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Morphology, molecular phylogeny and taxonomy of *Nitella comptonii* (Charales, Characeae)

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The taxonomic status of *Nitella comptonii* (Charales, Characeae) is not well understood because this species has not been recorded since the early 20th century, and detailed examinations of fully mature individuals have not been performed. Recently, we collected fully mature *N. comptonii* from the Okinawa Islands in Japan. These exhibited a distinctive fertile spherical head in the axils, and their vegetative and reproductive structures were consistent with those in the original description of the species. In 1965 in his 'A revision of the Characeae', R. D. Wood assigned *N. comptonii* as a subspecies of *N. pseudoflabellata.* In our study, scanning electron microscopic analysis of *N. comptonii* oospores revealed that external and internal oospore morphology of *N. comptonii* was clearly different from that of *N. pseudoflabellata.* In addition, multiple DNA phylogeny using the concatenated sequences of the chloroplast *rbc*L, *atp*B, and *psa*B genes, and nuclear 5.8S ribosomal RNA gene and internal transcribed spacer regions, clearly separated *N. comptonii* from *N. pseudoflabellata.* Therefore, *N. comptonii* should be classified as a separate species.

KEY WORDS: *atp*B gene, Charales, Charophyceae, Internal transcribed spacer region, Japanese taxa, Morphology, *Nitella comptonii,* Oospores, *psa*B gene, *rbc*L gene, Scanning electron microscopy, Taxonomy

INTRODUCTION

Species assigned to the genus *Nitella* C. Agardh (Charales, Characeae) are macroscopic green algae that inhabit fresh- and brackish-water environments worldwide (Wood 1965). The thalli are differentiated into a series of nodes and internodes, which alternate along the main axis. The nodes bear forked whorled branchlets of limited growth. In some species, the fertile whorled branchlets are compact and form a mass of branchlets (called a 'head').

Nitella (subgenus *Tieffallenia* R.D. Wood) *comptonii* J. Groves was originally described by Groves (1922) on the basis of material collected from New Caledonia in 1914 by Mr R. H. Compton. Subsequently, Groves & Allen (1935) reported that material collected from Australia in 1912 by Mr C. T. White could be identified as *N. comptonii.* Since then, however, no other records of *N. comptonii* have been reported. This species is characterized by having fertile spherical heads with mucus in the axils at the base of whorled branchlets (Groves 1922; Groves & Allen 1935). In his revision of the Characeae, Wood (1965) regarded *N. comptonii* as a subspecies of *N. pseudoflabellata* A. Braun on the basis of the similarities in vegetative morphology between these two species. Subsequently, he reported that the existence of the fertile heads in the type specimen was doubtful, and he treated *N. comptonii* as a synonym of *N. pseudoflabellata* (Wood 1966, 1972). Recently, studies of oospore morphology using scanning electron microscopy (SEM) integrated with molecular phylogenetic analyses demonstrated that some of the infraspecific taxa treated by Wood (1962, 1965) should be recognized as distinct species (Sakayama *et al.* 2002, 2004b, 2005). Therefore, re-assessment of morphology on the basis of fully mature material and molecular phylogenetic analyses are necessary to determine the taxonomic status of *N. comptonii.*

In this study, we collected fully mature thalli of *N. comptonii* from Okinawa Islands, Japan, and reassessed their vegetative morphology. To clarify the taxonomic status of *N. comptonii,* we also performed SEM examinations of oospores and molecular phylogenetic analyses using the concatenated sequences of the chloroplast genes encoding the large subunit of Rubisco (*rbc*L), beta subunit of ATP synthase (*atp*B), and photosystem I P700 chlorophyll *a* apoprotein A2 (*psa*B), and nuclear 5.8S ribosomal (r) RNA gene and internal transcribed spacer (ITS) regions.

MATERIAL AND METHODS

Samples of *N. comptonii* were collected from three localities in the Okinawa Islands of Japan (Table 1). The thalli of *N. comptonii* were preserved as dried specimens and they were deposited in the Department of Botany, National Science Museum, Tsukuba, Japan. The culture strains were also deposited in the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan. The methods for field collection, culture, and light microscopy (LM) and SEM were essentially the same as in our previous studies (Sakayama *et al.* 2002, 2004b, 2005). To examine the SEM oospore morphology of the original material, the type specimen of *N. comptonii* (BM000767159; deposited at the Department of Botany, Natural History Museum, London, UK) was exam-

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Table 1. Source of *Nitella comptonii* sequenced in this study.

