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Taxonomy, morphology, and genetic variation of *Nitella flexilis* var. *bifurcata* (Charales, Characeae) from Japan

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SUMMARY

The taxonomic status of *Nitella flexilis* var. *bifurcata* (Charales, Characeae), which is endemic to Japan, has remained unclear because there have been no records since its description in 1964, and the detailed morphology of fully mature individuals as well as its molecular phylogeny have not been studied. Recently, we collected a fully mature *N. flexilis* var. *bifurcata* from two lakes near the type locality in Japan. The morphological characteristics of the thalli agreed well with that in the original description of the taxon, with distinctive fertile and sterile branchlets that divided twice and unicellular dactyls. Scanning electron microscopy showed that the fine oospore structure of *N. flexilis* var. *bifurcata* was different from that of related taxa. In addition, molecular phylogenetic and haplotype network analyses using the chloroplast *rbc*L DNA sequences and the intergenic spacer regions between *atp*B and *rbc*L genes demonstrated that *N. flexilis* var. *bifurcata* and *N. flexilis* var. *flexilis* were distinct.

Key words: *atp*B-*rbc*L intergenic spacer region, Charophyceae, Japanese endemic taxa, oospores, *rbc*L gene, scanning electron microscopy.

INTRODUCTION

The genus *Nitella* C. Agardh (Charales, Characeae) is characterized by the morphological features of one to multiple forked branchlets composed of unicellular segments and single- to multiple-celled terminal segments ('dactyl'), as well as two tiers of coronal cells in the female reproductive organ, and laterally compressed oospores (Wood 1965). *Nitella flexilis* C. Agardh var. *bifurcata* Kasaki, which is an endemic taxon to Japan, was originally described by Kasaki (1964) based on specimens collected from Lake Chuzenji (Tochigi Prefecture, Japan) in 1958. There have been no records of the taxon since its description. This taxon is characterized by distinctive fertile and sterile branchlets that divide twice, and unicellular dactyls (Kasaki 1964). Wood (1965) considerably revised the taxonomy of *N. flexilis* and its related taxa in a monograph on Charales worldwide, but *N. flexilis* var. *bifurcata* was not included in the revision.

Recent scanning electron microscopy (SEM) and molecular phylogenetic studies have shown that the morphology of the fully mature oospores is a reliable character for elucidating the species-level taxonomy of the charalean taxa (John & Moore 1987; John *et al*. 1990; Leitch *et al*. 1990; Ray *et al*. 2001; Sakayama *et al*. 2002, 2005; Casanova 2005, 2009; Sakayama 2008; Urbaniak 2011a, b). However, the SEM oospore morphology and molecular phylogeny of *N. flexilis* var. *bifurcata* have not been studied. Therefore, re-examination of the detailed morphology of the oospore wall using fully mature individuals by SEM and genetic analyses are necessary to determine the taxonomic status of *N. flexilis* var. *bifurcata*.

In this study, we determined the morphology of the vegetative thalli and the fine oospore structures of *N. flexilis* var. *bifurcata* using fully matured specimens newly collected from Lakes Yuno and Suge-numa near Lake Chuzenji (type locality of this taxon). In addition, we conducted genetic analyses of the chloroplast *rbc*L DNA sequences and the intergenic spacer region between *atp*B and *rbc*L genes (*atp*B-*rbc*L IGS) using specimens of *N. flexilis* var. *flexilis* sensu Wood (1965) and var. *bifurcata* to elucidate their phylogenetic relationship.

MATERIALS AND METHODS

The collection localities are shown in Figure 1 (the map image was created using Generic Mapping Tools package; Wessel *et al*. 2013) and Table S1 in Supporting Information. The methods used for field collection, culture, light microscopy (LM), and SEM followed Sakayama *et al*. (2002, 2004, 2009), with the following modifications: LM observations of thalli and oospores were performed with SZ61, MVX10 and BX51 microscopes (Olympus, Tokyo, Japan); SEM observations of oospores were obtained using S-2150N and S-4800 SEMs (Hitachi, Tokyo, Japan) at 5–20 kV. The terms used to describe SEM oospore morphology followed Wood (1965), John and Moore (1987), Faegri and Iversen (1989), and Leitch *et al*. (1990). The thalli of *N. flexilis* var. *bifurcata* used in the present study were pressed onto herbarium sheets and preserved in formalin acetic acid alcohol (FAA) (formalin : acetic acid : 50% ethanol = 5 : 5 : 90) or 70% ethanol. The pressed specimens were deposited at the Herbarium, Depart-

