2 0 0 4年3月・国際藻類学会転載許可済

Taxonomic re-examination of *Nitella* (Charales, Charophyceae) from Japan, based on microscopical studies of oospore wall ornamentation and *rbc*L gene sequences

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H. Sakayama, H. Nozaki, H. Kasaki and Y. Hara. 2002. Taxonomic re-examination of *Nitella* (Charales, Charophyceae) from Japan, based on microscopical studies of oospore wall ornamentation and *rbc*L gene sequences. *Phycologia* 41: 397–408.

Nine taxa of *Nitella* (Charales, Charophyceae) from Japan, including five Japanese or east Asian endemics, were examined to improve understanding of their taxonomic status. The approaches used were light and scanning electron microscopy (SEM) of the oospores and sequencing of the gene (*rbc*L) encoding the large subunit of Rubisco. The species delineated were *N. pulchella* (= *N. dualis* f. *pulchella*), *N. furcata*, *N. inversa* (= *N. furcata* f. *inversa*), *N. gracilens* (= *N. furcata* f. *gracilens*), *N. pseudoflabellata*, *N. hyalina*, *N. spiciformis* (= *N. gracilis* f. *spiciformis*), *N. moriokae* (= *N. rigida* f. *moriokae*) and *N. axillaris* (= *N. translucens* var. *axillaris*). SEM observations showed that the oospore morphology of four taxa – *N. inversa*, *N. gracilens*, *N. spiciformis* and *N. axillaris* – was distinctly different from that of the species (*N. furcata*, *N. furcata*, *N. gracilis* and *N. translucens*, respectively) to which R.D. Wood assigned them as infraspecific taxa. Furthermore, the *rbc*L data showed that *N. gracilens* was separated phylogenetically from *N. furcata* and *N. axillaris* from *N. translucens*. This is the first integration of SEM oospore morphology and molecular phylogenetics in charalean taxonomy, demonstrating the efficiency of both approaches to address problems at lower taxonomic levels.

INTRODUCTION

Nitella is a charalean genus characterized by having once or repeatedly forked branchlets, two tiers of crown cells in the female organ, and laterally compressed oospores (Wood 1965). This genus exhibits extensive diversity, with around 200 species and infraspecific taxa and a worldwide distribution (Wood 1965). Fifty-six species and infraspecific taxa have been recorded from Japan, of which 36 appear to be endemic to Japan or east Asia (Imahori & Kasaki 1977; Han *et al.* 1994; Choi & Kim 1998).

Studies of oospore morphology using scanning electron microscopy (SEM) have shown their value for delineating species of the order Charales (John & Moore 1987; John *et al.* 1990; Leitch *et al.* 1990; Casanova 1991; Nozaki *et al.* 1998). Molecular phylogenetic analyses, based on the large subunit of Rubisco (*rbc*L) and 18S ribosomal RNA gene sequences from European, South American and Australian taxa of the Charales, have been carried out to resolve phylogenetic relationships at specific or generic levels (McCourt *et al.* 1996, 1999; Meiers *et al.* 1999). However, there have been no taxonomic or phylogenetic studies of Japanese or east Asian taxa based on these

modern methods, except for the SEM studies on oospores of *N. allenii* Imahori (John & Moore 1987) and *N. gracilens* Morioka (Nozaki *et al.* 1998). The present study was undertaken to evaluate species concepts in *Nitella*, especially Japanese taxa, based on a combination of SEM oospore morphology and molecular phylogenetic analysis. We examined nine taxa of *Nitella*, including five Japanese or east Asian endemics, collected from central and northern Japan (Table 1).

MATERIAL AND METHODS

Field collections and cultures

The localities from which material was collected are shown in Table 1. Thalli were collected using a handmade anchor, which was thrown into the water from the shores of ponds and lakes, or from a boat, and then dragged along the bottom. Part of the material was preserved as dried specimens. Unicharalean cultures were established for all the species at each locality.

Cultures were established by inoculating part of the thalli in a 2000 ml glass vessel or a 900 ml glass jar containing a soil–water medium. The soil–water medium consisted of tap water that had been left for at least a day and either muddy soil collected from the shore at Lake Haryu-numa (Table 1) or sandy soil used for gardens (TOSTEM VIVA, Tokyo, Ja-

Table 1. Species and culture strains used for the present *rbc*L gene phylogeny.

Species	Strain designation and collection information	Origin of *rbc*L gene sequence accession data	GenBank number
Chara globularis Thuillier	F124C, Germany	McCourt *et al.* (1999)	AF097163
	X751, Canada	McCourt *et al.* (1999)	AF097165
	X692, Australia	McCourt *et al.* (1999)	AF097164
C. connivens Salzmann *ex* A. Braun	JRM, unknown	Manhart (1994)	L13476
	X774, Spain	McCourt *et al.* (1999)	AF097160
	F140, Spain	McCourt *et al.* (1999)	AF097161
	X214, Israel	McCourt *et al.* (1999)	AF097162
C. vulgaris Linnaeus	X152, Denmark	McCourt *et al.* (1999)	AF097166
	F101, France	McCourt *et al.* (1999)	AF097167
C. rusbyana M. Howe	X066, Argentina	McCourt *et al.* (1999)	AF097168
	LG, unknown	McCourt *et al.* (1999)	AF097169
Lamprothamnium papulosum (Wallroth) J. Groves	F137, France	McCourt *et al.* (1999)	AF097170
	X695, Australia	McCourt *et al.* (1999)	U27534
Nitellopsis obtusa (Desvaux *in* Loiseleur-Deslongchamps) J. Groves	F131B, Germany	McCourt *et al.* (1999)	U27530
Lychnothamnus barbatus (Meyen) Leonhardi	Croa, Croatia	McCourt *et al.* (1999)	U27533
	Aus, Australia	McCourt *et al.* (1999)	AF097171
	Ger, Germany	McCourt *et al.* (1999)	AF097172
Nitella flexilis (Linnaeus) C. Agardh[1]	S007, Lake Yunoko, Nikkou-shi, Tochigi, Japan, 30 Jun. 2000	this study	AB076055
	S010, Lake Hibara, Urabandai highlands, Kitashiobara-mura, Fukushima, Japan, 8 Oct. 1998	this study	AB076056
N. opaca (Bruzelius) C. Agardh	F146, Poland	McCourt *et al.* (1999)	AF097174
N. praelonga A. Braun	P/CR7, Costa Rica	McCourt *et al.* (1999)	AF097173
N. pulchella[2,3] Allen	S011, Lake Goshiki-numa, Urabandai highlands, Kitashiobara-mura, Fukushima, Japan, 18 May 1999	this study	AB076057
N. furcata (Roxburgh *ex* Bruzelius) C. Agardh[2]	S003, Bog at Furukawa-shi, Miyagi, Japan, 25 Jun. 2000	this study	AB076058
	S037, Paddy field at Urabandai highlands, Kitashiobara-mura, Fukushima, Japan, 8 Oct. 1999	this study	AB076059
N. inversa Imahori[2,3]	S035, Lake Haryu-numa, Nishizao highlands, Yamagata-shi, Yamagata, Japan, 6 Sep. 2000	this study	AB076060
N. gracilens Morioka[2,3]	S017, Lake Haryu-numa, Nishizao highlands, Yamagata-shi, Yamagata, Japan, 24 Sep. 1998	this study	AB076061
	S018, Lake Futatsu-numa, Nishizao highlands, Yamagata-shi, Yamagata, Japan, 14 Sep. 1998	this study	AB076062
	KINU[4], Lake Ashinoko, Hakone-shi, Kanagawa, Japan, 18 Nov. 1995	this study	AB076063
N. pseudoflabellata A. Braun[2]	S031, Lake Haryu-numa, Nishizao highlands, Yamagata-shi, Yamagata, Japan, 28 Jul. 1998	this study	AB076064
	S032, Lake Godai-numa, Yawata-cho, Yamagata, Japan, 14 Oct. 1998	this study	AB076065
	S016, Paddy field at Urabandai highlands, Kitashiobara-mura, Fukushima, Japan, 8 Oct. 1999	this study	AB076066
N. hyalina (De Candolle) C. Agardh[2]	S012, Lake Ogawara, Misawa-shi, Aomori, Japan, 6 Sep. 1999	this study	AB076067
N. spiciformis Morioka[2,3]	S015, Lake Yuzawa-numa, Murayama-shi, Yamagata, Japan, 9 Jul. 1998	this study	AB076068
N. moriokae R.D. Wood[2,3]	S004, Lake Koryu, Nishizao highlands, Yamagata-shi, Yamagata, Japan, 19 Jun. 2000	this study	AB076069
N. translucens (Persoon) C. Agardh	F108, France	McCourt *et al.* (1999)	AF097745
N. axillaris A. Braun[2]	S005[5], unknown	this study	AB076070
Tolypella prolifera (Ziz *ex* A. Braun) Leonhardi	F142, Netherlands	McCourt *et al.* (1999)	U27532
	F150, France	McCourt *et al.* (1999)	AF097175