Strain	S091	S137	S138
Locality	creek at Aha, Kunigami-son, Okinawa, Japan	bog at Yamashiro, Kumejima, Okinawa, Japan	bog at Tancha, Onna-son, Okinawa, Japan (26°28′7.1″N, 127°49′59.63″E)
Collection date	3 Mar. 2005	22 Feb. 2005	3 Mar. 2005
Specimen no.	TNS-AL 160706	TNS-AL 160707	TNS-AL 160708
Accession no.			
*rbc*I gene	AB236669	AB236670	AB236671
*atp*B gene	AB236672	AB236673	AB236674
*psa*B gene	AB236675	AB236676	AB236677
ITS-5.8S rRNA gene	AB236678	AB236679	AB236680

ined. However, the thallus of the type specimen was sterile and lacked oospores.

The preparation of total DNA, amplification of DNA by polymerase chain reaction (PCR), direct sequencing of the PCR products, and phylogenetic analyses were conducted as described previously (Sakayama *et al.* 2002, 2004a, b, 2005), except for the addition of three samples of *N. comptonii*, and the use of a CEQ 2000XL DNA analysis system (Beckman Coulter, Tokyo, Japan) and a CEQ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter).

The *atp*B [1020 base pairs (bp), see Sakayama *et al.* 2004b], *rbc*L (1182 bp, see Sakayama *et al.* 2004b), *psa*B (1494 bp, see Sakayama *et al.* 2005), and ITS-5.8S rRNA gene sequences (857 bp, see Sakayama *et al.* 2005) were obtained for three samples of *N. comptonii* (Table 1). These three samples, which represent identical sequences in 4553 bp, were treated as a single operational taxonomic unit (OTU). Phylogenetic analyses were performed using a concatenated nucleotide data set composed of an OTU of *N. comptonii* and 31 OTUs previously analyzed in Sakayama *et al.* (2005, see EMBL-Align database accession number ALIGN_000771).

