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Molecular evidence of polyandry and polygyny in the mating system of the non-biting midge *Chironomus riparius* (Diptera: Chironomidae)

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Abstract: *Chironomus riparius* (Insecta: Diptera) is an ecological and environmental flagship species widely distributed in organically enriched waters throughout the temperate latitudes of the Northern Hemisphere. The present study elucidates the mating system of *C. riparius* using molecular evidence of the reproductive strategies employed by both sexes. A total of 760 larvae from 19 egg masses collected from two different wild populations (Sarayköy and Pamukkale) in Denizli, Türkiye, were genotyped at the MSC1 and MSC4 microsatellite loci, and the number of fathers per egg mass was inferred using GERUD and COLONY. Multiple paternity was detected in all sampled egg masses from both populations, with notable differences in the mean number of fathers between them. *C. riparius* exhibited skewed paternity in populations with male-biased breeding sex ratios. The species is polygynandrous, with both sexes mating with multiple partners. The high frequencies of multiple paternity observed in this study likely indicate substantial genetic diversity within these populations, supporting the species' persistence across diverse ecological conditions.

Keywords: *Chironomus riparius*; multiple paternity; effective mating frequency; paternity skew; breeding sex ratio

INTRODUCTION

Insects (Arthropoda: Insecta), the most species-rich class of Animalia [1], are distributed across all continents and occupy nearly every terrestrial habitat in diverse biogeographical regions [2]. One of the most abundant insect groups is chironomids, known as non-biting midges. Chironomidae is a worldwide-distributed family belonging to the suborder Nematocera, which is found in nearly all types of freshwater ecosystems [3,4]. They play a key role in many lake and river systems through detritus consumption and high abundance [3], serving as a significant food source for numerous fish species, as well as other vertebrates and invertebrates [3,5]. Chironomids are holometabolous organisms, with a life cycle comprising an egg stage, four larval stages (instars), a pupal stage, and typically all aquatic, followed by a terrestrial adult stage [6,7]. Adult males form aerial swarms that function as mating arenas to attract females [7-9]. Adult females gather in the vegetation near these swarms and enter them to pair with a male [10]. There is a strong association between swarm size and mating frequency; the formation of swarm flights facilitates elevated mating rates, particularly under conditions of low population density [7,8]. As a result, higher-density swarms are more effective at attracting females [7,10]. The pair then leaves the swarm, alights, and copulates for less than 60 s. Immediately after transferring the spermatophore, the male returns to the swarm to mate with another female, whereas the female moves to oviposit [10]. Most chironomids deposit their eggs as gelatinous masses in contact with water, attaching them to substrates [7].

Chironomus riparius, a common and widespread chironomid species, occurs extensively in organically enriched waters across temperate regions of the Northern Hemisphere [11-13]. Its larvae are sediment-dwelling [14] and occur in nearly all types of aquatic environments, tolerating extreme variations in temperature, pH, salinity, depth, and current velocity [6,9]. *C. riparius* plays a key role in the recycling of organic matter and energy, serving as an important link between aquatic and terrestrial environments [15]. The species has a short life cycle and can be easily cultured in the laboratory [12,16]. *C. riparius* is widely used in standard

laboratory toxicity tests to assess chemical effects in freshwater ecosystems [12,16,17]. It is also frequently used as a bioindicator in environmental monitoring studies [18]. Collectively, these traits underscore its ecological and environmental significance.

Insects exhibit a remarkable diversity of mating systems, primarily shaped by millions of years of evolutionary processes [19-21]. Investigating the mating systems across insect species provides key insights into the principles of reproductive biology and evolutionary ecology and has attracted considerable attention in recent decades alongside advances in molecular biology and technology. Numerous studies have employed molecular markers to clarify the mating strategies of various insect species, such as the Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae) [22], the small hive beetle *Aethina tumida* (Coleoptera: Nitidulidae) [23], the spotted lanternfly *Lycorma delicatula* (Hemiptera: Fulgoridae) [24], the honey bee *Apis mellifera* (Hymenoptera: Apidae) [25], the field cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae) [26], and the Egyptian cotton leaf-worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) [27]. However, despite extensive knowledge of its biology, no molecular study has yet examined the mating system of the ecological and environmental flagship species *C. riparius*. This constitutes a significant gap in understanding its reproductive biology. To address it, the present study genotyped randomly selected larvae from several egg masses collected from wild populations using microsatellite loci to determine maternal and paternal genotypes and assess the occurrence of both polyandry and polygyny. This study provides molecular evidence elucidating the mating system of *C. riparius*, with a focus on the reproductive strategies of both sexes.

MATERIALS AND METHODS

Sample collection

Adult females and egg masses of *C. riparius* were collected from two different wild populations: a natural river in the Sarayköy district ("Sarayköy population") and an artificial pond in the Pamukkale district ("Pamukkale population"), both in Denizli, Türkiye (Supplementary Fig. S1). Collections in each district were conducted on a single day (on 18 August 2023 in Sarayköy and 13 September 2023 in Pamukkale). A total of 19 egg masses (12 from the Sarayköy population and 7 from the Pamukkale population), attached to stones or aquatic plants, were carefully collected using forceps and placed individually in 15-mL Falcon tubes containing site water (Supplementary Fig. S2A). The Falcon tubes were transferred to the laboratory in an upright position without shaking. In addition, 40 adult females (20 per population) were captured with a sweep net, placed individually in cryotubes filled with 96% ethyl alcohol, transported to the laboratory, and stored at -20°C until DNA isolation.

