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PRIMARY RESEARCH PAPER

Establishment success of alien *Daphnia* in the ancient Lake Biwa: insights from sedimentary archives

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Abstract Invasive species pose serious threats to global biodiversity, prompting studies to identify factors underlying invasion and establishment success. However, it's difficult to discern these factors due to the absence of survey data since the initial invasion, because introduced species often remain unnoticed until their population becomes large enough. Here, we investigated the establishment process of the alien zooplankton, *Daphnia pulicaria*, in the past 30 years using sedimentary archives at Lake Biwa. We performed genetic analysis on mitochondrial DNA sequences of the control region and ND5 gene of ephippia in sediments, as well as present females from water samples. Furthermore, we investigated the relationships between the abundance of *D. pulicaria* and its major predator fish, Ayu, based on the catch data, as well as its competitor, *Daphnia galeata* whose abundance was inferred from claw remains. Our analyses showed the studied mitochondrial sequences were identical between all samples. The abundance of *D. pulicaria* was not correlated with that of a competitor but was negatively with that of a predator. These findings suggest the successful establishment of *D. pulicaria* was primarily influenced by reduced predation pressure, with a limited impact from competitive interactions and potential hindrance for adaptation caused by loss of genetic variation.

Keywords Invasion · Sedimentary egg bank · Zooplankton · Enemy-free space · mtDNA control region · mtDNA ND5 gene

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Introduction

Alien species occurring outside of their natural range have been unintentionally introduced into many ecosystems associated with human activities (ISSG, 2000; Heger et al., 2013). Among them, invasive species have caused habitat destruction, disease transmission, and the extinction of native species (ISSG, 2000; Richardson et al., 2011; Heger et al., 2013)

consequently, they pose serious threats to global biodiversity (Early et al., 2016). To date, many studies have been conducted to reveal the mechanisms underlying invasion success. Genetic variation has long been considered one of the critical determinants of invasion success under the premise that genetic diversity is required for adaptation (Drake, 2006). Given that the population size of introduced species is often very small at the time of introduction, these populations are expected to have low genetic diversity, and the subsequent genetic drift can then contribute to further decline in genetic variation (Sakai et al., 2001; Dlugosch & Parker, 2008). Owing to such genetic bottlenecks, introduced species can suffer from low adaptation potential (Schrieber & Lachmuth, 2017) or be vulnerable to infectious diseases (Spielman et al., 2004). Nonetheless, many introduced species adapt to new environments and become invasive, and this dilemma is referred to as the genetic paradox of biological invasion (Allendorf & Lundquist, 2003). This seems to reflect that genetic variance is not a major determinant of invasion success.

Invasion success can be explained by factors other than genetic variance. Two major hypotheses have been proposed, that is, enemy release (Keane & Crawley, 2002) and biotic resistance (Maron & Vilà, 2001). The enemy release hypothesis assumes that introduced species are released from natural enemies (e.g., predators, parasites), which facilitates their successful invasion. The biotic resistance hypothesis suggests that introduced species fail to establish owing to competitive interactions with native species (Maron & Vilà, 2001), and thus, the absence of competitors would enhance invasion success. However, in general, it is very difficult to test these hypotheses throughout the invasion process, especially during the initial invasion phase, which often passes without being noticed.

The *Daphnia pulicaria* Forbes, 1893, population that invaded Lake Biwa, Japan, provides a good model for investigating the relative importance of multiple factors for invasion success. This species suddenly appeared in the lake in 1999 (Urabe et al., 2003). Before this species appeared, *Daphnia galeata* Sars, 1864, was the dominant *Daphnia* species in Lake Biwa (Tanaka, 1992; Urabe et al., 2003; Liu et al., 2020). Cyclical parthenogenetic *Daphnia* spp. reproduce by parthenogenetically produced subitaneous eggs, but can switch to sexual reproduction resulting in the production of resting eggs under unfavorable conditions (Cáceres et al., 2004). The resting eggs are enveloped in thickened carapaces, referred to as ephippial cases, which can be preserved in sediments for centuries (Hairston et al., 1995; Hairston, 1996; Mergeay et al., 2006; Brede et al., 2009). The sedimentary resting eggs, together with remains (post-abdominal claw), can allow for an estimation of past population dynamics, including invasion timing (Brede et al., 2009; Monchamp et al., 2017). Based on the amount of their claw remains, Tsugeki et al. (2022) estimated the temporal abundance of *D. pulicaria* and *D. galeata* in Lake Biwa. Another important feature of sedimentary resting eggs is that DNA can be retrieved from them (Mergeay et al., 2006; Brede et al., 2009; Ohtsuki et al., 2015; Monchamp et al., 2017), which enables an estimation of past genetic diversity. DNA can also be recovered from ephippial cases, albeit less successfully, as shown by Ishida et al. (2012). Here, to unveil the factors underlying the invasion success of the alien zooplankton, *Daphnia pulicaria*, we first investigated the historical genetic variation in the introduced *D. pulicaria* population since their initial invasion of Lake Biwa. This was accomplished by using *D. pulicaria* resting eggs recovered from sediment cores and adult individuals collected from the water column at this lake. Mitochondrial DNA (mtDNA), multiple copies of which are contained in each cell, was targeted to increase detection probability. The mtDNA control region, renowned for its hypervariability, has often been utilized in population genetic studies (Fatsi et al., 2020; Marini et al., 2021) including *Daphnia pulicaria* studies (Dudycha, 2004). Therefore, we focused on the mitochondrial control region, which was chosen for its higher variation, and additionally the NADH dehydrogenase subunit 5 (ND5) gene, which has been used for phylogenetic lineage studies (Colbourne et al., 1998; Crease et al., 2012). Subsequently, to ascertain the effects of competitors and natural enemies on the population dynamics, we utilized the temporal abundance data of *D. pulicaria* and its competitor *D. galeata* from a previous study (Tsugeki et al., 2022) and the catchment data of *D. pulicaria*'s main fish predator. By combining these results, we discuss the factors responsible for the invasion success of *Daphnia*.