Table 1. Continued

Species	Strain designation and collection information	Origin of *rbcL* gene sequence accession data	GenBank number
T. nidifica (O.F. Müller) A. Braun	F138, France	McCourt *et al.* (1999)	U27531
T. glomerata (Desvaux *in* Loiseleur-Deslongchamps) Leonhardi	F131A, Germany	McCourt *et al.* (1999)	AF097176
Coleochaete nitellarum Jost	unknown	Nishiyama & Kato (1999)	AB013662
C. orbicularis Pringsheim	unknown	Manhart (1994)	L13477
Zygnema peliosporum Wittrock	unknown	McCourt & Karol (1995)	U38701

[1] Identified based on the vegetative and gametangial morphologies following Wood (1965).
[2] The oospores were observed by scanning electron microscopy in the present study (Figs 1–27).
[3] Japanese or eastern Asian endemic species.
[4] Oospore wall ornamentations were previously examined by Nozaki *et al.* (1998).
[5] Cultured material obtained from a greenhouse at University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan.

pan); it was autoclaved for 1 min. The cultures were maintained under controlled laboratory conditions at 20°C with a 12 : 12 h light–dark cycle and 140 μmol m^{-2} s^{-1} provided by fluorescent lamps; alternatively, they were kept in the greenhouse at the Faculty of Science, Yamagata University, Japan. The thalli were transferred to a new soil–water medium every 2–6 mo to maintain healthy cultures.

Light microscopy and SEM

When thalli with fully mature female reproductive organs were collected, the oospores were removed and fixed with 70% ethanol. Thalli with immature female reproductive organs were grown in culture for more than a month in order to mature the oospores. After the oospores had discoloured to dark brown within the organs, they too were fixed with 70% ethanol. Fully mature oospores that had already been released from their accessory cells were also collected from the detritus deposited on the surface of the soil layer of the soil–water medium.

Before examination, oospores were cleaned by incubation in 10% Triton-X 100 at 60°C for > 12 h. They were then subjected to acetolysis (Erdtman 1960; Faegri & Iversen 1989) or sonicated for c. 2 min (Casanova 1997). Because flanges on the surface of the oospore were fragile in some species, such acetolysis was not carried out, in order to observe the flanges. Subsequently, the oospores were transferred to a 15-ml centrifuge tube containing distilled water, which was agitated vigorously by a vortex mixer for 3 min. The oospores were then dehydrated in an ethanol series and mounted on brass or aluminium stubs with double-sided adhesive tape, air-dried, sputter-coated with gold and viewed with a JSM-5300LV scanning electron microscope (JEOL, To-

kyo, Japan) or a S-4500 scanning electron microscope (Hitachi, Tokyo, Japan) at 10–20 kV. For light microscope (LM) observations, the oospores were occasionally crushed and washed in 10% KOH to remove the abundant starch grains within the oospore walls. LM observations were carried out with a BX50 microscope (Olympus, Tokyo, Japan) equipped with Nomarski interference optics.

Molecular phylogenetic analysis

Field-collected or cultured thalli were checked under the dissection microscope for the presence of other photosynthetic organisms attached to their surface. A part of the thallus free of such organisms was collected, fixed with 70% ethanol, and stored at 4°C before DNA extraction.

For DNA extraction, a small piece of the thallus (1–2 internodes) was mixed with 1 ml CTE buffer (50 mM Tris buffer, pH 8.0, and 20 mM Na_2EDTA, pH 8.0) and ground using a mortar and a pestle. The ground material was transferred to a glass tube (10 × 180 mm) containing 130 μl of 20% (w/v) sodium dodecyl sulfate and 25 μl proteinase K (20 mg ml^{-1}), and further ground at room temperature using a wash brush (10 × 60 mm) in the glass tube (Nozaki *et al.* 1997). Then 1 ml CTE buffer was added to the mixture and incubated at 55°C for 1.5–12 h, during which 25 μl proteinase K (20 mg ml^{-1}) was added to the mixture. After the proteinase K treatment, the DNA samples were extracted and purified as described previously (Nozaki *et al.* 2000). Amplification of DNA by polymerase chain reaction (PCR) and direct sequencing of the PCR products were essentially the same as in a previous study (Nozaki *et al.* 2000), except for the primers used for amplifying and sequencing the *rbcL* genes (Table 2).

For phylogenetic analysis, a data matrix containing 1194 bp of unambiguously aligned *rbcL* gene sequences from 40 charalean operational taxonomic units (OTUs) and three other charophycean species (EMBL-Align database accession number ALIGN_000279) was subjected to unweighted maximum parsimony (MP) analysis by using PAUP* 4.0b8 (Swofford 2001). The MP trees were constructed based on the 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). From the same alignment as in the MP analysis, a

Table 2. Primers used for amplifications and sequencing of the *rbcL* genes in the present study.

Designations	Positions[1]	Sequence (5' to 3')
CHAR-RF-1	1–23	ATGTCACCACAGACAGAAACTAA
CHAR-RF-2	560–581	GAGCTGTATATGAATGTCTTCG
CHAR-RR-3	695–676[2]	GTTTCTGCTTGAGATTTATA
CHAR-RR-4	1250–1229[2]	GCTCCTGGAGCATTTCCCCAAG

[1] Coordinate number from the *Chara connivens* Salzmann *ex* A. Braun *rbcL* gene (Manhart 1994).
[2] Reverse primer.

distance matrix was calculated by applying the Kimura 2-parameter method (Kimura 1980) in PAUP* 4.0b8. A phylogenetic tree was then constructed by a neighbour-joining (NJ) algorithm (Saitou & Nei 1987), again using PAUP 4.0; the robustness of lineages was tested by a bootstrap analysis with 1000 replications. In these phylogenetic analyses, *Coleochaete nitellarum* Jost, *C. orbicularis* Pringsheim and *Zygnema peliosporum* Wittrock were designated as the outgroup because they belong to the Charophyceae *sensu* Mattox & Stewart (1984) and recent molecular phylogenetic studies (McCourt *et al.* 1999, 2000) give reasonably high bootstrap support for the monophyly of the Characeae within the Charophyceae.

RESULTS AND DISCUSSION

Taxonomic accounts

Nitella pulchella Allen 1895, p. 69

Figs 1–3

SYNONYM: *Nitella dualis* Nordstedt f. *pulchella* (Allen) R.D. Wood (1962, p. 17).

OOSPORE MORPHOLOGY: The oospores were oval in face view and had 6–7 flanged ridges; they were 290–320 μm long and 225–260 μm wide, and measured 40–60 μm across the fossa (Fig. 1). The walls of mature oospores were chestnut brown to dark brown. The fossa wall was strongly reticulate, with three to six meshes 7.5–15 μm in diameter across the fossa (Fig. 2). Under the SEM, the reticulate pattern of the fossa wall could be seen to be composed of a network of narrow and completely fused ridges and crater-like depressions outlined by the ridges (Fig. 3).

