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River dam prevents the invasion of non-native species of *Neocaridina*
Kubo, 1938 (Decapoda: Caridea: Atyidae) into native habitats: A case
study in the Yumesaki River system, Japan

Ryosuke Ishii^{1†} and Yusuke Fuke^{2,3}

¹ Graduate School of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan

²Animal Ecology Laboratory, Graduate School of Science, Kyoto University, Kyoto, Kyoto
606-8502, Japan

³Ecological Genetics Laboratory, National Institute of Genetics, Mishima, Shizuoka 411-
8540, Japan

[†]Ryosuke Ishii and Yusuke Fuke have equal contribution to this article.

Correspondence: Y. Fuke; e-mail: yfa73986@gmail.com

ABSTRACT

The range expansion of invasive species that threaten biodiversity is caused by repeated
cycles of their artificial introduction and subsequent dispersal. While dispersal can occur
without human intervention, its pattern is mainly determined by environmental factors and

species characteristics. Knowledge regarding the dispersal processes of invasive species and their subsequent interactions with native species is limited, which makes controlling invasive species and conserving native species difficult. We focused on the freshwater shrimps genus *Neocaridina* Kubo, 1938 in the Yumesaki River system, Japan, which is the habitat of the native species *N. denticulata* (De Haan, 1844) and has experienced the introduction of closely related invasive species. We examined the genetic population structures and morphological characteristics of the introduced species to elucidate their dispersal process. The results showed that populations comprising only the native species remained upstream of the Sugo Dam, whereas the native species was replaced by the introduced species *N. davidi* (Bouvier, 1904) at the other sites. Our results demonstrate that the distribution of invasive species can expand throughout the entire river system in rivers without structures that restrict the movement of aquatic organisms. Our findings highlight a rare example of a riverine structure that is generally detrimental to native species, acting as a bulwark for native species against invasive species.

KEY WORDS: Crustacea, freshwater shrimp, invasive species, MIG-seq, mitochondrial DNA, morphology

INTRODUCTION

Alien species can have significant impacts on native species, communities, ecosystems, and even human life via predation, resource competition, environmental modification, and the transport of parasites and diseases (Havel *et al.*, 2015; Diagne *et al.*, 2021). The expansion of the alien species' distribution results from repeated introductions and dispersal processes. Introductions can occur intentionally or unintentionally through human intervention (Levine, 2008; Havel *et al.*, 2015), while dispersal can occur without human intervention, with its pattern determined by the environmental factors and species characteristics (Pyšek *et al.*, 2010). We need to conduct case studies on the dispersal patterns of various invasive species to improve the management of invasive species and the conservation of native species.

Neocaridina davidi (Bouvier, 1904) is a freshwater shrimp with a landlocked life cycle that is native to China, South Korea, and Taiwan. It is also an invasive alien species that has been discovered in freshwater areas worldwide (Englund & Cai, 1999; Klotz *et al.*, 2013; Mitsugi *et al.*, 2017). *Neocaridina davidi* is a popular ornamental species in aquariums worldwide and also used as fishing bait. For this reason, its establishment outside its native range can be attributed to artificial events, such as the escape of aquarium stock and the release of surplus fishing bait (Niwa, 2010, 2011; Klotz *et al.*, 2013). This species has replaced the native freshwater shrimps (*Paratya improvisa* Kemp, 1917 and the closely related species *N. denticulata* (De Haan, 1844)) in some Japanese rivers, which has become a conservation issue (Katayama *et al.*, 2017; Onuki & Fuke, 2022). The specifics of its

dispersal process after introduction and interactions with native species, however, remain unclear, which hinders efforts to control and prevent the spread of the invasive species and conserve native species.

To clarify the invasion process by an alien species, we examined the distribution, morphological characteristics, and genetic population structure of species of *Neocaridina* in the Yumesaki River system, western Japan, where alien species have invaded the habitats of the native *N. denticulata*. The Yumesaki River basin was originally inhabited by *N. denticulata* (Niwa & Yokoyama, 1993a, b), but was later invaded by an unidentified species of *Neocaridina* assumed to be from China (Niwa *et al.*, 2005; Niwa, 2011). This unidentified species has been introduced since 1993 via escapees of fishing-bait stocks (Niwa, 2011). The consequences of the introduction of this species into the river remain unknown. This river system, therefore, presents an ideal location for examining the invasion processes of alien species and their interactions with native ones.

