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Phylogeny of Oedogoniales (Chlorophyceae, Chlorophyta) inferred from 18S rDNA sequences with emphasis on the relationships in the genus *Oedogonium* based on ITS-2 sequences

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Abstract. The phylogeny of Oedogoniales was investigated by using nuclear 18S rDNA sequences. Results showed that the genus *Oedocladium*, as a separated clade, was clustered within the clade of *Oedogonium*; whereas the genus *Bulbochaete* was in a comparatively divergent position to the other two genera. The relationship among the species of *Oedogonium* was discussed, focusing on ITS-2 phylogeny analyzed combining with some morphological characteristics. Our results showed that all the dioecious nannandrous taxa involved in this study were resolved into one clade, while all the monocious taxa were clustered into another clade as a sister group to the former. The report also suggests that the dioecious macrandrous taxa form a paraphyly and could be more basally situated than the dioecious nannandrous and the monoecious taxa by means of molecular phylogeny and morphotype investigations.

Key words: Oedogoniales, *Oedogonium*, *Oedocladium*, *Bulbochaete*, 18S rDNA, ITS-2, phylogeny, systematics.

Introduction

The Oedogoniales, comprised of three genera (*Oedogonium*, *Oedocladium* and *Bulbochaete*), can be found in freshwater all over the world (Hirn 1900, Tiffany 1937, Gemeinhardt 1939, Gauthiér-Lièvre 1963, Islam and Sarma 1963, Jao 1979, Mrozińska 1985). Their fascinating and distinctive features that are well known to phycologists set them apart into a very unusual order of green algae. While they have no obvious ancestors, several features common to other green algae (e.g. possession of the phycoplast) place them in the Chlorophyta (*sensu* Mattox and Stewart 1984). But the systematic position of this group has changed over time according to the criteria used by different authors, who considered that morphological characters and certain aspects of the life cycle were the principal diacritic features (Alberghina et al. 2006).

Oedogonium, *Bulbochaete* and *Oedocladium* are separated based on differences in their filaments and the presence or absence of hairs, however few characters in morphology and structure could be used to discuss the evolutionary course of the three genera (Jao 1979). Tiffany (1930) arranged them in *Bulbochaete*, *Oedocladium* and *Oedogonium*; Gauthiér-Lièvre (1963) chose the order *Oedocladium*, *Bulbochaete* and *Oedogonium*; Jao (1979) also agreed with Hirn's conclusion (1900) as *Oedogonium*, *Bulbochaete* and *Oedocladium*; However, recent illustration of Mrozińska (1985) sorted them in the order *Oedogonium*, *Oedocladium* and *Bulbochaete*. The relationships of the three genera are still uncertain.

During the past few years various phycologists used different characteristics as criterion for dividing in the three genera of Oedogoniales. Jao's monographic work (1979) based on sample collections from China, presented types of sexual differentiation as groundwork for classification below the rank of genus *Oedogonium* and genus *Bulbochaete*. Since living samples of genus *Oedocladium* had not been found, until Liu (1993) did report this new record in China. Mrozińska (1991, 1993) proposed to divide the genera *Oedogonium* and *Bulbochaete* into sections based on the number of spermatozoids produced in the antheridial cell respectively, which characteristics should be chosen as the criterion in taxonomical classification below the rank of genera. Yet the evolutionary relationships between the genera remained problematic.

To date, only a limited number of molecular phylogenetic studies have been based on sequences of the Oedogoniales. Correlative studies (Booton et al. 1998, Buchheim et al. 2001, Shoup and Lewis 2003, Krienitz et al. 2003) have taken not more than four species of this order into analyses, and have only drawn preliminary conclusions: The Oedogoniales was monophyletic; and *Bulbochaete* could be more basally situated phylogenetically than the other two genera. The latest research of Alberghina (2006) reported a further phylogenetic analysis using 18S rDNA of 10 *Oedogonium* species in Argentina, which also demonstrated the monophyly of Oedogoniales, and the *Oedogonium* group did not appear to be monophyletic. Since the traditional taxonomy of Oedogoniales did not define natural groups and the evolutionary position remained uncertain, it was necessary to clarify the systematic problem and confirm many evolutionary hypotheses in a deeper way. Except for 18S rDNA, internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA have been shown to provide good phylogenetic resolution in recently diverging lineages (Kooistra et al. 1992; Van Oppen et al. 1993, 1995; Leclerc et al. 1998). In this paper, more 18S rDNA sequences with an emphasis on ITS-2 of *Oedogonium* species were involved, which obtained a better understanding of the relationship among the three genera, and the phylogeny among *Oedogonium* species with a magnitude of sexual differentiation types furtherly. We limited the spacer analysis to ITS-2 because this part of sequence contained higher base conservation and it was proven to be most useful at species and genus level (Van Nues et al. 1995; Hershkovitz and Lewis 1996; Coleman and Mai 1997; Stiger et al. 2000, 2003; Coleman 2003; Hegewald and Wolf 2003). In addition, the ITS-2 region is informative and easy to be aligned guided by secondary structure (Mai and Coleman 1997). Morphological characters were also combined with the ITS-2 study to assess the relationships among *Oedogonium* taxa.