Larval rearing

All collected egg masses were examined under a dissecting microscope to confirm the absence of hatched larvae that might have originated from nearby egg masses of other females. Each egg mass was placed in a separate plastic container (18×10×10 cm) filled with settled, dechlorinated tap water (Supplementary Fig. S2B). Containers were labeled with the egg mass code and maintained at room temperature under a seasonal photoperiod. A small amount of shredded Whatman No. 1 filter paper was added to each plastic container to serve as a substrate for case construction and larval growth. Containers were covered with tightly woven mesh to exclude external organisms, and larvae were allowed to hatch. After hatching, larval development was monitored daily, with TetraMin® flake food provided every two days to meet nutritional requirements (Supplementary Fig. S2C). Settled, dechlorinated tap water was added as needed to maintain water levels.

Larvae sampling

Larvae were sampled upon reaching the fourth instar. Assuming an average egg mass contains approximately 400 eggs, 40 larvae were randomly selected from each container, representing 10% of the egg mass. A total of 480 larvae from 12 Sarayköy egg masses and 280 larvae from 7 Pamukkale egg masses were individually placed in cryotubes labeled with larva numbers and egg mass codes, containing 96% ethanol, and stored at -20°C until DNA extraction.

DNA isolation and microsatellite genotyping

Total DNA was isolated from the body tissues of the sampled adult females and larvae as previously reported [28]. Briefly, larvae were air-dried and incubated in 500 µL of STE solution with 12.5 µL proteinase K (19.6 mg/mL) and 25 µL 10% SDS at 55°C for 4 h. Following incubation, the phenol-chloroform method was applied, and DNA was precipitated with 3 M sodium acetate and 100% ice-cold ethanol. Subsequently, the DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 100 µL of Tris-EDTA buffer. The extracted DNA was assessed for integrity on 1% agarose gels and stored at -20°C for subsequent use.

For genotyping the sampled adult females and larvae, two microsatellite loci were selected for PCR amplification: MSC1, a dinucleotide repeat, and MSC4, a trinucleotide repeat. Both loci were specifically developed for *C. riparius* and shown to be polymorphic [29]. Forward primers of MSC1 and MSC4 were fluorescently labeled with 5'-FAM and 5'-TAMRA (oligonucleotide probes with fluorescein (FAM) as a 5' label and tetramethylrhodamine (TAMRA) as a 5' label), respectively, while the reverse primers were unlabeled. Both loci were amplified in a 25-µL total reaction volume with a final concentration of 0.3 mM total dNTP, 0.2 µM each of forward and reverse primers, 1.5 mM $MgCl_2$, 1X *Taq* buffer, 5 U *Taq* DNA polymerase, and 6 µL of the template DNA solution. For both loci, thermal cycling began with 5 min denaturation at 94°C, followed by 40 cycles of 40 s denaturation at 94°C, 40 s annealing at 60°C, and 40 s extension at 72°C, concluding with a final 6 min extension at 72°C. Prior to fragment analysis, PCR products were run on 2% agarose gels to check for their yield and quality and stored at -20°C in the dark. Fragment length analysis was performed using an ABI3730XL Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, CA, USA) with ROX350 as the internal size standard.

Data analysis

Raw data obtained from the Genetic Analyzer were visualized using Peak Scanner 2.0 (Applied Biosystems), and the allele sizes and genotypes of the samples were identified. The existence of allelic stutter, large allele dropout, and null alleles in the larval genotype dataset was checked using Micro-Checker 2.2.3 [30]. For the 20 sampled adult females from each population, the Hardy-Weinberg equilibrium was tested, and allele frequencies and expected heterozygosity (H_{exp}) at the two loci were calculated using Genepop 4.7.5 [31]. Linkage disequilibrium between two loci was tested using a Markov chain model in Genepop 4.7.5 [31]. Observed heterozygosity (H_{obs}) at each locus was calculated using the following formula [32]:

$$H_{obs} = 1 - \sum({p_i}^2)$$

where p is the frequency of the ith allele for n alleles.

The probability (q) that two unrelated individuals within each population share the same genotype at a locus was calculated for both females and males using the following unbiased formula [33]:

$$q_{(unbiased)} = \frac{[(n^3(2a_2^2 - a_4) - 2n^2(a_3 + 2a_2) + n(9a_2 + 2) - 6)]}{[(n-1)(n-2)(n-3)]}$$

where n is the sample size, $a_i = p_j^i$, and p_j is the frequency of the jth allele. The probability (Q) that two unrelated individuals share a common genotype across two loci was calculated for females and males by multiplying the probabilities from each locus [32,33].

The probabilities of detection per locus (d) and for the combination of two loci (D) were calculated using the following formulas [34]:

$$d = 1 - 2a_2 + a_3 + 3(a_2a_3 - a_5) - 2(a_2^2 - a_4)$$

$$D = 1 - \prod_{i=1}^{n}(1 - d_i)$$

where $a_n = \sum_{i=1}^{k} p_i^n$, p_i is the frequency of the ith allele, and k is the number of alleles.

To assess whether the dataset had sufficient power for paternity analysis, the probability of detecting multiple paternity with 40 larvae per egg mass was calculated for the 20 sampled adult females per population using PrDM, which incorporates the number of loci, allele frequencies, and offspring sample size [35]. PrDM analysis was conducted assuming varying contribution rates (equal, skewed, highly skewed) among 2, 3, or 4 potential fathers.

Paternity analysis was performed using GERUD2.0 [36], a program that determines the minimum number of fathers per progeny array, and COLONY 2.0.7.1 [37], a maximum likelihood-based program that determines the maximum number of fathers per progeny array. Allele frequencies were incorporated in GERUD to allow the program to determine the most likely genotype combinations for each progeny, based on Mendelian segregation patterns and expected population genotypic frequencies. For COLONY analysis, allelic dropout and genotyping error rates were set at 0.000 and 0.001, respectively. A polygamous mating system was assumed for both sexes in this diploid species, and three medium-length replicate runs were performed. To determine whether any male mated with multiple females, inferred male genotypes from both programs were compared. The breeding sex ratio was calculated as the total number of genetically distinct male genotypes (across both loci) divided by the total number of egg-laying females analyzed.