MATERIALS AND METHODS

Sampling

The collection of *Neocaridina* species was conducted on 2–3 September 2020 in the Sugo and Yumesaki rivers, Yumesaki River system (Himeji City, Hyogo Prefecture, Japan). Surveys were conducted at 10 sites (stn. 01–10; Fig. 1, Supplementary material Table S1)

along a section of the river 2.5–31.0 km from the estuary. Sampling was conducted by a single person (RI) at each site for 30 min using a 36 cm (width) hand net with a mesh size of approximately 2.5 mm. Sampling was conducted in an emerged plant zone. The collected shrimps were fixed in chilled 99.5% ethanol. The Yumesaki River system was surveyed for *N. denticulata* in 1985–1991, after which the presence of alien species was recorded (Niwa & Hamano, 1990; Niwa & Yokoyama, 1993a, b; Niwa, 2011). We assumed that habitat-use patterns were common across sexes and growth stages (Niwa & Hamano, 1990; Onuki, 2021).

Morphological analysis

The collected specimens were sorted according to sex based on the size and shape of the endopods of the first pleopods (Kubo, 1938). As *Neocaridina* shows sexual dimorphism in some traits (e.g., rostral length; Shih *et al.*, 2017) and one of the diagnostic traits is male-specific, only males were used in the following analyses. Measurements and counts of each trait were performed under a microscope (DSZT-44; Carton, Sagami-hara, Japan) using digital calipers (DN-150; Niigataseiki, Sanjo, Japan). The following trait values were obtained following Onuki & Fuke (2022): number of dorsal rostral teeth (RTD) and ventral teeth (RTV); rostral length (RL); postorbital carapace length (CL), an indicator of body size; and length and width of the endopod of the first pleopod (Supplementary material Table S2). The

RL:CL ratio and aspect ratio of the tip-to-base:width length of the endopod of the first pleopod (AE) effectively distinguish *N. denticulata* from *N. davidi* in Japanese populations (Onuki & Fuke, 2022). We also measured the excavation of the carpus of the first pereopod (FP) and the curvature of the propodus of the third pereopod (TP) for 24 individuals used in the genetic analyses (Supplementary material Table S3). FP was classified into three categories according to the degree of excavation (slightly excavated, excavated, and strongly excavated; Kemp, 1918; Onuki & Fuke, 2022). TP was calculated by subtracting the smaller angle at the center formed by the two lines connecting the center and each terminal of the propodus of the third pereopod from 180°. TP and FP also tend to differ between the two species (Kemp, 1918; Klotz *et al.*, 2013), but recent observations suggest that these traits are unsuitable for species identification (Onuki & Fuke, 2022).

All statistical analyses and visualizations were performed using R 4.1.1 (R Core Team, 2023). We used Kruskal–Wallis test to test differences for all morphological characteristics between populations. For traits with significant differences, we performed multiple comparisons using Fisher’s exact probability test for FP and the Steel-Dwass test for the other traits. The Steel-Dwass test was performed using the R package NSM3 (pSDCFlig function; method = "Monte Carlo"; seed = 0) (Schneider *et al.*, 2021). The significance level was set at 5%. A significant correlation was observed between AE and CL ($P = 0.002$, $\rho = -0.27$, Spearman's rank correlation test) and between RTV and CL ($P < 0.001$, $\rho = 0.38$,

Spearman's rank correlation test; Supplementary material Fig. S4). The corrected values, which excluded the effects of CL, were used for analysis and illustration. The residuals from a linear model analysis with AE as the dependent variable and CL as the independent variable for AE using the lm function and from the Poisson regression model estimated by a generalized linear model analysis, with RTV as the dependent variable and CL as the independent variable for RTV using the glm function, were used as the datasets. The residuals were obtained using the residuals function.

DNA extraction and mitochondrial DNA (mtDNA) sequencing

DNA was extracted from the fifth pereopod or pleopod of 24 males (5–9 individuals per site) using a Genomic DNA Purification kit (Promega, Madison, WI, USA). A fragment of the mitochondrial COI region was amplified by PCR using primers LCO-nc (5'–TTG TCA ACA AAC CAC AAA GAY ATT GG–3') and HCO-nc (5'–ATA CYT CAG GGT GYC CAA AGA ATC A–3') (Onuki & Fuke, 2022), with a 15- μ l reaction mixture containing 0.3 μ l of PrimeSTAR GXL DNA polymerase (Takara Bio, Kasatsu, Japan), 3.0 μ l of 5 $\times$ PrimeSTAR GXL buffer, 1.2 μ l of dNTP mixture, 0.9 μ l of each 5 μ M primer, 1 μ l of template DNA, and 7.7 μ l of ultrapure water. The PCR amplification conditions were as follows: initial denaturation (98 °C, 30 s); 30 cycles of denaturation (98 °C, 10 s), annealing (55 °C, 15 s), and extension (68 °C, 45 s), and a single final extension (68 °C, 30 s). The PCR products

were purified using Illustra ExoProStar (Cytiva, Tokyo, Japan) and sequenced from both the 5' and 3' ends. Sanger sequencing was outsourced to the Eurofins Genomics (Tokyo, Japan). The obtained sequences were confirmed and edited for base calls using Unipro UGENE 49.0 (Okonechnikov *et al.*, 2012). All sequences were deposited in the International Nucleotide Sequence Database via DNA Data Bank of Japan (DDBJ) (Accession numbers LC822759–LC822782; Supplementary material Table S3).