Methods

Sampling site, sediment cores, dating, and individual collection

Lake Biwa is the largest lake in Japan with a surface area of 674 km^2 and a maximum depth of 104 m. The lake has a long history of over 400 thousand years as one of the most ancient lakes in the world (Hampton et al., 2018). It has been eutrophic since around the 1960s (Hyodo et al., 2008; Tsugeki et al., 2010; Hsieh et al., 2011) and is now in a mesotrophic condition. To unveil historical variation in the *Daphnia pulicaria* population in Lake Biwa, we used ephippia both with and without resting eggs collected from two sediment cores (namely LB1 and LB4 cores: Table S1) and from surface sediments (EK-L: Table S2), for which detailed sampling methods have been previously described (Tsugeki et al., 2021, 2022). Three 30 cm long core samples (LB1 and LB4 for ephippia, and LB7 for chronology) and the surface sediments (EK-L) were collected on 17 August 2017 using a gravity corer (inner diameter = 10.9 cm) and Ekman Bottom Grab, respectively, at the pelagic site (35°15′02″ N, 136°04′01″ E; water depth = 71 m; Fig. S1) onboard the Research Boat *Hasu* operated by the Center for Ecological Research (CER), Kyoto University. Each core was carefully sliced at 1 cm intervals from the surface to the bottom, and surface sediment samples were stored at − 80 °C prior to further analyses. Additionally, resting eggs were collected from the surface sediments on 9 March (EK-3: Table S2) and from the lake water on 21 April 2021 (Ie-1: Table S2); each 20 female *Daphnia* individuals were collected on 5 June 2018 and 13 May 2019 onboard the Research Boat *Hasu* at the Ie site (35°12′58″ N, 135°59′55″ E; water depth = 75 m; Fig. S1) where the regular survey is operated by CER, Kyoto University. Individual *Daphnia* females were vertically sampled at a depth of 0–20 m using a plankton net (mouth diameter, 15 cm; mesh size, 72 µm; Rigosha, Tokyo, Japan). Sediment chronology was performed for the LB7 core based on the constant rate of supply method of ^{210}Pb dating (Appleby & Oldfield, 1978) and verified using the ^{137}Cs peak traced between 1962 and 1963 (Appleby, 2001). Details of the chronological method and results have been reported elsewhere (Tsugeki et al., 2021, 2022).

D. pulicaria appeared in Lake Biwa in the late 1990s (Urabe et al., 2003; Tsugeki et al., 2022). Thus, the ephippia of *D. pulicaria* were obtained from the layer above the depth of 8.5 cm (each layer was expressed as mid-depth; 8.5 cm for the 8–9 cm layer), covering the period of its appearance after the late 1990s, for cores LB1 and LB4, except for two layers (4.5 cm and 7.5 cm of the LB1 core), totaling 16 sediment samples in two cores (Table S1). Ephippia were collected from the sliced and surface sediment samples through sieving using a 100 µm mesh and picked under a dissecting microscope at × 20 to × 40 magnification (Tsugeki et al., 2021). Since *D. pulicaria* and *D. galeata* species in Lake Biwa can be distinguished based on ephippia size, with a boundary length of approximately 860 µm between them (Tsugeki et al., 2021), *D. pulicaria* was identified based on the ephippia length via microscopic observation. At least 16 ephippia lengths from each layer were measured based on photographs taken by a digital camera, excluding those from the samples in which fewer than 16 complete ephippia were detected (Tsugeki et al., 2022). Ephippia identified as *D. pulicaria* were placed in 0.2 ml tubes and stored at − 80 °C for further genetic analysis (Table S1, Table S2). These ephippia were checked for the presence/absence of resting eggs through photographs. The *Daphnia* post-abdominal claw is utilized as an indicator of their population abundance (Hann, 1989; Verschuren & Marnell, 1997; Tsugeki et al., 2009) due to their stable preservation for more than 10,000 years (Kadota, 1984; Korponai et al., 2011). As individual *D. pulicaria* females can be distinguished from *D. galeata* based on differences in the claw (Benzie, 2005), we also used the population abundance of *D. pulicaria* and *D. galeata* obtained from the microscopic analysis of post-abdominal claw remains preserved in the LB7 core (for a detailed method, please see Tsugeki et al., 2022).

