**Article**

**Morphology and Molecular Phylogeny of Genus *Oedogonium* (Oedogoniales, Chlorophyta) from China**

Qian Xiong \(^1\), Yangliang Chen \(^{1,2}\), Qingyu Dai \(^{1,2}\), Benwen Liu \(^1\) and Guoxiang Liu \(^{1,*}\)

\(^1\) Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

\(^2\) College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing 100039, China

\(^*\) Correspondence: liugx@ihb.ac.cn

**Abstract:** Oedogoniales comprises the three genera *Oedogonium*, *Oedocladium*, and *Bulbochaete*, which include more than 600 described species. The classification of Oedogoniaceae is currently based on morphology, and the complicated morphological characteristics make species identification difficult, with the limited molecular data also restricting the phylogenetic analysis. In the present study, we collected 47 *Oedogonium* specimens from China and sequenced 18S rDNA, ITS2, ITS (ITS1 + 5.8S + ITS2), and rbcL sequences to conduct phylogenetic analyses. We selected nine morphological characteristics, most of which were considered important in traditional systematics, for comparison with the molecular phylogeny results. All the topologies based on different datasets showed similar results; *Oedogonium* was a paraphyletic group, and *Oedocladium* and *Bulbochaete* clustered with *Oedogonium*. The morphological characteristics matching the phylogenetic results showed that the types of sexual differentiation, characteristics of the oogonium (including shape, types of aperture, and ornamentation of oospore wall), division types of antheridial, and number of sperm of each antheridial, which are considered the most important morphological characteristics in traditional taxonomy of *Oedogonium*, did not form monophyletic lineages respectively, indicating that traditional systematics may not reflect the real phylogeny of the genus *Oedogonium*. In addition, a new taxonomical classification of the genus *Oedogonium* was presented according to the shapes of basal cells, which matched well with the phylogenetic topologies. In addition, we propose to divide the genus *Oedogonium* into two sections, section *Globosum* and section *Elongatum*, representing the species with spherical or sub-hemispherical basal cells and elongated basal cells, respectively.

**Keywords:** Oedogoniales; morphological characteristics; molecular phylogeny; *Oedogonium*; paraphyletic group; basal cell shapes

**1. Introduction**

The order Oedogoniales belonging to the family Oedogoniaceae Chlorophyceae, Chlorophyta, includes three genera, *Oedogonium* Link ex Hirn, *Oedocladium* Stahl, and *Bulbochaete* Agardh [1–4]. More than 600 species have been described in this order, most of which can be found in fresh water throughout the world. Genus *Oedogonium* includes 444 species and 349 lower taxonomical units, genus *Bulbochaete* includes 113 species and lower taxonomical units, and genus *Oedocladium* includes 15 species [5]. The presence of branches and hairs are useful characteristics for distinguishing taxa at the genus level. *Oedogonium* has simple unbranched filaments; *Oedocladium* has branched filaments; and *Bulbochaete* forms bulb-based hairs [4–13]. Asexual reproduction occurs via zoospores with a complex flagellar apparatus that is formed by vegetative cells and germinate almost immediately. Sexual reproduction is by oogonia and spermatozoids; oogonia are single or in groups, arising as a result of division of a vegetative cell, opening by a pore or a split, through which the spermatozoid may pass; the oogonium when fertilized becomes the oospore, with a wall of one to three layers, which after a period of rest produces four zoospores [2,4,14–16].
During the past few years, phycologists have used different characteristics as criteria for dividing the genus *Oedogonium*. Sexual differentiation could be the criterion for taxonomic classification for all species of *Oedogonium*, according to the position and relation of the oogonium and the antheridia could be distinguished as monocious; dioecious, macrandrous; dioecious, and nannandrous [6,10]. Gauthier recognized that the different types of oogonium apertures (pore or circumcision) should be the first taxonomic characteristic for the genus *Oedogonium*. Mrozińska [17,18] proposed that the number of spermatozoids of the antheridial cell should be chosen as the criterion in taxonomical classification below the rank of genus for *Oedogonium*.

With the development of molecular phylogenetics, phycologists later conducted a series of phylogenetic studies of Oedogoniales. Oedogoniales are monophyletic, and *Bulbochaete* may be a sister to the other two genera [19–22]. Using nuclear 18S rDNA of 10 *Oedogonium* species, Alberghina et al. [23] suggested that *Oedogonium* might not be monophyletic and that the morphological characteristics may not define the phylogenetic groups. Using 18S rDNA sequences, Mei et al. [24] found that *Oedocladium* formed a separate clade within *Oedogonium*, whereas *Bulbochaete* was relatively distant from the other two genera. Xiong et al. [25–28] indicated that *Oedogonium* was paraphyletic based on chloroplast genome protein-coding genes [25,26], mitogenome protein-coding genes [27], and the single-copy orthogroups of the transcriptomes [28].