The observed mating frequency (m_o) was defined as the number of different fathers detected per egg mass. The effective mating frequency (m_e), reflecting the actual paternity contribution of each male, was calculated for females mated with multiple males using the following formula [38]:

$$m_e = 1/\sum {p_i}^2$$

where p_i is the percentage of the larvae in an egg mass sired by male i, with the following correction for sampling error [39]:

$$\sum {p_i}^2 = (N\sum {y_i}^2 - 1)/(N-1)$$

where y_i is the observed contribution by each father, and N is the number of the sampled larvae.

The Ryan-Joiner test was used to assess the normality of the paternity data (observed number of fathers), which were found to be normally distributed. Consequently, differences in the number of fathers per egg mass between the two populations were analyzed using a parametric two-sample t-test. All analyses were performed in Minitab 21 (Minitab Inc., State College, PA) with a significance threshold of $P<0.05$.

RESULTS

Molecular marker analysis

In the study, 40 adult females, 20 from each of the Sarayköy and Pamukkale populations, were genotyped at the MSC1 and MSC4 microsatellite loci. It was observed that both loci were highly polymorphic in both populations (Table 1). In the Sarayköy population, 10 and 7 different alleles were detected for MSC1 and MSC4, respectively. The H_{exp} values for both loci (0.90 for MSC1 and 0.78 for MSC4) were slightly higher than the H_{obs} values (0.88 for MSC1 and 0.76 for MSC4). The allele frequencies at both loci were within expectations of the Hardy-Weinberg equilibrium ($P>0.05$), and no linkage disequilibrium was detected between the two loci ($P>0.05$). The q value was calculated as 0.02 at MSC1 and 0.11 at MSC4, while the Q value was found to be 2.3×10^{-3} across two loci. The d value was calculated as 0.75 at MSC1 and 0.54 at MSC4. Combining the two loci, the overall D value was 0.89. In the Pamukkale population, nine and six different alleles were detected for MSC1 and MSC4, respectively. The H_{exp} values for both loci (0.88 for MSC1 and 0.77 for MSC4) were slightly higher than the H_{obs} values (0.86 for MSC1 and 0.75 for MSC4). The allele frequencies at both loci were within expectations of the Hardy-Weinberg equilibrium ($P>0.05$), and no linkage disequilibrium was detected between the two loci ($P>0.05$). The q values were 0.03 at MSC1 and 0.12 at MSC4, while the combined probability across both loci (Q) was 3.9×10^{-3}. The detection probabilities (d) were 0.71 at MSC1 and 0.52 at MSC4, with the combined detection probability (D) for both loci calculated as 0.86.

To assess the probability of detecting multiple paternity using the two loci in this study with 40 larvae sampled per egg mass, PrDM values were calculated under different scenarios of paternal contribution rates among multiple fathers (Table 2). For the Sarayköy population, the average PrDM value with a sample size of 40 larvae was 0.951 for two fathers, 0.998 for three fathers, and 1.000 for four fathers. For the Pamukkale population, these values were 0.934, 0.996, and 1.000, respectively. Thus, it was concluded that in both populations, the likelihood of failing to detect multiple paternity with these two microsatellite loci was very low, indicating that the dataset provides sufficient power for paternity analysis.

Table 1. Summary statistics of MSC1 and MSC4 microsatellite loci used for paternity analysis across populations.

Population	Locus	Number of alleles	Allele size (bp)	H_{exp}	H_{obs}	HW	*q*	*d*
Sarayköy	MSC1	10	90-108	0.90	0.88	0.21	0.02	0.75
	MSC4	7	203-221	0.78	0.76	0.06	0.11	0.54
Pamukkale	MSC1	9	92-108	0.88	0.86	0.68	0.03	0.71
	MSC4	6	203-218	0.77	0.75	0.14	0.12	0.52

H_{exp} – expected heterozygosity; H_{obs} – observed heterozygosity; HW – Hardy-Weinberg P value; *q* – the probability that two unrelated females share the same genotype at a locus; *d* – the probability of detection per locus.

Table 2. Population-based PrDM values calculated for a sample size of 40 larvae per egg mass under varying paternal contribution scenarios.

Possible number of fathers	Possible paternal contribution rate	Population	
		Sarayköy	Pamukkale
2	Equal (1:1)	0.970	0.954
	Skewed (2:1)	0.968	0.955
	Very skewed (9:1)	0.916	0.893
	Mean	**0.951**	**0.934**
3	Equal (1:1:1)	0.999	0.998
	Skewed (2:1:1)	0.999	0.998
	Very skewed (8:1:1)	0.995	0.991
	Mean	**0.998**	**0.996**
4	Equal (1:1:1:1)	1.000	1.000
	Skewed (2:1:1:1)	1.000	1.000
	Very skewed (7:1:1:1)	1.000	0.999
	Mean	**1.000**	**1.000**

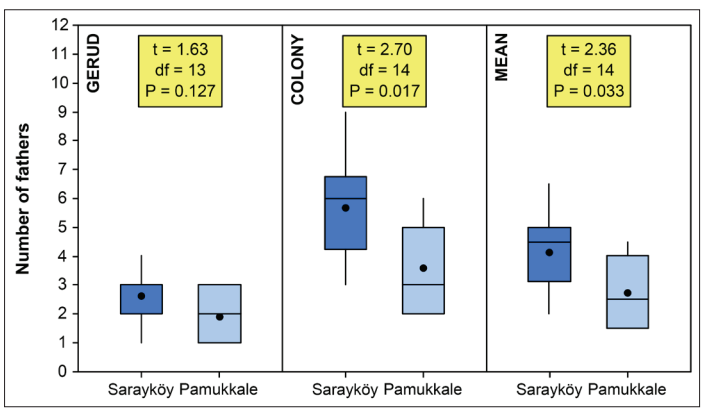

Fig. 1. Comparison of the number of fathers observed in the egg masses from the Sarayköy and Pamukkale populations. The box extends from the 25th percentile to the 75th percentile. The median is represented by the middle horizontal line and the mean by the black circle in the box, while the range of the distribution is indicated by the whiskers. Note that the statistical results of the two-sample t-test are included in the graph.

