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# *Chara zhengzhouensis* (Characeae, Charophyta), a New Freshwater Algal Species Described from North China

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**Abstract:** A new species of freshwater alga, *Chara zhengzhouensis*, is described and illustrated based on material collected in a lake at Zhengzhou, in Henan Province, China. Phylogenetic analysis of sequence data from the 18s rDNA, *rbc*L, and *atp*B indicated the separation between *C. zhengzhouensis* and other species of the genus *Chara*. Additionally, from a morphological point of view, *C. zhengzhouensis* differs from another closely related species, *C. connivens*, in its diplostichous cortex and corticate or ecorticate in based segments of branchlets. Therefore, the results based on both morphological observation and molecular evidence facilitated the proposal of this new species—*C. zhengzhouensis*. It represents another species in the charophyte diversity in China and the description of this new species provides more molecular data for phylogenetic analysis of the genus *Chara*.

**Keywords:** *Chara*; new species; morphological characteristics; molecular phylogenetics; secondary structure analysis



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## 1. Introduction

The phylum Charophyta comprises large freshwater submerged algal plants, which can grow in both fresh water and salt water and are widely distributed on all continents of the world. They can use photosynthesis to produce oxygen; therefore they may improve water quality. In addition, according to the relevant literature, charophytes also have certain applications in pesticides, agriculture, medicine, etc. [1].

Charophytes, often called stoneworts, are a group of autotrophic and macroscopic algae represented by more than 400 species assigned to the Characeae family within the Charophyta phylum (Schneider et al. 2015; Guiry and Guiry 2019).

*Chara* is a genus of Charophyta growing in alkaline freshwater. Its sexual reproduction is via oogamy. The male and female gametocyte spermatozoa and ovipositor are the unique reproductive methods of charophytes different from those of other small algae. The members of the genus are fixed on habitats and grow in relatively clear freshwater, but are not common in coastal habitats [2]. At present, there are more than 200 known taxa, which are distributed on all continents of the world (https://www.algaebase.org/browse/taxonomy/#7030 accessed on 10 September 2022).

In recent years, the research on charophytes has been very active, especially on their taxonomy. Some authors believe that charophytes are algae with potential relatives in terrestrial plants [3,4]. In order to more clearly determine the taxonomic status of charophytes, Bhattacharya et al. have performed related research on both morphology and genome [5]. The phylogenetic relationship between the main lineages of green algae was determined by DNA sequence molecular data analysis, and it was found that the charophyte was at the end of the branch of the green algal phylogenetic tree [6]. The latest research abroad found that *C. braunii*, like land plants, has a cell wall composed of cellulose embedded in a matrix of pectin and hemicellulose [7,8]. Charophytes are more strongly demonstrated to be close relatives of land plants [9].

The tribe *Chareae* was originally discovered and established by Braun [10], and it is crucial to incorporate a new species into an existing genus and to assess its taxonomy. In 2013,

a large number of charophytes found in the Himalayas included an undetermined species. In addition to its taxonomic identification, the taxonomic status of known algal species was replanned [11]. Changes in environmental conditions may cause certain changes in some morphological characters of charophytes [12], so it is important to combine morphological and molecular phylogenetic characters for their classification [13]. Among them, the nuclear-encoded 18S rDNA sequence has been the main data source for inferring phylogenetic relationships among green algae [14], and reconstructing ancestral characteristics can help reinterpret known specimens with uncertain affinities.

Microscopic features such as stipules and cortexes are often used for the classification of charophytes [15,16]. Molecular data allow for more accurate identification of new species. The members of *Chara* are mainly distributed in central and eastern China; their adaptability is stronger than that of the genus *Nitella*, and they are mostly found in subtropical and temperate regions [17]. A charophyte sample found in Ya'an, Sichuan, has the morphological characteristics of both *Chara* and *Nitella*. In this case, the role of molecular methods in identification is very important [18]. At the same time, the internal phylogeny of charophytes was also investigated, and the classification of charophyte was discussed at the molecular level [19].

In this paper, we report a new freshwater species collected from Henan Province based on morphological and molecular evidence. This research improves our knowledge of the genus *Chara* in China and provides information on the phylogenetic relationship of this freshwater algal genus.

## 2. Materials and Methods

### *2.1. Specimen Collection*

The samples were collected on 4 July 2021, in the Wetland Park of Zhengdong New District, Zhengzhou City, Henan Province, China (34°78′ N, 113°73′ E) (Figure 1). The alga grows in the slow-flowing water, and some physicochemical parameters are shown in Table 1.

