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TAXONOMIC REEXAMINATION OF 17 SPECIES OF *NITELLA* SUBGENUS *TIEFFALLENIA* (CHARALES, CHAROPHYCEAE) BASED ON INTERNAL MORPHOLOGY OF THE OOSPORE WALL AND MULTIPLE DNA MARKER SEQUENCES[1]

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In an attempt to reconstruct the natural taxonomic system for *Nitella*, 17 species of *Nitella* subgenus *Tieffallenia* were reexamined using SEM observations of the internal morphology of the oospore wall (IMOW) and phylogenetic analyses of 4553 base pairs from multiple DNA markers (*atp*B, *rbc*L, *psa*B, and ITS-5.8S rRNA genes). Our SEM observations identified three types of IMOW: homogeneous (HG), weakly spongy (W-SG), and strongly spongy (S-SG) types. Based on differences in the IMOW, species with reticulate or tuberculate oospore wall ornamentation in the external morphology of the oospore wall (EMOW) were subdivided into two distinct groups (characterized by the HG or S-SG types of IMOW, respectively), which were robustly separated from each other in our molecular phylogenetic analyses. In our molecular phylogeny, the subgenus *Tieffallenia* consisted of four robust monophyletic groups—three clades of the HG type and a spongy (S-SG and W-SG) type clade—that were characterized by differences in the IMOW and EMOW. In addition, our SEM observations and sequence data verified the distinct status of five species (*N. japonica* Allen, *N. oligospira* A. Braun, *N. vieillardii* stat. nov., *N. imperialis* stat. nov., and *N. morongii* Allen) that R. D. Wood had assigned as infraspecific taxa. Moreover, our SEM observations of the IMOW also suggested that *N. megaspora* (J. Groves) Sakayama originally identified by LM includes at least two distinct species, characterized by W-SG and S-SG types of IMOW, respectively.

Key index words: **Charales; Charophyceae; chloroplast DNA; morphology; *Nitella*; nuclear DNA; oospores; scanning electron microscopy; taxonomy; *Tieffallenia***

Abbreviations: ***atp*B, the gene encoding the beta subunit of ATP synthase; BI, Bayesian inference; BS, bootstrap value; EMOW, external morphology of the oospore wall; IMOW, internal morphology of the oospore wall; ITS, internal transcribed spacer; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony; PP, posterior provability; *psa*B, the gene encoding the PSI P700 chl *a* apoprotein A2; *rbc*L, the gene encoding the large subunit of RUBISCO; TBR, tree-bisection-reconnection**

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Nitella C. Agardh is a genus of the Charales characterized by having one or more forked branchlets that are composed of unicellular segments and single- or

multiple-celled terminal segments (called a "dactyl"), two tiers of coronal cells in the female reproductive organ, and laterally compressed oospores (Wood 1965); around 200 taxa have been described worldwide (Wood 1965). Agardh (1824) originally described this genus with 11 species. Subsequently, Braun (1867, 1882), Groves and Bullock-Webster (1920), Groves and Allen (1935), and Zaneveld (1940) classified the genus *Nitella* into subdivisions based on differences in vegetative morphology. However, these traditional classifications were based on limited numbers of specimens and species. Subsequently, Wood (1965) published a monograph of the worldwide Charales, and he divided *Nitella* into three subgenera: *Nitella*, *Hyella* R. D. Wood, and *Tieffallenia* R. D. Wood, based on the number of cells forming 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); the subgenus *Tieffallenia* is characterized by having multicellular dactyls, in which the end cell is very different from the penultimate cell (Wood 1965). The subgenus *Tieffallenia* contains most of the species of the genus and exhibits almost all the types of oospore wall ornamentation (Wood 1965). Recent molecular phylogenetic studies demonstrated that the subgenera *Hyella* and *Tieffallenia* are monophyletic, whereas the subgenus *Nitella* is paraphyletic (Sakayama et al. 2002, 2004b).

Wood (1962, 1965, 1966) characterized the sections and species of the genus *Nitella* based mainly on differences in vegetative morphology, such as the overall appearance of the thallus, furcation in branchlets, and the shape and number of cells in dactyls. By contrast, the external morphology of the oospore wall (EMOW) (especially oospore wall ornamentation), which had been used for species diagnoses (Allen 1892, 1894, 1896, Groves and Bullock-Webster 1920, Zaneveld 1940, Imahori 1954), was treated as diagnostic at the infraspecific level (Wood 1962, 1965, 1966). Therefore, Wood (1962, 1965, 1966) reduced many species of *Nitella* to infraspecific rank. Within the genus *Nitella*, he recognized 53 of 204 species previously described (Wood 1965). Subsequently, SEM studies of the EMOW showed the existence of distinct features that indicate that some of the infraspecific taxa recognized by Wood (1962, 1965) should be raised to species rank (Caceres 1975, John and Moore 1987, Leitch et al. 1990, Casanova 1991, Mukherjee and Ray 1993, Mandal et al. 1995, Nozaki et al. 1998, Mandal and Ray 1999, Ray et al. 2001). Recently, Sakayama et al. (2002, 2004b) demonstrated that the combined analyses of the EMOW by SEM and molecular phylogeny are useful for delimiting species within the genus *Nitella*. However, they only examined 15 taxa in the genus. Therefore, reexamination of additional taxa based on SEM oospore morphology and DNA sequences are necessary in the genus *Nitella*.

In the taxonomy of the Charales, SEM of the EMOW (i.e. the overall appearance, number of spiral ridges, and wall ornamentation) has been used as a taxonomic character. However, the internal morphology of the oospore wall (IMOW) has not been used for taxonomic studies of the Charales, although Caceres (1977), Leitch (1989), and Leitch et al. (1990) suggested the taxonomic significance of the IMOW, as revealed by fractured face characters.

In this study, we reexamined species of *Nitella* subgenus *Tieffallenia* by analyzing the EMOW and IMOW and conducting phylogenetic analyses of the concatenated sequences of the chloroplast *rbc*L, *atp*B, and *psa*B genes and nuclear 5.8S rRNA gene and internal transcribed spacer (ITS) regions. We used cultured materials of 31 samples of 13 species that were previously studied (Sakayama et al. 2002, 2004a,b) as well as 8 new samples, including 4 additional species that were collected from New Caledonia, Australia, and Thailand. The taxonomic significance of the IMOW and the natural taxonomic system of *Nitella* at and above the species level are discussed critically based on the detailed and robust phylogenetic relationships resolved.

MATERIALS 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 LM and SEM were essentially the same as those in our previous studies (Sakayama et al. 2002, 2004b), except in the following three respects. First, to observe the IMOW, the oospores were fractured manually in a longitudinal section, using a razor blade, before the Triton X-100 treatment. Second, SEM observations were made using S-4500 or S-4000 scanning electron microscopes (Hitachi, Tokyo, Japan) at 7–10 kV. Finally, the terms used to describe the EMOW and IMOW by LM or SEM were based on those of Wood (1965), John and Moore (1987), Leitch et al. (1990), and Faegri and Iversen (1989). For IMOW, we describe the overall appearance of the fractured face of the wall. Although this study only examined 17 taxa from sections in the subgenus *Tieffallenia*, they represent almost all the LM oospore wall types (granulate, papillate, fibrous, tuberculate, and reticulate) recognized by Wood (1965).

Molecular phylogenetic analysis. Preparation of total DNA, amplification of DNA by the PCR, and direct sequencing of the PCR products were essentially as described previously (Sakayama et al. 2002, 2004a,b), except for the primers used to amplify and sequence the *psa*B genes (Table 2). The *psa*B gene sequences analyzed corresponded to positions 247–1740 of the *Marchantia polymorpha* Linnaeus *psa*B gene (Ohyama et al. 1986).

Phylogenetic analyses of the concatenated *atp*B (Sakayama et al. 2004b), *rbc*L (Sakayama et al. 2004b), *psa*B, and ITS-5.8S rRNA gene sequences (4553 base pair [bp]) from 39 samples of the subgenus *Tieffallenia* and from *N. pulchella* Allen (subgenus *Hyella*) were carried out using distance, maximum parsimony (MP), maximum likelihood (ML), and Bayesian methods (Table 1). *Nitella pulchella* was used as the outgroup, because the subgenus *Hyella* is sister to the subgenus *Tieffallenia* (Sakayama et al. 2002, 2004b). Before concatenating the sequence data sets, their congruence was assessed using the partition-homogeneity test (Farris et al. 1994) based on 1000 replications of a heuristic search (with the tree-bisection-reconnection [TBR] branch-swapping algorithm), implemented in PAUP* 4.0b10 (Swofford 2002). The ITS, 5.8S rRNA, and partial 18S and 26S rRNA sequences of *Nitella* were aligned using the

TABLE 1. List of the charalean species/strains and DDBJ/EMBL/GenBank accession numbers used for the present phylogenetic analyses.

