

**(i) Title**

Genetic and growth differences in the outcrossings between two clonal strains of the self-fertilizing mangrove killifish

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**Abstract:** The only known self-fertilizing vertebrate *Kryptolebias marmoratus* (Poey, 1880) (Pisces: Rivulidae) populations usually consist of different homozygous lineages, however heterozygous individuals are found occasionally and ratios of homozygosity and heterozygosity in populations are dependent on the proportion of males. However, it is still unclear what impact male-mediated genetic diversity has on the phenotype of *K. marmoratus*. In order to clarify this, we attempted outcrossing between male and hermaphrodite of two different clonal strains with different life-history traits using artificial insemination, and examined the genotypes and growth of the hybrid F<sub>2</sub> generation. We detected genetic differences between the two clonal strains using Amplified Fragment Length Polymorphism (AFLP) analysis with 3 primer combinations, and then obtained 11 AFLP markers. From a total of 31 times artificial insemination with two clonal strains, 1 of 13 hatched fish clearly indicated heterozygosity. The hybrid F<sub>2</sub> generations were also heterozygous. Moreover, the growths of the hybrid F<sub>2</sub> generation were intermediate of the parental strains from days 0 to 30. Therefore, outcrossing changes genetic architecture and the new genotypes potentially result in new phenotypes of the subsequent generations of *K. marmoratus*. It may also play a role in adaptation to new environments and the facilitation of local adaptation.

## Introduction

The mangrove killifish *Kryptolebias marmoratus* Poey, 1880 (Pisces: Rivulidae; formerly *Rivulus marmoratus*; Costa 2004) is the only known self-fertilizing vertebrate (Harrington 1961; Vrijenhoek et al. 1989). This unique species is broadly distributed in coastal mangrove habitats from southern Brazil through the Caribbean Islands and Central America to the Yucatan and along the coast of Central Florida to North Florida (Taylor 2000). They are capable of synchronous self-fertilization, producing homozygous offspring as a consequence (Kallman and Harrington 1964; Harrington and Kallman 1968). *K. marmoratus* has three sexual phenotypes (Harrington 1975). The hermaphrodites have marbled brown bodies usually including a caudal ocellus, with gonads possessing both oocytes and spermatocytes. Primary males have orange bodies usually without a caudal ocellus, and their gonads only have spermatocytes. There are also secondary males which arise from hermaphrodites following the loss of the female function. In nature, the majority of the *K. marmoratus* population is composed of hermaphrodites, but males are occasionally observed. The proportion of males in the Belize Cays is higher (20-25%) than in other sites (1-2%, Taylor et al. 2001). Although males are rare in the wild, primary males are induced in laboratory rearing embryos at low temperatures (20°C, Harrington 1967). Hermaphrodites transform into secondary males upon exposure of hermaphrodites to high temperature (30°C, Harrington 1971). Recently, Kanamori et al. (2006) reported that androgen (17 $\alpha$ -methyltestosterone) treatment of the embryos efficiently induces primary males.

Previous molecular genetic surveys, such as multilocus DNA fingerprinting and microsatellite genotyping by microsatellite markers, have revealed high genetic diversity in wild populations of *K. marmoratus* suggesting that outcross events between hermaphrodites and males occur (Turner et al. 1990; Turner et al. 1992; Lubinski et al. 1995; Taylor et al. 2001; Mackiewicz et al. 2006a). Mackiewicz et al. (2006a) surveyed the genetic architecture of *K. marmoratus* individuals in Belize, Florida and the Bahamas. Their results showed that heterozygosity in the Belize populations was higher than in the other populations, suggesting that outcrossing in Belize is relatively common.

