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Rapid Communication

Genetic evidence for the invasion of *Cymbella janischii* (A. Schmidt) De Toni, 1891 in Japan

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Abstract

Cymbella janischii (A. Schmidt) De Toni 1891, an endemic diatom of the Pacific Northwest, was found in 2006 in Japan, and since then, its distribution has been expanding. Here, we analyzed *rbc*L, *psa*B, *psb*A, 18S rRNA, and 28S rRNA gene sequences (6526 bp in total) of the *C. janischii* specimens from several locations in Japan and explored their genetic relatedness with *C. janischii* from its country of origin (the United States) and its closely related species. We showed that all Japanese specimens had the same sequences, regardless of geographical distance, and formed a clade with the US *C. janischii*. The identities and the pairwise distance between the sequences of the Japanese and the US diatoms were 99.937% and 0.0003, respectively, indicating that these diatoms are extremely similar. These results provide potential genetic evidence of the recent invasion and rapid spread of *C. janischii* from the US in Japan.

Key words: diatom, genetic identities, molecular phylogeny

Introduction

Invasion of alien species and their impact on local ecosystems has become a major issue in recent years (Simberloff et al. 2013). Among diatoms, invasive species are also a serious problem, as exemplified by the well-known invasion of New Zealand by *Didymosphenia geminata* (Lyngbye) M. Schmidt 1899 (Kilroy et al. 2008; Blanco and Ector 2009).

Cymbella janischii (A. Schmidt) De Toni 1891 has an ability to produce copious extracellular polymeric substances that form the stalks and cause nuisance blooms as "rock snot", similar to that of *D. geminata* (Khan-Bureau et al. 2016) (Figure 1A, B). *Cymbella janischii* is considered an endemic diatom in the Pacific Northwest (Krammer 2002; Bahls 2007); however, it has recently been observed outside of its native range (e.g., Arizona, Colorado, New York, Oklahoma, and Connecticut in the US and in Japan) (Suzawa et al. 2011; Khan-Bureau et al. 2014, 2016).