Species	Strain designation and collection information	Accession number			
		ITS-5.8S rRNA gene	*rbc*L gene	*atp*B gene	*psa*B gene
Nitella furcata (Roxburgh *ex* Bruzelius) C. Agardh	S003, Japan	AB169926[a]	AB076058[b]	AB110842[c]	AB191748[d]
	S037, Japan	AB169927[a]	AB076059[b]	AB110843[c]	AB191749[d]
	S074, Japan	AB169928[a]	AB169966[a]	AB169958[a]	AB191750[d]
N. inversa Imahori	S035, Japan	AB169929[a]	AB076060[b]	AB110844[c]	AB191751[d]
N. japonica Allen	S077, Japan	AB169930[a]	AB169967[a]	AB169959[a]	AB191752[d]
	S083, Japan	AB169931[a]	AB169968[a]	AB169960[a]	AB191753[d]
	S090, Japan	AB169932[a]	AB169969[a]	AB169961[a]	AB191754[d]
N. tumulosa Zaneveld	S058, Thailand	AB169933[a]	AB110868[c]	AB110845[c]	AB191755[d]
	S060, Malaysia	AB169934[a]	AB110869[c]	AB110846[c]	AB191756[d]
N. oligospira A. Braun[e]	S095, Creek at Rhmerie near Dumbéa, New Caledonia, 15 May 2003	AB191724[d]	AB191732[d]	AB191740[d]	AB191757[d]
	S096, Creek along the road between Koumac and Ouegoa, New Caledonia, 17 May 2003	AB191725[d]	AB191733[d]	AB191741[d]	AB191758[d]
N. gracilens Morioka	S017, Japan	AB169935[a]	AB076061[b]	AB110847[c]	AB191759[d]
	S018, Japan	AB169936[a]	AB076062[b]	AB110848[c]	AB191760[d]
	KINU, Japan	AB169937[a]	AB076063[b]	AB110849[c]	AB191761[d]
	S049, Japan	AB169938[a]	AB110870[c]	AB110850[c]	AB191762[d]
	S050, Japan	AB169939[a]	AB110871[c]	AB110851[c]	AB191763[d]
N. pseudoflabellata A. Braun	S031, Japan	AB169940[a]	AB076064[b]	AB110852[c]	AB191764[d]
	S032, Japan	AB169941[a]	AB076065[b]	AB110853[c]	AB191765[d]
	S016, Japan	AB169942[a]	AB076066[b]	AB110854[c]	AB191766[d]
N. megaspora (J. Groves) Sakayama	S054 (complex 1), Japan	AB169943[a]	AB110872[c]	AB110855[c]	AB191767[d]
	S073 (complex 2), Japan	AB169944[a]	AB169970[a]	AB169962[a]	AB191768[d]
N. imperialis (Allen) Sakayama,[e] stat. nov.	S100, River connected to Thamphra Waterfall, Seka District, Nong Khai Prov., Thailand, 21 July 2003	AB191726[d]	AB191734[d]	AB191742[d]	AB191769[d]
N. vieillardii (A. Braun) Sakayama,[e] stat. nov.	S092, Creek along the road between Koumac and Ouegoa, New Caledonia, 19 May 2003	AB191727[d]	AB191735[d]	AB191743[d]	AB191770[d]
	S093, Creek along the road near Pouebo, New Caledonia, 17 May 2003	AB191728[d]	AB191736[d]	AB191744[d]	AB191771[d]
N. hyalina (De Candolle) C. Agardh	S012, Japan	AB169945[a]	AB076067[b]	AB110856[c]	AB191772[d]
	S061, unknown	AB169946[a]	AB110873[c]	AB110857[c]	AB191773[d]
	S094, Creek along the road near Pouebo, New Caledonia, 17 May 2003	AB191729[d]	AB191737[d]	AB191745[d]	AB191774[d]
N. gracillima Allen	S053, Japan	AB169947[a]	AB110874[c]	AB110858[c]	AB191775[d]
	S082, Japan	AB169948[a]	AB169971[a]	AB169963[a]	AB191776[d]
N. morongii Allen[e]	S089,[f] Michel River, Mutchilba, Queensland, Australia	AB191730[d]	AB191738[d]	AB191746[d]	AB191777[d]
N. spiciformis Morioka	S015, Japan	AB169953[a]	AB076068[b]	AB110859[c]	AB191778[d]
	S055, Japan	AB169954[a]	AB110875[c]	AB110860[c]	AB191779[d]
N. moriokae R. D. Wood	S004, Japan	AB169955[a]	AB076069[b]	AB110861[c]	AB191780[d]
	S052, Japan	AB169956[a]	AB110876[c]	AB110862[c]	AB191781[d]
N. axillaris A. Braun	S005, unknown	AB169949[a]	AB076070[b]	AB110863[c]	AB191782[d]
N. axilliformis Imahori	S056, Japan	AB169950[a]	AB110877[c]	AB110864[c]	AB191783[d]
	S085, Japan	AB169951[a]	AB169972[a]	AB169964[a]	AB191784[d]
	S087, Japan	AB169952[a]	AB169973[a]	AB169965[a]	AB191785[d]
	S097, Swamp at Ston Popidéry near La Foa, New Caledonia, 20 May 2003	AB191731[d]	AB191739[d]	AB191747[d]	AB191786[d]
N. pulchella Allen	S011, Japan	AB169957[a]	AB076057[b]	AB110840[c]	AB191787[d]

[a]Sakayama et al. (2004a).
[b]Sakayama et al. (2002).
[c]Sakayama et al. (2004b).
[d]This study.
[e]Species newly examined.
[f]Thalli provided by Dr. T. Yamada (University of Tokyo).

TABLE 2. Primers used for amplifications and sequencing of the *psa*B genes in the present study.

Designations	Positions[a]	Sequence (5′ to 3′)
*psa*B-F1[b]	205–224	GCITGGCA(AG)GGIAA(TC)TT(TC)GA
CH-*psa*B-F3[c]	1039–1060	TTAGTAGCTCAACATATGTATT
*psa*B-R2[b]	1133–1114[d]	AT(AG)TA(TC)TG(AG)TG(AG)TGIGT(AG)TA
*psa*B-R6[b]	1760–1741[d]	ATIGT(AG)TTIA(AG)CATCCA(AG)AA

[a]Coordinate number from the *Marchantia polymorpha* Linnaeus *psa*B gene (Ohyama et al. 1986).
[b]Nozaki et al. (2000).
[c]This study.
[d]Reverse primer.

program CLUSTAL X (Thompson et al. 1997) with the default options. Subsequently, the aligned data matrix was corrected manually based on the putative secondary structures described in Sakayama et al. (2004a). Gaps were removed from the aligned sequences of the 39 ingroup samples of the subgenus *Tieffallenia*; the remaining 857 bp were used for the phylogenetic analysis. Samples representing identical sequences in 4553 bp were treated as a single operational taxonomic unit. The concatenated nucleotide data set representing 31 operational taxonomic units (EMBL-Align database accession number ALIGN_000771) was subjected to unweighted 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 using the TBR branch-swapping algorithm. A bootstrap (BS) analysis (Felsenstein 1985) was carried out based on 1000 replications of the simple heuristic search (full heuristic type with the TBR branch-swapping algorithm). Following the guidelines for constructing a topology using the distance method (Nei and Kumar 2000), we selected Jukes-Cantor distances (Jukes and Cantor 1969) to construct minimum evolution (ME) trees. For the same alignment used in the MP analysis, a distance matrix was calculated using the Jukes-Cantor method (Jukes and Cantor 1969) in PAUP* 4.0b10. Based on a heuristic search using the stepwise addition of 100 random replications (with the TBR branch-swapping algorithm), ME trees were constructed, again using PAUP* 4.0b10; the robustness of lineages was tested by a BS analysis with 1000 replications of the simple heuristic search (full heuristic type with TBR branch-swapping algorithm) using PAUP* 4.0b10. The likelihood ratio test was applied to select an appropriate substitution model in the ML analysis using Modeltest 3.06 with a default individual alpha value of 0.01 (Posada and Crandall 1998). Using the same alignment data, a BS analysis of an ML method (with the GTR+G+I model selected by Modeltest 3.06, Posada and Crandall 1998) was carried out using PAUP* 4.0b10, based on 100 replications of the simple heuristic search (full heuristic type with the TBR branch-swapping algorithm). Using the same alignment data and an evolutionary model as the ML analysis, the Bayesian inference (BI) method was carried out using MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001). Four simultaneous Markov chains were run for 1,000,000 generations, sampling every 100 generations, for a total of 10,000 trees. The first 1000 trees (10%) from the run were ignored as "burn-in." Based on the remaining 9000 trees, we obtained the posterior probabilities (PP) for each node of the tree using MrBayes v3.0b4.

RESULTS

Internal morphology of the oospore wall. Based on our SEM observations of the IMOW as revealed by the fractured face of oospore walls from 17 species of *Nitella* subgenus *Tieffallenia*, three types of IMOW were recognized: homogeneous (HG), strongly spongy (S-SG), and weakly spongy (W-SG) (Figs. 1–8, Table 3). Eleven of the 17 species (*N. axillaris* A. Braun, *N. axilliformis* Imahori, *N. furcata* [Roxburgh *ex* Bruzelius] C. Agardh, *N. gracilens* Morioka, *N. gracillima* Allen, *N. inversa* Imahori, *N. japonica* Allen, *N. moriokae* R. D. Wood, *N. oligospira* A. Braun, *N. spiciformis* Morioka, and *N. tumulosa* Zaneveld) could be assigned to the HG type, which has a fossa wall with a homogeneous fractured face under SEM (Table 3). The outer margins of the fractured face in eight HG-type species (*N. axillaris*, *N. axilliformis*, *N. furcata*, *N. gracillima*, *N. inversa*, *N. japonica*, *N. oligospira*, and *N. tumulosa*) consisted of waved and connected ridges (Figs. 1, a–f, 4, and 5). Moreover, in *N. gracillima*, *N. inversa*, *N. japonica*, and *N. tumulosa*, papillae or projections were raised from the ridges (Figs. 1, b–d, and 4). By contrast, the other HG-type species had unwaved outer margins with very fine granules (*N. gracilens*, Fig. 1g) or tubercles (*N. spiciformis*, Fig. 1h, and *N. moriokae*, Fig. 1i).

Conversely, *N. hyalina* (De Candolle) C. Agardh, *N. imperialis* stat. nov., *N. megaspora* (J. Groves) Sakayama (complexes 1 and 2), *N. morongii* Allen, *N. pseudoflabellata* A. Braun, and *N. vieillardii* stat. nov. had a spongy texture in the fractured faces of the oospore wall and were subdivided into S-SG and W-SG types based on differences in the size or density of the openings forming the spongy texture (Table 3). All six species, with the exception of *N. megaspora* complex 1 and *N. pseudoflabellata*, exhibited an S-SG type texture in which the openings in the fractured faces of the fossa wall were dense and evident (Figs. 2 and 6–8). The longitudinal fractured face of the fossa wall of *N. megaspora* complex 2 (Fig. 2, a and b), *N. hyalina* (Fig. 2, c and d), and *N. vieillardii* (Fig. 7, e and f) exhibited a fibrous network, in which the openings were circular to oval (0.4–0.7, 0.08–0.3, and 0.3–2.0 μm in diameter, respectively, at the outermost side of the fossa wall) and gradually became smaller toward the inner side of the fossa wall. The fossa wall of *N. morongii* exhibited an alveolar fractured face, in which the openings were circular to oval (0.15–1.1 μm in diameter) and gradually became smaller toward the inner side of the wall (Fig. 8, e and f). In *N. imperialis*, the openings were compressed laterally (0.3–2.8 μm long) and formed a lamellate structure in the longitudinal fractured face of