Materials and methods

Sampling and cultivation. Twelve *Oedogonium* taxa and one *Oedocladium* taxon were collected respectively in Hubei Province 110.6°–115°E, 30.4°–31.7°N (PRC) and Zhangjiajie 110.4°E, 29.1°N (Hunan Province, PRC). Samples were isolated from field by the authors and cultivated at 20°C to 25°C at a 16:8-h light:dark cycle under 25 μmol photons $m^{-2} \cdot s^{-1}$, in modified Bold's Basal medium (BBM, Nichols and Bold 1965) supplemented with soilwater 70 $ml \cdot l^{-1}$.

Taxon selection. A total of 28 Oedogoniales taxa were selected in 18S rDNA or ITS-2 sequence analyses. All these organisms, their origins, strain number or reference and the GenBank accession numbers of each sequence are listed in Table 1. For

Table 1. Oedogoniales taxa used for 18S rDNA or ITS-2 sequence analyses, strain source, strain No. or reference, Genbank accession numbers and sexual reproduction type and sperm No. in each antheridium of the *Oedogonium* species used in ITS-2 analysis. Sequences marked with an asterisk were determind by this study. FACHB: Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Science

Taxon, strain source	18S rDNA		ITS-2 sequence		Type of sexual differentiation	No. of sperm per antheridium
	Strain reference	No. or Acc.No.	Strain reference	No. or Acc.No.		
Bulbochaete rectangularis var. *hiloensis* (Nordst) Tiffany	Booton et al. 1998	U83132	UTEX LB954.	AY962677		
Oedocladium carolinianum Beaney & Hoffman	Booton et al. 1998	U83135				
Oedocladium prescottii Islam * (Sandy soil, Zhangjiajie, China)	FACHB 993	DQ078298				
Oedogonium acrosporum De Bary	Alberghina et al. 2006	DQ115892				
Oedogonium angustistomum Hoffmann	Booton et al. 1998	U83134	UTEX LB1557	AY962676	Dioecious macrandrous	2
Oedogonium borisianum (Le Clerc) Wittr.,			UTEX LB2239	AY962670	Dioecious nannandrous	1
Oedogonium brevicingulatum Jao * (Pond at Institute of Hydrobiology, CAS, Wuhan, China)	FACHB 999	DQ413052	FACHB 999	DQ413058	Monoecious	2
Oedogonium cardiacum Wittr.	Booton et al. 1998	U83133	UTEX LB40	AY962675	Dioecious macrandrous	2
Oedogonium calliandrum Hoffm.			UTEX LB1554	AY962672	Dioecious macrandrous	2
Oedogonium crispum (Hass.) Wittr.			ACOI 1287	AY962680	Monoecious	2
Oedogonium cylindrosporum Jao * (Pond at Zhifang, Wuhan, China)	Present study	DQ078297	Present study	DQ078300	Dioecious macrandrous	2
Oedogonium eminens (Hirn) Tiff. * (Pond at Zhifang, Wuhan, China)			Present study	DQ078302	Dioecious nannandrous	1
Oedogonium fragile Wittr.			SCCAP K0093	AY962679	Monoecious	2
Oedogonium globosum Nordst. * (Pond at Zhifang, Wuhan, China)	FACHB 992	DQ413051	FACHB 992	DQ413057	Monoecious	2

Table 1. (Continued)

