Phycological Research

Phycological Research 2014; **62:** 222–227

RESEARCH NOTE

Occurrence of the endangered species *Nitellopsis obtusa* (Charales, Charophyceae) in western Japan and the genetic differences within and among Japanese populations

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SUMMARY

Nitellopsis obtusa (Charales, Charophyceae) are widely distributed from Europe to Asia; however, this species has been recorded in only the five lakes in central Honshu in Japan. This species was thought to be extinct in Japan, but was rediscovered in limited areas of Lake Kawaguchi in central Honshu. More recently, we discovered more Japanese populations of *N. obtusa* in Lake Biwa in western Honshu, and it became clear that the species had a broader distribution in Japan than originally believed. In addition, although only male or female thalli have been collected at each lake, both male and female thalli were found from Lake Biwa. This is the first report of a potentially sexual population of *N. obtusa* in Japan. The DNA sequences of three chloroplast DNA markers, including both coding and non-coding regions, were identical in all specimens from Lake Kawaguchi and Lake Nojiri (Central Honshu), and differed from those of Lake Biwa and German specimens. Although Japanese and German specimens were genetically similar, Japanese specimens displayed considerable genetic diversity according to locality.

Key words: Charales, conservation, endangered species, Japanese lakes, Lake Biwa, *Nitellopsis obtusa*.

Charalean algae (Charales, Charophyceae) are an important ecological element of inland water ecosystems. However, because their general habitats are highly sensitive to environmental changes due to anthropogenic factors (such as eutrophication, water abstraction, sediment mining and shoreline reinforcement), they have experienced massive diversity loss over the last century, especially in Europe (Schubert & Blindow 2003; Langangen 2007). Accordingly, many charalean taxa are considered threatened species (red lists) in a number of countries, including Japan (Blaženčić *et al*. 2006; Siemińska *et al*. 2006; Bryant & Stewart 2011; Azzella *et al*. 2013).

Although more than 80 charalean taxa (species, variety and form) in the four genera *Chara* L., *Lamprothamnium* J. Groves, *Nitellopsis* Hy and *Nitella* Agardh have been reported in Japan (Imahori 1954; Kasaki 1964; Imahori & Kasaki 1977; Sakayama *et al*. 2006, 2009; Kato *et al*. 2010), many are considered threatened or endangered due to eutrophication and reclamation of their habitats. Watanabe *et al*. (2005) reported that charalean algae are missing from 27 of the 39 Japanese lakes in which they were reported by Kasaki (1964). Moreover, the Japanese Red List includes three extinct, one 'extinct in the wild (EW)', 57 'critically endangered and endangered (CR+EN)', and one vulnerable taxon (Environmental Agency of Japan 2012).

The genus *Nitellopsis* currently contains two species: *N. obtusa* (Desvaux) Groves and *N. sarcularis* Zaneveld (Wood 1965; García 1990). *N. obtusa* is characterized by dioecious thallus lacking cortical cells and possesses elongated bract cells resembling branchlet segments, a coronula consisting of five cells in a single tier and star-shaped bulbils (Kasaki 1964; Wood 1965; Kato *et al*. 2005). Because of its preference for cold waters, *N. obtusa* has been classified as a boreal taxon (Corillion 1957). This species is distributed widely across Eurasia

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Received 25 January 2013; accepted 19 February 2014.
doi: 10.1111/pre.12057