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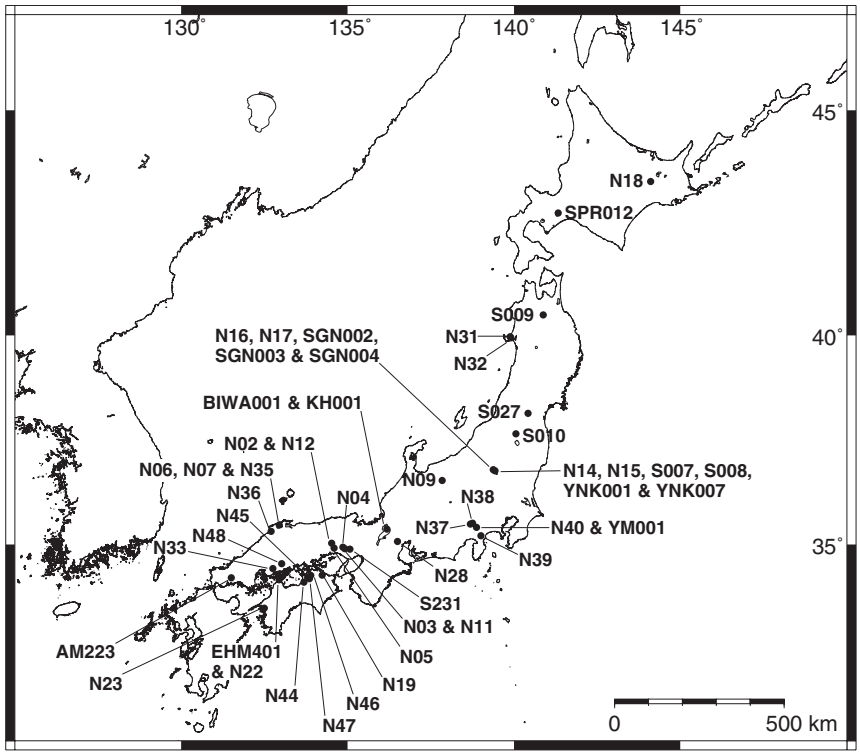


Fig. 1. Map showing the localities where the Japanese specimens of *Nitella flexilis* var. *flexilis* and var. *bifurcata* were collected. For the strain/specimen designations, see Table S1.

ment of Botany, National Science Museum (TNS), Tsukuba, Japan. To examine the SEM oospore morphology of the original material, the lectotype [MAK A12404; deposited at the Makino Herbarium (MAK), Tokyo Metropolitan University, Tokyo, Japan], isolectotypes (MAK A17611–MAK A17620 and MAK A22963; deposited at the MAK), syntypes (MAK A21063 and MAK A21587; deposited at the MAK) and isosyntypes (MAK A22180, MAK A21588, MAK A21589 and MAK A22946; deposited at the MAK) of *N. flexilis* var. *bifurcata* were examined. However, the thalli of the type specimens lacked mature oospores. Newly collected specimens of var. *bifurcata* from the Lakes Yuno and Suge-numa that lacked mature oospores were identified based on their *atp*B-*rbc*L IGS sequences.

The extraction of total DNA, amplification of DNA by polymerase chain reaction (PCR), direct sequencing of the PCR products, and phylogenetic analyses were conducted as previously described (Sakayama *et al*. 2002, 2006; Kato *et al*. 2008), except for the use of five *N. flexilis*-specific primers that we designed for the *atp*B-*rbc*L IGS (Table S2 in Supporting Information).

To determine the *rbc*L gene phylogeny, an aligned dataset of the *rbc*L sequences (1140 bp) that represented 143 operational taxonomic units (OTUs) (Tables S1 and S3 in Supporting Information; TreeBASE accession number S16281), where specimens with identical sequences were treated as a single OTU, were subjected to the Bayesian inference (BI) method using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The substitution models used for each codon position of the *rbc*L gene in the BI analyses were GTR+I+G (1st codon position), HKY+I (2nd codon position), and GTR+I+G (3rd codon position), which were estimated based on Akaike's Information Criterion (AIC) and selected by MrModeltest 2.3 (Nylander *et al*. 2004) using PAUP* 4.0b10 (Swofford 2002). BI analyses were performed using MrBayes 3.1.2, as previously described (Nakada & Nozaki 2009). The parameters of the substitution models for each codon position were unlinked. The Markov chain Monte Carlo iteration process was stopped at 1 000 000 generations. The first 25% of generations were discarded as burn-in, whereas the remaining trees were used to calculate a 50% majority-rule tree and to determine the posterior probabilities of individual branches. The average standard deviations of the split frequencies were below 0.01, indicating convergence of the iterations. Maximum likelihood (ML) analyses were conducted using PhyML 3.0 (Guindon & Gascuel 2003). Kakusan4 (Tanabe 2011) was used to identify the sequence evolution model that fit the dataset using AIC. The bootstrap proportions (Felsenstein 1985) used for ML analyses (with the GTR+I+G model selected by Kakusan4) were calculated based on 100 replicates of heuristic searches with the best of the nearest neighbor interchange (NNI) and subtree pruning regrafting (SPR) branch-swapping algorithms. In these phylogenetic analyses, two species of the genus *Tolypella* (Table S3) were selected as an outgroup because recent phylogenetic studies have demonstrated that the genus *Tolypella* constitutes the most basal lineage within the Charales (McCourt *et al*. 1996, 1999; Sakayama 2008).