DISTRIBUTION: Japan (Imahori 1954; Wood & Imahori 1959; Kasaki 1964; Wood 1965; Table 1) and China (Han *et al.* 1994).

REMARKS: Wood (1962) reduced *N. pulchella* to a form of *N. dualis*, based on morphological similarities, e.g. having allantoid and generally uniform end cells, dense mucus around fertile branchlets, sterile lowest branchlet nodes, and an often exaggerated primary branchlet ray. However, *N. pulchella* can be clearly distinguished from *N. dualis* via differences in the sexuality of the plants and the number of meshes across the fossa of the oospore. In *N. pulchella* the plants are monoecious and have three to six meshes across the fossa (Imahori 1954; Wood 1965) (Figs 1–3); *N. dualis*, on the other hand, is dioecious and has about 15 meshes across the fossa (Wood 1965). Therefore, *N. pulchella* is considered to be a distinct species from *N. dualis*. The oospore wall ornamentations of *N. dualis sensu* Wood (1965) have not been observed previously by SEM.

Nitella furcata (Roxburgh *ex* Bruzelius) C. Agardh 1824, p. 124

Figs 4–6

BASIONYM: *Chara furcata* Roxburgh *ex* Bruzelius (1824, p. 22).

OOSPORE MORPHOLOGY: The oospores were almost orbicular in face view and had 5–6 flanged ridges; they were 320–380 μm

long and 265–345 μm wide, and measured 47–64 μm across the fossa (Fig. 4). The walls of mature oospores were golden brown to chestnut brown. The fossa wall was reticulate, with about 20 meshes, each 1.5–3 μm in diameter, across the fossa (Fig. 5). By SEM, the fossa wall could be seen to exhibit an imperfect or irregular reticulate pattern, in which the reticulum was formed by swollen, waved and occasionally fused ridges (Fig. 6).

DISTRIBUTION: India, Myanmar and Japan (Wood 1965; Table 1), Korea (Choi & Kim 1998), China (Han *et al.* 1994), Australia (Wood 1965) and Argentina (Caceres 1975).

REMARKS: If the taxonomic system of Wood (1965) is followed, the present alga can be assigned to *N. furcata* f. *furcata*, on the basis of the robust appearance, the indistinguishable fertile and sterile branchlets, the predominantly abbreviated and generally two-celled dactyls, and the reticulate ornamentation on the oospore wall. Our SEM examination revealed that swollen and waved ridges form an imperfect or irregular reticulate pattern on the fossa wall (Fig. 6). SEM observations of oospores of *N. furcata* have also been carried out by Caceres (1975) and Mandal *et al.* (1995), using Argentinian and Indian plants, respectively. The oospore wall ornamentation of the Indian material was similar to that in the present Japanese material in having a reticulum formed by occasionally fused ridges on the fossa wall (Mandal *et al.* 1995). However, in Caceres' (1975) Argentinian material, the ridges differed from those observed in our study because they bore small papilla-like structures. In addition, the fossa breadth was narrower in the Argentinian material (Caceres 1975) than in the Japanese and Indian (Mandal *et al.* 1995) material.

#### *Nitella inversa* Imahori 1954, p. 125

Figs 7–9

SYNONYM: *Nitella furcata* f. *inversa* (Imahori) R.D. Wood (1965, p. 495).

OOSPORE MORPHOLOGY: The oospores were orbicular in face view and had 6–7 weakly flanged ridges; they were 300–350 μm long and 250–330 μm wide, and measured 40–75 μm across the fossa (Fig. 7). The walls of mature oospores were yellowish brown to light brown. The fossa wall was papillate, with up to *c.* 20 projections across the fossa (Fig. 8). Using SEM, the tapering papillae could be seen to be about 1.8 μm high and were located 1–4 μm from each other (Fig. 9).

DISTRIBUTION: Japan (Imahori 1954; Wood & Imahori 1959; Wood 1965; Table 1) and China (Han *et al.* 1994).