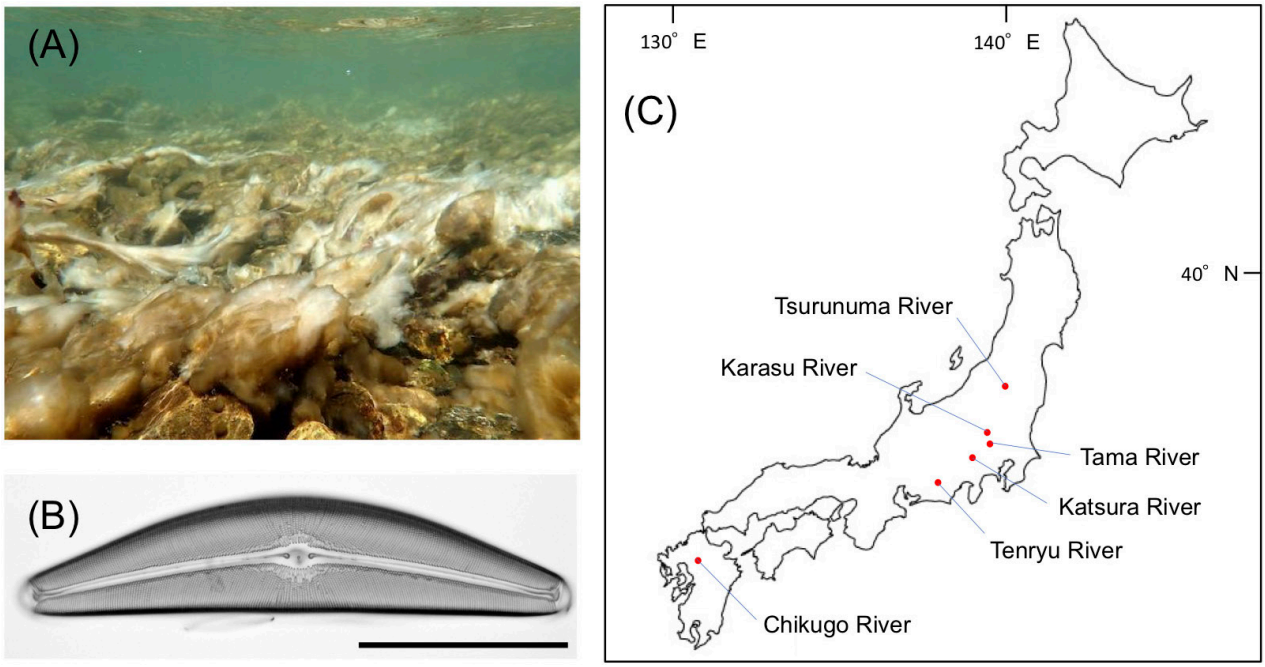

Figure 1. Images of the diatom and geographical collection sites. (A) State of the riverbed covered with *Cymbella janischii.* (B) Light microscopic image of *C. janischii* from the Tama River in Japan. Scale bar = 100 µm. (C) Map with locations of sample collection in Japan. Geographic coordinates of each sampling site are provided in Supplementary material Table S1. Photos by Seiji Sato (A) and AK (B).

Genetic analysis is useful in estimating the region of origin for invasive species, their evolution, and the pathway through which they were introduced (Cann et al. 1987; Gaut et al. 1992; Kato-Unoki et al. 2020). For example, the invasion of *D. geminata* in New Zealand was evidenced by the genetic distance based on a comparison of a 2230 bp sequence in the chloroplast genomes of strains from New Zealand and other countries (Kilroy and Novis 2018). For *C. janischii*, the genetic data from Idaho and Connecticut in the US were submitted in GenBank (Nakov et al. 2014; Khan-Bureau et al. 2016). Phylogenetic analysis based on the sequences of the V4 region of the small subunit rRNA (18S) from Khan-Bureau et al. (2016) showed a close relationship between the sequences from Idaho and Connecticut (KJ011622 and KJ160166–KJ160169).

In Japan, *C. janischii* was first reported from the Chikugo River in 2006 (Suzawa et al. 2011) and has expanded its distribution thereafter (Suzawa and Suzawa 2021). In the previous study, *C. janischii* was identified morphologically; however, genetic analyses were not carried out. Here, we determined the sequences of previously reported gene region of this species in Japanese samples (chloroplast encoded ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene [*rbc*L], photosystem I and II genes [*psa*B and *psb*A], and nuclear-encoded 18S and large subunit rRNA [28S] genes; Nakov et al. 2014), and confirmed species identification at the molecular level as "*C. janischii*". Additionally, we clarified their phylogenetic relationship with *C. janischii* from the country of origin (the US) and other closely related species.

Materials and methods

Cymbella janischii specimens were collected from six different geographical sites in Japan, in 2018–2019, as shown in Figure 1C and Table S1. Each single cell was washed with sterile water by pipetting under a stereo microscope (Olympus SZX10) and stored in a sample tube at −80 °C until further use. Genomic DNA was amplified from a single cell using REPLI-g Mini kit (Qiagen). The samples were incubated for 10 h at 30 °C. Amplified products were diluted 20 times with TE and then used as PCR templates.

The previously reported DNA fragments (encoding *rbc*L, *psa*B, *psb*A, 18S rRNA, and 28S rRNA genes; Nakov et al. 2014) were amplified with Phusion DNA polymerase (New England BioLabs) using the following primer sets (primer information shown in Table S2): rbcL66+ and rbcL1255– [annealing temperature: 56 °C], nd6+ and dp7 [52 °C], psaB22F and psaB2000R [58 °C], psbA78A and psbA997R [62 °C], SSU1 and ITS1DR [59.5 °C], and DIR and D2C [56 °C]. The PCR mixture consisted of 1 µL genomic DNA, 1× Phusion HF buffer (New England BioLabs), 0.2 mM of each dNTP, 0.5 µM of each primer, and 0.2 µL Phusion DNA polymerase in a total volume of 20 µL. The PCR conditions were as follows: one cycle at 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 52–62 °C for 10 s (the annealing temperature for each primer set is as written above), 72 °C for 40 s, and a final extension at 72 °C for 2 min. PCR products were sequenced with the primers listed in Table S2 after purification with NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL). The obtained sequences were assembled using the ATGC Ver. 4.3.5 software (GENETYX Co.). The determined sequences, except the primer region, were deposited in GenBank (accession number: LC568542–LC568546 for 28S, 18S, *rbc*L, *psb*A, and *psa*B).

