Can multilocus heterozygosity reveal inbreeding depression?

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Abstract

Inbred lines of fish have been widely exploited as model organisms to determine the effect of inbreeding, which is often closely related to fitness such as endurance and productivity compared to morphological traits. Until now, much is unknown about the effects of inbreeding to fish. In the present study, inbred lines of guppies were used to examine the inbreeding effect on morphological traits corresponding to genotype variation. Two strains, called AY and NA1, were selected from the closed culture system. Both strains showed different levels of inbreeding coefficients when compared to microsatellite markers. The AY strain was less inbred as compared to NA1 strain. However, correlation between the standard lengths with multilocus heterozygosity (MLH) at the individual level was observed in the AY, but not in the NA1 strain. This indicated that highly inbred animals have higher similarity in morphological traits as compared to less inbred ones. The inbreeding process showed the importance of heterozygosity, even in laboratory-reared animals. This experiment illustrated the effects of inbreeding towards morphological and genetic changes.

Key words

DNA, Guppy, Growth, Inbreeding depression, Microsatellite markers
Materials and Methods

Research on animals has been documented on various species including European alpine marmots (Da Silva et al., 2006), Brent geese (Harrison et al., 2011) and guppies (Herdegen et al., 2014), which have shown a positive correlation between heterozygosity and fitness. Nevertheless, a weak relationship was always observed when tested with the wild population (Taylor et al., 2010, Grueber et al., 2011, Herdegen et al., 2014). The main problem while measuring wild population is possibly due to high variation at the individual level (Taylor et al., 2010). This will reduce the chance of detecting inbreeding depression and comparisons between fitness related traits with multilocus-heterozygosity (MLH) that could lead to low correlation. However, for individuals in a small population which have a higher risk of inbreeding, might have higher chances of detecting a correlation between MLH with various traits. Alternative methods were developed to improve the correlation between MLH with traits, such as internal relatedness (IR) (Amos et al., 2001) and standardized heterozygosity (SH) (Coltman et al., 1999). IR were weighted on rare alleles existing in homozygotes, while SH is a method of calculating heterozygosity by the average of heterozygosity weight on each locus.

In the present study, correlations between IR and SH with standard length in inbred guppy strains. The objective of this study is to understand the changes and correlation between genetic information and morphological related traits and also to determine whether heterozygosity can be used to evaluate inbreeding depression.

Guppies were randomly selected from two strains that were cultured in a closed system at the Laboratory of Population Genetic Informatics, Faculty of Science Agriculture, Tohoku University. The strains consisted of AY and NA1 strain which originated from Thailand and Okinawa, Japan, respectively. Both the strains were kept in close culture system since 1999 for NA1 and 2009 for AY strains. The inbreeding experiment was initiated by parental selection which consisted of five pairs of males and females from the AY and NA1 strains. These pairs were allowed to breed and reproduce in a 2 l aquarium. The first generation

1. Five pairs of male and female guppy were selected

2. Obtaining new guppy seed and raised to adulthood

3. Male and female siblings guppy were randomly selected to produce next generation of inbred.

4. Obtaining new inbred guppy seed and raised to adulthood.

Fig. 1 : Illustrated diagram of full sibling mating process using guppy as a model organism. Started off with five pairs of parental generation (P) and were allowed to produce offspring of the 1st generation (F1). Then the F1 offspring we cultured until it reached maturity then randomly separated in a new parental generation. The second generation F2 were produce through full sibling mating between F1 generations. This cycle continue for several generations in order to obtain full sibling mating inbred line.
Can multilocus heterozygosity reveal inbreeding depression?

progeny (F₁) from the parental generations (P) were collected and separated into a small family. A maximum number of five individuals were randomly selected from F₁ progeny to be cataloged as a family. Each family was placed inside a 1 l container and maintained by weekly water changes. The F₁ progeny were measured for body length (mm) every 30 days until 180 days, old and were fed daily. New full sibling parental generations were created from F₁ generation by randomly pairing the family sibling to produce second generations (F₂) of inbred progeny. This cycle continued to produce several generations of inbred guppies, which is illustrated in Fig. 1.