REMARKS: Wood (1965) regarded *N. inversa* as a form of *N. furcata* because both species are monoecious, lack reproductive organs at the base of whorled branchlets and have indistinguishable fertile and sterile branchlets; the dactyls are predominantly abbreviated and two(–three) celled and the antheridia are 140–540 μm in diameter. He described the oospore wall ornamentations of *N. inversa* and *N. furcata* as being 'weakly or strongly reticulate' and 'reticulate', respectively (Wood 1965). However, our observations of the oospores of *N. inversa*, which agree with those of Imahori & Kasaki (1977), indicate that the oospore wall ornamentation is papillate (Fig. 9). In contrast, *N. furcata* exhibits an imper-
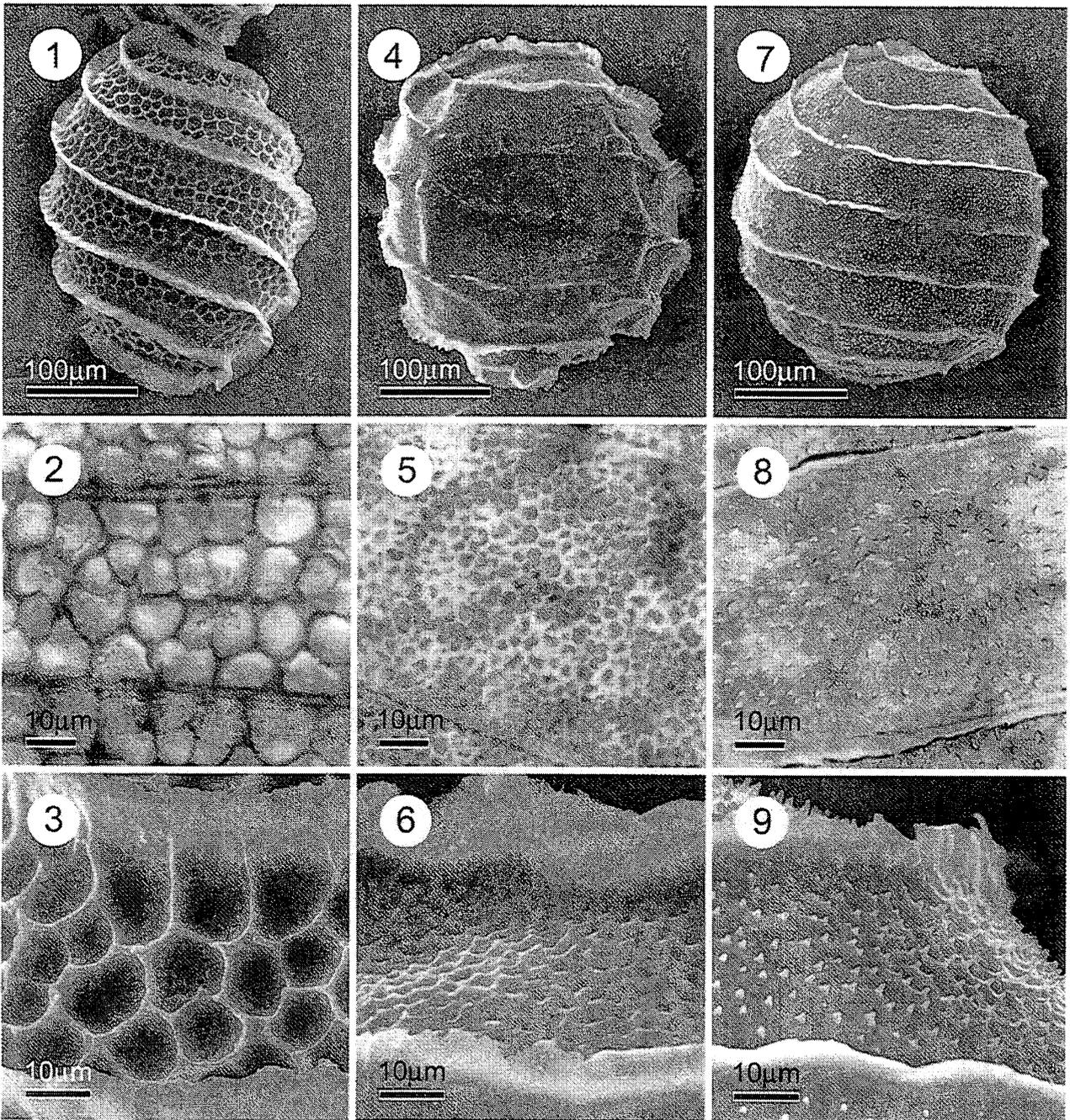
Figs 1–3. *Nitella pulchella* (S011).
  Fig. 1. Oospore with 6–7 flanged ridges on the surface, SEM.
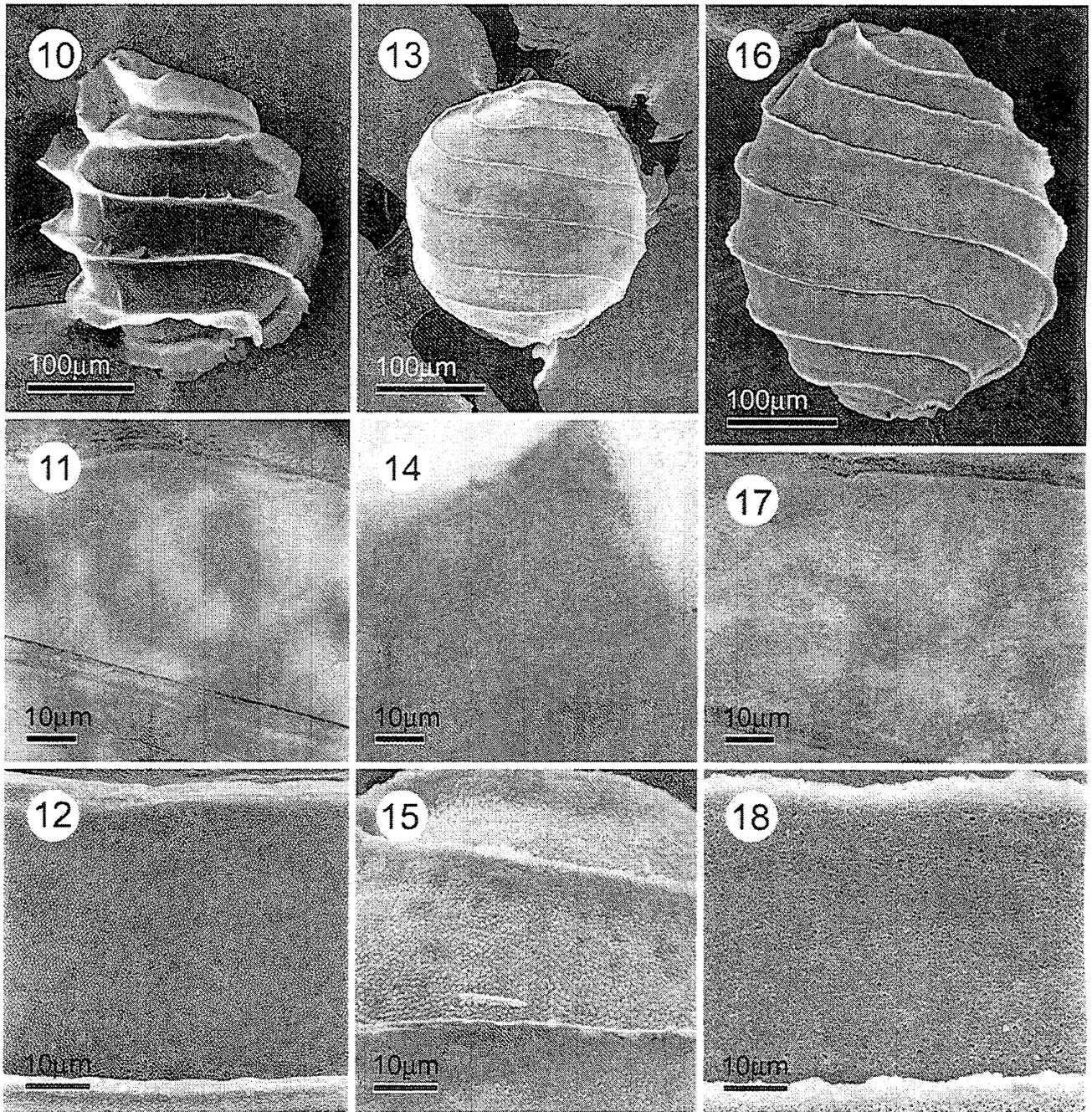 Fig. 2. Part of fossa wall, showing strongly reticulate ornamentation with large meshes, LM.
  Fig. 3. Part of fossa wall, showing a reticulate pattern consisting of completely fused ridges and crater-like depressions, SEM.
Figs 4–6. *Nitella furcata* (S037).
  Fig. 4. Oospore with 5–6 flanged ridges on the surface, SEM.
  Fig. 5. Part of fossa wall, showing reticulate ornamentation, LM.
  Fig. 6. Part of fossa wall, showing an imperfect or irregular reticulate pattern formed by swollen, waved and occasionally fused ridges, SEM.
Figs 7–9. *Nitella inversa* (S035).
  Fig. 7. Oospore with 6–7 weakly flanged ridges on the surface, SEM.
  Fig. 8. Part of fossa wall, showing papillate ornamentation, LM.
  Fig. 9. Part of fossa wall, showing papillate ornamentation consisting of tapering projections, SEM.

Figs 10–12. *Nitella gracilens* (S018).

Fig. 10. Oospore with 5–6 strongly flanged ridges on the surface, SEM.

Fig. 11. Part of fossa wall, showing smooth or finely granulate ornamentation, LM.

Fig. 12. Part of fossa wall, showing a finely granulate pattern in which the granules were arranged compactly, SEM.

Figs 13–15. *Nitella pseudoflabellata* (S031).

Fig. 13. Oospore with 6–8 unflanged ridges on the surface, SEM.

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

Fig. 15. Part of fossa wall, showing finely granulate pattern consisting of compactly arranged and flattened granules, SEM.

Figs 16–18. *Nitella hyalina* (S012).

Fig. 16. Oospore with 6–8 weakly flanged ridges on the surface, SEM.

Fig. 17. Part of fossa wall, showing finely granulate ornamentation, LM.

Fig. 18. Part of fossa wall, showing fibrous pattern, SEM.

fect reticulate ornamentation (Fig. 6; Caceres 1975; Mandal *et al.* 1995). 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. inversa*. However, the position of the antheridia in *N. inversa* is unique among the infraspecific taxa of *N. furcata sensu* Wood (1965) because they are predominantly positioned laterally on the branchlet nodes (Imahori 1954; Wood 1965), whereas in the other taxa the antheridia are borne terminally (Wood 1965). Therefore, *N. inversa* can be clearly distinguished from *N. furcata* and its related taxa.

Nitella gracilens Morioka 1941, p. 64

Figs 10–12

SYNONYM: *Nitella furcata* f. *gracilens* (Morioka) R.D. Wood (1965, p. 495).

OOSPORE MORPHOLOGY: The oospores were oval in face view and had 5–6 strongly flanged ridges; they were 230–268 µm long and 190–252 µm wide, and measured 40–55 µm across the fossa (Fig. 10). The walls of mature oospores were yellowish brown. The fossa wall was smooth or finely granulate (Fig. 11), with granules packed densely together (Fig. 12).

DISTRIBUTION: Japan (Imahori 1954; Wood & Imahori 1959; Kasaki 1964; Wood 1965; Table 1) and China (Han *et al.* 1994).

