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Facultative parthenogenesis in the burrowing mayfly, *Ephoron eophilum* (Ephemeroptera: Polymitarcyidae) with an extremely short alate stage

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Abstract. Facultative parthenogenesis is important for mayflies with short alate stages because females are able to reproduce without mating. We studied facultative parthenogenesis in *Ephoron eophilum*, a mayfly with an extremely short alate stage. We examined the survival rates of embryos from unfertilized eggs, in addition to investigating the number of chromosomes in parthenogenetic offspring and the mode of inheritance by nuclear genetic analyses using Exon-Primed Intron-Crossing markers. The survival rate of thelytokous embryos was 0–70.2% (16.7 ± 26.7%, mean ± S.D.). Sixteen chromosomes were present throughout most of the mitotic metaphase in parthenogenetic offspring, which was similar to the number recorded in diploid females. All parthenogenetic offspring were homozygous in nuclear genetic analyses, despite the presence of heterozygous mothers. These results indicate that *E. eophilum* has the ability to reproduce via facultative parthenogenesis, producing mostly diploid thelytokous offspring. The restoration of ploidy level occurs by automixis via terminal fusion or gamete duplication, and causes rapid reduction of heterozygosity. However, despite this, significant deviation from Hardy-Weinberg equilibrium (HWE) was not observed in the studied populations. This is because facultative parthenogenesis in these circumstances normally has little influence on population genetic structuring, even though parthenogenetic embryos exhibit a high survival rate. The lack of influence of parthenogenesis on the population structure of the natural population strongly suggests that parthenogenesis rarely occurs under natural circumstances.

INTRODUCTION

Cytological thelytokous parthenogenesis (i.e., when females are produced from unfertilized eggs) occurs as two main forms (Suomalainen et al., 1987): apomictic (or ameiotic) and automictic (or meiotic). In apomictic parthenogenesis, alleles do not undergo recombination; thus, offspring are "true clones" of the mother. In automictic parthenogenesis, the first stages of meiosis are similar to those in sexual reproduction, but fusion occurs between two nuclei originating from the same individual. Gene recombination may occur in automictic parthenogenesis. Therefore, the two types of parthenogenesis give rise to different patterns of heredity (Templeton, 1982; Pearcy et al., 2006).

Obligatory parthenogenesis is rare; it has been reported in only 0.1% of the hexapod species examined (Normark, 2014). However, obligatory parthenogenesis has been reported occurring at a relatively high frequency in Ephemeroptera (0.4% of described species), although is lower in other aquatic insects, i.e., Odonata (0.02%; Cordero et al., 2005), Plecoptera (0%), and Trichoptera (0.05%; Corbet, 1966). In contrast, facultative parthenogenesis has the potential to cause the evolution of obligatory parthenogenesis (Simon et al., 2003), and has been observed in sexual insect species, i.e., Odonata (Kato et al., 1997; Watanabe et al., 1999), Plecoptera (Harper, 1973; Fochetti & de Figueroa, 2008), Trichoptera (Salokannnel et al., 2010; Malicky & Pauls, 2012), and especially Ephemeroptera (e.g., Funk et al., 2010; Sekiné & Tojo, 2010a). In this context, it is possibly that the short alate stage of mayflies is closely related to parthenogenetic reproduction and could be an adaptive trait for females in the population that cannot mate due to the limited period of this stage.

The type of parthenogenesis is mainly thelytoky, whereby only female offspring are produced, although deuterotoky, producing both female and male offspring, occurs in a few cases. A previous study that analyzed the allozymes of seven mayfly species reported that facultative parthenogenesis generates female offspring and a few males via automixis and causes a decrease of heterozygosity (Funk et al., 2010).

The bisexual polymitarcyid burrowing mayfly, *Ephoron eophilum* Ishiwata, (Ephemeroptera: Polymitarcyidae) has a distribution limited to the Kanto Plain in Japan (Ishiwata, 1996; Sekiné et al., 2013b). Nymphs burrow U-shaped

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cavities in the clay of riverbeds and feed on particles of organic matter or detritus suspended in the water (Ishiwata, 1996; Aoyagi et al., 1998). The species has an extremely short alate stage (male subimago and imago and female subimago), lasting for a maximum of two hours. Females do not molt to the final imago stage, but lay eggs as subimagos.