Even though the genus *Oedogonium* has described a large amount of species based on morphology, the molecular phylogenetic studies included relatively limited samples, the molecular data in the public database about the order Oedogoniales are also limited, and the phylogeny of *Oedogonium* remains problematic. In addition, the relationships that the morphological characteristics used as criterion of *Oedogonium* with the molecular phylogenetic result have not been discussed comprehensively; more molecular data and a broader sampling is required for further study of this group.

In the present study, a total number of 47 *Oedogonium* specimens from China were sampled and the 18S rDNA, ITS, and nucleotide sequences of the chloroplast genome (cpDNA) rbcL gene of the majority of them were obtained for phylogenetic analyses. The relationships between the different morphological characteristics and phylogenetic results were comprehensively discussed. The objective of the present study was to explore the phylogenetic relationship of the genus *Oedogonium* based on additional taxa and to search for the morphological characteristics which could reflect the phylogeny of *Oedogonium*.

### 2. Results

#### 2.1. Morphological Observation of Voucher Specimens

Characteristics of the *Oedogonium* taxa included in this study are listed in Table 1. Morphological observations of these taxa were based on materials from the field because taxonomic identification of Oedogoniales species requires analysis of the reproductive structures for sexual reproduction. Inability to define taxa as concrete species was primarily due to a lack reproductive structures or limited samples. We were unable to induce sexual reproduction, but limited characteristics, such as shapes of vegetative cells, basal cells, and terminal cells, are listed.

| Terminal Cell: Obtuse (1); Apiculate or with Conical Apex (2); Hyaline (3); Taxa | Phenotypic Traits |
|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| *Oedogonium* sp. BBG18524 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| *Oedogonium* crispum CQ05 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 2 |
| *Oedogonium* sp. CQ1912_8 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 0 |
| *Oedogonium* sp. CQ1913 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 2 |
| *Oedogonium* sp. CQ1916_3 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 0 | 0 |
| *Oedogonium* sp. CQ1916_4 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 0 |

Table 1. Matrix of phenotypic traits scored for the five Oedogoniales strains. Character state definitions are below; unknown character states are notated as “0”. Polymorphic conditions are indicated with multiple state numbers.
Table 1. Cont.