REMARKS: *Nitella gracilens* was regarded as a form of *N. furcata* by Wood (1965), who characterized this taxon as having a reticulate oospore wall. In contrast, Nozaki *et al.* (1998), who re-examined the oospore wall ornamentation of this species on the basis of LM and SEM, using material from the type locality, Lake Ashinoko, pointed out that *N. gracilens* has a finely granulate oospore wall, as in the original description of Morioka (1941). In the present study, we collected additional material of *N. gracilens* from two different habitats in Yamagata Prefecture and here too there was a finely granulate oospore wall ornamentation and strongly flanged striae (Figs 10–12), as reported by Nozaki *et al.* (1998). Furthermore, molecular phylogenetic analyses demonstrated that *N. gracilens* from three localities in Japan is different from *N. furcata* (Fig. 28).

Nitella pseudoflabellata A. Braun 1882, p. 54

Figs 13–15

OOSPORE MORPHOLOGY: The oospores were almost orbicular in face view and had 6–8 unflanged ridges; they were 230–290 µm long and 200–265 µm wide, and measured 40–42 µm across the fossa (Fig. 13). The walls of mature oospores were dark brown to reddish brown. The fossa wall was finely granulate, with 29–46 granules across the fossa (Fig. 14). Using SEM, the granules could be seen to be compactly arranged and 0.5–1.0 µm in diameter (Fig. 15).

DISTRIBUTION: India, Indonesia, China, Japan and Australia (Wood 1965; Table 1).

REMARKS: This species demonstrates variability in vegetative and oospore morphology (Zaneveld 1940; Imahori 1954; Kasaki 1964; Wood 1965). In the plants we collected from three

localities in Japan, there were two types of vegetative morphology. The thalli originating from Lake Haryu-numa (S031) invariably had two-celled dactyls, whereas the two other specimens (S016 and S032) showed two- or three-celled dactyls. Both types can be assigned to *N. pseudoflabellata* var. *pseudoflabellata*, if the taxonomic system of Wood (1965) is followed. However, our SEM observations showed differences between the fossa walls of Japanese plants and those of the Australian plants studied by Casanova (1991). The fossa walls of the present Japanese plants exhibit finely granulate ornamentations consisting of compactly arranged granules (Fig. 15), whereas those of the Australian materials of *N. pseudoflabellata* have fused tuberculate ornamentations (Casanova 1991).

Nitella hyalina (De Candolle) C. Agardh 1824, p. 126

Figs 16–18

BASIONYM: *Chara hyalina* De Candolle (1815, p. 247).

OOSPORE MORPHOLOGY: The oospores were ovate in face view and had 6–8 weakly flanged ridges; they were 325–365 µm long and 260–320 µm wide, and measured 41–59 µm across the fossa (Fig. 16). The walls of mature oospores were brown to dark brown. The fossa wall was finely granulate under LM (Fig. 17), but under SEM, it could be seen to be fibrous (Fig. 18).

DISTRIBUTION: Cosmopolitan (Wood & Imahori 1959; Wood 1965; Table 1).

REMARKS: This species grows in fresh- and brackish water (Kasaki 1964; Wood 1965), and is characterized by having accessory branchlets and a fibrous ornamentation on the oospore wall (Fig. 18). The SEM oospore wall ornamentation in the Japanese material of *N. hyalina* was identical to that in the French (John & Moore 1987), Argentinian (Caceres & Garcia 1989) and Indian (Mandal & Ray 1999) material.

### *Nitella spiciformis* Morioka 1941, p. 63

Figs 19–21

SYNONYMS: *Nitella morongii* Allen var. *spiciformis* (Morioka) Imahori (1957, p. 59); *N. gracilis* (Smith) C. Agardh f. *spiciformis* (Morioka) R.D. Wood (1965, p. 644).

OOSPORE MORPHOLOGY: The oospores were oval in face view and had 5–6 strongly flanged ridges; they were 235–240 µm long and 200–235 µm wide, and measured 35–45 µm across the fossa (Fig. 19). The walls of mature oospores were yellowish brown to brown. In LM, the fossa wall appeared papillate or tuberculate, with 10–15 worm-like projections across the fossa (Fig. 20). SEM showed that the tuberculate ornamentations consist of connected cylindrical projections *c.* 3 µm high (Fig. 21).

DISTRIBUTION: Japan (Imahori 1954; Wood & Imahori 1959; Kasaki 1964; Wood 1965; Table 1), Korea (Choi & Kim 1998) and China (Han *et al.* 1994).

REMARKS: *Nitella spiciformis* was treated as a variety of *N. morongii* by Imahori (1957), whereas Wood (1965) reduced *N. spiciformis* to a form of *N. gracilis*. The papillate or tuberculate wall ornamentations of *N. spiciformis* were studied
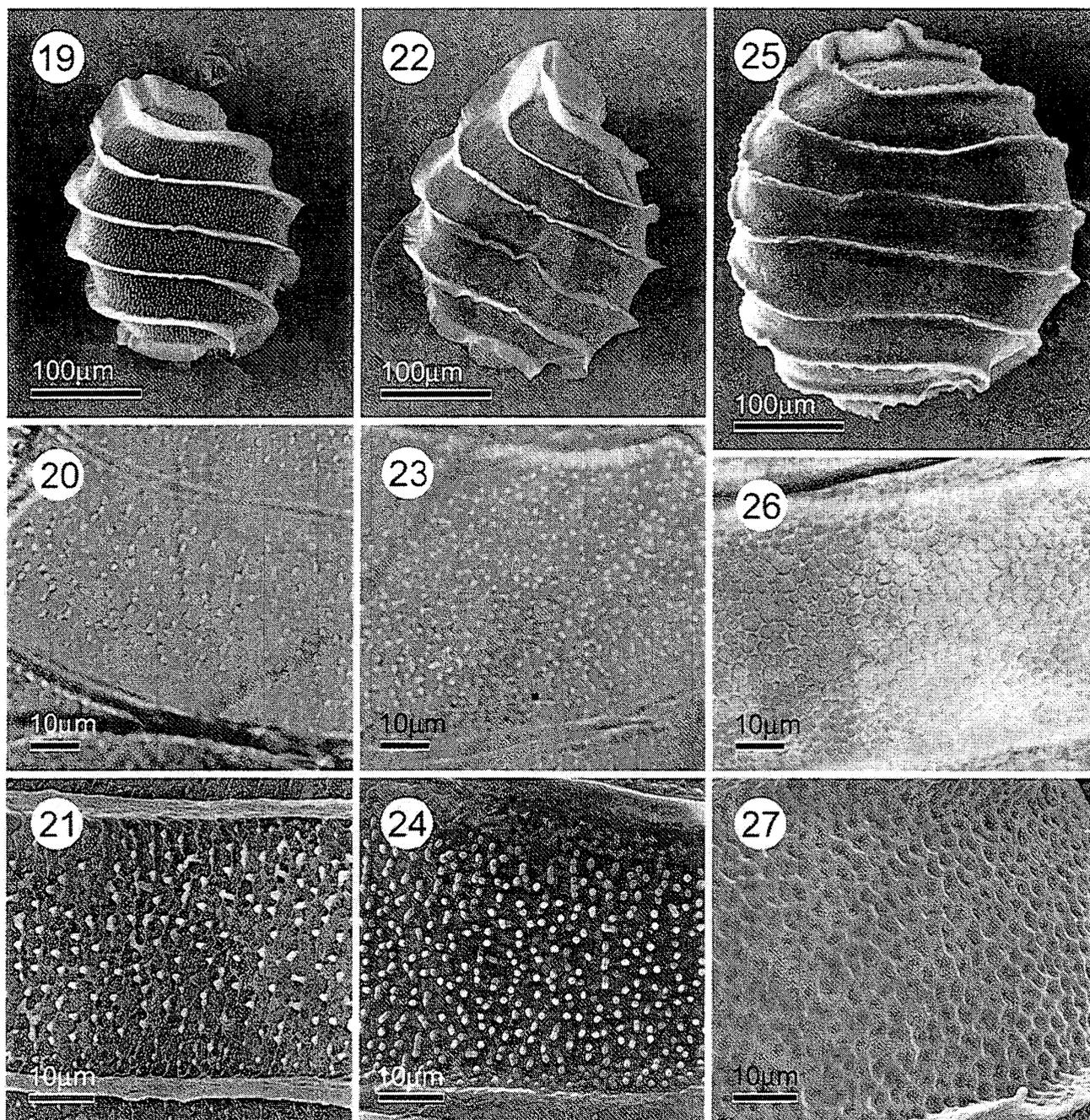
Figs 19–21. *Nitella spiciformis* (S015).