Ephoron eophilum is phylogenetically closely related to *E. shigae* (Takahashi) (Ishiwata, 1996; Sekiné et al., 2013a), a geographically parthenogenetic species with both unisexual and bisexual populations that is widely distributed throughout Japan, Korea, and Russian Far East (Watanabe & Ishiwata, 1997; Tojo et al., 2006). Although obligatory parthenogenesis is observed within unisexual populations of *E. shigae*, facultative parthenogenesis is also observed in the bisexual populations of this species (Sekiné & Tojo, 2010a, b). These studies showed that the survival rates of diploid thelytokous embryos were noticeably lower in the unfertilized eggs of *E. shigae* under facultative parthenogenesis (mean 14.1%) than obligatory parthenogenesis (89.2%). A previous study involving histochemical analyses clarified that obligatory parthenogenesis in this species is automictic, with terminal fusion occurring through the mature egg nucleus fusing with its sister second polar body nucleus to form a single embryonic nucleus (Sekiné & Tojo, 2010b).

The main objective of the present study was to evaluate the parthenogenetic capacity of *E. eophilum*, and the cytological mode of parthenogenesis (i.e., ploidy, sexuality, and automixis or apomixis). In the case of diploid thelytoky, we attempted to elucidate using exon-primed intron-crossing (EPIC) markers for nuclear genes whether apomixis produces only asexual lineages (clones), whilst automixis generates both homo- and heterozygotic offspring. Currently, unlike microsatellite markers, EPIC markers are relatively easily to develop and hence are the most widely used nuclear sequence markers for phylogeographic studies (Thomson et al., 2010). Lastly, we examined the bias of genetic structure expected following parthenogenetic reproduction on the characteristic patterns of heredity, i.e. to assess whether there was significant deviation from HWE expectations in terms of homo-and heterozygote numbers within the natural populations sampled.

MATERIAL AND METHODS

Embryonic development

We collected about 30 female last instar nymphs of *E. eophilum* from the Kinu-gawa River of the Tone-gawa River Basin on July 12, 2009 (Joso, Ibaraki Prefecture: 36°07′03″N, 139°58′03″E). Following the methods of Sekiné & Tojo (2010a) for collecting unfertilized eggs and estimating their survival rates of thelytokous embryos, we separately reared 30 nymphs in the laboratory of which only one grew to the adult stage. Thereafter, unfertilized eggs were collected from a single reared virgin female subimago (Mother -a). In addition, we dissected six final instar female nymphs (Mothers -b to -g) and obtained their unfertilized eggs. As a control, fertilized eggs were obtained from ten mated female subimagos (Mothers -A to -L) from the population collected from the Sakura-gawa River on August 20, 2008 (Tsuchiura, Ibaraki Prefecture: 36°05′27″N, 140°10′26″E) in the Tone-gawa River Basin.

The fertilized and unfertilized eggs were separately incubated in batches at 20 ± 0.5°C. During the embryogenesis of this mayfly species, diapause occurs at the last embryonic stage (Aoyagi et al., 1998; Nakamura & Endo, 2001), which is equivalent to stage 13 in embryos of the ephemerid burrowing mayfly, *Ephemera japonica* McLachlan (Ephemeroptera: Ephemeridae) (cf. Tojo & Machida, 1997). After about six months of incubation, embryo survival rates were measured under a microscope (SMZ1500, Nikon, Tokyo, Japan) for each egg batch of fertilized and unfertilized eggs.

After the nymphs began hatching, the number of chromosomes in each offspring were counted. The chromosome samples were prepared using Giemsa staining (Sekiné & Tojo, 2010a, b) and photographed under a light microscope (80i, Nikon, Tokyo, Japan) at 1000× magnification.