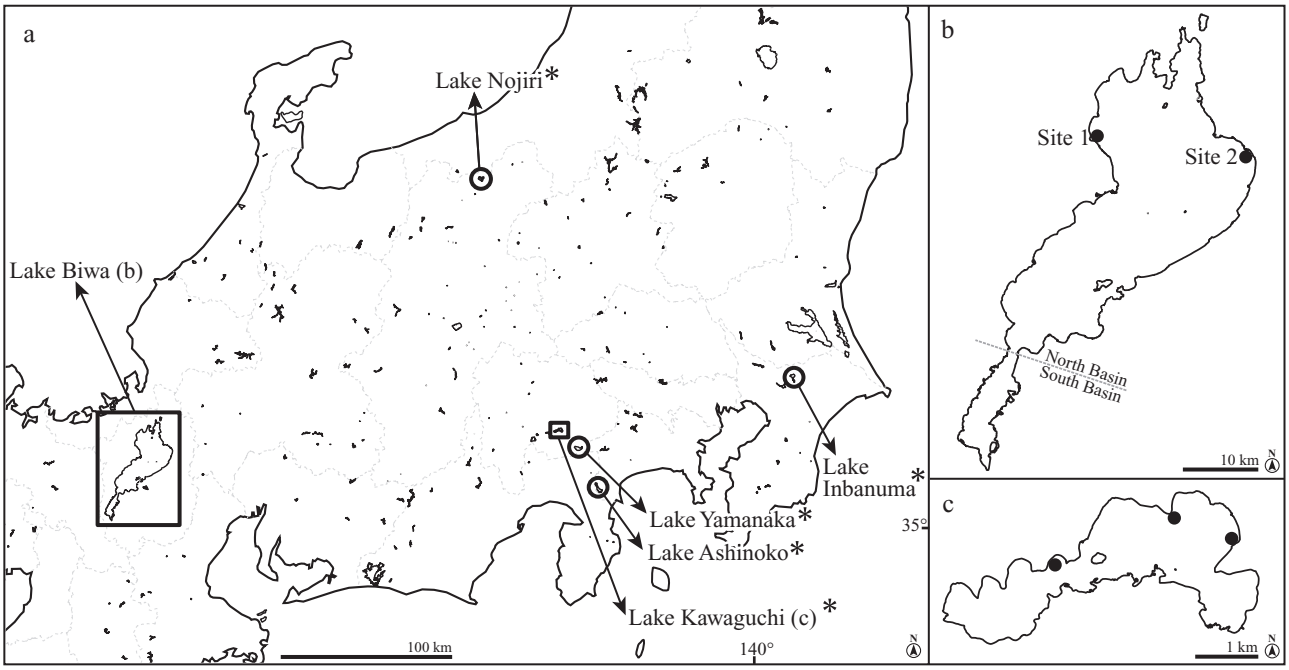

Fig. 1. Localities in Japan at which *Nitellopsis obtusa* were collected in the present and previous studies. Closed circles represent the sites of *N. obtusa* collection in this study. (a) Western to central Japan. Lakes marked with asterisks are localities reported previously (Imahori 1963; Kasaki 1964; Kato *et al*. 2005). Circled lakes indicate localities in which the species was thought to be extinct (Watanabe *et al*. 2005). (b) Lake Biwa. (c) Lake Kawaguchi.

from the western coast of Europe to Japan (18–65°N) (Wood 1965; Soulié-Märsche *et al*. 2002).

In Japan, *N. obtusa* has been recorded in only five lakes in central Honshu: Lake Ashinoko, Lake Kawaguchi, Lake Yamanaka, Lake Nojiri and Lake Inbanuma (Kasaki 1956, 1962, 1964; Imahori 1963, Watanabe & Morita 1977) (Fig. 1). However, *N. obtusa* was not found in these lakes in later surveys, and the species was considered extinct in Japan (Watanabe *et al*. 2005). Following these recent surveys, *N. obtusa* was listed as an EW species in Japan (Environmental Agency of Japan 2000). More recently, Kato *et al*. (2005) rediscovered this species in limited areas of Lake Kawaguchi, and thus its rank was changed from EW to CR+EN in the most recent Japanese Red List (Environmental Agency of Japan 2012).

In our recent field survey of water plants and charalean species in Lake Biwa (Shiga Prefecture, Japan), we discovered new populations of *N. obtusa*. During a scuba diving survey to investigate 109 sites using the line-transect method (transect lines perpendicular to the shoreline) covering large parts of the coast of Lake Biwa from May 2011 to September 2013, *N. obtusa* was collected at two sites on the eastern and western coasts of the lake (site 1: 35°22′ 57.69″ N, 136°2′ 40.33″ E and site 2: 35°21′ 52.10″ N, 136°15′ 45.70″ E) (Fig. 1). At site 1, vegetative thalli were collected on 6 July 2011 and 28–29 July 2012. At site 2, fertile thalli bearing both female and male reproductive organs were collected on 12 September 2013 (Fig. 2). The morphology of *N. obtusa* from Lake Biwa agreed with descriptions of the species in Kasaki (1956, 1964), Wood (1965), and Kato *et al*. (2005) (Fig. 2, Text S1 in Supporting Information).