| Terminal Cell: Obtuse (1); Apiculate or with Conical Apex (2); Hyaline (3); Taxa | Phenotypic Traits |
|---|---|---|---|---|---|---|---|---|---|
| Oedogonium sp. CQ1921_5 | 2 | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 2 |
| Oedogonium sp. CQ1923_5 | 3 | 1 | 1 | 2 | 0 | 0 | 1 | 1 | 1 |
| Oedogonium sp. CQ1923_8 | 2 | 1 | 0 | 0 | 1 | 2 | 1 | 1 | 3 |
| Oedogonium dentireticulatum CQ1925_1 | 2 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| Oedogonium sp. CQ1931 | 3 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Oedogonium sp. CQ1932_4 | 2 | 1 | 1 | 0 | 0 | 1 | 2 | 1 | 1 |
| Oedogonium sp. CQ1933_1 | 2 | 1 | 0 | 1 | 2 | 1 | 1 | 1 |
| Oedogonium sp. CQ1940_2 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium sp. FYT1801 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium sp. GDQY3 | 2 | 1 | 1 | 0 | 0 | 1 | 1 | 1 |
| Oedogonium sp. HH1810_2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium sp. HH1815_2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium varians TS191012 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 |
| Oedogonium sp. TS1907_1 | 1 | 1 | 2 | 0 | 1 | 2 | 1 | 1 |
| Oedogonium sp. TS1907_2 | 3 | 1 | 1 | 2 | 0 | 0 | 1 | 1 | 0 |
| Oedogonium sp. TS1907_4 | 0 | 2 | 1 | 2 | 0 | 0 | 1 | 1 | 0 |
| Oedogonium oblongum DQ178025 | 2 | 2 | 1 | 2 | 1 | 2 | 1 | 1 | 3 |
| Oedogonium capillare XQ1802 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 0 |
| Oedogonium sp. XQ1804 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium sp. XQ1819 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium sp. XQ1825 | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 |
| Oedogonium sp. XT1902_1 | 1 | 1 | 0 | 2 | 1 | 2 | 1 | 1 |
| Oedogonium sp. XT1902_3 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 |
| Oedogonium sp. XT1902_5 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | 0 |
| Oedogonium sp. ZWY1801_6 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| Oedogonium sp. ZWY1903_2 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 2 | 2 |
| Oedogonium subplagiostomum Ley FACHB989 | 2 | 1 | 1 | 1 | 2 | 1 | 2 | 1 |
| Oedogonium brevicingulatum Jao FACHB999 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 |
| Oedogonium nodulosum Witt. FACHB996 | 1 | 1 | 2 | 2 | 1 | 2 | 1 |
| Oedogonium hispanicum (Le Clerc) Witt. UTEX LB2239 | 3 | 2 | 1 | 1 | 1 | 0 | 1 | 2 | 3 |
| Oedogonium calliandrum Hofm. UTEX LB1554 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Oedogonium cardiaclum Witt. UTEX LB40 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Oedogonium angustistomum Hofmann UTEX LB1557 UTEX LB1557 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Oedogonium subplagiostomum Ley FACHB989 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Oedogonium brevicingulatum Jao FACHB999 | 1 | 1 | 1 | 1 | 2 | 1 | 2 |
| Oedogonium nodulosum Witt. FACHB996 | 1 | 1 | 2 | 2 | 1 | 2 | 1 |
| Oedogonium hispanicum (Le Clerc) Witt. UTEX LB2239 | 3 | 2 | 1 | 1 | 1 | 0 | 1 | 2 | 3 |
| Oedogonium calliandrum Hofm. UTEX LB1554 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Oedogonium cardiaclum Witt. UTEX LB40 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |
| Oedogonium angustistomum Hofmann UTEX LB1557 UTEX LB1557 | 2 | 1 | 1 | 1 | 2 | 1 |
| Oedogonium subplagiostomum Ley FACHB989 | 2 | 1 | 1 | 1 | 2 | 1 | 1 |

1. Sex differentiation: monoecious species (1); dioecious, macrandrous species (2); dioecious, nannandrous, gynandrosporous species (3); dioecious, nannandrous, idioandrosporous (4). 2. The shape of oogonium: globose or subglobose (1); ellipsoid (2). 3. Type of oogonium aperture: pore (1); circumcision (2). 4. Oospore wall ornamentation: smooth (1); with ornamentations (2). 5. The division way of antheridium: horizontal division (1); vertical division (2). 6. The number of sperm of each antheridium: 1 (1); 2 (2). 7. Vegetative cell: cylindrical or subcylindrical (1); noncylindrical (2). 8. Basal cell: elongated (1); subhemispherical or nearly spherical (2). The strains newly collected in this study are marked in bold.
2.2. Molecular Phylogeny

In the present study, we generated phylogenetic analyses by BI and ML methods based on ITS (ITS1 + 5.8S + ITS2), ITS2, and 18S + ITS (ITS1 + 5.8S + ITS2) + rbcL of 47 Oedogonium taxa newly sampled together with the sequences downloaded from the NCBI database. The ITS dataset consisted of 375 bp, of which 42.9% was variable informative and 32.5% was parsimony informative. The ITS2 sequence consisted of 213 bp, of which 67.61% was variable informative and 54.00% was parsimony informative. The concatenated dataset consisted of 3159 bp, of which 17.6% was variable informative and 9.5% was parsimony informative. The phylogenetic trees based on ITS sequences by both ML and BI showed the same results: separation of Oedogoniales into two clades with high support value (1/100 in BI and ML, respectively) (Figure 1). In the first clade, Bulbochaete clustered with Oedogonium and Oedocladium was separated into two clades, with Oe. prescottii clustering with Oedogonium and the four Oe. carolinianum strains forming the other clade; the remaining species of Oedogonium formed the second clade. We also used the ITS2 sequence with 213 bp to reconstruct the phylogenetic tree (Figure S1), and the general topology was almost consistent with the result based on the concatenated dataset by ITS1, ITS2, and 5.8S. We tried to concatenate the 18S rDNA, ITS, and rbcL of 44 Oedogoniales taxa including 40 taxa of Oedogonium and 4 taxa of Oedocladium to reconstruct the phylogenetic relationship. The topologies were basically identical by BI and ML analyses that Oedogonium formed three main clades (Figure 2). In the second clade, species of Oedocladium clustered with the small clade formed by Oedogonium species. In addition, the other two clades were both composed of Oedogonium taxa.