mtDNA analyses

A haplotype-based analysis of mtDNA was performed to estimate the population structure and phylogenetic positions of the examined specimens. COI haplotypes were detected using vsearch 2.23.0 (Rognes *et al.*, 2016). The haplotypes of *Neocaridina* species from previous studies in Japan were included in the analysis for comparison. Only those species identified based on morphological traits were used (Shih *et al.*, 2017; Fuke *et al.*, 2021, 2024; Onuki & Fuke, 2022). The new haplotypes we detected for the first time were assigned haplotype names according to Onuki & Fuke (2022). Using this dataset along with *N. spinosa* and *N. hofendopoda* as outgroups (Shih *et al.*, 2007; Chen *et al.* 2020), a phylogenetic tree estimation based on the maximum likelihood method using IQ-TREE 2.2.2.6 (Minh *et al.*, 2020) was performed with the codon partitions model. Substitution models were selected using ModelFinder (Kalyaanamoorthy *et al.*, 2017) based on the Bayesian information

criterion (BIC) (first codon TN+F+I, second codon TNe+G4, third codon TIM3+F+R2). The reliability of each branch was assessed using the ultra-fast bootstrap method (Hoang *et al.*, 2018). The obtained tree was visualized using FigTree 1.4.4 (<https://github.com/rambaut/figtree>). A haplotype network was constructed based on the TCS algorithm using PopART to visualize the distribution of haplotypes (Clement *et al.*, 2000; Leigh & Bryant, 2015).

MIG-seq analyses

We performed multiplexed inter-simple sequence repeat (ISSR) genotyping by sequencing method (MIG-seq; Suyama & Matsuki, 2015) to assess the genetic characteristics of the specimens based on genome-wide single nucleotide polymorphisms (SNPs). We prepared the MIG-seq library as described by Suyama *et al.* (2022) and Watanabe *et al.* (2020). The library was outsourced to Macrogen Japan (Tokyo) and sequenced using HiSeq X (Illumina, San Diego, CA, USA). The raw data were deposited in the DDBJ Sequence Read Archive (DRA) under Bioproject PRJDB18192 (RUN ID: DRR585202–DRR585225; Supplementary material Table S3).

Quality control and trimming of the obtained raw reads were performed using fastp 0.23.4 (Chen *et al.*, 2018). All reads were trimmed to 134 bases, the first 17 bases of Read 1 and Read 2 were trimmed, reads containing low-quality bases (<Q30) and adaptor sequences

were removed.

We performed SNP calls using the `denovo_map.pl` pipeline in Stacks 2.64 (Rochette *et al.*, 2019) with the following setting: minimum depth ($m = 3$), maximum genetic distance allowed between stacks ($M = 5$), minimum percentage of SNPs shared by all individuals ($R = 0.5$), minimum minor allele frequency ($\text{min-mac} = 2$), maximum observed heterozygosity ($\text{max-obs-het} = 0.75$), and only the first SNP per locus (`write-single-snp`). Downstream analyses were conducted on the files generated in PLINK format.

Principal component and clustering analyses were performed to assess genetic population structure. Principal component analysis (PCA) was performed using PLINK v1.90b5 (Purcell *et al.*, 2007). Clustering analysis was performed using ADMIXTURE 1.3.0 (Alexander *et al.*, 2009), for $K = 1-5$, with 100 iterations at different seed values. The results were summarized based on CV-error values. We performed a reanalysis for each K based on the seed value that showed the lowest CV-error to obtain the final output. These analyses and result visualizations were performed in R using custom scripts.

To assess the gene flow and dispersal ability of *Neocaridina* species, the migration rate (m) and direction among sites during recent generations were estimated using BayesAss 3.0.5 (Wilson & Rannala, 2003). The input data were the same as those used for ADMIXTURE analysis. Markov Chain Monte Carlo (MCMC) was run 50,000,000 times and sampled at 1,000 intervals. The mixed parameters for migration rate, allele frequency, and inbreeding

coefficient (m, a, f) were adjusted between 0.2 and 0.6, aiming for final adoption rates between 20% and 60%. The first 10% of the samples were excluded as burn-in, and the effective sample size (ESS) of all parameters was checked to be > 200 using Tracer 1.7.2 (Rambaut *et al.*, 2018). We interpreted that migration was present if $m > 0.1$.

RESULTS

Longitudinal distribution of *Neocaridina* in the Sugo River

Species of *Neocaridina* were collected from 8 of the 10 survey sites in the Yumesaki River system, excluding Stn. 01 at the downstream end and Stn. 10 at the upstream end. The total number of males was 136. The majority of individuals were collected from the midstream sampling sites (stns. 05 and 06), which together accounted for approximately 45% of the total collection (Supplementary material Table S1).

