

# Taxonomic re-examination of six species of *Nitella* (Charales, Charophyceae) from Asia, and phylogenetic relationships within the genus based on *rbcL* and *atpB* gene sequences

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Six taxa of *Nitella* (Charales, Charophyceae), collected from Asia, were investigated using light and scanning electron microscopy (SEM) for the oospores, and sequencing of the gene encoding the large subunit of Rubisco (*rbcL*), in order to improve our understanding of their taxonomic status. Our SEM observations demonstrated that the oospore morphology of four taxa belonging to the subgenus *Tieffallenia* [*N. megaspora* (J. Groves) Sakayama, *comb. nov.* (= *N. pseudoflabellata* f. *megaspora*), *N. tumulosa* (= *N. furcata* f. *tumulosa*), *N. gracillima* (= *N. gracilis* f. *gracillima*) and *N. axilliformis* (= *N. translucens* f. *axilliformis*)] is distinctly different from that of the species *N. pseudoflabellata*, *N. furcata*, *N. gracilis* and *N. translucens*, respectively, to which R.D. Wood assigned them as infraspecific taxa. Our *rbcL* sequence data showed that *N. megaspora* is separated phylogenetically from *N. pseudoflabellata*, *N. tumulosa* from *N. furcata* and *N. axilliformis* from *N. translucens*. In addition, to re-examine the taxonomic system of *Nitella* proposed by R.D. Wood, who treated oospore wall ornamentations as diagnostic at the infraspecific level, we carried out molecular phylogenetic analyses using the combined sequence data set for the gene encoding the beta subunit of ATP synthase (*atpB*) and the *rbcL* gene of these six species, as well as eleven species of *Nitella* studied previously. The combined sequence data resolved five robust clades within the subgenus *Tieffallenia* that were characterized by differences in oospore wall ornamentation. However, the species and sectional diagnoses of R.D. Wood, such as the form and cell number of dactyls in vegetative thalli, were variable within the clades. Therefore, R.D. Wood's taxonomic system appears unnatural, at least within the subgenus *Tieffallenia*.

## INTRODUCTION

Within the Charales, *Nitella* C. Agardh is the largest genus and exhibits the highest diversity of oospore morphology (Wood 1965; Khan & Sarma 1984); it is common in bodies of water in Asia, especially rice fields and bogs that are used for irrigation (Zaneveld 1940; Imahori 1954; Wood 1965; Han *et al.* 1994; Choi & Kim 1998). In his worldwide monograph of the Charales, Wood (1965) divided *Nitella* into three subgenera, *Nitella*, *Hyella* R.D. Wood, and *Tieffallenia* R.D. Wood, based on the number of cells forming a terminal branchlet ray (called a 'dactyl') and the shapes of the end cells. The subgenera *Nitella* and *Hyella* are characterized by having unicellular dactyls and multicellular dactyls, respectively, in which the end cell is similar to the penultimate cell (Wood 1965). Conversely, the subgenus *Tieffallenia* is characterized by multicellular dactyls, in which the end cell is very different from the penultimate cell; it contains most of the species in the genus *Nitella*, and is mainly distributed in Africa, Asia and Australia (Wood 1965; Khan & Sarma 1984).

Wood (1962, 1965) characterized the sections and species of the genus *Nitella* mainly on the basis of differences in vegetative morphology, for example the overall appearance of

the thallus, the furcation in a branchlet, the shape and number of cells in dactyls, and the number, position, or size of the gametangia. By contrast, oospore morphology, especially oospore wall ornamentation, was treated as diagnostic at the infraspecific level (Wood 1962, 1965). Therefore, he reduced many species of *Nitella* from Asia to infraspecific rank (Wood 1962, 1965). Within the genus *Nitella*, he recognized 53 species out of 204 species previously described. However, subsequent studies of oospore morphology (especially wall ornamentation) using scanning electron microscopy (SEM) have shown the existence of distinct features that suggested that some of the infraspecific taxa recognized by Wood (1962, 1965) should be raised to species rank (Caceres 1975; John & Moore 1987; Leitch *et al.* 1990; Casanova 1991; Mukherjee & Ray 1993; Mandal *et al.* 1995; Nozaki *et al.* 1998; Mandal & Ray 1999; Ray *et al.* 2001). Recently, Sakayama *et al.* (2002) examined nine species of *Nitella* collected from Japan; based on oospore morphology (by SEM) integrated with *rbcL* gene phylogeny, they demonstrated the phylogenetic validity of using oospore morphology for the diagnosis of some species of *Nitella*. In common with other phylogenetic studies of the Charales (McCourt *et al.* 1996, 1999), however, Sakayama *et al.* (2002) did not resolve phylogenetic relationships within *Nitella* reliably enough to test the taxonomic system of Wood (1962, 1965); this may have been because of the limited in-

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**Table 1.** List of the charalean species or strains and related species and DDBJ-EMBL-GenBank accession numbers used for the present phylogenetic analyses.