![Figure 1. Phylogenetic tree of the Oedogoniales algae based on 80 ITS (ITS1 + 5.8S + ITS2) sequences. Numbers on the left and right side of the branches represent ultrafast bootstrap inferred by Bayesian posterior probabilities (≥0.5) and RAxML (≥50%), respectively. Branch lengths are proportional to the genetic distances, which are indicated by the scale bar. The sequences downloaded from NCBI are marked in bold.](image-url)
Figure 2. Phylogenetic tree inferred from 44 18 S, ITS1 (ITS1 + 5.8 S + ITS2) rDNA and rbcL sequences. Numbers on the left and right side of the branches represent ultrafast bootstrap inferred by Bayesian posterior probabilities (≥0.5) and RAxML (≥50%), respectively. Branch lengths are proportional to the genetic distances, which are indicated by the scale bar. The sequences downloaded from NCBI are marked in bold.

Since all the topologies based on different datasets showed similar results, the phylogenetic tree based on ITS sequences including more species with complete morphological characteristics were used for the following analysis. The nine morphological characteristics (types of sexual differentiation; types of oogonium aperture; ornamentation of the oospore wall; division types of the antheridial cell; number of sperm of each antheridial cell; shape of the vegetative cell; shape of the basal cell; and shape of the terminal cell) were selected to match the phylogenetic tree based on ITS sequences to search for the characteristic reflecting the phylogeny of Oedogonium (Figure 3 and Figure S2). Our results showed that monoeccious; dioecious, macrandrous; and dioecious, nannandrous Oedogonium taxa did not form monophyletic lineages, and, similarly, the type of oogonium aperture; type of oogonium opening; division type of the antheridial cell; number of sperm of each antheridial cell; shape of the vegetative cell; and shape of the terminal cell did not form monophyletic lineages. Instead, Oedogoniales was separated into two clades according to the shape of the basal cell (elongated or spherical and sub-hemispherical) with a strong supported value. In the first clade, the taxa possessed elongated basal cells, and in the second clade, they possessed spherical or sub-hemispherical basal cells (Figure 3). Hence, we suggested that the genus Oedogonium can be divided into two sections based on the two kinds of basal cell shapes, namely section Globosum and section Elongatum, representing the species with spherical or sub-hemispherical basal cells and elongated basal cells, respectively. The descriptions of the two sections as follows:
Figure 3. Phylogenetic tree inferred from 80 ITS (ITS1 + 5.8 S + ITS2) rDNA sequences with morphological traits. Numbers on the left and right side of the branches represent ultrafast bootstrap inferred by Bayesian posterior probabilities (≥0.5) and RAxML (≥50%), respectively. The main morphological traits including sex differentiation, the shape of oogonium, oogonium aperture, oospore wall ornamentation, the division way of antheridium, the number of sperm of each antheridium, vegetative cell, basal cell, and terminal cell displayed and represented by circles, asterisks, rectangles, lozenges, semi-circles, hexagons, triangles, ticks, and crosses, respectively.

**Globosum sect.**
The basal cell spherical or sub-hemispherical; the filaments are relatively small with the width almost less than 10 um; the oogonium operculate, most division median, smooth oospore wall.

Type species: *Oedogonium capitellatum* Wittrock ex Hirn (1900, 149, pl. XXIII).

**Elongatum sect.**
The basal cell is elongated, and the other characteristics are the same as the description of genus *Oedogonium* (Link ex Hirn, 1900).

Type species: *Oedogonium grande* Kützing ex Hirn (1900, 143, pl. XXI).
3. Discussion

In the present study, the phylogenetic results based on ITS, ITS2 and 18 S + ITS + rbcL by both BI and ML methods showed that Oedogonium was polyphyletic, and Oedocladium was separated into two clades clustering with Oedogonium, which were identified with our previous studies [25–28], and both Bulbochaete and Oedocladium clustered with Oedogonium. Oedocladium is not considered an independent lineage; this was explained by studies by Liu et al. [13], in which Oedogonium pakistanense, one of the few terrestrial species belonging to the genus Oedogonium, showed apical growth considered as a typical characteristic of Oedogonium. The authors proposed that Oedogonium pakistanense represents an evolutionary transition between Oedogonium and Oedocladium [13]. Based on our morphological observations with indoor cultures and field samples of Oedogonium, apical growth also occurs in many aquatic species, which showed a cladogenic pattern and obscured phylogenetic signals between the two genera. Even though exact topologies differed based on ITS, ITS2 and 18 S + ITS + rbcL, they showed a basal clade including identical taxa (clade II and clade III, respectively).