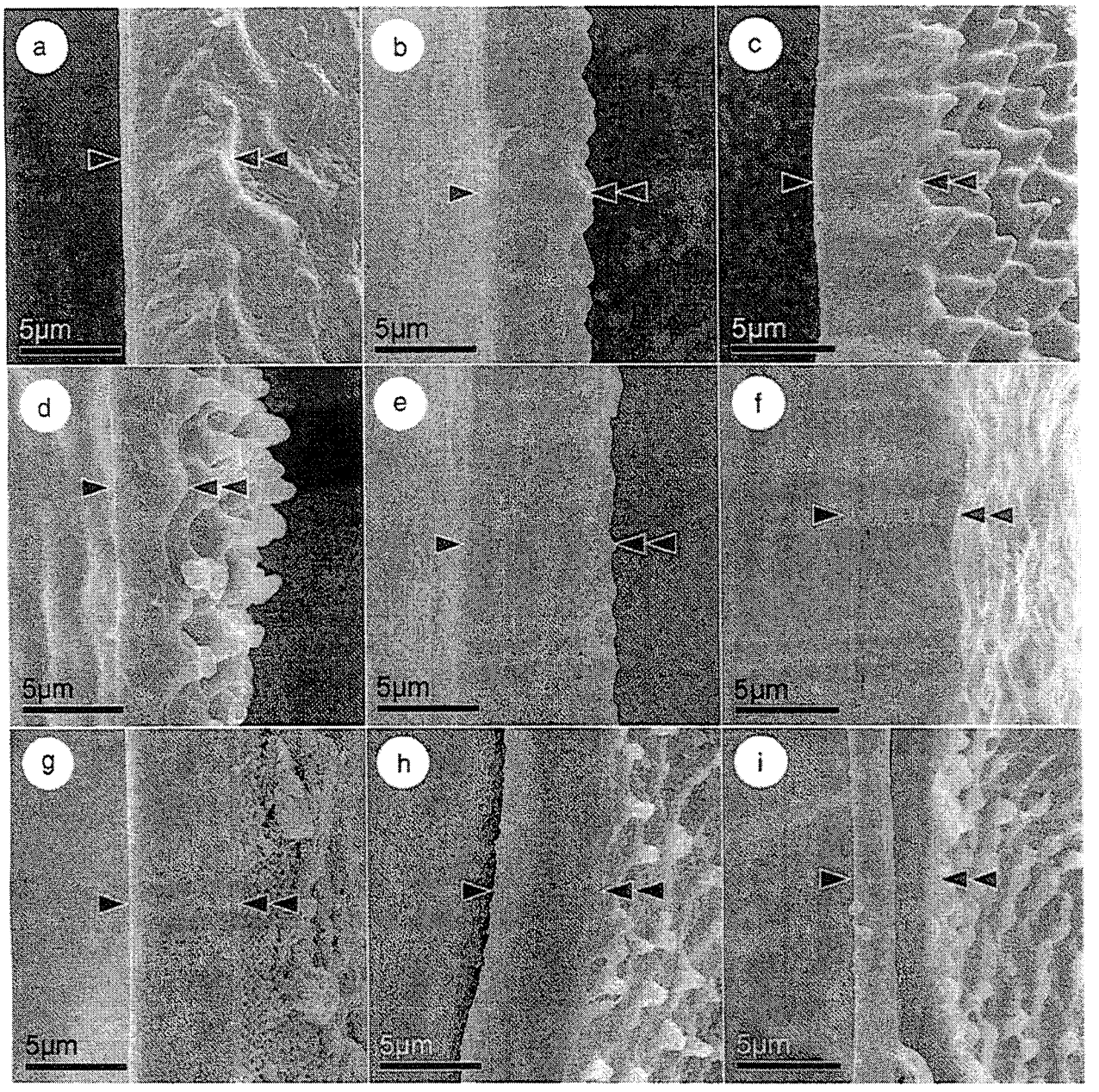

FIG. 1. Longitudinal fractured faces of fossa wall of nine *Nitella* species, showing homogeneous textures. SEM. Single- or double-arrowhead indicates the inner or outer side of the wall, respectively. (a) *Nitella furcata* (S037). (b) *N. inversa* (S035). (c) *N. tumulosa* (S060). (d) *N. gracillima* (S053). (e) *N. axillaris* (S005). (f) *N. axilliformis* (S056). (g) *N. gracilens* (S018). (h) *N. spiciformis* (S015). (i) *N. moriokae* (S004).

the fossa wall (Fig. 6, e and f). Conversely, *N. pseudoflabellata* (Fig. 3, a and b) and *N. megaspora* complex 1 (S054) (Fig. 3, c and d) exhibited a W-SG type texture consisting of sparse small openings in the fractured faces of the fossa wall.

Congruency of the four DNA marker data sets. The *P* values resulting from partition-homogeneity tests of the four DNA marker data sets are shown in Table 4. None of the combinations of data partitions tested resulted in significantly incongruent trees. The *rbc*L versus ITS-5.8S rRNA sequences were congruent ($P = 1.000$). The *atp*B versus *rbc*L, *atp*B versus *psa*B, *atp*B versus ITS-5.8S rRNA, *rbc*L versus *psa*B, and *psa*B versus ITS-5.8S rRNA sequences were also congruent with $P \geq 20\%$. The concatenated sequence data set of the three chloroplast genes (*atp*B, *rbc*L, and *psa*B) versus the nuclear ITS-5.8S rRNA sequence data set exhibited high congruency with $P = 0.967$.

Multiple DNA marker phylogeny. The MP analyses of the concatenated *atp*B, *rbc*L, *psa*B, ITS, and 5.8S

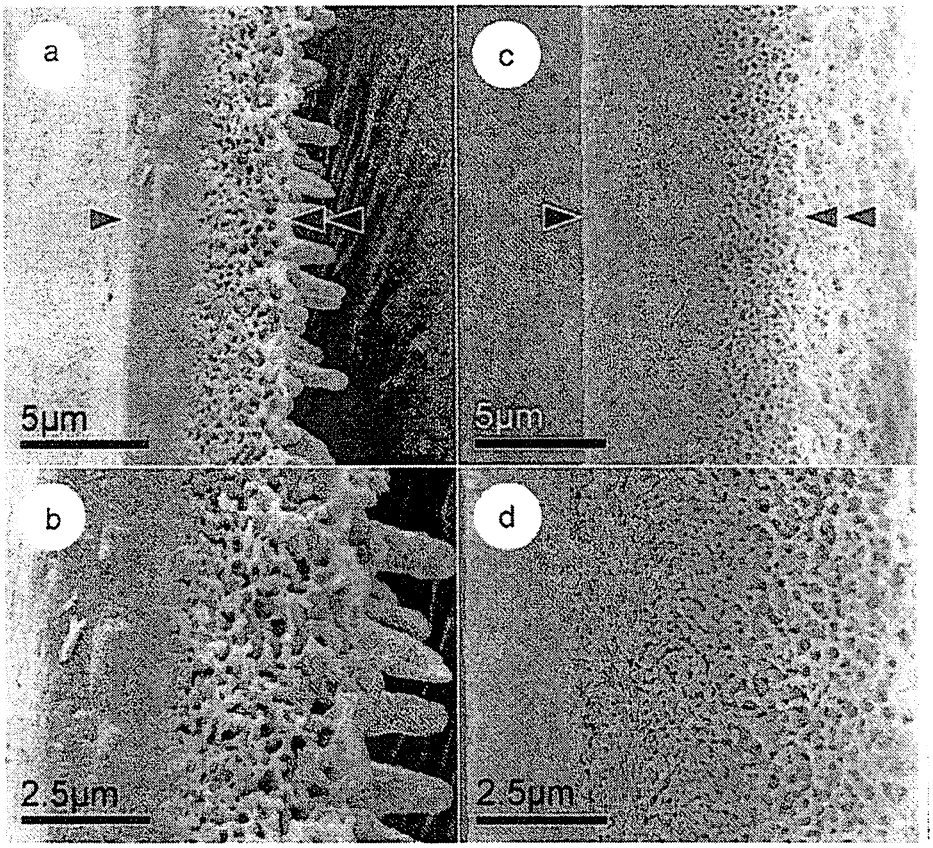

FIG. 2. Longitudinal fractured faces of fossa wall of *Nitella megaspora* complex 2 (S073) (a and b) and *N. hyalina* (S012) (c and d), showing strongly spongy texture. SEM. Single or double arrowhead indicates the inner or outer side of the wall, respectively.

rRNA sequences yielded six equally parsimonious trees, one of which is shown in Figure 9. The phylogenetic relationships we report here for the subgenus *Tieffallenia* are consistent with, but are better resolved than, those revealed by previously reported phylogenies (Sakayama et al. 2002, 2004a,b). Four robust clades were resolved with ≥ 97% BS and 1.00 PP in the MP, ME, ML, and BI analyses: 1) *N. furcata*, *N. inversa*, *N. japonica*, *N. tumulosa*, *N. gracillima*, *N. oligospira*, *N. axillaris*, and *N. axilliformis*; 2) *N. pseudoflabellata*, *N. megaspora* complex 1, *N. megaspora* complex 2, *N. vieillardii*, *N. imperialis*, *N. morongii*, and *N. hyalina*; 3) *N. gracilens*; and 4) *N. spiciformis* and *N. moriokae*. Moreover, the sequence data resolved two monophyletic groups within clade 1: one composed of *N. furcata*, *N. inversa*, *N. japonica*, *N. tumulosa*, and *N. gracillima* and the other containing *N. oligospira*, *N. axillaris*, and *N. axilliformis*. In the former group, *N. japonica* was sister to the *N. furcata–N. inversa* clade, supported by ≥ 99% BS and 1.00 PP in the MP, ME, ML, and BI analyses. In addition, a robust monophyly was resolved for *N. pseudoflabellata* with ≥ 74% BS and 1.00 PP in the MP, ME, ML, and BI analyses. Almost all the phylogenetic relationships at the species level were resolved with ≥52% BS or ≥ 1.00 PP in two or more of the phylogenetic methods used, except for relationships between *N. furcata* and *N. inversa*.

TAXONOMIC ACCOUNTS

The SEM oospore morphology (EMOW and IMOW) of the following five species was examined for the first time, and taxonomic accounts are provided here.

Nitella* (subgen. *Tieffallenia*) *japonica Allen (1893, p. 120)

(Fig. 4)

Synonym: Nitella furcata (Roxburgh *ex* Bruzelius) C. Agardh f. *japonica* (Allen) R. D. Wood (1965, p. 493).

Oospore morphology: The oospores are oval in face view and have five to six flanged spiral ridges; they are 281–305 μm long, 260–295 μm wide, and 55–76 μm across the fossa (Fig. 4a). The walls of mature oospores are brown to light brown. The fossa wall is papillate or beaded imperfect reticulate, with approximately 22–35 papillae across the fossa (Fig. 4b). Under SEM, there is a papillate or beaded imperfect reticulate pattern (with 8–12 meshes across the fossa) (Fig. 4c), in which the reticulum is formed by waved ridges decorated by tapering projections about 0.3–1.7 μm high and about 0.5–1.0 μm in diameter (Fig. 4, d and e). The papillae extend onto the spiral ridges but are absent from the flanges (Fig. 4c). The longitudinal fractured face of the fossa wall is homogeneous (Fig. 4, e and f).