Paternity analysis

A total of 760 larvae from 19 egg masses were genotyped at two microsatellite loci for paternity analysis in the Sarayköy and Pamukkale populations. No allelic stutter, large allele dropout, or null alleles were detected at either locus in the larval dataset for both populations. Genotyping of the females that laid the sampled egg masses using GERUD confirmed that none of the egg masses originated from the same female. Paternity analysis results are presented in Table 3. According to GERUD results, the number of fathers per egg mass in the Sarayköy population ranged from 1 to 4. Multiple paternity was detected in 10 out of 12 egg masses (83.33%), with a mean m_o value of 2.58±1.00 males per egg mass (Table 3). In the Pamukkale population, the number of fathers for the egg masses ranged from 1 to 3. Multiple paternity was determined in four of seven egg masses (57.14%), with a mean m_o value of 1.86±0.90 males per egg mass (Table 3). Although the mean number of fathers in the Sarayköy population was higher than that in the Pamukkale population, this difference was not statistically significant (P>0.05) (Fig. 1).

Based on COLONY results, multiple paternity was observed in all 12 egg masses (100%) from Sarayköy, with the number of fathers ranging from 3 to 9 (Table 3). The mean m_o value was 5.67±1.83 males per egg mass (Table 3). In Pamukkale, multiple paternity was also found in all 7 egg masses (100%), with 2 to 6 fathers detected, and a mean m_o value of 3.57±1.51 males (Table 3). The mean number of fathers was significantly higher in the Sarayköy population than in the Pamukkale population (P<0.05) (Fig. 1).

Combining results from both programs, all egg masses (100%) from both populations exhibited multiple paternity, with a mean of 4.13 ± 1.37 contributing males per egg mass in the Sarayköy population and 2.71 ± 1.19 in the Pamukkale population (Table 3). The mean number of fathers in the Sarayköy population was found to be significantly higher than that in the Pamukkale population (P<0.05) (Fig. 1).

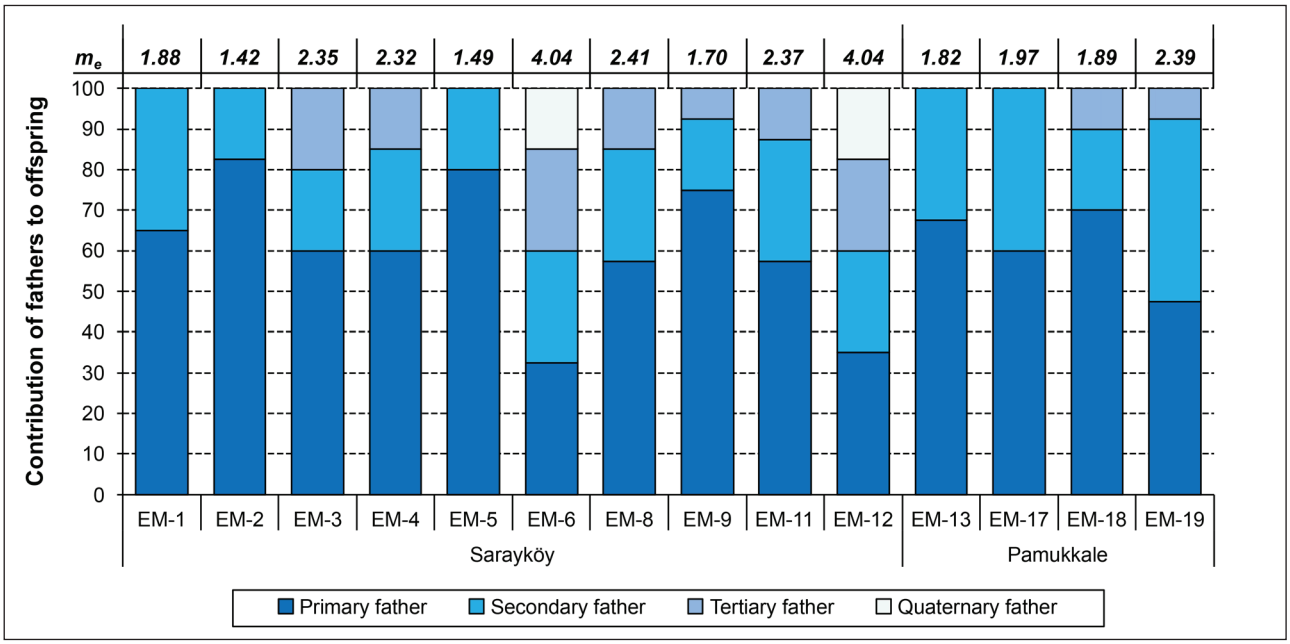

Fig. 2. GERUD results for the relative contribution of fathers to offspring from egg masses sired by multiple males and the calculated effective mating frequency (number of effective mates) (m_e) for each of these egg masses.