Morphological analyses

Analysis of all individuals revealed differences between sites for all four traits: RTD, RTV, RL/CL ratio, and AE ($P < 0.036$, Kruskal-Wallis rank sum test). Traits other than AE were statistically significant only between one population pair: RTD differed between stns. 03 and 04, RTV differed between stns. 05 and 09, and the RL/CL ratio differed between stns. 02 and 09 ($P = 0.043, 0.046$, and 0.026 , respectively; Steel-Dwass test; Fig. 2). The AE showed

distinct differences between the upstream (stns. 08, 09) and middle and downstream (stns. 03–07) populations ($P < 0.021$, Steel-Dwass test; Fig. 2). The elongated endopods found in the upstream populations are characteristic of *N. denticulata* on Honshu (Onuki & Fuke, 2022).

For the 24 individuals used in the genetic analyses, differences were also found between sites for FP and TP ($P = 0.010$ and 0.015 , respectively; Kruskal-Wallis rank sum test). Multiple comparisons revealed significant differences between upper and lower site populations for both FP (stn. 02 vs. stn. 09, $P = 0.036$; Fisher's exact probability test) and TP (stn. 02 vs. stn. 08, $P = 0.037$; stn. 02 vs. stn. 09; $P = 0.005$, Steel-Dwass test) (Supplementary material Fig. S5).

mtDNA analyses

The mtDNA analysis based on a 669 bp COI revealed 12 COI haplotypes (six haplotypes of *N. denticulata* and six haplotypes of *N. davidi*) in 24 individuals from four sites on the Yumesaki River system. *Neocaridina denticulata* haplotypes were detected at stns. 08 and 09, whereas *N. davidi* haplotypes were found at stns. 02, 06, and 08 (Fig. 3A). All *N. denticulata* haplotypes were common to those from the Lake Biwa Basin (Onuki & Fuke, 2022). We also identified three new haplotypes of *N. davidi* (dvA07, dvB24, and dvC02). Phylogenetic analysis showed that *N. davidi* haplotypes, including the novel ones, were grouped into three

clades: A, B, and C (Fig. 3B).

MIG-seq analyses

The PCA showed two clusters based on 561 genome-wide SNPs for the 24 individuals from four sites. One cluster included all individuals from stn. 09, a site above the dam, and part of stn. 08, just below the dam. The other included individuals from downstream sites. One individual from stn. 08 was located between two clusters (Fig. 4A).

The ADMIXTURE analysis, consistent with PCA results, indicated that the optimal number of clusters (K) based on CV-error values was two (Supplementary material Fig. S6). The distribution of clusters at $K = 2$ was divided into upstream and downstream groups around stn. 08, with both clusters present at this site (Fig. 4B). One individual from stn. 08 contained components from both clusters and could be considered a putative hybrid between clusters. BayesAss estimates of migration rates between populations over the past few generations detected only migration from stn. 09, above the dam to stn. 08, downstream of the dam ($m = 0.11$; Fig. 4C).

DISCUSSION

Species identification based on DNA and morphology

We utilized genomic and morphological analyses to examine difficult-to-distinguish shrimps,

revealing the distribution patterns of native and non-native species at the water-system level. We detected *N. denticulata* upstream and *N. davidi* downstream of the dam using the mtDNA and genome-wide SNP data, and showed that the two species co-occurred at a site immediately downstream of the dam. All six mtDNA haplotypes found in *N. denticulata* were shared with the populations around Lake Biwa (Onuki & Fuke, 2022). Despite the Yumesaki and Yodo river systems (to which Lake Biwa belongs) being approximately 100 km apart in a straight line, both locations fall within the same biogeographical region for many freshwater fish species (Tominaga *et al.*, 2016; Kitanishi *et al.*, 2016; Miyake *et al.*, 2021). This distribution pattern is explained by the paleo-watersheds being thought to have existed during the last glacial maximum containing both river systems (Sato & Yasuda, 2022). The sharing of haplotypes between water systems is, therefore, compatible with *N. denticulata* being native to the Yumesaki River system.

Six haplotypes were identified in *N. davidi* samples, and included in three clades (Clades A, B, and C; Onuki & Fuke, 2022). Clade C was consistent with the mtDNA sequences of specimens previously identified as *N. palmata* (Chen *et al.* 2020; Jiang *et al.* 2023). Our results and those of Onuki & Fuke (2022), which examined genome-wide SNPs and morphology, indicated no separation between Clades C and A or B, so we classified these three clades as *N. davidi*. Some *N. davidi* haplotypes detected in Japan are shared with Chinese populations, indicating that Clades A, B, and C in Japan are of exotic origin (Onuki

& Fuke, 2022; Cui *et al.*, 2023).