Species	Strain designation and collection information	Accession number	
		<i>atpB</i> gene	<i>rbcl</i> gene
<i>Chara connivens</i> Salzmänn ex A. Braun	F140, Spain	AF408782 <sup>1</sup>	AF097161 <sup>2</sup>
<i>Lamprothamnium macropogon</i> (A. Braun) Ophel	X695, Australia	AF408783 <sup>1</sup>	U27534 <sup>3</sup>
<i>Nitellopsis obtusa</i> (Desvaux in Loiseleur-Deslongchamps) J. Groves	F131B, Germany	AF408785 <sup>1</sup>	U27530 <sup>3</sup>
<i>Lychnothamnus barbatus</i> (Meyen) Leonhardi	159, Australia	AF408784 <sup>1</sup>	AF097171 <sup>2</sup>
<i>Nitella flexilis</i> (Linnaeus) C. Agardh	S010, Japan	AB110837 <sup>4</sup>	AB076056 <sup>5</sup>
<i>N. opaca</i> (Bruzellius) C. Agardh	F146, Poland	AF408786 <sup>1</sup>	AF097174 <sup>2</sup>
<i>N. mirabilis</i> Nordstedt ex J. Groves <sup>6</sup>	S040, Bog at Yokawa-cho, Hyogo, Japan, 2 Jun. 2001	AB110838 <sup>4</sup>	AB110865 <sup>4</sup>
<i>N. acuminata</i> A. Braun ex Wallman <sup>6</sup>	S057, <sup>7</sup> Bog at Temerloh, Pahang Darul Makmur, Malaysia, 28 Nov. 2001	AB110839 <sup>4</sup>	AB110866 <sup>4</sup>
<i>N. praelonga</i> A. Braun	P/CR7, Costa Rica	AY093930 <sup>8</sup>	AF097173 <sup>5</sup>
<i>N. pulchella</i> Allen <sup>9</sup>	S011, Japan	AB110840 <sup>4</sup>	AB076057 <sup>5</sup>
	S051, Konita-ike, Yashiro-cho, Hyogo, Japan, 3 Jun. 2001	AB110841 <sup>4</sup>	AB110867 <sup>4</sup>
<i>N. furcata</i> (Roxburgh ex Bruzelius) C. Agardh <sup>9</sup>	S003, Japan	AB110842 <sup>4</sup>	AB076058 <sup>5</sup>
	S037, Japan	AB110843 <sup>4</sup>	AB076059 <sup>5</sup>
<i>N. inversa</i> Imahori <sup>9</sup>	S035, Japan	AB110844 <sup>4</sup>	AB076060 <sup>5</sup>
<i>N. tumulosa</i> Zaneveld <sup>6</sup>	S058, <sup>10</sup> Haew Loam waterfall (9°45'35.0"N, 98°40'38.3"E), Thailand, 24 Dec. 2001	AB110845 <sup>4</sup>	AB110868 <sup>4</sup>
	S060, <sup>7</sup> Bog at Temerloh, Pahang Darul Makmur, Malaysia, 28 Nov. 2001	AB110846 <sup>4</sup>	AB110869 <sup>4</sup>
<i>N. gracilens</i> Morioka <sup>9</sup>	S017, Japan	AB110847 <sup>4</sup>	AB076061 <sup>5</sup>
	S018, Japan	AB110848 <sup>4</sup>	AB076062 <sup>5</sup>
	KINU, Japan	AB110849 <sup>4</sup>	AB076063 <sup>5</sup>
	S049, Ojiga-ike, Yashiro-cho, Hyogo, Japan, 2 Jun. 2001	AB110850 <sup>4</sup>	AB110870 <sup>4</sup>
	S050, Konita-ike, Yashiro-cho, Hyogo, Japan, 3 Jun. 2001	AB110851 <sup>4</sup>	AB110871 <sup>4</sup>
<i>N. pseudoflabellata</i> A. Braun <sup>9</sup>	S031, Japan	AB110852 <sup>4</sup>	AB076064 <sup>5</sup>
	S032, Japan	AB110853 <sup>4</sup>	AB076065 <sup>5</sup>
	S016, Japan	AB110854 <sup>4</sup>	AB076066 <sup>5</sup>
<i>N. megaspora</i> (J. Groves) Sakayama, <sup>6</sup> <i>comb. nov.</i>	S054, <sup>11</sup> Wetland at Watarase-yusui, Fujioka-machi, Tochigi, Japan	AB110855 <sup>4</sup>	AB110872 <sup>4</sup>
<i>N. hyalina</i> (De Candolle) C. Agardh <sup>9</sup>	S012, Japan	AB110856 <sup>4</sup>	AB076067 <sup>5</sup>
	S061, <sup>12</sup> unknown	AB110857 <sup>4</sup>	AB110873 <sup>4</sup>
<i>N. gracillima</i> Allen <sup>6</sup>	S053, Bog at Yokawa-cho, Hyogo, Japan, 2 Jun. 2001	AB110858 <sup>4</sup>	AB110874 <sup>4</sup>
<i>N. spiciformis</i> Morioka <sup>9</sup>	S015, Japan	AB110859 <sup>4</sup>	AB076068 <sup>5</sup>
	S055, <sup>11</sup> Wetland at Watarase-yusui, Fujioka-machi, Tochigi, Japan	AB110860 <sup>4</sup>	AB110875 <sup>4</sup>
<i>N. moriokae</i> R.D. Wood <sup>9</sup>	S004, Japan	AB110861 <sup>4</sup>	AB076069 <sup>5</sup>
	S052, Bog at Yokawa-cho, Hyogo, Japan, 2 Jun. 2001	AB110862 <sup>4</sup>	AB110876 <sup>4</sup>
<i>N. translucens</i> (Persoon) C. Agardh	F108, France	—	AF097745 <sup>2</sup>
<i>N. axillaris</i> A. Braun <sup>9</sup>	S005, unknown	AB110863 <sup>4</sup>	AB076070 <sup>5</sup>
<i>N. axilliformis</i> Imahori <sup>6</sup>	S056, <sup>11</sup> Wetland at Watarase-yusui, Fujioka-machi, Tochigi, Japan	AB110864 <sup>4</sup>	AB110877 <sup>4</sup>
<i>Tolypella prolifera</i> (Ziz ex A. Braun) Leonhardi	F150, France	AF408787 <sup>1</sup>	AF097175 <sup>2</sup>
<i>Coleochaete orbicularis</i> Pringsheim	UTEX LB 2651	AF408788 <sup>1</sup>	L13477 <sup>13</sup>
<i>Zygnema peliosporum</i> Wittrock	UTEX LB 45	AF408799 <sup>1</sup>	U38701 <sup>14</sup>
<i>Klebsormidium flaccidum</i> (Kützinger) P.C. Silva et al.	UTEX LB 2017	AF408801 <sup>1</sup>	L13478 <sup>13</sup>
<i>Chlorokybus atmophyticus</i> Geitler	UTEX LB 2591	AF408805 <sup>1</sup>	AF408255 <sup>1</sup>
<i>Arabidopsis thaliana</i> (Linnaeus) Heynhold		AP000423 <sup>15</sup>	AP000423 <sup>15</sup>
<i>Ginkgo biloba</i> Linnaeus		AJ235481 <sup>16</sup>	D10733 <sup>17</sup>

Table 1. Continued.

Species	Strain designation and collection information	Accession number	
		<i>atpB</i> gene	<i>rbcL</i> gene
<i>Sphagnum palustre</i> Linnaeus		AF313557 <sup>18</sup>	L13485 <sup>13</sup>
<i>Marchantia polymorpha</i> Linnaeus		X04465 <sup>19</sup>	X04465 <sup>19</sup>

<sup>1</sup> Karol *et al.* (2001).<sup>2</sup> McCourt *et al.* (1999).<sup>3</sup> McCourt *et al.* (1996).<sup>4</sup> This study.<sup>5</sup> Sakayama *et al.* (2002).<sup>6</sup> Species identification by the present SEM observations of the oospores (Figs 1–25).<sup>7</sup> Thalli provided by Mr T. Yamada (University of Tokyo).<sup>8</sup> Cimino & Delwiche (2002).<sup>9</sup> Species identification by Sakayama *et al.* (2002).<sup>10</sup> Thalli provided by Prof. M. Kato (University of Tokyo).<sup>11</sup> Cultured material provided by Dr S. Arai (University of Tokyo).<sup>12</sup> Cultured material provided by Mr T. Takahashi (St Margaret's Junior and Senior High Schools).<sup>13</sup> Data from Manhart (1994).<sup>14</sup> McCourt *et al.* (1995).<sup>15</sup> Sato *et al.* (1999).<sup>16</sup> Savolainen *et al.* (2000).<sup>17</sup> Hasebe *et al.* (1992).<sup>18</sup> Pryer *et al.* (2001).<sup>19</sup> Ohyama *et al.* (1986).

formation from the single gene used and the small number of species analysed. Therefore, additional taxa and genes are needed to resolve robust, detailed phylogenetic relationships within the genus *Nitella*.

The chloroplast-encoded *atpB* gene is one of the photosynthetic genes and has been used in the phylogeny of certain algae and land plants (Lockhart *et al.* 1992; Hoot 1995; Hoot & Crane 1995; Hoot *et al.* 1995a, b; Wolf 1997). Wolf (1997) and Nozaki *et al.* (1999) showed that the divergences of *atpB* genes among the fern genera and the colonial Volvocales, respectively, are similar to those of the *rbcL* genes. Therefore, combined data from *atpB* and *rbcL* gene sequences seem to be efficient for resolving detailed phylogenetic relationships within the Charales.

In this study, six additional taxa of *Nitella* were collected from Asia, and SEM oospore morphology and *rbcL* gene phylogeny were examined to improve the understanding of their taxonomic status. Furthermore, we carried out molecular phylogenetic analyses using a combined sequence data set that consisted of the beta subunit of the adenosine triphosphate synthase (*atpB*) and *rbcL* genes of these six species and of 11 species of *Nitella* studied previously (Mccourt *et al.* 1999; Karol *et al.* 2001; Cimino & Delwiche 2002; Sakayama *et al.* 2002). Our phylogenetic analyses resolved detailed phylogenetic relationships that conflict with the taxonomic system of Wood (1962, 1965) within the subgenus *Tieffallenia*.