Fig. 19. Oospore with 5–6 strongly flanged ridges on the surface, SEM.

Fig. 20. Part of fossa wall, showing papillate or tuberculate ornamentation with worm-like projections, LM.

Fig. 21. Part of fossa wall, showing tuberculate ornamentation consisting of connected cylindrical projections, SEM.

Figs 22–24. *Nitella moriokae* (S004).

Fig. 22. Oospore with 5–6 strongly flanged ridges on the surface, SEM.

Fig. 23. Part of fossa wall, showing granulate, tuberculate or moniliform ornamentation, LM.

Fig. 24. Part of fossa wall, showing granulate, tuberculate or moniliform pattern consisting of occasionally fused ridges with granules or projections (or both), SEM.

Figs 25–27. *Nitella axillaris* (S005).

Fig. 25. Oospore with 6–7 weakly flanged ridges on the surface, SEM.

Fig. 26. Part of fossa wall, showing strongly reticulate ornamentation, LM.

Fig. 27. Part of fossa wall, showing a reticulate pattern consisting of completely fused ridges and crater-like depressions, SEM.
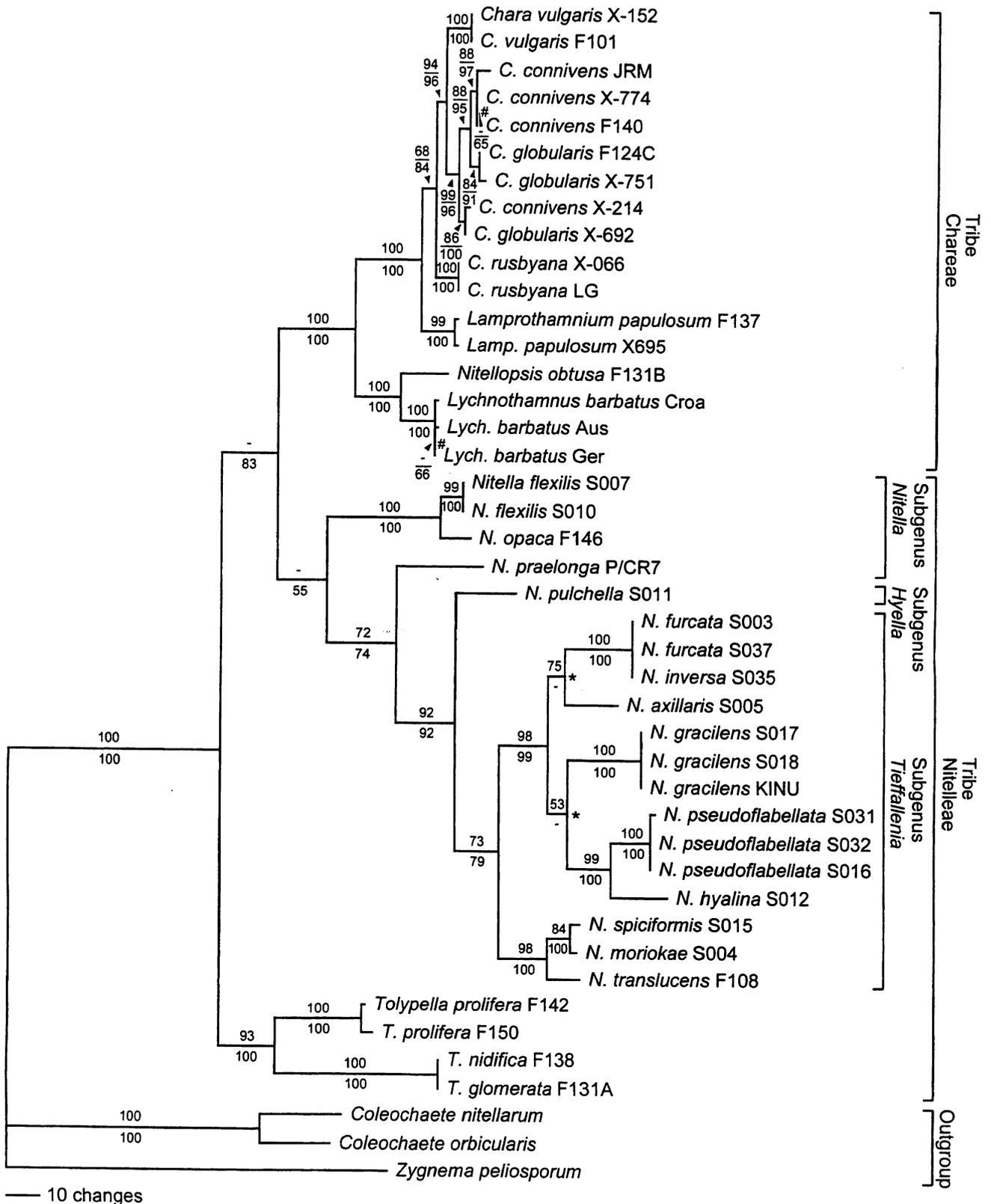
— 10 changes

Fig. 28. One of the four equally maximum parsimonious (MP) trees based on 1194 base pairs in the coding regions of the *rbcL* genes of 40 strains of the charalean genera, *Chara* (*C.*), *Lamprothamnium* (*Lamp.*), *Lychnothamnus* (*Lych.*), *Nitellopsis*, *Nitella* (*N.*) and *Tolypella* (*T.*) as well as three outgroups (Table 1). The MP trees were found by PAUP* 4.0b8 (Swofford 2001), based on a heuristic search using the stepwise addition of 100 random replications. The tree is 876 steps long with a consistency index of 0.6119 and a retention index of 0.8477. Branch lengths are proportional to the nucleotide changes, which are indicated by the scale bar below the tree. Numbers above or below branches are bootstrap values (50% or more) based on 1000 replications of the MP analysis or neighbour-joining (NJ) analysis, respectively. Branches not resolved in the strict consensus tree of the four MP trees or NJ tree are indicated by # or asterisk, respectively.

in LM by Imahori (1957), Kasaki (1964) and Imahori & Kasaki (1977), and the present SEM study has demonstrated that the tuberculate ornamentation consists of connected cylindrical projections (Fig. 21). However, the oospores of *N. gracilis* have a fibrous ornamentation (John & Moore 1987) and *N. morongii* has a sponge-like ornamentation on the oospore wall (Leitch *et al.* 1990). *Nitella spiciformis* is similar to *N. leptosoma* Nordstedt [= *N. gracilis* f. *leptosoma* (Nordstedt) R.D. Wood] in its tuberculate ornamentation (John & Moore 1987). However, *N. spiciformis* is clearly distinguished from *N. leptosoma* in having axillary fertile branchlets with terminal spike-like dactyls, in which mucus is absent (Kasaki 1964; Wood 1965).

Nitella moriokae R.D. Wood 1952, p. 335

Figs 22–24

SYNONYMS: *Nitella moniliformis* Morioka (1941, p. 61) *non* Zaneveld (1940, p. 79); *N. rigida* Allen f. *moriokae* (R.D. Wood) R.D. Wood (1962, p. 21).