DNA extraction

DNA extraction from individual *Daphnia* females was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), whereas DNA extraction for ephippia, regardless of the presence or absence of resting eggs, was performed in accordance with the methods of previous studies (Ishida et al., 2012), which have been specifically developed

for the ephippium. Ephippia were individually transferred to 200 µl PCR tubes, to which 50 µl of alkaline lysis buffer (100 mM NaOH and 50 mM of disodium EDTA, pH 12) was added, and then, samples were subjected to five cycles of thermal shock, consisting of 5 min at − 80 °C and 20 s at 70 °C. The samples were then sonicated for at least 1 min using an HD 2070-U ultrasonic homogenizer (BANDELIN Electronic, Berlin, Germany) and then incubated at 95 °C for 30 min. Immediately thereafter, samples were stored on ice for > 3 min, after which a further 50 µl of neutralizing buffer (Tris–HCl, pH 5) was added to each tube and the tubes were stored at − 80 °C until further analysis. The DNA concentrations in the DNA extracts from the ephippia were quantified using a Qubit 4 Fluorometer with a dsDNA HS Assay Kit (quantification range: 0.1 to 120 ng per measurement; Thermo Fisher Scientific). Each measurement utilized 2 µl of DNA extract, with a lower quantification limit of 0.05 ng/µl, and the DNA concentrations ranged from under the quantification limit to 0.167 ng/µl. To avoid sample consumption, the DNA extracts below the quantification limit were not quantified.

Amplification of the mtDNA control region and their sequencing

We designed a nested PCR to amplify the mtDNA control region from resting eggs. Initially, DNA fragments were amplified using 12S (5′-TAACCGCGA CGGCTGGCAC-3′) and fMet (5′-GGGCATGAA CCCACTAGCTT-3′) primers, which bind to adjacent sequences of the control region (Lehman et al., 1995). The amplified PCR fragments were further subjected to nested PCR. Internal primers for the nested PCR were designed as follows: we obtained the sequence between the 12S and fMet primers by sequencing the DNA fragments (ca. 900 bp) amplified from a resting egg DNA sample of *D. pulicaria* in Lake Biwa, previously used to obtain ND5 sequences (DDBJ/GenBank/EMBL accession no. LC534943; Tsugeki et al., 2021). Based on the obtained sequence (accession no. 727786), the following internal primer pair was designed using the Primer3 function in DNADynamo (Blue Tractor Software, North Wales, UK) as follows: Dpl_12S (5′-ACTAGGCCCAAATATTTGTTTCCT -3′) and Dpl_tRNA-Ile (5′-CAACAAGTTGCATCA TCTACCCT-3′). Since only one sequence was available for *D. pulicaria* for the internal primer-binding regions (accession no. LS991509), we retrieved 28 additional complete mtDNA genome sequences of closely related *Daphnia pulex* Leydig, 1860 from the DDBJ/GenBank/EMBL database to check for compatibility of the Dpl_12S and Dpl_tRNA-Ile primers. The Dpl_12S and Dpl_tRNA-Ile primers had no mismatches with the *D. pulicaria* sequence and 26 *D. pulex* sequences; the remaining two *D. pulex* sequences had only one mismatch within five bases of the 3′-ends of the primers. Thus, we used Dpl_12S and Dpl_tRNA-Ile primers in the second PCR.

First PCR reactions were carried out in a 40 µl reaction volume containing 10 µl of DNA extract and 300 nM each of the 12S and fMet primers in 1 × KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, CA, USA); the thermal cycling conditions were as follows: 2 min at 95 °C; 40 cycles of 20 s at 98 °C, 15 s at 65 °C, and 1.5 min at 72 °C, and 2 min at 72 °C. Using 1 µl of the first PCR product as the template, additional PCR reactions were carried out in a 20 µl reaction volume containing 300 nM each of the Dpl_12S and Dpl_tRNA-Ile primers in 1 × KAPA HiFi HotStart ReadyMix using thermal cycling conditions of 2 min at 95 °C, 35 cycles of 20 s at 98 °C, 15 s at 60 °C, 1 min at 72 °C, and 2 min at 72°C. The same nested PCR protocol was used for adult samples, except that the first PCR was carried out in a 20 µl reaction volume using 4 µl of DNA extract as the template.