## MATERIAL AND METHODS

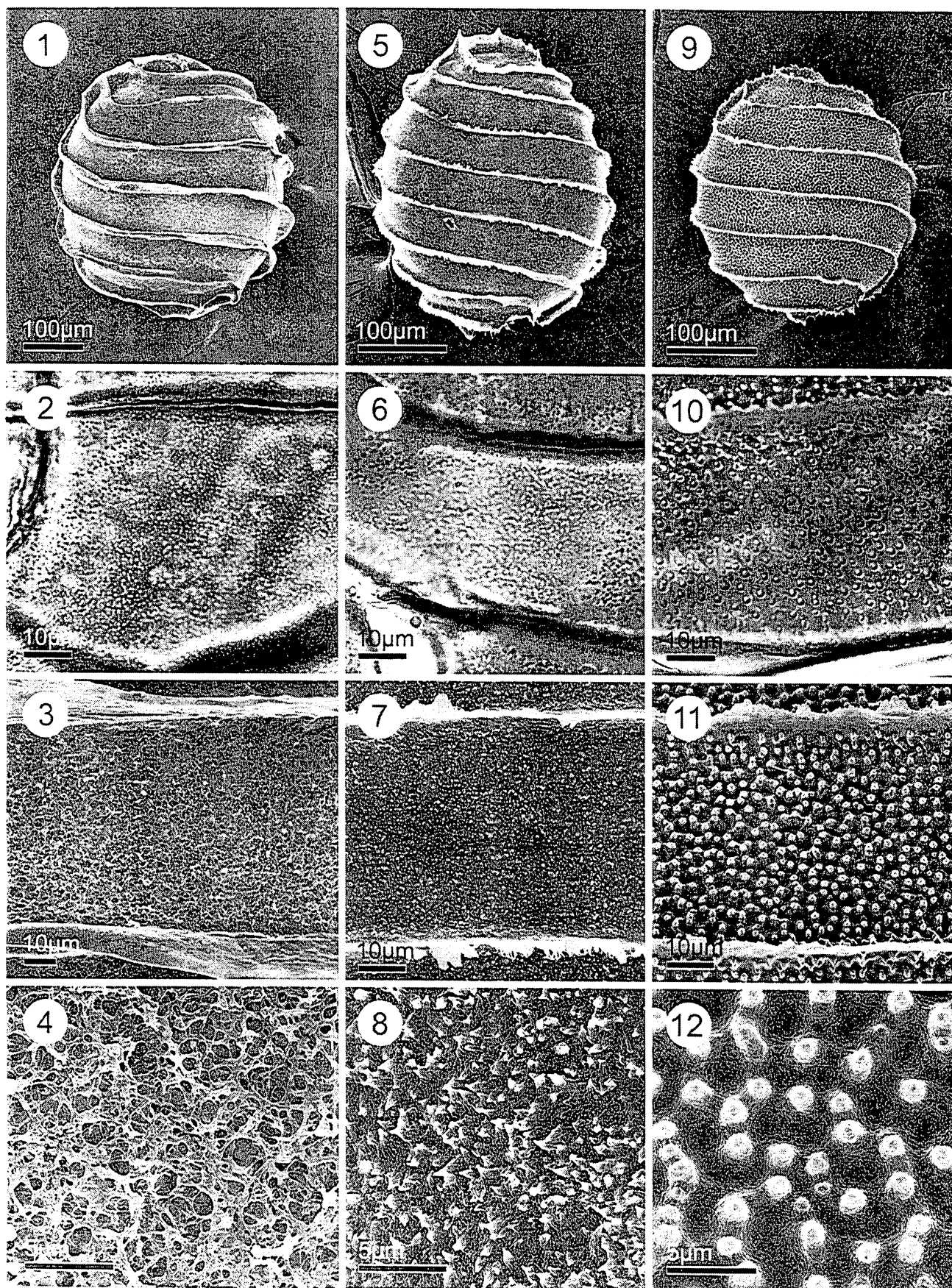
### Culture and morphological methods

The localities from which material was collected are shown in Table 1. The methods for field collection, culture, and light microscopy (LM) and SEM were essentially the same as in our previous study (Sakayama *et al.* 2002), except in the following five respects. (1) Cultures were established by inoculating part of each thallus in a 2000 ml glass vessel or 900 ml glass jar containing soil–water medium for the Charales (SWC-1 or SWC-2). SWC-1 consisted of deionized water and two soil layers composed of leaf mould in the bottom layer and black soil in the surface layer; SWC-2 consisted of deionized water and a river sand layer (Sungreen, Chiba, Japan) containing particles of calcium carbonate fertilizer (Toyo-kogyo, Tochigi, Japan). (2) The glass vessel or jar containing the soil layers and deionized water was autoclaved separately for 20 min before cultivation. (3) The cultures were maintained under controlled laboratory conditions at 20–25°C with a 16:8 h light–dark cycle and 10–40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  illumination provided by fluorescent lamps. (4) LM observations were made with a BX60 microscope (Olympus, Tokyo, Japan) equipped with Nomarski interference optics, and SEM observations were made with an S-4500 scanning electron microscope (Hitachi, Tokyo, Japan) at 10–20 kV. (5) The terms used

Table 2. New primers used for amplifications and sequencing of the *atpB* genes in the present study.

Designations	Positions <sup>1</sup>	Sequence (5' to 3')
CH- <i>atpB</i> -F4	227–248	CTATGAGTGCTACTGATGGTCT
CH- <i>atpB</i> -F5	722–743	CAGGTGCTCGCATGAGAGTTGG
CH- <i>atpB</i> -R6	884–863 <sup>2</sup>	CCTACTGCTGAAGGCATTCTAC
CH- <i>atpB</i> -R7	1322–1301 <sup>2</sup>	CCAGTAAAGACTTCTGCTACAA

<sup>1</sup> Coordinate number from the *Marchantia polymorpha atpB* gene (Ohyama *et al.* 1986).<sup>2</sup> Reverse primer.



in this study to describe the LM and SEM oospore morphology are based on those of Wood (1965), John & Moore (1987) and Leitch *et al.* (1990).

### Molecular phylogenetic analysis

Preparation of total DNA, amplification of DNA by the polymerase chain reaction (PCR) and direct sequencing of the PCR products were essentially as described previously (Nozaki *et al.* 2000; Sakayama *et al.* 2002), except for the primers used to amplify and sequence the *atpB* genes. Primers *atpB*-F1 and *atpB*-R3 (Nozaki *et al.* 1999) and four Charales-specific primers that we designed (Table 2) were used. The *atpB* gene sequences analysed in this study were 1020 bp and corresponded to positions 256–1275 of the *Marchantia polymorpha atpB* gene (Ohyaama *et al.* 1986).

For phylogenetic analysis, a data matrix containing 1182 bp of unambiguously aligned *rbcL* gene sequences (see Sakayama *et al.* 2002) from 36 charalean operational taxonomic units (OTUs) and eight related species (EMBL-Align database accession number ALIGN\_000550) was subjected to unweighted maximum parsimony (MP) analysis using PAUP\* 4.0b10 (Swofford 2002). The MP trees were constructed using a heuristic search with the stepwise addition of 100 random replications [with the tree bisection–reconnection (TBR) branch-swapping algorithm]. A bootstrap analysis (Felsenstein 1985) was carried out based on 1000 replications of the general heuristic search (full heuristic type with TBR branch-swapping algorithm). Based on the guidelines for constructing a topology using the distance method (Nei & Kumar 2000), we selected Jukes–Cantor (JC) distances (Jukes & Cantor 1969) to construct minimum evolution (ME) trees. For the same alignment used in the MP analysis, a distance matrix was calculated using the JC method (Jukes & Cantor 1969) in PAUP\* 4.0b10. Based on a heuristic search using stepwise addition of 100 random replications (with the TBR branch-swapping algorithm), an ME tree was constructed, again using PAUP\* 4.0b10; the robustness of lineages was tested by a bootstrap analysis with 100 replications of the general heuristic search (full heuristic type with TBR branch-swapping algorithm) using PAUP\* 4.0b10. Based on the same alignment data, a maximum likelihood (ML) analysis [with the Hasegawa–Kishino–Yano 85 model (Hasegawa *et al.* 1985)] was carried out using

PAUP\* 4.0b10 to estimate ‘quartet puzzling support (QPS) values’, which have the same practical meaning as bootstrap values, for internal branches of the phylogenetic tree with 1000 ‘puzzling steps’ (comparable to the number of bootstrap replicates) (Strimmer & von Haeseler 1996). In addition, phylogenetic trees based on 2202 bp of the combined sequence data set from the *atpB* (1020 bp) and *rbcL* (1182 bp) genes were constructed using the same methods as used to construct the *rbcL* gene trees (EMBL-Align database accession number ALIGN\_000554), except that *N. translucens* was excluded (Table 1) because no *atpB* gene sequences are available for this species. In these phylogenetic analyses, four charophyte species (*Coleochaete orbicularis*, *Zygnema peliosporum*, *Klebsormidium flaccidum* and *Chlorokybus atmophyticus*) and four land plants (*Arabidopsis thaliana*, *Ginkgo biloba*, *Sphagnum palustre* and *Marchantia polymorpha*) were designated as the out-group because recent molecular phylogenetic studies (McCourt *et al.* 1999, 2000; Karol *et al.* 2001) give reasonably high bootstrap support for the monophyly of the Characeae within the Streptophyta [Charophyceae *sensu* Mattox & Stewart (1984) and land plants].

## RESULTS AND DISCUSSION

### Taxonomic accounts

*Nitella* (subgen. *Nitella*) *mirabilis* Nordstedt ex J. Groves (1924, p. 364)

Figs 1–4

**OOSPORE MORPHOLOGY:** The oospores are oval in face view and have six to seven robust and strongly flanged spiral ridges; they are 420–440 µm long, 407–425 µm wide and 61–74 µm across the fossa (Fig. 1). The walls of mature oospores are brown to yellowish brown. The fossa wall is finely granulate under LM (Fig. 2), but appears fibrous under SEM (Fig. 3), with the fibrils forming an anastomosing network (Fig. 4). Fibrils are absent from both the spiral ridges and laterally extended walls (called flanges, Fig. 3).