Morphological analysis also supported the results of the mtDNA analysis, which showed the coexistence of the two species in the Sugo River. AE, the diagnostic characteristics of the two species, showed that the populations at stn. 08, just below the dam, and stn. 09, upstream of the dam, differed from the other populations, suggesting that they were belonged to the native species *N. denticulata*. Although elongated endopods are considered a character of *N. koreana*, previous studies have shown that the Honshu population of *N. denticulata* also exhibits this characteristic (Kamita, 1951, 1953; Onuki & Fuke, 2022), which is supported by our results. The downstream populations showed characteristics of the invasive species *N. davidi*, such as a short rostrum and broad endopods (Klotz *et al.* 2013; Onuki & Fuke, 2022). Although there were differences in the rostral teeth among some populations, they were not useful for distinguishing species. Rostral length and endopod shape are useful for species identification, suggesting that the populations not genetically analyzed (i.e., stns. 03–05 and 07) are also composed of the alien species, as are the population at stn. 02.

Origin and expansion process of alien species

The first survey of the distribution of *Neocaridina* in the Yumesaki River system in about 30 years provided insights into the expansion of the distribution of an alien species. The native species *N. denticulata* was found throughout the Yumesaki River system (Niwa & Yokoyama,

1993a, b) in 1990, but today it has been replaced by the invasive species *N. davidi*, except in the upper reaches of the Sugo Dam. According to interviews with residents along the river by Niwa (2011), the escape of Chinese *Neocaridina* species, that had been stocked downstream of a dam, occurred around 2000. Although escapes and releases of invasive species may have occurred in other locations at other times, our results suggest that *N. davidi* can rapidly expand its distribution throughout a water system, displacing native species in river channels lacking structures that block migration. River dams generally have significant adverse effects on freshwater organisms due to habitat fragmentation and environmental modification (Morita *et al.*, 2009; Kano *et al.*, 2016; Reid *et al.*, 2019; Wu *et al.*, 2019). Freshwater shrimps, such as the species of *Neocaridina*, *Caridina* H. Milne Edwards, 1837, and *Macrobrachium* Spence Bate, 1868 tend to migrate upstream in rivers, and river structures act as barriers to migration (Hamano & Hayashi, 1992; Niwa & Yokoyama, 1997; Hongjamrassilp *et al.*, 2021; Bauer, 2023). Our results provide a rare example of a structure (the Sugo Dam) protecting a native species by preventing the invasion of alien species (Zaccara *et al.*, 2021). Because gene flow from upstream to downstream of the dam was detected, preventing the spread of alien species would not be possible if they had been introduced upstream.

Our results suggest that hybridization with native species may have occurred only partially during the spread of *N. davidi*. Our data showed that the hybridization zones were

limited to the contact zone (Stn. 08), where the migration of native species occurred in one direction. The lack of evidence for hybridization between native and non-native species, except in the contact zone, suggests that the spread of non-native species occurred without interspecific hybridization or that the genetic regions of native species were rapidly purged. Differences in the morphological characters of non-native populations across sites may be explained by phenotypic plasticity or past hybridization with native species. Future high-resolution analyses should enable examining the extent and role of interspecific hybridization in the species-replacement process.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Crustacean Biology* online.

S1 Table. Survey sites and number of samples.

S2 Table. Morphological data (all individual dataset).

S3 Table. Morphological data and DNA accession numbers (24 individual dataset).

S4 Figure. The relationships between each trait and CL.

S5 Figure. Results of the morphological analysis of 24 individual dataset.

S6 Figure. Plot of the CV-error for the ADMIXTURE analysis.

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List of captions

Figure 1. Study area for the distribution of species of *Neocaridina*. The closed and open circles represent the locations where shrimps were present and absent, respectively.

Figure 2. Four morphological trait values of adult male *Neocaridina* species from eight sites in the Yumesaki River system. The number of ventral rostral teeth and aspect ratios of the endopod of the first pleopod correlated with carapace length were used as differences from the regression model. The letters above the violin plots summarize the results of the Steel-

Dwass test, with different letters representing statistically significant differences at $P < 0.05$.

Figure 3. Population structure analysis based on mtDNA COI region (669 bp). Haplotype network estimated using the TCS algorithm (**A**). The bars and numbers represent the number of base substitutions; the dots represent the hypothetical haplotypes. Phylogenetic tree estimated using the maximum-likelihood method (**B**). Haplotypes identified in this study are highlighted in blue at the tips. The numbers on the branches represent the support values calculated by the ultrafast bootstrap.

Figure 4. Population structure analysis for *Neocaridina* species in Yumesaki River system based on 561 SNPs obtained by MIG-seq. Stn. 09 is upstream of Sugo Dam; the other stations are downstream sites. Principal component analysis (**A**), bar plots of ADMIXTURE analysis (**B**), and chord diagram of migration flows (**C**). The ADMIXTURE analysis was repeated 100 times for $K = 1-5$, each time with a different seed value. The results show the runs with the lowest CV-error values. The top panel represents mitochondrial clades. Migration rate (m) between populations estimated by BayesAss. Values with $m > 0.1$ are shown in arrows.

