Genetic confirmation of “egg parasitism” in androgenetic freshwater Corbicula clams by paternity testing using microsatellite DNA markers

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Abstract: Corbicula leana and C. fluminea are hermaphroditic and ovoviviparous freshwater clams. Although they are considered to reproduce using self-fertilization, the possibility of outcrossing was suggested due to lineage discordance between mitochondrial and genomic DNA. In these species, outcrossing means “egg parasitism” by the spermatozoa from one or more other clams, because they reproduce by androgenesis in which only the nucleus of spermatozoa is transmitted to the progeny. Moreover, the presence of males in these species was reported in the previous study, and they were estimated to reproduce by egg parasitism of hermaphrodites. In this study, we investigated the paternity of juveniles in the brood pouches of six hermaphrodites by comparing the genotypes of the brooded juveniles, brooding clams, and neighboring adult clams using two microsatellite DNA markers. Brooded juveniles showed either identical genotypes to the parental clam or different genotypes from their parent in one clam with brooding. The genotypes of brooded juveniles were identical to those of neighboring hermaphrodites and males. These results indicate that androgenetic Corbicula reproduce not only by self-fertilization but also by egg parasitism, with outcrossing among hermaphrodites and from males to hermaphrodites.

Key words: Corbicula fluminea, androgenesis, hermaphrodite, self-fertilization, ovoviviparity

The clam genus Corbicula provides us an interesting model system to study clonal reproductive strategy in asexual (androgenetic) eukaryotic organisms. Corbicula leana Prime, 1864 and C. fluminea (Müller 1774) are hermaphroditic (Ikematsu & Shigaki 1976; Kraemer 1978), either diploid or triploid, and reproduce by androgenesis (Komaru et al. 1998; Ishibashi et al. 2003). Oogenesis and spermatogenesis proceed simultaneously in C. leana (Ikematsu & Yamane 1977) and C. fluminea (Kraemer 1978), and oviparity and ovoviviparity coexist in some populations; released oocytes were all fertilized ovipariously in C. leana (Ikematsu & Shigaki 1976). In the case of ovoviviparity, the oocytes and spermatozoa are released through the gonoduct into a brood pouch in the inner demibranch, where fertilized zygotes develop into D-shaped larvae (Kraemer 1978). Another characteristic of C. leana and C. fluminea reproduction is androgenesis (Komaru et al. 1998; Ishibashi et al. 2003). During androgenesis in Corbicula clams, all chromosome extrusions are embodied in two polar bodies at meiosis and only one incorporated male pronucleus develops into the zygote nucleus. Consequently, only the nucleus DNA of non-reductional spermatozoa is transmitted to the progeny in androgenetic clams (Komaru et al. 1998; Ishibashi et al. 2003).

Androgenetic Corbicula are considered to be self-fertilizing, due to the mode of reproduction described above. This is supported by the observation that single individuals of C. leana can reproduce under experimental conditions (Ikematsu & Shigaki 1976; Ikematsu & Yamane 1977). However, discordance in the lineage between mitochondrial and genomic DNA has been observed in Corbicula populations (Lee et al. 2005; Hedtke et al. 2008, 2011; Pigneur et al. 2011, 2012; Tiemann et al. 2017). Lee et al. (2005) suggested that this discordance is caused by the fertilization of eggs by the sperm of other individuals, which means that outcrossing also exists in Corbicula reproduction. Egg parasitism has been suggested to explain this complete replacement of an unclear genome by outcrossing (Hedtke et al. 2008, 2011; Pigneur et al. 2011, 2012).

Corbicula fluminea was introduced to Japan in the 1980s...
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(Ishibashi & Komaru 2003). Japanese populations of C. flu- 
minea had been thought to be exclusively constituted of her- 
maphrodites (Ishibashi & Komaru 2003; Yamada et al. 2010).
However, males have been recently found in one area (Houki et al. 2011). The males have produced non-reductional spermatozoa and shared the same mitochondrial haplotypes as the sympatric hermaphrodites. Houki et al. (2011) suggested that oocytes of hermaphrodites might be fertilized by the spermatozoa of the sympatric males, leading to the replacement of nuclear DNA in zygotes. In other words, males reproduce by egg parasitism of hermaphrodites. However, egg parasitism in Corbicula has not been confirmed by genetic paternity testing. To confirm male reproduction and egg parasitism in Corbicula, detection of outcrossing using molecular markers is necessary. In this study, we verified the paternity of juveniles brooded in brood pouches of hermaphrodites by comparing the genotypes of the brooded juveniles, brooding clams, and neighboring adult clams, using microsatellite DNA markers.