Genetic analyses

In addition to samples used for observation of embryonic development, we collected female subimagos and male imagos of *E. eophilum* for genetic analysis from Kinu-gawa (N = 15) and Sakura-gawa Rivers (N = 13) of the Tone-gawa River Basin on August 25, 2007 and August 20, 2006, respectively. Specimens were fixed in 95% ethanol and included maternal subimagos (N = 11), last instar nymphs (N = 6), and first instar nymphs (N = 31) which hatched from unfertilized eggs. Total genomic DNA was extracted from the whole body or tissues of the ethanol-preserved specimens and purified using a standard cetyltrimethylammonium bromide (CTAB) protocol (Wang & Wang, 2012). Total RNA was extracted from the last instar nymphs using an RNeasy Mini kit (Qiagen, Venlo, Netherlands). The RACE-ready cDNA libraries were prepared using a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, US) and the total RNA.

We designed the EPIC primers for the nuclear *elongation factor-1 alpha 1* (*EF-1α*) and *boule* genes conserved among animals. Primers of *EF-1α* were designed based on sequences of *Hexagenia limbata* (Serville) (Accession No. AY305469) and *Ephemerella inconstans* Traver (GQ886698) in Ephemeroptera (Ephemeridae and Ephemerellidae, respectively) and *Drosophila melanogaster* Meigen (X06870) in Diptera (Drosophildae). Degenerate primer PCR to obtain partial cDNA of *boule* gene orthologues was performed using primers (5'-GTGCCGAAYCGCGTGTTTGTGG-3' and 5'-TCTCRAASGTGATGAARCCGTARCC-3') corresponding to highly conserved regions of respective genes of the mayfly *Ephemera danica* Müller (Ephemeridae) (GAUK01089074), the sawfly *Athalia rosae* (L.) (Hymenoptera: Tenthredinidae) (AB719982) the red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (EFA05679), and the fruit fly *D. melanogaster* (AAF50316), as in previous studies (Sekiné et al., 2015). Subsequently, the adjacent 5' and 3' regions identified by using the RACE-ready cDNA libraries and gene-specific primers (5'-GCGGACTTTCTGCAGACACAACAG-3' and 5'-TTTGGAGACTCCTGCGCGATCAC-3').

These were partially amplified by polymerase chain reaction (PCR) using the following primer sets: *EF-1α*: 5'-CCTGGGTGTTGGACAAGCTCAAG-3' and 5'-AAGACGCAGGGGCTTCTCTG-3'; *boule*: 5'-GTGCCGAAYCGCGTGTTTGTGG-3' and 5'-GGTAAGAGTAATTCTGCGGAGTG-3'. PCR products were then purified using ExoSap-IT (GE Healthcare, Buckinghamshire, UK), and the purified DNA fragments sequenced directly on an automated sequencer (Applied Biosystems, ABI 3130*xl* DNA Analyzer, Waltham, USA) using an automated method involving BigDye Terminator v1.1 or 3.1 Cycle Sequencing Kits (Applied Biosystems).

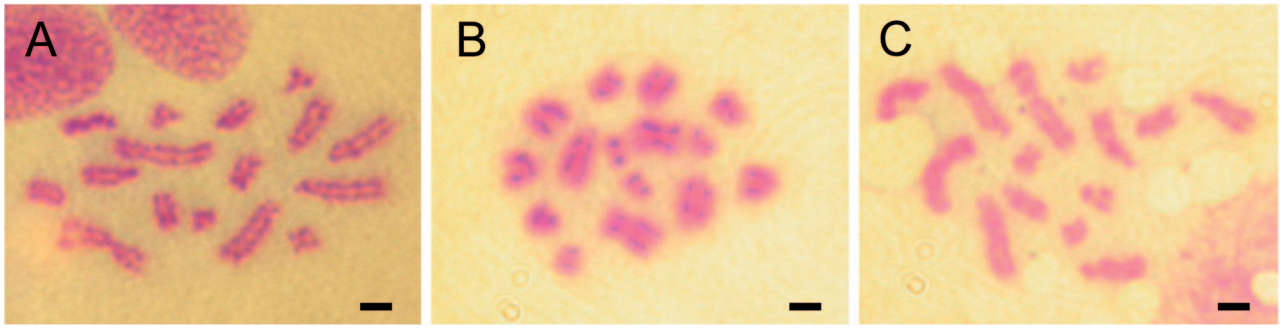

Fig. 1. Mitotic metaphase chromosomes prepared from parthenogenetically developed nymphs. Chromosomes of 2n = 16 (A), 15 (B), and 14 (C). Scale bars = 1 μm.