RESULTS

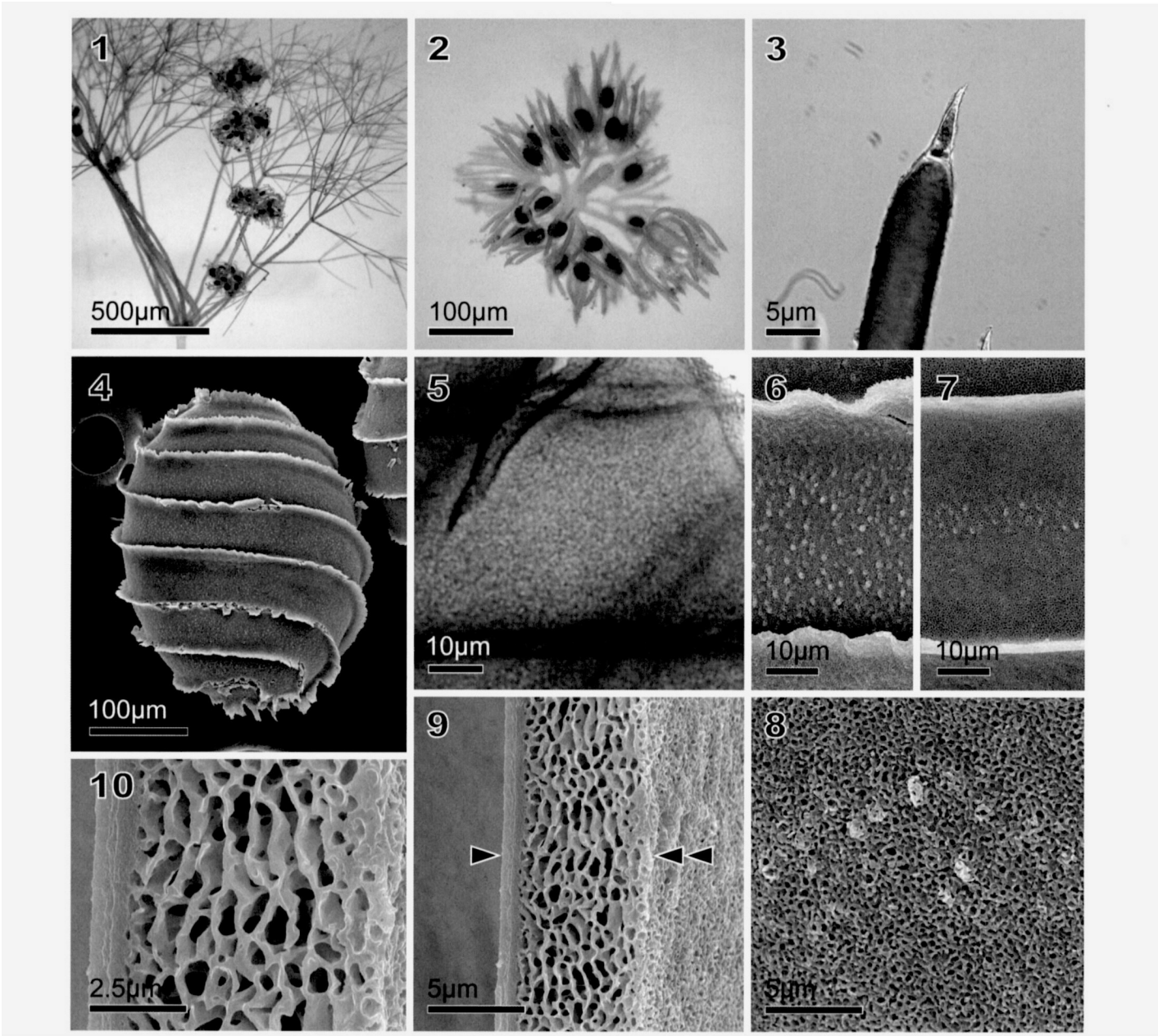
Morphological observations

Thalli of *N. comptonii* from Okinawa Islands were monoecious, bright green, and up to *c.* 23 cm high. The axes were 250–400 μm in diameter; the internodes were *c.* 0.5–2.6 cm long. The sterile branchlets were seven or eight in a whorl, *c.* 1–3 cm long and bi- or trifurcate; the primary rays were more than three-fifths of total branchlet length; with seven to eight secondaries, three to five tertiaries, and two to five quaternaries. The fertile branchlets were compact and formed a spherical head (Fig. 1), eight in a whorl, *c.* 100–200 μm long, single or bifurcate; the primary rays were about two fifths of total branchlet length; with six to seven secondaries and four to five tertiaries (Fig. 2). The dactyls were two-celled (Figs 2, 3); the end cells were long conical and acute, 50–78 μm long, and 21–27 μm wide at the base (Fig. 3). Mucus was present at fertile branchlets (Fig. 1). The gametangia were conjoined or sejoined at the primary or secondary branchlet nodes. The oogonia were solitary, 423–470 μm long (including coronula) and 303–321 μm wide, and had seven to nine convolutions. The coronula were 25–30 μm high, and 50–56 μm wide at the base; the upper cells of the coronula were as long as the lowers. The antheridia were 202–236 μm in diameter. These vegetative and reproductive characters were essentially consistent with those in the original description of Groves (1922).

The oospores were oval in face view, and had six to eight flanged spiral ridges; they were 252–321 μm long, 217–260 μm wide, and about 31–53 μm across the fossa (Fig. 4). The walls of mature oospores were dark brown. The fossa wall was finely granulate under LM (Fig. 5). The overall appearance of oospores and LM wall ornamentations were consistent with those in the original description of Groves (1922). Moreover, detailed internal and external morphology of the oospore wall (IMOW and EMOW) were observed for the first time. SEM revealed that the minute fused fibrils form a fibrous or spongy pattern with about 1–12 irregularly arranged granules across the fossa (Figs 6–8); the granules were 0.7–1.8 μm in diameter, and were located 0.5–6.2 μm from each other (Figs 6–8). The fibrils extended onto the spiral ridges and flanges, but the granules were absent there (Figs 6, 7). The longitudinal fractured face of the fossa wall exhibited a strongly spongy texture, in which the openings were almost circular or laterally compressed (0.2–2.5 μm long and 0.2–0.6 μm wide), and formed a lamellate pattern (Figs 9, 10).

