



Molecular taxonomic report of *Nitella megacephala* sp. nov. (Characeae, Charophyceae) from Korea

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Abstract

We report a new taxonomic entity of *Nitella megacephala* sp. nov. (Charales, Charophyceae) from Korea. The characean algae collected from two sites (Haenam-gun and Kangjin-gun) had distinctive morphological characteristics representing a new *Nitella* species. Those samples showed a light-green color in gross morphology and a plant body length up to 13 cm. Moreover, the two-celled dactyls and head formation differed clearly from closely related *Nitella* species (*N. moriokae*, *N. spiciformis*, and *N. translucens*). From a molecular phylogenetic analysis of *rbcL* DNA sequences, *Nitella megacephala* sp. nov. formed a single clade with *N. translucens*, *N. moriokae* and *N. spiciformis*, and was distantly related to those three species as a sister taxon. In the terms of interspecific sequence variation, *Nitella megacephala* showed 3.2–5.5% pairwise distance values with sister groups in phylogenetic tree (*N. translucens*, *N. moriokae* and *N. spiciformis*) and 3.2–9.1% with other of *Nitella* species. In contrast, its sister group species differed 0.3–1.7% at the interspecific level. These unique morphological and molecular taxonomic characteristics clearly support the establishment of this taxonomic entity as a new species in the genus *Nitella* (*Nitella megacephala* sp. nov.)

Keywords: Characeae, *Nitella megacephala* sp. nov., *rbcL*, Korea, new species

Introduction

Most characean algae are sensitive to environmental changes and show the restricted distribution patterns (Graham et al 2009). In the genus *Nitella*, eight species are reported as endangered species on the IUCN Red List (<https://www.iucnredlist.org>). Therefore, biogeographic data about *Nitella* species may provide useful information for the ecological conservation of rare characean species.

In terms of characean plant morphology, the family Characeae is divided into two tribes- Chareae and Nitelleae. Tribe Nitelleae consists of two genera *Nitella* and *Tolypella* (Wood and Imahori 1965). The genus *Nitella* is characterized by an incrustated body, one to three furcated branches, a two-layered coronula in the oogonia, and lateral growth of oogonia and antheridia. About 280 *Nitella* species are known from worldwide, and 22 taxa in the genus *Nitella* (14 species, 1 subspecies, 6 varieties, and 1 variety and forma) have been reported in Korea (Imahori and Choe 1963; Choi et al 1996; Choi and Kim 1998; Guiry and Guiry 2019). The genus *Nitella* is composed of three subgenera *Nitella*, *Hyella*, and *Tieffallenia*, according to features such as the number of cells in the dactyls and their branching patterns (Wood and Imahori 1965).

From a phylogenetic perspective, characean algae have placed at the basal group from which land plants are derived and they share many characteristics with land plants (reproductive structures, feature of cytokinesis and genetic compositions) (McCourt et al 1996; Hall et al 2010; Nishiyama et al. 2018). Molecular phylogenetic analyses of characean species have mainly been carried out using *rbcL* (ribulose biphosphate carboxylase large subunit) gene sequences (e.g. Sakayama et al. 2002, 2004, 2005, 2008). Taxonomic revisions including description of new species have also been conducted using *rbcL* gene sequence analysis (e.g., Sakayama et al. 2006; Lee et al. 2016).

Sakayama *et al.* (2002) re-examined the morphological characteristics (oospore wall ornamentation) and reconstructed the phylogenetic relationships among *Nitella* species using *rbcL* sequences (Sakayama *et al.* 2002). They reported nine taxa of Japanese *Nitella* species including species endemic to Japan and East Asia. Based on the integrative analysis of scanning electron microscope (SEM) oospore morphology and molecular phylogenetic relationships among *Nitella* species, they found an efficient combination of morphological and molecular phylogenetic approaches for resolving the taxonomic problems at the species level.