The second PCR products were subjected to 1% agarose gel electrophoresis using the E-Gel System (Thermo Fisher Scientific, Waltham, MA, USA). Target bands (ca. 800 bp) were excised from the gels, purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and sequenced from both directions using the Dpl_12S and Dpl_tRNA-Ile primers. Sequencing was outsourced to Eurofins Genomics (Tokyo, Japan). Forward and reverse sequences were assembled using SeaView (Gouy et al., 2010) to obtain sequences of the control region.

Amplification of mtDNA ND5 and its sequencing

This experiment was conducted exclusively on samples from which the mtDNA control region sequences were successfully obtained. Note that certain ephippia samples had insufficient amounts remaining and were consequently not subjected to ND5 amplification and

sequencing. The mtDNA ND5 gene was amplified with the primers DpuND5a (5′-ATAAAACTCCAA TCAACCTTG-3′) and DpuND5b (5′-GGGGTGTAT CTATTAATTCG-3′) (Colbourne et al., 1998) in a 20 µl reaction volume containing 6 µl (for ephippia samples) or 4 µl (for adult samples) of DNA extract and 300 nM of each primer in 1 × KAPA HiFi HotStart ReadyMix. The thermal cycling conditions were as follows: 2 min at 95 °C; 40 cycles of 20 s at 98 °C, 15 s at 54.5 °C, 1 min at 72 °C, and 2 min at 72 °C. For the ephippia samples, additional PCR reactions were carried out using 1 µl of the first PCR product as a template, with the same PCR conditions as the initial PCR reaction, except that the thermal cycling number was reduced to 35 cycles.

The PCR products were purified using illustra ExoProStar (Cytiva) and sequenced from both directions using the internal primers Dpl_ND5_seqF1 (5′-CAAAACTTAAGTAAGTAAGCCGAAC-3′) and Dpl_ND5_seqR1 (5′-GGGCTTAGCTTGAATTTT CC-3′) designed in this study. The sequencing was outsourced to Eurofins Genomics (Tokyo, Japan).

Haplotype network analysis

A haplotype network was constructed separately for the mtDNA control sequences and ND5 sequences to visualize the genetic relationships among the haplotypes identified in Lake Biwa in this study and known haplotypes from other populations.

As for the control region, all available sequences of *D. pulicaria* ($n = 30$) were retrieved from the DDBJ/EMBL/GenBank databases, wherein three short sequences (266–542 bp) and three sequences containing more than three undetermined bases (N) were eliminated from further analysis. The haplotype sequences, including the Lake Biwa sequence obtained in this study, were aligned using CLUSTALW (https://www.genome.jp/tools-bin/clustalw) and trimmed to the same length, specifically 667 bp including gaps, using DNA Dynamo. Then, two duplicate sequences from the same populations were removed. The aligned sequences in FASTA format (Supplementary File S1) were imported into R ver. 4.1.2 (R Core Team, 2021) using the ape package ver. 5.6.2 (Paradis & Schliep, 2019). A haplotype network was generated with the HaploNet function of the pegas package (Paradis, 2010).

Similarly, we retrieved all the available ND5 sequences of *D. pulicaria* ($n = 230$) from the DDBJ/EMBL/GenBank databases. After aligning them using CLUSTALW, the sequences were trimmed to 605 bp using DNA Dynamo, resulting in 101 sequences. Subsequently, we used these sequences, along with the sequence of *D. galeata* as an outgroup (Supplementary File S2), to construct a neighbor-joining tree using the Tajima-Nei model with 1000 bootstrap replicates by MEGA 11 (Stecher et al., 2020). From the resultant phylogenetic tree (Fig. S2), we extracted 20 sequences within the same clade as a haplotype from Lake Biwa (Supplementary File S3). These sequences were then employed to construct a haplotype network in the same manner used for the control region.

Competitor and enemy of *Daphnia pulicaria* and data analysis

In Lake Biwa, the 11 main fish species (Ayu *Plecoglossus altivelis* (Temminck & Schlegel, 1846), *Oncorhynchus* sp., *Cyprinus carpio* Linnaeus, 1758, *Carassius cuvieri* Temminck & Schlegel, 1846, *Carassius buegeri grandoculis* Temminck & Schlegel, 1846, *Gnathopogon caerulescens* (Sauvage, 1883), *Opsariichthys uncirostris* (Temminck & Schlegel, 1846), *Tribolodon hakonensis* (Günther, 1877), *Opsariichthys platypus* (Temminck & Schlegel, 1846), *Gymnogobius isaza* (Tanaka, 1916), and *Anguilla japonica* Temminck & Schlegel, 1846) composed about 75% on average of the total catch abundance for all the species (Fujioka, 2020). Biwa Ayu reached an abundance of over 75% among the main species for the last several decades (Fujioka, 2020) and mainly preyed on *Daphnia* species in the zooplankton community (Kawabata et al., 2020). *Daphnia galeata* and *Daphnia pulicaria* have been dominant *Daphnia* species in the zooplankton community for the last several decades (Liu et al., 2020). Therefore, we selected *Daphnia galeata* and Biwa Ayu as the main competitor and predator for *Daphnia pulicaria*, respectively.

