Genetic Variation in Released Gametes Produces Genetic Diversity in the Offspring of the Broadcast Spawning Coral *Acropora Tenuis*

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Abstract

All coral species in the genus Acropora are broadcast-spawning hermaphrodites. Fertilization in the ocean requires sufficient numbers of gametes from conspecifics and the contact time for fertilization is thought to be limited by the rapid diffusion of sperm. Many studies have reported a positive correlation between sperm concentration and fertilization success, but it is not clear how released gametes are fertilized in nature (in situ) and how genetic diversity arises at a fertilization event. To elucidate this, we analyzed the changes in sperm concentration in situ after spawning and genotyped sperm and fertilized eggs from seawater using seven microsatellite (MS) markers. This showed that the sperm concentration in situ was suboptimal (<106 sperm/mL), but most of the eggs were fertilized. MS genotyping showed that the alleles of released sperm were diverse and those alleles also appeared in the fertilized eggs. In addition, the MS fragment peak height in released sperm, which presumable reflects the allele frequency of the sperm, was positively correlated with the allele frequencies of the fertilized eggs. Collectively, the genetic diversity of colonies with high fecundity dictates the genetic diversity of their descendants.

Introduction

Sexual reproduction produces genetic diversity in offspring, which enables the selection of genotypes associated with higher fitness and adaptation.[1-3] Reef-building corals in the genus *Acropora* release gametes into seawater synchronously and this promiscuous mating system gives rise to genetic diversity in their offspring; eggs of one colony can potentially mate with sperm from many other colonies.

Spawning events in *Acropora* may be a response to climate change by ensuring the genetic diversity of offspring. Coral reefs are now under threat,[4] and reef degradation from bleaching events has increased.[4-6] The remaining corals have a higher thermal threshold for bleaching.[7] Although the importance of sexual reproduction is known, knowledge of how the process from spawning to fertilization generates genetic diversity in nature (*in situ*) is still limited.

Fertilization success is crucial for sexual reproduction in broadcast spawning marine species. Many marine invertebrates are benthic or sessile, releasing their gametes into the water column and spawning synchronously to facilitate fertilization.[8,9] Spawning synchronism in marine invertebrates has been widely reported,e.g., [10,11-13] and synchronism is associated with fertilization success.[14]

In broadcast-spawning *Acropora* corals, fertilization success is associated with sperm concentration, which is affected by spawning synchrony, gamete number, and other factors. For example, sympatric *Acropora* species release their gamete bundles synchronously,[10] and *in vitro* experimentse.g., [15,16] have shown that the fertilization rate depends on sperm concentrations, which in nature are expected to be associated with spawning synchrony,[17] water currents,[18] colony densities,[19,20] and the distance from the sperm source.[21] The timing of spawning and variation in water currents lead to high variation in sperm concentrations that affects the selection of fertilization-related traits. However, data on sperm concentrations *in situ* after spawning are lacking.

Examining sperm concentrations and fertilization success in *Acropora* corals *in situ* is difficult because of the spawning synchrony of heterospecifics. However, in Okinawa, Japan, *Acropora tenuis* spawns at sunset, about 2.5 h earlier than most other *Acropora* species.[22] Moreover, at Sesoko Island, Okinawa, sunset spawning is dominated by *A. tenuis*,[23] making it a good model for investigating the time course from spawning to fertilization.

In this study, we obtained *in situ* data for *A. tenuis* on the time course of sperm concentrations, the genetic diversity of released sperm, and the non-biased fertilization success of released gametes. We discuss the relationship between the genetic diversity of the fertilized eggs that leads to the next generation, and the amount and genetic diversity of the released gametes.

Materials And Methods

Underwater observation of Acropora tenuis

We observed spawning of the scleractinian coral *A. tenuis* (n=11) on a section of the fringing reef of Sesoko Island, Okinawa, Japan (26°37′43.9″N 127°51′43.3″E), by snorkeling or SCUBA diving for six nights from May 26 to May 31, 2018. Floats were released from 4 of the 11 tagged colonies (ten15, ten21, ten27, and ten31). The area we where we monitored the floats measured approximately 1×1 km^2.