Sakayama *et al.* (2008) analyzed the phylogenetic relationships among *N. axilliformis*, *N. jurcata*, *N. megaspora*, *N. pseudojiabellata*, *N. translucens*, and *N. tumulosa* using *rbcL* and *atpB* gene sequences. Moreover, Sakayama *et al.* (2005) examined 17 species of *Nitella* subgenus *Tieffallenia* using multiple DNA marker sequences and morphological characteristics. Those *rbcL* reference sequences provide a useful database clarifying the taxonomic entity of poorly described *Nitella* species (e.g., *Nitella comptonii* in Sakayama *et al.* 2006). Therefore, we used *rbcL* gene sequences to examine new taxonomic entity with distinctive morphological characteristics found in Korea.

Traditional studies of Korean *Nitella* species have been conducted based on morphological features. Imahori and Choe (1963a, 1963b) first studied Korean characean flora. Chung (1968), Choi *et al.* (1996), and Choi and Kim (1998) examined species of the genus *Nitella* from Korea, with Choi and Kim (1998) reporting 13 species and 6 varieties. In this study, we examined the morphology of a new taxonomic entity in the genus *Nitella* and analyzed its phylogenetic position using plastid *rbcL* sequences. We established *Nitella megacephala* sp. nov from Korea based on these morphological and molecular taxonomic characteristics.

Materials and Methods

Field sampling and morphological observation

We monitored the Korean flora of characean algae starting in 2014 (Lee *et al.* 2016) and recently observed individuals with a distinct morphology at two sites in Korea Haenam-gun (6 May 2014) and Kangjin-gun (31 Aug. 2017). Two Korean *Nitella* samples were found in narrow water supply systems alongside rice paddy fields.

Samples were collected with a rake and a hook with a long rope from the soil of the channel bottom (Lee *et al.* 2016). We examined a total of ten samples, including one specimen (NIBRCR0000100441) from Haenam-gun and nine specimens (NIBRCR0000100442–450) from Kangjin-gun. All specimens were deposited at the algal herbarium of the National Institute of Biological Resources (NIBR) in Incheon, Korea. Morphological observation was conducted using a light microscope (Olympus BX50, Japan) and a stereomicroscope (Zeiss, Germany).

Molecular analysis followed previously described methods (Lee *et al.* 2016; Lee and Lee 2018). Total genomic DNAs were extracted from dried *Nitella* specimens using a DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen, USA). The *rbcL* region was selected for molecular phylogenetic analysis using the primers listed in Sakayama *et al.* (2002). PCR was performed with an initial denaturation step at 94°C for 3 min followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, with a final extension step at 72 °C for 7 min. *AmfiXpand* PCR Master Mix (GenDEPOT, USA) was used for PCR and the products were sequenced by a commercial sequencing service (Genotech, Korea).

Chromatograms were assembled with the program Sequencher 5.4.6 (Gene Codes, USA). We downloaded *rbcL* sequences from the GenBank maintained by the National Center for Biotechnology Information (NCBI) and those sequences were used for molecular phylogenetic analyses (Fig. 1). Phylogenetic analysis and pairwise distance calculations were accomplished using MEGA ver. 6 (Tamura *et al.* 2013) with the neighbor-joining method and 2,000 bootstrap replicates.

Results

3.1. Morphology

We examined ten specimens collected from two sites. The plants are light-green, and somewhat slender in gross morphology (Fig. 2A). Dactyls are bicellulate and penultimate, tapering distally to the base of the end cell (Fig. 2B). Lower whorls are furcated or simple (Fig. 2C). Upper whorls are bi- or tri-furcated. Fertile heads form axillary or terminally, and are numerous. Heads are composed of reduced branches and gametangia. Rhizoids are long and bushy

(Fig. 2D). Their cells are connected with swollen ends (Fig. 2E). The plants are monoecious (Fig. 2F). Gametangia are formed at the basal site of a secondary ray without a stipe (Fig. 2G). The antheridia are formed inside the nodes of furcated branchlets (Fig. 2H). The antheridium is orange to red in color, and 210–283 µm in diameter. The oogonium is surrounded by five tubal ridges with two-layered coronula (Fig. 2I).