Although all previously reported populations of *N. obtusa* were restricted to central Honshu (Fig. 1), the new locality in Lake Biwa was located in western Honshu. Thus, this species has a broader geographic distribution in Japan than initially predicted. Kasaki (1964), Nozaki *et al*. (1997) and the Japan Water Agency (2009) did not find *N. obtusa* from Lake Biwa; indeed, this species was identified in only two of the 109 sites. Therefore, the distribution and population size of *N. obtusa* in Lake Biwa were thought to be sporadic and small, respectively.

Kasaki (1962, 1964, 1994) reported that Japanese populations of *N. obtusa* were composed of only female or male thalli in each lake, and so considered these populations likely to reproduce asexually by bulbils. In Europe, it was rare that both female and male thalli are collected from one lake, and there have been few reports of the occurrences of matured oospores (Migula 1897; Olsen 1944, Willén 1960, Langangen 2007). Therefore, sexual reproduction was believed to be rare in this species. In contrast, our finding of both female and male thalli in a lake

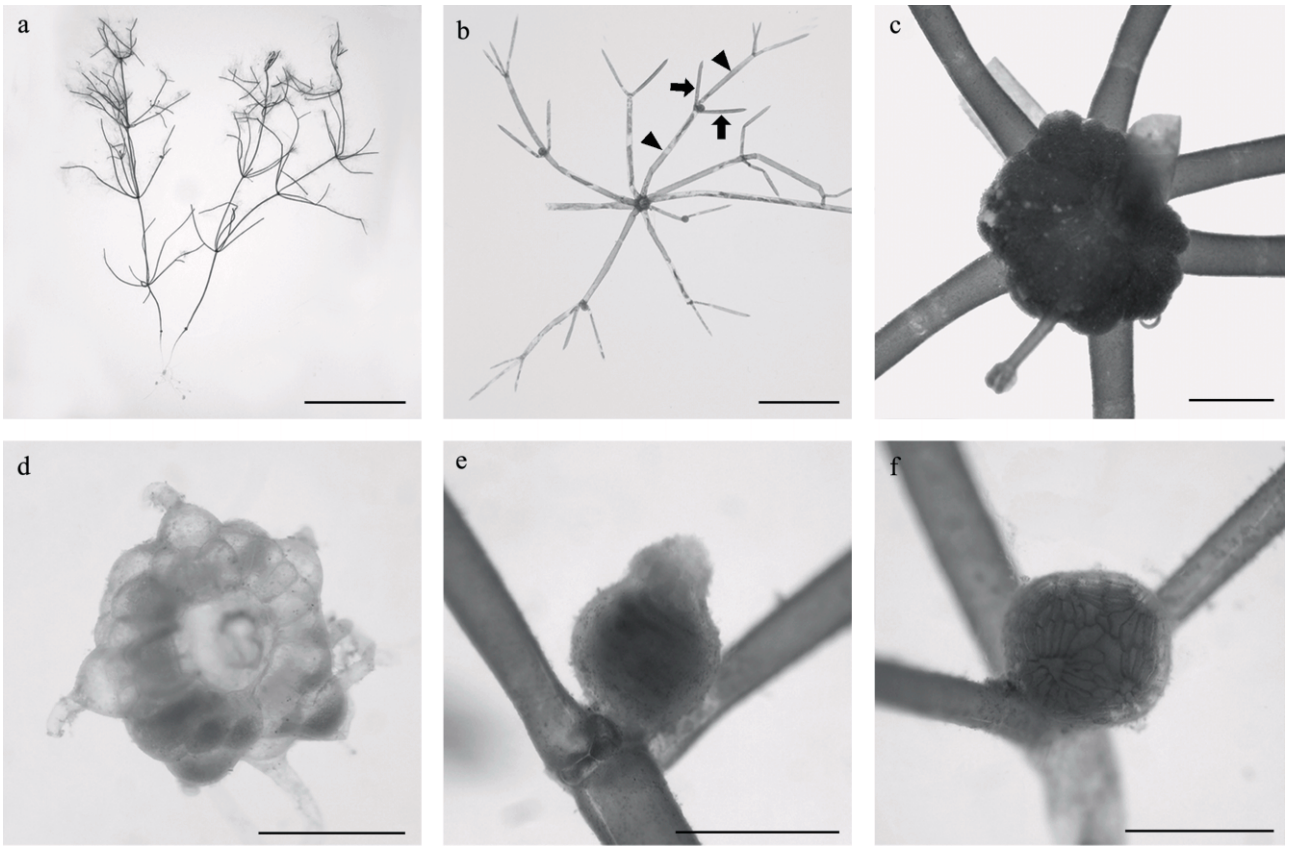

Fig. 2. Morphology of *Nitellopsis obtusa* collected from Lake Biwa. (a) Habit of thallus. (b) An isolated node and its whorl of branchlets (basal view). Arrows or arrowheads indicate bract-cells or branchlet segments, respectively. (c) Thickened axial nodal cells (basal view). (d) Star-shaped bulbil. (e) Female organ (Oogonium). (f) Male organ (Antheridium). Scale bar = 5 cm for a; 1 cm for b; 1 mm for c, d, e and f.

