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The First Establishment of "Hand-Pairing" Cross-Breeding Method for the Most Ancestral Wing Acquired Insect Group

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Insects are the most diverse organisms in the world and have been in existence since ca. 480 Ma; given this, they can provide profound insights into evolution. Among them, the order Ephemeroptera is one of the most basal clades of winged insects. This makes Ephemeroptera a significant key taxon in understanding the macro-evolution or the insect groundplan. In the development of biological evolutionary studies of this taxon, it is important to establish a technique for cross-breeding. Furthermore, the establishment of these techniques also makes a great contribution in the fields of micro-evolution. In a non-model taxon, the mayfly, subcultivation in the laboratory has been thus far considered impossible. With the exception of some parthenogenetic strains, it is extremely difficult to mate these insects in artificial environments. In this study, we established a successful artificial mating technique, i.e., a "hand-pairing" based cross-breeding method for mayflies. Furthermore, we also succeeded in clearly verifying by a genotyping method that the offspring reproduced by hand-pairing were in fact derived from the actual male and female which were used for hand-pairing. We established a reproductive experimental technique for hand-pairing of *Dipteromimus tipuliformis* and verified this technique by means genotyping. This technique could allow the artificial control of fertilization timing, and result in offspring which can be verified as to their status by means of genotyping. This achievement will be extremely important in the future for both the macro- and micro-evolutionary studies of insects.

Key words: artificial copulation, cross-breeding, Ephemeroptera, Paleoptera, verification of reproductive

INTRODUCTION

As the most diverse group of organisms in the world, insects provide profound insights into evolution (Muller and Wagner, 1991; Grimaldi and Engel, 2005). It is estimated that insects originated around 479 Ma, and insects that acquired wings appeared around 406 Ma (Engel and Grimaldi, 2004; Misof et al., 2014; Tojo et al., 2017). That is, insects were the first organisms to be able to fly in Earth's history. The order Ephemeroptera is one of the most basal clades of winged insects, of which the ancestral groups of mayflies originated approximately 400 Ma (Engel and Grimaldi, 2004; Misof et al., 2014). Therefore, Ephemeroptera is a significant key taxon in understanding the macro-evolution or the groundplan of insects, e.g., their highly functional body plan and their acquisition of wings, which in turn lead to their adaptive radiation (Tojo and Machida, 1998; Niwa et al., 2010). In terms of the development of their evolutionary biological studies, it is important to establish the technique of cross-breeding". The establishment of these techniques also makes a great contribution in the fields of micro-evolution.

Among Ephemeropterans, there are groups which have developed specific reproductive strategies, such as those observed in geographically parthenogenetic (Sekiné and Tojo, 2010; Sekiné et al., 2015) or ovoviviparous (Degrange, 1959) species; it has been suggested that such species are also important when considering an approach to the question of what constitutes a "species". However, it is extremely difficult to mate mayflies in artificial environments. To date, in Ephemeroptera, reproductive experiments in the laboratory have been limited to very few examples (Clarke and Sheppard, 1956; Huff and McCafferty, 1974). In one case, Funk et al. (2006) conducted hybridization experiments and showed that produced offspring were derived from the actual male and female involved. But this study used only allozymes analysis, meaning it was tested by electrophoresis, and they did not show raw data. However, on this matter, we

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have succeeded in a big breakthrough. We have established a successful artificial mating technique, hand-pairing, for cross-breeding Ephemeroptera in the laboratory. Furthermore, we have clearly proven by means of a genotyping method that the reproduced offspring were in fact derived from the actual male and female which were used in "hand-pairing." That is, it was shown that each oocyte was fertilized by the hand-paired male's spermatozoon. Specifically, we verified by means of molecular identification that the hand-pairing method is an effective technique, and this is the first report that this has been achieved for mayflies. So far, the hand-pairing of insects was an extremely limited technique that had only been attempted for a few taxa [i.e., Lepidoptera (Scriberl and Lederhousel, 1988; Nagasaki, 2011)]; Odonata (Oppenheimer and Waage, 1987; Battin, 1993; Tsubaki, 2003); Hymenoptera (Laidlaw, 1949; Woyke, 1976); Diptera (Davidson, 1964).