Distribution: Japan (Allen 1893, Imahori 1954, Wood 1965, Sakayama et al. 2004a) (Table 1),

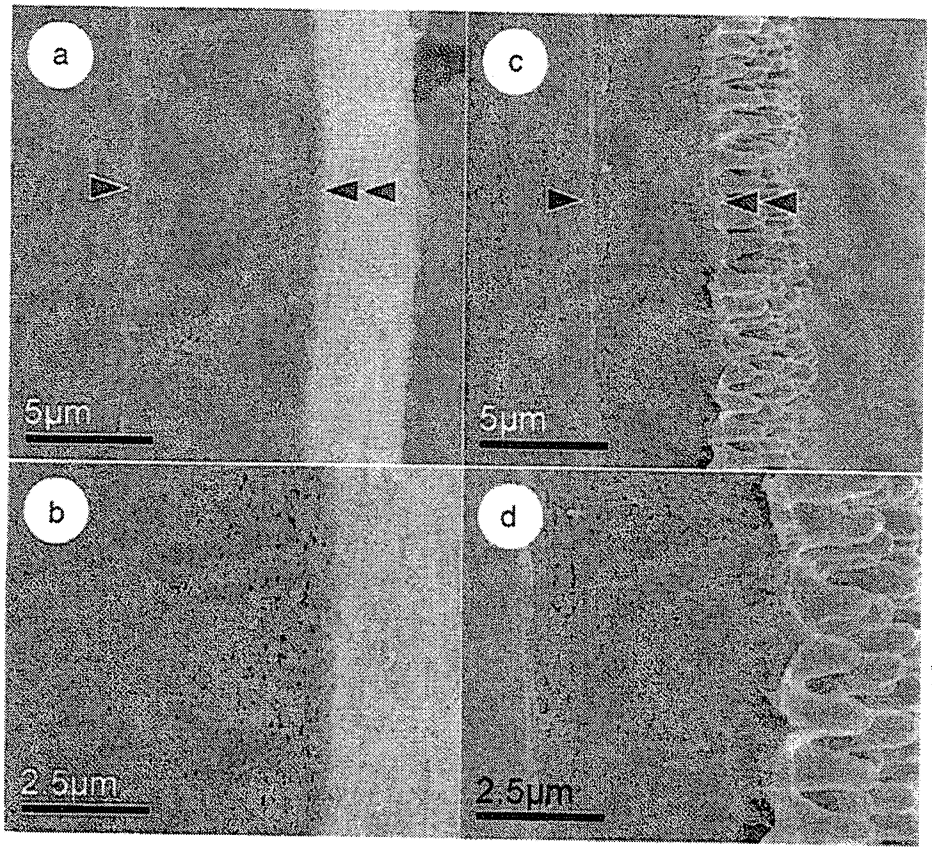

FIG. 3. Longitudinal fractured faces of fossa wall of *Nitella pseudoflabellata* (S031) (a and b) and *N. megaspora* complex I (S054) (c and d), showing weakly spongy texture. SEM. Single or double arrowhead indicates the inner or outer side of the wall, respectively.

Korea (Choi and Kim 1998), and China (Han et al. 1994).

Remarks: Wood (1965) reduced *N. japonica* to a form of *N. furcata*, based on similarities in vegetative morphology, such as the morphology of the branchlet, sexuality, and position and size of the reproductive organ as well as the presence or absence of the fertile head. However, these two species can be clearly distinguished, based on the difference in SEM oospore morphology. Under SEM, *N. furcata* exhibits an imperfect reticulate ornamentation (Caceres 1975, Mandal et al. 1995, Sakayama et al. 2002), whereas *N. japonica* has a papillate or beaded imperfect reticulate pattern (Fig. 4c). By contrast, the oospores of *N. inversa* are similar to those in *N. japonica* under LM (Sakayama et al. 2002). However, our SEM study showed that these two species could be distinguished by the difference in the number of papillae across the fossa (Fig. 4, a–c) (Sakayama et al. 2002). The fossa walls of *N. inversa* have up to 20 papillae across the fossa (Sakayama et al. 2002), whereas *N. japonica* has more than 22 papillae across the fossa (Fig. 4, a–c). Additionally, the molecular phylogenetic analyses separated *N. inversa* from *N. japonica* (Fig. 9) (Sakayama et al. 2004a).

According to Wood (1965), *N. furcata* f. *megacarpa* (Allen) R. D. Wood (= *N. megacarpa* Allen) has papillate ornamentation on the fossa wall, as in *N. japonica*. However, the dactyls of *N. japonica* are uniformly composed of two cells, whereas *N. furcata* f. *megacarpa* has two- or three-celled dactyls (Imahori 1954, Wood 1965).

Nitella* (subgen. *Tieffallenia*) *oligospira A. Braun (1858, p. 357)

(Fig. 5)

Synonym: Nitella furcata (Roxburgh *ex* Bruzelius) C. Agardh f. *oligospira* (A. Braun) R. D. Wood (1965, p. 505).

Oospore morphology: The oospores are oval in face view and have five to six robust flanged spiral ridges; they are 275–305 μm long, 250–285 μm wide, and 50–65 μm across the fossa (Fig. 5a). The walls of mature oospores are yellowish brown to light brown. The fossa wall is imperfect reticulate, with about 6–10 meshes, each about 1.6–8.3 μm in diameter, across the fossa (Fig. 5b). Under SEM, the fossa walls exhibit a papillate pattern or have imperfect reticulate ornamentation (Fig. 5c), due to slightly waved ridges between the papillae (Fig. 5d); the papillae are about 1.5–2.6 μm in diameter (Fig. 5d). The ridges forming a slight reticulum extend onto the spiral ridges but are absent from the flanges (Fig. 5c). The longitudinal fractured face of the fossa wall is homogeneous (Fig. 5, e and f).

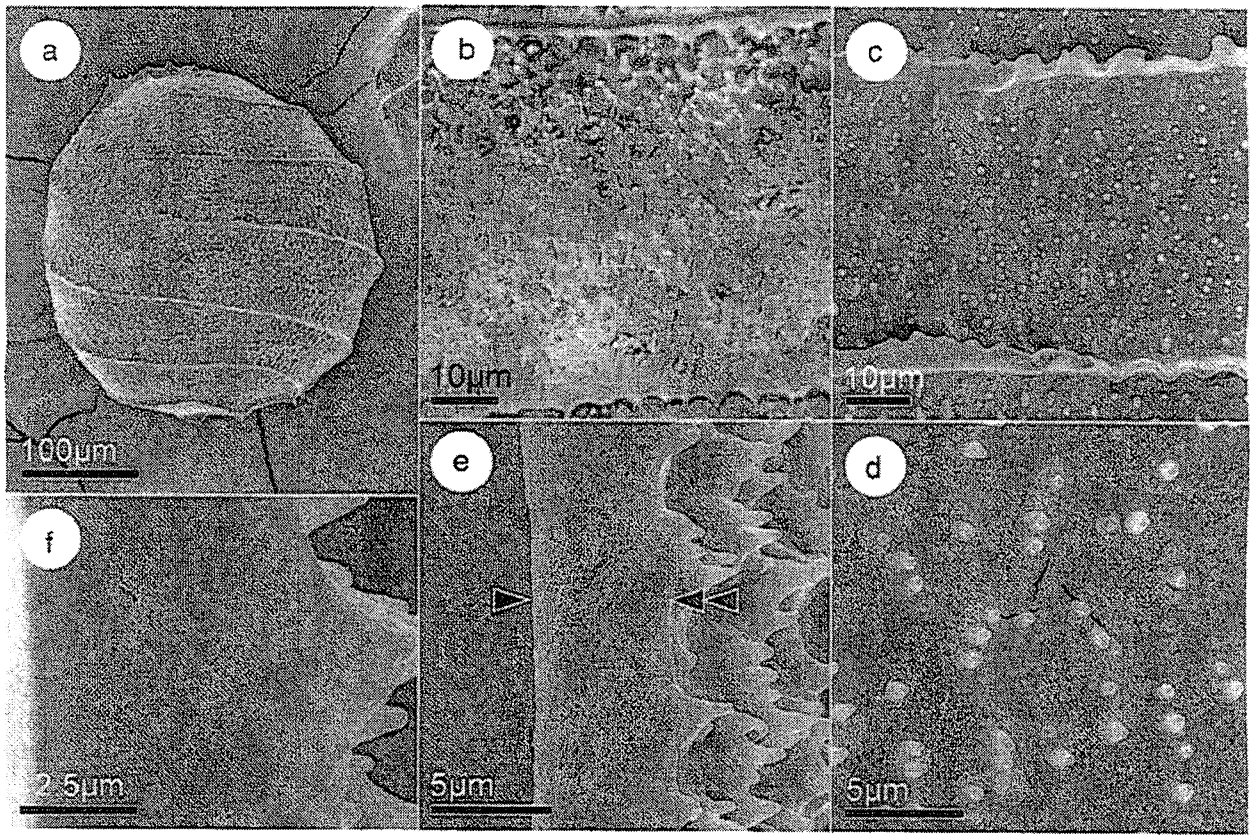

FIG. 4. Oospores of *Nitella japonica* (S077). Single or double arrowhead indicates the inner or outer side of the wall, respectively. (a) Oospore with five to six flanged spiral ridges on the surface. SEM. (b) Part of fossa wall, showing papillate or beaded imperfect reticulate ornamentation. LM. (c) Part of fossa wall, showing papillate or beaded imperfect reticulate ornamentation, with flanged spiral ridges. SEM. Note the papillae extend onto the spiral ridges but are absent from the flanges. (d) Detail of fossa wall, showing papillate or beaded imperfect reticulate pattern. SEM. Note the reticulum is formed by waved ridges with tapering projections. (e) Longitudinal fractured face of fossa wall, showing homogeneous texture. SEM. (f) Close view of longitudinal fractured face of fossa wall, showing homogeneous texture. SEM.

Distribution: Asia, North America, South America, Oceania, and Africa (Wood 1965) (Table 1).

Remarks: Wood (1965) reduced *N. oligospira* to a form of *N. furcata* based on vegetative morphology, as he did with *N. japonica*. Under LM, the oospores of *N. oligospira* (Fig. 5b), *N. furcata* (Sakayama et al. 2002), and *N. mucronata* A. Braun (= *N. furcata* subsp. *mucronata* [A. Braun] R. D. Wood) (Wood 1965) have similar reticulate ornamentation. In addition, Wood (1965) characterized *N. mucronata* and *N. oligospira* as having elongate and two- or three-celled dactyls, in which the end cells are mucronate. However, our SEM observations showed that the fossa walls of *N. oligospira* have a papillate pattern (Fig. 5, c and d). By contrast, the SEM oospore ornamentations of *N. furcata* and *N. mucronata* are imperfect reticulate (Caceres 1975, Mandal et al. 1995, Sakayama et al. 2002) and strongly reticulate (John and Moore 1987, Mandal et al. 1995), respectively. Our DNA phylogeny clearly separated *N. oligospira* from *N. furcata* (Fig. 9).

Nitella* (subgen. *Tieffallenia*) *imperialis (Allen) Sakayama, stat. nov.

(Fig. 6)

Basionym: Nitella pseudoflabellata A. Braun var. *imperialis* Allen (1898, p. 78).

Synonym: Nitella pseudoflabellata A. Braun f. *imperialis* (Allen) R. D. Wood (1965, p. 587).

