

# Distribution and parthenogenetic fecundity of obligate and facultative parthenogenetic strains of the mayfly *Ephoron shigae*

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**Abstract:** The polymitarcyid mayfly *Ephoron shigae* (Takahashi, 1924) generally propagates via sexual reproduction, but a parthenogenetic-dominant monophyletic strain derived from western Japanese sexual-dominant strains is widely distributed in Japan. This species may provide a case study on distribution patterns of genetic divergence in sexual-dominant and parthenogenetic-dominant strains of aquatic insects. In this study, we sought to characterize the distribution of parthenogenetic-dominant and sexual-dominant strains of *E. shigae* in eastern Japan and to assess whether there was sex ratio bias among local populations with and without the parthenogenetic strain, as well as the parthenogenetic reproductive success of both strains. We found that in eastern Japan, the parthenogenetic-dominant strain with the cytochrome c subunit I haplotype C1 is more widespread than sexual-dominant strains and that there are sites where both parthenogenetic-dominant and sexual-dominant strains coexist. There was no sex ratio bias at sites where the parthenogenetic-dominant strain was not observed, but at sites with both reproductive strains, the sex ratio was skewed toward females (64–99%), though the degree of bias depended on the relative frequency of the 2 strains. We found no evidence that females of the parthenogenetic-dominant strain mated after emergence; thus, in eastern Japan, females of the parthenogenetic-dominant strain may not mate even when males are present, and they pass through generations by parthenogenesis. The success rates of unfertilized oocytes of the parthenogenetic-dominant strain varied, with developmental success rates of 70% or higher. Oocyte batches with extremely low success rates (<10%) were also observed at 2 of the sites with both strains. Embryo developmental success of unfertilized oocytes in sexual-dominant strains was generally low (<30%), but exceptionally high rates of >90% were observed. At sites with both strains, the degree of developmental success of unfertilized oocytes in the parthenogenetic-dominant strain may be related to its relative frequency. Examining the causes of the distribution of these strains, and how they may change in the future, will provide new perspectives on reproductive modes and geographic parthenogenesis.

**Key words:** geographic parthenogenesis, diploid thelytoky, sexual reproduction, high-resolution melting analysis, automixis, *Ephoron shigae*, Polymitarcyidae

Parthenogenesis, an asexual mode of reproduction by a sole female that does not require fertilization by a male partner, has been reported across a variety of eukaryotes. Although sexual reproduction is more common by far, the presence of parthenogenesis in a given population effectively doubles fertility because all individuals in the population are potentially fertile (Maynard Smith 1978). Parthenogenetic species or strains often have geographically distinct distributions from sexual relatives, a tendency known as geographic par-

thenogenesis (Vandel 1928). These parthenogenetic populations are often reported in marginal ranges, at higher latitudes and altitudes, and on islands or in island-like areas. Because parthenogenesis does not require males and leads to high fecundity, species or strains capable of this asexual reproductive mode are well suited for colonization. In some cases, parthenogenetic populations successfully expand or compete as invasive species or agricultural pests (Mergeay et al. 2006, Hoffmann et al. 2008, Gutkunst et al. 2018, Yano

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et al. 2021). In addition, ecological differentiation between parthenogenetic and sexual relatives has also been reported to cause coexistence within the same habitat (Verduijn et al. 2004, Agh et al. 2007, Lehto and Haag 2010, Schmit et al. 2013, Rossi et al. 2017).

In bisexual populations (i.e., mixed-sex populations in which both males and females occur), there can be facultative parthenogenesis, a reproductive mode in which a female produces offspring either by parthenogenesis (laying unfertilized oocytes without mating) or by sexual reproduction (mating with a male). Examples of geographic parthenogenesis through facultative parthenogenesis have been reported for mayflies, stick insects, and harvestmen (Sweeney and Vannote 1987, Burns et al. 2018, Nakano et al. 2019, Liegeois et al. 2021). In the geographically parthenogenetic stick insect *Clitarchus hookeri* (White, 1846) in New Zealand, females in unisexual populations are highly parthenogenetic, but females in bisexual populations have also been reported to exhibit a similar parthenogenetic capacity (Morgan-Richards et al. 2010). Further, facultative parthenogenesis can lead to sex ratios that are female biased. Assuming a 50:50 sex ratio in populations with solely sexual reproduction, a female-biased sex ratio would be expected in populations containing both parthenogenetic-dominant and sexual-dominant strains, with the degree of bias depending on the relative frequency of the 2 strains. Depending on the population and site of the river or stream, the sex ratio may be biased (any deviation from 50:50) or almost or completely female only, and this bias can affect the parthenogenetic capability of a population. For example, it has been reported that for mayflies, the more female-biased the sex ratio, the greater the developmental success of parthenogenetic embryos (Liegeois et al. 2021). It is also possible that for mayflies, sex ratios could shift along a river or stream and over time. As such, the mayfly could be an important and valuable subject for studying the evolutionary maintenance of sexual reproduction.

*Ephoron shigae* (Takahashi, 1924) is a polymitarcyid burrowing mayfly that inhabits the midreaches of rivers and streams. This mayfly is distributed in the Russian Far East, Northeast China, the Korean Peninsula, and Japan (Ishiwata 1996, Quan et al. 2002). In Japan, some rivers have both male and female populations, whereas others have unisexual (female-only) populations (Watanabe and Ishiwata 1997, Tojo et al. 2006). The species is therefore considered to exhibit geographic parthenogenesis, but the distribution of unisexual populations has no clear geographic trend among rivers in Japan. Unisexual populations of *E. shigae* primarily generate diploid females (Sekiné and Tojo 2010a) and, rarely, males (Sekiné et al. 2015a) by parthenogenesis. The females in bisexual populations also exhibit parthenogenesis (i.e., facultative parthenogenesis), but the developmental success of their unfertilized oocytes is considerably lower than in unisexual populations (Tojo et al. 2006, Sekiné and Tojo 2010b).

Molecular phylogenetic analyses using mitochondrial DNA cytochrome c subunit I (COI) show apparent genetic differentiation between eastern and western Japanese bisexual populations (Sekiné et al. 2013, 2015a). In contrast, all females from unisexual populations across Japan have a common haplotype, C1, and are included in a monophyletic parthenogenetic-dominant strain that originated in western Japanese bisexual populations (Sekiné et al. 2015a). Thus, it is likely that females of the parthenogenetic-dominant strain dispersed from western Japan and subsequently colonized eastern Japan. Recent research suggests that the expansion of the parthenogenetic-dominant strain has led to a transition from bisexual to unisexual populations in the downstream area of the Abukuma-gawa River in eastern Japan within the past 20 y (Sekiné and Tojo 2019). However, many males and females of sexual-dominant strains continue to inhabit the upstream areas of the river, and the coexistence of both strains can be observed in the middle area of the river (Sekiné and Tojo 2019).