Phylogenetic analysis combined with morphological studies revealed the morphological characters (the types of sexual differentiation; the characters of oogonium, including the shape, the types of the aperture, and the ornamentation of oospore wall; the division types of antheridial and the number of sperm of each antheridial) important in the traditional taxonomy of Oedogonium did not form monophyletic lineages, respectively, implying that the traditional morphological character used for the taxonomy of Oedogoniales may not reflect the real phylogeny of this group. The phenomenon of different types of sexual differentiation evolving multiple times has also been reported in bryophytes and plants [29–37]. In addition to these morphological characteristics recognized as important in traditional systematics, we also discussed vegetative, terminal, and basal cell shapes. Vegetative and terminal cell shape were not consistent with an evolutionary relationship for poor matching. Most of the species of Oedogoniales possessed cylindrical vegetative cells; a minority presented undulate or nodulose, punctate or granulate, distinctly capitellate and subhexagonal or subellipsoid shapes. In the present study, we collected the taxa with cylindrical and distinctly capitellate shapes to further evaluate this characteristic; more species with other types are needed for further analysis. Terminal cell shapes showed no accordance with evolutionary relationships mainly for the different environmental conditions; the same species may show different types of terminal cells according to the studies of Jao [10] and our observation during the past few years; for example, Oedogonium pringsheimii presented with obtuse or apiculate terminal cells in the field or in culture. However, basal cell shapes were found to match well with both of the topologies based on ITS, ITS2, and 18 S + ITS + rbcL, suggesting that basal cell shape is more consistent with the molecular phylogeny results. The basal cell of the filament of Oedogonium is most frequently elongated, and the attached lower end may be simple or lobed. In addition, the developing basal cell of the other Oedogonium species may be flattened into a subhemispherical cell or rarely appear nearly spherical. Based on samples of Jao [10] collected from a large area in China, Oedogonium included about 440 species, and the number of the species with subhemispherical or nearly spherical basal cells was 33. According to the statistics of the morphological characteristics of these 33 species, we found that they included the three kinds of sexual differentiation types, which showed again that the sexual differentiation types could not reflect the real phylogeny of the genus Oedogonium. In addition, all the 33 species also showed some common characteristics; for example, the filaments are relatively small, with the width almost less than 10 um; and with the oogonium subdepressed-globose; circumcision; and smooth oospore wall, and these characteristics were considered more primitive in the studies based on phenetic- and cladistics taxonomy methods by Mrozińska [17,18], implying that these features may be genetic linkage.
4. Materials and Methods

4.1. Sampling, Morphological Observation, and Culture Procedures

The 47 specimen strains described in this study were isolated from water samples and deposited in the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB collection), Wuhan, Hubei province, China. Voucher numbers are shown in Table S1. Samples were isolated from the field by the authors and primarily examined under a stereo microscope and an Olympus BX53 (Tokyo, Japan) light microscope equipped with an Olympus DP80 digital camera. CellSens standard image analysis software (Tokyo, Japan) was used for morphological examination. Characteristics of the 47 specimens are summarized in Table 1.

4.2. DNA Extraction, PCR Amplification, and Sequencing

To obtain unialgal cultures, clean filaments were selected and transferred onto solid BG11 medium (1.5% BG11 agar) once or twice. All strains were grown at 20–25 °C in solid BG11 medium under a 12 h-12 h light-dark cycle at an intensity of 15–30 μmol/(m²·s). Once a unialgal culture was obtained, total genomic DNA was extracted using a Universal DNA Isolation Kit (AxyPrep, Suzhou, China) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) amplification of partial 18 S rDNA, whole ITS rDNA regions, and rbcL were performed using 5 μL template DNA, 0.1 μM of each primer, and 25 μL 2 × Tap Master Mix (ExTa: Takara) in a 50 μL reaction volume. Nuclear-encoded 18 S rDNA sequences were amplified using the primers 18 F (5′-TGGTTGATCCTGCCAGT-3′) and 18 R (5′-TGATCCTTCTGCAGGTTCACC-3′; [38]). The amplification conditions were as follows: 5 min at 94 °C, 35 cycles of 50 s at 94 °C, 60 s at 55 °C, and 90 s at 72°C, and a final 10 min extension step at 72 °C. The ITS sequence was amplified using the primer pair designed by Primer5 ITS A1 (5′-TGATCCTTCTGCAGGTTCACC-3′) and ITS S1 (5′-GAACCTGCGCGACCCGTTAG-3′). The amplification conditions for ITS were as follows: 5 min at 94 °C, 32 cycles of 50 s at 94 °C, 60 s at 52 °C, and 90 s at 72 °C, and a final 10 min extension step at 72 °C. The rbcL sequence was amplified using the primer pair designed by Primer5 rbcL A3 (5′-CGATTTGAATCCATGTTAGG-20) and rbcL S3 (50-AGTTCTCGAGACCATTTTG-18). The amplification conditions for rbcL were as follows: 5 min at 94 °C, 35 cycles of 50 s at 94 °C, 50 s at 56.5 °C, and 70 s at 72 °C, and a final 10 min extension step at 72 °C. PCR products were sequenced by TSINGKE Biotechnologies (China) and assembled using Seqman [39] and deposited in GenBank under the accession numbers provided in Table S1.