OOSPORE MORPHOLOGY: The oospores were ovate in face view and had 5–6 strongly flanged ridges; they were 240–285 μm long and 220–240 μm wide, and measured 40–60 μm across the fossa (Fig. 22). The walls of mature oospores were yellowish brown. The fossa wall was granulate or tuberculate with 17–25 granules or projections (0.8–1.2 μm in diameter) (or both) across the fossa; occasionally it showed a moniliform pattern, because of the interconnection of the granules (Figs 23, 24); the granules or projections (or both) were 0.5–1.2 μm high (Fig. 24).

DISTRIBUTION: Japan (Imahori 1954; Wood & Imahori 1959; Wood 1965; Table 1) and China (Han *et al.* 1994).

REMARKS: *Nitella moriokae* was regarded as a form of *N. rigida* by Wood (1962). However, *N. moriokae* can be clearly distinguished from other taxa assignable to *N. rigida sensu* Wood (1965) [*N. rigida* f. *rigida* (Allen) R.D. Wood, *N. rigida* f. *saitoiana* (Allen) R.D. Wood and *N. rigida* f. *tanakiana* (Allen) R.D. Wood] by the morphology of the oospores. In *N. moriokae*, the fossa walls are granulate, tuberculate or moniliform, and this can be seen even in LM (Morioka 1941; Wood 1965) (Fig. 23), whereas the fossa walls of *N. rigida* f. *rigida*, *N. rigida* f. *saitoiana* and *N. rigida* f. *tanakiana* exhibit papillate ornamentation (Imahori 1957; Wood 1965).

Nitella axillaris A. Braun 1858, p. 356

Figs 25–27

SYNONYM: *Nitella translucens* (Persoon) C. Agardh var. *axillaris* (A. Braun) R.D. Wood (1962, p. 22).

OOSPORE MORPHOLOGY: The oospores were orbicular in face view and had 6–7 weakly flanged ridges; they were 335–340 μm long and 330–340 μm wide, and measured 50–62.5 μm across the fossa (Fig. 25). The walls of mature oospores were brown to bright brown. The fossa wall was strongly reticulate, with 13–17 meshes, each 2–4 μm in diameter, across the fossa (Fig. 26). SEM revealed that the reticulate pattern is composed of a network of completely fused ridges and crater-like depressions (Fig. 27).

DISTRIBUTION: Asia, Pacific Islands, North America and South America (Wood 1965).

REMARKS: *Nitella axillaris* was treated as a variety of *N. translucens* by Wood (1965). Recently, based on the SEM observations, John & Moore (1987) reported that oospores of *N. translucens* are elongate-ovoid and have a fine reticulate ornamentation and 5–6 strongly flanged ridges on the fossa wall. In contrast, our observations revealed that the oospores of *N. axillaris* are broadly ovoid, and exhibit a strongly reticulate ornamentation on the fossa and 6–7 weakly flanged ridges (Figs 25, 27). In addition, *N. axillaris* is robustly separated from *N. translucens* in our *rbc*L gene tree (Fig. 28). Therefore, *N. axillaris* should be regarded as a distinct species of *Nitella*.

Molecular phylogenetic analysis

Four equally parsimonious trees were found in MP analyses based on a heuristic search using the stepwise addition of 100 random replications. One of the four MP trees is presented in Fig. 28, in which we show all the branches supported by ≥ 50% bootstrap values in MP or NJ analyses. The tree is 876 steps long, with a consistency index of 0.6119 and a retention index of 0.8477. The phylogenetic relationships within the Characeae resolved in the present study are essentially the same as those of McCourt *et al.* (1999), who also used *rbc*L sequences, in that the Characeae are subdivided into a monophyletic tribe Chareae and a paraphyletic tribe Nitelleae. However, detailed phylogenetic relationships within the genus *Nitella* were resolved in the present study, which includes a larger number of OTUs of *Nitella* (Table 1).

The genus *Nitella* was resolved as a monophyletic group only in the NJ analysis, and then only with low bootstrap values (55%), and appeared to be subdivided into a monophyletic group composed of the subgenera *Hyella* and *Tieffallenia*, and a basal paraphyletic group containing three species of the subgenus *Nitella* [*N. flexilis* (Linnaeus) C. Agardh, *N. opaca* (Bruzelius) C. Agardh and *N. praelonga* A. Braun].

The morphological species of *Nitella* analysed in this study formed robust clades or had distinct *rbc*L gene sequences, except for *N. furcata* and *N. inversa*, which had identical *rbc*L gene sequences (Fig. 28). Two further features emerge from the molecular analyses: (1) three OTUs of *N. gracilens* (= *N. furcata* f. *gracilens*) originating from the type locality Lake Ashinoko, Kanagawa Prefecture (Nozaki *et al.* 1998), and two localities in Yamagata Prefecture (Lake Haryu-numa and Lake Futatsu-numa) had the same *rbc*L gene sequence and were separated from *N. furcata* examined in this study (Fig. 28); and (2) *N. axillaris* (= *N. translucens* var. *axillaris*) was clearly separated from a robust clade composed of *N. translucens*, *N. spiciformis* and *N. moriokae* (Fig. 28).

The present study focuses on Japanese and east Asian taxa to address some questions of taxonomic positions of certain taxa, and only 13 taxa out of around 200 *Nitella* taxa (Wood 1965) were sampled. Therefore, addition of further taxa may well resolve phylogenetic relationships between species and infraspecific taxa within the genus *Nitella*.

The species concept in *Nitella*

Wood (1962, 1965) delineated species of *Nitella* mainly on the basis of differences in vegetative morphology and used

oospore morphology, especially the oospore wall ornamentations under LM, as a diagnostic character at the infraspecific level. He, therefore, reduced many charalean taxa that had previously been accepted as species to the infraspecific rank. John & Moore (1987), however, suggested that the morphology of oospores should be used to delineate morphological species of *Nitella* because oospore morphology as observed by SEM seems to be quite stable. They proposed that some of the infraspecific taxa recognized by Wood (1962, 1965) should be raised to species rank, based upon the SEM oospore wall ornamentation.

The present SEM study of various Japanese taxa of *Nitella* has also demonstrated that six taxa previously treated as infraspecific taxa by Wood (1962, 1965) have distinct oospore ornamentation and seem to represent distinct species; these comprise *N. pulchella, N. inversa, N. gracilens, N. spiciformis, N. moriokae* and *N. axillaris* (Figs 1–3, 7–12, 19–27). In addition, our molecular phylogenetic analysis, based on *rbc*L gene sequences (Fig. 28), clearly supports the distinct status of two of the six species (*N. gracilens* and *N. axillaris*). Therefore, an integrated approach, involving both SEM studies of oospore morphology and molecular phylogenetic analyses based on *rbc*L gene sequences, seems appropriate to address problems at lower taxonomic levels within the genus *Nitella*. However, *N. inversa* and *N. furcata* exhibited identical *rbc*L gene sequences, although they are clearly distinguished from each other by differences in oospore wall ornamentation (Figs 4–9), as well as in the position of antheridia on the branchlet nodes. Therefore, *N. inversa* and *N. furcata* may be closely related, but nevertheless distinct natural entities. Further morphological and molecular analyses of these species (using more variable genes than *rbc*L), using plants collected from localities throughout the world, are needed to resolve fully their phylogenetic and taxonomic relationships.

ACKNOWLEDGEMENTS

REFERENCES

AGARDH C.A. 1824. *Systema algarum.* Berling, Lund, Sweden. 312 pp.

ALLEN T.F. 1895. Japanese Characeae – II. *Bulletin of the Torrey Botanical Club* 22: 68–71.

BRAUN A. 1858. Über die in Columbien und Guyana aufgefundenen

Characeen. *Monatsberichte der Königlichen Preussischen Akademie der Wissenschaften zu Berlin* 1859: 349–368.