All sequences were aligned and checked for double peaks using CLC Workbench software (CLC bio, Aarhus, Denmark), the results being cross-checked using the Clustal W (Larkin et al., 2007) algorithm, implemented in MEGA5.10 (Tamura et al., 2011). *EF-1α* and *boule* sequences were submitted to the DNA data Bank of Japan (DDBJ database) with accession numbers LC013981–LC014119. The PHASE algorithm (Stephens et al., 2001; Stephens & Donnelly, 2003), as implemented in DnaSP v5 (Librado & Rozas, 2009), was used to reconstruct putative alleles of the nuclear *EF-1α* and *boule* genes, and to calculate statistical parsimony trees (network tree) using TCS, version 1.21 (Clement et al., 2000).

The exact tests of HWE (Wigginton et al., 2005) were used to test *EF-1α* and *boule* genes for single nucleotide polymorphisms (SNPs) in parthenogenetic progenies reared in the laboratory and individuals from the Kinu-gawa and Sakura-gawa populations.

RESULTS

The overwhelming majority of embryos from all 10 fertilized egg batches developed to the final stage (94.9 ± 6.7%, mean ± SD; maximum: 99.7%, minimum: 78.1%; Table 1). In contrast, eggs from only four out of the seven unfertilized batches developed to the final stage (16.7 ± 26.7%, mean ± SD; maximum: 70.2%; minimum: 0%). Most nymphs hatched from unfertilized eggs had 16 mitotic metaphase chromosomes (15 individuals out of 19; Fig. 1A), but four individuals had different chromosome numbers (i.e., 14 and 15 chromosomes were observed in 1 and 3 individuals, respectively; Fig. 1B, C).

In the Sakura-gawa and Kinu-gawa populations (Fig. 2A, B), and using the PHASE algorithm, it was estimated that 15 and seven genotypes from the *EF-1α* (512 bp; Intron-I 58, Exon-I 168, Intron-II 190, and Exon-II 96) and *boule* (694 bp; Exon-I 205, Intron-I 283, and Exon-II 206) sequences with introns, respectively, occured within the 68 individuals tested, excluding parthenogenetic offspring. However, the genotype IV and genotype I predominated in *EF-1α* and *boule*, respectively, thirteen and five substitutions were observed, with 55 and 53 out of the 68 individuals being heterozygous at one or more positions in *EF-1α* and *boule*, respectively. In addition, no genetic differentiation between the Sakura-gawa and Kinu-gawa populations was found.

Table 1. Survival rate of embryos from fertilized or unfertilized eggs of *Ephoron eophilum*.

	Mother individual	Number of eggs examined	Number of embryos developing to the final embryonic stage	Rate of embryos developing to the final embryonic stage
Fertilized eggs	A	725	686	94.6%
	B	105	82	78.1%
	C	270	264	97.8%
	D	915	910	99.5%
	G	33	30	90.9%
	H	549	542	98.7%
	I	866	857	99.0%
	J	831	762	91.7%
	K	385	382	99.2%
	L	287	286	99.7%
Unfertilized eggs	a	409	142	34.7%
	b*	1511	1061	70.2%
	c*	626	66	10.5%
	d*	1437	19	1.3%
	e*	1865	0	0.0%
	f*	1683	0	0.0%
	g*	776	0	0.0%

*Unfertilized eggs obtained from final instar female nymphs by dissection.