Since the taxonomy and phylogenetic relationships of C. flu-
minea and C. leana remain unresolved (Yamada et al. 2010; Pigneur et al. 2011; Komaru et al. 2013), we treated our specimens as Corbicula spp. Adult clams were collected from an irrigation ditch that flows from the Yasu River, a river that flows into Lake Biwa in Ritto, Shiga Prefecture, Japan, on 27 July 2018. These were collected from the area where Houki et al. (2011) reported the sympatry of hermaphrodites and males. Based on shell color, specimens were classified into two types, “Yellow” and “Green,” which correspond to C. flu-
ninea color type I and the C. leana color type, respectively, in Houki et al. (2011). The collected clams consisted of 30 Yellow specimens and 40 Green specimens. They were dis-
sected and the brooding larvae in their gills were checked un-
der a stereo microscope. The clams with larvae were treated as brooding clams, while those without larvae were taken as their neighboring clams in subsequent analyses. Larvae in gills were recognized in the Yellow type only (n=6), but not the Green type.

The sex (hermaphrodite or male) of the neighboring clams was judged by histological observation on the gonad and clams were classified into three phenotypes: Yellow herma-
phrodite (n=24), Green hermaphrodite (n=20), and Green male (n=20). The ploidy levels of brooding and neighboring clams were estimated using flow cytometry. Gill tissue was dis-
sected from adult clams and incubated for 40 minutes in phosphate-buffered saline (PBS) containing 50 μg/mL trypsin with gently agitation using a shaker at room temperature (ap-
proximately 20 to 25°C) to isolate the cells. After incubation, the cell suspension was washed three times with PBS and fixed in 2% paraformaldehyde solution for 10 minutes at room temperature. Following washing, the cell suspension was stained with propidium iodide/RNAse solution (Immunostep, Salamanca, Spain) for 15 minutes at room temperature. Ploidy levels were examined using a FACSCalibur flow cytometer with CELLQuest software (BD Biosciences, Franklin Lakes, NJ, USA). Fluorescence was excited using an argon laser at a 488 nm wavelength. Cells showing fluorescence were col-
lected at FL-2 (564–606 nm). The dioecious species C. ja-
ponica was used as a diploid control. Flow cytometry analysis results are shown in Fig. 1. FL-2 intensity peaked at 140 in estimated 2n clams and 210 in estimated 3n clams. The foot muscles of brooding and neighboring clams were dissected and processed to extract DNA using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer’s protocols.

Almost all larvae (shell length was approximately 200 μm) were collected from the gills of six Yellow type clams. Lar-
vae were reared together in mesh containers corresponding to each brooding clam and all containers were placed into a 20 L beaker at 23°C. Larvae (or juveniles) were fed with Chlorella homosphaera for 2 months, until their shell length reached approximately 700 μm, which is large enough for DNA analy-
ysis. Up to 20 grown juveniles were collected from each brood. Consequently, 20, 19, 20, 11, 20, and 17 were collected from each brooding clam. Juvenile DNA was extracted from the entire body using the HotSHOT method (Truett et al. 2000). Each juvenile was placed in a polymerase chain reaction (PCR) tube containing 18 μL of 50 mM NaOH, and then heat-
ed at 95°C for 10 minutes and chilled on ice. After addition of 2 μL of 1 M tris-HCl (pH 8.0), each tube was centrifuged at 12,000 rpm for 10 minutes, and supernatant was collected as a crude DNA solution.

Two microsatellite primers, cf8 and cf35 (Table 1), were selected from the 97 microsatellite loci reported by Peñarrubia et al. (2015). These two primers amplified microsatellite loci in all our specimens and detected some genetic poly-
morphisms between specimens. For each locus, amplification was performed with a 10 μL total volume, including 50 ng of template DNA or 0.25 μL of crude DNA solution, 1×PCR reaction buffer, 200 nM of dNTPs (Takara, Shiga, Japan), 200 μM of both primers, and 0.5 U of TaKaRa Taq DNA poly-
merase (Takara). Forward primers were tagged with a 5’ fluo-
rescently labeled CAG-tag 5’-CAG TCG GGC GTC ATC A-3’. Amplification by PCR was performed using touchdown ther-
mal cycling programs (Don et al. 1991). The fragments were analyzed using an ABI 3730XL Genetic Analyzer with Ge-

![Fig. 1. Flow cytometry analysis of the gill cells of the clams. A, estimated ploidy level was 2n. B, estimated ploidy level 3n.](image-url)
neScan-500 (LIZ) size standard (Applied Biosystems, Foster City, CA, USA). Results were analyzed using GeneMapper 4.0 software (Applied Biosystems), considering ploidy.

