water bodies threatened from both land and sea sides are one of the most endangered and shrinking habitats world-wide [68,69]. There is no assessment of charophyte habitat loss at the coast of the Caspian Sea are available, but long-term negative trend is notable for the Black Sea region, where it is already resulted in significant decrease of charophytes biomass and area of their communities [70–73]. The evident loss of coastal habitat can be recognized as too late for the restoration of initial state [74] and may result in irreversible disappearance of brackish water charophytes. Therefore, *Chara globata*, *C. neglecta*, *Tolypella nidifica* and their habitats are suggested here as a primary target for charophyte protection in Dagestan.

The territory of Dagestan is recognized as a floristic province at the beginning of study of its flora. It is one of key areas for speciation of xerophytic flora at the Caucasus, which can be proven with a high number of local endemics of magnoliophytes [75]. The absence of endemic species among charophytes from Dagestan and the Caucasus contrasts with the flora of terrestrial magnoliophytes, which have a large number of endemic species [76–78]. In contrast, the flora of the submersed aquatic magnoliophytes of this area consists of mostly widely distributed species [51,77–80]. Therefore, the aquatic ecosystems of this region do not seem to be an area with ongoing diversification and speciation of submersed aquatic macroscopic plants. This paradox looks similar to Tajikistan, which has no unique species of charophytes, in contrast with its original flora of terrestrial magnoliophytes [81].

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, 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.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. In addition, the data that support the findings of this study are openly available in GenBank.

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