**DISTRIBUTION:** China (Zaneveld 1940; Wood 1965; Han *et al.* 1994), India (Zaneveld 1940; Pal *et al.* 1962; Wood 1965) and Japan (Table 1).

**REMARKS:** If the taxonomic system of Wood (1965) is fol-

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Figs 1–4. Oospores of *Nitella mirabilis* (S040).

Fig. 1. Oospore with six to seven robust and strongly flanged spiral ridges on the surface; SEM.

Fig. 2. Part of fossa wall, showing finely granulate ornamentation; LM.

Fig. 3. Part of fossa wall, showing fibrous pattern, with strongly flanged spiral ridges lacking fibrils on the surface; SEM.

Fig. 4. Detail of fossa wall, showing fibrous pattern in which the fibrils formed an anastomosing network; SEM.

Figs 5–8. Oospores of *N. acuminata* (S057).

Fig. 5. Oospore with six to seven flanged spiral ridges on the surface; SEM.

Fig. 6. Part of fossa wall, showing scattered granulate ornamentation; LM.

Fig. 7. Part of fossa wall, showing scabrous ornamentation, with flanged spiral ridges; SEM. Note that the projections extend onto the base of the spiral ridges, but are absent from the flanges.

Fig. 8. Detail of fossa wall, showing scabrous pattern consist of minute projections that are irregularly arranged; SEM.

Figs 9–12. Oospores of *N. tumulosa* (S060).

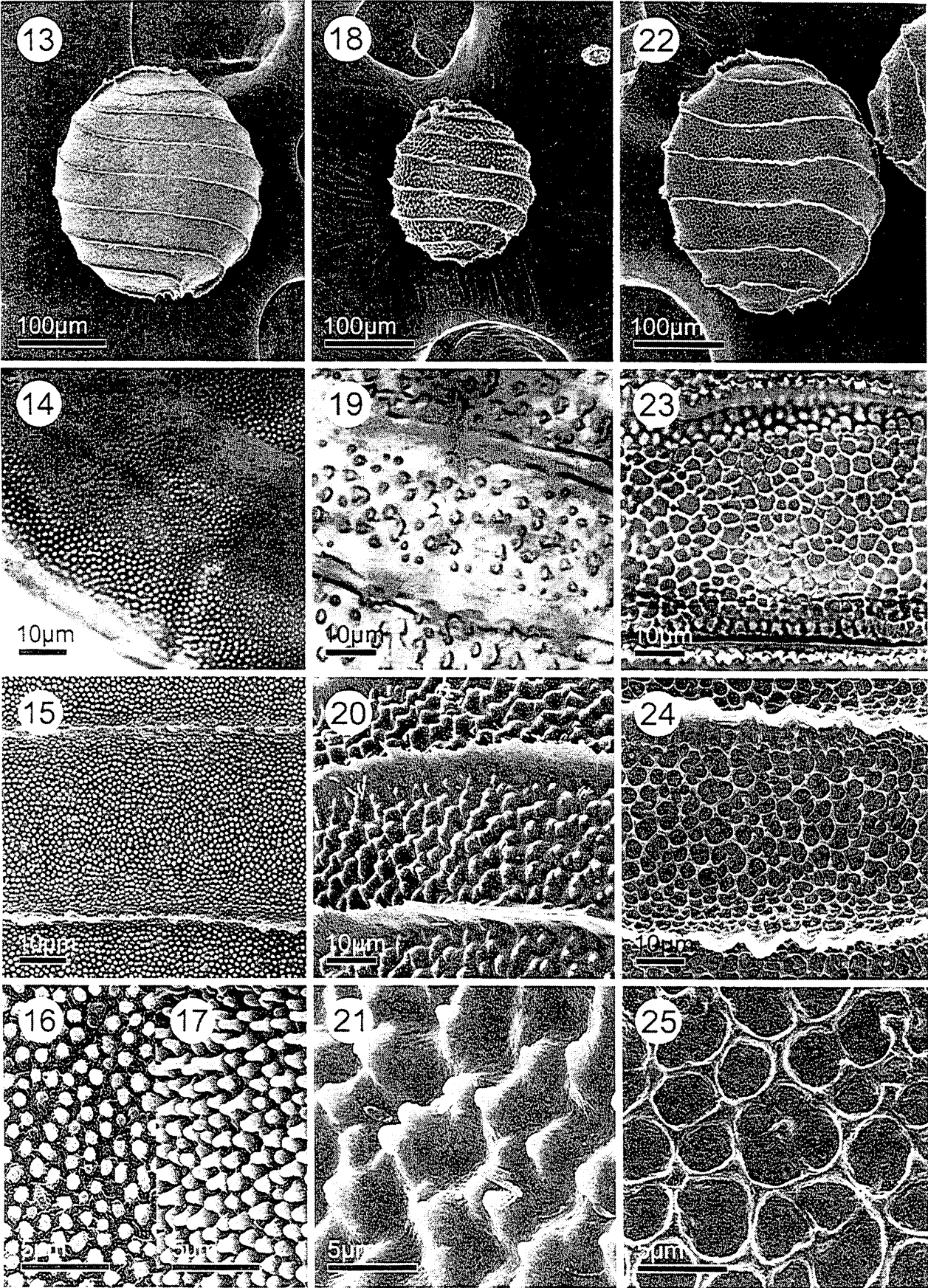
Fig. 9. Oospore with five to six flanged spiral ridges on the surface; SEM.

Fig. 10. Part of fossa wall, showing papillate ornamentation; LM.

Fig. 11. Part of fossa wall, showing papillate or beaded imperfect reticulate ornamentation, with flanged spiral ridges; SEM. Note that the papillae extend onto the base of the spiral ridges, but are absent from the flanges.

Fig. 12. Detail of fossa wall, showing papillate or beaded imperfect reticulate pattern; SEM. Note that the reticulum is formed by waved ridges with obtuse papillae.





lowed, this alga can be assigned to *N. mirabilis* var. *mirabilis*, based on its dioecism, the once-forked branchlets with unicellular dactyls, the compact fertile heads covered with mucus, the aggregated gametangia at the branchlet node or at the base of whorled branchlets, and the short antheridial stalk. The oospore wall ornamentations of this alga are finely granulate under LM (Fig. 2), as described by Wood (1965) for *N. mirabilis* var. *mirabilis*. However, our SEM observations revealed that the ornamentation of *N. mirabilis* var. *mirabilis* is fibrous (Figs 3, 4). Two other varieties of *N. mirabilis* – *N. mirabilis* var. *inokasiraensis* (Kasaki ex R.D. Wood) R.D. Wood and *N. mirabilis* var. *libera* R.S. Han & H.L. Fu – have been described from Japan (Wood 1965) and China (Fu & Han 1990), respectively, and their oospore wall ornamentations are also finely granulate under LM. However, no SEM study has been carried out for these two varieties. *Nitella mirabilis* var. *mirabilis* has not been previously reported from Japan.

***Nitella* (subgen. *Nitella*) *acuminata* A. Braun ex Wallman (1853, p. 35)**

Figs 5–8

**OOSPORE MORPHOLOGY:** The oospores are ovate in face view and have six to seven flanged spiral ridges; they are 288–297  $\mu\text{m}$  long, 255–263  $\mu\text{m}$  wide and 41–59  $\mu\text{m}$  across the fossa (Fig. 5). The walls of mature oospores are dark brown to brown. In LM, the fossa wall is scattered granulate with 30–50 granules (0.5–1.2  $\mu\text{m}$  in diameter) across the fossa (Fig. 6). SEM showed that the scabrous ornamentations consist of minute projections (Fig. 7). The irregularly arranged projections are 0.4–1.8  $\mu\text{m}$  high and extend barely onto the base of the spiral ridges; they are absent from the flanges (Figs 7, 8).

**DISTRIBUTION:** North and South America, India and Asia (Wood 1965; Table 1).

**REMARKS:** This alga can be assigned to *N. acuminata* var. *acuminata sensu* Wood (1965), based on the slender axis of less than 500  $\mu\text{m}$ , the once-forked branchlets lacking mucus, the unicellular dactyls that taper gradually and have an acuminate tip, and the obscure or compact fertile heads that rarely formed in the axils at the base of whorled branchlets. Leitch *et al.* (1990) reported that *N. acuminata* var. *greenii* R.D. Wood has a scabrous pattern on the fossa wall. Our SEM observations revealed that the fossa wall ornamentations of *N.*

*acuminata* var. *acuminata* (Figs 7, 8) are quite similar to those of *N. acuminata* var. *greenii*. However, *N. acuminata* var. *acuminata* is clearly distinguished from *N. acuminata* var. *greenii* by differences in the fertile heads and the size of the oospore. In *N. acuminata* var. *acuminata*, the fertile heads are absent or compact (Wood 1965), and the oospores are less than 300  $\mu\text{m}$  long (Fig. 5). By contrast, *N. acuminata* var. *greenii* has dense fertile heads that locate in the axils at the base of whorled branchlets, and the oospore exceeds 320  $\mu\text{m}$  (Wood 1965).