**DNA extraction and analysis:** Total genomic DNA from all the individuals were extracted using the Wizard® Genomic DNA Purification Kit (Promega) and stored at 4°C prior to PCR. Screening was done using 19 microsatellite marker consisting of AC₃, AGAT₁₁, ATCC₂ (Olendorf et al., 2004), Pret-₄₆, Pret-₄₉, Pret-₆₉, Pret-₇₁, Pret-₇₂, Pret-₈₀ (Watanabe et al., 2003), G₈₂, G₂₈₉, G₃₅₀ (Shen et al.,

![Fig. 2: The body length at 180 days of AY(A) and NA1(B) of guppy strains separated gender and generations. Error bars; standard deviation of body length at 180 days. Significant changes were compared in gender and generations. The symbol “a” by the letters denote differences that were significant.](image-url)
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848

Table 1: Selected microsatellite markers for this experiment

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat motif</th>
<th>Primer sequences</th>
<th>GenBank Accession no.</th>
<th>No. of alleles</th>
<th>Allele range (bp)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATCC2 (AATG)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>BV097133</td>
<td>15</td>
<td>234-319</td>
<td>Olendorf et al. (2004)</td>
</tr>
<tr>
<td>2</td>
<td>AC3 (CA)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>BV097135</td>
<td>22</td>
<td>236-288</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AGAT11 (AGAT)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>BV097141</td>
<td>27</td>
<td>213-381</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pret-46 (CA)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>AB100334</td>
<td>7</td>
<td>170</td>
<td>Watanabe et al. (2003)</td>
</tr>
<tr>
<td>5</td>
<td>Pret-49 (CA)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>AB100336</td>
<td>7</td>
<td>251</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Pret-69 (GT)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>AB100347</td>
<td>6</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Pret-71 (GA)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>AB100348</td>
<td>4</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Pret-72 (GA)$_n$</td>
<td>F: AGTCACGAGTTATTTGACATCATCAGAGGCCCTG&lt;br&gt;R: CATTCACACTAGTGGACGCG</td>
<td>AB100349</td>
<td>4</td>
<td>116</td>
<td></td>
</tr>
</tbody>
</table>

cycles consisting of denaturation for 30 sec at 94°C, annealing for 30 sec at a specific annealing temperature (please refer Table 1.1) and extension for 30 sec at 72°C.

Table 2: Genotyped changes in inbred AY and NA1 strain guppy

<table>
<thead>
<tr>
<th>Strains</th>
<th>N</th>
<th>Ho (SE)</th>
<th>He (SE)</th>
<th>Fst</th>
<th>f</th>
</tr>
</thead>
<tbody>
<tr>
<td>AY-1</td>
<td>30</td>
<td>0.522 (0.062)</td>
<td>0.626 (0.064)</td>
<td>0.102</td>
<td>0.250</td>
</tr>
<tr>
<td>AY-2</td>
<td>60</td>
<td>0.452 (0.127)</td>
<td>0.582 (0.057)</td>
<td>0.158</td>
<td>0.375</td>
</tr>
<tr>
<td>AY-3</td>
<td>45</td>
<td>0.382 (0.057)</td>
<td>0.508 (0.057)</td>
<td>0.233</td>
<td>0.500</td>
</tr>
<tr>
<td>NA1-1</td>
<td>45</td>
<td>0.479 (0.055)</td>
<td>0.643 (0.062)</td>
<td>0.245</td>
<td>0.250</td>
</tr>
<tr>
<td>NA1-2</td>
<td>75</td>
<td>0.371 (0.075)</td>
<td>0.601 (0.072)</td>
<td>0.375</td>
<td>0.375</td>
</tr>
<tr>
<td>NA1-3</td>
<td>34</td>
<td>0.285 (0.075)</td>
<td>0.531 (0.065)</td>
<td>0.453</td>
<td>0.500</td>
</tr>
</tbody>
</table>

Notes: N= sampled individuals, Ho= observed Heterozygosity, He= expected heterozygosity, Fst= Fixation index, AY-x = AY strain x generations, NA1-x = NA1 strain x generations. SE= standard error.

2007), Pr 21, Pr 40, Pr 80 (Becher et al., 2002), Pr1, Pr13 and Pr15 (Paterson et al., 2005). Nine microsatellite markers selected for analysis comprised of Pret-46, Pret-49, Pret-69, Pret-71, Pret-72, Pret-80, AC3, AGAT11 and ATCC2 (Table 1.1) and other microsatellite markers were excluded due to low allele number count and high existence of null alleles. All of the loci were amplified with a fluorescently labeled forward primer. PCR amplification was carried out with a Thermal Cycler (TaKaRa) in 10 µl reaction mixtures containing 20-50 ng of genomic DNA, 1.5 mM MgCl$_2$, 1x PCR buffer, 0.1 µM dNTPs, 0.2 µM of each primer and 0.8U of Taq DNA polymerase (Promega). PCR was preset for denaturation at 94°C for a min, followed by 33 cycles consisting of denaturation for 30 sec at 94°C, annealing for 30 sec at a specific annealing temperature (please refer Table 1.1) and extension for 30 sec at 72°C. PCR products were kept at 4°C for storage and were used for further analysis. A volume of 0.5 µl GeneScan$^*$ 400HD (ROX) size standard were mixed with 1 µl PCR product and 10 µl HI-Di$^{TM}$ formamide. The mixture were given 2 min of heat shock treatment at 94°C using a thermal cycler and cold shock at 4°C before being electrophoresed using an ABI 3500xl Genetic Analyzer (Applied Biosystems).