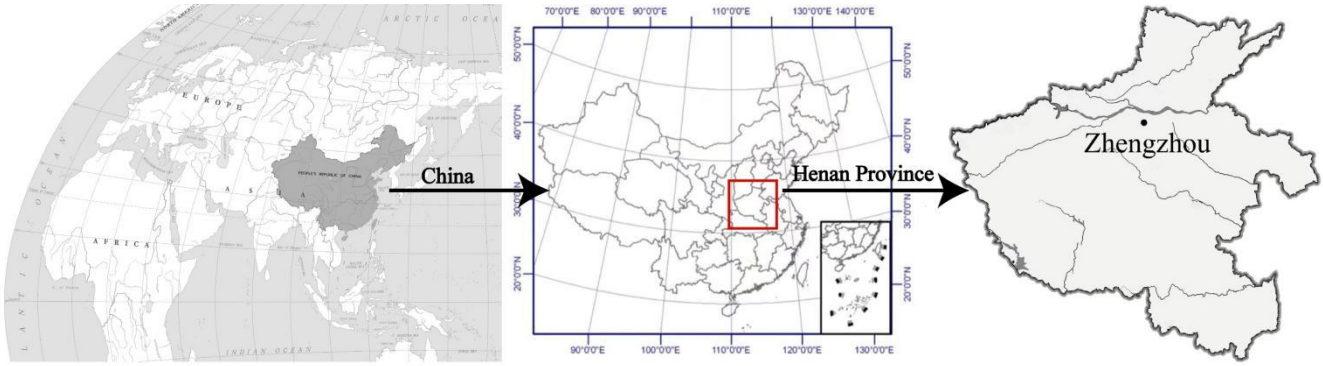


**Figure 1.** Distribution of sampling location in Henan Province, China.

**Table 1.** Physicochemical parameters of water in habitat.

| Parameters | Water Temperature | pH | Turbidity | Conductivity | Water Velocity |
|---|---|---|---|---|---|
| value | 16 °C | 7.5 | 1.9 ftu | 1042 μs.cm$^{-1}$ | 0.15 m.s$^{-1}$ |
| **Parameters** | **Dissolved oxygen** | **Total nitrogen** | **Total phosphorus** | **Ammonia nitrogen** | **Water depth** |
| value | 13.4 mg.L$^{-1}$ | 234.1 mg.L$^{-1}$ | 6.4 mg.L$^{-1}$ | 32.6 mg.L$^{-1}$ | 0.5 m |

Materials were repeatedly rinsed with deionized water to remove epiphytes and sediment. Specimens for DNA extraction were stored in silica gel, whereas the material for morphological examination was placed in centrifuge tubes and fixed with 4% formalin solution.

### 2.2. Morphological Observation

For morphological observations, both fresh and formalin preserved thalli (unstained) were observed with an optical microscope (BX51, Olympus), outfitted with a digital camera (DP72) for microphotography.

The specimens (No. SXU-HN210704) were placed in a bottle and fixed with 4% formalin solution and deposited in the herbarium of Shanxi University (SXU), Taiyuan, Shanxi Province, China.

### 2.3. Herbarium

The preparation of the herbarium of *Chara zhengzhouensis* was according to the guidelines [20–22]. The algal sample was blotted with absorbent paper to remove moisture and put carefully on the card. The sheet was then provided with an herbarium label. The sample was attached to the labeled sheet using Drobnik's method [20]. A voucher specimen (Figure 2) was deposited in the herbarium of Shanxi University (SXU), Taiyuan, Shanxi Province, China.

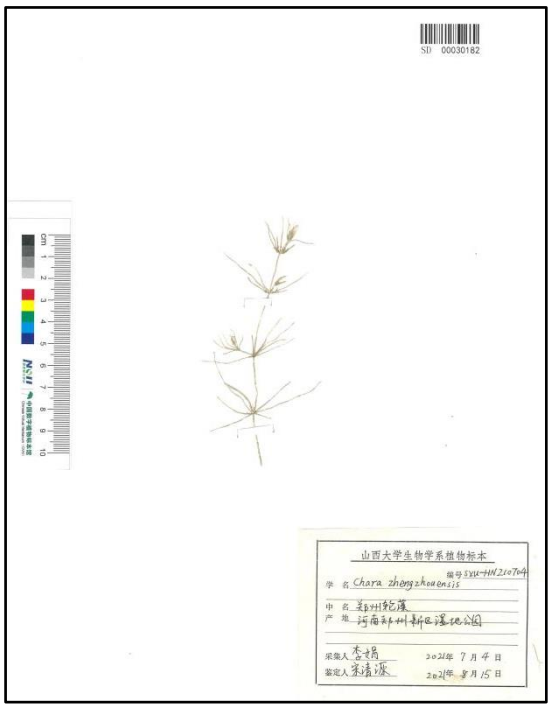


**Figure 2.** Herbarium specimen of *Chara zhengzhouensis* from Henan, China (deposited in SXU herbarium). Prepared and scanned by Q. Y. Song.

### 2.4. Sequence Amplification

Total DNA was extracted from algal material. About 0.5 g sample (including silica gel powder) was taken, and added with liquid nitrogen to a clean mortar for thorough grinding, then the Easy Pure Plant Genomic DNA Kit (Quanshijin Biotechnology Co., Ltd., Beijing, China) was used according to the instructions for extraction.