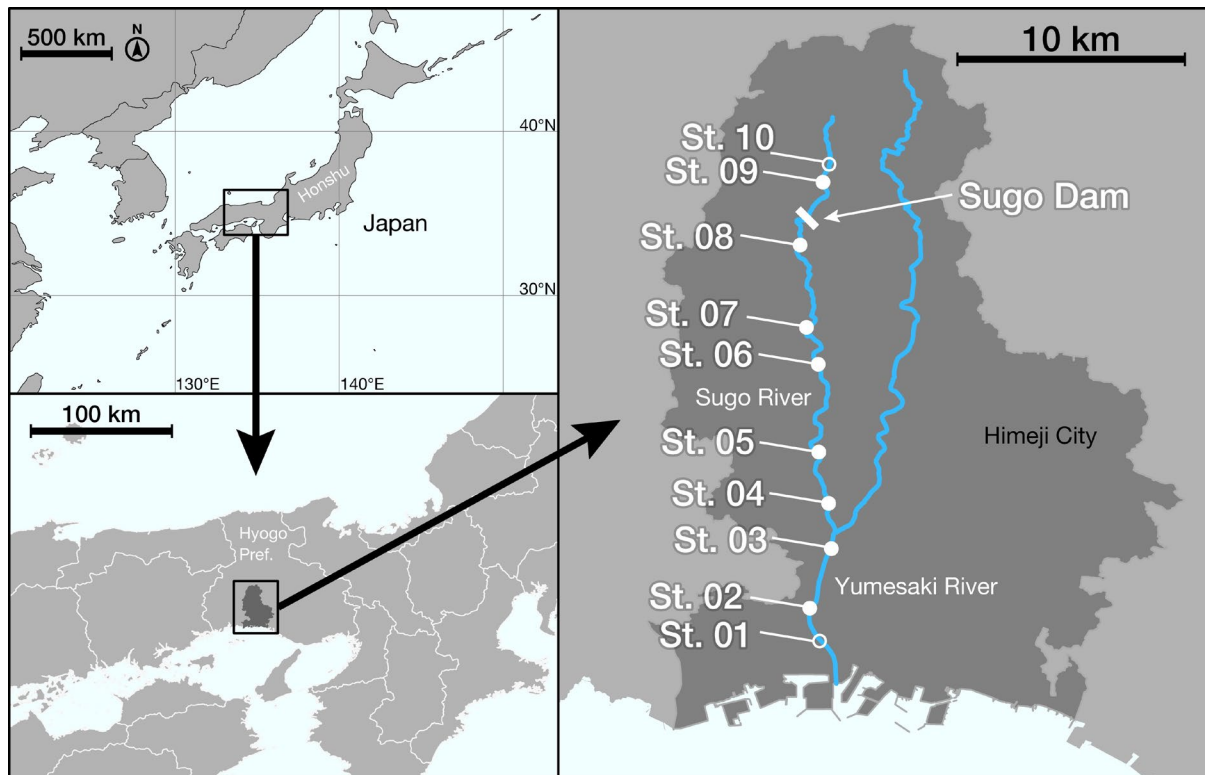


Figure 1