In this study, we sought to understand the evolutionary maintenance of bisexual and parthenogenetic reproduction among *E. shigae* in rivers in eastern Japan. Specifically, we wanted to characterize the distribution of sexual-dominant and parthenogenetic-dominant populations across downstream, middle, and upstream areas of the Abukuma-gawa River, and we asked 1) whether sex ratios would be female-biased in bisexual populations and 2) whether the development of parthenogenetically produced oocytes would differ between bisexual and unisexual populations. In addition, it is assumed that where a parthenogenetic-dominant strain is present, such as downstream areas of the Abukuma-gawa River, that parthenogenetic females reproduce mainly by parthenogenesis, but it is not known whether facultative parthenogenesis occurs in these situations (i.e., whether females of the parthenogenetic-dominant strain are able to mate with males and reproduce by sexual reproduction). Therefore, we investigated whether parthenogenetic females reproduce via both sexual reproduction and parthenogenesis at sites where both sexual-dominant and parthenogenetic-dominant strains coexist.

## METHODS

From 2000 to 2021, we collected data on the distribution and sex ratios of the mayfly *E. shigae* along rivers in eastern Japan. We also reviewed recently published literature and records of distribution, sex ratio, and genetic data since 2000. We determined the sex ratios of *E. shigae* at 60 total sites, which included additional data from previous publications (Sekiné et al. 2015a, Sekiné and Tojo 2019), and we used genetic analysis to determine the distribution of reproductive strains at the river system level. Finally, we assessed differences in embryo development success between river systems and years, as well as between bisexual and

parthenogenetic reproduction and between sexual-dominant and parthenogenetic-dominant strains.

### Mayfly field collection

We qualitatively sampled mayfly nymphs between 2008 and 2020 at 27 sites distributed along 10 river systems in eastern Japan: Kitakami-gawa River, Natori-gawa River, Abukuma-gawa River, Shinano-gawa River, Tone-gawa River, Ara-kawa River, Tama-gawa River, Obitsu-gawa River, Yoro-gawa River, and Isumi-gawa River (Table S1). We selected the sites based on distribution records of previous studies and a comprehensive survey of river reaches, and they covered a distance of ~450 km from the northernmost to the southernmost point of the river network. We collected nymphs by placing a D-frame net (1.0 × 1.0-mm mesh) in contact with the river bottom and disturbing stones with our hands or feet to dislodge nymphs and allow them to float into the net. In addition, we collected female nymphs with black wing buds just before emergence in 2 sites along the Abukuma-gawa River (site 14:  $n = 3$ ; site 15:  $n = 10$ ) in September 2020.

We also sampled adult female *E. shigae* and oocyte masses (i.e., egg batches). To attract adult females, we used a high-intensity discharge lamp (55W) or fluorescent lantern (9W), and we collected them by hand from 12 sites along 9 river systems in September between 2006 and 2021. In September 2020, we collected oocytes at 3 sites along the Abukuma-gawa and Ara-kawa rivers. In September 2021, we also collected oocytes from 2 sites along the Ara-kawa River (site 44:  $n =$  oocytes from 25 adults; site 46:  $n = 23$ ), and at 1 site each along the Kiryu-gawa, Kamanashi-gawa, Sagami-gawa, and Isumi-gawa rivers (site 36:  $n = 20$ ; site 52:  $n = 17$ ; site 53:  $n = 20$ ; site 59:  $n = 16$ ) (Table S2). In the field, we fixed the whole bodies of nymphs, adults, and ~100 oocytes in ~99% ethanol. Adults were fixed in ethanol after they were allowed to lay eggs in the field. To assess the success of embryogenesis, the remaining oocytes were brought back to the laboratory and incubated at  $20 \pm 0.5^\circ\text{C}$  (see below). We collected additional oocytes in the laboratory by dissecting final-instar nymphs and retrieving their oocytes from the lateral oviducts. The adults and nymphs were kept for high-resolution melting (HRM) analysis to discriminate their strains in the laboratory (see below).

### Sex ratios

To determine mayfly sex, we examined nymphs ( $n = \sim 200$  ind./site; Table S1) under a microscope in the laboratory. Under magnification we assessed the presence or absence of visible primordia of male forceps and penes and recorded the incidence of males and females. We also identified previous studies for which larval data were available (Tojo et al. 2006, Sekiné et al. 2015a, Sekiné and Tojo 2019). We then tested for the presence or absence of skewed sex

ratios with a 2-tailed binomial test, separately for each site (71 sites including those sampled for this study and those reported in previous studies), and adjusted  $p$ -values with a Bonferroni correction (R version 4.2.2; R Project for Statistical Computing, Vienna, Austria). Some sites were sampled in multiple years. In these cases, data were combined from all sampling events.

### Determining strains through genetic analysis and HRM

No morphological differences are found between eastern and western Japanese populations of *E. shigae*, so the only way to discriminate between parthenogenetic-dominant and sexual-dominant strains is through genetic analysis by sequencing the mitochondrial COI or 16S rRNA regions (Sekiné et al. 2015a, Sekiné and Tojo 2019). However, there are several factors that make such a method both time consuming and costly. First, because sex ratios can vary within a river system, multiple sites need to be sampled at both river system and stream scales. In addition, the data can vary from year to year, requiring consistent monitoring over time. Finally, a large number of samples must be processed for genetic analysis. Sequencing is a relatively complex procedure, and reagents are expensive, making it costly in both time and money. Alternatively, HRM analysis is a convenient closed-tube method for genotyping and mutation scanning using real-time polymerase chain reaction (PCR) (Wittwer et al. 2003, Liew et al. 2004). An inexpensive DNA helix-intercalating dye is now available, making HRM analysis cost effective, with a simpler and faster workflow than other genotyping technologies, such as sequencing and TaqMan single nucleotide polymorphism typing (e.g., Li et al. 2010). Several studies have suggested that HRM analysis is useful for species discrimination based on the COI barcode, which is widely used to identify animal species (Fernandes et al. 2017, 2018, Everman and Wang 2019). Therefore, in the current study, we established a new method using HRM analysis to identify reproductive strains based on sequence variants, which we validated via sequencing.

We conducted COI sequencing and HRM analysis on the *E. shigae* we collected as well as *E. shigae* collected for 2 prior studies: Sekiné et al. (2015a) and Sekiné and Tojo (2019), with a total of 120 ind. First, we tested whether the *E. shigae* nymphs and adults belonged to sexual-dominant or parthenogenetic-dominant strains by performing genetic analysis of their mitochondrial DNA COI region with a 3500 Genetic Analyzer (Applied Biosystems<sup>SM</sup>, Waltham, Massachusetts) according to the methods of Sekiné et al. (2015a) and Sekiné and Tojo (2019). The parthenogenetic-dominant strain has a specific mitochondrial DNA COI haplotype (C1 haplotype), and the sexual-dominant strains have other haplotypes. Next, we used the SplitsTree4 program (Huson and Bryant 2006) to construct a median-joining network based on the COI sequences with accession numbers LC635021

to LC635085 in this study and AB874307 to AB874386 and AB985382 to AB985390 from Sekiné et al. (2015a) and Sekiné and Tojo (2019). We used the Primer 3Plus software (Untergasser et al. 2007) to design the primers as Es\_COI\_F9: 5'-ACCAGTATTAGCAGGAGCCA-3' and Es\_COI\_R11: 5'-CTCCTCCTGCTGGGTCAAAG-3'. We designed the size of the product to be 80 bp because previous studies recommended short fragments for routine work in HRM analysis (Vossen et al. 2009, Słomka et al. 2017).