Taxon, strain source	18S rDNA		ITS-2 sequence		Type of sexual differentiation	No. of sperm per antheridium
	Strain reference	No. or Acc.No.	Strain reference	No. or Acc.No.		
Oedogonium nodulosum Wittr. * (Pond at Macheng city, Hubei Province, China)	FACHB 996	DQ018735	FACHB 996	DQ078301	Monoecious	2
Oedogonium oblongum Wittr.,			ACOI 1118	AY962681	Monoecious	2
Oedogonium pakistanense Islam & Sarma * (Sandy soil, Xiantao County, Hubei Province, China)	FACHB 995	DQ076244	FACHB 995	DQ413060	Monoecious	2
Oedogonium pseudohirnii Jao * (Soil, Wuyuan Country, Jiangxi Province, China)	FACHB 994	DQ413053	FACHB 994	DQ413059	Monoecious	2
Oedogonium pusillum kirchner	Alberghina et al. 2006	DQ115898				
Oedogonium rugulosum Nordstedt	Alberghina et al. 2006	DQ115901				
Oedogonium sp. * (Pond at Qinglong village in Zhifang, Wuhan, China)	FACHB 990	DQ413048	FACHB 990	DQ413055	Dioecious macrandrous	2
Oedogonium sp. * (Pond at Institute of Hydrobiology, CAS, Wuhan, China)	FACHB 991	DQ413049	FACHB 991	DQ413056	Monoecious	2
Oedogonium sp. * (Humid soil, Moshan village, Wuhan, China)	FACHB 997	DQ418462				
Oedogonium sp. * (Stream in Shennongjia, Hubei Province, China)	FACHB 998	DQ413050	FACHB 998	DQ178023	Dioecious nannandrous	1
Oedogonium subplagiostomum Ley * (Pond at Qinglong village in Zhifang, Wuhan, China)	FACHB 989	DQ078295	FACHB 989	DQ413054	Dioecious macrandrous	2
Oedogonium tenerum Jao * (Pond at Macheng city, Hubei Province, China)	Present study	DQ078296				
Oedogonium vaucherii (Le Clrec) A. Braun,			SCCAP K0094	AY962678	Monoecious	2
Oedogonium undulatum (Brébisson) A. Braun * (Pond at Macheng city, Hubei Province, China)			Present study	DQ178025	Dioecious nannandrous	1

the taxa involved in ITS-2 sequence analyses, we also listed their sexual reproduction type and member of sperms in each antheridium in the table. There are 13 new 18S rDNA sequences and 12 new ITS-2 sequences were generated for the investigation.

DNA extration. Algal cultures were harvested by centrifugation at 7,400 rpm for 2 min and then resuspended in 0.8 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 3% SDS). The algal mass was added to 1 ml of 0.5 mm glass beads, and the algal cells were lysed by bead beating at 4,800 rpm for 2 min in a mini-beadbeater (model 3110BX, Biospec Products, Bartlesville, OK). Lysates were pelleted, and 1 ml new lysis buffer was added and samples were incubated at 70°C for 20 min. Then lysates were extracted with an equal volume of equilibrated phenol:chloroform:isoamyl alcohol (25:24:1), and the aqueous phase was recovered by centrifugation at 13200 rpm for 5 min. DNA was precipitated using 2.5 volumes of isopropanol and 0.1 volume of 3M NaOAc. DNA pellets were washed in 70% ethanol, air dried at room temperature, and dissolved in 30 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