Type II regression models, along with the standardized major axis, were used to determine the relationship between the abundance of *Daphnia pulicaria* and the predominant planktivorous fish Ayu, in addition to the abundance of competing native *D. galeata*. Historical catchment data for Ayu (https://

www.pref.shiga.lg.jp/ippan/shigotosangyou/suisan/18693.html) were obtained from the database of the Shiga prefecture. While the abundance of *D. galeata* was taken from sedimentary records (Tsugeki et al., 2022). Since the time resolution of sediment samples used for *D. pulicaria* population abundance differed depending on the period (Tsugeki et al., 2022), prior to statistical analysis, annual catchment data of Ayu were recalculated based on the average values within the year corresponding to the sedimentation period for each sample. Prior to Type II regression analysis, distribution was tested by performing the Shapiro–Wilk test (significance level $P < 0.05$). We then used Type II regression models to determine the relationship of *D. pulicaria* abundance with Ayu and *D. galeata*. All statistical analyses were performed using R ver. 4.1.2 (R Core Team, 2021) with the package "smatr" ver. 3.4.8.

Results

In total, 19 mtDNA control region sequences were obtained from the ephippia from the surface to 9 cm depth layers, covering the period of their initial introduction after the 1990s in two sediment cores (Table S1). The sequence data were obtained from 10 of 33 ephippia in the LB1 core and 9 of 63 ephippia in the LB4 core (Figs. S1, S3, Table S1). The percentage of extracted ephippia samples with identified sequences was 19.8% (19 identified among a total of 96 ephippia in two cores), which is indicative of a relatively low chance of acquiring sequence data. This could be explained by the observation that the majority of ephippia preserved in the sediment cores did not contain resting eggs; almost 80% (73 among a total of 96 ephippia: Table S1) lacked resting eggs. Conversely, among the ephippia for which sequence data were obtained, the majority contained resting eggs (15 out of the 19 ephippia with available data, constituting 78.9%). To expand the genetic data collection, we investigated ephippia collected from the surface sediment (EK-3, 15; EK-L, 7; Table S2) and lake water (Ie-1, 23; Table S2) including adult *Daphnia* females collected from water samples. Consequently, the sequence data were obtained from 45 total ephippia and 40 adult *Daphnia* females. All the 104 sequences obtained were identical, and thus belonged to a single haplotype. The sequence of this haplotype has been deposited in the DDBJ database under the accession number LC727786 (Table S3). Haplotype network analysis showed that this single haplotype from Lake Biwa had 12 point mutations to the most closely related haplotype detected in Lawrence Lake, Michigan, USA, and was more closely related to the haplotypes detected from populations in Michigan than those in Oregon (Fig. 1).

We obtained sequence data for the mtDNA ND5 gene from a total of 15 ephippia collected from the sediment cores, with 8 and 7 ephippia sourced from the LB1 and LB4 cores, respectively. Additionally, ND5 sequence data were acquired from a total of 27 ephippia (Ie-1, 23; EK-3, 4; EK-L, 0), and 40 adult *Daphnia* females. All 82 sequences obtained were identical to the previously reported ND5 sequences of *D. pulicaria* from Lake Biwa (accession nos. AB512006, LC534943, LC534944). A haplotype network showed that the single haplotype from Lake Biwa was closely related to the haplotypes detected from populations in Quebec and Manitoba, Canada, and Michigan, USA (Fig. 2, Table S4).

A significant negative correlation was detected between *D. pulicaria* abundance and Ayu (Type II regression model: $R^2 = 0.67$, $P < 0.005$, $n = 10$; Fig. 3a). Conversely, no significant relationship was observed between the abundance of *D. pulicaria* and that of *D. galeata* ($R^2 = 0.012$, $P = 0.76$, $n = 10$; Fig. 3b).