Sperm concentration in situ

To trace gamete bundles released from the tagged colonies, floats with fluorescent light bars (Fig. 1 A) were released directly above the spawning colonies approximately 10 min after the colonies started spawning. We followed the floats by kayak, and collected 1 L seawater near each float at 9 min intervals for approximately 1 h after the release of the floats (Fig. 1 B). The collected seawater was brought back to Sesoko Station within 30 min, and the sperm concentrations were measured with a Thoma hemocytometer according to a previous study.[12] The sperm in the collected seawater was measured five times in 200×200 μm. When no sperm was found, the concentration was described as being below 2.5×10^4 sperm/mL. It took about 1 h from collecting the seawater to the start of sperm concentration measurement, and by this time most of the eggs in the seawater had already been fertilized.

Genetic diversity of spermatozoa in seawater and fertilized eggs

The genotypes of sperm and fertilized eggs were determined using microsatellite markers developed for *Acropora*.[24] Eggs in the collected seawater were transferred to fresh 0.22 μm filtered seawater, and fertilized eggs that had completed embryogenesis were fixed with 99.5% ethanol 3 days after collection. The remaining seawater was filtered and sperm were trapped on a membrane (mixed cellulose ester

gridded at 0.45 µm, Merck Millipore, MA, USA). The membranes were soaked with 1 mL CHAOS solution (4 M guanidine thiocyanate, 0.1% [v/v] N-lauryl sarcosine sodium salt, 0.1 M β-mercaptoethanol, 10 mM Tris-HCl pH 8.0) and the DNA was extracted using a Wizard SV genomic DNA purification system (Promega, WI, USA). DNA was extracted from the fertilized eggs following a previous study with some modifications.[12] Fertilized eggs were kept in filtered seawater for 2 days and fixed in 99.5% EtOH. The fixed larvae were treated with 20 µL f lysis buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 0.3% [w/v] Triton X-100, 0.3% [w/v] Tween 20) containing 1 g/mL proteinase K for 2.5 h at 55°C and heated at 95°C for 5 min. The supernatant was used for PCR reactions for genotyping. As a negative control for sperm detection in seawater, seawater was collected in the daytime following the same protocol. We sampled eight times at 9 min intervals after a float was released.

The sperm in seawater and fertilized eggs were genotyped with seven microsatellite markers with FAM or BIC (12406m3, 11543m5, 11401m4, 441m6, 11292m4, 10366m5, and 12130m5; Supplementary Information 1[24]). The allele diversity of these seven markers was marked and the fragment amplification was stable. Subsequently, we used the seven markers to verify the presence of the alleles. Fragments were analyzed with a DNA sequencer (Applied Biosystems 3730xl or 3130xl) with GeneScan 500 LIZ dye size standard (Thermo Fisher, MA, USA). Peaks were measured with Microsatellite Analysis v1.0 software (Applied Biosystems by Thermo Fisher, MA, USA). For analyses of seawater containing sperm, peaks below 100 were excluded as alleles (Supplementary Fig. 1). The ratio of each peak in the samples (Rx) was calculated as $R_{x1} = (H_{x1}/H_1 + H_2 + \ldots + H_x)$. Here, H_{x1} indicates the height of one peak among the others, and $H_1 + \ldots H_x$ is the sum of the heights of all peaks. In the negative seawater control, marker 12406m3 was used and three alleles (176, 179, and 185) were detected in one of the seven samples. These alleles were not detected in sperm in this study (153–174). Thus, the alleles detected in sperm from seawater were treated as those from the released sperm.

Statistical analyses

The Welch *t*-test was used to examine the differences in allele ratios. A GLMM was performed with the glmmML package in R ver. 3.1.[25] Each allele was treated as a random effect and the binominal distribution was used as a family.

Results

Spawning of tagged colonies

In all, 10 of the 11 tagged *A. tenuis* colonies spawned on 29 May and 2 colonies spawned on 30 May. Most *A. tenuis* colonies released >1000 gamete bundles each.