3.2. Characterization of plastid *rbcL* sequences

We successfully identified 1,205 bp of *rbcL* sequences from two individuals collected from Haenam-gun (accession number MK649946) and Kangjin-gun (accession number MK649945). No sequence variation was found between these two Korean individuals. *Nitella megacephala* sp. nov. showed 3.2–9.1% pairwise distances with other of *Nitella* species at the interspecific level (Fig. 1). Moreover, *Nitella megacephala* sp. nov. showed 3.2–5.5% of pairwise distance values with its sister groups in the phylogenetic tree (*N. translucens*, *N. moriokae*, and *N. spiciformis*), while those three sister group species had differences 0.3–1.7% among them. On the other hand, *Nitella* species showed 0–0.9% differences at the intraspecific level (Fig. 1: e.g., *N. acuminata*, *N. axilliformis*, *N. megaspora* and *N. mirabilis*). *Nitella moriokae* which is closely related with *Nitella megacephala* sp. nov., represented 0–0.1% divergence at the intraspecific level (Japanese AB110876, KJ395936 [NIES-1633], AB076069 [Japan], and AB110876 [*N. rigida* var. *moriokae*]).

Discussion

We conducted ecological monitoring of the Korean flora of characean algae since 2014 and found a novel species of Characeae (Lee *et al.* 2016). Most Korean characean algae are rarely found in restricted ecological habitats. In this study, samples with distinctive morphological characteristics were collected from only two sites in Jeollanam-do near the paddy fields.

The genus *Nitella* consists of numerous species of Characeae, with high morphological variations at the species level. The morphological characteristics used for species identification in the genus *Nitella* are gross morphology, furcation pattern of branches, dactyl features, and oospore wall ornamentation. However, these characteristics show large variations at the intraspecific level. Morphological ambiguity prevents the accurate species identification of the genus *Nitella*. Thus, the molecular approach offers a practical alternative method for species identification (e.g., Sakayama *et al.* 2004).

The genus *Nitella* contains three subgenera, differentiated according to the form of the dactyl or end cell and the number of cells forming a dactyl. Wood and Imahori (1965) suggested that *Nitella* has two evolutionary groups according to differing dactyl forms. One is subgenus *Nitella* with one-celled dactyls; the other contains subgenera *Hylla* and *Tiffallenia* with dactyls that have two or more-celled. This plant has two-celled dactyl form and, placed in subgenus *Tieffallenia* through molecular analysis. In our molecular phylogenetic analysis (Fig. 1), *Nitella* sp. nov. showed a close relationships with *N. translucens*, *N. moriokae* and *N. spiciformis*.

Wood and Imahori (1965) described *Nitella moriokae* and *N. spiciformis* as formas of species. Following that taxonomic study, Sakayama *et al.* (2002) examined those taxa in terms of oospore morphology and treated *N. moriokae* and *N. spiciformis* as species level taxa. *Nitella megacephala* sp. nov. showed closed a relationship to these taxa, as their sister group in the phylogenetic tree. *Nitella moriokae* and *N. spiciformis* are reported to lack a head feature. *Nitella megacephala* sp. nov. differs from these taxa in having distinctly large and compact gametangia and reduced branches. Moreover, *N. megacephala* sp. nov. was different from *N. translucens* in having dactyls as wide as the apex of the penultimate cell and being small to medium in size.

Nitella megacephala sp. nov. formed a single clade with *N. translucens*, *N. moriokae*, and *N. spiciformis*, and this clade was supported by a bootstrap value of 81 (Fig. 1). Three species of sister group showed 3.2–5.5% pairwise distances with *Nitella megacephala* sp. nov. (*Nitella translucens* [AF097745] 3.6%, *N. moriokae* 3.2–3.3%, and *N. spiciformis* [AB076068] 3.4%). In contrast, the pairwise distances among the species *N. moriokae*, *N. spiciformis*, and *N. translucens* were 0.3–1.7%. From these pairwise distance values and phylogenetic relationships, *Nitella megacephala* sp. nov. showed clear separation from *N. translucens*, *N. moriokae*, and *N. spiciformis*. Based on the results of our morphological examination and molecular phylogenetic analysis, we determined that the new taxonomic entity collected from two sites in Korea should be treated as a new species (*Nitella megacephala* sp. nov.).