We performed PCR and melting curve analysis with CFX96 Touch™ Real-Time PCR (Bio-Rad, Hercules, California). Initial melting was performed at 98°C for 2 min, followed by 40 cycles of melting at 98°C for 5 s, annealing and extension at 60°C for 5 s, and final extension at 95°C for 1 min. We used SsoFast™ EvaGreen® Supermix (Bio-Rad) as a PCR reagent. We performed HRM analysis immediately after PCR completion. After 60°C for 1 min, we acquired sample fluorescence from 60 to 95°C in 0.2°C increments 5 s after each temperature increase was reached. Finally, we examined whether the sexual-dominant strains (eastern haplotypes) and parthenogenetic-dominant (C1 haplotype) distinguished by COI sequencing were also clearly distinguishable in the melting curve of the HRM analysis (Fig. S1).

### Embryo developmental success by reproductive mode

To assess the developmental success of embryos via bisexual and parthenogenetic reproduction for sexual-dominant and parthenogenetic-dominant strains, we first determined if oocyte batches contained sperm, indicating bisexual reproduction. For ~30 oocytes/ind. (i.e., adults and nymphs; range 8–70 oocytes/ind.), we removed the oocytes from the 99% ethanol solution and placed them into a series of 75, 50, and 25% ethanol in P-buffered saline (PBS; pH 7.4) for ~5 min for each solution and finally with PBS alone. We then stained the fixed oocytes with 4', 6-diamidino-2-phenylindole at a concentration of 0.5 µg/mL in PBS for 10 min and washed them twice with PBS. We mounted stained samples on glass slides with 10% glycerol in PBS and observed them at 400× magnification under a Carl Zeiss™ Axio Lab™ A1 fluorescence microscope (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany) to detect the presence of sperm on the oocyte surface and among the micropyles (Fig. S2).

Next, we incubated the remaining oocytes to assess developmental success of embryos. We cultured each batch of oocytes (mean 664, range 20–2992/ind.) separately at 20 ± 0.5°C. After ~2 mo of incubation, we assessed developmental success (i.e., the percentage of embryos that developed) for each batch of oocytes. During embryogenesis of *E. shigae*, diapause occurs at the last embryonic stage (Nakamura et al. 1999, Watanabe and Ohkita 2000,

Nakamura and Endo 2001). We considered an embryo as having developed successfully if it reached diapause.

Finally, we compared developmental success between groups: river systems, fertilization type (i.e., bisexual vs parthenogenetic reproduction as determined by the presence/absence of sperm), strain (i.e., sexual dominant vs parthenogenetic dominant), and year. We examined the normality and equal variances of each group by a Shapiro–Wilk test and a Levene's test, respectively, with the *car* package (version 3.2-2; Fox and Weisberg 2019) in R. We used averaged developmental success at the same site in the tests for fertilization type, strain, and year to prevent pseudoreplication and built models separately for each predictor. For data for which normality and equal variances were not rejected, we performed a 1-way analysis of variance, and for data for which they were rejected, we performed a Kruskal–Wallis test. If the analysis of variance or Kruskal–Wallis test had a *p*-value of <0.05, we used the *nparcomp* package (version 3.0; Konietzschke et al. 2015) in R to run the Steel–Dwass test to determine pairwise differences between groups. In this case, we used data from individual oocyte batches. Prior to running tests, we excluded groups for which there were ≤6 oocyte batches.

## RESULTS

### Distribution and sex ratio

The presence of nymphs or adults of *E. shigae* has been confirmed at 60 different sites along 33 rivers or channels of 21 river systems in eastern Japan (Tables 1, S1, Fig. 1A–C). Sixteen of these sites (sites: 15, 35, 36, 38–40, 42, 43, 45–48, 55, 56, 58, and 59; Tables 1, S1, Fig. 1A–C) are new to this study, and 44 sites are from previous publications (Sekiné et al. 2015a, Sekiné and Tojo 2019). Overall, there was a strong female bias among sites. The sex ratio of nymphs was biased toward females (binomial test, all *p* < 0.047) at 34 sites, 6 of which were new sites. Twenty-three of the sites with a female bias were female-only sites. Five additional sites were extremely female-biased, with ~2.55% males (i.e., almost female-only site). One site where only female nymphs were observed, but a few male adults were observed, was designated as almost female only. Seven sites where only female adults were observed were categorized as female only, as were 6 sites where only female nymphs were observed and the number of observations was small.

Along the Abukuma-gawa River, the sex ratios in the upstream areas were 1:1, and moving downstream, the sex ratio of females steadily increased until populations became exclusively female in downstream areas. In contrast, along the Ara-kawa River, the upstream areas were solely occupied by females, and the ratio of males increased along the downstream gradient. That is, the sex ratio and distribution pattern of the parthenogenetic-dominant and sexual-dominant strains between the 2 rivers were reversed.

Table 1. Localities, sex ratios, and cytochrome c subunit I (COI) haplotypes of *Ephoron shigae* in eastern Japan from 2000 to 2021. Almost female only indicates that there were  $\leq 2.55\%$  males identified at a site (see Table S1 for additional details). Note that the number of genetically analyzed individuals is shown here. The total number of individuals is available in Table S1. HRM = high-resolution melting.