***Nitella* (subgen. *Tieffallenia*) *tumulosa* Zaneveld (1940, p. 86)**

Figs 9–12

**SYNONYM:** *Nitella furcata* (Roxburgh ex Bruzelius) C. Agardh f. *tumulosa* (Zaneveld) R.D. Wood (1965, p. 526).

**OOSPORE MORPHOLOGY:** The oospores are oval in face view and have five to six flanged spiral ridges; they are 260–270  $\mu\text{m}$  long, 245–260  $\mu\text{m}$  wide and 45–60  $\mu\text{m}$  across the fossa (Fig. 9). The walls of mature oospores are yellowish brown to light brown. The fossa wall is papillate, with up to c. 20 papillae across the fossa (Fig. 10). Under SEM, there is a papillate or beaded imperfect reticulate pattern (Fig. 11), in which the reticulum is formed by waved ridges with obtuse papillae about 0.7–1.4  $\mu\text{m}$  in diameter (Fig. 12). The papillae extend onto the base of the spiral ridges but are absent from the flanges (Fig. 11).

**DISTRIBUTION:** Indonesia (Zaneveld 1940; Wood 1965), Malaysia (Table 1), Thailand (Table 1) and India (Mandal & Ray 1999).

**REMARKS:** *Nitella tumulosa* was regarded as a form of *N. furcata* by Wood (1965) because both species are monoecious, lack reproductive organs at the base of whorled branchlets and have indistinguishable fertile and sterile branchlets in which the dactyls are predominantly abbreviated and two- or three-celled. However, SEM showed that the oospore wall ornamentations of *N. tumulosa* are clearly different from those of *N. furcata* [= *N. furcata* f. *furcata sensu* Wood (1965)] or *N. inversa* [= *N. furcata* f. *inversa sensu* Wood (1965)]. The oospore wall ornamentation of *N. tumulosa* is a papillate or beaded imperfect reticulate pattern composed of obtuse papillae (Figs 11, 12). In contrast, oospores of *N. furcata* have an imperfect reticulate ornamentation (Mandal *et al.* 1995; Sakayama *et al.* 2002), whereas those of *N. inversa* have a

**Figs 13–17. Oospores of *Nitella megaspora* (S054).**

Fig. 13. Oospore with six to seven unflanged spiral ridges on the surface; SEM.

Fig. 14. Part of fossa wall, showing finely granulate ornamentation; LM.

Fig. 15. Part of fossa wall, showing finely granulate ornamentation, with unflanged spiral ridges; SEM. Note that the granules barely extend onto the spiral ridges.

Figs 16, 17. Two detailed views of fossa wall, showing prominent and elongate granules; SEM.

**Figs 18–21. Oospores of *N. gracillima* (S053).**

Fig. 18. Oospore with five to seven flanged spiral ridges on the surface; SEM.

Fig. 19. Part of fossa wall, showing papillate or tuberculate ornamentation; LM.

Fig. 20. Part of fossa wall, showing papillate or beaded imperfect reticulate ornamentations, with flanged spiral ridges; SEM. Note that the papillae/tubercles fuse with the base of the spiral ridges and are absent from the flanges.

Fig. 21. Detail of fossa wall, showing papillate or beaded imperfect reticulate pattern; SEM. Note that the reticulum is formed by fused or waved ridges with papillae/tubercles.

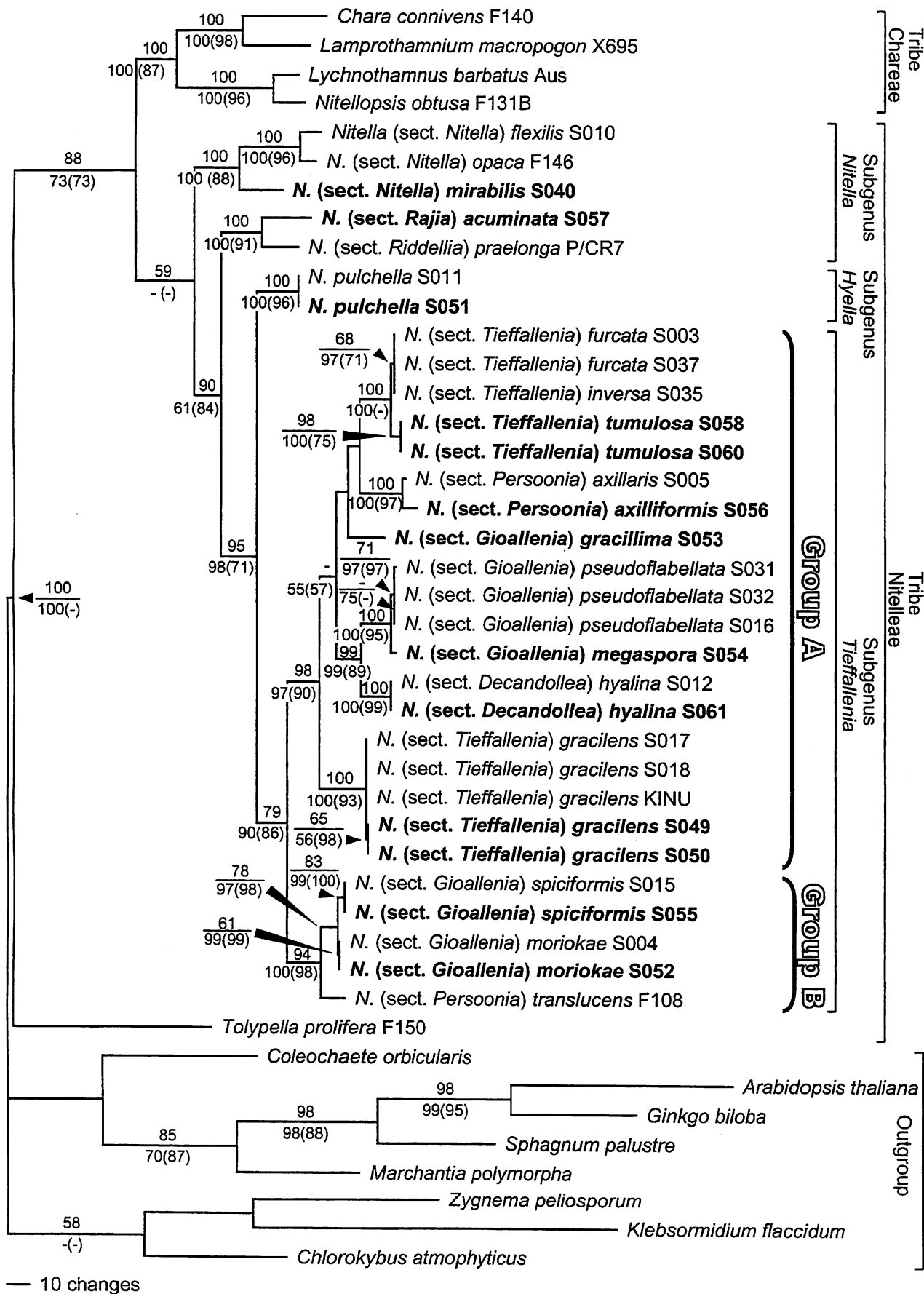
**Figs 22–25. Oospores of *N. axilliformis* (S056).**

Fig. 22. Oospore with six to seven weakly flanged spiral ridges on the surface; SEM.

Fig. 23. Part of fossa wall, showing strongly reticulate ornamentation; LM.

Fig. 24. Part of fossa wall, showing reticulate ornamentation, with weakly flanged spiral ridges; SEM. Note that the reticula extend onto the base of the spiral ridges, but are absent from the flanges.