Molecular phylogenetic analysis

On the basis of the concatenated *atp*B, *rbc*L, *psa*B, ITS, and 5.8S rRNA sequences of 4553 bp aligned characters (with 649 potentially parsimony-informative characters), six equally parsimonious trees were found in maximum parsimony (MP) analyses on the basis of a heuristic search using the stepwise addition of 100 random replications. One of the six MP trees is shown in Fig. 11, in which we show branches supported by ≥ 50% bootstrap values (BS) and ≥ 0.95 posterior probabilities (PP) in the MP, minimum evolution (ME), maximum likelihood (ML) and Bayesian inference (BI) analyses. The tree is 1434 steps long, with a consistency index of 0.7134 and a retention index of 0.8802. The phylogenetic relationships within the subgenus *Tieffallenia* resolved in the concatenated *atp*B-*rbc*L-*psa*B-ITS-5.8S rRNA analyses were essentially the same as those in the previous multiple DNA marker phylogenies (Sakayama *et al.* 2004a, b, 2005), except for relationships regarding *N. comptonii* newly examined here. Three strains of *N. comptonii* had identical sequences in 4553 bp and were located within the spongy (SG) oospore clade, where *N. comptonii* and *N. vieillardii* (A. Braun) Sakayama form a robust clade (with 100% BS and 1.00 PP in the MP, ME, ML, and BI analyses) that is sister to *N. imperialis* (Allen) Sakayama (Fig. 11).



Figs 1–10. Thalli and oospores of *Nitella comptonii* (S091).
Fig. 1. Part of thallus, showing fertile spherical heads in the axils.
Fig. 2. Close view of fertile head, in which an axial node bears seven whorled branchlets with oogonia.
Fig. 3. Part of dactyl, consisting of an end cell and a penultimate cell. Note the penultimate cell is tapering distally to the base of the end cell.
Fig. 4. Oospore with 7–8 flanged spiral ridges on the surface, SEM.
Fig. 5. Part of fossa wall, showing finely granulate ornamentation, LM.
Figs 6, 7. Parts of fossa wall, showing a fibrous ornamentation with irregularly arranged granules, with flanged spiral ridges, SEM. Note the fibrils extend onto the spiral ridges and flanges, but the granules are absent there.
Fig. 8. Detail of fossa wall, showing minute fused fibrils forming a fibrous or spongy pattern with irregularly arranged granules, SEM.
Fig. 9. Longitudinal fractured face of fossa wall, showing strongly spongy texture, SEM. Single or double arrowhead indicates the inner or outer side of the wall, respectively.
Fig. 10. Close view of longitudinal fractured face of fossa wall, showing strongly spongy texture with almost circular or oval openings, SEM.

DISCUSSION

Wood (1962, 1965) reduced 23 taxa to infraspecific taxa of *N. pseudoflabellata* on the basis of the following morphological similarities: a medium- to large-sized thallus, a slender axis, branchlets forked two to five times, and two- to three-celled dactyls with the base of the end cell the same width as the apex of the penultimate cell. In his classification of *N. pseudoflabellata,* he regarded *N. comptonii* as a subspecies *'comptonii'*, and separated it from the other varieties or forms, which were included in the other subspecies *'pseudoflabellata'*, because *N. comptonii* had only spherical fertile heads at the axils of the whorled branchlets. Subsequently, he treated *N. comptonii* as a synonym of the type form of *N. pseudofla-*