Refer to the literature to determine the polymerase chain reaction (PCR) amplification system and primers (Table 2) [18,23]. PCR was performed in the thermal cycler mainframe using designed primers. The total volume of 20 μL included: 2.0 μL 10 × buffer ($Mg^{2+}$), 2.0 μL dNTPs, 2.0 μL each of forward primer and reverse primer, 0.2 μL Taq DNA polymerase (all from BGI Biotechnology Co., Ltd., Shenzhen, China), 1.0 μL of extracted algal DNA, and 10.8 μL of autoclaved double-distilled water. The samples were amplified in separate PCR reactions to prevent sequencing confusion.

**Table 2.** Summary of oligonucleotide primers used for polymerase chain reaction (PCR) and sequencing.

| Primer | Sequence | Target | Direction |
|---|---|---|---|
| *Chara*-18s1 | 5′-CCGGAAAGCTGCAGGTCTAT-3′ | 18S rDNA | Forward |
| *Chara*-18s2 | 5′-CGGATAACTGGTGCGTCAAA-3′ | 18S rDNA | Reverse |
| *Chara*-r3 | 5′-CGGGCGAAATCAAAGGACAT-3′ | *rbc*L | Forward |
| *Chara*-r4 | 5′-GAAGAAGTAGGCCGTTGTCG -3′ | *rbc*L | Reverse |
| *Chara*-a5 | 5′-CTCGTGACTGGGATCAAGGT-3′ | *atp*B | Forward |
| *Chara*-a6 | 5′-GACTGATAACTCCGGAGCCA-3′ | *atp*B | Reverse |

A typical PCR amplification profile for this experiment consisted of initial denaturation at 94 °C for 5 min, followed by denaturation for 30 s and extension at 72 °C for 1 min (18S rDNA and *atp*B) or 30 s (*rbc*L) for 35 cycles; then, final denaturation was carried out at 72 °C for 10 min longer. The annealing temperature was 55 °C. After the PCR product was amplified, it was analyzed by 1% agarose gel electrophoresis (120 V, 20 min) [19,24–26]. The clear and bright bands were selected and sent to BGI (Beijing) for bidirectional sequencing by Sanger dideoxy termination method.

### 2.5. Construction of Phylogenetic Tree

We selected 18S rDNA, *atp*B, and *rbc*L of *Chara* and other related sequences from NCBI. The sequences obtained by forward and reverse sequencing were spliced and sorted by Contig Express [27], and then aligned with the downloaded sequences. These sequences were manually corrected and edited in Bio-Edit to facilitate the next steps [28]. In order to make the identification more accurate, three methods were used to construct the phylogenetic trees: maximum likelihood method (ML), Bayesian inference method (BI), and neighbor-joining method (NJ) [29,30].

After using the Clustalw module in MEGA6.1.0 to perform multiple sequence alignment analysis, the conserved sites, variant sites, and parsimony information sites were analyzed [31]. After the FASTA sequences were aligned in MEGA6.1.0, an NJ tree was constructed and saved in EMF format for further analysis. The BI tree was constructed using the software Mrbayes-3.1.22, and the ML tree was constructed using two software packages, PAUP*4.0b10 and PhyML-3.0 [32,33].

Modeltest3.7 [34] was used to determine the parameters and the best surrogate model for each marker (Table 3).

**Table 3.** Nucleotide substitution model parameter estimates for Modeltest 3.7 analyses.

| Gene | Model Selected | Base Frequencies | Rate Matrix |
|---|---|---|---|
| 18S rDNA | TrN+I+G<br>−lnL = 4623.7183<br>K = 7<br>AIC = 9261.4365<br>(I) = 0.5183<br>(G) = 0.7373 | freqA = 0.2429<br>freqC = 0.2113<br>freqG = 0.2840<br>freqT = 0.2617 | R(a) [A-C] = 1.0000<br>R(b) [A-G] = 2.3888<br>R(c) [A-T] = 1.0000<br>R(d) [C-G] = 1.0000<br>R(e) [C-T] = 10.4735<br>R(f) [G-T] = 1.0000 |
| *rbc*L | GTR+I+G<br>−lnL = 4215.2666<br>K = 10<br>AIC = 8450.5332<br>(I) = 0.4206<br>(G) = 0.8503 | freqA = 0.2972<br>freqC = 0.1468<br>freqG = 0.2121<br>freqT = 0.3439 | R(a) [A-C] = 2.6430<br>R(b) [A-G] = 5.7966<br>R(c) [A-T] = 2.4732<br>R(d) [C-G] = 1.9510<br>R(e) [C-T] =15.1225<br>R(f) [G-T] = 1.0000 |
| *atp*B | GTR+G<br>−lnL = 3441.4927<br>K = 9<br>AIC = 6900.9854<br>(I) = 0<br>(G) = 0.1286 | freqA = 0.3583<br>freqC = 0.1199<br>freqG = 0.1549<br>freqT = 0.3669 | R(a) [A-C] = 3.7343<br>R(b) [A-G] = 8.3657<br>R(c) [A-T] = 0.7536<br>R(d) [C-G] = 5.5236<br>R(e) [C-T] = 14.4017<br>R(f) [G-T] = 1.0000 |