To annotate the genetic classification, BLASTN homology search was conducted on the nucleotide collection (nr/nt) database. To confirm the phylogenetic relationship with *C. janischii* from the US (Idaho) and its closely related species as previously reported by Nakov et al. (2014), each gene sequence was aligned with the corresponding genes from the following most closely related taxa using MAFFT ver. 7 (Katoh and Standley 2013): *C. tumida*, *C. proxima*, *C. baicalensis*, *C. stuxbergii*, *C. mexicana*, *C. janischii*, *D. dentata*, *D. geminata*, *D. siberica*, and *Encyonema triangulum* (used for the outgroup). Aligned sequences were concatenated after removing ambiguous aligning regions (total 5863 bp), and processed via the maximum likelihood (ML) analysis with partitioning by genes and codons using RAxML ver. 8.2.11 (Stamatakis 2014), with the GTR gamma model and 1,000 bootstraps. The pairwise distance (*p*-distance) between the sequences of specimens from Japan and the US was calculated with MEGA ver.7 (Kumar et al. 2016) using concatenated and aligned sequences with gaps removed.

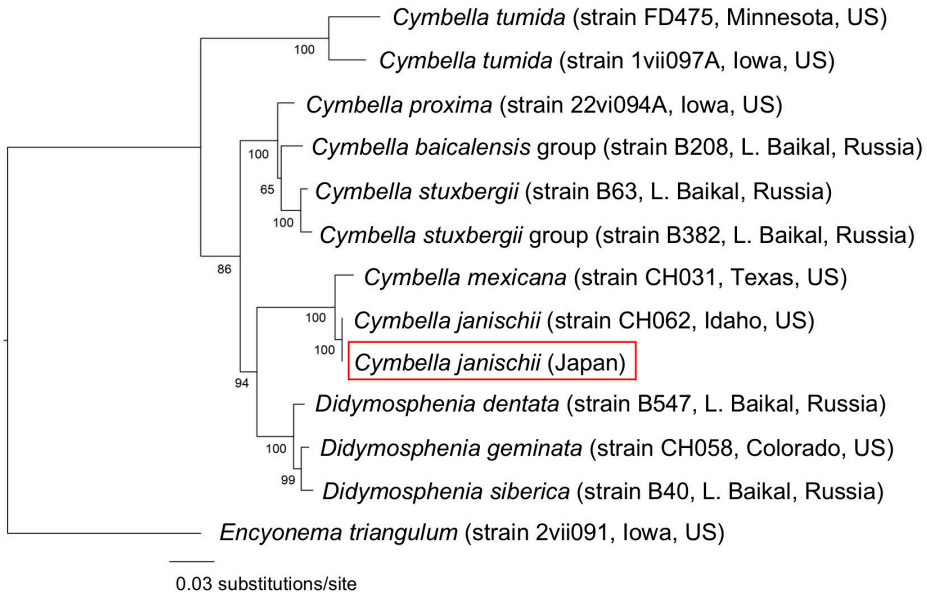

Figure 2. Maximum likelihood (ML) tree based on five genes (28S, 18S, *rbc*L, *psb*A, and *psa*B) of *Cymbella janischii* in Japan and its country of origin (Idaho, US), and of related species. Numbers on branches indicate ML bootstrap values. Strain number and origin are shown in parentheses.