Table 3. Summary information and paternity analysis results of the egg masses collected from each population

Population	Egg mass ID	Number of fathers				Contribution of fathers to offspring (%) (based on the GERUD results)			
		GERUD	COLONY	Mean		Primary	Secondary	Tertiary	Quaternary
Sarayköy	EM-1	2	4	3.0		65.0	35.0	-	-
	EM-2	2	5	3.5		82.5	17.5	-	-
	EM-3	3	6	4.5		60.0	20.0	20.0	-
	EM-4	3	6	4.5		60.0	25.0	15.0	-
	EM-5	2	5	3.5		80.0	20.0	-	-
	EM-6	4	6	5.0		32.5	27.5	25.0	15.0
	EM-7	1	3	2.0		100.0	-	-	-
	EM-8	3	7	5.0		57.5	27.5	15.0	-
	EM-9	3	8	5.5		75.0	17.5	7.5	-
	EM-10	1	3	2.0		100.0	-	-	-
	EM-11	3	6	4.5		57.5	30.0	12.5	-
	EM-12	4	9	6.5		35.0	25.0	22.5	17.5
	Total	**31**	**68**	**49.5**					
	Mean±SD	**2.58±1.00**	**5.67±1.83**	**4.13±1.37**					
Pamukkale	EM-13	2	3	2.5		67.5	32.5	-	-
	EM-14	1	2	1.5		100.0	-	-	-
	EM-15	1	2	1.5		100.0	-	-	-
	EM-16	1	3	2.0		100.0	-	-	-
	EM-17	2	4	3.0		60.0	40.0	-	-
	EM-18	3	5	4.0		70.0	20.0	10.0	-
	EM-19	3	6	4.5		47.5	45.0	7.5	-
	Total	**13**	**25**	**19**					
	Mean±SD	**1.86±0.90**	**3.57±1.51**	**2.71±1.19**					

SD – standard deviation

Paternal contribution

In egg masses sired by multiple males, the genetic contributions of detected fathers were assessed using GERUD. Contributions were generally unequal in both populations (Fig. 2 and Table 3). In all egg masses of multiply mated females, the majority of the offspring was sired by a single male (primary father), with the remainder fathered by one, two, or three additional males (secondary, tertiary, and quaternary fathers, respectively). In the Sarayköy population, primary fathers contributed a mean of 60.5% (range: 32.5%–82.5%) of offspring, and secondary fathers 24.5% (17.5%–35.0%). Tertiary fathers in seven egg masses contributed 7.5%–25.0%, while quaternary fathers in two egg masses contributed 15.0% and 17.5%. In the Pamukkale population, primary and secondary fathers contributed 61.3% (47.5%–70.0%) and 34.4% (20.0%–45.0%), respectively, with tertiary fathers in two egg masses contributing 7.5% and 10.0%.

Using GERUD results on paternal contributions (Table 3), the m_e for each multiply mated female was calculated (Fig. 2). In the Sarayköy population, m_e ranged from 1.42 to 4.04, and in the Pamukkale population from 1.82 to 2.39. Generally, m_e values were lower than the m_o, except in two egg masses (EM-6 and EM-12), where m_e (4.04) slightly exceeded m_o (4.00).

Breeding sex ratio

Using the paternal contribution rates of the fathers provided by the GERUD program, the q value was calculated as 0.03 at MSC1 and 0.12 at MSC4 for the Sarayköy population and as 0.05 at MSC1 and 0.12 at MSC4 for the Pamukkale population. The Q value across the two loci was 3.8×10^{-3} for Sarayköy and 6.5×10^{-3} for Pamukkale. Based on the results from GERUD, 31 male genotypes for the Sarayköy population and 13 for the Pamukkale population were identified as genetically contributing to the larvae (Table 3). Two male genotypes in the Sarayköy population were identical, indicating that one male mated with two females and sired offspring in both, while the remaining 29 males each mated with only one female (Supplementary Table S1). In contrast, all 13 male genotypes identified in Pamukkale were distinct, suggesting that each male mated with only one female (Supplementary Table S1). The polygyny rate was 3.33% in the Sarayköy population, based on 30 distinct males and one polygyny event, and 0.00% in the Pamukkale population. The breeding sex ratio was estimated at 2.50 males per female (30 males to 12 females) in Sarayköy and 1.86 males per female (13 males to 7 females) in Pamukkale.

COLONY analysis identified 68 male genotypes contributing to offspring in the Sarayköy population and 25 male genotypes in the Pamukkale population (Table 3). In Sarayköy, 16 of 42 distinct males mated with multiple females: eight mated with two females, six with three, and two with four (Supplementary Table S1). In Pamukkale, 4 of 20 distinct males mated with multiple females: three mated with two females, and one with three (Supplementary Table S1). The polygyny rate was 38.10% in the Sarayköy population and 20.00% in the Pamukkale population. The breeding sex ratio was 3.50 males per female (42 males to 12 females) in Sarayköy and 2.86 males per female (20 males to 7 females) in Pamukkale.

DISCUSSION

The evolution of mating systems is a fundamental topic in ecology. Revealing insect mating behavior is essential for understanding their ecology and evolution, and characterizing a species' mating system is a key step in comprehending its life history. Mating systems can affect a species' genetic effective population size and evolutionary trajectory [40], making them particularly important in small populations. Studying the mating systems and behaviors of insect species, particularly those with broad distributions and high ecological or economic significance, provides insights into their current status and potential future resilience against environmental threats such as pollution, global warming, and human activities. Such studies can also resolve outstanding questions by enabling verification and correction of observations on species biology. This study represents the first molecular investigation of the *C. riparius* mating system, providing comprehensive evidence of the reproductive strategies of both sexes and underscoring its significance.

Molecular marker analyses in this study showed that the MSC1 and MSC4 loci were highly polymorphic in both populations. The H_{exp} and H_{obs} values were relatively high, the allele frequencies at both

loci conformed to Hardy-Weinberg equilibrium expectations, and there was no linkage disequilibrium between the two loci. The *D* values were higher than 0.85 with the combination of both loci. These findings indicate that MSC1 and MSC4 are highly informative and effective molecular markers for studying mating systems in *C. riparius* populations. Additionally, PrDM values calculated for a sample size of 40 larvae per egg mass, across different potential paternal contribution scenarios, ranged from 0.893 to 1.000. These high values indicate that, despite the limited number of egg masses and use of only two loci, the dataset provides sufficient power for paternity analysis, with a high probability of detecting multiple paternity. Consequently, the marker analyses offer strong evidence that the study's results are accurate and reliable, underscoring its significance.