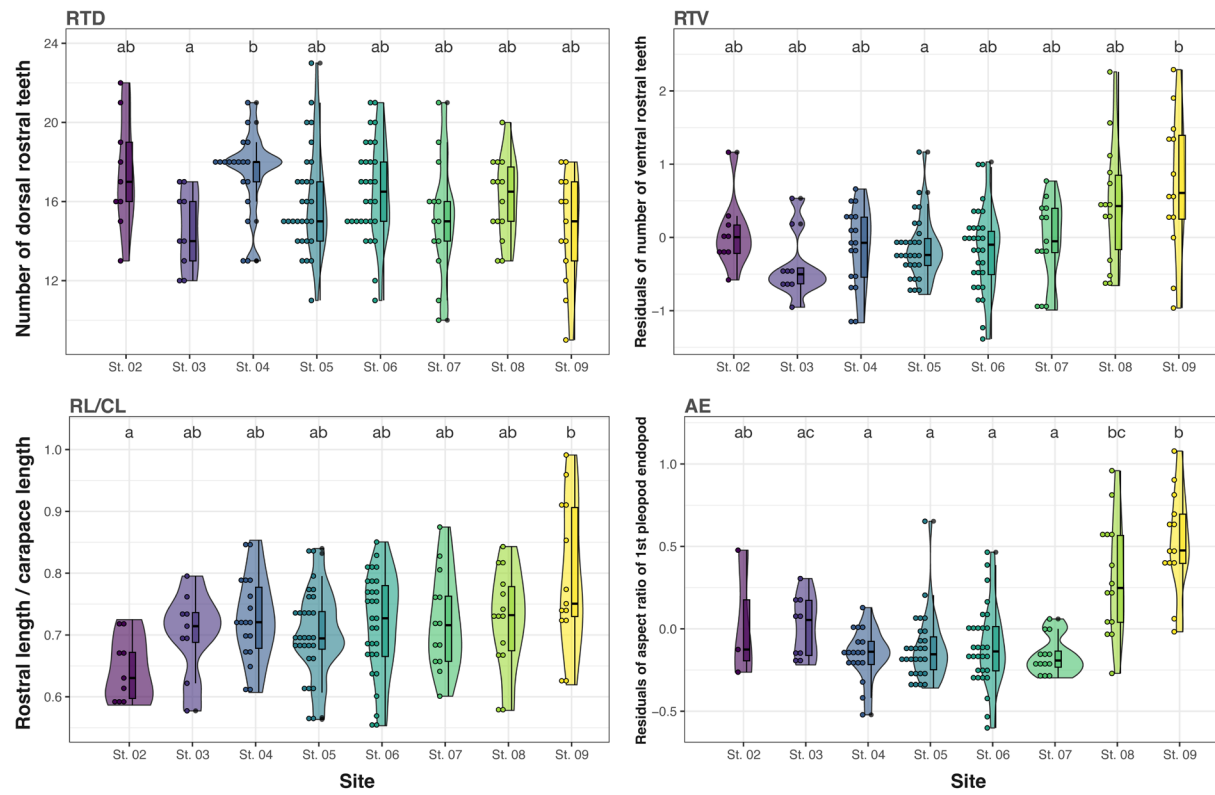


Figure 2

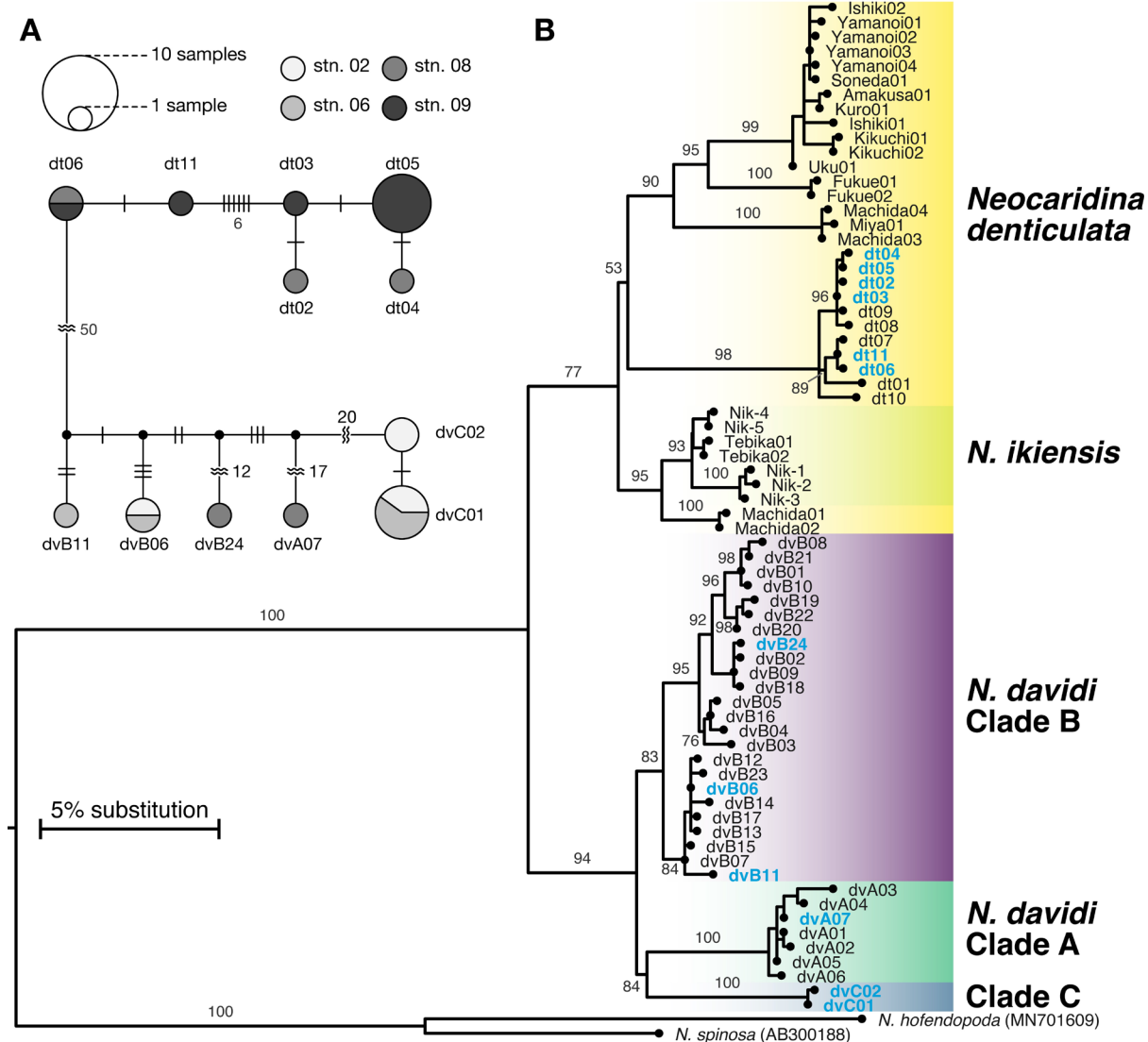