Results

The sequences of samples from geographically distinct sites, morphologically identified as *C. janischii* in Japan, were determined with the *rbc*L (1473 bp), *psa*B (1958 bp), *psb*A (895 bp), 18S (1667 bp), and 28S (533 bp) genes (total 6526 bp). The sequences of all samples were identical. The BLASTN search for each gene sequence showed the highest homology to the sequences of *C. janischii* registered in Genbank with the following identity rates: *rbc*L 100%, *psa*B 99.95%, *psb*A 99.88%, 18S 99.88%, and 28S 100%. The degenerate nucleotides of existing sequence, "R" in *psa*B (KJ011751) and "Y" in 18S (KJ011622), were single nucleotides "A" and "C" in samples from Japan, respectively. The phylogenetic tree resulting from BLASTN search showed that each gene sequence in the Japanese specimens belonged to the same branch as *C. janischii* (Figure S1). The ML tree based on the multigene analysis also showed the Japanese sequence to be in the same branch as *C. janischii* with 100% bootstrap support (Figure 2). Hence, our genetic analysis confirmed that all diatoms in question from Japan were *C. janischii*. The identities and the *p*-distance between the aligned sequences of specimens from Japan and the US were 99.952% and 0.0002 in the chloroplast genome (total 4200 bp of *rbc*L, *psa*B, and *psb*A), 99.907% and 0.0004 in the nuclear genome (total 2146 bp of 18S and 28S), and 99.937% and 0.0003 in the five gene sequences (total 6346 bp), respectively.

Discussion

This study provides molecular genetic confirmation that the stalked diatoms recently found in Japanese streams are *C. janischii*, and enables an estimation for the invasion from the country of origin and the recent

dispersal in Japan. Geographical distance and/or isolation along with environmental differentiation can lead to genetic diversity (Sexton et al. 2014; Fernández et al. 2017). In case of *D. geminata*, genetic divergence (*p*-distance up to 0.005 in a total 2230 bp region of chloroplast genome [*atp*F–*atp*H, *rbc*S–*rbc*L, and *sec*A–*rbc*R]) was observed in the indigenous areas in the Northern Hemisphere, whereas genetic homogeneity was found within New Zealand, which was compatible with specimens from several indigenous areas (Kilroy and Novis 2018). The variation in the US (Colorado and Montana) specimens of *D. geminata* in that region corresponded to a *p*-distance of 0.001 (Kilroy and Novis 2018). In this study, all Japanese *C. janischii* specimens showed the same sequence, regardless of geographical distance, and were extremely similar in sequence to isolates from the country of origin, the US. Between the Japanese and the US specimens, the *p*-distance is 0.0002 in the chloroplast genome regions (*rbc*L, *psa*B, and *psb*A) and 0.0003 in the total region (6346 bp) including the nuclear genomes regions (18S and 28S). Since the genomic regions analyzed in this study and in *D. geminata* are not the same, it is not possible to make a strict comparison; however, the Japanese *C. janischii* specimens have higher similarity than that observed in *D. geminata*. These results indicate that *C. janischii* from the country of origin invaded and spread widely in Japan in recent times. Most likely, this occurred via some object or biotic material (e.g., fishing gear, the transport of fish, their eggs, and water), such as has been discussed in the case of *D. geminata* (Kilroy and Novis 2018). Future studies will be needed to confirm if the observed genetic differences are due to intraspecific polymorphisms, since the genetic information comes from a limited number of specimens from the country of origin and from a single cell in each Japanese specimen.

In rivers invaded by *C. janischii*, the entire surface of the riverbed is covered with a yellowish-brown gelatinous substance (Figure 1A) as reported from rivers/in habitats invaded by *D. geminata*, which changes the aquatic ecosystem. Damage to fishing for *Plecoglossus altivelis*, a prominent Japanese fish which feeds on algae, has been reported (Ashizawa and Kaji 2019). An understanding of invasion characteristics and ecology of this species is needed. Detection and monitoring of *C. janischii* using highly sensitive methods such as environmental DNA analysis might be useful for river management and future research on *C. janischii,* e.g., clarifying the invasion characteristics and ecology of this species. The genetic data obtained here will contribute to the design of a highly sensitive detection system for this species.

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Author contribution

Conceptualization: YK. Sample design and methodology: YK, AK, SM. Investigation and data collection: YK, AK, TK, YS (Suzawa), SM. Data analysis and interpretation: YK, AK, SM. Funding provision: YK. Writing – original draft: YK. Writing – review and editing: YK, AK, TK, YS (Shimasaki), YS (Suzawa), SM.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Collection site information.

Table S2. List of primers used in this study.

Figure S1. Phylogenetic trees resulting from BLASTN search.

This material is available as part of online article from:
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