In addition, unrooted statistical parsimony networks for haplotypes of the full-length sequences of *atp*B-*rbc*L IGS from 51 specimens of *N. flexilis* var. *flexilis* and var. *bifurcata* were constructed based on insertions/deletions (indels) and single base mutations (see Table 1) using statistical parsimony (Templeton *et al*. 1992) with a 95% confidence limit in TCS 1.21 (Clement *et al*. 2000). Sequence alignment was performed using MUSCLE (Edgar 2004a, b) with default options.

Table 1. Polymorphic sites and cpDNA haplotypes based on the full-length sequences of *atp*B-*rbc*L IGS from *Nitella flexilis* var. *flexilis* and var. *bifurcata*

Haplotype	Aligned position 63 (indel A1[1])	85	87	91	94 (indel B1[2])	94 (indel B2[3])	168 (indel C[4])	253 (indel D[5])
IGS-A	–	A	A	A	–	+	–	+
IGS-B	–	T	A	A	–	+	–	+
IGS-C	+	A	A	A	–	+	–	–
IGS-D	–	A	A	A	–	+	–	+
IGS-E	–	A	A	A	?[10]	–	–	+
IGS-F	–	A	A	A	+	+	–	+
IGS-G	–	A	A	A	–	+	+	+
IGS-H	–	A	A	A	–	+	–	+
IGS-I	–	A	T	T	–	+	–	+

Haplotype	Aligned position 303	415 (indel E1[6])	438 (indel E2[7])	438 (indel E3[8])	655	693	783	1028 (indel F[9])
IGS-A	T	+	+	+	C	G	T	–
IGS-B	T	+	+	+	C	G	T	–
IGS-C	T	+	+	+	C	G	T	–
IGS-D	T	+	–	+	C	G	T	–
IGS-E	T	+	?[10]	–	C	C	G	–
IGS-F	T	+	?[10]	–	C	C	G	–
IGS-G	T	+	?[10]	–	T	C	G	–
IGS-H	A	–	?[10]	+	C	G	T	+
IGS-I	A	–	?[10]	+	C	G	T	+

[1]Indel A1 = TAAAT; [2]Indel B1 = TTAAATATAAATA; [3]Indel B2 = TTAAATATAAATATAA; [4]Indel C = ATAAAATGATAATAATA; [5]Indel D = TTTAGTATTTAAA; [6]Indel E1 = CACAATTTAAATAATTAAATTAATTATCATAATTAAATTAATTATGATAA; [7]Indel E2 = TTATCATAATTAAATTAA; [8]Indel E3 = TTATCATAATTAAATTAATTATGATAATTAATTTAATTAT; [9]Indel F = ATAATAGATAATAGAA; [10]Treated as 'Missing data' based on the simple gap coding method (Simmons & Ochoterena 2000).

Subsequently, the aligned data matrix was manually corrected using SeaView 4.0 (Gouy *et al*. 2010). To treat each indel as a single mutation event, SeqState 1.32 (Müller 2005) was used to code indels with the simple gap coding method (Simmons & Ochoterena 2000). The gaps coded as binary states '0' or '1' were coded again as binary states 'A' or 'T,' respectively, for use with TCS 1.21 and the 'gaps missing' option was activated (Watanabe *et al*. 2006; Chen *et al*. 2012).