Fig. 25. Detail of fossa wall, showing sharply developed fused ridges forming a reticulate pattern; SEM.





papillate ornamentation consisting of tapering papillae (Sakayama *et al.* 2002). Furthermore, our molecular phylogenetic analyses showed that *N. tumulosa* from two localities is robustly separated from a clade composed of *N. furcata* and *N. inversa* (Figs 26, 27). According to Wood (1965), *N. furcata* f. *megacarpa* (Allen) R.D. Wood (= *N. megacarpa* Allen) has papillate ornamentations on the fossa wall in LM, very similar to those in *N. tumulosa*. However, *N. tumulosa* can be distinguished from *N. furcata* f. *megacarpa* by the form of the dactyls (Wood 1965). *Nitella tumulosa* has unique two-celled dactyls, in which the penultimate cells are cylindrical and taper at the apex and have the same width as the base of the conical and curved end cells (Wood 1965). Conversely, in *N. furcata* f. *megacarpa* the dactyls are two- or three-celled, and commonly reduced and mucronate (Wood 1965).

***Nitella* (subgen. *Tieffallenia*) *megasporea* (J. Groves)  
Sakayama, comb. nov.**

Figs 13–17

BASIONYM: *Nitella leptodactyla* J. Groves var. *megasporea* J. Groves (1928, p. 132).

SYNONYM: *Nitella pseudoflabellata* A. Braun f. *megasporea* (J. Groves) R.D. Wood (1965, p. 584; “*megacarpa*”).

OOSPORE MORPHOLOGY: The oospores are oval in face view and have six to seven unflanged spiral ridges; they are 252–269  $\mu\text{m}$  long, 216–232  $\mu\text{m}$  wide and 30–50  $\mu\text{m}$  across the fossa (Fig. 13). The walls of mature oospores are dark brown. The fossa wall is finely granulate, with about 28–35 granules across the fossa (Figs 14, 15). Under SEM, the granules are prominent (Fig. 16) or, rarely, elongated (Fig. 17); they are located 0.3–1.5  $\mu\text{m}$  from each other and are 0.4–0.8  $\mu\text{m}$  in diameter (Figs 16, 17). The granules barely extend onto the spiral ridges (Fig. 15).

DISTRIBUTION: Madagascar (Groves 1928; Wood 1965) and Japan (Imahori 1954; Wood 1965; Table 1).

REMARKS: This alga was originally described as a variety of *N. leptodactyla* by Groves (1928) because *N. megasporea* is distinguished from *N. leptodactyla* only by oospore size (Groves 1922, 1928). Subsequently, Wood (1965) regarded *N. megasporea* and *N. leptodactyla* as forms of *N. pseudoflabellata*, based on vegetative similarities, for example, a medium-sized thallus of 20–30 cm and a slender axis of up to 800  $\mu\text{m}$  in diameter, with branchlets forked two to five times. However, our SEM observations showed differences in the fossa walls of *N. megasporea* (Figs 15–17) and *N. pseudoflabellata* (Sakayama *et al.* 2002). The fossa walls of *N. megasporea* have prominent granulate ornamentations composed of compact papilla-like granules that are rarely elongate (Figs 15–17). In contrast, *N. pseudoflabellata* has compressed granulate ornamentations composed of obscure granules on the fossa wall (Sakayama *et al.* 2002). Moreover, *N. megasporea* was phylogenetically separate from the clade composed of three sam-

ples of *N. pseudoflabellata* in our phylogenetic analyses (Figs 26, 27). Although the oospore wall ornamentations of *N. leptodactyla* seen in SEM (Mandal & Ray 1999) are very similar to those of *N. megasporea* (Figs 15, 16), these two species are clearly distinguished from each other by differences in oospore size and morphology of the fertile branchlets. The oospores of *N. leptodactyla* are smaller (180–230  $\mu\text{m}$ ) (Groves 1922; Wood 1965) than those of *N. megasporea* (252–269  $\mu\text{m}$ ) (Fig. 13). One of the secondary rays of a fertile branchlet in *N. megasporea* is robust and positioned centrally (Imahori & Kasaki 1977), whereas *N. leptodactyla* lacks central secondary rays in the fertile branchlets (Mandal & Ray 1999). Therefore, *N. megasporea* should be regarded as a distinct species.

***Nitella* (subgen. *Tieffallenia*) *gracillima* Allen (1898, p. 76)**

Figs 18–21

SYNONYM: *Nitella gracilis* (Smith) C. Agardh f. *gracillima* (Allen) R.D. Wood (1962, p. 21).

OOSPORE MORPHOLOGY: The oospores are oval to almost orbicular in face view and have five to seven flanged spiral ridges; they are 159–200  $\mu\text{m}$  long, 150–175  $\mu\text{m}$  wide and 23–38  $\mu\text{m}$  across the fossa (Fig. 18). The walls of mature oospores are light brown to brown. By LM, the fossa wall appears to be papillate or tuberculate, with five to six projections across the fossa (Fig. 19). SEM showed a papillate or beaded imperfect reticulate pattern, in which the reticulum is formed by fused or waved ridges with papillae or tubercles (Figs 20, 21). The papillae or tubercles are fused with the base of the spiral ridges and are absent from the flanges (Fig. 20).

DISTRIBUTION: Japan (Allen 1898; Imahori 1954; Wood 1965; Table 1), Korea (Choi & Kim 1998) and China (Han *et al.* 1994).

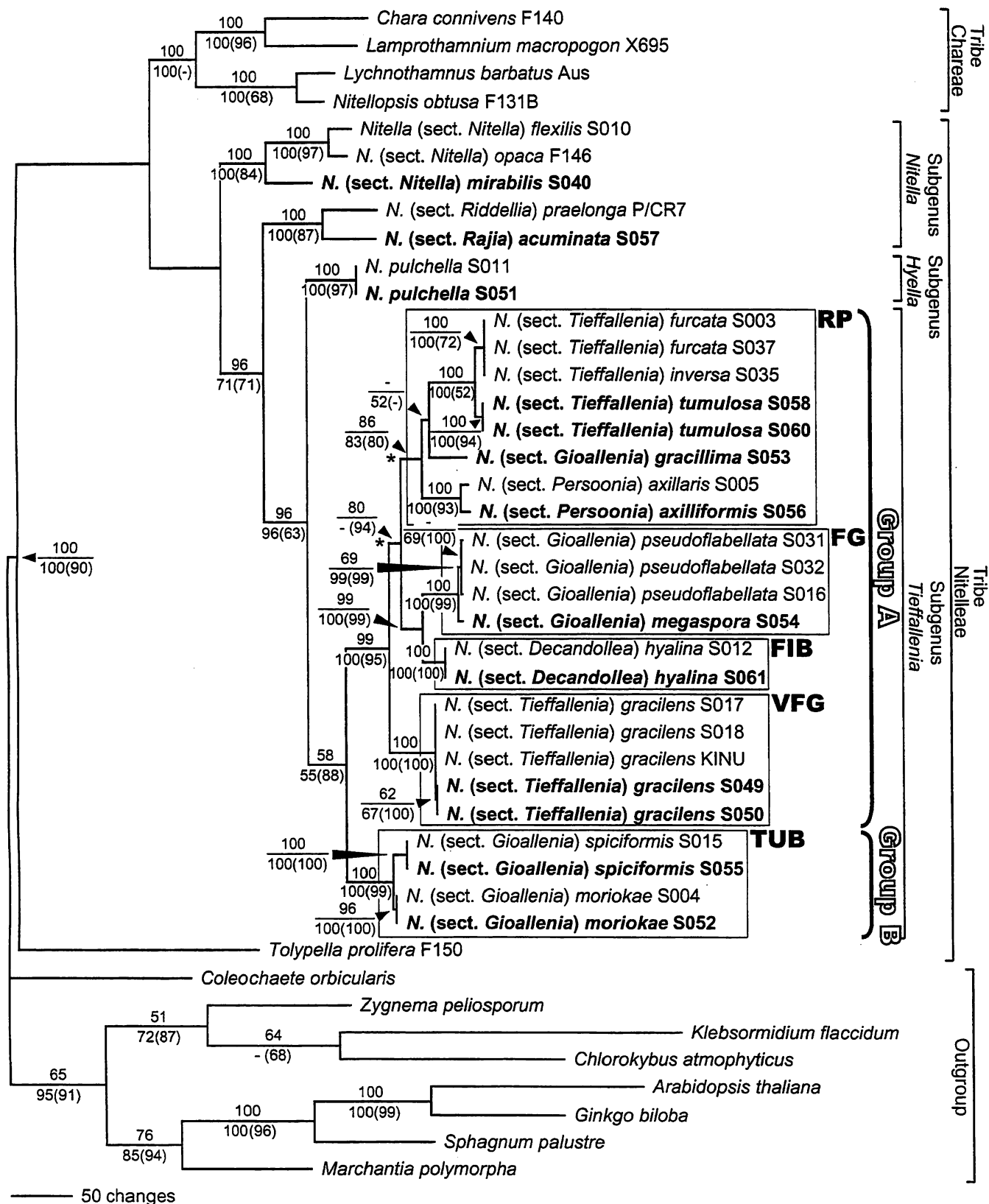
REMARKS: *Nitella gracillima* was treated as a form of *N. gracilis* by Wood (1962), based on their similarities, for example a small thallus, 5–15 (or 20) cm long, a slender axis 90–800  $\mu\text{m}$  in diameter, an unisolated thallus apex and a non-reticulate oospore wall. However, *N. gracillima* is clearly distinguished from *N. gracilis* by the difference in oospores (John & Moore 1987) (Figs 18, 20, 21). In *N. gracillima*, the oospores are 159–200  $\mu\text{m}$  long and have five to seven flanged spiral ridges (Fig. 18); our SEM observations showed papillate or beaded imperfect reticulate ornamentations on the fossa wall (Figs 20, 21). By contrast, the oospores of *N. gracilis* are 250–320  $\mu\text{m}$  long and have about six unflanged spiral ridges (Wood 1965), exhibiting fibrous ornamentations on the fossa wall under SEM (John & Moore 1987). Therefore, *N. gracillima* should be regarded as a distinct species.