Oospore morphology: The oospores are oval in face view and have six to seven (or eight) flanged spiral ridges; they are 306–335 μm long, 232–270 μm wide, and about 40–48 μm across the fossa (Fig. 6a). The walls of mature oospores are dark brown. The fossa wall is irregular or obscure finely granulate, with about 15–32 granules across the fossa under LM and SEM (Fig. 6, b and c). Under SEM, there are minute openings on the surface of the fossa wall and granules (Fig. 6, c and d). The granules are prominent to obscure (Fig. 6, c and d); they are located 0.3–4.5 μm from each other and are 0.7–1.6 μm in diameter (Fig. 6, c and d). The granules extend onto the spiral ridges and flanges (Fig. 6, a and c). The longi-

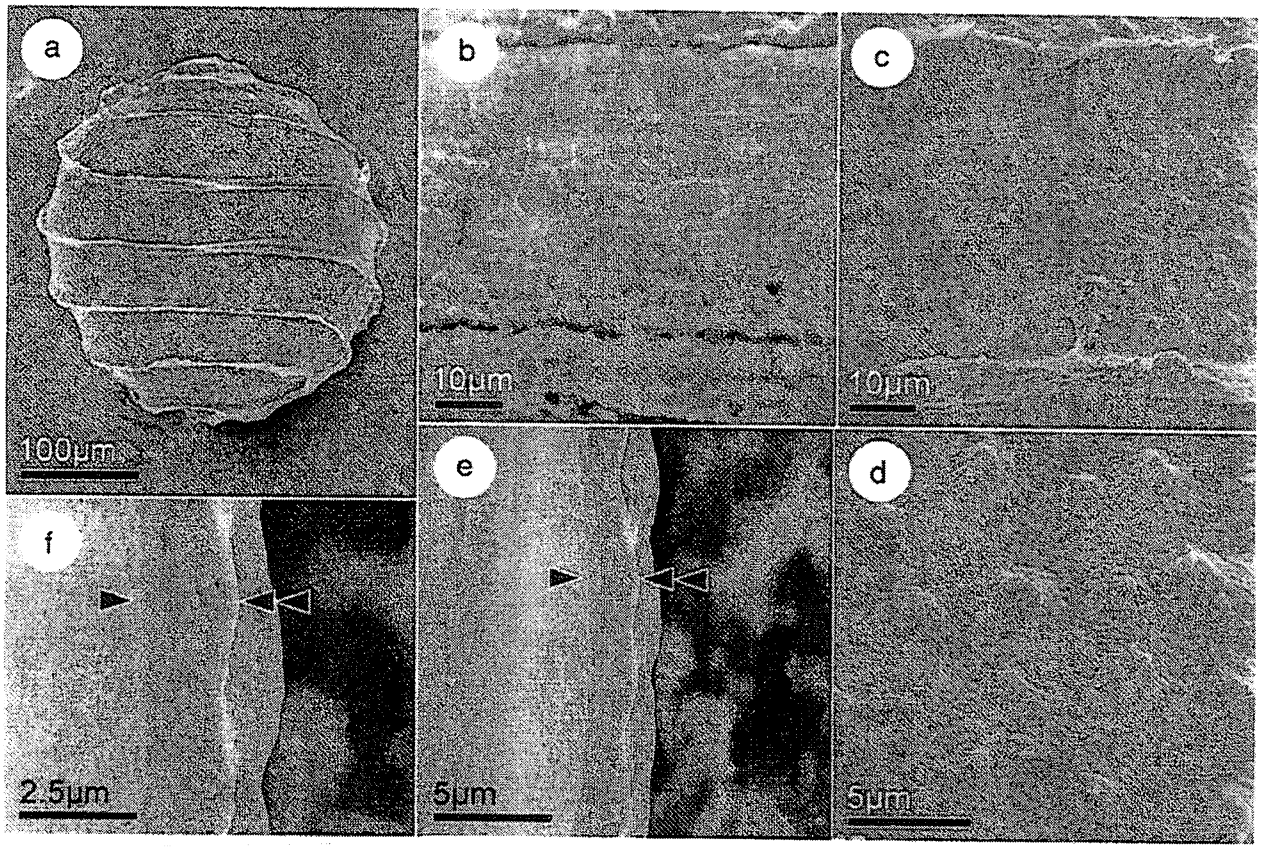

FIG. 5. Oospores of *Nitella oligospira* (S095). Single or double arrowhead indicates the inner or outer side of the wall, respectively. (a) Oospore with five to six robust flanged spiral ridges on the surface. SEM. (b) Part of fossa wall, showing imperfect reticulate ornamentation. LM. (c) Part of fossa wall, showing papillate or slightly beaded imperfect reticulate ornamentation, with robust flanged spiral ridges. SEM. Note the ridges forming slight reticulum extend onto the spiral ridges but are absent from the flanges. (d) Detail of fossa wall, showing papillate or slightly beaded imperfect reticulate pattern. SEM. Note the reticulum is formed by slightly waved ridges between obtuse papillae. (e) Longitudinal fractured face of fossa wall, showing homogeneous texture. SEM. (f) Close view of longitudinal fractured face of fossa wall, showing homogeneous texture. SEM.

tudinal fractured face of the fossa wall has a strongly spongy texture, in which the openings are laterally compressed (0.3–2.8 µm long) and form a lamellate pattern (Fig. 6, e and f).

Distribution: Japan (Imahori 1954, Wood 1965), China (Han et al. 1994), and Thailand (Table 1).

Remarks: This alga was originally isolated from Japan and described as a variety of *N. pseudoflabellata*, a taxon featuring á felt-like oospore wall composed of fine hairs and mucus covering the thallus (Allen 1898). By contrast, Imahori (1954) assigned this taxon as a synonym of *N. pseudoflabellata* var. *mucosa* (Nordstedt) F. M. Bailey, because fully mature oospores of his material exhibited a spongy-like ornamentation, with elongated granules on the fossa wall under LM, as in *N. pseudoflabellata* var. *mucosa* (Imahori 1954). However, our LM observations showed that the fossa walls of *N. imperialis* exhibit an irregular finely granulate ornamentation (Fig. 6b) as reported by Wood (1965). Moreover, *N. imperialis* is distinguished from *N. pseudoflabellata* var. *mucosa* by differences in the branchlet (Wood 1965). In *N. imperialis*, one of the secondary rays of a fertile branchlet is robust and positioned centrally, (Wood 1965). Conversely, *N. pseudoflabellata* var. *mucosa* lacks central secondary rays in the fertile branchlets (Wood 1965).

Wood (1965) assigned *N. imperialis* as a form of *N. pseudoflabellata* because both species are characterized as having a medium-sized thallus and a slender axis with branchlets forked two to five times (Wood 1965). Under LM, the oospore walls of both *N. imperialis* (Fig. 6b) and *N. pseudoflabellata* (Sakayama et al. 2002) are finely granulate. However, our SEM observations of the EMOW and IMOW demonstrated differences between *N. imperialis* (Fig. 6) and *N. pseudoflabellata* (Sakayama et al. 2002). In *N. imperialis*, the oospore wall ornamentations exhibit a spongy-like pattern with irregularly arranged granules (Fig. 6, c and d), and the longitudinal fractured face of the fossa wall has a strongly spongy texture with laterally compressed openings (Fig. 6, e and f). Conversely, *N. pseudoflabellata* has compressed finely granulate ornamentations (Sakayama

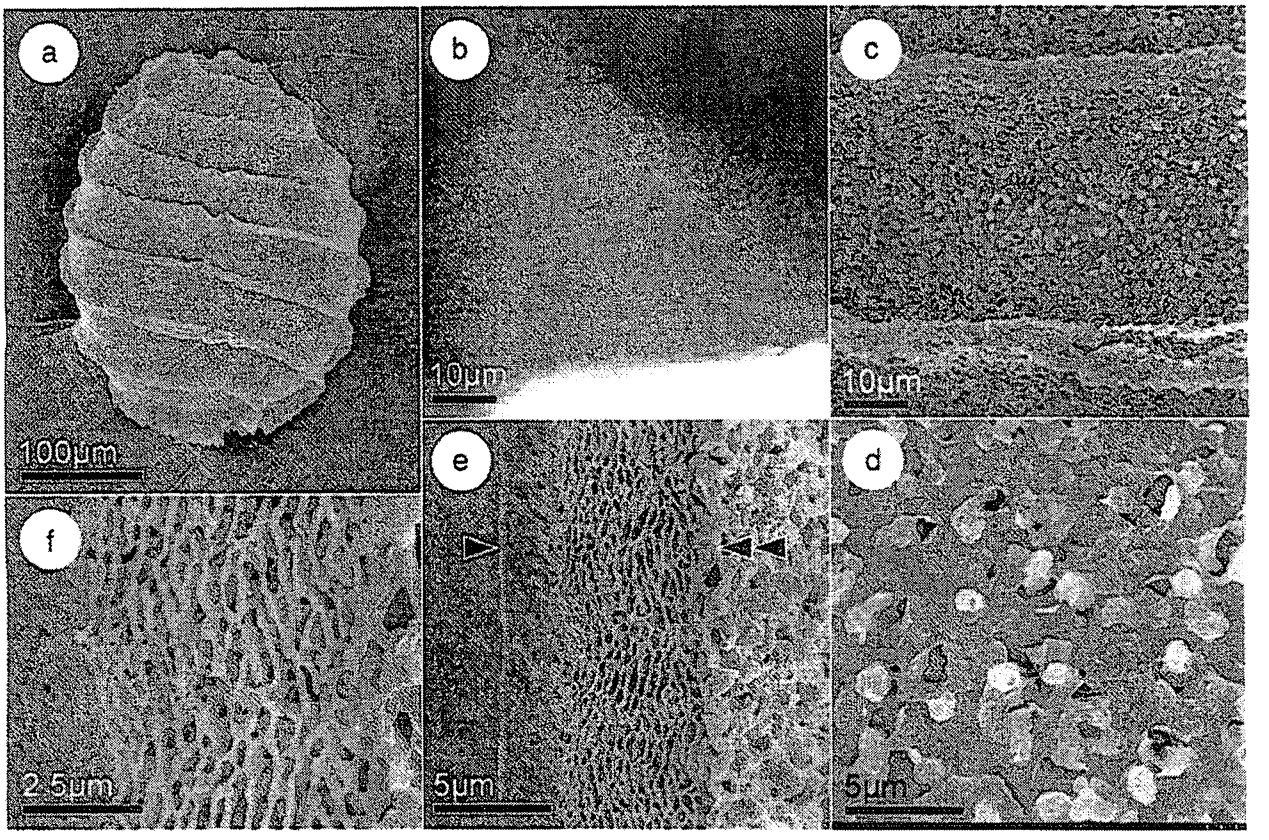

FIG. 6. Oospores of *Nitella imperialis* (S100). Single or double arrowhead indicates the inner or outer side of the wall, respectively. (a) Oospore with six to seven flanged spiral ridges on the surface. SEM. (b) Part of fossa wall, showing irregular or obscure finely granulate ornamentation. LM. (c) Part of fossa wall, showing irregular finely granulate ornamentation, with flanged spiral ridges. SEM. Note the granules extend onto the spiral ridges and flanges. (d) Detail of fossa wall, showing irregular finely granulate pattern composed of prominent to obscure granules. SEM. Note the surface of the fossa wall and granules has minute openings. (e) Longitudinal fractured face of fossa wall, showing strongly spongy texture. SEM. (f) Close view of longitudinal fractured face of fossa wall, showing strongly spongy texture. SEM. Note the openings are laterally compressed and form a lamellate pattern.

et al. 2002) and the fractured face of the fossa wall exhibits a weakly spongy texture (Fig. 3, a and b). Furthermore, our DNA sequences clearly separated *N. imperialis* from *N. pseudoflabellata* (Fig. 9).