In this study, the establishment of an experimental reproductive method by hand-pairing in non-model taxa, Ephemeroptera, constitutes an extremely important step, and makes available an extremely important technique in the study of phylogenetic evolution and species diversity creation mechanisms. Furthermore, it is expected that this achievement will contribute to the development of various studies in biology, from macro-evolution through to micro-evolution.

MATERIALS AND METHODS

In this study, we focused on the dipteromimid mayfly, *Dipteromimus tipuliformis* (Ephemeroptera, Dipteromimidae). Dipteromimidae, consisting of 2 species within one genus, is endemic to the Japanese Islands (Tojo and Matsukawa, 2003; Takenaka and Tojo, 2019), typically inhabiting headwater streams, and go through one full life cycle per year. Generally, mayflies copulate in the air where males conduct a swarming flight for mating. However, in the case of the mayfly species *D. tipuliformis*, mating behavior is performed on leaves. Therefore, it is easy to observe and verify the mating behavior and pose (Takenaka and Tojo, 2014), making this species the most suitable mayfly for reference to establish hand-pairing methods.

Reproductive experiments

In the present study, we collected individuals in the last instar nymph or sub-imago stage, virgin females, and copulated females of *D. tipuliformis* from a stream in a field (Fig. 1A). In the laboratory, the nymphs or sub-imagos were separated from each other into individual cases and were reared up to the adult stage. Nymphs were reared in plastic cases (78 mm × 114 mm× height 42 mm; Inomata, Osaka), and sub-imagos were reared in 30 mL cylindrical container named "Push Vials" (diameter 30 mm× height 59.2 mm; Nikko Hansen, Tokyo). Since nymphs mainly eat detritus, we raised them in stream water containing detritus. Almost all the last instar nymphs emerged within one week from the start of rearing. The emerged sub-imago once again molt and become imagos, even when combining these sub-imago and imago stages, that period amounts to at most 2–3 days in nature (Takenaka and Tojo, 2014). However, we rearing the sub-imagos following emergence under dark and low temperature conditions where able to extend the combined sub-imago and imago stage to about five days. In this manner, we tried to spread the timing at which the artificially bred specimens became available for hand-pairing by adjusting the various times after their emergence that they took to reach the adult stage.

We conducted three types of reproductive experiments, as follows. We investigated: (1) the rate of embryos developing to the final embryonic stage and hatching rates of egg batches that were oviposited by the artificially hand-paired females, (2) the rate of embryos developing to the final embryonic stage and hatching rates of egg batches that were oviposited by copulated females collected in the field, and (3) the egg batches oviposited by virgin females (not hand-paired females) as a control check for the potential of parthenogenesis. After 30 days of incubation at 20–25°C, the rate of embryos developing to the final embryonic stage and percentage of hatching success were measured under a microscope (SMZ1500, Nikon, Tokyo, Japan) for each egg batch. In this study, the developmental incidence was calculated based on the ratio of