***Nitella* (subgen. *Tieffallenia*) *axilliformis* Imahori  
(1951, p. 215)**

Figs 22–25

SYNONYM: *Nitella translucens* (Persoon) C. Agardh f. *axilliformis* (Imahori) R.D. Wood (1965, p. 689).

Fig. 26. One of the 48 equally most parsimonious (MP) trees based on 1182 bp in the coding regions of the *rbcL* genes of 31 strains representing 19 species of the genus *Nitella*, five species of other charalean genera and eight out-group species (Table 1). The MP trees were found by PAUP\* 4.0b10 (Swofford 2002), based on a heuristic search using the stepwise addition of 100 random replications. The tree is 1617 steps long with a CI of 0.4576 and an RI of 0.6442. Branch lengths are proportional to the nucleotide changes, which are indicated by the scale bar below the tree. Numbers above branches are bootstrap values (50% or more) based on 1000 replications of the MP analysis. Branches resolved with 50% or more bootstrap values (based on 100 replications) or QPS values (based on 1000 replications) by ME (based on JC distance) or ML analyses are also shown by numbers under the branches without or with parentheses, respectively. Thirteen OTUs examined in the present study are indicated in boldface. Two robust sister clades within the subgenus *Tieffallenia*, groups A and B, are shown.



**OOSPORE MORPHOLOGY:** The oospores are oval in face view and have six to seven weakly flanged spiral ridges; they are 275–285  $\mu\text{m}$  long, 235–260  $\mu\text{m}$  wide and 40–55  $\mu\text{m}$  across the fossa (Fig. 22). The walls of mature oospores are light brown. The fossa wall is strongly reticulate, with 9–11 meshes across the fossa, each 1.2–4.4  $\mu\text{m}$  in diameter (Figs 23, 24). SEM revealed that the fossa wall has sharply developed fused ridges forming a reticulate pattern with deep depressions (Figs 24, 25). The reticula extend onto the base of the spiral ridges but are absent from the flanges (Fig. 24).

**DISTRIBUTION:** Taiwan (Imahori 1951; Wood 1965), Japan (Imahori 1954; Wood 1965; Table 1), Korea (Choi & Kim 1998) and China (Han et al. 1994).

**REMARKS:** Wood (1965) reduced *N. axilliformis* to a form of *N. translucens* because both species have uniformly two-celled dactyls, obscure terminal coroneae composed of reduced dactyls of the sterile branchlets and tiny and compact fertile heads at the base of whorled branchlets. Based on SEM observations, John & Moore (1987) reported that oospores of *N. translucens* are elongate-ovoid and have a fine reticulate pattern and five to six strongly flanged spiral ridges on the fossa wall. By contrast, our SEM observations revealed that the oospores of *N. axilliformis* are broadly ovoid, and have a coarse reticulate pattern and six to seven weakly flanged spiral ridges (Figs 22, 24, 25). Conversely, the reticulate oospore wall ornamentations of *N. axilliformis* (Fig. 23) are quite similar to those of *N. axillaris* A. Braun [= *N. translucens* var. *axillaris* (A. Braun) R.D. Wood] (Sakayama et al. 2002) under LM. In SEM, however, the fossa walls of *N. axilliformis* exhibit sharply developed fused ridges forming a reticulate pattern (Figs 24, 25), whereas *N. axillaris* has obtuse fused ridges on the fossa wall (Sakayama et al. 2002). Moreover, the oospores of *N. axilliformis* are smaller (275–285  $\mu\text{m}$ ) (Fig. 22) than those of *N. axillaris* (335–340  $\mu\text{m}$ ) (Sakayama et al. 2002). Our *rbcL* gene phylogeny showed that *N. translucens* is phylogenetically separated from *N. axilliformis* and *N. axillaris* (Fig. 26). Therefore, *N. axilliformis* should be regarded as a distinct species of *Nitella*.

#### Molecular phylogenetic analyses based on the *rbcL* gene sequence data

Based on the *rbcL* gene sequence data set of 1182 aligned characters (with 400 potentially parsimony-informative characters), 48 equally parsimonious trees were found in the MP analyses based on a heuristic search using the stepwise addition of 100 random replications. One of the 48 MP trees is shown in Fig. 26, in which we show the branches supported by  $\geq 50\%$  bootstrap/QPS values in MP, ME or ML analyses. The tree is 1617 steps long, with a consistency index (CI) of 0.4576 and a retention index (RI) of 0.6442.

The phylogenetic relationships within the Characeae and genus *Nitella* resolved in these *rbcL* analyses were essentially the same as those of Sakayama et al. (2002), except for relationships regarding the six *Nitella* species newly examined. Five species of the subgenus *Nitella* formed a basal paraphyletic lineage within the genus *Nitella*, where three species belonging to the section *Nitella* (*N. flexilis*, *N. opaca* and *N. mirabilis*) constituted the most basal clade, and *N. (sect. Rajia) acuminata* and *N. (sect. Riddellia) praelonga* A. Braun formed the second most basal clade (Fig. 26). The subgenus *Hyella* was the next basal lineage and sister to the large monophyletic group corresponding to the subgenus *Tieffallenia* (Fig. 26). The latter subgenus was subdivided into two robust sister clades, one (group A) composed of *N. furcata*, *N. inversa*, *N. tumulosa*, *N. pseudoflabellata*, *N. megaspora*, *N. hyalina*, *N. gracillima*, *N. axillaris*, *N. axilliformis* and *N. gracilens* and the other (group B) consisting of *N. spiciformis*, *N. moriokae* and *N. translucens* (Fig. 26). Group A was composed of six lineages: (1) *N. furcata*, *N. inversa* and *N. tumulosa*; (2) *N. pseudoflabellata* and *N. megaspora*; (3) *N. hyalina*; (4) *N. gracillima*; (5) *N. axillaris* and *N. axilliformis*; and (6) *N. gracilens* (Fig. 26). However, the phylogenetic relationships between these six lineages were ambiguous in the *rbcL* gene tree (Fig. 26).

Within group A, two OTUs of *N. tumulosa* with the same *rbcL* sequence were separated from a robust clade composed of *N. furcata* and *N. inversa* (Fig. 26). *Nitella megaspora* was separated from a robust clade composed of three OTUs of *N. pseudoflabellata* (Fig. 26). Two sections of the subgenus *Tieffallenia*, *Gioallenia* R.D. Wood and *Persoonia* R.D. Wood, were resolved as polyphyletic groups with species belonging to both groups A and B (Fig. 26). The section *Gioallenia* was composed of three separate lineages: *N. pseudoflabellata*–*N. megaspora*, *N. gracillima* and *N. spiciformis*–*N. moriokae* (Fig. 26). Within the section *Persoonia*, *N. axilliformis* and *N. axillaris* formed a clade within group A, whereas *N. translucens* was positioned most basally within group B (Fig. 26).