RESULTS AND DISCUSSION

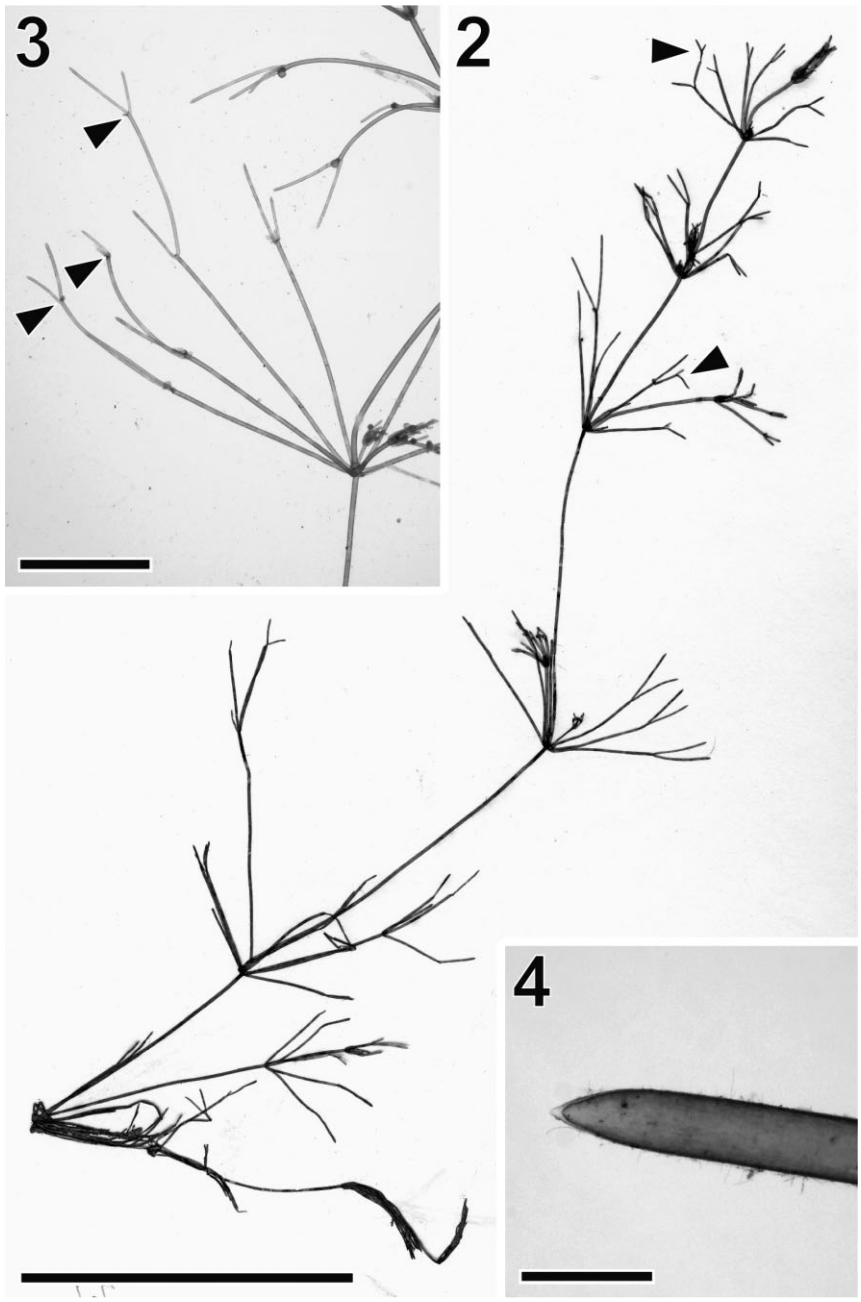
Morphological observations

The thalli of *N. flexilis* var. *bifurcata* collected from Lakes Yuno and Suge-numa were monoecious, bright green, and up to approximately 30 cm in height (Fig. 2). The axes were approximately 460–700 μm in diameter and the internodes were up to approximately 7.0 cm in length (Fig. 2). The sterile and fertile branchlets were similar to each other, six in a whorl, up to approximately 4.0 cm in length and 1- or 2-furcate; the primary rays comprised more than half of the total branchlet length, with two or three secondaries and two or three tertiaries (Figs 2, 3). The dactyls were unicellular, acuminate (Fig. 4) and 0.2–1.4 cm in length. Mucus was absent from sterile and fertile thalli. The gametangia were conjoined at the primary branchlet nodes. The oogonia were solitary, approximately 400–600 μm in length (excluding coronula) and 370–530 μm in width, with six to seven convolutions. The coronula were deciduous and missing. The antheridia were 360–590 μm in diameter. These vegetative and reproductive characters were essentially consistent with those in the original description of Kasaki (1964).