## 3. Results

### *3.1. Morphological Description*

***Chara zhengzhouensis*** Q. Song, X. Liu, F. Nan, Q. Liu, J. Lv, J. Feng and S. Xie. sp. nov. (Figure 3) .

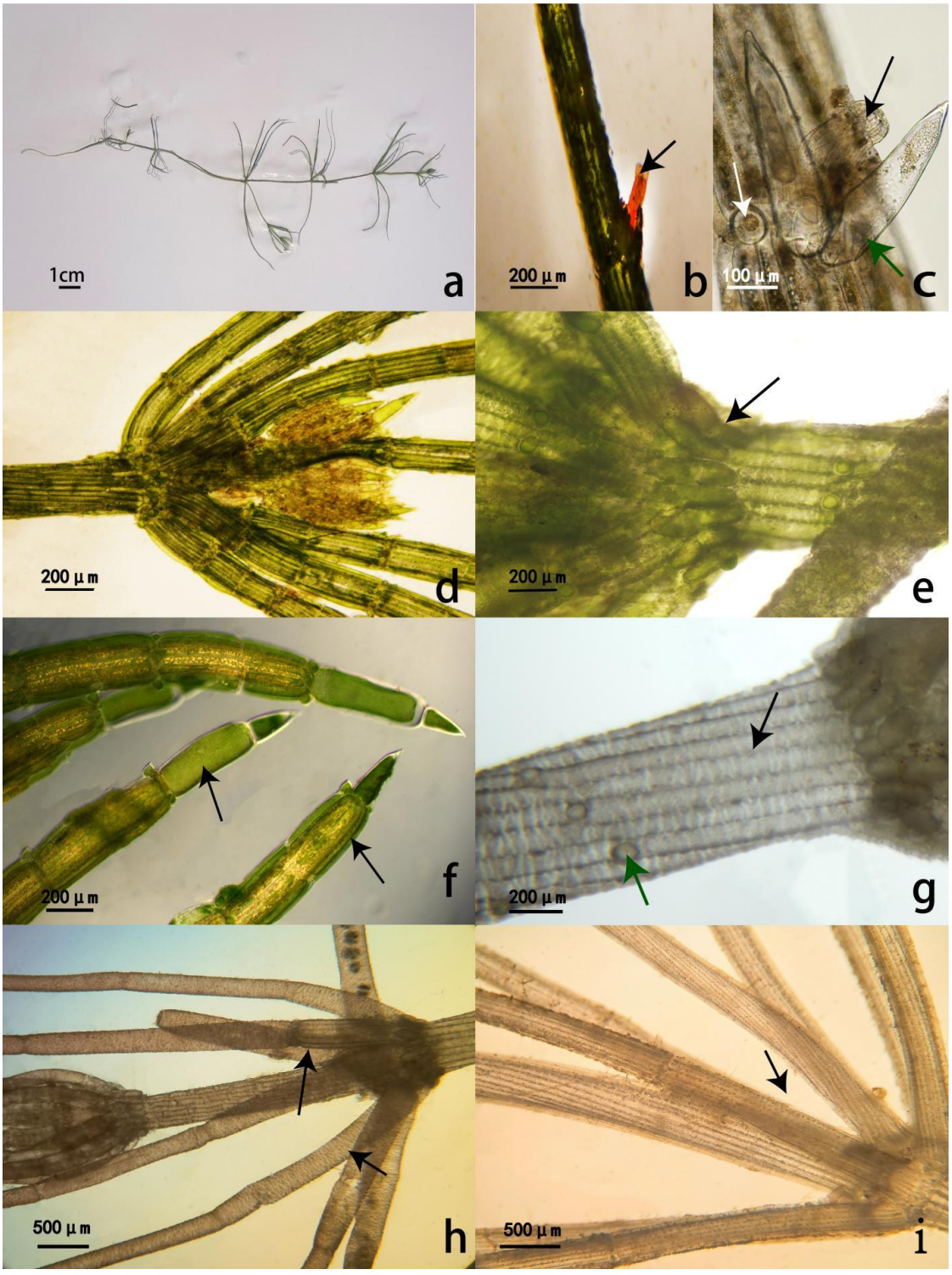


**Figure 3.** Morphological characteristics of *Chara zhengzhouensis* collected in Zhengzhou, China. (**a**) Thallus; (**b**,**c**) oogonium (black arrow), bract cells (white arrow) and bracteoles (green allow); (**d**,**e**) whorl with stipulodes; (**f**) ultimate segments of branchlets; (**g**) stem with cortex (black arrow) and spine cells (green arrow); (**h**,**i**) based segments of branchlets corticate or ecorticate.