Genotypes of three microsatellite loci of brooding clams and their juveniles are shown in Table 2. Brood A, B, C, E, and F juveniles had only the same genotype as their brooding clams. Juveniles with a genotype different to that of their brooding clam were detected in brood D. Because Corbicula spp. juveniles inherit only the nuclear genome of spermatozoa due to androgenesis (Komaru et al. 1998; Ishibashi et al. 2003), the mismatch of alleles between brooding clams and their juveniles means fertilization by spermatozoa from other individuals has occurred. Accordingly, this suggests that Corbicula spp. reproduce not only by self-fertilization but also by outcrossing (egg parasitism). The outcrossing rate in brood D was 18% (2/11; Table 2). The outcrossing rate in this study is likely to be an underestimate due to the sharing of the same genotype in two loci between brooding clams and their neighbors. For instance, brooding clams D and E shared the same genotype as Y01 in the neighborhood (Tables 2 and 3). Brooding juvenile genotype d01 coincided with neighboring clam genotypes Y02, G08, and M04, and juvenile genotype d02 coincided with the genotype of brooding clams B, C, and F (Tables 2 and 3). These results mean that outcrossing occurs not only with neighboring hermaphrodites but also with neighboring males. This supports the possibility that males of Corbicula spp. may be able to reproduce by egg parasitism (Houki et al. 2011).

Corbicula leana and C. fluminea have been considered to reproduce solely by self-fertilization because they are both hermaphrodites. Additionally, histological studies have suggested that the gonoduct opening in the inner demibranch was suited to self-fertilization in C. leana (Ikematsu and Yamane 1977) and C. fluminea (Kraemer 1978). However, egg parasitism caused by outcrossing in androgenic Corbicula has been suggested to explain the discordance between mitochondrial and genomic DNA (Lee et al. 2005; Hedtke et al. 2008, 2011; Pigneur et al. 2011, 2012). This study confirmed egg parasitism, with direct evidence of outcrossing in the gills of brooding clams.

In the present study, three genotypes of juveniles in brood D showed that outcrossing occurs with multiple neighbors (Table 2). The multiple paternities caused by egg parasitism provides risk diversifications for sperm-releasing clams, be-
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cause larvae inheriting their genes could be brooded by other clams as well as by them. Conversely, there would be no benefit to parasitized brooding clams, except that the sperm releaser and parasitized clam share the same genotype. Among hermaphrodites, individuals behave as both sperm releasers and parasitized clams so they can obtain both the risks and benefits. However, parasitism by male clams would be a risk only to hermaphrodite clams. In the future, accurate paternity tests based on more detailed genetic analysis will likely reveal the actual state of egg parasitism under the coexistence of males and hermaphrodites in androgenetic *Corbicula*.

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| Genotype           | Microsatellite locus | Number of clams | Ploidy of clams |
|--------------------|----------------------|-----------------|-----------------|
|                    | cf8                  | cf35            | 2n/3n           |
| Yellow hermaphrodites |                     |                 |                 |
| Y01                | 352                  | 328             | 3/0             |
| Y02                | 340                  | 331             | 0/3             |
| Y03                | 343                  | 328             | 0/1             |
| Y04                | 334/337              | 331             | 1/0             |
| Y05                | 340/352              | 328             | 4/0             |
| Y06                | 334                  | 331             | 0/1             |
| Y07                | 334/343              | 322/328         | 2/0             |
| Y08                | 340/346              | 331             | 0/1             |
| Y09                | 340/352              | 328             | 3/0             |
| Y10                | 337/340/346          | 331             | 0/1             |
| Y11                | 334/343              | 322/328         | 1/0             |
| Y12                | 340/343              | 322/331         | 0/1             |
| Y13                | 331/343              | 328             | 1/0             |
| Y14                | 337/340              | 331             | 0/1             |
| Green hermaphrodites |                     |                 |                 |
| G01                | 343                  | 328             | 5/0             |
| G02                | 325                  | 328             | 1/0             |
| G03                | 337/340              | 322/328/331     | 0/1             |
| G04                | 343/349              | 331             | 1/0             |
| G05                | 304/322              | 328/373         | 1/0             |
| G06                | 340                  | 328/331         | 1/0             |
| G07                | 349/352              | 331             | 1/0             |
| G08                | 340                  | 331             | 1/0             |
| G09                | 304/331              | 331             | 0/2             |
| G10                | 304/319              | 328/373         | 1/0             |
| G11                | 346/349              | 328/331         | 1/0             |
| G12                | 304/322              | 328/331         | 1/0             |
| G13                | 304                  | 328/373         | 1/0             |
| G14                | 340/349              | 331/334         | 1/0             |
| G15                | 331                  | 331/373         | 1/0             |
| Green males        |                      |                 |                 |
| M01                | 340                  | 328             | 5/0             |
| M02                | 340                  | 328/331         | 1/11            |
| M04                | 340                  | 331             | 2/1             |
| M05                | 340                  | 322             | 0/1             |

Table 3. Genotypes of two microsatellite DNA in the neighboring clams of *Corbicula* spp.
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