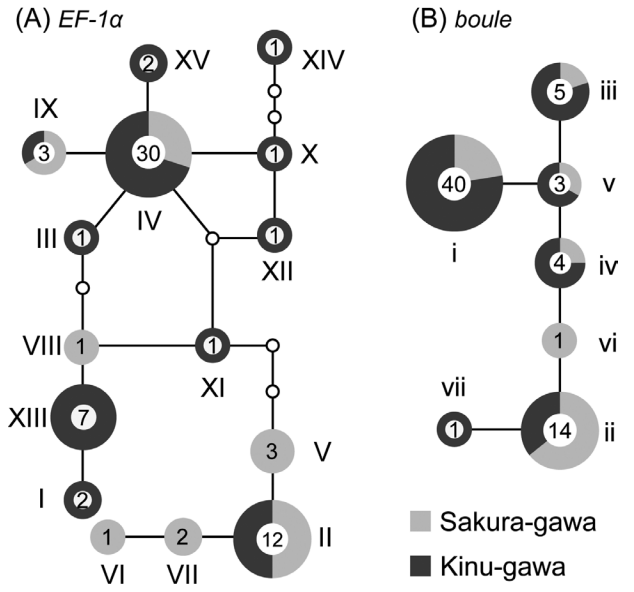

Fig. 2. Statistical parsimony network for the *EF-1α* (A) and *boule* (B) of *E. eophilum* in the Sakura-gawa and Kinu-gawa River populations. Solid lines connect genotypes with a single step (missing intermediates are shown by an open circle). The size of the circle and Arabic numerals indicates the number of genotypes, whilst the division within the circle shows the proportion of two populations in which the genotype was detected. Roman numerals represent the genotype names presented in Table 2.

Four and two genotypes for *EF-1α* and *boule*, respectively, were identified in offspring parthenogenetically generated from mothers -a, -b, and -c (Table 2). Mothers -a and -d were homozygous, while -b, -c, -e, -f, and -g were heterozygous at the 7 and 5 sequence positions in *EF-1α* and *boule*, respectively. All parthenogenetic offspring analyzed were homozygous in both genes independent of whether the mother was homo- or heterozygous.

In the exact tests of HWE for the SNPs of *EF-1α* and *boule* genes, significant deviations from expectations were observed in parthenogenetic progenies for almost all SNPs (N = 33), but not in the Kinu-gawa and Sakura-gawa populations (N = 33, Table 3). Although there was no observed significant deviation from HWE for position 140 in the *boule* sequences, all parthenogenetic progenies were homozygous for guanine.

DISCUSSION

This study confirmed that *E. eophilum* is capable of facultative parthenogenesis. This species mostly has a diploid chromosome number of 2n = 15 (♂) or 2n = 16 (♀), and rarely variant chromosome numbers (Sekiné et al., 2013a). Our results indicate that when unfertilized *E. eophilum* eggs undergo parthenogenesis, they usually generate diploid female offspring (2n = 16), i.e., diploid thelytoky. In addition, some parthenogenetic offspring had different

Table 2. Partial sequences of *EF-1α* and *boule* genes in offspring developed from unfertilized eggs and mothers.

EF-1α (512 bp)

	Number of individuals	Sequence position							Genotype*
		4	10	175	260	265	335	412	
Mother-a	1	A	G	A	C	G	C	C	I / I
offspring	10	A	G	A	C	G	C	C	I / I
Mother-b	1	A / G	A / G	A / G	C / T	A / G	C / G	C / T	II / III
offspring	7	G	G	G	C	G	C	C	II / II
offspring	5	A	A	A	T	A	G	T	III / III
Mother-c	1	A / G	A / G	A / G	C / T	A / G	G	C / T	III / IV
offspring	7	G	G	G	C	G	G	C	IV / IV
offspring	2	A	A	A	T	A	G	T	III / III
Mother-d	1	G	G	G	C	G	G	C	IV / IV
Mother-e	1	A / G	A / G	A / G	C / T	A / G	G	C / T	III / IV
Mother-f	1	A / G	A / G	A / G	C / T	A / G	G	C / T	III / IV
Mother-g	1	A / G	A / G	A / G	C / T	A / G	G	C / T	III / IV

boule (694 bp)