Mackiewicz et al. (2006c) confirmed hybrids between a hermaphrodite and a male from different inbred laboratory strains by microsatellite analysis. They concluded that male-mediated outcrossing could produce a new recombinant clonal strain. It is now becoming clear that genetic diversity in wild populations is caused by males. However, how male-mediated increase in genetic diversity impacts the phenotype of *K. marmoratus* is still unclear. In order to find out, we chose two different strains with identifiable genetic and phenotypic characteristics and established genetic tools to assess changes in genetic diversity. The Aquaculture Biology Laboratory, Nagasaki University, Japan keeps two clonal strains, PAN-RS and DAN, which were originally collected near Bocas del Toro, Republic of Panama and Dangriga, Belize, respectively (Grageda et al. 2005). These strains do not have significant differences in morphometric or meristic characteristics, but they do have different life-history traits such as growth rate, fecundity and sex ratio (Grageda et al. 2005). The

PAN-RS strain showed significantly higher growth and fecundity than the DAN strain, and the DAN strain produced more primary males than the PAN-RS strain. Thus, these well-characterized strains are useful for revealing changes of genotype and phenotype after male-mediated outcrossing.

We hypothesized that genotypic diversity generated by outcrossing in *K. marmoratus* incurs significant phenotypic diversity. When outcrossing occurred in these two strains, we can expect heterozygosity in genotype and heterosis in growth. In this study, we used AFLP (Amplified Fragment Length Polymorphism) analysis to determine the genetic differences between two clonal strains and confirmed that male-mediated outcrossing can indeed occur using artificial insemination of two clonal strains. Furthermore, we investigated the genotypes and growth as a phenotypic characteristic of the hybrid F<sub>2</sub> generation, and compared them to the hybrid F<sub>1</sub> generation and the two parental clonal strains because we can expect more genetic variance in F<sub>2</sub> than F<sub>1</sub>.

### **Materials and methods**

Genetic differences between the two clonal strains were determined using AFLP analysis. Afterwards, unfertilized eggs of each strain were artificially inseminated and a hybrid strain was obtained, confirmed by success of hybridization with AFLP markers of the parental strains. The genotype and growth of the F<sub>2</sub> generation from the hybrid strain were compared with the parental strains.

Molecular markers have been useful for the analysis of genetic diversity. As the southern blot technique revealed that both PAN-RS and DAN were homozygous but could not detect the

genetic differences between strains due to the low resolution of detected bands using chemical luminescence (Grageda 2004) instead of radio-active marker (Turner et al. 1990), a more sensitive marker was needed. AFLP is a PCR-based, multi-locus fingerprinting technique that combines the advantages of the RFLP (Restriction Fragment Length Polymorphism) and RAPD (Random Amplified Polymorphic DNA) methods (Vos et al. 1995). AFLP does not require any previous molecular information, and can therefore even be applied to species that have not been well-studied. Another advantage of AFLP is the capability of producing multi-locus fingerprints in a single analysis, and thus AFLP is powerful for revealing genomic polymorphisms (Liu and Cordes 2004). The application of AFLP has progressed in Pisces species such as the giant mottled eel *Anguilla marmorata* (Ishikawa et al. 2004), the channel catfish *Ictalurus punctatus* (Mickett et al. 2003) and the gilthead sea bream *Sparus auratus* (Miggiano et al. 2005). Recently, killifish (*Fundulus heteroclitus*), the same order as *K. marmoratus*, was successfully used in AFLP analysis to measure population differentiation and diversity (McMillan et al. 2006).

## **Experimental fish**

We used two clonal strains, PAN-RS and DAN, obtained from Dr. W. P. Davis of the U.S. Environmental Protection Agency, Gulf Breeze, Florida, U.S.A. The PAN-RS individuals were the descendants of a single hermaphrodite originally collected near Bocas del Toro, Republic of Panama, in 1994, while DAN were the descendants of a single fish collected in Dangriga, Belize (Grageda et