Nitella* (subgen. *Tieffallenia*) *vieillardii (A. Braun) Sakayama, stat. nov.

(Fig. 7)

Basionym: Nitella gracilis (Smith) C. Agardh var. *vieillardii* A. Braun (1882, p. 61; "*viellardi*").

Synonym: Nitella pseudoflabellata A. Braun var. *vieillardii* (A. Braun) R. D. Wood (1966, p. 28).

Oospore morphology: The oospores are oval in face view and have seven (to eight) flanged spiral ridges; they are 300–315 μm long, 228–243 μm wide, and about 42–48 μm across the fossa (Fig. 7a). The walls of mature oospores are dark brown. The fossa wall is imperfect reticulate, with about 13–16 meshes, each about 0.4–3.8 μm in diameter, across the fossa (Fig. 7b). The SEM revealed that the fossa wall has sharply developed fused fibrils (with papilla-like projections) forming an irregular reticulate pattern with deep depressions (Fig. 7, c and d); the fibrils are 0.6–0.8 μm in diameter (Fig. 7d). The reticula or fibrils extend onto the spiral ridges and flanges (Fig. 7, a and c). The longitudinal fractured face of the fossa wall has a strongly spongy texture, in which the openings are almost circular to oval and 0.3–2.0 μm in diameter (Fig. 7, e and f).

Distribution: Endemic to New Caledonia (Braun 1882, Wood 1966) (Table 1).

Remarks: Braun (1882) originally described this alga as a variety of *N. gracilis* based on the vegetative similarities, especially in having two- or three-celled dactyls (Braun 1882). Subsequently, Wood (1966) regarded this alga as a variety of *N. pseudoflabellata*, because *N. vieillardii* has central secondary rays in the fertile branchlets, submucronate end cells of the dactyl, and a reproductive organ positioned at the lowest branchlet nodes (Wood 1965). However, the oospore wall ornamentations of *N. vieillardii* (Fig. 7, b–d) clearly differ from those in *N. gracilis* (John and Moore 1987, Ray et al. 2001) and *N. pseudoflabellata*

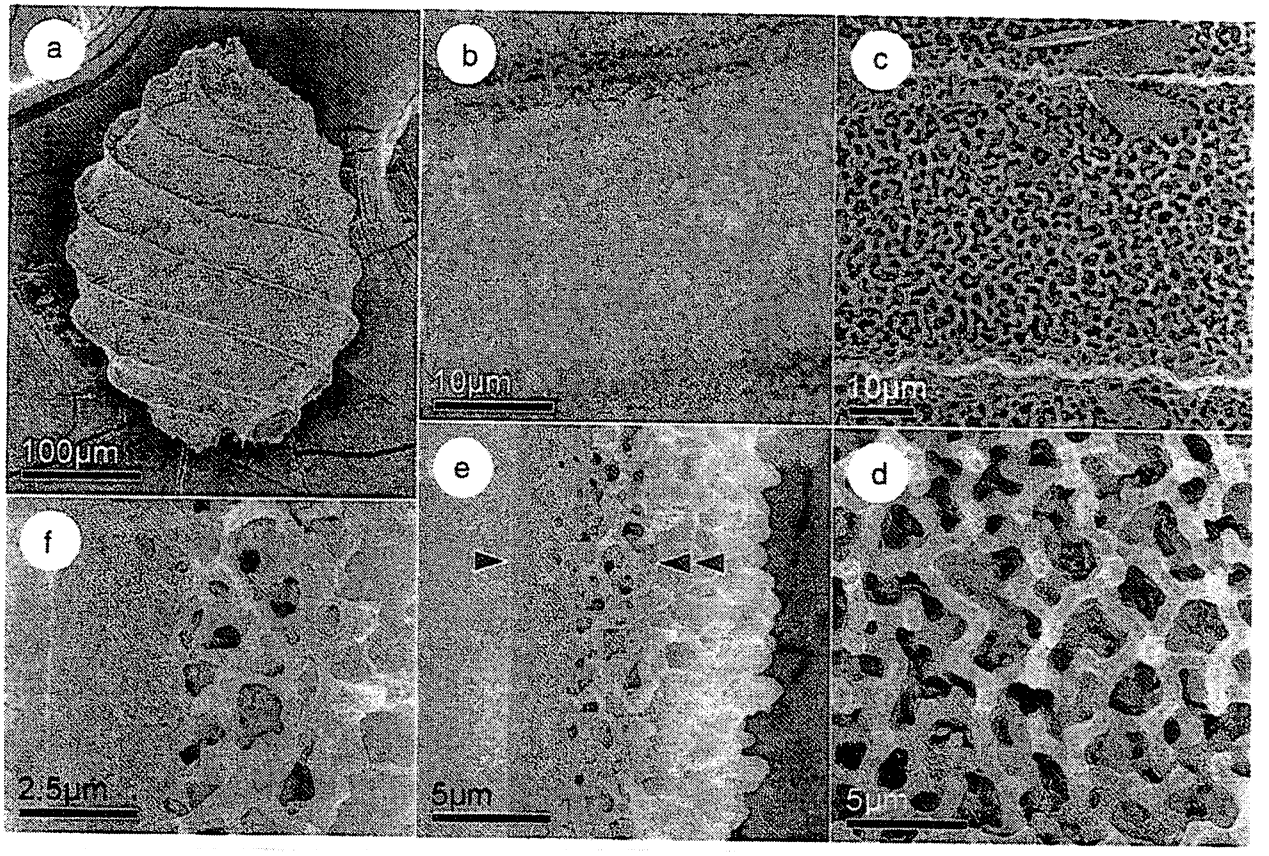

FIG. 7. Oospores of *Nitella vieillardii* (S092). Single or double arrowhead indicates the inner or outer side of the wall, respectively. (a) Oospore with seven to eight flanged spiral ridges on the surface. SEM. (b) Part of fossa wall, showing imperfect reticulate ornamentation. LM. (c) Part of fossa wall, showing irregular or imperfect reticulate ornamentation with deep depressions, with flanged spiral ridges. SEM. Note the reticula or fibrils extend onto the spiral ridges and flanges. (d) Detail of fossa wall, showing irregular or imperfect reticulate pattern. SEM. Note the sharply developed fused fibrils form an irregular reticulate pattern with deep depressions. (e) Longitudinal fractured face of fossa wall, showing strongly spongy texture. SEM. Note the papilla-like projections arise from the surface of the fibrils forming the reticulate pattern. (f) Close view of longitudinal fractured face of fossa wall, showing strongly spongy texture with almost circular to oval openings. SEM.

(Sakayama et al. 2002). Our SEM analyses also revealed that the IMOW differs markedly in *N. vieillardii* (Fig. 7, e and f) and *N. pseudoflabellata* (Fig. 3, a and b). The longitudinal fractured face of the fossa wall of *N. vieillardii* has a strongly spongy texture (Fig. 7, e and f), whereas that of *N. pseudoflabellata* is weakly spongy (Fig. 3, a and b). Moreover, *N. vieillardii* is separated phylogenetically from *N. pseudoflabellata* in our molecular phylogeny (Fig. 9). Therefore, *N. vieillardii* should be raised to species rank.

The morphological characteristics of our *N. vieillardii* material agreed with those of the type material reported by Wood (1966), except for the dactyls. Our specimens possessed two-celled dactyls uniformly. Conversely, Braun (1882) and Wood (1966) reported that the dactyls of this taxon were composed of two or three cells.

Nitella* (subgen. *Tieffallenia*) *morongii Allen (1887, p. 214)

(Fig. 8)

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

Oospore morphology: The oospores are oval in face view and have seven to eight flanged ridges; they are 262–277 μm long, 230–243 μm wide, and measure 32–48 μm across the fossa (Fig. 8a). The walls of mature oospores are dark brown. In LM, the fossa wall is papillate or tuberculate with a finely granulate background; there are four to six papillae or tubercles across the fossa (Fig. 8b). SEM showed tuberculate patterns with the spongy background formed by minute openings on the surface of the fossa wall (Fig. 8, c and d). The tubercles are cylindrical, occasionally connected, 1.2–4.5 μm wide and 1.7–4.2 μm high (Fig. 8d). Tubercles or papillae are absent from the spiral ridges and flanges, whereas the spongy background extends onto both the spiral ridges and flanges (Fig. 8, a and c). The longitudinal fractured face of the fossa wall has a strongly spongy texture that appears to be alveolar, in which the openings are