River system	River	Locality	Site No.	Sex ratio	COI (sequencing or HRM)				
					Female		Male		
					C1	Eastern	C1	Eastern	
Kitakami-gawa	Kitakami-gawa	Oshu, Iwate	1	Female only	7	0	0	0	
		Ichinoseki, Iwate	2	Female only	0	0	0	0	
		Tome, Miyagi	3	Female only	8	0	0	0	
Nanakita-gawa	Nanakita-gawa	Sendai, Miyagi	4	Female only	3	0	0	0	
Natori-gawa	Hirose-gawa	Sendai, Miyagi	5	Female only	0	0	0	0	
			6	Female only	9	0	0	0	
			7	Female only	8	0	0	0	
	Natori-gawa	Sendai, Miyagi	8	Almost female only	8	0	0	0	
			9	Almost female only	18	0	0	3	
			10	Almost female only	24	0	0	0	
			11	Almost female only	20	0	1	0	
Abukuma-gawa	Surikami-gawa	Fukushima, Fukushima	11	Almost female only	20	0	1	0	
	Abukuma-gawa	Fukushima, Fukushima	12	Almost female only	56	2	0	5	
		Motomiya, Fukushima	13	Bisexual	47	1	2	32	
		Koriyama, Fukushima	14	Bisexual	33	18	1	45	
	Yamanoiri-gawa	Nihonmatsu, Fukushima	Sukagawa, Fukushima	15	Bisexual	31	42	1	17
			Nakajima, Fukushima	16	Bisexual	31	16	0	46
			17	Female only	1	0	0	0	
Agano-gawa	Agano-gawa	Agano, Niigata	18	Female only	8	0	0	0	
	Aga-gawa	Aizuwakamatsu, Fukushima	19	Female only	5	0	0	0	
Niida-gawa	Niida-gawa	Minamisoma, Fukushima	20	Female only	10	0	0	0	
Ukedo-gawa	Ukedo-gawa	Namie, Fukushima	21	Female only	7	0	0	0	
Natsui-gawa	Natsui-gawa	Iwaki, Fukushima	22	Female only	8	0	0	0	
Kuji-gawa	Kuji-gawa	Tanakura, Fukushima	23	Female only	8	0	0	0	
		Hitachi, Ibaraki	24	Female only	9	0	0	0	
Naka-gawa	Naka-gawa	Hitachiohmiya, Ibaraki	25	Female only	8	0	0	0	
Same-gawa	Same-gawa	Iwaki, Fukushima	26	Female only	7	0	0	0	
Shinano-gawa	Shinano-gawa	Ojiya, Niigata	27	Female only	26	0	0	0	
			28	Female only	0	0	0	0	
	Chikuma-gawa	Nagano, Nagano	29	Female only	0	0	0	0	
			30	Almost female only	2	0	1	0	
			31	Female only	0	0	0	0	
			32	Female only	0	0	0	0	
			33	Female only	1	0	0	0	
			34	Female only	8	0	0	0	
			35	Female only	10	0	0	0	
			36	Female only	24	0	0	0	
Tone-gawa	Kiryu-gawa	Kiryu, Gunma	37	Female only	8	0	0	0	
			38	Female only	2	0	0	0	
	Kokai-gawa	Chikusei, Ibaraki	37	Female only	8	0	0	0	
	Watarase-gawa	Ashikaga, Tochigi	38	Female only	2	0	0	0	
	Karasu-gawa	Kamisato, Saitama	39	Female only	8	0	0	0	
	Kanna-gawa	Kamikawa, Saitama	40	Female only	4	0	0	0	
Ara-kawa	Ara-kawa	Shimozuma, Ibaraki	41	Female only	10	0	0	0	
		Hachigata, Yorii, Saitama	42	Female only	38	0	0	0	

Table 1. (Continued)

River system	River	Locality	Site No.	Sex ratio	COI (sequencing or HRM)			
					Female		Male	
					C1	Eastern	C1	Eastern
Tama-gawa	Oppe-gawa Iruma-gawa Hino-yosui Channel	Akahama, Yorii, Saitama	43	Female only	27	0	1	0
		Kumagaya, Saitama	44	Bisexual	31	28	0	21
		Kitamoto, Saitama	45	Bisexual	5	20	0	16
		Okegawa, Saitama	46	Bisexual	29	35	0	16
		Kawajima, Saitama	47	Bisexual	8	0	0	1
		Sayama, Saitama	48	Bisexual	2	3	0	3
		Hino, Tokyo	49	Bisexual	0	16	0	0
			50	Bisexual	0	16	0	3
			51	Bisexual	0	16	0	0
		Fuji-kawa	Kamanashi-gawa	Kai, Yamanashi	52	Female only	25	0
Sagami-gawa	Sagami-gawa	Sagamihara, Kanagawa	53	Female only	24	0	0	0
Tenryu-gawa	Tenryu-gawa	Iida, Nagano	54	Female only	8	0	0	0
Obitsu-gawa	Obitsu-gawa	Tawarada, Kimitsu, Chiba	55	Bisexual	0	12	0	0
		Kururi, Kimitsu, Chiba	56	Bisexual	0	12	0	0
		Ichihara, Chiba	57	Bisexual	0	7	0	0
Yoro-gawa	Yoro-gawa	Kuzufuji, Otaki, Chiba	58	Bisexual	0	32	0	0
		Uehara, Otaki, Chiba	59	Bisexual	0	63	0	0
Isumi-gawa	Isumi-gawa	Horinouchi, Otakicho, Chiba	60	Bisexual	0	14	0	6

### Genetic analysis and HRM

Sequencing analyses showed that the parthenogenetic-dominant strain was present in female-only, almost female-only, and bisexual sites, whereas sexual-dominant strains were only found in almost female-only and bisexual sites (Tables 1, S1, Figs 1A–C, 2, S2). In all, 66 ind. from 4 rivers belonged to the sexual-dominant eastern Japan strains, and 54 ind. from 5 rivers belonged to the parthenogenetic-dominant strain (Tables 1, S1, S2).

Along the midstream area of the Abukuma-gawa River and downstream of the Ara-kawa River, the parthenogenetic-dominant strain coexisted at the same sites where moderate numbers of males were observed, although there was a female bias at these sites. No specimens of the parthenogenetic-dominant strain were observed along the Hino-yosui Channel (sites 49–51; Fig. 1C), upstream areas of the Abukuma-gawa River (site 16; Fig. 1B), or within the Boso Peninsula (sites 55–60; Fig. 1C). Sexual-dominant strains were found at extremely restricted sites along the Hino-yosui Channel, in relatively upstream areas of the Abukuma-gawa River, and along 3 rivers within the Boso Peninsula.

In addition to the 89 haplotypes already discovered in Japan (of which 42 are unique to eastern Japan; Sekiné et al. 2015a, Sekiné and Tojo 2019), sequencing identified 65 new haplotypes (Fig. 2). We found 3 types of variants in the amplified region of the eastern haplotypes (40 bp excluding primers), including 3 new haplotypes, differing

from the C1 haplotype by 2, 3, and 3 bases, respectively (Table 2).

### Embryo developmental success by reproductive mode

We found unfertilized oocytes from both parthenogenetic-dominant and sexual-dominant strains and fertilized oocytes only from the sexual-dominant strains (Table 3). Unfertilized oocytes without sperm from the parthenogenetic-dominant strain were observed from 111 adult females at 4 female-only and 3 bisexual sites along 5 rivers (sites 15, 36, 42, 44, 46, 52, and 53). Unfertilized oocytes without sperm from the sexual-dominant strains were observed from 23 adult females at 3 bisexual sites along 2 rivers (sites 44, 46, and 59). Fertilized oocytes with sperm from the sexual-dominant strains were observed from 30 adult females at 4 bisexual sites along 3 rivers (sites 15, 44, 46, and 59). In addition, we found unfertilized oocytes from final-instar nymphs for the parthenogenetic-dominant (1 female nymph) and sexual-dominant (6 female nymphs) strains at site 14 and parthenogenetic-dominant (3 nymphs) and sexual-dominant (7 nymphs) strains at site 15, both which are bisexual sites.