al. 2005). These strains have been reared for over 9 generations in our laboratory. Fertilized eggs and fish were individually cultured at  $25\pm 1^{\circ}\text{C}$  with a light and dark photoperiod of 14h:10h, which were established rearing conditions by Koenig and Chaser (1984) and the same rearing conditions applied in the former study using those 2 strains (Grageda et al. 2005). They were kept in plastic containers with a diameter of about 5.8 cm in 30 mL of 17 ppt artificial brackish water until hatching. The larvae and adult fish were individually reared in plastic containers with 60 mL of 17 ppt artificial brackish water and fed newly hatched *Artemia franciscana* nauplii until satiation 5 times a week. This study was carried out under the Guide to the Care and Use of Experimental Animals by the Canadian Council on Animal Care and the guidelines from Animal Care and Use Committee of Nagasaki University, Japan.

### **Genomic DNA isolation**

Sample tissues were collected from PAN-RS ( $N = 5$ ) and DAN ( $N = 5$ ) by slicing the caudal fin from each fish after anesthesia with 400 mg/L of MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO, USA). The strains used were originated by only one parental hermaphrodite of both PAN-RS and DAN. The tissues were digested with 20 mg/mL proteinase K in a buffer of 10 mM Tris HCl (pH 7.5) with 125 mM NaCl, 10 mM EDTA and 10% sodium dodecyl sulfate overnight at  $37^{\circ}\text{C}$ . Proteins and lipids were removed with phenol-chloroform-isoamyl alcohol (25:24:1).

## **AFLP analysis**

AFLP analysis was performed using the AFLP™ Plant Mapping Kit (Applied Biosystems, 2000). MSE I and ECO RI enzymes were used for digestion of the genomic DNA which was isolated from each individual. The digested DNA fragments were ligated to the MSE I and ECO RI adaptors. Preselective and selective amplifications were carried out following the protocol of the AFLP™ Plant Mapping Kit (Applied Biosystems, 2000). A total of 3 primer combinations were used for selective amplifications: MSE I CAC/ECO RI ACC, MSE I CAG/ECO RI ACC and MSE I CTC/ECO RI ACC, respectively. Amplified DNA fragments were visualized on the Genetic Analyzer 310 (Applied Biosystems, U.S.A.) and their sizes determined using the Gene Scan 500 [ROX] size standard (Applied Biosystems, U.S.A.) and GENESCAN 3.7 software (Applied Biosystems, U.S.A.). The fragments with a peak height of more than 50 were characterized using the GENESCAN software (Applied Biosystems, 2000). Detected fragment sizing is often unclearly described, because different fragments are usually visually characterized as one fragment and one fragment as noise (Applied Biosystems, 2000). Therefore, we determined the fragment numbers visually. Fragments in the 50 to 450 bp range were considered. Moreover, only fragments that were detected on all of the individuals were defined as AFLP markers.



## **Artificial insemination**

We carried out artificial inseminations between the eggs of a hermaphrodite and the sperm of a primary male combining 2 different strains (Fig. 1). Since ovulated eggs are self-fertilized in the gonadal lumen in *K. marmoratus* (Sakakura et al. 2006), we surgically removed ovulated eggs from the hermaphrodite and fertilized them for 10 minutes in a sperm suspension from a primary male after euthanasia with 400 mg/L of MS-222. Artificial insemination was conducted using different PAN-RS hermaphrodites ( $N = 10$ ) versus DAN primary males ( $N = 3$ ), and reverse pairs using different DAN hermaphrodites ( $N = 2$ ) versus a PAN-RS primary male ( $N = 1$ ) with slight modification of isotonic method for Japanese medaka *Oryzias latipes* (Iwamatsu 1984). Two sperm suspensions were used: 1) 17 ppt artificial sea water and 2) Ringer medium. In the first method, 14 eggs from 4 PAN-RS hermaphrodites were immersed in the sperm suspension of one DAN primary male, and 10 embryos were obtained. In the second method, 8 gonads from hermaphrodites (6 PAN-RS and 2 DAN) were individually immersed in the sperm suspension from 3 primary males (1 PAN-RS and 2 DAN), with 3 embryos were obtained from 17 eggs.