The number of fathers detected by GERUD is considered conservative: it cannot be overestimated, but it may be underestimated [41]. In contrast, COLONY tends to overestimate the putative number of fathers in a progeny [42]. The programs GERUD and COLONY were used in this study for parental reconstruction/paternity analysis, providing an estimated range for each population, with GERUD giving the minimum and COLONY the maximum number of fathers. The GERUD results showed that the frequency of multiple paternity was 83.33% for the Sarayköy population and 57.14% for the Pamukkale population. COLONY, however, indicated a 100% frequency of multiple paternity in both populations. Statistical analyses further showed significant differences in the mean observed number of fathers between the two populations. These differences between the two populations could be due to the unequal numbers of egg masses collected from the populations. However, this is unlikely, as the frequency of multiple paternity is known to exhibit intraspecific variation [43]. The higher frequency of multiple paternity in the Sarayköy population may be attributed to its larger population size compared to the Pamukkale population. In larger populations, females are less likely to avoid male swarms to prevent re-mating and may enter the same or different swarms, increasing mate choice [7,8], which results in high polyandry and multiple paternity rates. Further research across different *C. riparius* populations is needed to identify the factors driving population-level differences in multiple paternity frequency. The relatively high frequencies of multiple paternity in both populations suggest large effective population sizes and high genetic diversity, which are critical for adaptability and survival under environmental changes [44]. Thus, the genetic diversity generated by multiple paternity may underlie *C. riparius*' wide distribution and ecological resilience.

Extensive studies have shown that multiple paternity occurs in various insect species, with reported frequencies reaching up to 100% [23,26,41,45-47]. However, only one study on multiple insemination in chironomids reported that multiple paternity is rare in *Chironomus oppositus*, occurring in just one of five egg masses, with offspring sired by at least two males [48]. The frequencies of multiple paternity observed in this study were higher than those reported for *C. oppositus* but fall within the range documented for other insect species, confirming that multiple paternity is common in nature and varies among species [43].

Several studies report polygynandry as the mating system in various insect species, including the Wellington tree wētā *Hemideina crassidens* (Orthoptera: Anostostomatidae) [49], the small hive beetle *A. tumida* [23], and the ponerine ant *Gnamptogenys striatula* (Hymenoptera: Formicidae) [50]. Consistent with the above, the detected levels of polyandry (GERUD: 83.33% for the Sarayköy population and 57.14% for the Pamukkale population; COLONY: 100% for both populations) and polygyny (GERUD: 3.33% for the Sarayköy population; COLONY: 38.10% for the Sarayköy population and 20.00% for the Pamukkale population) indicate that *C. riparius* has a polygynandrous mating system, with both sexes engaging in multiple mating. Multiple mating in insects is known to entail costs, including time and energy expenditure, as well as increased risks of injury, predation, and infection [51]. Despite these costs, both sexes often engage in multiple mating, suggesting adaptive benefits. In *C. riparius*, males likely mate multiply to enhance reproductive success, as male reproductive success is positively correlated with mating frequency [49]. Polygyny enables males to mate with females carrying different genetic traits, increasing the likelihood of passing on their genes. Multiple mating appears to benefit females more, providing both direct and indirect advantages [51,52], including sperm replenishment for high fertility, male-provided guarding, enhanced progeny genetic diversity, and avoidance of defective sperm or genetic incompatibility [53]. These

benefits likely apply to *C. riparius* females; however, the advantages of polyandry and polygyny in this species require further detailed investigation.

Even when a female mates with multiple males, not all males may successfully fertilize eggs due to factors such as premature termination of mating or male infertility. When multiple mating is successful, sperm from different males may compete to fertilize eggs, so not every mating results in genetic contribution to the offspring. Sperm competition is a significant driver in the evolution of reproduction of many organisms. In this study, male contributions within each egg mass from multiply mated females were uneven in both populations. Additionally, m_e values closely approximated m_o values for EM-1, EM-6, and EM-12 in the Sarayköy population and for EM-13 and EM-17 in the Pamukkale population, implying minimal paternity skew in these egg masses. In contrast, m_e values were much lower than m_o values for EM-2, EM-3, EM-4, EM-5, EM-8, EM-9, and EM-11 in the Sarayköy population and for EM-18 and EM-19 in the Pamukkale population, indicating highly skewed paternity in these cases. The results suggest that sperm competition, allowing multiply mated females to exercise post-copulatory sexual selection, may occur in both populations, particularly in Sarayköy. Since low male contribution may stem from less competitive sperm, the high genetic contribution of primary fathers indicates their success in fertilizing egg masses of polyandrous females and thus achieving reproductive success.

Considering male individuals as a demographic component has important implications for assessing the survival potential of a population. The breeding sex ratio, defined as the ratio of sexually receptive males to females actively seeking mates [54,55], reflects partner availability and mating opportunities [55]. This ratio varies both between and within species, both spatially and temporally throughout an individual's flight period [56]. In this study, both populations exhibited male-biased breeding sex ratios (GERUD: 2.50 males to 1 female for the Sarayköy population and 1.86 males to 1 female for the Pamukkale population; COLONY: 3.50 males to 1 female for the Sarayköy population and 2.86 males to 1 female for the Pamukkale population), indicating a male skew adult composition in both populations. Sampling egg masses from different females may bias results: it allows detection of polyandry and its direct outcome, multiple paternity, but limits the ability to confirm polygyny. The proportion of sexually active males may be overestimated if only a few egg masses are sampled. This risk decreases as sampling approaches the total number of females, improving the detection of paternal sibships. Therefore, further studies with larger egg mass samples are needed to obtain more accurate breeding sex ratios for these populations.