Figure 3

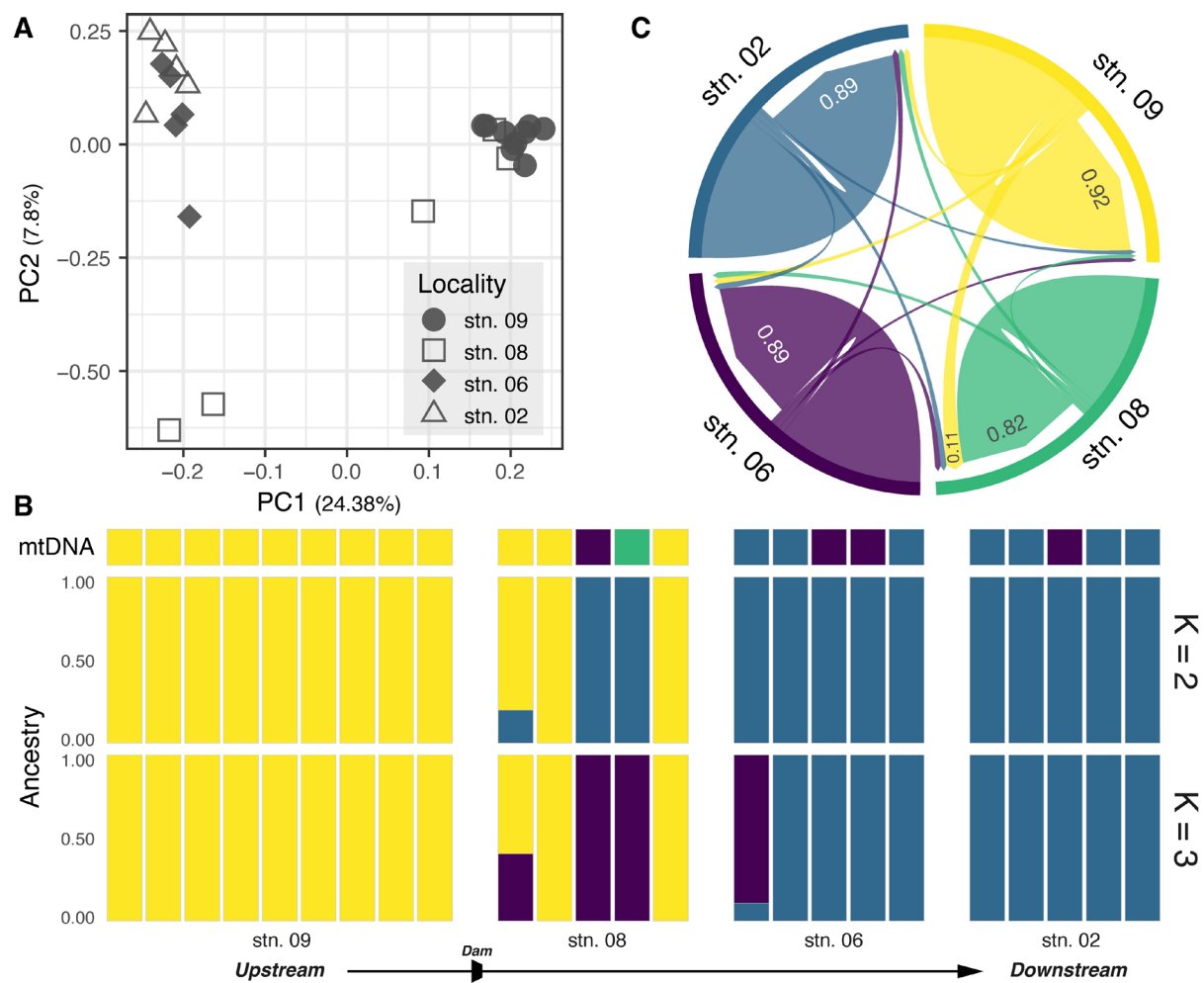


Figure 4