A total of 13 fertilized eggs from the above procedure were individually cultured in a plastic container as described above, and after hatching they were fed newly hatched *Artemia franciscana* nauplii until satiation 5 times a week.

## **Confirmation of hybridization using AFLP markers**

The genomic DNA of the individuals obtained by artificial insemination was isolated by slicing the caudal fin and/or anal fin from each fish and then analyzed using PAN-RS and DAN AFLP markers to determine the success of hybridization.

### **F<sub>2</sub> generation of hybrid**

This experiment was carried out to examine differences in genotype and the characteristics of the hybrid F<sub>2</sub> generation relative to the F<sub>1</sub> generation. F<sub>1</sub> from artificial insemination that had been kept after the AFLP analysis produced F<sub>2</sub> progeny. The hybrid was named the PDHy strain. As a control, three PAN-RS individuals produced by self-fertilization, which came from the same individual as the parent of the hybrid and a DAN individual of the same generation as the hybrid, were reared in plastic containers in the same rearing conditions as described above, respectively.

The genomic DNA of F<sub>2</sub> generations of PDHy ( $N = 12$ ), PAN-RS ( $N = 14$ ) and DAN ( $N = 13$ ) for controls were isolated by slicing the caudal fin and/or anal fin from each fish, after analysis of the variation of the genotype using AFLP markers of PAN-RS and DAN. All fish were measured to determine standard length (SL) by a digital microscope (VH6300, Keyence, Osaka, Japan) at 0, 10, 20 and 30 days after hatching. On each measurement day, the fish were anaesthetized with 200 mg/L of MS-222 and then SL was measured individually.

## **Data analysis**

We compared the total number of fragments and genetic similarity in PAN-RS and DAN in each primer combination. We also compared the difference between PAN-RS and DAN in each primer combination. Genetic similarity was compared by the band share index (BSI) (Wetton et al. 1987). BSI is defined for two individuals (a and b) by the following formula:  $BSI = 2N_{ab} / (N_a + N_b)$ .  $N_{ab}$  is the number of shared fragments by both individuals, and  $N_a$  and  $N_b$  are the total number of fragments in each individual, respectively. BSI was calculated as the inverse sine transformation before analysis, and compared using the Tukey-Kramer test after a one-way analysis of variance (ANOVA). We accepted  $P < 0.05$  as indicating significant differences between means. For the AFLP markers, completely specific fragments were scored as positive (+) or negative (-) between PAN-RS and DAN. Then, we checked whether dominant markers in F2 progeny fit into Mendelian ratios (3:1) using Fisher's Exact Probability test. The growth of each strain was expressed as the SL plot from day 0 to day 30 after hatching. Each strain was compared at the same age using a Tukey-Kramer test after a one-way ANOVA.

## **Results**

### **Genetic variation and AFLP markers**

The result of the AFLP analysis using the MSE I CAC/ECO RI ACC primer combination showed that BSI between the two different strains showed a significant difference (ANOVA,  $df=2$ ,  $F=408.729$ ,  $P < 0.0001$ , Tukey-Kramer test,  $P < 0.05$ , Table 1). We confirmed four specific fragments

between two different strains and determined them to be AFLP markers (Table 2). The result of the AFLP analysis using the MSE I CAG/ECO RI ACC primer combination showed that the BSI between the two different strains was significantly different (ANOVA,  $df=2$ ,  $F=267.650$ ,  $P<0.0001$ , Tukey-Kramer test,  $P<0.05$ , Table 1). We confirmed four specific fragments between two different strains and determined them to be AFLP markers for their primer combination (Table 2). The result of AFLP analysis using the MSE I CTC/ECO RI ACC primer combination showed that the BSI between the two different strains showed a significant difference (ANOVA,  $df=2$ ,  $F=74.111$ ,  $P<0.0001$ , Tukey-Kramer test,  $P<0.05$ , Table 1). We confirmed three specific fragments between the two different strains and determined them to be AFLP markers (Table 2).