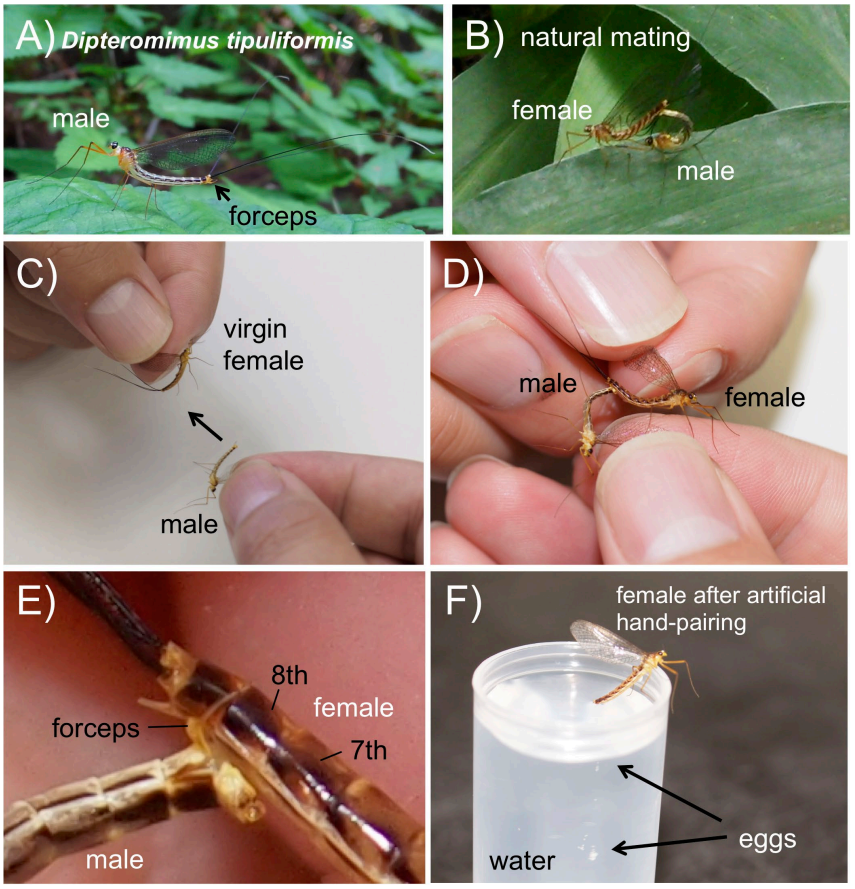

Fig. 1. Male of *Dipteromimus tipuliformis* **(A)**; Natural mating in a field of *D. tipuliformis* **(B)**; Hand-pairing method shown by grasping the wings of the virgin female and the male and then bringing the individuals together **(C)**: male tightly grasped female abdomen between 8th and 9th abdominal segments, when male forceps closed onto the female abdomen (**(D)**; **(E)** is enlarged); male genitalia inserted to female genitalia from subgenital plate between 7th and 8th abdominal segments **(E)**; female started to oviposit when female terminal abdominal segments made contact with water surface **(F)**. these processes were corresponding Table 1.

the total number of observed eggs and the number of eggs for which embryogenesis proceeded. When the embryogenesis reached "stage 13 (cf. Tojo and Machida, 1997, 1998)", it was counted as a developed egg. The hatching rate was calculated from the percentage of eggshells left after hatching.

Statistical analysis

To compare the rate of embryos developing to the final embryonic stage and hatching rates of the egg batches by females that the artificially hand-paired and females that had copulated in the field and non-mating females, we performed nonparametric multiple comparison by using the Steel–Dwass test of the 'Steel-Dwass' function (http://aoki2.si.gunma-u.ac.jp/R/Steel-Dwass.html) in R version 3.1.1 (R Core Team, 2014).

DNA sequencing

We conducted DNA analysis for each of the males and females that were used in hand-pairing experiments, and also that of their corresponding offspring (i.e., hatched nymphs) from these experiments. The extraction of total genomic DNA was conducted according to the same methods as used in our previous studies (Sekiné et al., 2013; Suzuki et al., 2014; Saito and Tojo, 2016a, b). Each total genomic DNA was used to amplify DNA fragments [the nuclear DNA (nDNA) PEPCK regions] by polymerase chain reaction (PCR) used to sets of primers: 5′-AGTGATGGCGGAGTCTTCTG-3′ and 5′-ATCACCTTGCCCTGTGTAATTC-3′ (own design). The purified PCR products, sequencing, and alignment was conducted according to the same methods as previously mentioned. Sequence data of the nuclear DNA PEPCK regions have been submitted to the GenBank (accession numbers: LC441034–LC441038).

RESULTS

Establishment of a hand-pairing method

In all of the examinations, only virgin *Dipteromimus tipuliformis* females and males were used (Fig. 1A). In addition, as a control experiment, individuals which had completed copulation under natural outdoor conditions were also used (Fig. 1B).

The detailed hand-pairing process is shown in Table 1. In this hand-pairing method, all of the examined males tightly grasped their paired female's abdomens, and penetrated the female genitalia from her subgenital plate (i.e., between 7th and 8th abdominal segments). This mating style (copulation style) was the same as the style under natural conditions observed in the field (Takenaka and Tojo, 2014). We established a reliable technique for artificial mating experiments, using hand-pairing. In these experiments, it was confirmed that the examined male indeed penetrated the female's genitalia (Please refer to the video documentation, as a supplementary data). Our involvement at this point was only to bring the male and female close to each other. When the male recognized the presence of the female by its forceps making contact (contact stimulus), he shifted himself into the mating posture. This results show the importance of the male's recognition of the female for successful mating.