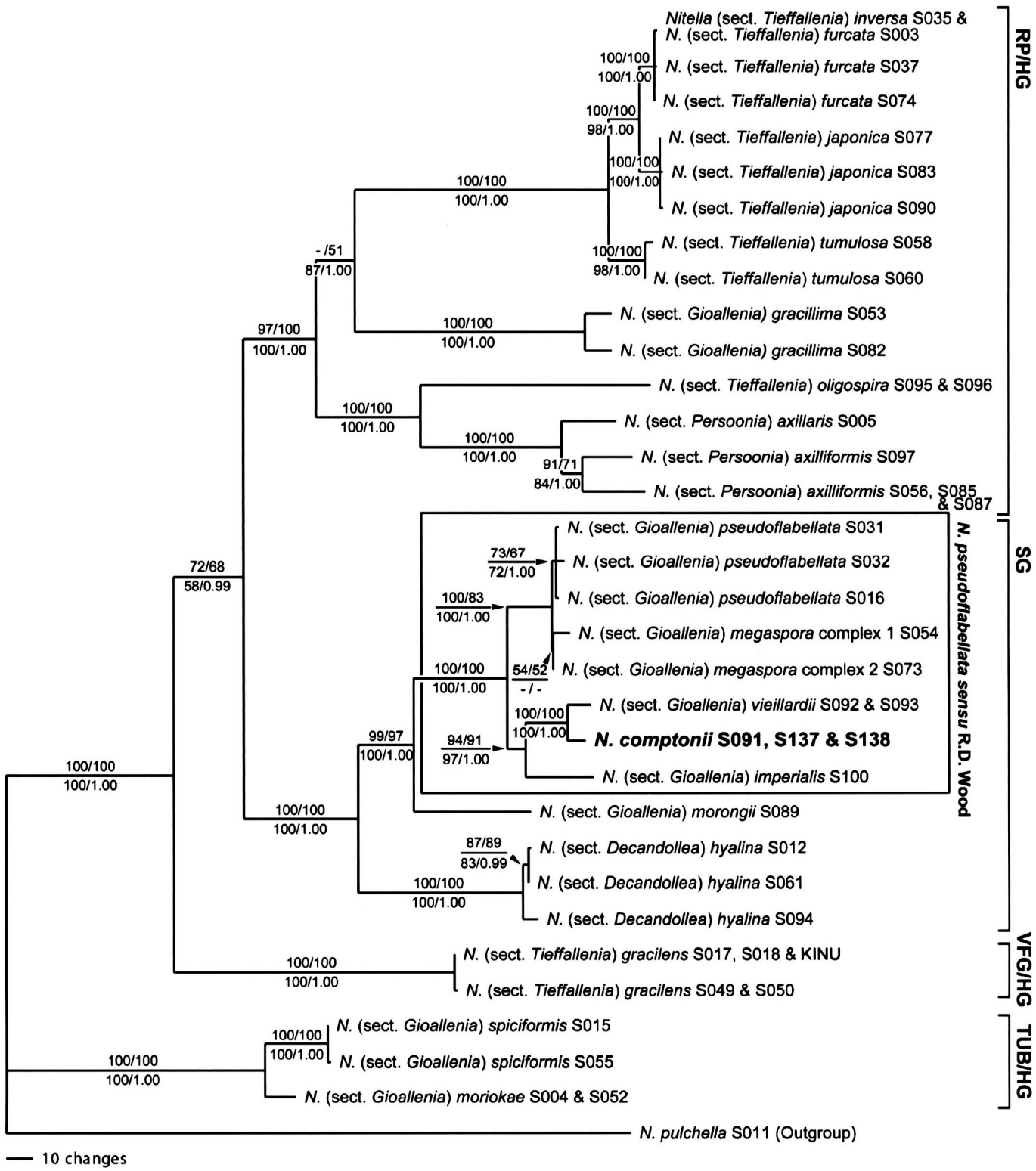


Fig. 11. One of the six equally MP trees based on 4553 base pairs of the concatenated sequences dataset from the *atp*B, *rbc*L, *psa*B and 5.8S rRNA genes and ITS regions of 43 strains of the genus *Nitella* (*N.*) representing 18 species of subgenus *Tieffallenia* and *N. pulchella* belonging to the subgenus *Hyella*. The MP trees were found by PAUP* 4.0b10 (Swofford 2002), on the basis of a heuristic search using the stepwise addition of 100 random replications. 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, based on Jukes-Cantor distance) analyses. Branches resolved with 50% or more BS values (based on 100 replications) or with 0.95 or more PP (based on 9000 trees) by ML [left, based on general time-reversible (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 the identical sequences of 4553 base pairs are treated as a single OTU. Four robust clades, the RP/HG, SG, VFG/HG and TUB/HG oospore clades, identified on the basis of IMOW and EMOW, and species corresponding to *N. pseudoflabellata sensu* R.D. Wood, are shown.