Discussion

The present study revealed the 20-year dynamics, specifically from the initial species introduction to the present, of the genetic structure of the introduced population of *D. pulicaria* in Lake Biwa. A total of 104 mtDNA control region sequences recovered from the ephippia collected from sediment cores, surface sediment involving lake water, as well as from adult females in lake water, were identical despite the fact that the mtDNA control region is known for its high variability (Straughan & Lehman, 2000; Dudycha, 2004; So et al., 2015). This single haplotype detected in Lake Biwa was novel and was most closely related to the haplotypes detected in *D. pulicaria* populations from North America (Michigan, Fig. 1). Consistent with the control region findings, all the mtDNA ND5 sequences retrieved from both the ephippia and

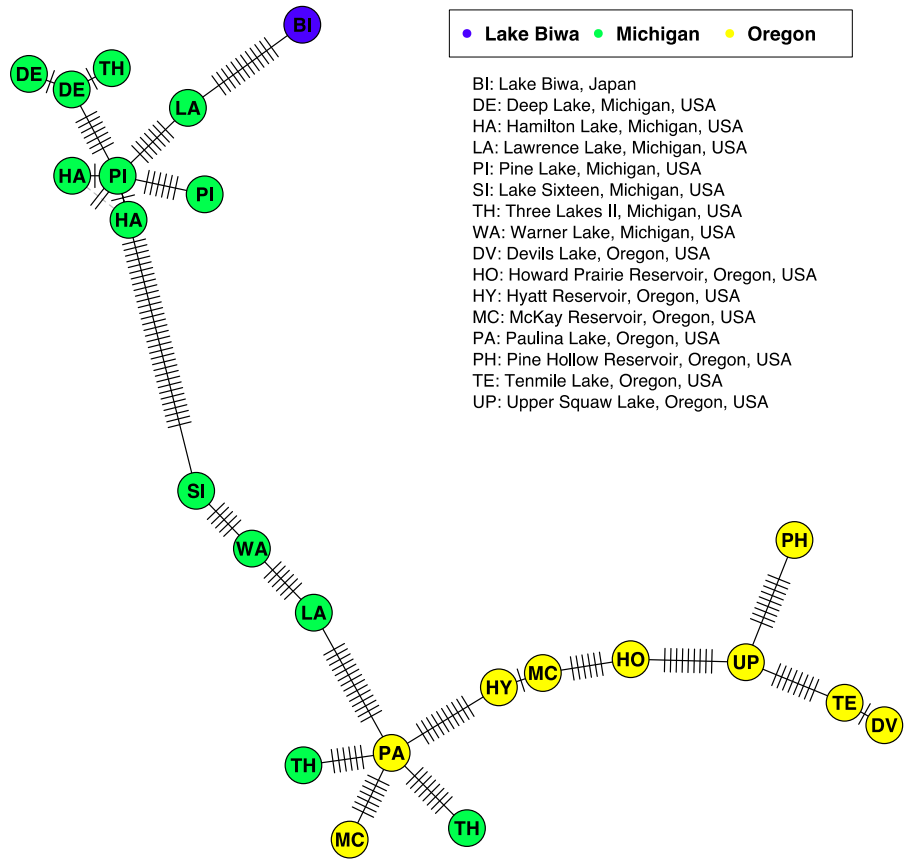

Fig. 1 Haplotype network based on partial sequences (667 bp including gaps) of the mitochondrial DNA control region. The haplotype from the *Daphnia pulicaria* population in Lake Biwa is shown with a blue circle and those from the populations in Michigan and Oregon, USA, are shown with green and yellow circles, respectively. The short lines on the branches represent mutations. The names of the populations are abbreviated by two letters as indicated in the legend within the figure. The accession numbers for the haplotypes are provided in Table S3

females were ascribed to a single haplotype, which was closely related to the haplotypes found in North America (Quebec, Manitoba, and Michigan, Fig. 2). A previous finding based on the mtDNA ND5 gene showed that *D. pulicaria* in Lake Biwa is more closely related to populations of North America than those of South America and Europe (Urabe et al., 2003; Tsugeki et al., 2021). Together, the Lake Biwa population might originate from a single clone, which was possibly derived from a region around North America, via a one-time introduction event. Thus, it is likely that genetic variance would not have affected the invasion success of the *D. pulicaria* population at Lake Biwa. In support of our speculation, a mesocosm experiment provided evidence that the establishment success of *Daphnia* species is not critically enhanced by multiple introduction events, unlike that for other zooplankton, such as copepods (Sinclair & Arnott, 2017). Moreover, some empirical studies have shown that introduced *Daphnia* populations are composed of one or a few genotypes at the early phase of establishment (Mergeay et al., 2006; Otake et al., 2022). Therefore, genetic variance in general might not affect the establishment of *Daphnia* in introduced areas, although our mtDNA sequence data cannot rule out that there was genetic variation in the nuclear DNA.