**Taxonomy:** Class Charophyceae, Order Charales, Family Characeae.

**Etymology:** The species epithet refers to the type locality (Zhengzhou, China).

**Type locality:** Zhengzhou City (34°78′ N, 113°73′ E), Henan Province, China.

**Holotype:** SXU-HN210704, collected by Juan Li, July 2021 and deposited in the SXU, Shanxi University, Taiyuan, Shanxi Province, China. Voucher number No. SXU-HN210714.

**Authentic strain:** SXU-HN210714.

**Habitat**: Plants grow fixedly on the sand and stone at a water depth of 0.5 m.

**Iconotype:** Figure 3a–i.

**Representative DNA Barcode:** GenBank OP286990 for 18S rDNA, OP286950 for *rbc*L and OP286951 for *atp*B.

**Description:** The plant is a large alga, around 20 cm high. The slightly encrusted and slender thalli are delicate green. Stems are medium thick and 400–600 μm in diameter and with the complete cortex, which is regularly dichotomous and the primary series was larger than the secondary. The internode is 1–1.5 times as long as the branchlet. Spine cells are solitary and papillate, 50–100 μm long. The stipulodes are in both rows well developed, about 190–240 μm long and 40–65 μm wide, opposite to the branchlets. Some of the stipulodes degenerate into papillates, about 40–60 μm long, and there are twice as many of these as the branchlets. Branchlets are 6–8 in whorls 2–3 cm long, straight or somewhat incurved, of 6–8 segments, corticate or ecorticate, and the ultimate 1–2 ecorticate and acute. The bract cells are usually 5 in number, papillate, 10–17 μm long. Bracteoles are 2, to 250–300 μm long. Plant is dioecious, and only immature oogonia are found, 300–400 μm long (excl. coronula) and 150–250 μm wide.

**Diagnosis:** This species is similar to *Chara connivens*, but the cortex of the latter is triplotichous and obviously different from this species [35–38]. It is also related to *Chara virgata* (=*C. delicatula*), but the latter is of triplotichous cortex and monoecious [36,39,40] (Table 4).

**Table 4.** Morphological comparisons of *Chara zhengzhouensis* with two other similar species.

| Character | *C. zhengzhouensis* | *C. connivens* | *C. virgata* |
|---|---|---|---|
| stipulodes | 2 rows, well developed, 190–240 μm long, sometimes degenerate | 2 rows, papillous | 2 rows, upper row well developed and acute, lower row short to papillous |
| cortex | dichotomous | triplostichous | triplostichous |
| spine cells | solitary, papillate | solitary, papillate | solitary, papillate |
| cortex of based segments of branchlets | corticate or ecorticate | corticate | corticate |
| bract cells | usually 5, papillate | 5–7, papillate | 5–6, inside well developed, outside papillate |
| ♀or ♂/♀ | dioecious | dioecious | monoecious |
| habitat | freshwater; ditch; oligotrophic water | freshwater or brackish water; ponds, ditches, and rice fields; mesotrophic to eutrophic water | freshwater or or occasionally brackish water; lakes to ditches and streams; oligotrophic water |
| distribution | Asia (China) | Asia, Africa, and Europe | Asia, Europe, North America, South America, Australia and New Zealand |

### *3.2. Phylogenetic Analysis*

The length of the 18S rDNA fragment used for phylogenetic analysis in this study was 1430 bp, of which 281 bp (19.7%) were variable sites and 173 bp (12.1%) were informative for parsimony. The length of the *rbc*L fragment used for phylogenetic analysis in this study was 1039 bp, of which 330 bp (31.8%) were variable sites and 189 bp (18.2%) were informative for parsimony. The length of the *atp*B fragment used for phylogenetic analysis in this study was 832 bp, of which 251 bp (30.2%) were variable sites and 158 bp (19.0%) were informative for parsimony. The genetic distances of three genes were calculated by

MEGA6.0, which showed that the transition and transversion between *Chara zhengzhou* and *C. connivens* have not reached saturation [41].

All three methods, maximum likelihood, neighbor joining, and Bayesian inference, produced similar topologies. Therefore, only the Bayesian trees are shown in Figures 4–6, respectively, with all the supporting values included on the nodes (greater than 50%). In the 18S rDNA phylogenetic tree (Figure 4), there were 30 accessible sequences of the genus *Chara* in the GenBank database. *Coleochaete nitellarum* and *Zygnema peliosporum* of the Chlorophyta were selected as the outgroups. *Chara zhengzhouensis* formed a self-contained branch, and the supporting values were 640/0.82/66. In *rbc*L phylogenetic tree (Figure 5), there were 30 accessible sequences of the genus *Chara* in the GenBank database. *Chara zhengzhouensis* and *C. connivens* gathered as sister clades (685/0.99/69). In the *atp*B phylogenetic tree (Figure 6), there were 21 accessible sequences of the genus *Chara* in the GenBank database. *Chara zhengzhouensis* was clustered with *C. connivens* and *C. virgata* (799/0.79/81).