4.3. Molecular Phylogenetic Analyses

The nuclear-encoded 18 S rDNA and ITS (ITS1 + 5.8 S + ITS2) sequences, and nucleotide sequence of the cpDNA rbcL gene were used for phylogenetic analyses. Sequences of related species were obtained from GenBank (Table S2). In addition, 18 S rDNA, ITS, and ITS2 sequences were aligned using MAFFT 7.0 [40] and adjusted manually using MEGA7 [41]. The alignment result of the ITS sequences was shown in Figure S3. For nucleotide sequences of rbcL, the genes were additionally aligned using the MUSCLE function of MEGA7 with the option “align codons” [41,42] and were adjusted manually using MEGA7 [41]. Phylosuite [43] was used to concatenate 18 S rDNA, ITS, and rbcL sequences. Phylogenetic calculations were performed using maximum likelihood analysis (ML) by RAxML v8.2.10 [44]. A bootstrap analysis with 1000 replicates of the dataset was performed with ML to estimate statistical reliability. MrBayes v3.2.6 [45] was used for Bayesian inference (BI) with modeling of the GTR + I + G suggested by jModelTest2 [46]. Markov chain Monte Carlo (MCMC) analyses were run with four Markov chains (three heated, one cold) for 3,000,000 generations, and trees were sampled every 1000 generations. In each round of calculation, a fixed number of samples (burn-in = 1000) was discarded from the beginning of the chain.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11182422/s1, Figure S1: Phylogenetic tree of the Oedogoniales algae based on 80 ITS2 sequences by MrBayes; Figure S2: The microphotos of the eight morphological traits used to match phylogenetic results; Figure S3: The alignment result of the ITS sequences. Supplementary Table S1 Location of the collecting sites and GenBank accession number of isolates. Supplementary Table S2 Strains whose sequences were downloaded from GenBank.

Author Contributions: Q.X.: original concept, sampling, culture experiments, data analysis, writing and editing manuscript; Y.C.: editing manuscript; Q.D.: editing manuscript; B.L.: editing manuscript; G.L.: sampling, editing manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Key Research Program of Frontier Sciences, CAS (Grant No. QYZDY-SSW-SMC029-6) and the National Natural Science Foundation of China (Grant No. 31750001).

Acknowledgments: This research was supported by the Wuhan Branch, Supercomputing Center, Chinese Academy of Sciences, China.