Calculation of heterozygosity : Internal relatedness (IR) and standardized heterozygosity (SH) were assessed using
Can multilocus heterozygosity reveal inbreeding depression?

Rhh as implemented in R statistical software (Alho et al., 2010). SH measure heterozygosity loci at one individual was divided by average heterozygosity of loci that were successfully genotyped for each individual (Coltman et al., 1999.) IR was calculated by the following formula:

$$IR = 2H - \sum f_i / (2N - \sum f_i)$$

Where, H is the loci number that are homozygous; N is the number of loci and fi is the frequency of the i-th allele contained in the genotype. This is a measure of genetic diversity within each individual (an estimate of parental relatedness and we averaged over individuals for every generations in every strains). The IR score from -1 to 1; sample closer -1 were assumed to be originated from unrelated parent, while 1 were assumed to be originated from highly related parent. The individual heterozygosity was measured by number of heterozygosity divided by total microsatellite markers used. The observed heterozygosity (Ho), expected heterozygosity (He) and fixation index (Fst) were calculated using software GenAlex version 6.501 (Peakall and Smouse, 2012).

**Statistical analysis:** Correlation between guppy standard length and MLH were calculated by Pearson Correlation using SPSS version 21 software. One-way ANOVA was used to demonstrate significant differences between the standard length in generations and strains with confidence at p<0.05.

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**Fig. 3:** Correlation between individual heterozygosity (IH) with body length (mm) of AY strains in three generation of full sibling mating. (a) First generation, (b) Second generation and (c) third generation of full sibling mating. Linear continuous line (-----) = female and dotted line (......) = male, $R^2$ = female, $R^2$ = male, ♦ = individual female, ×=individual male, p= significant, two tailed

**Fig. 4:** Correlation between standardized heterozygosity (SH) with body length (mm) of AY strains in three generation of full sibling mating. (a) First generation, (b) Second generation and (c) third generation of full sibling mating. Linear continuous line (-----) = female and dotted line (......) = male, $R^2$ = female, $R^2$ = male, ♦ = individual female, ×=individual male, p= significant, two tailed
Results and Discussion

Physical development of inbred guppy: The standard lengths of AY strain were measured every 30 days starting from birth until it reached 180 days (Figure 1.2). Standard lengths were separated by gender in different groups due to significant differences in length. The average standard length for AY female of first generation was 18.45±3.83 mm (mean body size ± standard deviation), while NA1 had an average of 16.25±2.69 mm. NA1 female had smaller body sizes as compared to AY female, however it was insignificant. Average body size of NA1 female increased as the inbreeding generation's progressed. The average NA1 female at third generation was at 17.64±3.56 mm. However, it was not significant. The AY strains female showed dropp in body size in second generation, however as inbreeding progressed, the mean body size remained similar and significant changes were not observed throughout the generations. The decreased standard length in AY male in second generation were significant (p<0.05) from 18.45±3.38 mm to 16.37±1.37 mm as compared to first generation. The mean body size of NA1 male in first generation was 15.52±2.27 mm and in third generation the mean body size was 14.86±1.2 mm. Significant changes were not observed throughout the generations. No abnormal morphology was noted in any individual fish in AY and NA1 strains.

Genotyped: Guppy samples were genotyped using highly polymorphic microsatellite markers. Strains and generations of inbred samples were separated. All the samples showed lower observed heterozygosity as compared to expected heterozygosity until third generation (Table 1.2).
Can multilocus heterozygosity reveal inbreeding depression?

indicated that both the strains were undergoing inbreeding program. In AY strain, observed heterozygosity was relatively higher as compared to NA1 strain. The observed heterozygosity for AY strains started of with Ho=0.522 and decreased in third generation at Ho=0.382. These probably indicated that inbreeding occurred as mating between sibling progressed. Similar to AY, the NA1 showed significant drop in observed heterozygosity from Ho=0.479 in first generation, while in third generation the observed heterozygosity was Ho=0.285. Increments of fixation index (Fst) were observed in the experiment as the inbred generation increased. The AY strains had lower Fst compared to NA1 strain. Increased in Fst indicated that population were undergoing inbreeding process. The AY strain started off with Fst=0.102 and in third generation it increased up to Fst=0.233. The NA1 strain were at Fst=0.245 in first generation of inbred guppy and increased drastically to Fst=0.453 in third inbred generation. Inbreeding proved to increase Fst possibly due to decrease in allelic diversity as inbreeding generation progressed.