Developmental and hatching rates of egg batches

The rate of embryos developing to the final embryonic stage and hatching rates of egg batches that were oviposited from the seven females that had copulated in the field were indicated to be at particularly high rates (i.e., the rate of embryos developing to the final embryonic stage was 95.7 ± 7.5% and the hatching rate was 91.5 ± 13.2%; Table 2). The rate of embryos developing to the final embryonic stage and hatching rates of egg batches that were oviposited by the artificially hand-paired females were also indicated to be particularly high (i.e., the rate of embryos developing to the final embryonic stage was 94.4 ± 4.8% and the hatching rate was 91.5 ± 6.0%; Table 2). On the other hand, the egg

Table 1. Hand-pairing procedure for the mayfly, *Dipteromimus tipuliformis* in laboratory.

Stages	Operation in "Hand-pairing" cross-breeding	Corresponding Fig. 1
1	Preparation of the virgin female and the male (which were reared up to the adult stage into individual case from nymph or sub-imago)	Fig. 1
2	It was grasping wings of the virgin female and the male	Fig. 1B, C
3	Male forceps was brought close to female abdomen between 8th and 9th abdominal segments similar to natural mating in field	Fig. 1C
4	When male forceps touched female abdomen, male grasped female abdomen by own forceps. Then, male genitalia inserted to female genitalia from subgenital plate between 7th and 8th abdominal segments (about 1 minute)	Fig. 1D, E
5	It was separated male and female	
6	Female started to oviposit when female terminal abdominal segments was contacted water surface	Fig. 1F
7	It was incubated oviposited egg batches at 20–25°C	
8	After 30 days, developmental rate and percentage of hatched successfully were counted	

Table 2. The rate of embryos developing to the final embryonic stage of each egg batch that oviposited by the artificially hand-paired females, natural mated females (copulated females collected in the field) and virgin females (not hand-paired females).

	No. of females examined	No. of eggs examined (average ± SD)	Developmental rate (average ± SD)	Percentage of hatching success (average ± SD)
Virgin females	6	211.5 ± 77.9	0.0 ± 0.0%	0.0 ± 0.0%
Natural mated females	7	169.6 ± 170.1	95.7 ± 7.5%	91.5 ± 13.2%
Hand-paired females	18	828.1 ± 507.1	94.4 ± 4.8%	91.5 ± 6.0%

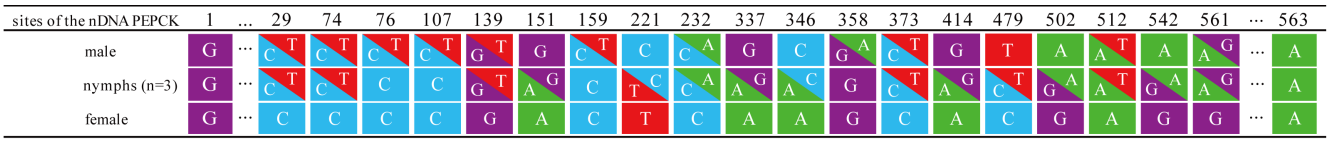

Fig. 2. Comparison of the nucleotide sequences (i.e., the genotype of the nuclear DNA PEPCK region, 563-bp) of the male and female pair used in the hand-pairing experiment, and the genotype of the randomly selected offspring (hatched nymphs) from the hand-paired females. Only the first and last nucleotides, and the sites where the polymorphism between targeted specimens was observed, are indicated. Three nymphs share the same genetic sequences.

batches that were oviposited by virgin females (not hand-paired females) that served as a control for the potential for parthenogenesis did not develop nor hatch (i.e., both the rate of embryos developing to the final embryonic stage and hatching rate were 0.0%; Table 2). The rate of embryos developing to the final embryonic stage and hatching rates of egg batches by the artificially hand-paired females were significantly high compared with the egg batches that were oviposited by virgin females (Steel–Dwass test, $P < 0.01$). On the other hand, the rate of embryos developing to the final embryonic stage and hatching rates of egg batches by the artificially hand-paired females were not significantly different compared with the egg batches by females that had copulated in the field (Steel–Dwass test, $P = 0.83$).