bellata because of only a few morphological differences, its rare occurrence, and the ambiguousness of the existence of spherical fertile heads in the type specimen (Wood 1966, 1972). In this study, we examined fully mature material of *N. comptonii* to elucidate its taxonomic status. Our morphological observations revealed that the thalli of *N. comptonii* exhibited a distinctive spherical fertile head (Figs 1, 2), which is consistent with that in the original description by Groves (1922). Under SEM, moreover, the oospore of *N. comptonii* (Figs 4–10) is clearly different from that of the type form of *N. pseudoflabellata* (Sakayama *et al.* 2002, 2005). The fossa wall of *N. comptonii* has minute fused fibrils forming a fibrous or spongy pattern with irregularly arranged granules in the EMOW, and exhibits a strongly spongy fractured face in the IMOW. Conversely, the type form of *N. pseudoflabellata* has a finely granulate EMOW pattern composed of the compressed granules and a weakly spongy IMOW. In our molecular phylogeny (Fig. 11), *N. comptonii* is separated phylogenetically from *N. pseudoflabellata* and three related taxa [*N. megaspora* (J. Groves) Sakayama, *N. vieillardii* and *N. imperialis*], which were previously reduced to the infraspecific rank of *N. pseudoflabellata* by Wood (1962, 1965, 1966). Therefore, *N. comptonii* should be classified as a separate species from *N. pseudoflabellata*.

On the basis of the combined EMOW and IMOW characters and molecular phylogenetic analyses, Sakayama *et al.* (2005) subdivided 17 species of the *Nitella* subgenus *Tieffallenia* into four groups: a clade of the SG type of IMOW (SG oospore clade) and three clades of the homogeneous (HG) type of IMOW [the reticulate or papillate (RP/HG), very finely granulate (VFG/HG), and tuberculate (TUB/HG) oospore clades]. Within the SG oospore clade, *N. morongii* and *N. comptonii* have a fertile head in the axils of the whorled branchlets, whereas the other species lack such fertile heads. In our molecular phylogeny, *N. morongii* and *N. comptonii* are in the intermediate and distal phylogenetic positions, respectively (Fig. 11). In addition, two species of the RP/HG oospore clade (*N. axillaris* and *N. axilliformis*) and all species of the TUB/HG oospore clade (*N. spiciformis* and *N. moriokae*) also have the axillary fertile head. Therefore, the axillary fertile head is not conserved phylogenetically, despite its usefulness for identifying taxa, and it evolved at least twice within the SG oospore clade.

On the other hand, all seven species of the SG oospore clade are characterized by a slender overall appearance, a fertile whorled branchlet with mucus, and an elongate and confluent dactyl consisting of two cells (Wood 1965). Conversely, eight species consisting of the RP oospore clade exhibit diversity in these vegetative morphologies (Wood 1965). For example, *N. gracillima* has a slender main axis and a compact and small branchlet, whereas the main axes and branchlets of the other species of the RP oospore clade are robust and large. The six species of this clade (*N. inversa, N. furcata, N. japonica, N. tumulosa, N. axillaris,* and *N. axilliformis*) have abbreviated dactyls, whereas the dactyls of *N. gracillima* and *N. oligospira* are elongate. This evidence suggests that taxa of the SG oospore clade are conservative in these vegetative morphologies as well as in oospore morphology. Within the SG oospore clade, however, only *N. hyalina* has the distinctive accessory branchlets compactly arranged. Therefore, *N. hyalina* seems to have the distinct evolutional trend within the SG oospore clade. Within the subgenus *Tieffallenia*, 12 taxa belonging to the sections *Earthya* and *Decandollea*, which include *N. hyalina*, exhibit the accessory branchlets (Wood 1965). Thus further examination of these taxa is necessary to clarify the evolutional and diversification processes within the SG oospore clade.

Nitella (subgen. *Tieffallenia*) *comptonii* J. Groves (1922, p. 69)

SYNONYM: *Nitella pseudoflabellata* A. Braun subsp. *comptonii* (J. Groves) R.D. Wood (1962, p. 20).

DISTRIBUTION: New Caledonia (Groves 1922; Wood 1965, 1966), Australia (Groves & Allen 1935; Wood 1965) and Japan (Table 1).

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