Developmental success differed between fertilization types but not between river systems, years, or strains (Tables S3–S6). Although developmental success did not differ between strains when considering both fertilization types, sexual strains had consistently high success for fertilized

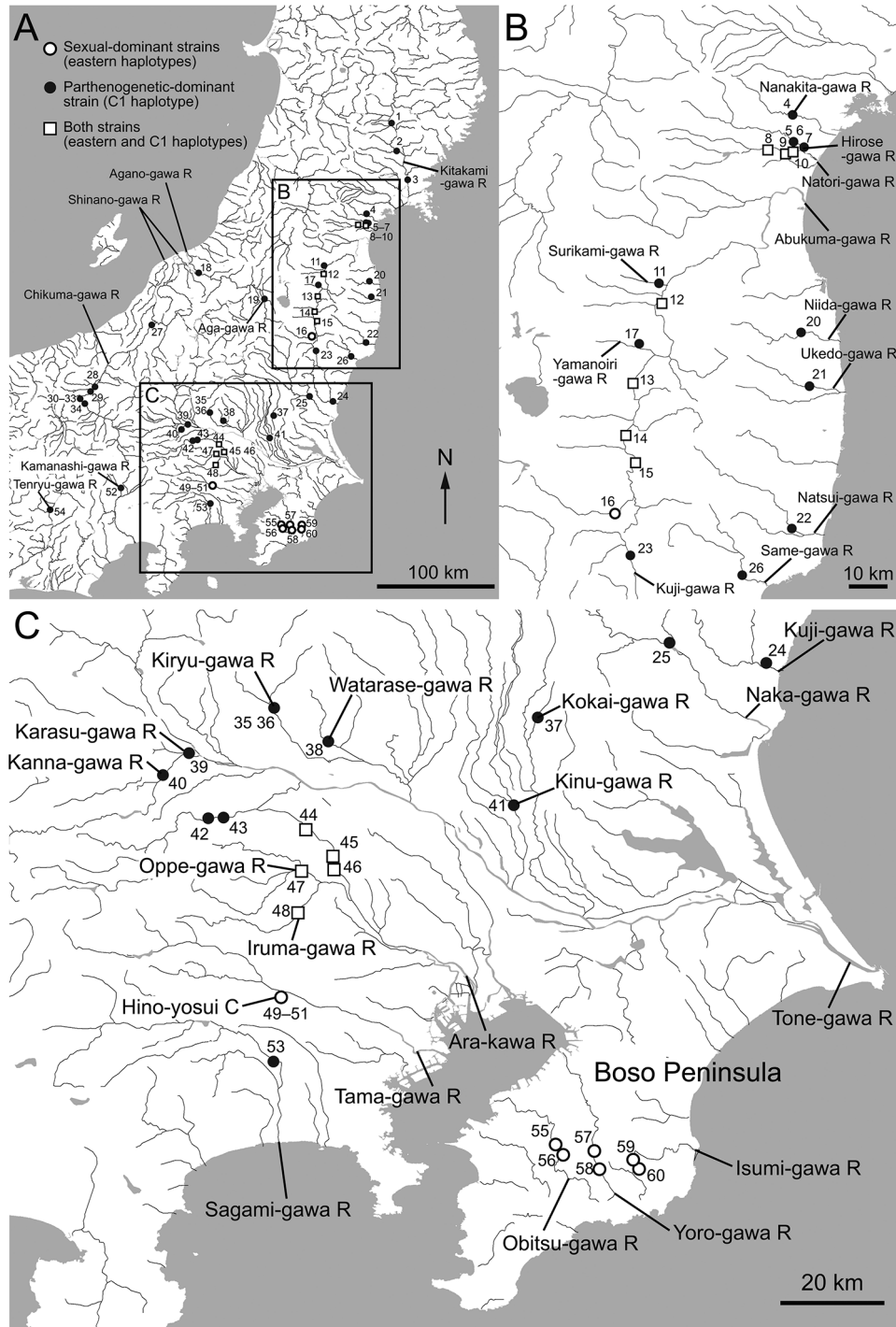


Figure 1. Distribution of *Ephoron shigae* in eastern Japan (A). Open circles, black-filled circles, and open squares indicate sites where the sexual-dominant strains (eastern haplotypes), parthenogenetic-dominant strain (C1 haplotype), and both are found, respectively. Enlarged views of sites in the northeastern (B) and southeastern (C) portions of the study. Numbers correspond with site numbers in Table 1. C = channel, R = river.

oocytes and generally low success for unfertilized oocytes (i.e., without sperm), whereas the parthenogenetic strain's developmental success varied across all categories (Fig. 3). Developmental success rates for unfertilized oocytes varied among sites at both female-only and bisexual sites (Table 3,

Fig. 3). At female-only sites, success rates ranged from a mean of 33.1% (SD: 23.5%,  $n = 20$  batches, site 53) to 90.2% (SD: 6.3%,  $n = 12$  batches, site 42), and there were differences in developmental success among sites (Table S7). At bisexual sites, unfertilized oocytes collected from adult females

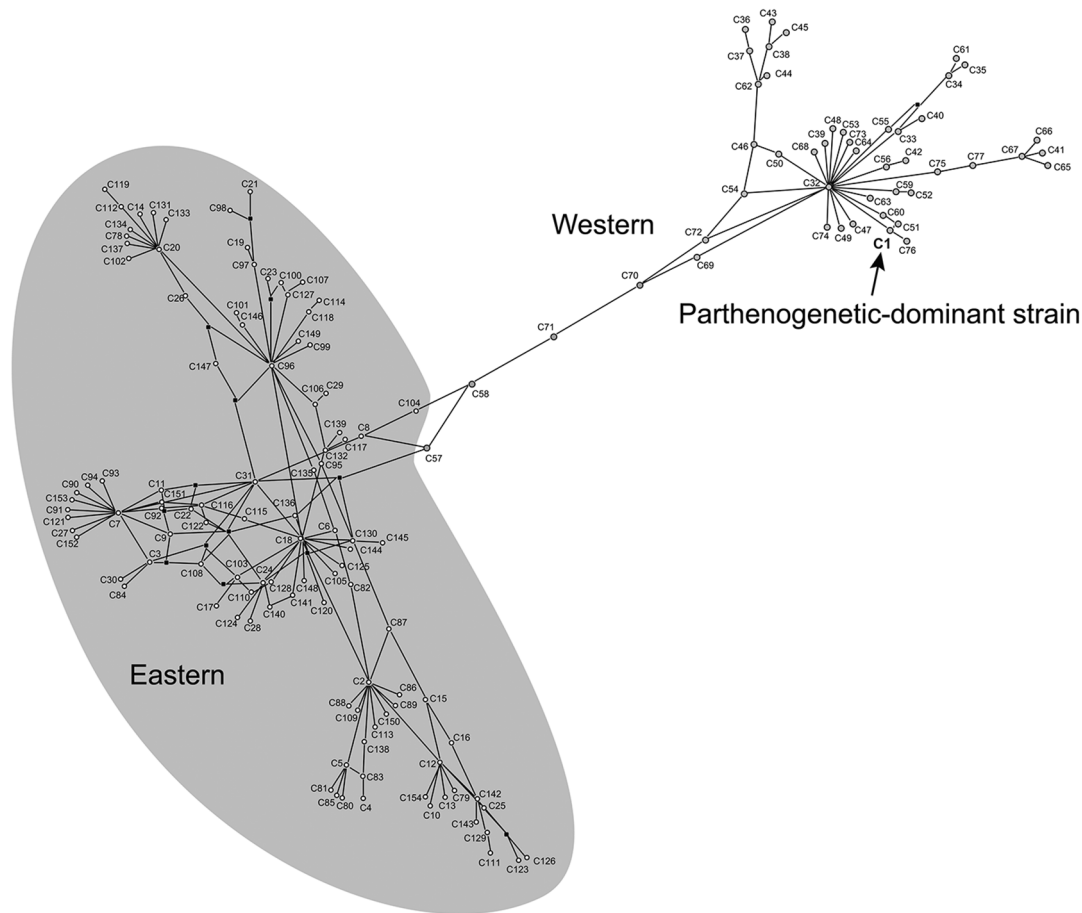


Figure 2. A median-joining network of the mitochondrial cytochrome c subunit I gene (636 bp). C1-154 haplotypes have accession numbers LC635021–LC635085 (this study), AB874307–AB874386 (Sekiné et al. 2015a), and AB985382–AB985390 (Sekiné and Tojo 2019). Open circles and black-filled circles indicate eastern haplotypes and western haplotypes, respectively.