CONCLUSIONS

This study provides the first direct evidence of the mating system of *C. riparius*, which is an ecologically and environmentally important species with a wide global distribution. High levels of polyandry (>55% according to GERUD; 100% according to COLONY) and low levels of polygyny (<5% according to GERUD; <40% according to COLONY) were detected in the Sarayköy and Pamukkale populations. As a direct consequence of polyandry, both populations exhibited high frequencies of multiple paternity (>55% according to GERUD; 100% according to COLONY), with notable differences in the mean observed number of fathers between populations. These high multiple paternity frequencies likely indicate elevated genetic diversity within the populations. Additionally, both populations exhibited male-biased breeding sex ratios. These results indicate that *C. riparius* individuals are promiscuous, with both sexes mating with multiple partners, supporting the conclusion that the species has a polygynandrous mating system. While these findings provide robust molecular evidence, further comprehensive studies are recommended to confirm and extend the results. Conducting similar research across different regions of *C. riparius* distribution will enable broader conclusions about its reproductive biology and offer a more complete understanding of the species.

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Data availability: The raw data underlying this article are available as an online research dataset: https://www.serbiosoc.org.rs/NewUploads/Uploads/Sari_Dataset.pdf

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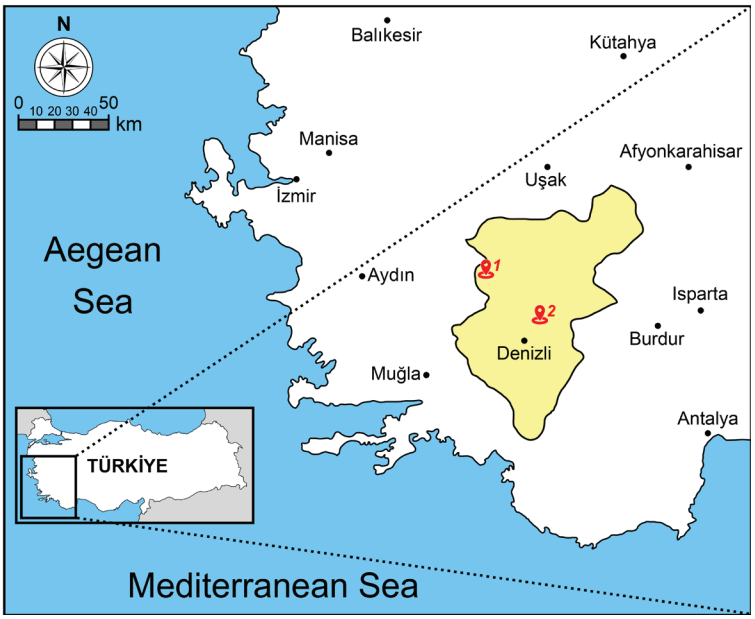
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Male genotypes that were identified as genetically contributing to the larvae based on the analyses in the GERUD and COLONY programs

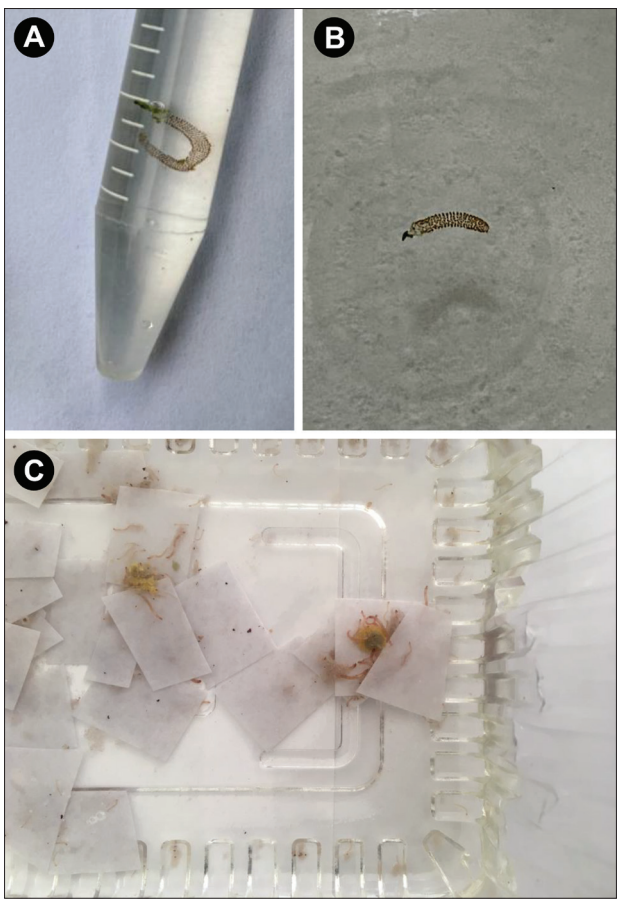
Population	Egg mass ID	GERUD					COLONY			
		Male	Male genotype		Male ID*		Male	Male genotype		Male ID*
			MSC1	MSC4				MSC1	MSC4	
Sarayköy	EM-1	#1	96/96	206/212	GS-1		#1	96/96	212/212	CS-1
		#2	96/102	215/218	GS-2		#2	96/96	215/215	CS-2
							#3	96/96	206/206	CS-3
							#4	96/102	218/218	CS-4
	EM-2	#1	102/106	206/209	GS-3		#1	92/92	209/212	CS-5
		#2	92/100	209/212	GS-4		#2	102/102	209/209	CS-6
							#3	100/100	209/209	CS-7
							#4	102/102	206/206	CS-8
							#5	106/106	209/209	CS-9
	EM-3	#1	90/100	209/212	GS-5		#1	100/100	209/215	CS-10
		#2	98/98	209/209	GS-6		#2	100/100	212/212	CS-11
		#3	90/98	215/218	GS-7		#3	98/98	209/209	CS-12
							#4	90/90	209/209	CS-13
							#5	98/98	215/215	CS-14
							#6	90/90	212/218	CS-15
	EM-4	#1	96/102	209/212	GS-8		#1	96/96	209/209	CS-16
		#2	98/100	209/209	GS-9		#2	100/100	209/215	CS-10
		#3	96/96	203/206	GS-10		#3	102/102	212/212	CS-17
							#4	98/98	209/215	CS-18
							#5	102/102	209/209	CS-6
							#6	96/96	203/206	CS-19
	EM-5	#1	92/100	203/209	GS-11		#1	100/100	209/209	CS-7
		#2	94/98	209/212	GS-12		#2	92/106	203/203	CS-20
							#3	94/94	209/209	CS-21
							#4	92/106	209/209	CS-22
							#5	98/98	212/212	CS-23
	EM-6	#1	92/94	203/209	GS-13		#1	96/98	209/209	CS-24
		#2	90/94	206/206	GS-14		#2	92/92	215/215	CS-25
		#3	96/98	209/209	GS-15		#3	96/96	215/215	CS-2
		#4	92/96	212/215	GS-16		#4	96/96	212/212	CS-1
							#5	92/94	203/209	CS-26
							#6	92/94	206/206	CS-27
	EM-7	#1	96/98	209/212	GS-17		#1	96/96	209/209	CS-16
							#2	98/98	209/209	CS-12
							#3	94/98	212/212	CS-28
	EM-8	#1	96/98	206/215	GS-18		#1	98/98	212/212	CS-23
		#2	98/102	212/212	GS-19		#2	98/98	215/215	CS-14
		#3	96/104	212/212	GS-20		#3	94/102	209/212	CS-29
							#4	96/98	206/206	CS-30
							#5	96/96	209/212	CS-31
							#6	104/104	212/212	CS-32
							#7	96/96	215/215	CS-2
	EM-9	#1	92/100	209/215	GS-21		#1	94/94	209/212	CS-33
		#2	94/96	209/212	GS-22		#2	100/100	209/209	CS-7
		#3	90/98	209/209	GS-23		#3	90/100	209/209	CS-34