In situ time course of sperm concentration and sperm genetic diversity

We followed the floats and collected seawater containing gametes. Most sperm concentrations in the collected seawater were < 10^5 sperm/mL (Fig. 2). We found no sperm in several samples although microsatellite analyses showed amplification of fragments in seawater in which no sperm were present (see below). The sperm concentrations varied over the course of sampling. Variation in sperm concentrations might have occurred because of differences between water currents and the movement of the floats, which might not represent precise water movements of the gametes from tagged colonies.

Microsatellite genotyping showed that several alleles were amplified in each seawater sample. The numbers of alleles were indicated by the fragment lengths (bp). The allele numbers varied after spawning (Table 1, supplementary information 2). Although sperm were not found in many observation with the hemocytometer, microsatellite fragments were amplified from these samples and the eggs in these samples were fertilized. Most alleles were detected in fertilized eggs (Tables 1, alleles marked with *). The alleles detected only in seawater had lower MS fragment peak heights in the fragment analyses (Fig. 3).

Genetic diversity of sperm and fertilized eggs in seawater

When genotyping the sperm samples, many alleles were detected for each marker, and the numbers varied by MS markers, sperm, and fertilized eggs (Table 1, supplementary information 2 and 3). We released floats from the spawning colonies to follow released gametes, but the alleles found in the collected seawater often did not match those of the spawned colony (Table alleles from tagged colonies are marked with †).

We examined 1 to 60 fertilized eggs for floats 1 to 3, while we could not analyze eggs were found for float 4 because of loss of the samples. Many alleles were detected and most of these matched those in the same seawater sample. The alleles present changed over the sampling time (Table 1, Supplementary Information 3). Several alleles in sperm samples were not detected in the fertilized eggs (Table 1, alleles are not marked), and there were alleles that found only in the fertilized eggs (Table 1, alleles marked with ¶).

When comparing the fragment peaks of the alleles, MS fragment peak height was positively correlated with the frequency of the alleles in the fertilized eggs (Figs. 3 and 4, Supplementary information 2 and 3). Several alleles were detected only in sperm samples and these had smaller peaks (Fig. 3, Supplementary Information 2 and 3). However, we could not distinguish which alleles in the fertilized eggs were derived from sperm or eggs. Alleles in the fertilized eggs were positively correlated with the peak heights in the fragment analyses (GLMM *P*<0.001, coefficient = 6.9; Fig. 4).

Discussion

The genetic variations of the fertilized eggs *in situ* was associated with that of the released gametes. After spawning, the sperm concentrations *in situ* varied and were often lower (<10^5 sperm/mL) than the "ideal" level (10^6 sperm/mL)[15,16], but fertilization was accomplished *in situ*. Microsatellite analyses showed that many alleles of the fertilized eggs matched those of sperm *in situ*. In addition, the frequencies of the alleles in fertilized eggs were positively correlated with MS fragment peak heights, which corresponds to the amount of sperm with those alleles. Therefore, more sperm have a greater chance to successfully fertilize eggs, while a smaller amount of released sperm had less chance *in situ*. This is the first evidence that inheritance in reef-building *Acropora* depends on the number of gametes released *in situ*.

The sperm concentration *in situ* was lower than we expected, but most eggs in the samples were fertilized. It took at least 1.5 h to examine egg fertilization. Many studies have indicated that sperm concentrations < 10^5 sperm/mL are insufficient for fertilization *in vitro*[15,16]. The sperm and eggs were kept in containers after collection, and so the condition for fertilization is not precisely represented *in situ*. The fertilization ratio is often dependent on the combination of colonies. For example, the fertilization rates of *A. tenuis* around Sesoko Station vary according to the combination of gametes from conspecific colonies; several combinations of colonies have very low fertilization rates.[23] While this needs to be examined, the alleles found in the fertilized eggs were correlated with the peak height of each allele, which is representative of sperm numbers.