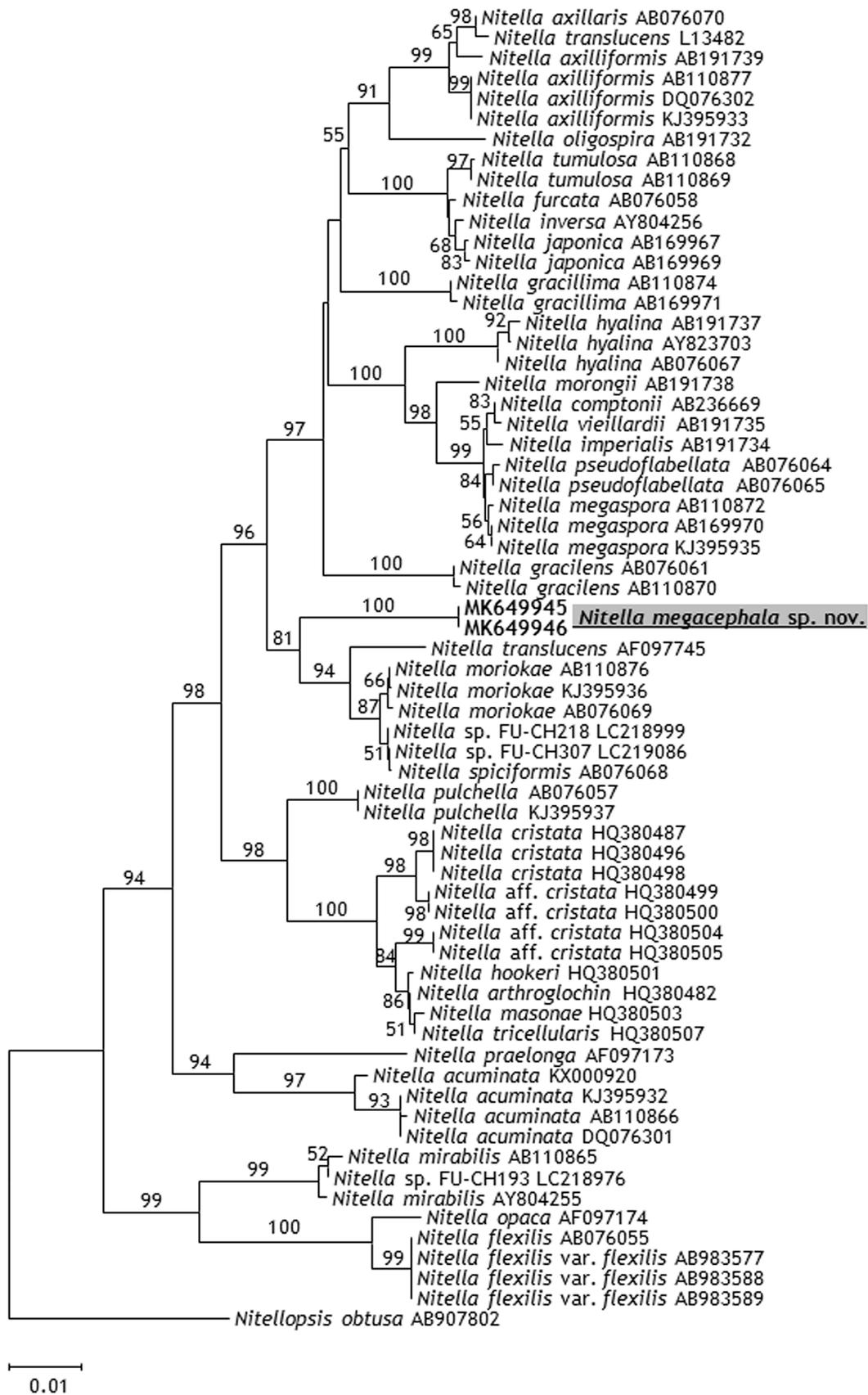


FIGURE 1. Molecular phylogenetic relationship of *Nitella megacephala* sp. nov. among other *Nitella* species. The phylogenetic tree was constructed from *rbcL* sequences. The neighbor-joining method (Kimura two-parameter model) was used and bootstrap values (2,000 replicates) are shown on branches (>50). *Nitellopsis obtusa* (AB907802) was used as an outgroup.