Further, the secondary structure of 18S rDNA of *Chara zhengzhouensis* and *C. connivens* were predicted and compared (http://rna.tbi.univie.ac.at/ (accessed on 10 September 2022)). It can be found that both have great differences in base position and arrangement sequence (Figure 7).

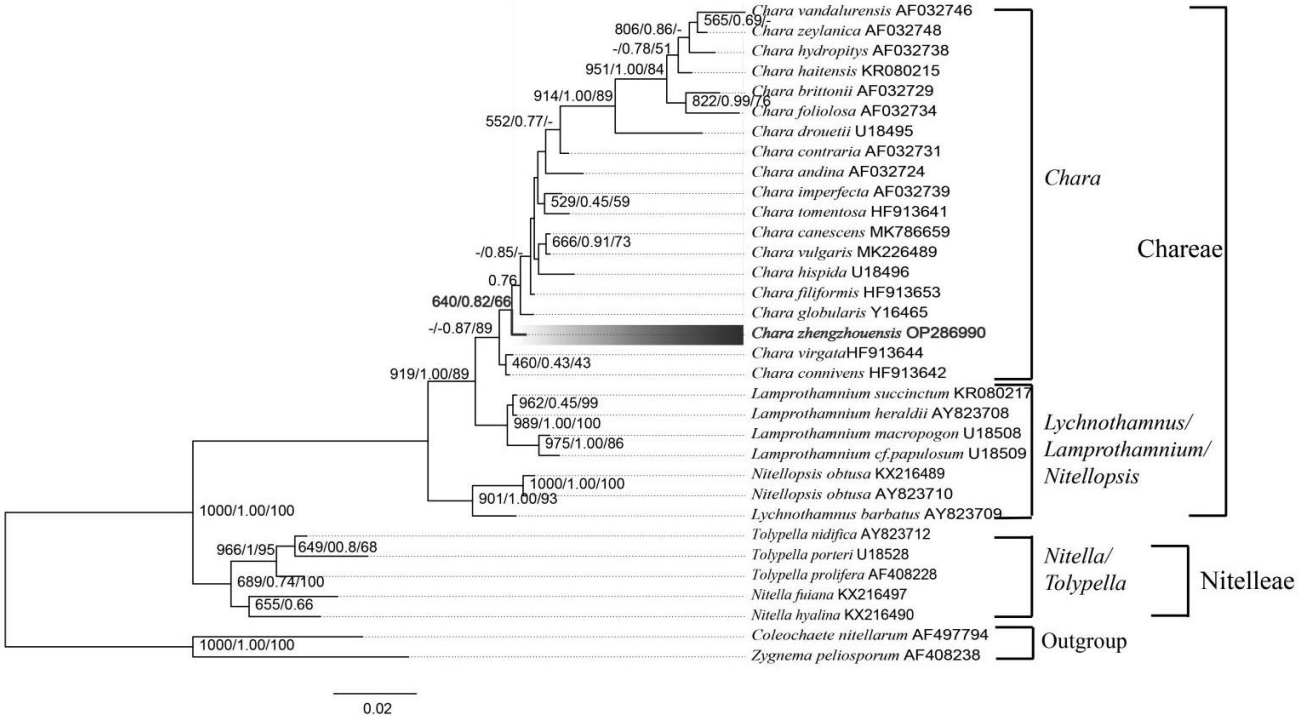


**Figure 4.** The phylogenetic tree constructed from 18S rDNA sequences. The numbers on the branch nodes represent the maximum likelihood bootstrap tree support values, Bayesian posterior probabilities, and neighbor joining bootstrap tree support values. Supporting values below 50% were noted with "-".