Although the fertilization ratio *in situ* was potentially overestimated, fertilization might be accomplished soon after sperm and eggs are mixed.[26] Moreover, sperm−egg interactions due to chemoattractants may contribute to their successful fertilization at lower sperm concentrations.[27] If sperm can efficiently interact with unfertilized eggs with the assistance of chemoattractants and fertilization finishes quickly, fertilization with many different sperm from many colonies may occur, even at low sperm concentrations. However, we have no practical observations or information about gamete interactions, such as details of the release of bundles from colonies, bundles separated into sperm and eggs, and diffusion of these gametes.

The genetic inheritance of fertilized eggs, number of alleles, and their frequencies were positively correlated with sperm alleles found in the water. Most alleles found in the fertilized eggs were also found in the seawater containing sperm (Tables 1, alleles marked with *). Presumably, most alleles found in both seawater and eggs represent the alleles in the fertilizing sperm, but several alleles were found only in the fertilized eggs (Table 1, alleles marked with ¶) and these alleles may be from both sperm and eggs. Although we could not distinguish the origin of alleles (sperm or eggs), the MS fragment peak heights of alleles found in seawater and fertilized eggs were slightly higher than those of alleles found only in seawater (Fig. 3), suggesting that the alleles found in more sperm in seawater completed fertilization *in situ*. There is almost no information on which alleles are passed to the next generation in corals.

Genetic inheritance in *Acropora* needs more study regarding the fertilization of gametes according to sperm concentration. In a previous study, eggs preferred conspecific sperm, while the proportion of

fertilization by heterospecific sperm increased at lower sperm concentrations.[12] This implies that fertilization matching conspecific sperm and eggs is complicated and lower sperm concentrations might be associated with hybridization. In this study, we collected gametes in the ocean off Sesoko Island to follow the fertilization process in *A. tenuis*. Near this island, *A. donei*, which can potentially mate with *A. tenuis*, does not release a large number of eggs.[23] In addition, *A. tenuis* sperm does not fertilize eggs of *A. donei* in the presence of conspecifics.[22,23] Therefore, hybridization between *A. tenuis* and *A. donei* rarely occurs. We need to consider the breeding success of conspecifics or hybridization in later-spawning intercrossing species such as *A. intermedia* and *A. florida*. Fertilization *in situ* among intercrossing species may be associated with hybridization, which could be associated with adaptation to climate change.[28] Future studies need to address this.

Declarations

Acknowledgments

Author contributions

MM conceived and designed the experiments. SK and ST performed the experiments. MM, SK wrote the paper. All authors approved the final version of the manuscript.

Competing interests:

The authors declare no competing or financial interests.