The genotypes of offspring (i.e., hatched nymphs)

After the hand-pairing experiments, we determined the genotypes of the male and female that were used in each experiment. We also analyzed the genotype of the offspring (i.e., hatched nymphs) from each of the hand-paired females. For this analysis, we utilized the nuclear DNA PEPCK region, which is observed to be a comparatively genetic polymorphism rich region (Fig. 2).

It was revealed that the offspring (hatched nymphs) from hand-pairing method had heterozygotic genotypes derived from both the male and female used in each hand-pairing experiment (e.g., the locus 151-bp: hand-paired male had “G” homo-type, on the other hand the hand-paired female had “A” homo-type, and the hatched nymph oviposited by the artificially hand-paired female had the corresponding “A/G” hetero-type; Fig. 2). In other words, it was clearly indicated that the hatched nymph had derived its genotype from both the male and female that were used in each experiment (Fig. 2). We conducted the verification of such hand-pairing for multiple pairs, but we have shown only the result of one pair of these because all results of genetic analyses indicated the same outcome (Fig. 2).

DISCUSSION

Although some mayflies are known to have potential for parthenogenesis (Huff and McCafferty, 1974; Funk et al., 2006; Sekiné and Tojo, 2010; Sekiné et al., 2015), *Dipteromimus tipuliformis* do not have the ability of parthenogenetic reproduction. Furthermore, the egg batches oviposited by the artificially hand-paired females indicated both significantly high developmental and also significantly high percentage of hatching success rates.

The hand-paired females oviposited more eggs than the virgin females. With regard to the number of ovipositions of natural females, as we may have collected females already started oviposition, the number of eggs that could be observed was small. On the other hand, virgin females were reluctant to oviposition. Even if we encourage eggs to females that have not completed copulation in this way, the tendency to not oviposit is commonly found in many other mayflies (e.g., *Ephemera strigata*, *Ephemera japonica*, *Potamanthus formosus*). For these reasons, we suggest that artificial mating using hand-pairing is an effective method in experiments regarding both embryonic development and also hatching. However, there may have been a possibility that stimulation of artificial mating might have induced parthenogenetic reproduction. Therefore, in this study, we verified by using molecular markers as to whether or not male sperm contributed to insemination. As a result of the genetic analyses of the pairs used in the artificial hand-pairing experiment and of the randomly selected nymphs from the hand-paired females, it was revealed that all these nymphs exhibited a heterozygotic genotype derived from both the male and female used in each hand-pairing experiment. This finding verifies without doubt that male sperm transferred to the female, and the eggs oviposited by the hand-paired females achieved fertilization by the corresponding paired male. By means of this, we have achieved for the first time extremely important knowledge of a hand-pairing method verified by genotyping for the most ancestral wing acquired insect group and non-model taxa, i.e., Ephemeroptera.

In addition, we were also able to have success using the hand-pairing method with two other mayfly families (i.e., Baetidae and Ephemeridae). Both of these two families along with the family Dipteromimidae, are each systematically distant (Ogden and Whiting, 2005). For this reason, we are considering that our hand-pairing method will likely prove to be useful for various mayflies, and that this technique will be applicable to many other mayflies. Also, this technique enables the artificial control of fertilization timing, because this technique is completely artificially. This is an essential step in the development of experiments from the viewpoints of macro-evolution. It is important to control fertilization timing for the study of evolutionary embryology, developmental ecology, and/or developmental genetics. In particular, the order Ephemeroptera is one of the most basal clades of wing acquired insects, so this group is a key taxon for the study of the evolutionary origin of insect wings. Moreover, cross-breeding can be further developed for the study of speciation. Not only in these studies, but we also expected that the hand-pairing method will contribute to the further development of many macro-evolutionary studies.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

M.T., K.S. and K.T. designed, managed the study, and performed sample collection; M.T. mainly performed laboratory work and phylogenetic analyses; M.T., K.S. and K.T. wrote and reviewed the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available online. (URL: http://www.bioone.org/doi/suppl/10.2108/zs180169).

Supplementary movie.

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