### **Artificial insemination**

The genomic DNA of 13 individuals obtained from artificial insemination was analyzed using 11 AFLP markers by AFLP analysis in order to determine the success of hybridization. AFLP analysis revealed that 12 out of 13 individuals displayed the homozygous form, and therefore 12 individuals must have come from their sole parental strain. One individual (namely PDHy) from artificial insemination using sperm suspension of artificial seawater was heterozygous between the PAN-RS hermaphrodite and the DAN male (Table 2).

### **F<sub>2</sub> generation of PDHy**

The F<sub>2</sub> generation of PAN-RS and DAN as controls only had their own AFLP markers, respectively. However, PDHy had a completely different genotype compared with their parental strains and the F<sub>1</sub> generation of PDHy (Table 2). Occurrence of AFLP bands in F<sub>2</sub> progeny of PDHy corresponded to 3:1 ratio (Fisher's Exact Probability test,  $P>0.21$ ), as expected for dominant markers.

The PDHy body size in terms of SL ranged from  $4.39\pm 0.17$  mm (mean $\pm$ SD,  $N = 12$ ) at day 0 to  $11.86\pm 0.70$  mm at day 30. The growth of PDHy from day 20 ( $9.21\pm 0.56$  mm) to day 30 ( $11.86\pm 0.70$  mm) showed a significant difference between that of PAN-RS ( $9.93\pm 0.38$  mm,  $12.61\pm 0.60$  mm,  $N = 14$ ) and DAN ( $8.58\pm 0.69$  mm,  $10.72\pm 0.91$  mm,  $N = 13$ , Fig. 2).

## **Discussion**

Artificial insemination using sperm from males of two different clonal strains of the mangrove killifish, which have different life-history traits, was performed and successfully produced one hybrid (F<sub>1</sub>) from the parental strains. The hybrid F<sub>1</sub> matured and produced F<sub>2</sub> generations, which showed intermediate growth to those parental strains and had completely different patterns in genotypes compared with the hybrid F<sub>1</sub> and the two parental clonal strains. Therefore, it was concluded that changes in the genetic structures caused by outcrossing affect not only genotype but also phenotype, and that outcrossing in *K. marmoratus* may have important ecological roles for generating biological diversities in this species.

AFLP analysis was applied to discriminate the genotypes of the experimental fish, and

detected genetic differences between the two clonal strains, subsequently confirming that male-mediated outcrossing was possible using artificial insemination. Thus, AFLP is applicable for detecting genetic differences in *K. marmoratus* both for laboratory studies and field surveys. We compared the banding pattern in two clonal strains using BSI (Wetton et al. 1987). The BSI value varies from zero, when there are no bands in common, to 1, when band patterns are identical. The value is approximately 0.5 for the first degree relatives, 0.25 for the second degree relatives, and so forth (Wetton et al. 1987). Taniguchi et al. (1996) compared the genetic similarity of DNA fingerprints by digesting with restriction enzyme among clonal strains in the gynogenetic diploid fish ayu, *Plecoglossus altivelis*, by BSI. They showed that the BSI value was 1 for identical clones and between 0.20-0.33 in different clonal strains. The BSI values of our results were above 0.9 in each parental strain and significantly lower values were detected between the two clonal strains. The reason why the BSI value of each clonal strain did not match as 1 might be due to non-specific amplifications.