	Number of individuals	Sequence position					Genotype*
		112	300	306	437	689	
Mother-a	1	G	A	C	A	T	i / i
offspring	10	G	A	C	A	T	i / i
Mother-b	1	G	A / C	C / T	A / T	C / T	i / ii
offspring	7	G	A	C	A	T	i / i
offspring	5	G	C	T	T	C	ii / ii
Mother-c	1	G	A / C	C / T	A / T	C / T	i / ii
offspring	7	G	A	C	A	T	i / i
offspring	2	G	C	T	T	C	ii / ii
Mother-d	1	G	A	C	A	T	i / i
Mother-e	1	G / T	C	C / T	A / T	C / T	ii / iii
Mother-f	1	G	A / C	C / T	A / T	C / T	i / ii
Mother-g	1	G	A / C	C / T	A / T	C / T	i / ii

* Genotype names are reffered in Fig. 2.

Table 3. The exact tests of Hardy-Weinberg equilibrium for SNPs of *EF-1α* and *boule* genes in individuals in the Kinu-gawa and Sakura-gawa populations and in parthenogenetic progenies.

Sequence position	Nucleotide	Kinu-gawa and Sakura-gawa populations				Parthenogenetic progenies			
		HETERO	HOMO 1	HOMO 2	*P*-value	HETERO	HOMO 1	HOMO 2	*P*-value
EF-1α (512 bp)									
4	A / G	17	6	11	1.00	0	18	14	< 0.001
10	A / G	11	3	20	0.39	0	7	25	< 0.001
175	A / G	16	7	11	0.74	0	18	14	< 0.001
260	C / T	12	19	3	0.66	0	25	7	< 0.001
265	A / G	12	3	19	0.66	0	7	25	< 0.001
335	C / G	7	1	26	0.46	0	18	14	< 0.001
412	C / T	8	23	3	0.11	0	25	7	< 0.001
boule (694 bp)									
112	G / T	5	29	0	1.00	0	32	0	1.000
300	A / C	13	14	7	0.28	0	25	7	< 0.001
306	C / T	11	21	2	0.64	0	25	7	< 0.001
437	A / T	10	21	3	0.34	0	25	7	< 0.001
689	C / T	12	4	18	0.41	0	7	25	< 0.001

HETERO, HOMO 1, and HOMO 2 indicate individual numbers of heterozygosity, homozygosity (left nucleotide in Nucleotide column), and homozygosity (right nucleotide), respectively.

chromosome numbers (2n = 14 and 15). Normal development requires a diploid complement of chromosomes as shown in other insects (Corley et al., 1999). However, abnormal (i.e. malformed) phenotypes were not observed in the 1st instar nymphs of 2n = 14 and 15 forms of the insect (data not shown), although post-embryological development was not followed by us. In *E. eophilum*, individuals with chromosome number 2n = 15 (X0) and 16 (XX) are male and female, respectively, (Sekiné et al., 2013a). In species with XX : X0 sex determination (i.e. males and females have 1 and 2 X chromosomes, respectively), parthenogenetically derived males may develop as a consequence of accidental sex chromosome loss due to non-disjunction (cf. van der Kooi & Schwander, 2014). Indeed, it has been proposed that deuterotoky, which generates both parthenogenetic males and females from unfertilized eggs, occurs in some mayflies (Huff & McCafferty, 1974; Mingo, 1978; Funk et al., 2010). Although it is unclear whether *E. eophilum* parthenogenetic nymphs with chromosome number 2n = 15 in this study lost a sex chromosome, deuterotoky could occur in *E. eophilum*. Alternatively, different chromosome numbers may arise because of the presence of B chromosomes, as suggested by Sekiné et al. (2013a).