of the parthenogenetic-dominant strain had success rates ranging from a mean of 2.9% (SD: 2.4%,  $n = 15$  batches, site 44) to 70.9% (SD: 26.0%,  $n = 9$  batches, site 15; Tables 3, S2, Fig. 3), and there were, again, differences in developmental success among sites (Tables S6). In contrast, the unfertilized oocytes of the sexual-dominant strains were generally not highly viable, with a developmental success rate of <9.6% (SD: 13.8%,  $n = 8$  batches, site 44). However, fertilized oocytes of the sexual-dominant strains were gener-

ally highly viable, with developmental success rates ranging from 77.4% ( $n = 1$  batch, site 46 in 2020) to 97.8% (SD: 0.2%,  $n = 2$  batches, site 44).

### DISCUSSION

In this study we sought to understand the evolutionary maintenance of bisexual and parthenogenetic reproduction among *E. shigae* in rivers in eastern Japan. We found

Table 2. Sequence type of amplicon for the high-resolution melting analysis of the mitochondrial DNA cytochrome c subunit I (COI) region. Primer parts in the amplicon are excluded.

COI haplotype	Nucleotide position from 5' to 3'			
	9	26	27	35
Parthenogenetic-dominant strain: C1	C	C	C	G
Sexual-dominant strains: eastern (C2 and many others)	C	T	C	T
Sexual-dominant strains: eastern (C23 and C100)	T	T	C	T
Sexual-dominant strains: eastern (C90 and C111)	C	T	T	T



Table 3. Embryo developmental success rates of fertilized or unfertilized oocytes (eggs). Corresponding site names are shown in Fig. 1 and Tables 1 and S1. Reproductive mode was identified by cytochrome c subunit I high-resolution melting analysis. FE = fertilized eggs, max = maximum, min = minimum, P = parthenogenetic-dominant strain, S = sexual-dominant strains, UFE = unfertilized eggs. Site number followed by a year in parentheses indicates a site that was sampled twice. – indicates that there was no minimum or maximum because there was only 1 adult female examined.

Mayfly life stage	River	Site	Oocyte fertilization status	Reproductive mode	No. females examined	No. eggs examined	% embryos that developed to the final embryonic stage	
							Mean ± SD	Min–max
Adult	Abukuma-gawa	15	UFE	P	9	3702	70.9 ± 26.0	26.4–96.2
	Abukuma-gawa	15	FE	S	9	5918	89.1 ± 11.9	67.7–99.2
	Ara-kawa	42	UFE	P	12	7097	90.2 ± 6.3	79.0–97.2
	Ara-kawa	44	UFE	P	15	9782	2.9 ± 2.4	0.2–7.8
	Ara-kawa	44	UFE	S	8	2961	9.6 ± 13.8	0–37.8
	Ara-kawa	44	FE	S	2	818	97.8 ± 0.2	96.7–98.8
	Ara-kawa	46 (2020)	UFE	P	9	3099	52.8 ± 36.4	0.7–95.2
	Ara-kawa	46 (2020)	UFE	S	3	1484	7.5 ± 8.1	0–16.2
	Ara-kawa	46 (2020)	FE	S	1	186	77.4	–
	Ara-kawa	46 (2021)	UFE	P	9	4234	6.3 ± 6.7	0–22.0
	Ara-kawa	46 (2021)	UFE	S	2	2273	7.8 ± 0.3	7.6–8.0
	Ara-kawa	46 (2021)	FE	S	12	12,706	92.4 ± 7.3	74.7–99.3
	Kiryu-gawa	36	UFE	P	20	7998	39.2 ± 20.4	8.4–72.8
	Kamanashi-gawa	52	UFE	P	17	10,770	39.5 ± 19.5	1.7–93.1
	Sagami-gawa	53	UFE	P	20	10,675	33.1 ± 23.5	4.2–84.4
Isumi-gawa	59	UFE	S	10	9,514	25.6 ± 27.3	1.7–93.1	
Isumi-gawa	59	FE	S	6	10,537	97.8 ± 1.4	95.1–99.1	
Last instar nymphs	Abukuma-gawa	14	UFE	P	1	173	68.2	–
	Abukuma-gawa	14	UFE	S	2	813	9.4 ± 11.6	1.2–17.6
	Abukuma-gawa	15	UFE	P	3	2487	65.6 ± 27.2	39.7–93.9
	Abukuma-gawa	15	UFE	S	7	6446	2.1 ± 1.8	0.6–4.4

that 1) the parthenogenetic-dominant strain was widely distributed, whereas sexual-dominant strains were geographically restricted and dominated only in limited regions, such as the Boso Peninsula and the Hino-yosui Channel; 2) both parthenogenic-dominant and sexual-dominant strains coexisted in some areas, such as in the Abukuma-gawa and Ara-kawa river systems; and 3) fertility of the parthenogenetic-dominant strain varied widely compared with bisexual fertility of the sexual-dominant strain, which was consistently successful. In addition to characterizing the distribution and oocyte developmental success of parthenogenic-dominant and sexual-dominant strains of *E. shigae*, we also tested HRM and found it to be an efficient alternative identification method to genetic sequencing for both strains. In all, our findings indicate that although the parthenogenetic-dominant strain may be expanding its distribution, its fertility was unstable. Fu-

ture research is needed to determine whether this instability in fertility allows both strains to coexist or whether the distribution of the strains will change in the future.

#### Expansion of a parthenogenetic-dominant strain and coexistence with sexual-dominant strains

The parthenogenetic-dominant strain of *E. shigae* is considered to be obligatorily parthenogenetic (Tojo et al. 2006, Sekiné and Tojo 2010b, Sekiné et al. 2015a), whereby females do not mate even when males are present but instead pass through generations by parthenogenesis and almost never produce offspring by bisexual reproduction. There are known cases where organisms that have gone through many generations of exclusively parthenogenetic reproduction lose the ability to reproduce sexually (Schwander et al. 2013, van der Kooi and Schwander 2014, Jaron et al.