Growth of the hybrid F<sub>2</sub> generation was intermediate of the parental strains, indicating that changes in the phenotype from the parents had occurred. Although we only compared differences in growth between the hybrid F<sub>2</sub> generations and parental strains, there are more phenotypically different characteristics, such as fecundity and sex ratio, between the parental strains used in this study (Grageda et al. 2005). Comparison of these phenotypic characteristics in the hybrid F<sub>2</sub> generation will reveal additional phenotypic diversity. Mackiewicz et al. (2006c) detected hybrids by

male-mediated outcrossing between two different inbred laboratory strains using 36 microsatellite markers, and indicated that new inbred strains will be produced by heterozygous progeny after several generations of self-fertilization. Our results confirm the statement of Mackiewicz et al. (2006c), because new strains are being formed by artificial insemination between two different clonal strains which will eventually lead to different inbred strains, and there is loss of heterozygosity already in F2 and heterozygosity will be decreasing in each subsequent generation of *K. marmoratus*.

The mangrove killifish inhabits mangrove containing stagnant pools, sloughs or ditches (often intermittently dry) and some fossorial niches (inside or under damp logs, debris, leaf litter) and survives in unstable environments with salinities ranging from 0 to 68 ppt and temperatures from 7 to 38°C (Taylor 2000). Lin and Dunson (1995) found that changing environmental factors, such as salinity and food level, impacted reproductive fitness differently in five strains of *K. marmoratus*. It is possible that the androdioecious (populations comprised of hermaphrodites and males) mixed-mating system of *K. marmoratus* may allow survival in a highly fluctuant environment. Weibel et al. (1999) suspected that outcrossing evolved as a phenotypically plastic character and its expression in *K. marmoratus* may be dormant unless triggered by some ecological factor such as low temperature. In addition, the genetic architecture of Florida populations where males are rare seems to reflect a mixed mating strategy of predominantly selfing with occasional outcrossing (Mackiewicz et al. 2006b). If this is the case, then the androdioecious mixed mating system may be

extending gradually in the northern populations of *K. marmoratus* and may be facilitated adaptation in response to environmental variables. The ability to produce offspring by self-fertilization may generate reproductive assurance whereas outcross events with males may promote genetic variation into the lineage to produce different phenotypes and to prevent the accumulation of deleterious mutations. Thus, this reproductive mechanism may allow *K. marmoratus* to make physiological adaptations as the climate changes at the mangrove swamps.

Functional hermaphrodites sometimes emit unfertilized eggs that could be available for fertilization by males (Harrington 1963). However, previous studies have not documented mating behavior and the experimental conditions for mating. To find out whether the unfertilized eggs emitted by functional hermaphrodites could be available for fertilization by males, the environmental factors that trigger the mating behavior of *K. marmoratus* will be studied in the future.

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Table 1. Genetic similarity (BSI) in PAN-RS and DAN strains using the 3 primer combinations of Mse I and Eco RI. Fragments in 50 to 450 base pair (bp) were compared.

Primer combinations	strain	<i>N</i>	total number of fragments (Mean ± SD.)	BSI (Mean ± SD.)
Mse I CAC-Eco RI ACC	PAN-RS	5	27.4 ± 0.6	0.96 ± 0.02 <sup>a</sup>
	DAN	5	30.4 ± 1.1	0.94 ± 0.02 <sup>a</sup>
	PAN-RS, DAN	10	28.9 ± 1.8	0.79 ± 0.03 <sup>b</sup>
Mse I CAG-Eco RI ACC	PAN-RS	5	31.2 ± 2.1	0.93 ± 0.03 <sup>a</sup>
	DAN	5	28.4 ± 1.1	0.92 ± 0.02 <sup>b</sup>
	PAN-RS, DAN	10	29.8 ± 2.2	0.76 ± 0.02 <sup>c</sup>
Mse I CTC-Eco RI ACC	PAN-RS	5	25.6 ± 3.1	0.90 ± 0.04 <sup>b</sup>
	DAN	5	23.6 ± 0.9	0.94 ± 0.02 <sup>a</sup>
	PAN-RS, DAN	10	24.6 ± 2.4	0.82 ± 0.03 <sup>c</sup>