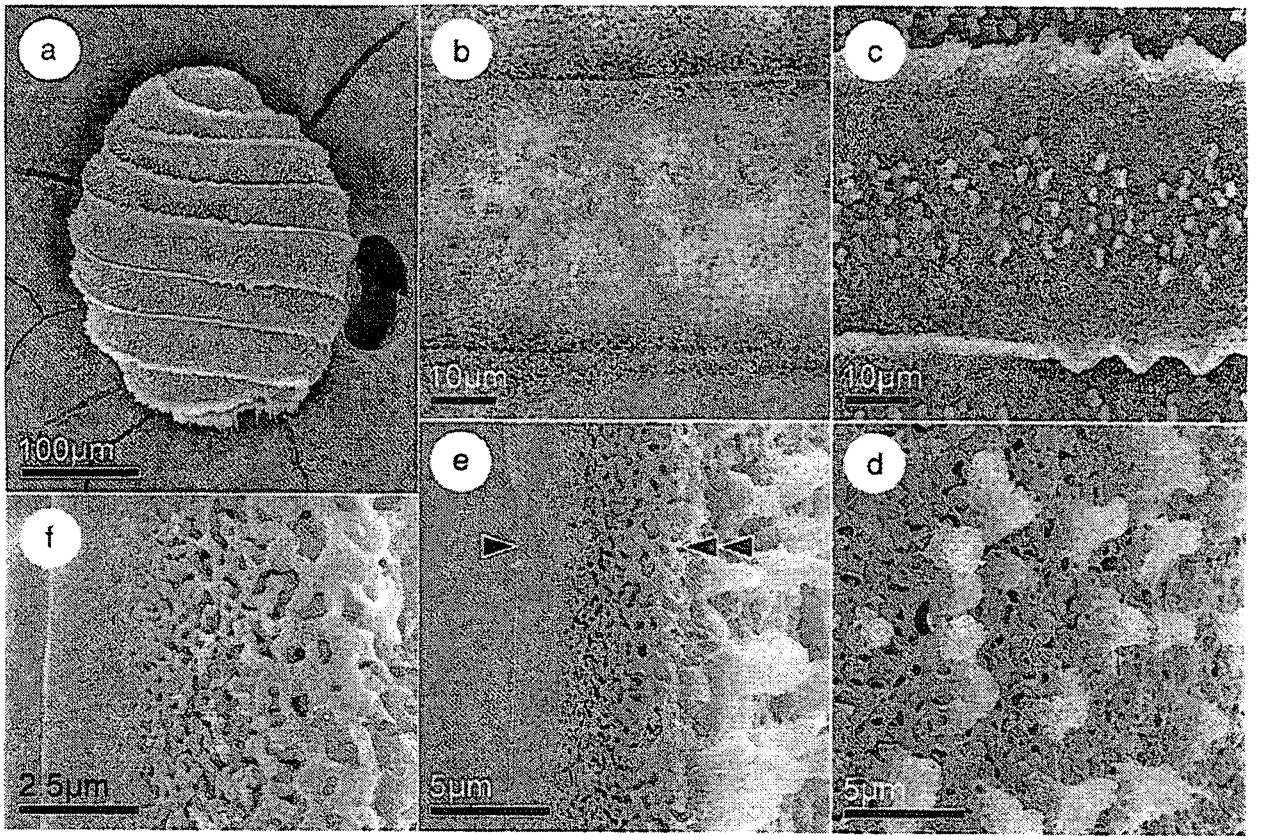

FIG. 8. Oospores of *Nitella morongii* (S089). Single or double arrowhead indicates the inner or outer side of the wall, respectively. (a) Oospore with seven to eight flanged spiral ridges on the surface. SEM. (b) Part of fossa wall, showing finely granulate ornamentation with papillae or tubercles. LM. (c) Parts of fossa wall, showing tuberculate pattern with spongy background, with flanged spiral ridges. SEM. Note the tubercles or papillae are absent from the spiral ridges and flanges, whereas the spongy background extends onto the spiral ridges and flanges. (d) Detail of fossa wall, showing tuberculate patterns. SEM. Note the minute openings on the surface of the fossa wall form spongy pattern, from which cylindrical and occasionally connected tubercles arise. (e) Longitudinal fractured face of fossa wall, showing strongly spongy texture. SEM. (f) Close view of longitudinal fractured face of fossa wall, showing strongly spongy texture. SEM. Note the openings are circular to oval and gradually become smaller toward the inner side of the wall.

circular to oval; the openings gradually become smaller toward the inner side of the wall (Fig. 8, e and f).

Distribution: United States (Allen 1887, 1894, Wood 1965) and Australia (Table 1).

Remarks: Wood (1962) assigned *N.morongii* as a form of *N. gracilis* because both species have a medium to small thallus, a slender axis, an un-isolated thallus apex, and a nonreticulate oospore wall (Wood 1962, 1965). John and Moore (1987) reported that the oospore wall ornamentations of *N. gracilis* are fibrous under SEM. By contrast, in our SEM examinations, the oospore walls of *N. morongii* have a tuberculate pattern with a spongy background (Fig. 8, c and d). According to Wood (1962, 1965), *N. morongii* is morphologically very similar to *N. gracilis* f. *asagrayana* (Schaffner *ex* Nordstedt) R. D. Wood and *N. gracilis* f. *annularis* (Allen) R. D. Wood, which have a spongy oospore wall ornamentation under SEM (Leitch et al. 1990). However, *N. morongii* is distinguished from *N. gracilis* f. *asagrayana* and *N. gracilis* f. *annularis* by the existence of tubercles on the fossa walls (Fig. 8, b–d). *Nitella morongii* has not been reported from Australia previously.

DISCUSSION

Taxonomic significance of the IMOW. Based on the differences in the IMOW seen with SEM, the reticulate and tuberculate oospore walls characterized by the EMOW were classified into two distinct types within the subgenus *Tieffallenia*. Of the four species with a reticulate EMOW, three (*N. furcata*, *N. axillaris*, and *N. axilliformis*) have a homogeneous IMOW (reticulate [R]/HG type). By contrast, *N. vieillardii* has a spongy fractured face (R/SG type). Of the three species with a tuberculate EMOW, *N. spiciformis* and *N. moriokae* have a homogeneous fractured face (tuberculate [TUB]/HG type), whereas the fractured face of the wall of *N. morongii* has a spongy texture (TUB/SG

TABLE 3. SEM oospore morphology of 17 species of *Nitella* subgenus *Tieffallenia* examined in the present study.

Species	External morphology of the oospore wall: Fossa wall ornamentations	External morphology of the oospore wall: Franges	Internal morphology of the oospore wall: Longitudinal fractured faces of the fossa wall
RP/HG oospore clade			
Nitella furcata (Roxburgh *ex* Bruzelius) C. Agardh	Imperfect or irregular reticulate (R/HG type)	Low	HG type
N. inversa Imahori	Papillate or beaded imperfect reticulate	Low	HG type
N. japonica Allen	Papillate or beaded imperfect reticulate	Low	HG type
N. tumulosa Zaneveld	Papillate or beaded imperfect reticulate	Low	HG type
N. gracillima Allen	Papillate or beaded imperfect reticulate	Low	HG type
N. oligospira A. Braun	Papillate or beaded imperfect reticulate	Low	HG type
N. axillaris A. Braun	Strongly reticulate (R/HG type)	Low	HG type
N. axilliformis Imahori	Strongly reticulate (R/HG type)	Low	HG type
SG oospore clade			
N. pseudoflabellata A. Braun	Finely granulate	Unflanged	W-SG type
N. megaspora (J. Groves) Sakayama			
Complex 1	Finely granulate	Unflanged	W-SG type
Complex 2	Finely granulate	Unflanged	S-SG type
N. imperialis (Allen) Sakayama, stat. nov.	Irregular or obscure finely granulate with openings	Low	S-SG type
N. vieillardii (A. Braun) Sakayama, stat. nov.	Irregular reticulate (R/SG type)	Low	S-SG type
N. hyalina (De Candolle) C. Agardh	Fibrous	Low	S-SG type
N. morongii Allen	Tuberculate with spongy background (TUB/SG type)	Low	S-SG type
VFG/HG oospore clade			
N. gracilens Morioka	Very finely granulate	High	HG type
TUB/HG oospore clade			
N. spiciformis Morioka	Tuberculate (TUB/HG type)	High	HG type
N. moriokae R.D. Wood	Tuberculate (TUB/HG type)	High	HG type

type). Moreover, our molecular phylogenetic analyses separated the R/HG and R/SG types and the TUB/HG and TUB/SG types (Fig. 9). Therefore, the differences in the IMOW revealed by the fractured faces of the wall are important phylogenetically, and these characteristics are useful for taxonomic studies of *Nitella* at and above the species level.

Our SEM observations of the IMOW also suggested that the algae identified as *N. megaspora* by LM include at least two distinct species (*N. megaspora* complex 1 and *N. megaspora* complex 2). The fractured face of the wall of *N. megaspora* complex 1 is weakly spongy, whereas *N. megaspora* complex 2 has a strongly spongy texture in the fractured face. Furthermore, these two complexes had differences in the DNA sequences, although they are sister to each other in the phylogenetic tree (Fig. 9). Therefore, the IMOW characters must provide valuable information for delimiting closely related taxa. Examinations of further samples of *N. megaspora* (including the type specimen) are needed to clarify whether these two IMOW types represent distinct species or morphological variation within a single species.

TABLE 4. Probability values from partition-homogeneity test with 1000 replications for various partitions of the data.

Data sets	*P* values
*atp*B vs. *rbc*L	0.336
*atp*B vs. *psa*B	0.336
*atp*B vs. ITS-5.8S rRNA	0.942
*bbc*L vs. *psa*B	0.778
*bbc*L vs. ITS-5.8S rRNA	1.000
*psa*B vs. ITS-5.8S rRNA	0.289
*atp*B-*rbc*L-*psa*B vs. ITS-5.8S rRNA	0.967

Subdivision of the subgenus Tieffallenia. Wood (1962, 1965) recognized eight sections within the subgenus *Tieffallenia* based on differences in vegetative morphology: *Tieffallenia*, *Gioallenia* R. D. Wood, *Earthya* R. D. Wood, *Decandollea* R. D. Wood, *Muelleria* R. D. Wood, *Vogania* R. D. Wood, *Persoonia* R. D. Wood, and *Migularia* R. D. Wood. Recently, Sakayama et al. (2004b) demonstrated that SEM EMOW characters are phylogenetically more conservative than the vegetative morphology Wood (1962, 1965) used for the section and species delimitations within the subgenus *Tieffallenia*. They also suggested the efficiency of using the EMOW to revise sections within the subgenus *Tieffallenia*. However, our SEM examinations of the IMOW and DNA phylogeny demonstrated that the two morphological groups characterized by the EMOW only—the reticulate EMOW group (the R/HG and R/SG type species) and the tuberculate EMOW group (the TUB/HG and TUB/SG type species)—are polyphyletic; the R/HG type species (*N. furcata*, *N. axillaris*, and *N. axilliformis*) are separated from the R/SG type species (*N. vieillardii*) and the TUB/HG type species (*N. spiciformis*