The oospores of the new *N. flexilis* var. *bifurcata* specimens were oval in face view, with five to six flanged spiral ridges, and were 380–520 μm in length, 350–440 μm in width, and 40–77 μm across the fossa (Figs 5, 6). The walls of the mature oospores were dark brown to black. The fossa wall was granulate to scabrous with large circular openings under LM (Fig. 8). Using LM, the overall appearances of the oospores and wall ornamentations were essentially consistent with those in the original description by Kasaki (1964). SEM observations detected a pitted pattern with large and minute circular openings on the fossa wall (Figs 6, 7), where the large and minute openings measured up to ca. 1.3 μm in diameter, which were usually located at a distance of more than approximately 0.32 μm from each other (Figs 6, 7). These openings extended onto the spiral ridges (Fig. 6).

Molecular phylogeny and genetic variations

The *rbc*L DNA sequences of 1140 base pairs from our 49 specimens of var. *flexilis* and var. *bifurcata* from Japan and Germany (haplotype *rbc*L-a) were identical to two previously published sequences of the taxon from Japan (S007 and S010 for the specimens from Lake Yuno and Lake Hibara, respectively) (Fig. 9, Table S1).



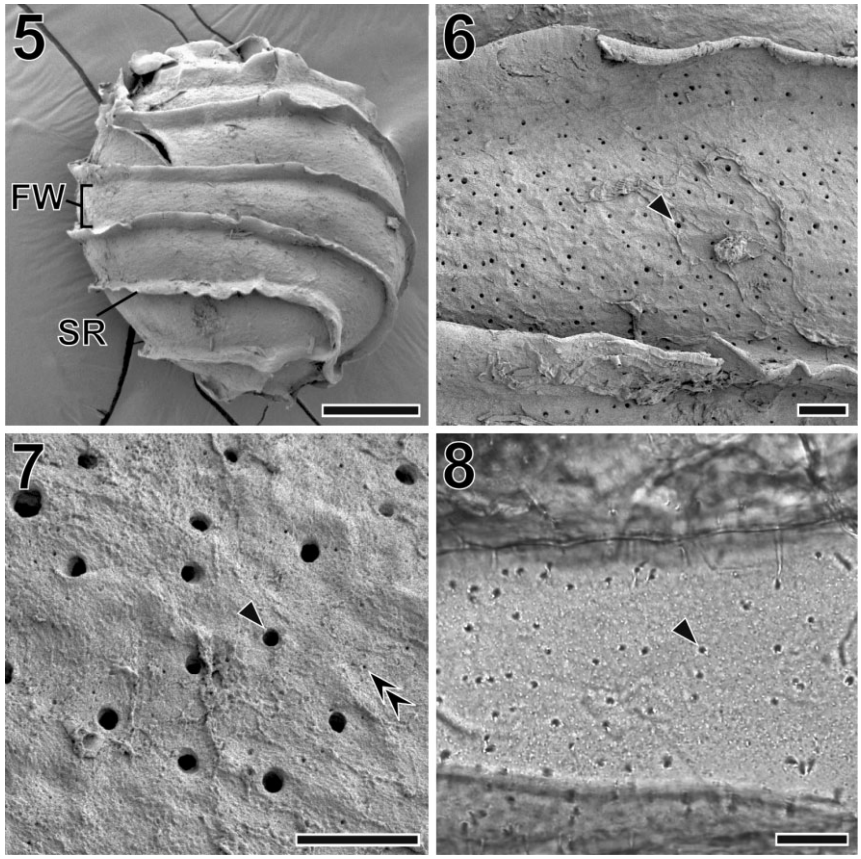
Figs 2–4. Thalli of *Nitella flexilis* var. *bifurcata* (SGN003). 2. Overall appearance of thallus. Arrowheads indicate twice-forked branchlets. Scale bar = 5 cm. 3. Part of a thallus that comprises a main axis and whorled branchlets. Arrowheads indicate twice-forked branchlets. Scale bar = 1 cm. 4. Apical part of dactyl showing the acuminate apice. Scale bar = 500 μm.

Based on the *atp*B-*rbc*L IGS sequences, nine haplotypes were detected within the 51 specimens of var. *flexilis* and var. *bifurcata* examined in the present study (Fig. 10). Statistical parsimony analysis at 95% confidence level resolved a single network based on the sequences. In the network, all haplotypes were distinguished from each other by 14 mutational steps (indels or single base mutations) (Fig. 10 and Table 1). Sakayama *et al*. (2002) assigned the strain S007 that was collected from Lake Yuno as *N. flexilis* but did not classify it to a variety level. All six specimens collected from Lake Yuno (YNK001, YNK007, N14, N15, S007 and S008) lacked mature oospores but they exhibited the IGS-H haplotype in the present analyses (Fig. 10 and Table S1). Therefore, the strain S007 was re-identified as var. *bifurcata* based on the *atp*B-*rbc*L IGS sequences.