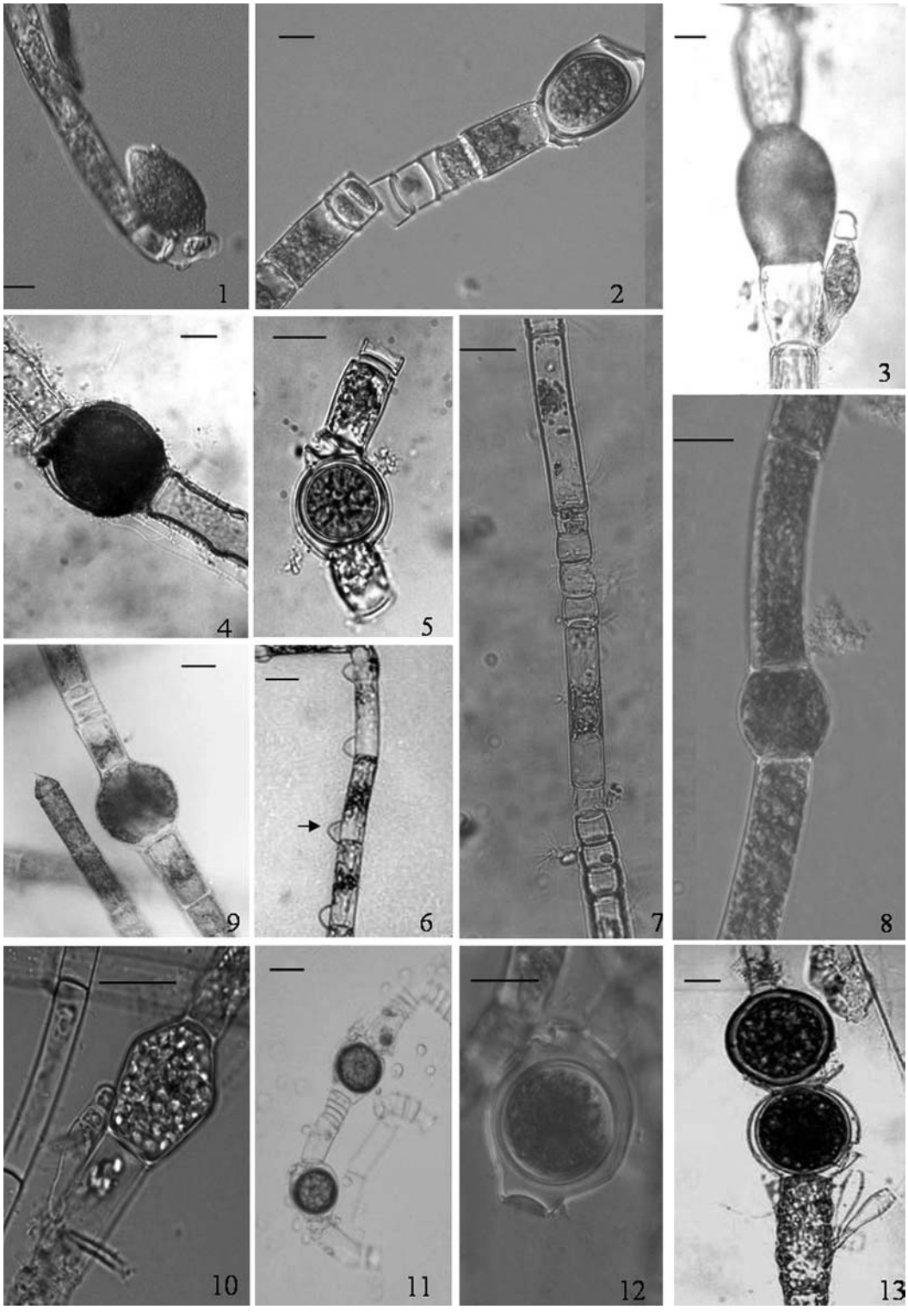
PCR amplification and sequencing. The 18S rRNA genes were amplified from total genomic DNA with eukaryote-specific synthetic oligonucleotide amplification primers, as presented in Medlin et al. (1988). The ITS sequence were PCR amplified and sequenced using the same primer (ITS5 & ITS4) (Pillmann et al. 1997). PCR was performed as described previously (Booton et al. 1998, Buchheim et al. 2001, Hegewald et al. 2001, Wolf et al. 2002, Shoup and Lewis 2003, Krienitz et al. 2003). The amplified fragments were purified with a Biostar glassmilk DNA purification kit following the manufacturer's instruction. The purified fragments were sequenced using ABI 3100 avant genetic analyzer. All sequences were deposited in GenBank.

Alignment. Both the 18S rDNA sequences file and the ITS-2 sequences file were initially aligned with the ClustalX 1.83 multiple alignments program (Thompson et al. 1997), and refined manually. Homology of sites and accuracy of the alignments were determined by examination secondary structure calculated by using the RNAstructure program 4.11 of Mathews et al. (1999). An 18S rDNA sequence for *Oedogonium nodulosum* was used for 18S rDNA sequence homologous site comparisons. The secondary structures of ITS-2 sequences were compared with reference to the result mentioned by Coleman (2003). The final to the 18S rDNA sequence alignment was used to produce a data matrix of 1,535 sites, which corresponds to the range between bases 49 and 1,582 of the published *Chlamydomonas reinhardtii* 18S rDNA sequence (Gunderson et al. 1987). The final alignments of ITS-2 sequence contained a matrix of 20 *Oedogonium* sequences with *Bulbochaete rectangularis* var. *hiloensis* as an outgroup taxon (231 bp long). The alignments are available from the authors on request.

Phylogenetic analyses. Mutational saturation was evaluated in the variable positions of the 18S rDNA and ITS-2 sequence alignments by plotting pair-wise distances (uncorrected for multiple substitutions) against model-corrected distances for Tamura and Nei (1993) and Kimura (1980) model and estimated in Modeltest 3.06 (Posada and Crandall 1998).