In the nuclear analyses of *EF-1α* and *boule* genes as EPIC markers, various genotypes were observed, although there was a weak bias in terms of genotype distribution. In addition, there was no evidence of population genetic differentiation due to a restriction of gene flow between the Sakura-gawa and Kinu-gawa populations. Hence, our observation that all parthenogenetic offspring were homozygous despite having heterozygous mothers, is a result obtained without the application of time-consuming histochemical methods and development of microsatellite markers. This finding indicates that the restoration of the ploidy level occurs via automixis during meiosis. Obligatory parthenogenesis in *E. shigae* is also automictic, with terminal fusion occurring through the mature egg nucleus fusing with its sister second polar body nucleus to form a single embryonic nucleus (Sekiné & Tojo, 2010b). Central fusion and gamete duplication are also known as a consequence of other automictic modes (Suomalainen et al., 1987). In central fusion, diploidy is recovered either by the fusion of the egg nucleus with one of the non-sister nuclei derived from the first polar body, or by the fusion of two of the non-sister polar bodies. In gamete duplication, diploidy in homozygous condition is recovered only after the first cleavage following the fusion of two blastomere nuclei. Terminal fusion and gamete duplication rapidly cause heterozygosity to decrease in automixis, even though the rates of transition to homozygosity of heterozygous loci differ according to the distance from the centromere. In the case of terminal fusion, the expected rates are 1.00 close to the centromere (no recombination) and 0.33 far from the centromere (substantial recombination) (Templeton, 1982; Pearcy et al., 2006). In this study, the facultative parthenogenesis of *E. eophilum* reduced heterozygosity so rapidly that the rates of transition from heterozygosities to homozygosities were 1.00 at both the *EF-1α* and *boule* loci. Thus, it is possible that in *E. eophilum* either terminal fusion (in which the *EF-1α* and *boule* loci are close to the centromere) or gamete duplication cause the restoration of the ploidy level during diploid thelytoky.

A previous study indicated that facultative parthenogenesis is an important trait that allows mayflies with a short adult stage to reproduce and form new populations, even if males are not available for mating (Funk et al., 2010). Most *Ephoron* adults have an extremely short lifespan (maximum ~2 h), with females of *E. eophilum* and *E. shigae* ovipositing with minimal stimulus (e.g. moisture or touch) even without mating (Sekiné & Tojo, 2010a). *E. shigae* in Japan and *E. album* (Say) in North America also have facultatively parthenogenetic ability; 0.3–34.7% (Sekiné

& Tojo, 2010a) and 8–10% (Britt, 1962), respectively, of the unfertilized eggs developed to the final stage. Like *Ephoron*, *Palingenia longicauda* Olivier (Ephemeroptera: Palingeniidae) in Europe also has an extremely short adult stage (< 2 h: Russev, 1987) and has about 50% parthenogenetic ability (Landolt et al., 1997). In comparison, the facultative parthenogenetic ability in the Japanese mayflies in the sister family Ephemeridae is relatively low, as shown in *Ephemera strigata* Eaton (mean 0.9%), *Ephemera japonica* (1.1%), and *Ephemera orientalis* McLachlan (0.6%), whose adult stages last a few days (Sekiné & Tojo, 2010a). Therefore, facultative parthenogenetic ability may be related to adult life span as well as mating and oviposition behaviour.

Even so, there was no evidence of deviation from HWE in the Kinu-gawa and Sakura-gawa populations, although in theory, parthenogenesis rapidly reduces the level of heterozygosity. From this it may be concluded that facultative parthenogenesis in these circumstances normally has little influence on population genetic structuring. Hence, the apparent lack of influence of parthenogenesis on the population structure of the natural population of *E. eophilum* strongly suggests that parthenogenesis rarely occurs in the wild, where both male and female individuals are abundant. This result is clearly at variance with previous views arguing that facultative parthenogenesis is important for mayflies that have a short reproductive alate stage, during which time females may fail to find males to mate with in the extremely brief "window of opportunity" available to them (Funk et al., 2010). In light of this, the reproductive/genetic characteristics of *E. eophilum* – in theory at least – doubtless contribute to maintaining small populations in harsh environments or populations that have been disrupted by ecological disturbance.

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