In the haplotype network, *N. flexilis* var. *flexilis* and var. *bifurcata* exhibited seven and two haplotypes, respectively (Fig. 10). These two taxa were distinguished from each other by more than three mutational steps (Fig. 10 and Table 1). Moreover, two groups (groups I and II) were identified in the haplotype network within var. *flexilis*, but the morphological differences between these two groups remain unclear.

Taxonomic accounts

Wood (1962, 1965) classified *N. flexilis* into two varieties, var. *flexilis* and var. *spanioclema* (J. Groves & Bullock-Webster ex Bullock-Webster) R. D. Wood, based on differences in the



Figs 5–8. Oospores of *Nitella flexilis* var. *bifurcata* (SGN002). 5. Overall appearance of oospore by scanning electron microscopy (SEM), showing five to six flanged spiral ridges (SR) on the surface and fossa walls (FW) between the spiral ridges. Scale bar = 100 μm. 6. Part of the fossa wall by SEM, showing the pitted pattern with approximately 9–12 circular openings across the fossa. Arrowhead indicates circular openings on the surface of the fossa wall. Scale bar = 10 μm. 7. Detail of the fossa wall by SEM, showing large and minute circular openings on the surface. Single or double arrowheads indicate large or minute circular openings, respectively. Scale bar = 5 μm. 8. Part of the fossa wall by light microscopy, showing the pitted pattern with circular openings. Arrowhead indicates circular openings on the surface of the fossa wall. Scale bar = 10 μm.

furcation of their branchlets. The branchlets fork once in var. *flexilis*, while var. *spanioclema* has once- or twice-forked branchlets. *Nitella flexilis* var. *bifurcata* resembles var. *spanioclema* in branchlet morphology (once- or twice-forked branchlets), but these two taxa clearly differ in terms of the fine structures of their oospores. In var. *bifurcata*, the fossa wall exhibited pitted patterns, which were characterized by prominent surface openings (Figs 6, 7). In contrast, var. *spanioclema* has a spongy fossa wall (Leitch *et al*. 1990).

The oospores of var. *flexilis* exhibited pitted fossa wall patterns that resembled those in var. *bifurcata* (Frame 1977; Leitch *et al*. 1990; Ray *et al*. 2001) (Figs 6, 7). In var. *bifurcata*, the surface openings on the fossa wall are prominent and almost circular and up to approximately 1.3 μm in diameter. In contrast, the fossa wall of var. *flexilis* has imperfectly or irregularly arranged surface openings, which measure up to approximately 0.85 μm in diameter (Frame 1977; Leitch *et al*. 1990; Ray *et al*. 2001; A. Kai and H. Sakayama, unpublished observations). In addition, genetic analyses based on the *rbc*L and *atp*B-*rbc*L IGS sequences demonstrated the close relationship between var. *bifurcata* and var. *flexilis* (Figs 9, 10 and Table 1), supporting the classification of var. *bifurcata* by Kasaki (1964).

The SEM observations of oospores and the genetic analyses of 11 specimens of var. *bifurcata* and 40 specimens of var. *flexilis* demonstrated their phylogenetic relationship and the unique phylogenetic position of var. *bifurcata* in Japan. We only analyzed specimens collected from Japan and Germany because this study focused on the Japanese endemic taxa. However, 12 forms have been recognized worldwide within var. *flexilis* and var. *spanioclema* by Wood (1965). Therefore, in a future taxonomic revision, further morphological and molecular analyses of these taxa, using a large number of specimens collected from localities throughout the world, are needed to resolve their natural relationship. As to the species or variety level taxonomy of *N. flexilis*, although the independence of var. *bifurcata* was supported, we suspend the revision of the taxonomic position until more data become available. Therefore, in the present study, var. *bifurcata* described by Kasaki (1964) was placed within *N. flexilis* sensu Wood (1965) as a variety.

Nitella (subgen. *Nitella*) *flexilis* C. Agardh var. *bifurcata* Kasaki (1964, p. 225)

Lectotype (here designated): MAK A12404 [deposited at the Makino Herbarium (MAK), Tokyo Metropolitan University, Tokyo, Japan]. This lectotype specimen was originally collected from Lake Chuzenji, Tochigi Prefecture, Japan (Aug. 30, 1958; see Kasaki 1964, p. 225; specimen no. 3310).

Isolectotype (here designated): MAK A17611–MAK A17620 and MAK A22963 (deposited at MAK; see Kasaki 1964, p. 225; specimen no. 3310).