The phylogenetic trees were inferred by distance (neighbor-joining; NJ), maximum parsimony (MP) and maximum likelihood (ML) criteria using PAUP*4.0b10 (Swofford 2002), and by Bayesian inference (BI) using MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001). To determine the evolutionary model that best fitted our data sets the program Modeltest 3.06 (Posada and Crandall 1998) was used, and the best models were selected by the hierarchial LRT (likelihood ratio test) for each set. For the 18S rDNA data set, the best model was the Tamura-Nei model (TrN; Tamura and Nei 1993), with consideration of the proportion of invariable sites (I) and the gamma shape parameters (G). For ITS-2, the Kimura model was deemed best by Modeltest.

Unrooted phylogenetic trees (Fig. 14) were inferred from 18S rDNA sequence data by MrBayes using BI with the model TrN + I + G. Bayesian phylogenetic analyses were conducted using four simultaneous Markov Chains running 1,000,000 generations, sampling every 100 generations. A 50% majority-rule consensus tree was calculated from the 10,000 trees saved during the analysis, excluding the first 1,000 that preceded the stabilization of the likelihood value. Bayesian posterior probabilities were determined by a 50% majority-rule consensus. The tree topology for ITS-2 sequence data file (Fig. 15) was calculated by



PAUP using the ML criterion with the Kimura model. The confidence of branching in all methods for each data set was assessed using 1000 bootstrap resamplings of the data set (except for ML analysis, which was assessed using 100 bootstrap resamplings; Felsenstein 1985).

Figs. 1–13. Some species involved in present study, light micrographys. Scale bars = 20μm. **1** *Oedocladium prescottii*, monoecious, showing the oogonium; **2** *Oedogonium brevicingulatum*, monoecious, showing the oogonium and an antheridium with two horizontally dividing sperms in it; **3** *Oedogonium eminens*, dioecious nannandrous, showing the budding oogonium and the dwarf male; **4** *Oedogonium nodulosum*, monoecious, showing the oogonium and a vegetative cell with a nodule on it; **5-6** *Oedogonium pakistanense*, monoecious, showing the oogonium and lateral apical caps (arrowheads) in a row along filament; **7–8**. *Oedogonium* sp. FACHB990, dioecious macrandrous, **7** the male strain; **8** the female strain; **9** *Oedogonuim* sp. FACHB991, monoecious, showing the oogonium, the antheridia and two horizontaly dividing sperms, **10** *Oedogonium* sp. FACHB998, dioecious nannandrous; **11** *Oedogonuim globosum*, monoecious, showing the mature oogonia and the antheridia; **12** *Oedogonuim pseudohirnii*, monoecious, showing the matrure oogonium; **13** *Oedogonium undulatum*, dioecious nannandrous, showing the continuous oogonia and the dwarf males

Results

Description of some Oedogoniales taxa presented in this study. Light micrographs of eleven taxa involved in the present study were presented in Figs. 1–13. Except for the monoecious *Oedocladium* taxon in Fig. 1, the others were all *Oedogonium* taxa. The taxa in Figs. 1, 2, 4–6, 9, 11, and 12 were monoecious, and the taxa in Figs. 3, 10, and 13 were dioecious and nannandrous. More about