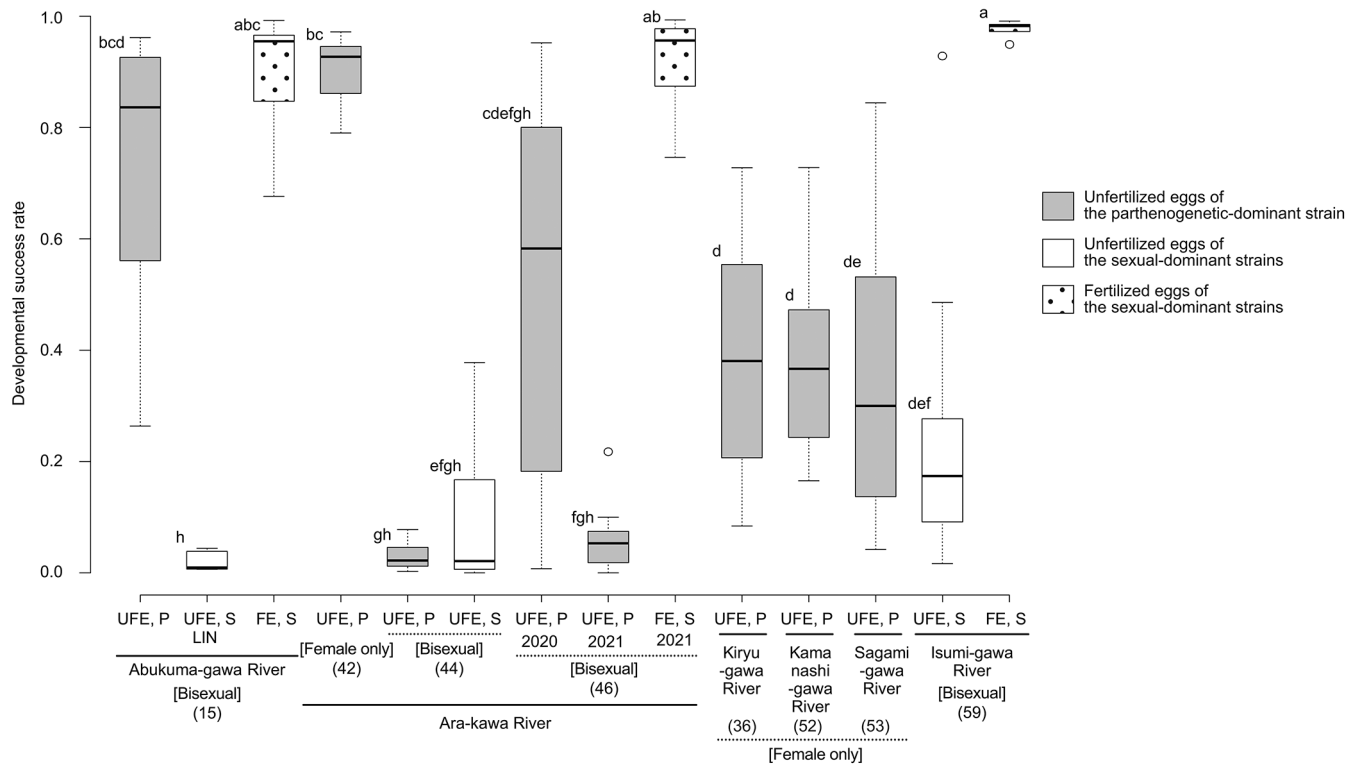


Figure 3. Boxplots of developmental success rates of fertilized and unfertilized oocytes (eggs) of adults or last-instar nymphs (LIN). The reproductive strains, parthenogenetic-dominant (P), or sexual-dominant (S) were identified via high-resolution melting analysis. Numbers in parentheses indicate site number (Table 1, Fig. 1). Gray boxes, open boxes, and hatched boxes indicate unfertilized eggs of the parthenogenetic-dominant strain, unfertilized eggs of sexual-dominant strains, and fertilized eggs of sexual-dominant strains, respectively. Lowercase letters above the boxes indicate differences (Tukey–Kramer test,  $p < 0.05$ ) between groups (i.e., strains, fertilization types, sites, and years where applicable). FE = fertilized eggs, UFE = unfertilized eggs.

2021, Burke and Bonduriansky 2022). It is possible that the parthenogenetic-dominant strain of *E. shigae* may also have lost the ability to mate. For example, females of the parthenogenetic-dominant strain may avoid mating, or males of sexual-dominant strains may not select parthenogenetic-dominant females as mates. Indeed, all 42 parthenogenetic-dominant adult females we examined had unfertilized oocytes (oocytes without sperm among micropyles). Thus, it appears that gene flow between the parthenogenetic-dominant and sexual-dominant strains does not occur in eastern Japan, even when the 2 types coexist.

Because the parthenogenetic-dominant strain of *E. shigae* uses only parthenogenesis to reproduce and primarily produces females, the presence of parthenogenetic-dominant individuals in a population is likely to bias the sex ratio toward females. Indeed, we did find sex ratio bias in several bisexual sites where the parthenogenetic-dominant strain was found, such as along the Abukuma-gawa River. Interestingly, we observed relatively high parthenogenetic fecundity in the bisexual population along the Isumi-gawa River, where sites were dominated by the sexual-dominant strain, but did not observe any sex ratio bias along this river.

Individuals of sexual-dominant strains are facultatively parthenogenetic and primarily reproduce bisexually or have low parthenogenetic posthatchling survival rates, which may explain the lack of a sex ratio bias. Similarly, a closely related bisexual species, *Ephoron eophilum* Ishiwata, 1996, exhibits high developmental success rates of its unfertilized oocytes, yet no sex ratio bias has been observed for the species (Sekiné et al. 2015b). Thus, the loss of bisexual reproductive capacity in parthenogenetic-dominant females is likely a major factor in biasing the sex ratio.

Although there are few examples of parthenogenetic-dominant and sexual-dominant strains coexisting in the same habitat, previous cases of coexistence have been reported in both animal (e.g., ostracods [Rossi et al. 2017], daphnids [Lehto and Haag 2010], stick insects [Larose et al. 2018]) and plant species (e.g., rockcress [Rushworth et al. 2018]). It has been suggested that the coexistence of strains is mainly due to ecological differentiation between breeding types. In the freshwater ostracod *Heterocypris barbara* (Gauthier & Brehm, 1928), the coexistence of parthenogenetic-dominant and sexual-dominant strains in temporary freshwater ponds was achieved by seasonal niche partitioning

(Rossi et al. 2017). In the genus *Boecheira* (rockcress), parthenogenetic reproduction was strongly associated with greater disturbances, reduced slope, and greater environmental variability (Rushworth et al. 2018). Along rivers or streams, niche segregation often leads to habitat gradients among related species of lotic insects (e.g., Ogitani et al. 2011, Saito and Tojo 2016, Saito et al. 2018, Okamoto and Tojo 2021). We observed similar niche segregation between sexual-dominant and parthenogenetic-dominant strains in *E. shigae*. However, the pattern of upstream/downstream, sexual-/parthenogenetic-dominant strain dominance was opposite between the Abukuma-gawa River and Ara-kawa River. We do not know why there is an opposite pattern between rivers, but this difference raises questions about how dispersal, niche segregation, etc. may differ between the rivers or may affect gene flow.