BRAUN A. 1882. Fragmente einer Monographie der Characeen. Nach den hinterlassenen Manuscripten A. Braun's herausgegeben von Dr. Otto Nordstedt. *Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin* 1882: 1–211.

BRUZELIUS A. 1824. *Observationes in genus* Charae. Lund, Sweden. 24 pp. [Paper not seen; we referred to a free German translation by A.E. Fürnrohr, which appeared in *Flora* 9: 481–494 (1826).]

CACERES E.J. 1975. Novedades carologicas Argentinas I. Una nueva especie de *Nitella* y tres adiciones al genero para la flora Argentina. *Kurtziana* 8: 105–125.

CACERES E.J. & GARCIA A. 1989. *Nitella hyalina* (DC.) Ag. (Characeae, Charophyta) in Argentina. *Nova Hedwigia* 48: 383–390.

CASANOVA M.T. 1991. An SEM study of developmental variation in oospore wall ornamentation of three *Nitella* species (Charophyta) in Australia. *Phycologia* 30: 237–242.

CASANOVA M.T. 1997. Oospore variation in three species of *Chara* (Charales, Chlorophyta). *Phycologia* 36: 274–280..

CHOI K.C. & KIM Y.H. 1998. Taxonomic study on the Charophytes in Korea II. *Nitella. Algae* 13: 283–321 [in Korean with English abstract].

DE CANDOLLE A.P. 1815. Famille des Nayades. In: *Flore Francaise* (Ed. by J.B.A.P. De Lamarck & A.P. De Candolle), vol. 6, pp. 246–247. Desray, Paris.

ERDTMAN G. 1960. The acetolysis method. *Svensk Botanisk Tidskrift* 54: 561–564.

FAEGRI K. & IVERSEN J. 1989. *Textbook of pollen analysis,* ed. 4. John Wiley, Chichester. 328 pp.

FELSENSTEIN J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* 38: 16–24.

HAN F., LI Y.-Y., LI Y.-J., LING Y. & WANG Y. 1994. *Flora algarum sinicarum aquae dulcis,* vol. 3, Charophyta. Science Press, Beijing. 267 pp [in Chinese].

IMAHORI K. 1954. *Ecology, phytogeography and taxonomy of the Japanese Charophyta.* Kanazawa University Press, Kanazawa, Japan. 234 pp.

IMAHORI K. 1957. Study on the Charophytes-flora in Hokkaido (Japan) and its phytogeographical characteristics a preliminary note. *Science Reports of Kanazawa University* 5: 55–83.

IMAHORI K. & KASAKI H. 1977. Class Charophyceae. In: *Illustrations of the Japanese fresh-water algae* (Ed. by H. Hirose & T. Yamagishi), pp. 761–829. Uchida Rokakuho Publishing, Tokyo [in Japanese].

JOHN D.M. & MOORE J.A. 1987. An SEM study of the oospore of some *Nitella* species (Chlorophyta, Charales) with descriptions of wall ornamentation and an assessment of its taxonomic importance. *Phycologia* 26: 334–355.

JOHN D.M., MOORE J.A. & GREEN D.R. 1990. Preliminary observations on the structure and ornamentation of the oosporangial wall in *Chara* (Charales, Chlorophyta). *British Phycological Journal* 25: 1–24.

KASAKI H. 1964. The Charophyta from the lakes of Japan. *Journal of the Hattori Botanical Laboratory* 27: 215–314.

KIMURA M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.

LEITCH A.R., JOHN D.M. & MOORE J.A. 1990. The oosporangium of the Characeae (Chlorophyta, Charales). *Progress in Phycological Research* 7: 213–268.

MANDAL D.K. & RAY S. 1999. Taxonomic significance of scanning electron microscopic study of oospore wall ornamentations in Charophyceae. *Phytomorphology* 49: 425–432.

MANDAL D.K., RAY S. & MUKHERJEE A. 1995. Scanning electron microscopic study of compound oospore wall ornamentations in some taxa under *Nitella furcata* complex (Charophyta) from India. *Phytomorphology* 45: 39–45.

MANHART J. 1994. Phylogenetic analysis of green plant *rbc*L sequences. *Molecular Phylogenetics and Evolution* 3: 114–127.

MATTOX K.R. & STEWART K.D. 1984. Classification of the green al-

gae: a concept based on comparative cytology. In: *Systematics of the green algae* (Ed. by D.E.G. Irvine & D.M. John), pp. 29–72. Academic Press, London.

McCourt R.M. & Karol K.G. 1995. Using *rbcL* sequences to test hypotheses of chloroplast and thallus evolution in conjugating green algae (Zygnematales, Charophyceae). *Journal of Phycology* 31: 989–995.

McCourt R.M., Karol K.G., Guerlesquin M. & Feist M. 1996. Phylogeny of extant genera in the family Characeae (Charales, Charophyceae) based on *rbcL* sequences and morphology. *American Journal of Botany* 83: 125–131.

McCourt R.M., Karol K.G., Casanova M.T. & Feist M. 1999. Monophyly of genera and species of Characeae based on *rbcL* sequences, with special reference to Australian and European *Lychnothamnus barbatus* (Characeae: Charophyceae). *Australian Journal of Botany* 47: 361–369.

McCourt R.M., Karol K.G., Helm-Bychowski J.B.K.M., Grajewska A., Wojciechowski M.F. & Hoshaw R.W. 2000. Phylogeny of the conjugating green algae (Zygnemophyceae) based on *rbcL* sequences. *Journal of Phycology* 36: 747–758.

Meiers S.T., Proctor V.W. & Chapman R.L. 1999. Phylogeny and biogeography of *Chara* (Chlorophyta) inferred from 18S rDNA sequences. *Australian Journal of Botany* 47: 347–360.

Morioka H. 1941. Charophyta Japonica (II). *Journal of Japanese Botany* 17: 57–70.

Nishiyama T. & Kato M. 1999. Molecular phylogenetic analysis among bryophytes and tracheophytes based on combined data of plastid coded genes and the 18S rRNA gene. *Molecular Biology and Evolution* 16: 1027–1036.

Nozaki H., Ito M., Watanabe M.M., Takano H. & Kuroiwa T. 1997. Phylogenetic analysis of morphological species of *Carteria* (Vol-

vocaceae, Chlorophyta) based on *rbcL* gene sequence. *Journal of Phycology* 33: 864–867.

Nozaki H., Kodo M., Miyaji M., Kato M., Watanabe M.M. & Kasaki H. 1998. Observations on the morphology and oospore wall ornamentation in culture of the rediscovered Japanese endemic *Nitella gracilens* (Charales, Chlorophyta). *European Journal of Phycology* 33: 357–359.

Nozaki H., Misawa K., Kajita T., Kato M., Nohara S. & Watanabe M.M. 2000. Origin and evolution of the colonial Volvocales (Chlorophyceae) as inferred from multiple, chloroplast gene sequences. *Molecular Phylogenetics and Evolution* 17: 256–268.

Saitou N. & Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406–425.

Swofford D.L. 2001. *PAUP* – phylogenetic analysis using parsimony (* and other methods),* version 4.0b8. Sinauer Associates, Sunderland, Massachusetts.

Wood R.D. 1952. The Characeae, 1951. *Botanical Review* 18: 317–353.

Wood R.D. 1962. New combinations and taxa in the revision of Characeae. *Taxon* 11: 7–25.

Wood R.D. 1965. Monograph of the Characeae. In: *A revision of the Characeae* (Ed. by R.D. Wood & K. Imahori), vol. 1. J. Cramer, Weinheim. 904 pp.

Wood R.D. & Imahori K. 1959. Geographical distribution of Characeae. *Bulletin of the Torrey Botanical Club* 86: 172–183.

Zaneveld J.S. 1940. The Charophyta of Malaysia and adjacent countries. *Blumea* 4: 1–224.