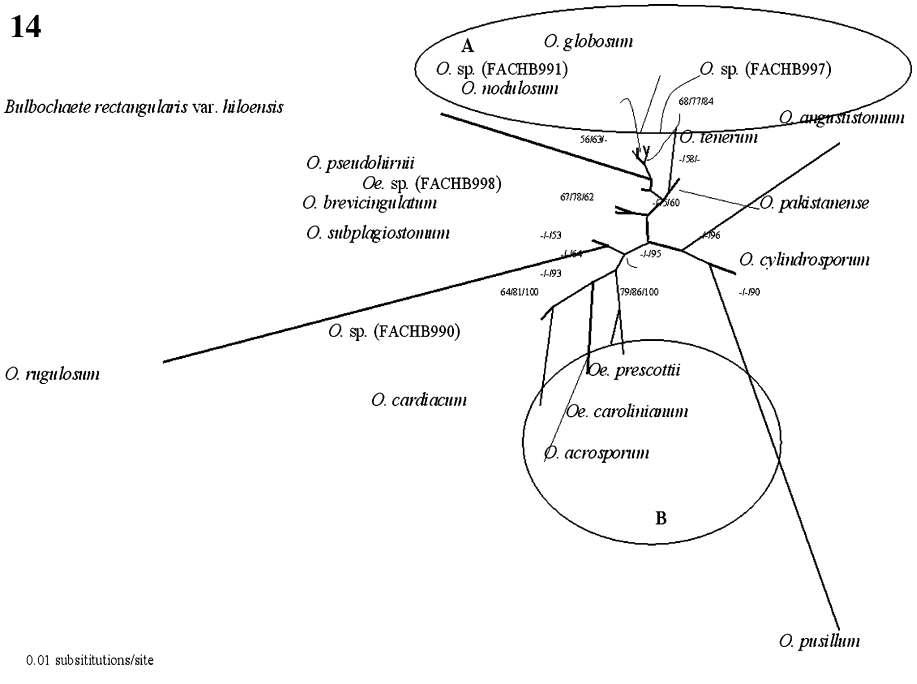


Fig. 14. Fifty percent majority-rule consensus tree derived from Bayesian analysis of 20 aligned 18S rDNA data (Base = 0.2620 0.2096 0.2707, Nst = 6, Rmat = 1.0000 1.9421 1.0000 1.0000 3.4930, Rates = gamma, Shape = 0.7355, Pinvar = 0.6867) Bootstrap percentages NJ (left), MP (middle) as well as Bayesian a posterior probabilities (right) are indicated for each node if higher than 50%. **A**: a group formed by four monoecious *Oedogonium taxa*; **B**: the *Oedocladium* taxa and their closest *Oedogonium* taxon. Note: Branch lengths are proportional to the number of the expected nucleotide substitutions (see scale in lower left). Unless otherwise noted, internodes without bootstrap values denote branches that were resolved in fewer than 50% of all bootstrap replicates. Some bootstrap values are noted using lines that refer to the appropriate internode

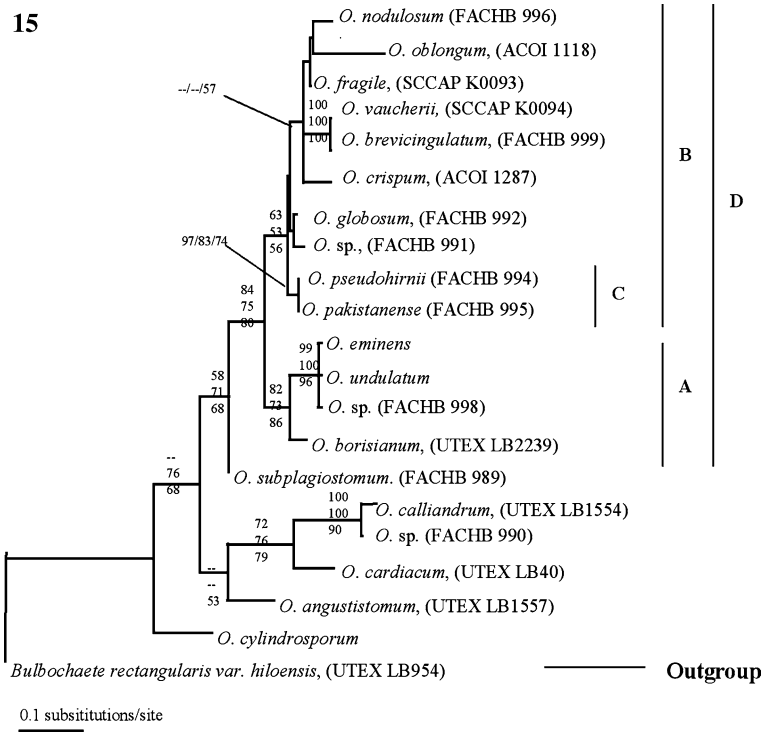


Fig. 15. Maximum likelihood tree of score 1316.25028 constructed from ITS-2 sequence data (Base = equal, Nst = 2, TRatio = 1.5351, Rates = gamma, Shape = 0.3107, Pinvar = 0). Bootstrap percentages of NJ (top) and MP (middle) as well as ML (bottom) are indicated for each node if higher than 50%. See note in Fig. 3. **A**: A strong supported clade of the dioecious nannandrous taxa; **B**: A group formed by the monoecious taxa; **C**: A clad of two terrestrial monoecious taxa; **D**: A group formed by the monoecious and the dioecious nannandrous taxa

morphology characters is presented in the figure legend.