Previous studies have suggested that eutrophication is a driving force of the invasion success of *Daphnia* spp. (Brede et al., 2009; Ohtsuki et al., 2015; Monchamp et al., 2017). However, this seems unlikely in the case of *D. pulicaria* in Lake Biwa because this species appeared in the 1990s when eutrophication had already been greatly mitigated in the lake (Hyodo et al., 2008; Hsieh et al., 2011). It is known that the population size of *D. galeata*, which has been in Lake Biwa for the past century, greatly increased during the 1960s when eutrophication progressed in the lake (Tsugeki et al., 2003, 2022). Since eutrophication would affect the increase in zooplankton in general through the enrichment of primary production in the lake, biotic resistance would be another

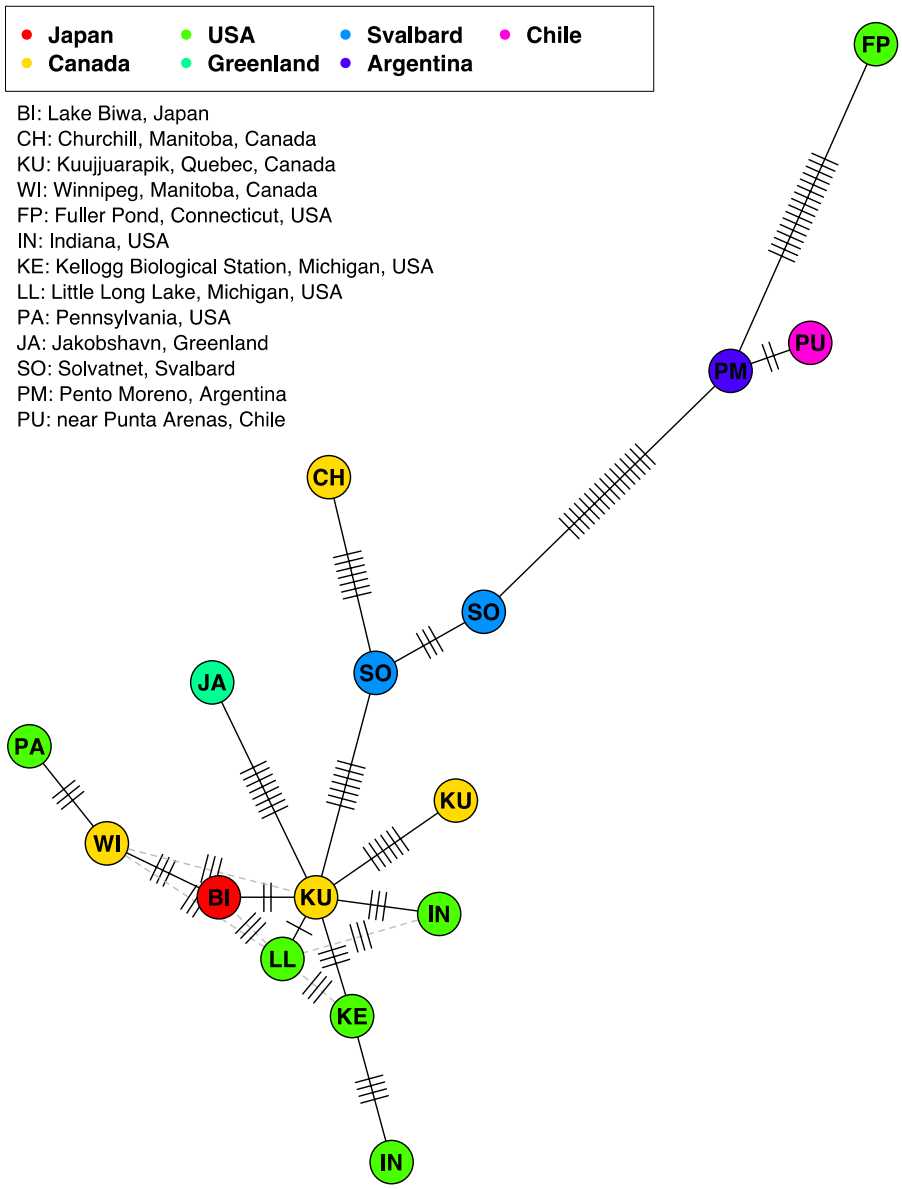

Fig. 2 Haplotype network based on partial sequences (605 bp) of the mitochondrial DNA ND5 gene. The short lines on the branches represent mutations. Alternative links are shown for a maximum of 3 mutations in dotted lines. The haplotype from the *Daphnia pulicaria* population in Lake Biwa is shown with a red circle. The names of the populations are abbreviated by two letters as indicated in the legend within the figure. The accession numbers for the haplotypes are provided in Table S4

possible mechanism underlying the invasion success of *D. pulicaria*. However, we did not find any significant relationships between the abundance of *D. pulicaria* and that of its competitor *D. galeata* (Fig. 3b). In addition, the abundance of the most dominant zooplankton in Lake Biwa, the copepod *Eodiaptomus japonicus* (Burckhardt, 1913), increased after the late 1990s (Liu et al., 2021; Nakane et al., 2023), suggesting an increase in competitive interactions. Therefore, the possibility of declining biotic resistance contributing to the spread of *D. pulicaria* in Lake Biwa is low.