a>b>c, Tukey-Kramer test,  $P < 0.05$



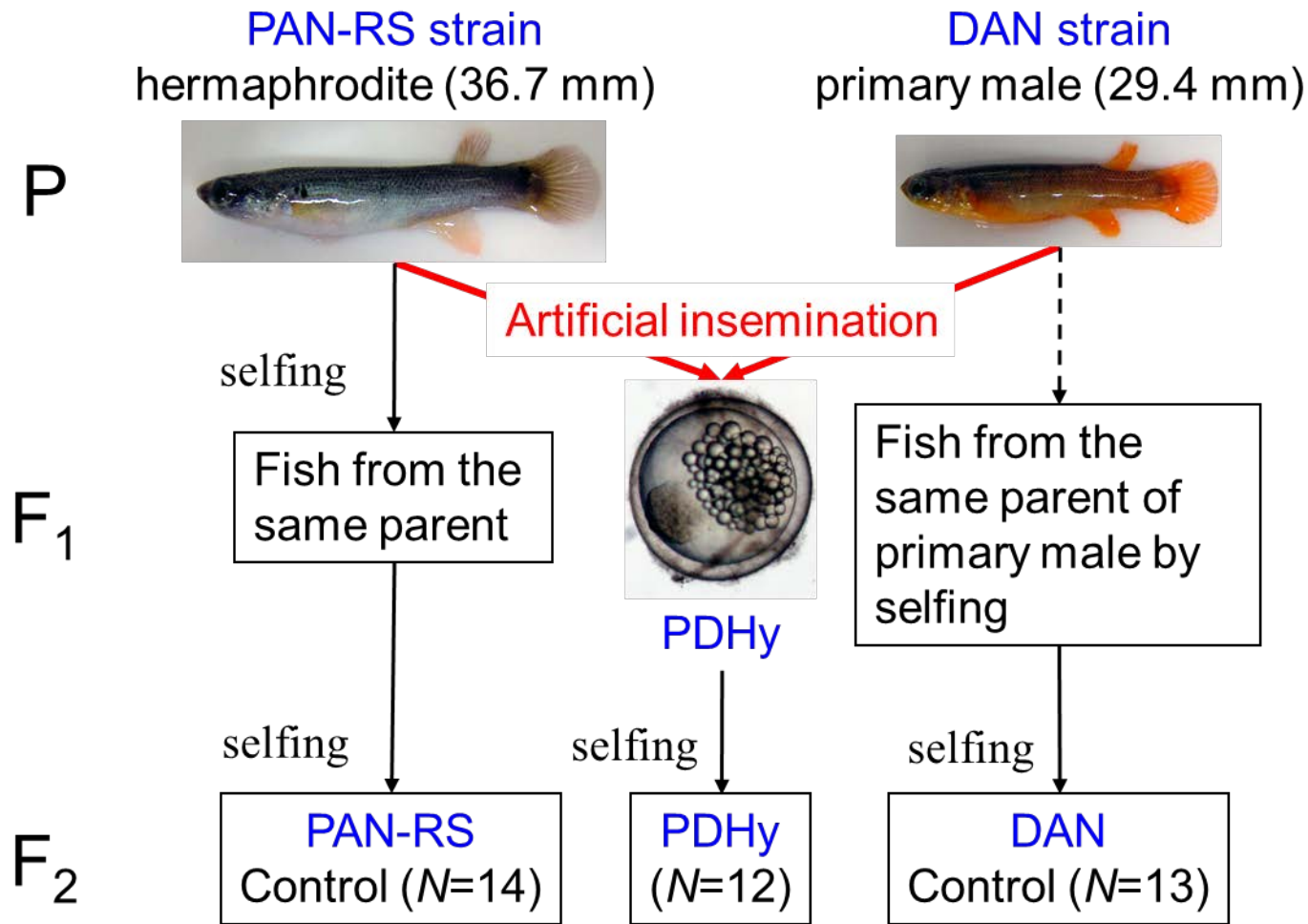


Figure 1. Experimental procedure of producing hybrid (PDHy) between 2 genetically different strains (PAN-RS and DAN) of the mangrove killifish. F<sub>2</sub> generations of each strain were used for the comparison of genetic architecture and growth.