Analyses of 18S rDNA data. After alignment, there were 1,535 sites of which 174 were variable and 33 were parsimony informative sites in the 18S rDNA sequence file. Pairwise-inferred substitutions comparing with TN93 distance showed that neither transitions nor transversions had reached saturation, which indicated that they could be used for phylogenetic analyses. The Bayesian phylogeny generated from this data (Fig. 14), was compared with one of the 675 equally parsimonious trees (L = 192, CI = 0.8177, RI = 0.5833, RC = 0.4470, HI = 0.4667) from MP analyses and the tree from NJ analyses (Farris 1989). A monophyletic group of *Oedocladium* taxa was well resolved with bootstrap support. This clade and some *Oedogonium* taxa (*O. acrosporum*, *O. cardiacum*, *O.* sp. FACHB 990) chosen in the present investigation formed a strong supported group (marked B) in Bayesian phylogeny while the *Bulbochaete* taxon and some *Oedogonium* taxa only obtained a weak bootstrap support to form a clade. Phylogenetic analyses of the 18S data also resolved four monoecious *Oedogonium* taxa (*O. globosum*, *O. nodulosum*, *O.* sp. FACHB 991 and *O.* sp. FACHB 997, marked A) as a monophyletic group with a well bootstrap resolution. Two Monoecious *Oedogonium* isolates of *O.* sp. (FACHB 998) and *O. brevicingulatum* were resolved as a group with bootstrap support 62–78%. The three dioecious macrandrous *Oedogonium* taxa (*O. cylindrosporum*, *O. pusillum* and *O. angustistomum*) formed a monophyletic group with strong bootstrap support in Bayesian analysis.

ITS-2 analyses of Oedogonium taxa. There were 231 sites of which 98 were variable and 64 were parsimony informative sites in the ITS-2 sequence file, and *Bulbochaete rectangularis* var. *hiloensis* was chosen as an outgroup taxon. Pairwise-inferred substitutions comparing with Kimura distance showed that neither transitions nor transversions had reached saturation. The optimal tree from ML analyses of ITS-2 data (Fig. 15) was compared with one of the 18 equally parsimonious trees (L = 216, CI = 0.6296, RI = 0.7143, RC = 0.4497, HI = 0.4469; not shown) from MP analyses and the tree from NJ analyses. Results from analyses of the ITS-2 data identify all the four dioecious nannandrous *Oedogonium* taxa (*O. eminens*, *O. undulatum*, *O.* sp. FACHB 998 and *O. borisianum*), formed a strong supported monophyletic group (marked A). In addition, analyses of the ITS-2 data placed all the monoecious *Oedogonium* taxa chosen in the present investigation as a poorly supported monophyletic group (marked B). Within this group, *O. pseudohirnii* and *O. pakistanense* formed a strong supported group (marked C). The results also resolved a monophyletic clade including all dioecious nannandrous and monoecious *Oedogonium* taxa with bootstrap support 84% for NJ, 75% for MP and 80% for ML (marked D). The dioecious macrandrous *Oedogonium* taxa were basal to this clade. Three isolates of the macrandrous taxa formed a group with 72–79% bootstrap support, and two of them showed a very close phylogenetic relationship.

Discussion

Oedogoniales phylogeny inferred from 18S rDNA sequences. Former correlative molecular phylogeny studies (Booton et al. 1998, Alberghina et al. 2006) have indicated that Oedogoniales constitute a monophyletic, taxonomically isolated clade and also suggested that *Bulbochaete* could be more basally situated phylogenetically than the other two genera.

In the present research a total of twenty 18S rDNA sequences data of the Oedogoniales were analyzed. The results provided by this study affirm the conclusion drawn by previous molecular investigations. In addition, as 16 new sequences were added by this study, the results also showed that the clade of *Oedocladium* was clustered into the members of *Oedogonium*. Morphological characters and the complexity of individual growth of *Bulbochaete*, have shown good correlation to results. The appearance of branches and terminal or interstitial cells bearing long hair cells with a bulbous base, of the basel cell of successive mediacy cell division, and the complex cell divisions that form the hair cell had obviously disjoined the *Bulbochaete* from *Oedocladium* and *Oedogonium* (Fritsch 1948, 1956; Pickett Heaps 1975). On the other hand, the features that the basal terminal cell of *Oedocladium* was not differentiated into a holdfast and the apical cell of *Oedocladium* divided and formed a cap structure, was found in some terrestrial taxa in *Oedogonium* (Chacko 1970, Jao 1979, Liu and Hu 2004, Luo et al. 2002). Therefore, the result that *Bulbochaete* is comparatively distant to the other genera in this order, whereas *Oedocladium* and some *Oedogonium* taxa are closely related to each other is also well supported by morphological studies. In a recent work, Alberghina et al. (2006) obtained a similar result, and they also suggested that *Bulbochaete* would not necessarily have been derived from a simpler *Oedocladium*-like ancestor. We compared the characters of the species of *Oedocladium* (Pickett Heaps 1977, Markowitz 1978, Mrozińska 1985, Liu and Bi 1993) and their most closely related *Oedogonium* species and found that these species may be similar in some characters of oogonium. For instance, both of the two *Oedocladium* species and their closest related *Oedogonium acrosporum* share the characters that the oogonium is terminal and solitary, with an epigynous circumscission on it. The *Oedogonium* species which are close to the *Oedocladium* species revealed by shorter branches all have solitary oogonia, while *O.*