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

References
1. Agardh, C.A. Synopsis Algarum Scandinavie: Adjecta Dispositione Universalii Algarum; Kessinger Publishing, LLC: Whitefish, MT, USA, 1817.
2. De Bary, A. Ueber die Algengattungen Oedogonium und Bulbochaete. Naturf. Ges. Frankf. 1854, 1, 29–105.
3. Stahl, E. Oedocladium protonema, eine neue Oedogoniaceen-Gattung. Jahrb. Der Wiss. Bot. 1891, 23, 339–348.
4. Hirn, K.E. Monographie und Iconographie der Oedogoniaceen. Acta Soc. Scient. Fennici 1900, 27, 1–395.
5. Mrozińska, T. Algal periphyton on higher-plants. Ekol. Pol. Pol. J. Ecol. 1986, 34, 457–472.
6. Tiffany, L.H. North American Flora: Oedogoniales; Botanical Garden: New York, NY, USA, 1937.
7. Gemeinhardt, K. Oedogoniales. In Dr. L. Rabenhorst’s Kryptogamen-Flora von Deutschland, Oesterreich und der Schweiz; Rabenhorst, L., Ed.; Akademische Verlagsgesellschaft: Leipzig, Germany, 1939.
8. Gauthier-Liévre, L. Oedogoniáceas Africaines; Verlag von J. Cramer: Stuttgart, Germany, 1964.
9. Islam, N.A.K.M.; Sarma, P. Two new species of terrestrial Oedogonium from east Pakistan. Trans. Am. Microsc. Soc. 1963, 82, 74–77. [CrossRef]
10. Jao, C.C. Monographia Oedogoniales Sinicae; Science Press: Beijing, China, 1979.
11. van den Hoek, C.; Mann, D.G.; Jahns, H.M.; van den Hoek, C.; Mann, D.G.; Jahns, H.M. Algae: An Introduction to Phycology; Cambridge University Press: Cambridge, UK, 1995.
12. Graham, L.E.; Wilcox, L.W. The systematics of coccoid green algae: 18s rrna gene sequence data versus morphology. Biologia 2004, 43, 669–671. [CrossRef]
13. Liu, G.; Hu, Z. Predominant occurrence of apical cell divisions in Oedogonium pakistanense and its phylogenetic significance. Phycologia 2004, 39, 437–446. [CrossRef]
14. Pringsheim, N. Beiträge zur Morphologie und Systematik der Algen. I. Morphologie der Oedogonien; Pringsheim’s Jahrbücher für Wissensc, 1858.
15. Wittrock, V.B. Protodromus Monographiae Oedogoniearum. Nova Acta Regiae Soc. Sci. Ups. 1874, 3, 64.
16. Collins, F.S. The Green Algae of North America; Tufts College, Mass, 1909.
17. Mrozińska, T. A preliminary investigation of the taxonomical classification of the genus Oedogonium link (oedogoniales) based on the phylogenetic relationship. Arch. Fur Protistenkd. 1991, 139, 85–101. [CrossRef]
18. Mrozińska, T. A preliminary investigation of the taxonomical classification of the genus Bulbochaete agardh (oedogoniales, chlorophyta) based on the phylogenetic relationship. Arch. Fur Protistenkd. 1993, 143, 113–123. [CrossRef]
19. Booton, G.C.; Floyd, G.L.; Fuerst, P.A. Origins and affinities of the filamentous green algal orders chaetophorales and oedogoniales based on 18s rna gene sequences. J. Phycol. 1998, 34, 312–318. [CrossRef]
20. Buchheim, M.A.; Michalopulos, E.A.; Buchheim, J.A. Phylogeny of the chlorophyceae with special reference to the sphaeropleales: A study of 18s and 26s rdna data. J. Phycol. 2001, 37, 819–835. [CrossRef]
21. Krienitz, L.; Hegewald, E.; Hepperle, D.; Wolf, M. The systematics of coccoid green algae: 18s rna gene sequence data versus morphology. Biologia 2003, 58, 437–446.
22. Shoup, S.; Lewis, L.A. Polyphyletic origin of parallel basal bodies in swimming cells of chlorophycean green algae (chlorophyta). J. Phycol. 2003, 39, 789–796. [CrossRef]
23. Alberghina, J.S.; Vigna, M.S.; Confalonieri, V.A. Phylogenetic position of the oedogoniales within the green algae (chlorophyta) and the evolution of the absolute orientation of the flagellar apparatus. Plant Syst. Evol. 2006, 261, 151–163. [CrossRef]
24. Mei, H.; Luo, W.; Liu, G.X.; Hu, Z.Y. Phylogeny of oedogoniales (chlorophyceae, chlorophyta) inferred from 18s rdna sequences with emphasis on the relationships in the genus Oedogonium based on its-2 sequences. Plant Syst. Evol. 2007, 265, 179–191. [CrossRef]
25. Xiong, Q.; Hu, Y.; Liu, B.; Zhu, H.; Liu, G.; Hu, Z. Chloroplast genomes and phylogenetic analysis of two species of Oedocladium (oedogoniales, chlorophyta). *Eur. J. Phycol.* 2021, 56, 403–415. [CrossRef]

26. Xiong, Q.; Hu, Y.; Lv, W.; Wang, Q.; Liu, G.; Hu, Z. Chloroplast genomes of five Oedogonium species: Genome structure, phylogenetic analysis and adaptive evolution. *BMC Genom.* 2021, 22, 707. [CrossRef]

27. Xiong, Q.; Wang, J.; Hu, Y.; Wang, Q.; Liu, G.; Hu, Z. Mitochondrial genome structure, phylogenetic analyses and substitution rate estimation of the Oedogoniales. *Eur. J. Phycol.* 2022, 1–12. [CrossRef]