Syntypes (here designated): MAK A21063 and MAK A21587 (deposited at MAK). These syntype specimens were originally collected from Lake Chuzenji, Tochigi Prefecture,

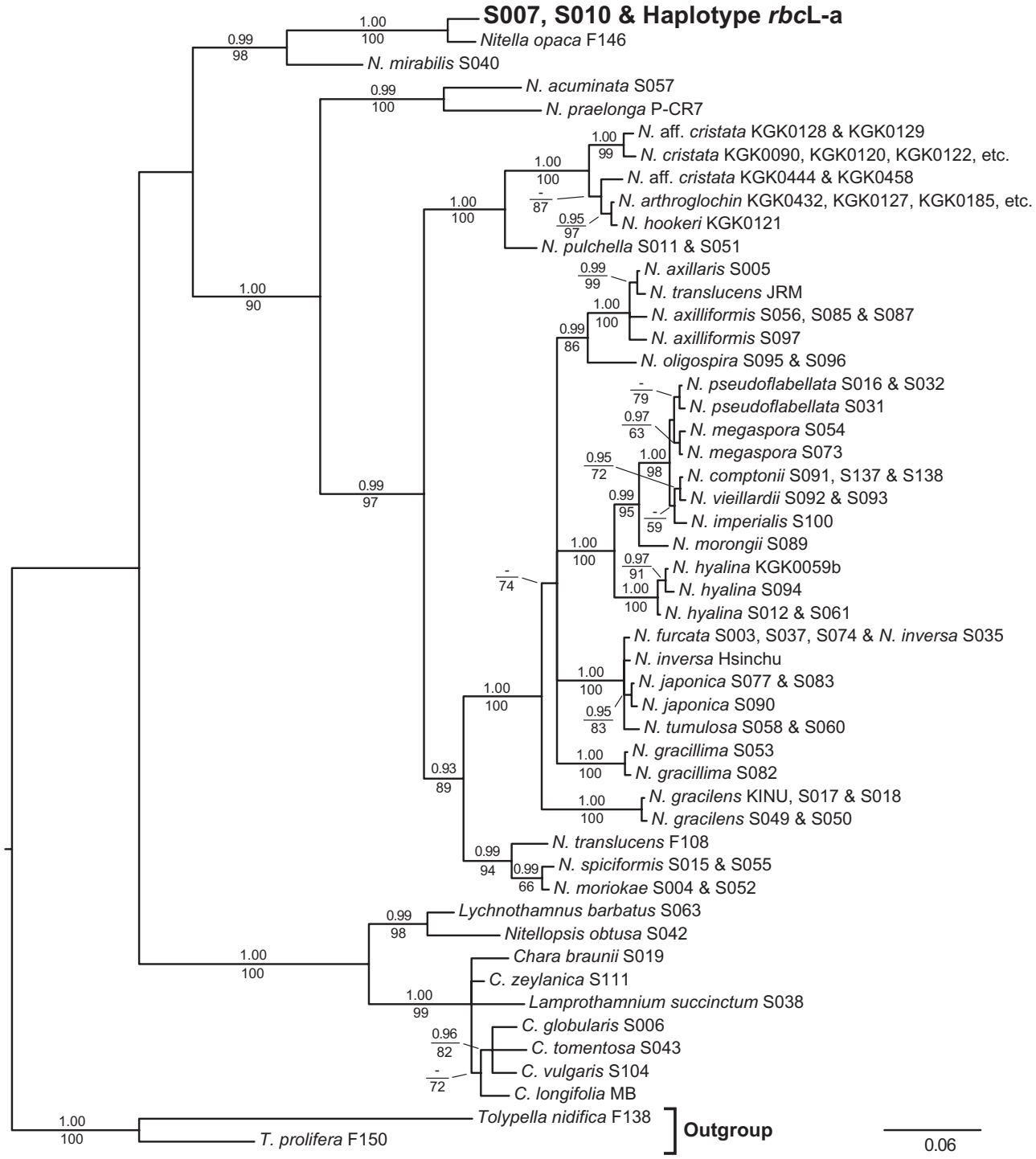


Fig. 9. Bayesian phylogenetic tree based on 1140 bp of the *rbc*L DNA sequences of 143 operational taxonomic units (OTUs) (Tables S1 and S3). The corresponding posterior probabilities (≥0.90) and the bootstrap proportions (≥50%) from the maximum likelihood analysis are shown above and below branches, respectively. The branch lengths and the scale bar represent the expected number of nucleotide substitutions per site. Specimens with identical sequences were treated as a single OTU.