Accordingly, we speculated the potential reason for the establishment success of *D. pulicaria* in Lake Biwa. We found a significant negative correlation between the abundance of *D. pulicaria* and that of the dominant planktivorous fish Ayu in Lake Biwa (Fig. 3a). Thus, reduced predation pressure would best explain the increase in *D. pulicaria* in Lake Biwa. The survival of large *Daphnia*, such as *D. pulicaria*, is known to be greatly affected by predation pressure since planktivorous fish generally prey preferentially on larger zooplankton (Vanni et al., 1990; Černý & Bytel, 1991). Such size-selective predation

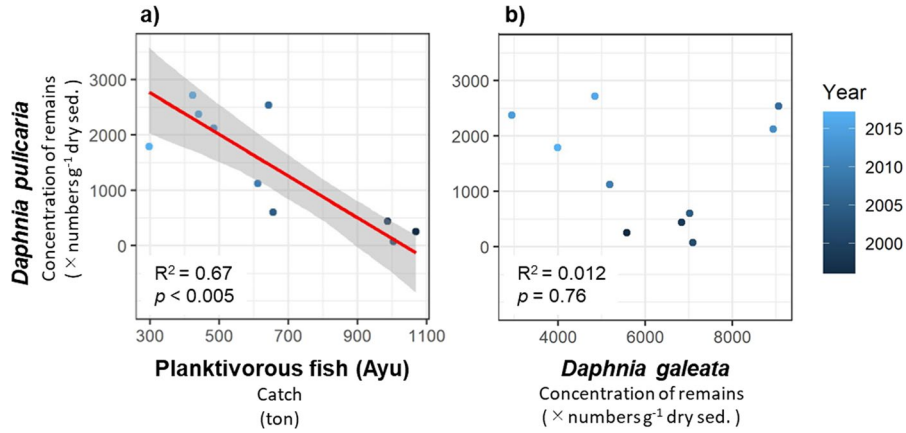

Fig. 3 Relationships among remaining *Daphnia pulicaria* (numbers dry sediment^{-1}) and **a** catchment of Ayu (ton year^{-1}) and **b** remaining abundance of *Daphnia galeata* (numbers dry sediment^{-1}). The red line denotes a Type II regression model with a 95% confidence interval indicated by the gray zone

is considered a reason for large *Daphnia* showing diel vertical migration, where they stay in the deeper layer of the water column during the day to escape predation (Lampert, 1993). A similar phenomenon was observed in Lake Biwa, where *D. pulicaria* tends to be distributed in deeper layers, as compared to the location of small *D. galeata* (Urabe et al., 2003). In fact, the abundance of planktivorous fish, including Ayu, has decreased since around the 1990s in Lake Biwa (Fujioka, 2020). *D. pulicaria* could thus successfully invade Lake Biwa by exploiting enemy-free space.

Resting eggs have been previously proposed to play a key role in the successful establishment of zooplankton, especially at the early phase of invasion (De Meester et al., 2002; Walsh et al., 2016). It is known that individuals of *Daphnia,* which hatched from resting eggs of the initial genotype, shifted to parthenogenetic reproduction and exhibited a rapid growth rate. This led to its successful establishment and thereby providing an advantage over subsequent genotypes (De Meester et al., 2002). For *D. pulicaria* in Lake Biwa*,* resting eggs also probably contributed to the maintenance of the population at the initial introduction phase because *D. pulicaria* temporarily disappeared from the water column immediately after its invasion in 1999 (Urabe et al., 2003). In line with our estimation, an empirical study of invasive zooplankton species (*Bythotrephes longimanus* Leydig, 1860) highlights that the resting egg in sediments allowed the species to endure severe conditions in the early invasion period, erupt, and then stabilize its population abundance under favorable conditions (Walsh et al., 2016). After approximately 2010, the resting egg production of *D. pulicaria* greatly decreased despite their population abundance remaining high (Tsugeki et al., 2022). Since predation pressure is recognized as a factor that stimulates resting egg production (Ślusarczyk, 1995, 2001), this decreasing phenomenon at Lake Biwa might suggest that the resting stage, which is an escape mechanism from predation pressure, is now less necessary than it was previously. Alternatively, deterioration in quantity and quality of available food might negatively affect resting egg production of *D. pulicaria*, as suggested previously (Smith et al., 2009). Further studies will be needed to test the stimulating signal of the resting stage.

In conclusion, our findings demonstrated that the *D. pulicaria* population in Lake Biwa originated from one genotype, possibly introduced from the North American region. *D. pulicaria* benefited from reduced predation pressure from planktivorous fish, which helped their establishment. Enemy-free space might thus be very vulnerable to invasion by *Daphnia*.

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contributed to the data analysis; K.U. contributed to primer design; N.T., I.H., and K.U. wrote the first draft of the manuscript; all authors revised and commented on the manuscript.

Data availability All data generated during this study are included in this published article.

Declarations

Conflict of interest The authors declare no conflict of interest.

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