angustistomum, *O. cylindrosporum*, *O. pusillum*, *O. rugulosum*, *O. subplagiostomum*, which revealed long branches, have solitary and continuous oogonia.

Differentiation into clades as supported group four monoecious taxa group, and a group three dioecious macrandrous taxa, and another group consist of a monoecious taxon and a dioecious nannandrous taxon further suggested that the relationships between *Oedogonium* taxa might be attributable to their sexual differentiation. In previous studies, most authors (Hirn 1900, Tiffany 1930, Jao 1979, Mrozińska 1985) divided the *Oedogonium* taxa according to their type of sexual reproduction, but they were not accordant with evolutionary relationships among the taxa belonging to groups with different sexual reproduction.

The ITS2 analyses of the Oedogonium taxa combining with sexual differentiation. In order to clarify the relationships between species within the genus *Oedogonium*, data from ITS-2, which was proven to be the more variable region and most useful at species and genus level (Van Nues et al. 1995; Hershkovitz and Lewis 1996; Coleman and Mai 1997; Stiger et al. 2000, 2003; Coleman 2003), were chosen for this analysis. According to the correlative results of the 18S rDNA analyses, *Bulbochaete rectangularis* var. *hiloensis* was used to root the trees. This is the first time that ITS-2 along with typical characters are involved into systematics of this interesting group *Oedogonium*.

So far, as is known, Jao (1979), based on his sample collecting from a large area in China, presented the types of sexual differentiation as the groundwork for classification below the rank of genus on *Oedogonium* in his monographic workon. This criterion (Wittrock 1874, Hirn 1900, Tiffany 1930) was used by many phycologists before and it was also well accepted by modern researchers (Mrozińska 1985). In the 1990's, Mrozińska proposed to divide the genera *Oedogonium* into sections based on the number of spermatozoids produced in the antheridial cell. We compared these characteristics with the result of the molecular phylogeny to compare the hypotheses.

Within the strongly supported monophyletic group marked A in Fig. 15, the four species have two common features as all are dioecious nannandrous and monospermatozoid species. In the group marked B in Fig. 15, the species are all monoecious and dispermatozoid. All ingroup species outside of the well resolved clade D consisting of the former two groups are dioecious nannandrous and dispermatozoid, and they form a paraphyly. These results suggest that classification based on type of sexual differentiation is more consistent with results of molecular phylogeny.

Our research support the type of sexual reproduction is better suited to characterize species. First, this character is of steady heredity, it will not change under the influence of habitat; secondly, species in each of the three genera can be grouped under the criterion of the type of sexual reproduction. In most cases, the monoeciousones are wave based than the dioeciousones. This consistency reveals the evolution of species within the genera.

We also found that both the monoecious taxa and dioecious nannandrous taxa are of independent origin, and they have a closer relationship with each other. The dioecious macrandrous taxa formed a paraphyly and could be more separately situated than the dioecious nannandrous and the moecious species, which is coincident with the result (a monoecious taxon *O. brevicingulatum* and a dioecious nannandrous taxon *O.* sp. FACHB 998 and were grouped) of the 18S rDNA analyses. Facts that support the result can also be found together in classical taxonomy. First, the dioecious macrandrous have obvious sexual differentiation; all the cells in their male strains are male. On the other hand, the dwarf males developed from the androsporangium, and the latter of the dioecious nannandrous are produced by "male" cells in plants consisting of two sexual cells. It is only the differentiation of some cells in the plants, not the sexual differentiation of the whole plant. There is no essential difference between the dioecious nannandrous and the monoecious species (Jao 1979).

In addition, the strongly supported group formed by two terrestrial monoecious isolates *O. pseudohirnii* and *O. pakistanense* is in a basal position relative to the other monoecious species analyzed. This fact gave the information that the two terrestrial taxa are more closely related compared with their relation to other hydrophytic taxa and that the terrestrials possibly evolved from the hydrophytics.

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