#### Molecular phylogenetic analyses using the combined *atpB* and *rbcL* data set

Using the combined *atpB* and *rbcL* sequence data set of 2202 aligned characters (with 766 potentially parsimony-informative characters), two equally parsimonious trees were found in MP analyses based on a heuristic search using the stepwise addition of 100 random replications. One of the two MP trees is shown in Fig. 27, in which we show branches supported by

Fig. 27. One of the two equally most parsimonious (MP) trees based on 2202 bp in the coding regions of the *atpB* (1020 bp) plus *rbcL* (1182 bp) genes from 30 strains representing 18 species of the genus *Nitella*, five species of other charalean genera and eight out-group species (Table 1). The MP trees were found by PAUP\* 4.0b10 (Swofford 2002), based on a heuristic search using the stepwise addition of 100 random replications. The tree is 3145 steps long with a CI of 0.4738 and an RI of 0.6571. Branch lengths are proportional to the nucleotide changes, which are indicated by the scale bar below the tree. Numbers above branches are bootstrap values (50% or more) based on 1000 replications of the MP analysis. Branches resolved with 50% or more bootstrap values (based on 100 replications) or QPS values (based on 1000 replications) by ME (based on JC distance) or ML analyses are also shown by numbers under the branches without or with parentheses, respectively. Thirteen OTUs examined in the present study are indicated in boldface. The detailed phylogenetic relationships within the subgenus *Tieffallenia*, only resolved in this multigene analyses, are indicated by asterisks. Two robust sister clades within the subgenus *Tieffallenia*, groups A and B, are shown, along with five robust oospore clades, identified on the basis of the oospore wall ornamentations within the subgenus *Tieffallenia*: the reticulate or papillate (RP), finely granulate (FG), fibrous (FIB), very finely granulate (VFG) and tuberculate (TUB) oospore clades.

$\geq 50\%$  bootstrap/QPS values in MP, ME or ML analyses. The tree is 3145 steps long, with CI = 0.4738 and RI = 0.6571.

The phylogenetic relationships within the genus *Nitella* resolved in the *atpB*–*rbcL* gene analyses were essentially the same as those in the *rbcL* gene analyses (Fig. 26), except for some detailed phylogenetic relationships within group A of the subgenus *Tieffallenia* (Fig. 27). Three lineages resolved in the *rbcL* gene phylogeny [(1) *N. furcata*, *N. inversa* and *N. tumulosa*; (4) *N. gracillima*; and (5) *N. axillaris* and *N. axilliformis*] formed a robust clade supported by  $\geq 80\%$  bootstrap/QPS values in the MP, ME and ML analyses, and this clade was resolved as a sister group to a robust clade comprising *N. pseudoflabellata*, *N. megaspora* and *N. hyalina*, with 80% and 94% bootstrap/QPS value in the MP and ML analyses, respectively (Fig. 27). Therefore, *N. gracilens* was the most basal lineage within group A (Fig. 27).

### Re-examination of the taxonomic system of R.D. Wood

Based on the differences in the oospore wall ornamentations seen with SEM, and the combined analyses of *atpB* and *rbcL* gene sequences from a large number of OTUs of the genus *Nitella*, the subgenus *Tieffallenia* was subdivided into five robust clades, the reticulate or papillate (RP), finely granulate (FG), fibrous (FIB), very finely granulate (VFG) and tuberculate (TUB) oospore clades (Fig. 27). In the RP oospore clade (*N. furcata*, *N. inversa*, *N. tumulosa*, *N. gracillima*, *N. axillaris* and *N. axilliformis*), the oospore wall ornamentations are formed by swollen, waved or fused ridges, and exhibit reticulate or papillate patterns (Sakayama *et al.* 2002) (Figs 11, 12, 20, 21, 24, 25). Both *N. pseudoflabellata* and *N. megaspora*, which belong to the FG oospore clade, have finely granulate patterns consisting of compressed, prominent or elongate granules (Sakayama *et al.* 2002) (Figs 15–17). In the FIB (*N. hyalina*) and VFG (*N. gracilens*) oospore clades, the oospore wall ornamentations have fibrous and very finely granulate patterns, respectively (Sakayama *et al.* 2002). The oospore wall ornamentations of *N. spiciformis* and *N. morioka*, both belonging to the TUB oospore clade, are decorated by worm-like projections or granules and have tuberculate patterns (Sakayama *et al.* 2002). According to the taxonomic system of Wood (1962, 1965), the form of the dactyl or end cell and the number of cells forming a dactyl are important characters at the sectional and specific levels in the subgenus *Tieffallenia*, whereas oospore wall ornamentations were diagnostic at the infraspecific level. However, our molecular phylogenetic analyses showed that the three sections within the subgenus *Tieffallenia* – *Tieffallenia*, *Gioallenia* and *Persoonina* – are polyphyletic, with species belonging to more than one oospore clade (Figs 26, 27). In addition, two oospore clades (RP and TUB) exhibit variable morphology in the dactyls. Within the RP oospore clade, the dactyls of *N. furcata*, *N. inversa*, *N. tumulosa*, *N. axillaris* and *N. axilliformis* have an abbreviated form, whereas *N. gracillima* has elongate dactyls (Wood 1965). Although *N. inversa* has two- to three-celled dactyls (Imahori 1954; Wood 1965), the dactyls of the other species of this oospore clade are uniformly composed of two cells (Wood 1965). Dactyls of *N. spiciformis* and *N. morioka* (TUB oospore clade) are uniformly two-celled and two- to three-celled, respectively (Morioka 1941; Wood 1965). Therefore, the section and species delimitations of

Wood (1965), which are based mainly on vegetative morphology (with oospore wall ornamentation used for infraspecific diagnosis), should be disregarded, at least within the subgenus *Tieffallenia*. Because our study demonstrated that under SEM the oospore wall ornamentations are phylogenetically conservative within the subgenus *Tieffallenia*, these characters should be used to construct a natural taxonomic system at the sectional level.

Of the seven sections containing 39 taxa recognized within the subgenus *Nitella* (Wood 1965), this study examined only three sections represented by five species (Table 1; Figs 26, 27). Horn af Ranzien (1959) characterized *N. flexilis* as an atypical species of the genus *Nitella* based on oospore morphology. Subsequently, Kasaki (1964) suggested that the subgenus *Nitella* [= subsection Anarthrodactylae (J. Groves & Bullock-Webster) Zaneveld *sensu* Kasaki (1964) (= subsection *Nitella*)] is an independent genus, based on differences in the morphological and histological characters. Wood (1965) distinguished the section *Nitella* from the other sections of the subgenus *Nitella* by differences in the vegetative morphology. The section *Nitella* is characterized by having obtuse, acute or apiculate apices of the dactyls, and swelling oogonial tube cells at maturity (Wood 1965). In contrast, the other sections of the subgenus *Nitella* have an acuminate apex of the dactyl and nonswelling oogonial tube cells at maturity (Wood 1965). Our molecular phylogenetic analyses demonstrated that these three species of the section *Nitella* are clearly separated from the other species of the genus *Nitella* (Fig. 27). Therefore, we suggest that the section *Nitella* be recognized as a distinct subgenus or genus, after studies of other taxa within the subgenus *Nitella*.

Based on morphological and cytotaxonomic studies, Kasaki (1964) proposed that the species belonging to the subgenera *Hyella* and *Tieffallenia* [= subsections Arthrodactylae (J. Groves & Bullock-Webster) Zaneveld plus Heterodactylae (J. Groves & Stephens) Zaneveld *sensu* Kasaki (1964)] are evolutionarily advanced within the genus *Nitella*, except for *N. hyalina*, which was thought to be in a separate lineage of the genus *Nitella* (Kasaki 1964). Recently, Mandal & Ray (2001) investigated the karyotype of the six taxa of the subgenus *Tieffallenia*, and suggested a primitive status for these taxa. Our molecular phylogenetic analyses are consistent with those of Kasaki (1964) concerning the advanced status of the subgenus *Tieffallenia*, although *N. hyalina* was resolved as a sister species to the FG oospore clade within the subgenus *Tieffallenia* in the molecular phylogeny (Fig. 27).

The results of SEM observations of oospores and molecular phylogenetic analyses of 19 species of the genus *Nitella* (Sakayama *et al.* 2002) (Figs 1–27) have demonstrated the essential problems in the taxonomic system of the genus *Nitella* proposed by Wood (1962, 1965). However, the number of taxa examined was very limited, especially in the subgenera *Nitella* and *Hyella*. Further morphological studies of the oospores and molecular phylogenetic analyses using a larger number of *Nitella* species exhibiting distinct oospore wall ornamentations are needed to construct a natural taxonomic system within the genus *Nitella*.

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