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Table

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.
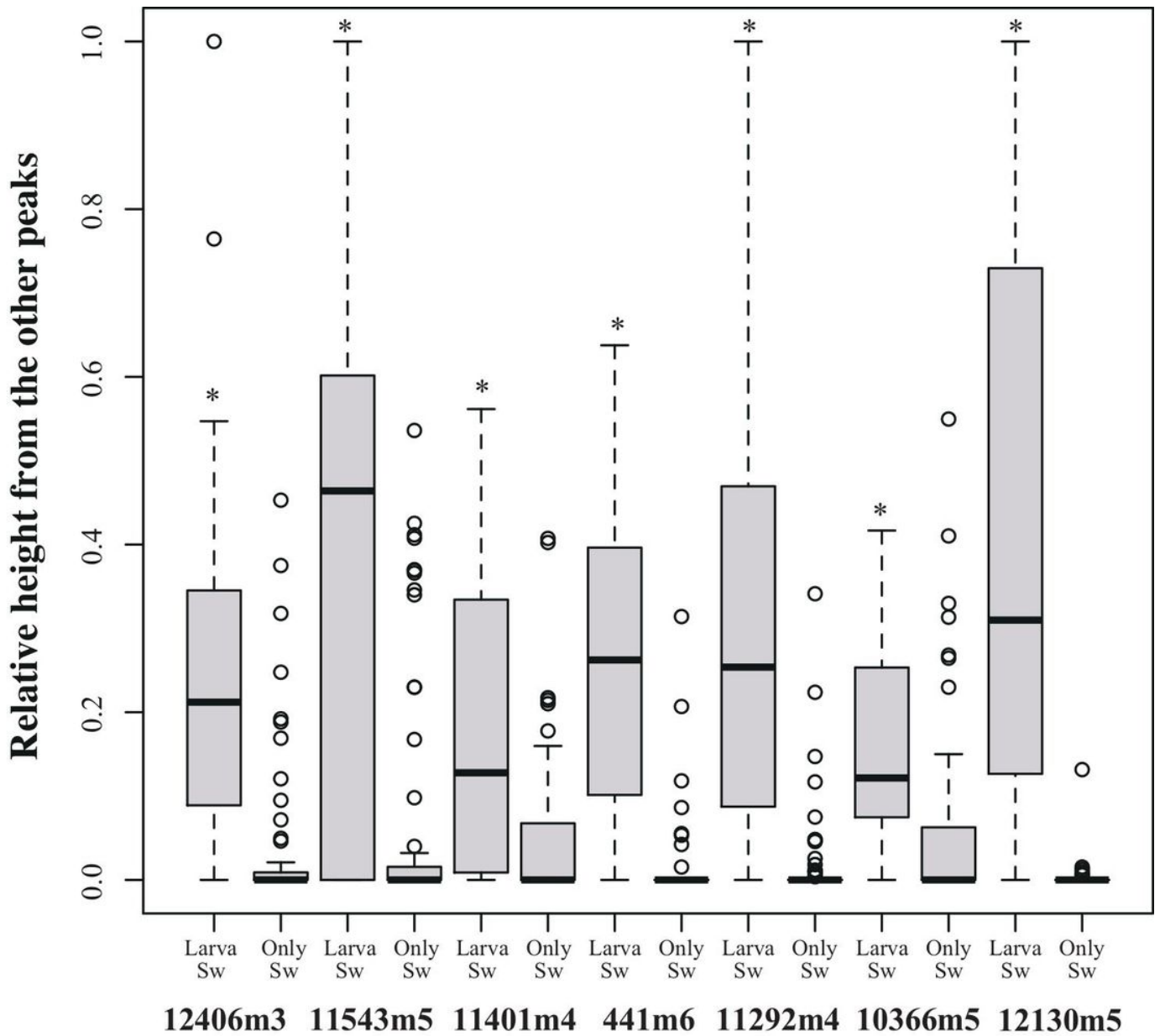
Figures
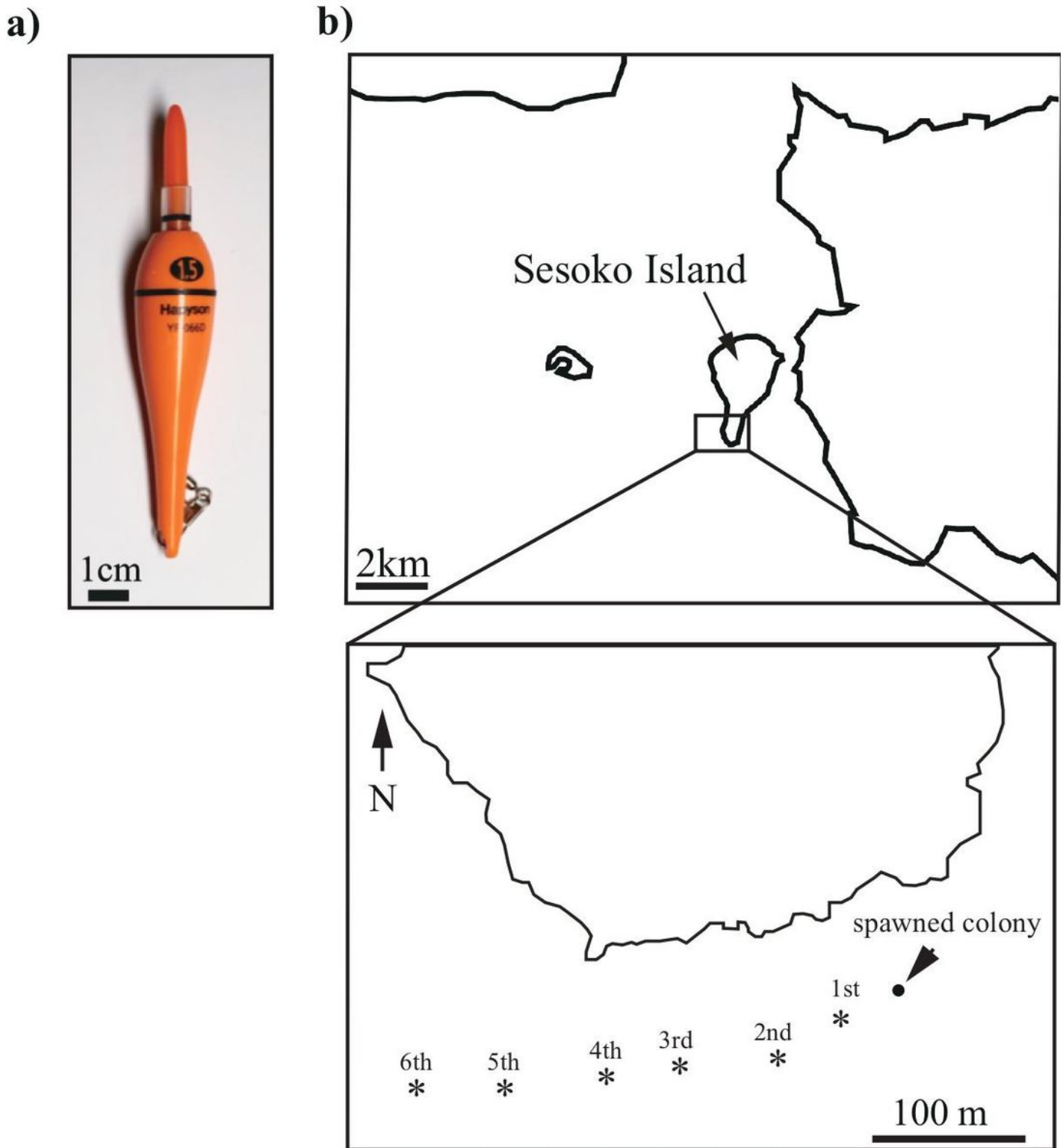
Figure 1