28. Xiong, Q.; Hu, Y.; Dong, X.; Chen, Y.; Liu, G.; Hu, Z. Phylotranscriptomic and evolutionary analyses of Oedogoniales (chlorophyceae, chlorophyta). *Diversity* 2022, 14, 157. [CrossRef]

29. Devos, N.; Renner, M.A.M.; Gradstein, R.; Shaw, A.J.; Laenen, B.; Vanderpoorten, A. Evolution of sexual systems, dispersal strategies and habitat selection in the liverwort genus radula. *New Phytol.* 2011, 192, 225–236. [CrossRef]

30. Shaw, B.; Crandall-Stotler, B.; Vana, J.; Stotler, R.E.; von Konrat, M.; Engel, J.J.; Davis, E.C.; Long, D.G.; Sova, P.; Shaw, A.J. Phylogenetic relationships and morphological evolution in a major clade of leafy liverworts (phylum marchantiophyta, order jungermanniales): Suborder jungermanniinae. *Systematic Bot.* 2015, 40, 27–45. [CrossRef]

31. Zhang, J.-Q.; Meng, S.-Y.; Wen, J.; Rao, G.-Y. Phylogenetic relationships and character evolution of rhodiola (crassulaceae) based on nuclear ribosomal its and plastid trnl-f and psba-trnh sequences. *Systematic Bot.* 2014, 39, 441–451. [CrossRef]

32. Torices, R.; Mendez, M.; Maria Gomez, J. Where do monomorphic sexual systems fit in the evolution of dioecy? *Insights from the largest family of angiosperms. New Phytol.* 2011, 190, 234–248. [PubMed]

33. Weiblen, G.D.; Oyama, R.K.; Donoghue, M.J. Phylogenetic analysis of dioecy in monocotyledons. *Am. Nat.* 2000, 155, 46–58. [CrossRef] [PubMed]

34. Barrett, S.C.H. Understanding plant reproductive diversity. *Philos. T. R. Soc. B.* 2010, 365, 99–109. [CrossRef] [PubMed]

35. Vamosi, J.C.; Otto, S.P.; Barrett, S.C.H. Phylogenetic analysis of the ecological correlates of dioecy in angiosperms. *J. Evol. Biol.* 2003, 16, 1006–1018. [CrossRef] [PubMed]

36. Medlin, L.; Elwood, H.J.; Stickel, S.; Sogin, M.L. The characterization of enzymatically amplified eukaryotic 16s-like rRNA-coding regions. *Gene* 1988, 71, 491–499. [CrossRef]

37. Swindell, S., R.; Plasterer, T.N. *SEQMAN: Contig Assembly. Sequence Data Analysis Guidebook. Methods in Molecular Medicine; Volume 70*, Springer: New York, NY, USA, 1997; pp. 75–89.

38. Medlin, L.; Elwood, H.J.; Stickel, S.; Sogin, M.L. The characterization of enzymatically amplified eukaryotic 16s-like rRNA-coding regions. *Gene* 1988, 71, 491–499. [CrossRef]

39. Swindell, S., R.; Plasterer, T.N. *SEQMAN: Contig Assembly. Sequence Data Analysis Guidebook. Methods in Molecular Medicine; Volume 70*, Springer: New York, NY, USA, 1997; pp. 75–89.

40. Medlin, L.; Elwood, H.J.; Stickel, S.; Sogin, M.L. The characterization of enzymatically amplified eukaryotic 16s-like rRNA-coding regions. *Gene* 1988, 71, 491–499. [CrossRef]

41. Kumar, S.; Stecher, G.; Tamura, K. Mega7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 2016, 33, 1870–1874. [CrossRef] [PubMed]

42. Edgar, R.C.; Sog, I.C. Muscle: Multiple sequence alignment with improved accuracy and speed. In *Proceedings of the IEEE Computational Systems Bioinformatics Conference*, Stanford, CA, USA; 2004; pp. 728–729.

43. Zhang, D.; Gao, F.; Jakovlic, I.; Zou, H.; Zhang, J.; Li, W.X.; Wang, G.T. Phylousite: An integrated and scalable desktop platform for streamlined molecular sequence data management and evolutionary phylogenetics studies. *Mol. Ecol. Resour.* 2020, 20, 348–355. [CrossRef]

44. Stamatakis, A. Raxml version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014, 30, 1312–1313. [CrossRef]