							#4	100/100	215/215	CS-35
							#5	92/92	209/209	CS-36
							#6	92/92	215/215	CS-25
							#7	96/96	212/212	CS-1
							#8	98/98	209/209	CS-12
	EM-10	#1	92/102	209/212	GS-24		#1	102/102	212/212	CS-17
							#2	92/92	209/209	CS-36
							#3	102/102	209/209	CS-6
	EM-11	#1	92/106	209/212	GS-25		#1	106/106	215/215	CS-37
		#2	106/106	215/218	GS-26		#2	102/106	212/218	CS-38
		#3	98/100	209/215	GS-27		#3	92/92	209/212	CS-5
							#4	106/106	209/209	CS-9
							#5	100/100	215/215	CS-35
							#6	98/98	209/212	CS-39
	EM-12	#1	92/102	209/209	GS-28		#1	102/102	215/215	CS-40
		#2	96/98	209/212	GS-17		#2	92/92	209/209	CS-36
		#3	90/102	215/215	GS-29		#3	102/102	209/209	CS-6
		#4	92/94	206/212	GS-30		#4	98/98	212/212	CS-23
							#5	92/94	206/206	CS-27
							#6	98/98	209/209	CS-12
							#7	96/96	209/209	CS-16
							#8	90/104	215/215	CS-41
							#9	94/94	212/212	CS-42
	EM-13	#1	102/106	212/215	GP-1		#1	102/102	212/215	CP-1
		#2	100/100	212/212	GP-2		#2	100/100	212/212	CP-2
							#3	106/106	212/212	CP-3
	EM-14	#1	100/106	206/215	GP-3		#1	100/100	206/206	CP-4
							#2	106/106	215/215	CP-5
	EM-15	#1	98/106	212/215	GP-4		#1	106/106	212/215	CP-6
							#2	98/98	212/212	CP-7
	EM-16	#1	100/106	212/215	GP-5		#1	106/106	215/215	CP-5
							#2	100/100	212/212	CP-2
							#3	92/106	212/212	CP-8
	EM-17	#1	92/98	206/212	GP-6		#1	98/98	215/215	CP-9
		#2	98/98	215/215	GP-7		#2	98/98	212/212	CP-7
Pamukkale							#3	98/98	206/206	CP-10
							#4	92/92	212/212	CP-11
	EM-18	#1	104/108	215/218	GP-8		#1	108/108	218/218	CP-12
		#2	102/106	206/206	GP-9		#2	100/104	209/209	CP-13
		#3	100/100	209/209	GP-10		#3	104/104	215/215	CP-14
							#4	100/104	218/218	CP-15
							#5	102/106	206/206	CP-16
	EM-19	#1	100/104	215/218	GP-11		#1	100/108	218/218	CP-17
		#2	92/108	212/218	GP-12		#2	100/100	215/215	CP-18
		#3	98/98	212/212	GP-13		#3	92/104	218/218	CP-19
							#4	104/104	212/212	CP-20
							#5	92/92	212/212	CP-11
							#6	98/98	212/212	CP-7

*Male IDs were assigned to distinct male genotypes within each population based on the results of each program. The first and second letters in a male ID represent the program name and population, respectively.



Supplementary Fig. S1. Map showing the locations of Sarayköy (1) and Pamukkale (2) districts of Denizli, Türkiye, from which *Chironomus riparius* adult females and egg masses were sampled in the study.



Supplementary Fig. S2. *Chironomus riparius* egg masses and their larvae. **A** – An egg mass placed in a Falcon tube filled with water from the sampling locality for transferring to the laboratory. **B** – An egg mass placed in a plastic container in the laboratory after checking it for the presence of any hatched larvae possibly from other egg masses. **C** – The larvae from an egg mass reared in a plastic container in the laboratory.