suggest that *N. obtusa* undergoes a sexual life cycle in Japan, although additional collections of thalli bearing zygotes are required to confirm this hypothesis.

Nitellopsis obtusa is thought to be a key indicator species of mesotrophic to oligotrophic lakes (Kasaki 1962). Lake Biwa was oligotrophic before the 1950s (Petts 1988), but has become meso- to eutrophic due to anthropogenic eutrophications (Pollingher 1990; Nakanishi & Sekino 1996). Therefore, *N. obtusa* in Lake Biwa is still considered threatened by extinction, although COD (chemical oxygen demand), T-N (total nitrogen), and T-P (total phosphorus) values have decreased slightly in the last several years (Shiga Prefectural Government 2012).

Reliable morphological identification of charalean species generally requires examination of fertile thalli with mature oospores (John & Moore 1987; John *et al*. 1990; Sakayama 2008; Sakayama *et al*. 2009), but oospores were not present in the thalli we collected. In addition, no genetic study within or among populations of this threatened species has been reported in any region. Therefore, to confirm species and infra-species level identification, and to assess genetic diversity of the specimens, we conducted molecular phylogenetic analyses using multiple genetic markers; namely, the DNA sequence of the large subunit of the ribulose bisphosphate carboxylase/oxygenase (*rbc*L) gene and its two flanking non-coding regions (intergenic spacer (IGS) regions between the beta subunit of the ATP synthase (*atp*B) and the *rbc*L genes (*atp*B-*rbc*L IGS), and between *rbc*L and the tRNA-Arg (CCG) genes (*trn*R) (*rbc*L-*trn*R IGS)). For genetic analyses, we used 25 *N. obtusa* samples, including 23 newly collected samples from Lakes Biwa and Kawaguchi (Figs 1,4). The methods were essentially identical to those described previously (Sakayama *et al*. 2002; Kato *et al*. 2011), except four newly designed species-specific primers for *atp*B-*rbc*L IGS and the protocol for phylogenetic analyses based on the *rbc*L gene were used (Table S1 and Text S2 in Supporting Information).

Based on phylogenetic analyses of the 1194-bp *rbc*L gene, the previously published sequences of *N. obtusa* and the samples from Lake Biwa formed a robust monophyletic group within the order Charales (Fig. 3). The *rbc*L gene sequences of Lake Biwa

samples (Accession No. AB907802, AB907803 and AB907808) were identical to those of the four samples from Japan (Lake Kawaguchi and Lake Nojiri), China, and Germany (Lake Nehmitz) (Fig. 3), while the sequence of another German sample (found from Lake Stadtwaldsee) differed by a single nucleotide substitution.