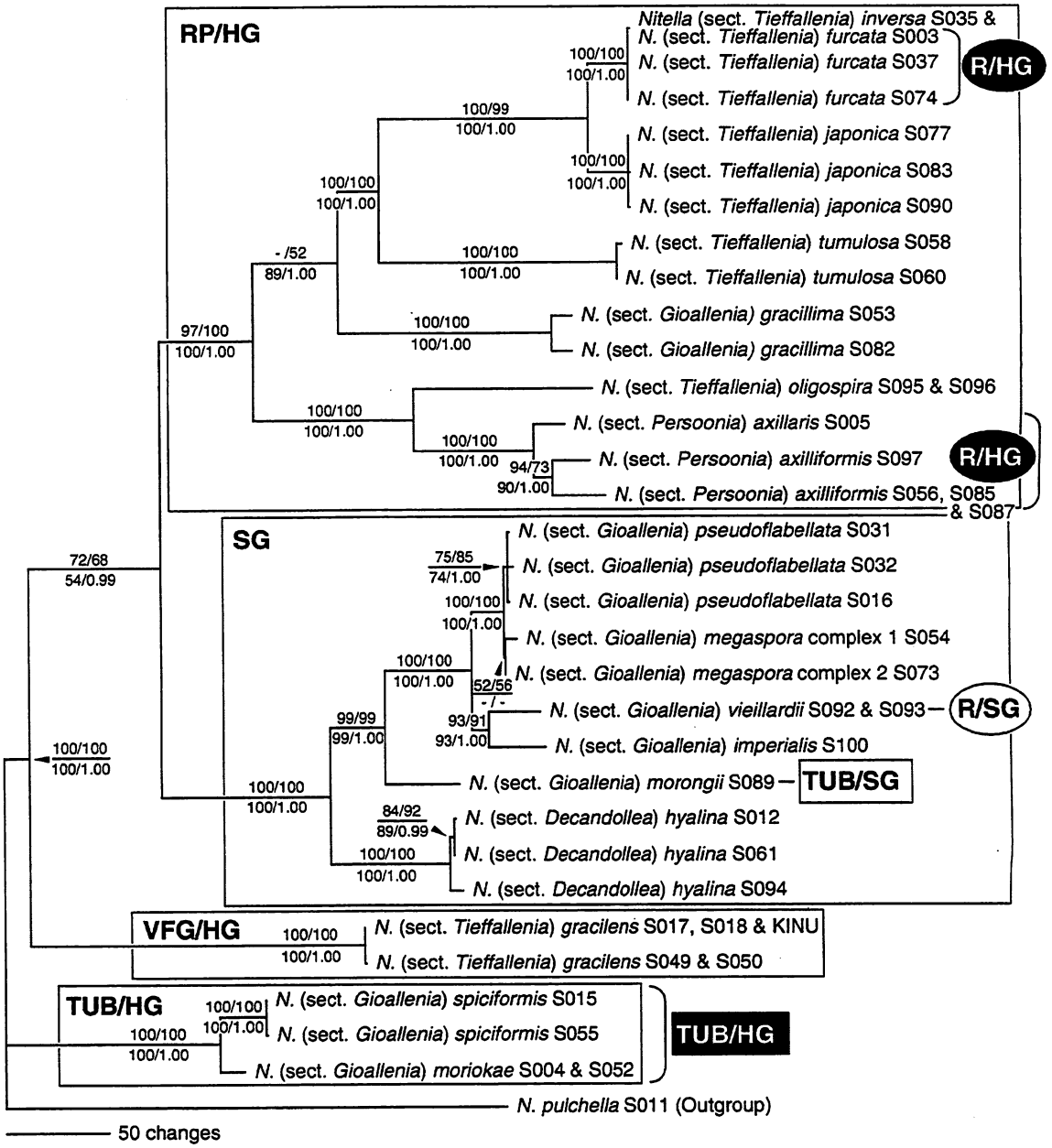

FIG. 9. One of the six MP trees based on 4553 bp (with 642 potentially parsimony-informative characters) of the concatenated sequences data set from the *atp*B, *rbc*L, *psa*B, and 5.8S rRNA genes and ITS regions of 40 strains representing 18 species of the genus *Nitella* (*N.*) (Table 1). The MP trees were found by PAUP* 4.0b10 based on a heuristic search using the stepwise addition of 100 random replications. The tree is 1169 steps long with consistency index of 0.7536 and retention index of 0.9077. Branch lengths are proportional to the nucleotide changes, which are indicated by the scale bar below the tree. Numbers above branches are BS values (50% or more) based on 1000 replications of the MP (left) and ME (right) analyses. Branches resolved with 50% or more BS values (based on 100 replications) or with 0.95 or more PPs (based on 9000 trees) by ML (left, based on GTR+G+I model) or BI (right, based on GTR+G+I model) analyses, respectively, are also shown by numbers under the branches. Samples representing identical sequences in 4553 bp are treated as a single operational taxonomic unit. Four robust oospore clades, the RP/HG, SG, VFG/HG, and TUB/HG oospore clades, identified based on the IMOW and EMOW and two types of the reticulate (R/HG and R/SG) or tuberculate (TUB/HG and TUB/SG) EMOW are shown.

and *N. moriokae*) from the TUB/SG type species (*N. morongii*). Based on the combined EMOW and IMOW characteristics, the 17 species examined (belonging to four sections *Tieffallenia*, *Gioallenia*, *Persoonia*, and *Decandollea*) are clearly subdivided into four robust monophyletic groups that were supported by ≥ 97% BS and 1.00 PP in the MP, ME, ML, and BI analyses: three clades of the HG type of IMOW (the reticulate

or papillate [RP/HG], very finely granulate [VFG/HG], and tuberculate [TUB/HG] oospore clades) and a spongy (SG) oospore clade consisting of species of the S-SG and W-SG types of IMOW. In the RP/HG oospore clade (*N. furcata*, *N. inversa*, *N. japonica*, *N. tumulosa*, *N. gracillima*, *N. oligospira*, *N. axillaris*, and *N. axilliformis*), the EMOW has a reticulate or papillate pattern that is formed by swollen, waved, or fused ridges, and the IMOW has a homogeneous texture. The SG oospore clade includes four types of EMOW: the granulate (*N. pseudoflabellata*, *N. megaspora* complex 1, *N. megaspora* complex 2, and *N. imperialis*), reticulate (*N. vieillardii*), tuberculate (*N. morongii*), and fibrous (*N. hyalina*) types. By contrast, in the IMOW, all the species of the SG oospore clade have the weak or strong spongy texture. The VFG/HG (*N. gracilens*) and TUB/HG (*N. spiciformis* and *N. moriokae*) oospore clades have very finely granulate and tuberculate patterns in the EMOW, respectively, although these two oospore clades have a homogeneous texture in the IMOW. In addition, our multiple DNA marker analyses robustly resolved the RP/HG oospore clade into two sister monophyletic groups, which can be distinguished by differences in the SEM oospore morphology: One is composed of *N. oligospira*, *N. axillaris*, and *N. axilliformis* and the EMOW has weak surface sculptures, whereas the other consists of *N. furcata*, *N. inversa*, *N. tumulosa*, *N. japonica*, and *N. gracillima* and the EMOW possesses relatively strong surface sculptures. These results strongly indicate that the detailed EMOW and IMOW examined under SEM is efficient for characterizing phylogenetic groups within the subgenus *Tieffallenia*. However, we did not examine the four minor sections of this subgenus (*Earthya*, *Muelleria*, *Vogania*, and *Migularia*) (Wood 1962, 1965), which are distributed mainly in Africa and Australia (Wood 1965). Therefore, further comprehensive taxonomic reexamination, including species of these four minor sections, is needed to reconstruct the natural taxonomic system within the subgenus *Tieffallenia*.

Phylogeny and evolution of oospore morphology within the subgenus Tieffallenia. In the subgenus *Tieffallenia*, the spongy type of IMOW (W-SG and S-SG types) was recognized in six of the 17 species we studied, and these six species formed the SG oospore clade, which is positioned distally within the subgenus in our molecular phylogeny. Moreover, *N. megaspora* complex 1 and *N. pseudoflabellata*, which possess the W-SG type of IMOW, occupy distal phylogenetic positions within the SG oospore clade. In vegetative morphology, species consisting of the SG oospore clade are also characterized by having elongate dactyls and exhibiting thalli covered with thick or thin mucus, except for three strains of *N. pseudoflabellata* (Wood 1965, 1966). This suggests that the spongy texture of the IMOW evolved once in the subgenus *Tieffallenia* and the W-SG type of IMOW evolved recently via the S-SG type of IMOW within the SG oospore clade.

In the EMOW, species with the reticulate or tuberculate oospore wall ornamentation were polyphyletic. Conversely, the other EMOW patterns (papillate, finely granulate, very finely granulate, and fibrous) were conservative phylogenetically. Therefore, overall EMOW features are apparently homoplasious at least in the species with the reticulate or tuberculate EMOW.

Based on SEM observations (Sakayama et al. 2002, 2004b), two types of flange on the spiral ridge of oospores are recognized within the 17 species of the subgenus *Tieffallenia* examined. Oospores of the species in the TUB/HG oospore clade (*N. spiciformis* and *N. moriokae*) and VFG/HG oospore clade (*N. gracilens*) possess high thin flanges on the spiral ridge. Conversely, species of the RP/HG and SG oospore clades lack such flanges. Our molecular phylogenetic analyses demonstrated that the VFG/HG and TUB/HG oospore clades form two branches in a paraphyletic assemblage that is basal to the large monophyletic group composed of the RP/HG and SG oospore clades. Therefore, the high thin flanges seen in the VFG/HG and TUB/HG oospore clades appear primitive, and flanges might have become lower and thicker with evolution within the subgenus *Tieffallenia*.

Multiple DNA marker analyses. In previous combined *atp*B-*rbc*L analyses of 12 species of the subgenus *Tieffallenia* (Sakayama et al. 2004b), the phylogenetic position of *N. gracillima* within the RP oospore clade (corresponding to the RP/HG oospore clade in our phylogenetic tree) was ambiguous. Recently, Sakayama et al. (2004a) demonstrated that *N. gracillima* is sister to a robust clade composed of *N. axillaris* and *N. axilliformis* based on 3060 bp of the concatenated chloroplast *atp*B and *rbc*L genes and nuclear 5.8S rRNA gene and ITS sequences. However, our DNA phylogeny based on 4553 bp of the concatenated *atp*B-*rbc*L-*psa*B-ITS-5.8S rRNA sequences from the 17 species of the subgenus *Tieffallenia* resolved *N. gracillima* as sister to the robust clade composed of *N. furcata*, *N. inversa*, *N. japonica*, and *N. tumulosa*, with 52% and 89% BS or 1.00 PP in the ME, ML, or BI analyses, respectively. Although the support for this sister relationship is not strong except for the BI analysis, this phylogenetic result is consistent with the differences in SEM oospore wall morphology. In *N. furcata*, *N. inversa*, *N. japonica*, *N. tumulosa*, and *N. gracillima*, projections/ridges on the fossa wall are elongate or strongly developed, whereas *N. oligospira*, *N. axillaris*, and *N. axilliformis* exhibit weakly developed projections/ridges on the fossa wall. Therefore, *N. gracillima* is thought to be phylogenetically related to a robust clade consisting of *N. furcata*, *N. inversa*, *N. japonica*, and *N. tumulosa*.

Our multiple DNA marker phylogeny demonstrated that all the species examined represent distinct lineages, except for *N. furcata* and *N. inversa*. Conversely, the phylogenetic relationships between *N. axillaris* and *N. axilliformis* were not resolved in the single DNA marker trees (not shown). Therefore, the concatenated

*atp*B-*rbc*L-*psa*B-ITS-5.8S rRNA analyses, which support the taxonomic decision based on the difference in SEM oospore morphology (EMOW and IMOW), seem to be efficient for species delimitation within the subgenus *Tieffallenia*. After accumulating such molecular and morphological data for a large number of taxa, the multiple DNA marker analyses should be helpful for species identification when morphological data of samples are insufficient or unavailable. Although *N. furcata* and *N. inversa* are clearly distinguished using oospore morphology (Sakayama et al. 2002), there are no differences in their concatenated 4553-bp sequences. Therefore, further morphological and molecular analyses (using rapidly evolving DNA markers) of these species, using a large number of samples collected from localities throughout the world, are needed to resolve their natural relationship.

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