FIGURE 2. *Nitella megacephala* sp. nov. (accession numbers MK649945-6). A: Live *Nitella megacephala* sp. nov. plant. B: Dactyls, acute distally to the end cell. C: Pattern of head and branch formation. D: Rhizoids of the plant. E: Rhizoidal cell with swollen end part. F: Gametangia at the base of sterile branchlets and heads. G: Laterally formed gametangia. H: Antheridium composed of tetra-scutate cells. I: Oogonium surrounded by spiral ridges. Scale bars represent: A, 1 cm; B, 200 μ m; C–D, 1 mm; E, 200 μ m; F, 500 μ m; G, 200 μ m; and H–I, 100 μ m.

Taxonomic treatment

Nitella megacephala sp. nov. E.-Y. Lee et S.-R. Lee (Figs. 1 and 2)

The plant is light-green, fertile, and 9.5–13 cm in height. The main axes are slender, with up to 8 whorls, 0.4–0.5 mm in width. Main internodes are 12–15 cm or 20–22 cm in length. The 1st branch axes are generally unbranched, rarely 2–3 furcate, 0.4–0.5 mm in width, and 10–15 mm in length. The 2nd branch axis is bifurcate, 0.2–0.3 mm in width, and 2–5 mm in length. The 3rd branch axis is 0.2 mm in width, 2 mm in length. Dactyls are 2-celled, acute, 220 µm in length. Heads are terminal or axillary. Rhizoid length is up to 190 µm. Gametangia are monoecious, conjoined at the node of a fertile branchlet. Oogonia are dark brown to almost black, 310–375 µm, rarely 250 µm wide and 300–330 µm long. Antheridia are 210–283 µm in diameter.

Holotype: NIBRCR0000100450, Chilyang-myun, Kangjin-gun, Jeollanam-do, Korea. 31 Aug. 2017 by Sang-Bong Lee.

Molecular sequences of typical material (NIBRCR0000100450): *rbcL* (GenBank accession MK649945)

Etymology: The specific epithet refers to having the large heads which form gametangial structure.

Korean name: Song-i-gal-re-mal (Song-i refers to the cluster like morphology and gal-le-mal is the Korean name of the genus *Nitella*)

Additional specimens examined (paratypes): NIBRCR0000100442, NIBRCR0000100443, NIBRCR0000100444, NIBRCR0000100445, NIBRCR0000100446, NIBRCR0000100447, NIBRCR0000100448, and NIBRCR0000100449 - Collection sites of specimens are near paddy fields in Kangjin-gun, Jeollanam-do, Korea.

NIBRCR0000100441 (*rbcL*, GenBank accession MK649946) - Collection site of specimen is near paddy fields in Haenam-gun, Jeollanam-do, Korea.

TABLE 1. Morphological characteristics of *N. megacephala* sp. nov. and three closely related species, with data from the literature (Morioka 1941; Imahori and Wood 1965; Sakayama *et al.* 2002).

Characters	<i>N. megacephala</i> sp. nov.	<i>N. translucens</i>	<i>N. moriokae</i>	<i>N. spiciformis</i>
Height	9.5–13 cm	15–45 cm	5–15 cm	10–15 cm
branches	up to 8	3–8		5–6
Width of branches	400–500 µm	400–1900 µm	400–500 µm	460–510 µm
Width of oogonia	(250)310–375 µm	270–430 µm	310–340 µm	180–210 µm
Width of Antheridium	210–283 µm	160–375 µm	250–290 µm	175–210 µm
Dactyl	2-celled	2-celled, Tiny dactyls	2–3 celled	2–3 celled
Head formation	Distinct	Numerous, axillary, around nodes	Not formed	Not formed

Acknowledgements

We thank Mr. Sang Bong Lee for sample collection. This work was supported by a grant from the NIBR, funded by the Ministry of Environment of the Republic of Korea (NIBR201701103 and NIBR201902103).

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