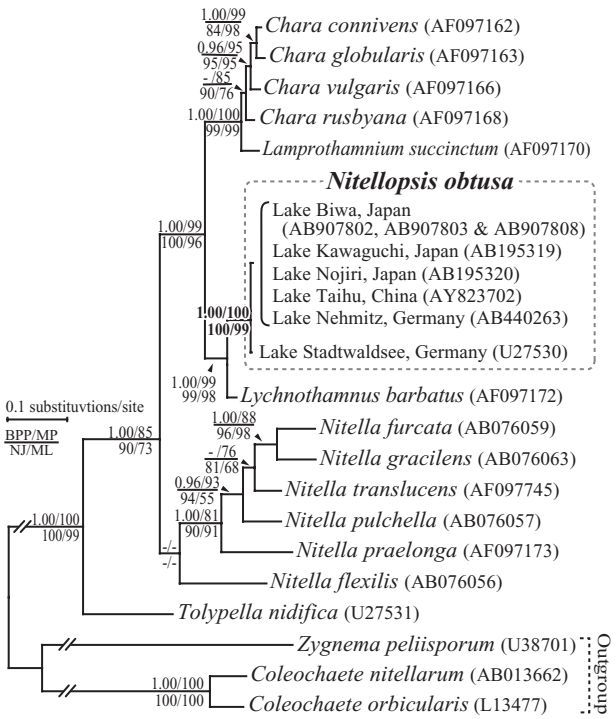

Fig. 3. Bayesian phylogenetic tree based on *rbc*L gene sequences (1194 bp) of 14 charalean species and three outgroup species. The GeneBank accession numbers are in parentheses. Numbers above the branches indicate Bayesian posterior probabilities (BPP, left) and bootstrap values from maximum parsimony (MP, right) analyses. Numbers below the branches indicate bootstrap values from neighbour-joining (NJ, left) analyses and maximum likelihood (ML, right) analyses. Only posterior probabilities ≥ 0.95 and bootstrap values ≥ 50% are shown. Branch lengths and the scale bar represent the expected number of nucleotide substitutions per site.

The *rbc*L-*trn*R IGS sequences (284 bp) were identical in all *N. obtusa* samples analyzed in this study (data not shown, Accession No. AB907827-AB907851). Based on *atp*B-*rbc*L IGS sequences (1437-71 bp), four haplotypes differing by repetitive insertion/deletions (indels) were identified (types 1–4; Fig. 4, Accession No. AB907802-AB907826). The *N. obtusa* samples from Lake Biwa were distinguishable from those from other Japanese lakes and from the German strain (Lake Nehmitz) (Fig. 4). Two haplotypes (types 1 and 2) were found from Lake Biwa, whereas all samples from Lakes Kawaguchi and Nojiri were of an identical haplotype (type 3) (Fig. 4). In other dioecious charalean species, Schaible *et al*. (2009) found an indel in the flanking region of a microsatellite locus in *C. canescens* Desvaux & Loiseleur. In this species, the indel was linked to a reproduction mode (sexual or parthenogenetic), and can also be used as a marker to distinguish populations within the same species. Although the reproduction mode of Japanese *N. obtusa* remains unclear, the genetic differences found in the present study suggested that *N. obtusa* in Lake Biwa may have originated separately from those in other Japanese lakes or that those haplotypes have diverged in Japan. It is possible that Japanese *N. obtusa* is not a clonal population and has a certain genetic diversity. Further molecular analyses using more samples/populations collected globally based on rapidly evolving DNA markers – such as nuclear microsatellite regions – may increase our understanding of the geographical genetic diversity patterns in *N. obtusa*.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 22770083 and 24570100 to H.S.) and a Grant-in-Aid for Scientific Research Fellows (No. 24·1993 to S.K.) from the Japan Society for the Promotion of Science, and Nippon Life Insurance Foundation, and conducted as a part of the Environment Research and Technology Development Fund of the Ministry of the Environment, Japan (No. RFd-1102 to H.S.).

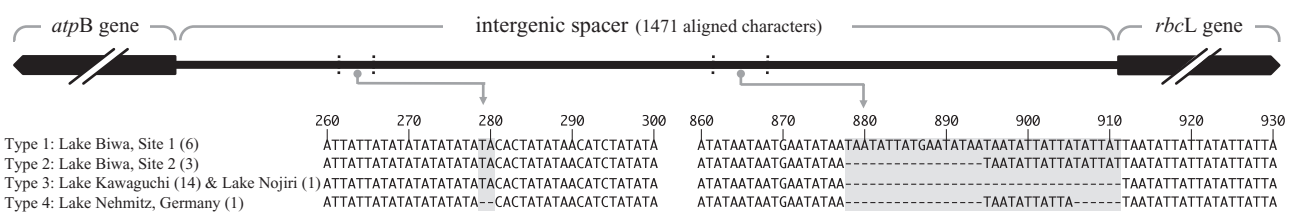

Fig. 4. Four haplotypes distinguished by insertion/deletions of intergenic spacer (IGS) regions between *atp*B and *rbc*L genes (*atp*B-*rbc*L IGS) in 25 samples of *Nitellopsis obtusa* from lakes in Japan and Germany. The numbers in parentheses after the lake name indicate the number of samples analyzed.

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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. New primers used for amplifications and sequencing of *atp*B-*rbc*L IGS in the present study.
Text S1. Morphological observations of *N. obtusa* from Lake Biwa.
Text S2. Supplementary methods.