In some geographically parthenogenetic organisms, parthenogenetic-dominant strains have been observed at the geographic or ecological margins of species' distributions (e.g., Tilquin and Kokko 2016). Different researchers have interpreted these margins as indicative of low-stability, transient, or disclimax habitats (Cuellar 1977, Bell 1982, Lynch 1984), a metapopulation structure with colonization–extinction cycles (Haag and Ebert 2004), a low amount or diversity of resources (Glesener and Tilman 1978, Gaggiotti 1994), low population productivity (Peck et al. 1999), low density (Hörandl 2006), and higher abiotic than biotic selection pressures (Glesener and Tilman 1978). However, we found that in eastern Japan, the geographic range of the parthenogenetic-dominant strain of *E. shigae* is clearly wider than those of sexual-dominant strains. Similarly, there are other cases where parthenogenetic organisms occupy a much larger geographic range than their bisexual relatives, such as the buttercup *Ranunculus auricomus* complex (Hörandl 2009) and the weevil *Otiorhynchus scaber* (Linnaeus, 1758) (Stenberg et al. 2003). It is reasonable that the parthenogenetic-dominant strain of *E. shigae* has a wider distribution than the sexual-dominant strains because of environmental factors, such as water temperature, chemical factors, physical factors, or biotic interactions. However, it may also be the case that individuals of the parthenogenetic-dominant strain enter a niche that is unsuitable for individuals of the sexual-dominant strains, or that is vacant, and thereby increase their population.

It is also possible that the population simply shifted from being sexual-dominant to parthenogenetic-dominant. The Abukuma-gawa River previously supported a bisexual population, for example at site 12, where surveys show that the population has been replaced by females over <20 y (Sekiné and Tojo 2019). Furthermore, at sites within the range of the Natori-gawa River, which closely neighbors the Abukuma-gawa River to the north, most of the females sampled were parthenogenetic-dominant, although several males of sexual-dominant strains were found (Tables 1, S1; Sekiné et al. 2015a, 2020). The increasing prevalence of parthenogenetic

females and increasingly biased sex ratio we identified along the downstream gradient of the Abukuma-gawa River lead us to predict that sex ratios along the Abukuma-gawa River will follow a similar trajectory as the Natori-gawa River. Long-term monitoring is needed to identify fluctuations in sex ratios and reproductive modes along these rivers.

### Variable parthenogenetic fecundity of the parthenogenetic-dominant strain

This study showed that even within the parthenogenetic-dominant strain, there is marked variation in the level of parthenogenetic fecundity between local populations and individuals. In previous studies, the developmental success of unfertilized oocytes in female-only sites along the Chikuma-gawa, Sakura-gawa, and Shonai-gawa rivers ranged from 69.3 to 96.9%, indicating high success rates among all individuals (Tojo et al. 2006, Sekiné and Tojo 2010b). We only found similar success rates at a single female-only site, site 42 (mean: 90.2%, SD: 6.3%), whereas other female-only sites had more variable success rates. In addition, the same was true for the bisexual sites, where higher and lower success rates of unfertilized oocytes in parthenogenetic-dominant strain were observed.

Similar to geographically parthenogenetic mayflies, populations with a greater sex ratio bias toward females appear to have greater parthenogenetic fecundity in female individuals (e.g., Liegeois et al. 2021). For example, in a geographically parthenogenetic ephemeropterid, *Eurylophella funeralis* (McDunnough, 1925), the females of unisexual populations have greater parthenogenetic fecundity than populations with a low sex ratio bias toward females (Sweeney and Vannote 1987). We observed a similar trend in *E. shigae* along the Ara-kawa River system, where the developmental success rate of unfertilized oocytes of the parthenogenetic-dominant strain was high at the female-only site 42, where we did not find any individuals of sexual-dominant strains. On the other hand, the developmental success rates of unfertilized oocytes of the parthenogenetic-dominant strain were lower at the bisexual sites 44 and 46, where individuals of sexual-dominant strains also existed. However, in the other rivers, developmental success was not necessarily higher at the female-only sites (sites 36, 52, and 53; Table 3, Fig. 3).

Why is there so much variation in the developmental success rates of unfertilized oocytes of the parthenogenetic-dominant strain? We propose 2 possible explanations relating to 1) genetic factors and 2) environmental factors. The parthenogenetic-dominant strain of *E. shigae* exhibits extremely low genetic diversity compared with the bisexual strains. In addition, the early development mode of the parthenogenetic-dominant strain is automixis (Sekiné and Tojo 2010a), in which the female pronucleus and polar nucleus fuse after meiosis to form a diploid organism. Therefore, the genotype of the resulting offspring is different from

that of the mother and may be homozygous (Suomalainen et al. 1987, Jaron et al. 2021). Reduced parthenogenetic developmental success could be caused by the expression of recessive deleterious genes due to homozygosity. Second, environmental factors, such as water temperature, water chemistry, physical factors, and biotic interactions, could affect parthenogenetic fecundity. Oocyte batches from maternal individuals stressed by environmental factors may also show reduced developmental success. Further research is needed to better understand the reasons for variable parthenogenetic reproductive success rates beyond female-biased sex ratios.

### Identification of parthenogenetic-dominant and sexual-dominant strains by HRM analysis

The genetic structure of COI sequences shows clear differentiation between eastern and western Japan except for the parthenogenetic-dominant strain (Sekiné et al. 2013, 2015a, Sekiné and Tojo 2019). Previous studies recognized 46 western haplotypes and the C1 haplotype (parthenogenetic-dominant strain) in western Japan (Sekiné et al. 2013, 2015a) (Fig. 2). The parthenogenetic-dominant strain originated within the western Japanese sexual-dominant strains (western haplotypes) and is similar to these sequences. The COI region of the HRM analysis in the parthenogenetic-dominant strain is common to 2 western haplotypes, C51 and C76, in western Japan (Fig. 2). Therefore, the HRM analysis could not directly identify the 2 reproductive strains in western Japan. However, this issue was not limited to HRM analysis—COI sequencing also had the same outcome—and it is possible that some sexual-dominant strains share the C1 haplotype with the parthenogenetic-dominant strain. Therefore, we suggest it is necessary to combine examinations of sex ratio and parthenogenetic fecundity in addition to COI sequencing or HRM analysis to identify reproductive strains of *E. shigae* in western Japan.

Reproductive modes in the animal kingdom, including for aquatic insects, have long been studied. Parthenogenesis is advantageous in terms of reproduction, and it is assumed that the prevalence of this reproductive mode will increase over time (Mergeay et al. 2006, Hoffmann et al. 2008, Gutekunst et al. 2018, Yano et al. 2021). Indeed, in eastern Japan, the parthenogenetic-dominant strain of *E. shigae* is more widely distributed than the sexual-dominant strains, and the parthenogenetic-dominant strain has replaced sexual reproduction in some sites. However, we found that the fecundity of the parthenogenetic strain was unstable. Although we were able to characterize the distribution of the sexual-dominant and parthenogenetic-dominant strains in eastern Japan and to compare the relative fecundity of bisexual and parthenogenetic reproduction, there are several questions that remain. Why is the parthenogenetic strain more widely distributed than the sexual-dominant strains? How will the distribution of these strains change in the future,

and will the parthenogenetic-dominant or sexual-dominant strains dominate? Examining these questions will provide new perspectives on reproductive modes and geographic parthenogenesis.

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Author contributions: KS generated the idea for the study, analyzed the data, interpreted the results, and wrote the 1<sup>st</sup> draft of the manuscript. KT improved the interpretation of results from the 1<sup>st</sup> draft. KS and KT drafted the final manuscript together.

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