Map of study site a) Floats used in this study had fluorescent light bars (⌀13.0 x 122 mm, Hapyson) attached. b) Trajectory of the float measured in daytime via GPS. Filled circle indicates location of spawning colony where float was released. Asterisks indicate location of water sampling at 9 min intervals.
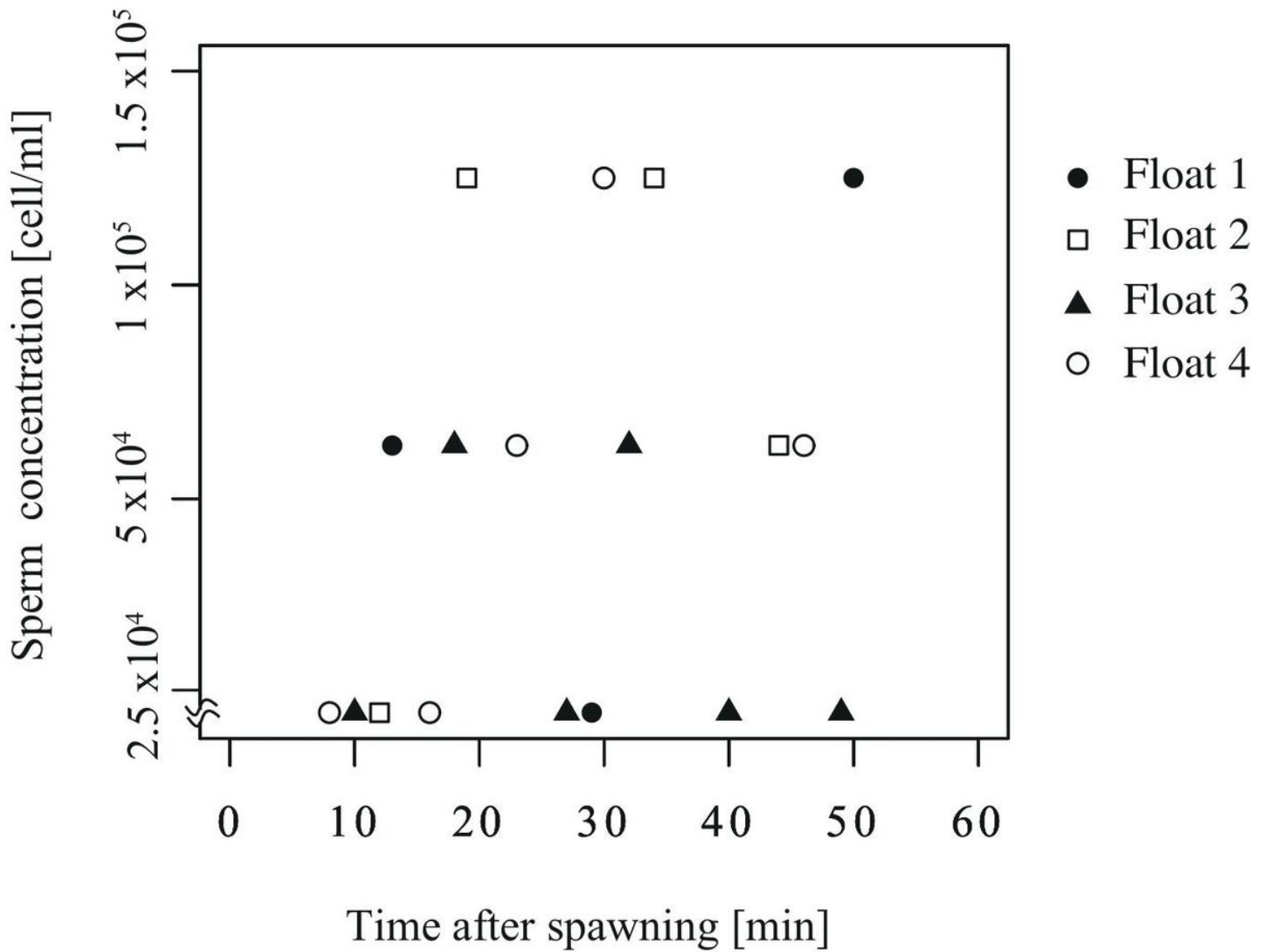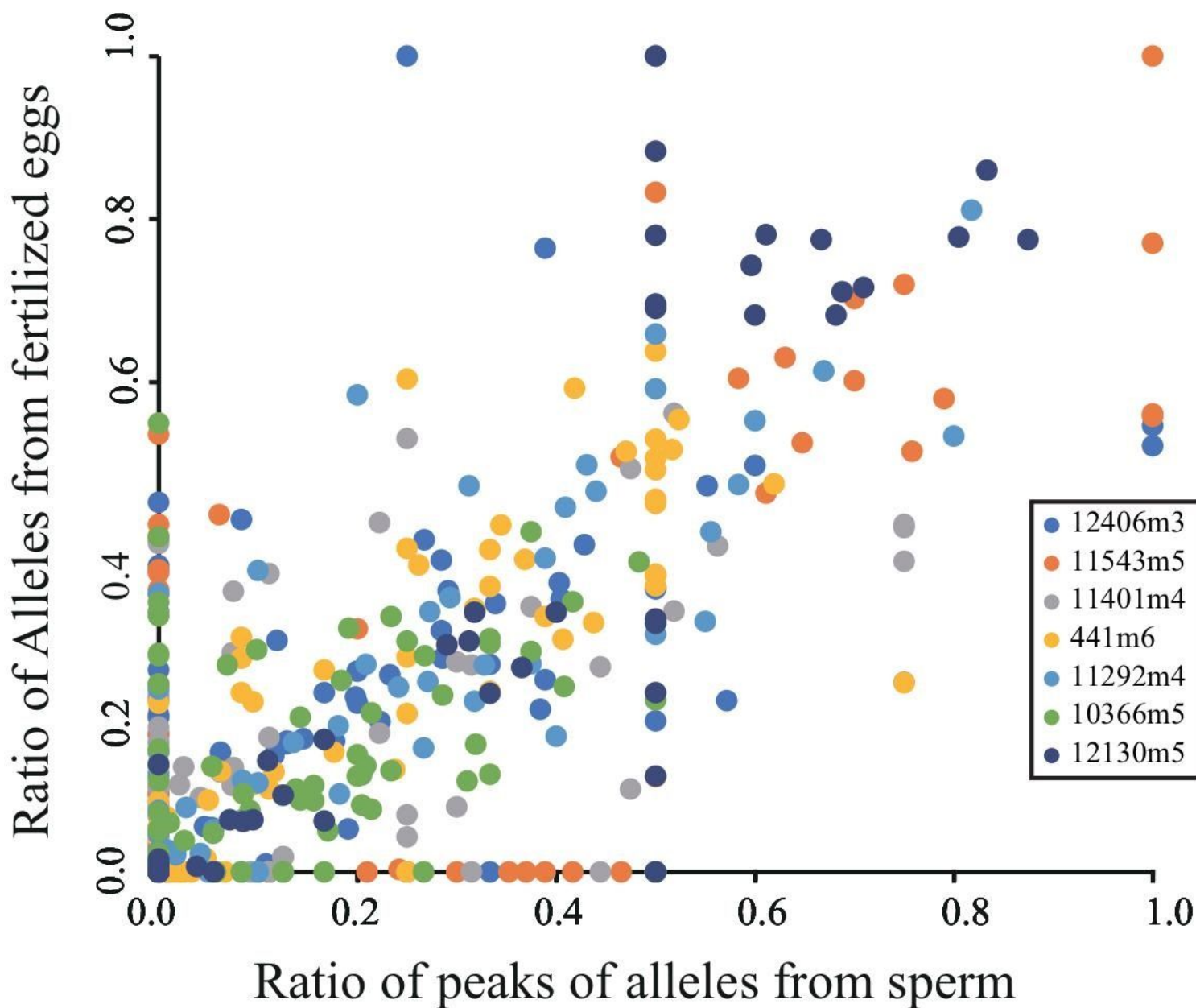
Figure 2