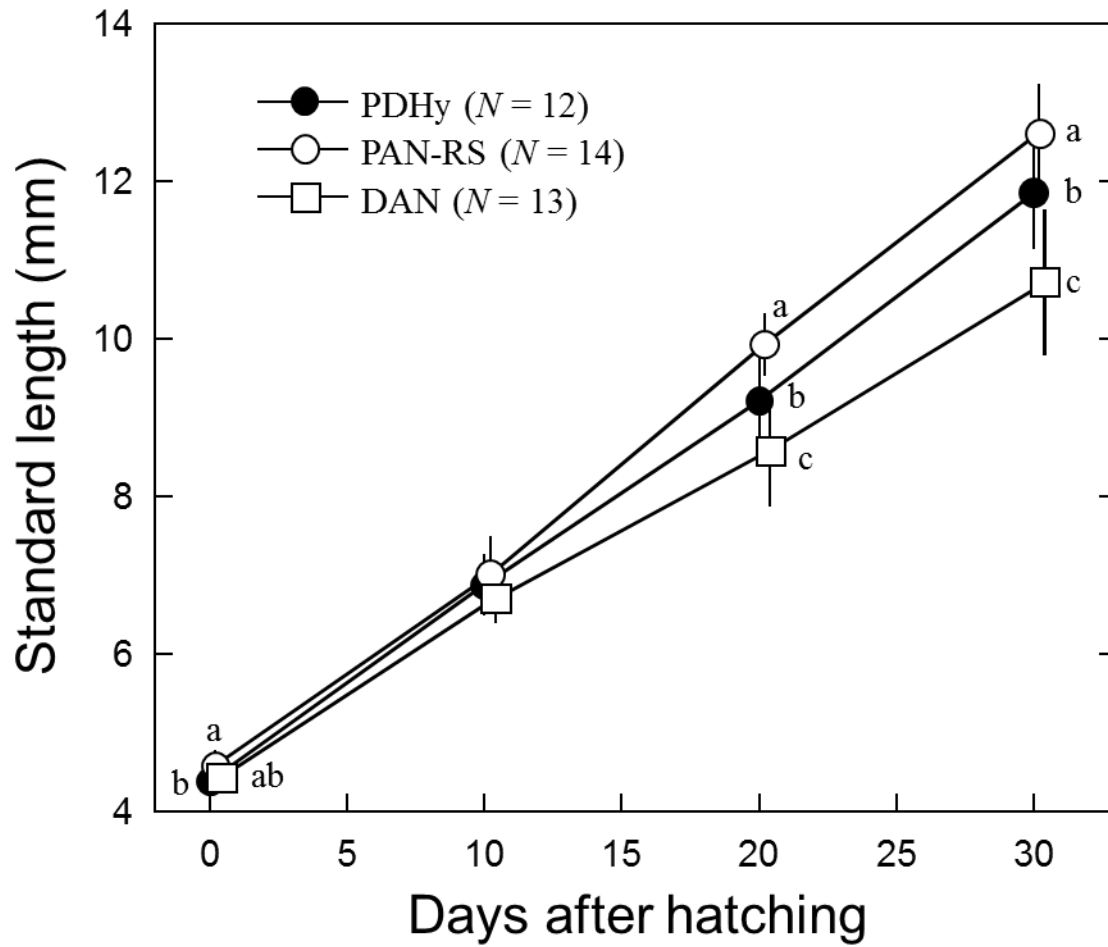


Figure 2. Growth of F<sub>2</sub> generation of PDHy compared with parental (PAN-RS and DAN) strains.

Plots and bars indicate average and standard deviations, respectively. Letters on each plot indicate significant difference in the same age group (a>b>c, Tukey-Kramer post-hoc test,  $P < 0.05$ ).