Japan (Aug. 30, 1958; see Kasaki 1964, p. 225; specimens no. 3308 and 3309 correspond to MAK A21063 and MAK A21587, respectively).

Isosyntypes (here designated): MAK A22180, MAK A21588, MAK A21589 and MAK A22946 (deposited at MAK; see Kasaki 1964, p. 225; specimens no. 3308 and 3309 correspond to MAK A22180, MAK A21588 and MAK A21589; and MAK A22946, respectively).

Distribution: Endemic to Japan (Fig. 1) (Kasaki 1964; Imahori & Kasaki 1977).

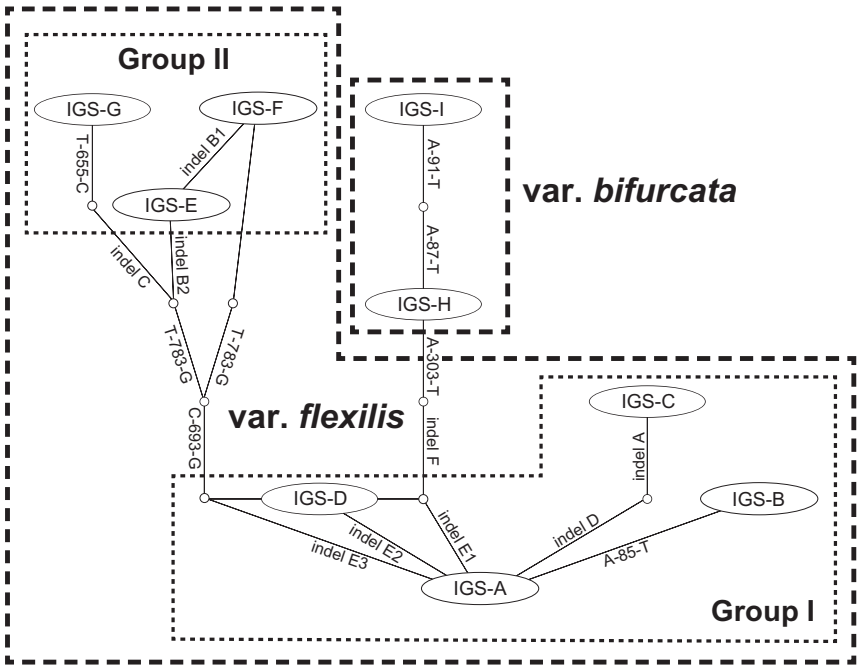


Fig. 10. Statistical parsimony network inferred from the intergenic spacer regions between the *atp*B and *rbc*L genes (*atp*B-*rbc*L IGS) in *N. flexilis* var. *flexilis* and var. *bifurcata*. The text inside the circles corresponds to the haplotypes in Tables 1 and S1. Missing intermediate haplotypes are indicated by small open circles. Each line connecting nine haplotypes represents a single mutation step with a 95% probability of being parsimonious. The letters on each line represent the types of indel or single base mutations (Table 1).

Specimen examined for the SEM oospore morphology: TNS-AL 189784 (H. Sakayama and S. Kato, specimen SGN002; collected from Lake Suge-numa; see Figs 5–8 and Table S1).

Other specimens examined in this study: TNS-AL 189785 and TNS-AL 189786 (H. Sakayama and S. Kato, specimens SGN003 and SGN004; collected from Lake Suge-numa); TNS-AL 189780 and TNS-AL 189781 (H. Sakayama and H. Nozaki, strains S007 and S008; collected from Lake Yuno); TNS-AL 189778 and TNS-AL 189779 (A. Naka and H. Sakayama, specimens YNK001 and YNK007; collected from Lake Yuno); TNS-AL 189782 and TNS-AL 189783 (M. Nishiyama and M. M. Watanabe, strains N14 and N15; collected from Lake Yuno); and TNS-AL 189787 and TNS-AL 189788 (M. Nishiyama and M. M. Watanabe, strains N16 and N17; collected from Lake Suge-numa) (Table S1).

Key to the varieties of *Nitella flexilis*

1a. Branchlets 1-furcate, rarely appearing simple and two-celled: var. *flexilis*
1b. Branchlets occasionally 2-furcate
2a. Oospores with spongy fossa wall patterns: var. *spanioclema*
2b. Oospores with pitted fossa wall patterns: var. *bifurcata*

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Source of samples of *Nitella flexilis* var. *flexilis* and var. *bifurcata* sequenced in this study.
Table S2. Primers used for amplifications and sequencing of the full-length *atp*B-*rbc*L IGS in the present study.
Table S3. Accession numbers of *rbc*L gene sequences of other species analyzed in this study.