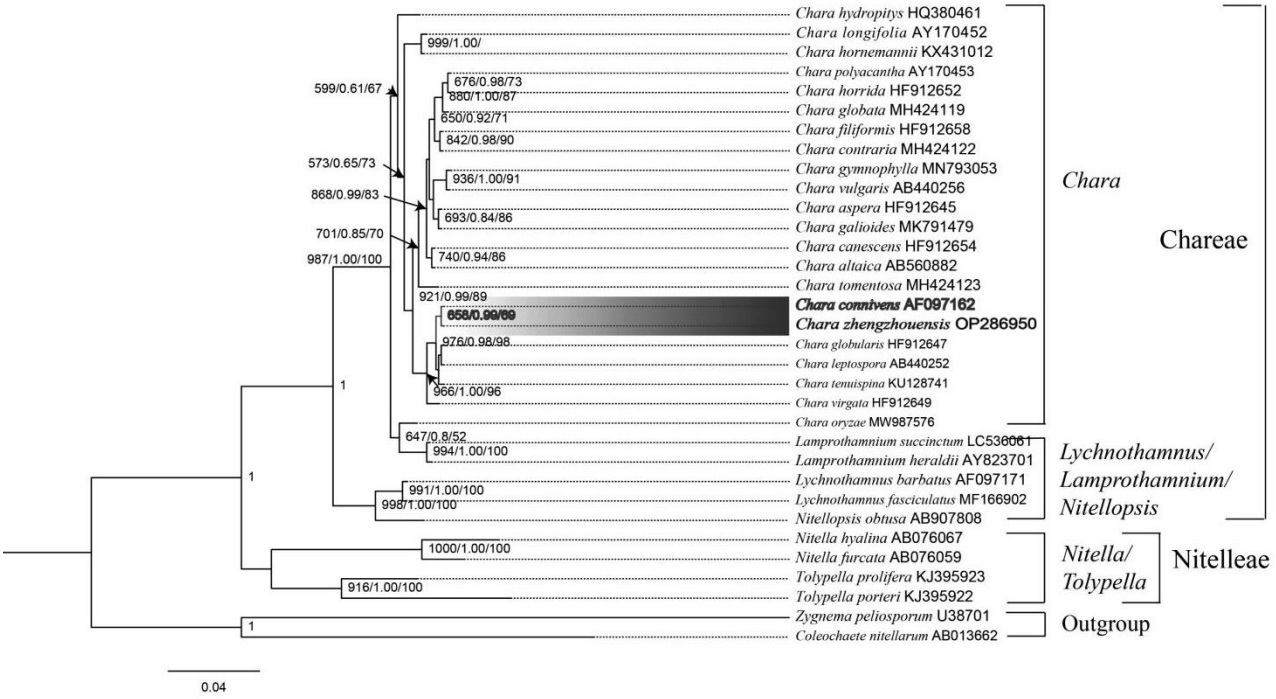


**Figure 5.** The phylogenetic tree constructed from *rbc*L sequences. The numbers on the branch nodes represent the maximum likelihood bootstrap tree support values, Bayesian posterior probabilities, and neighbor joining bootstrap tree support values. Supporting values below 50% were noted with "-".

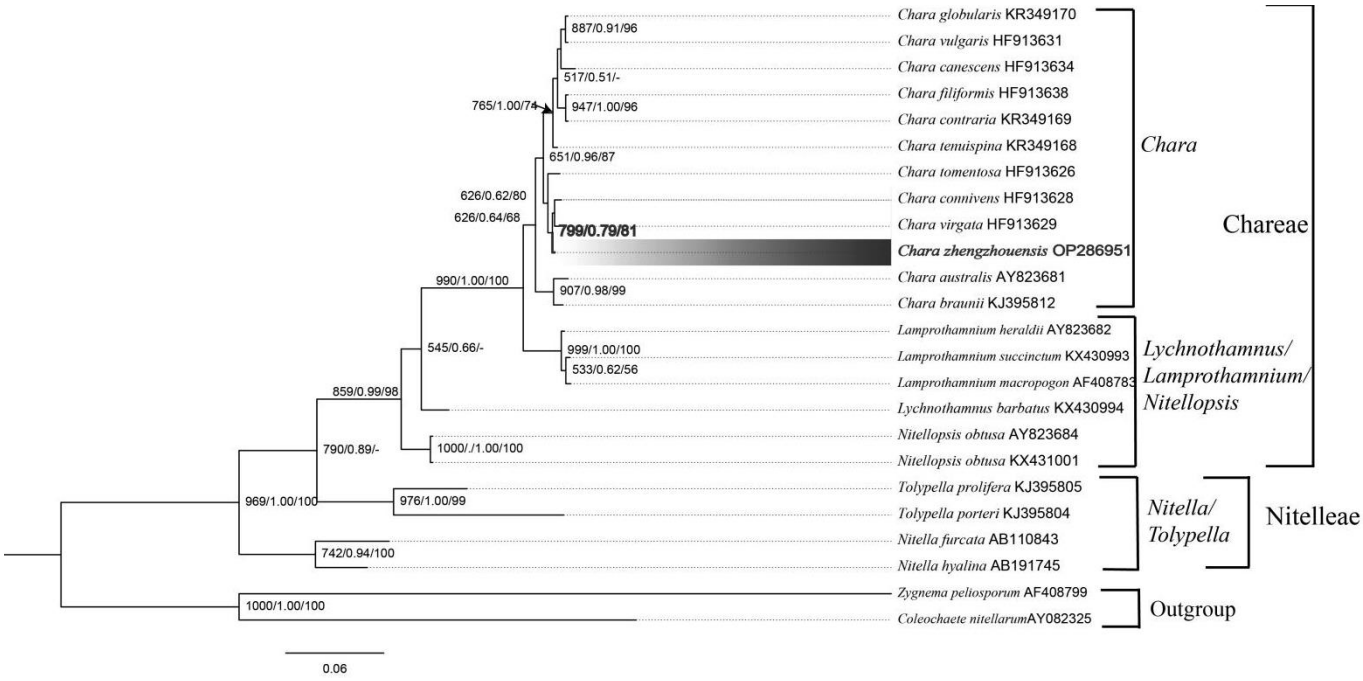


**Figure 6.** The phylogenetic tree constructed from *atp*B sequences. The numbers on the branch nodes represent the maximum likelihood bootstrap tree support values, Bayesian posterior probabilities, and neighbor joining bootstrap tree support values. Supporting values below 50% were noted with "-".