Sperm concentration time course after the spawning in situ Sperm concentrations were measured from seawater collected at 9 min intervals after the floats were released. Floats were released from the spawned colony approximately 10 min after they started releasing gametes.

Figure 3

Fragment peak comparison between alleles appeared in fertilized eggs or not Height of fragment peaks in each MS marker were compared. Alleles found in sperm/fertilized eggs (Larva/SW) or only in sperm (only SW) were plotted. *P<0.0001 (Welch t-test, M4 t = 3.95, df = 91.8, M5 t = 4.88, df = 30.5, M7 t = 4.89, df = 47.9, M8 t = 8,39, df = 69.5, M9 t = 7.52, df = 45.0, M12 t = 3.22, df = 85.0, M13 t = 7.71, df = 34.1)

Figure 4

Relationship between alleles in the fertilized eggs and sperm in situ Ratio of numbers of the each MS allele (ex. 191) and those of all MS alleles (ex. 191 and 197) in the fertilized eggs in each seawater sample (vertical line) (ex. Allele 191 in float1 3rd collection is 2/12=0.167 Supplementary information 1), and horizontal line indicates ratio of peak heights of fragment analyses in each sampled seawater (ex. ratio of allele 191 in float 1 3rd collection was = 0.16). Alleles in each MS in this study were indicated by different marks.

Supplementary Files

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- Suppmentaryinformation1MSinfo.xlsx
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