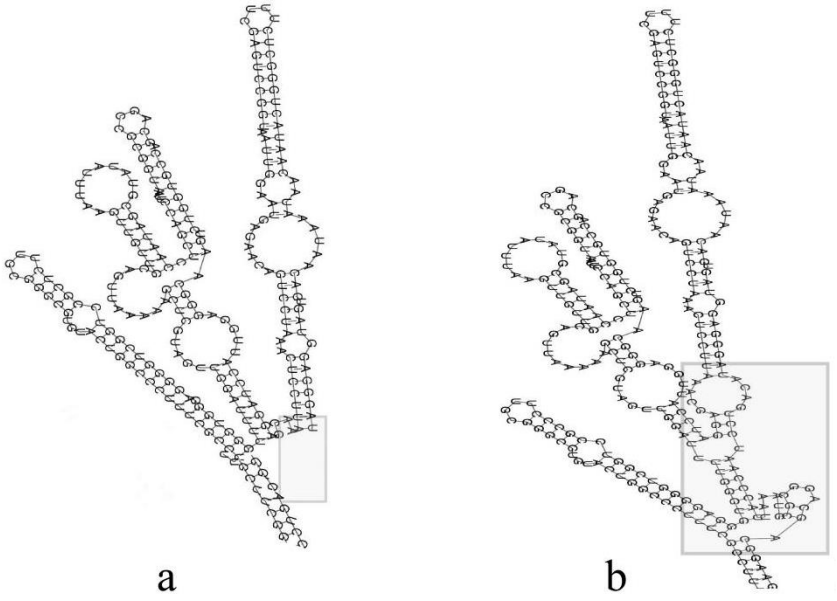


**Figure 7.** Prediction of the secondary structure of 18S rDNA. (**a**) *Chara zhengzhouensis*; (**b**) *C. connivens*.

## 4. Discussion

In this study, the morphological structures and molecular phylogenetic data indicate that the *Chara zhengzhouensis* is a member of the genus *Chara*.

The results of all three phylogenetic trees based on 18S rDNA, *rbc*L, and *atp*B showed that the new species *C. zhengzhouensis* formed an independent clade, supporting its independent position in *Chara*. Therefore, we propose *C. zhengzhouensis* as a new species. In addition, the secondary structure of 18S rDNA of *Chara zhengzhouensis* and *C. connivens* were predicted and compared, which further provided evidence to support the proposal of the new species [42].

In recent years, in order to reevaluate the taxonomic status of some plants, more and more studies have provided molecular data; 18S rDNA, *rbc*L, and *atp*B were all widely used genes [43–47]. It is generally believed that the nuclear gene 18S rDNA is more conservative and has a lower degree of variation and is more accurate for use at different taxonomic levels compared with the chloroplast genes *rbc*L and atpB in analyses of *Chara* [48]. This also showed that the results of this study are convincing.

The new species obviously differs from the closely related species *C. connivens*. The new species *C. zhengzhouensis* has a dichotomous cortex and *C. connivens* has a triplotichous cortex. In addition, the based nodes of branchlets of the new species sometimes were ecorticate, and multiple structures were also shorter or smaller than those of *C. connivens*. According to some references [35–38], *C. connivens* habitats in mesotrophic to eutrophic freshwater or brackish water, but the new species was found only in one type of locality, oligotrophic freshwater.

The new species was related to *Chara virgata* according to the results of phylogenetic analysis, but the latter is of triplotichous cortex and monoeciou. This also means that the importance of the arrangement of the cortex and monoecious/dioecious in taxonomy need further discussion. In addition, *C. virgata* habitats in oligotrophic freshwater or occasionally brackish water and differs from the new species [35,39,40].

## 5. Conclusions

Detailed morphological observations and molecular phylogenetic reconstructions enabled us to define a new species, *Chara zhengzhouensis*. This new species increases the total number of recognized species of the genus *Chara*. Further sampling, morphological observations, and molecular data are needed for a more accurate assessment of the evolution of this genus.

**Author Contributions:** Q.S. performed the analysis and wrote the article. X.L., F.N., Q.L., J.L., J.F., and R.L. gave technological help. S.X. designed the experiments and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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