Correlation between multilocus heterozygosity (MLH) and guppy body sizes AY strain: The body length of AY strain guppy were measured at 180 days after hatching. To determine whether multilocus heterozygosity can be used for assessing inbreeding depression, we correlated individual heterozygosity (IH), standardized heterozygosity (SH) and internal relatedness (IR) with body length. The male and female were separated into groups and generations (Fig. 1.3). Negative correlation was observed in the first generation of AY female with IH (R^2=0.333, p=0.02, n=15) (Fig.1.3a). In second generation, significant positive correlation was observed (R=0.216, p=0.00, n=31)(Fig. 1.3b). However, significant correlation was not observed in third generation. Male displayed significant positive correlation in first (R^2=0.278, p=0.04, n=15) and third (R^2=0.379, p=0.00, n=20) generations, respectively.

The standardized heterozygosity (SH) was highly correlated with body size in each generation (Fig.1.4). Significant positive correlations were observed in male and female inbred guppy. However, negative correlation was observed in first generation of female body length with SH (R^2=0.426, p=0.00, n=15) similar to IH (Fig. 1.4a). In male inbred, increase in correlation was observed in each generation, from F, (R^2=0.333, p=0.00, n=15) to F, (R^2=0.498, p=0.00, n=20). Internal relatedness (IR) was scored from -1 to 1. Individual with score close to number one (1) were assumed to be originated from highly related parent. In the study, significant negative correlation was observed between IR and body length indicating that individual from highly related parent tends to produce offspring with shorter body length (Fig. 1.5), but in the first generation of NA1 females, significant positive correlation was observed (Fig. 1.5a). It is clear that SH and IR had high sensitivity as compared to IH In Fig. 1.3b, male on the second generation did not illustrate significant correlation, whereas SH (Fig. 1.4b) and IR (Fig. 1.5b) showed otherwise. This experiment demonstrated the possibility for MLH to be used as early detection on inbreeding depression.

NA1 strain: The MLH method was also applied to the NA1 inbred strain. However, high correlation as illustrated in AY strain could not be detected using this method. Only first generation of inbred NA1 female showed a significant correlation between body length and IH, SH and IR (Fig. 1.6). The males did not show significant correlation in all the generations, even using the MLH methods.

The current studies demonstrate that microsatellite markers can be used to detect the probability of early inbreeding depression at an individual level. The present study was conducted using guppy (Poecilia reticulata) as a model organism after several years in a closed culture system. The effect of inbreeding was demonstrated using two strains (AY and NA1) and each strain illustrated unique patterns when correlated with MLH. The AY strains were reported to be less inbred as compared to the NA1 strains (Table 1.2), and the differences in inbreeding levels and variation in correlation between strains could be based on Fst. This demonstrates that inbred fish might be able to survive in control conditions, however individuals with higher heterozygosity showed better growth performances. This event is commonly documented in hatchery fish such as salmon indicating that hatchery strains have weaker fitness in comparison to wild population (Kallio-Nyberg et al., 2011; Beamish et al., 2012).

The NA1 strain in the study expressed a low correlation using the MLH method. This strain has been kept in a closed culture system for more than 30 generations, and the probability of alleles being fixed was high due to inbreeding. The experiment showed that the NA1 strains had highest Fst indicating that inbreeding processes might have increased the loci fixation rate. When the population were highly fixed, finding correlation between MLH might be challenging due to ineffectiveness of microsatellite markers (Chistiakov et al., 2006). Fst is important to detect possible early inbreeding depression in small population. In the
ornamental fish industry, fixed population are useful since the strain will have similar physiological patterns for subsequent generations and high levels of inbreeding will remove lethal alleles, thus creating a stable population under control conditions. However, due to low variation, it might not be able to cope with other environmental stressors, and this event would rapidly increase the rate of mortality and, at worse, species extinction (Martins et al., 2012; Sleadd et al., 2014).

In natural population, correlation between MLH and fitness related traits were reported to be low or insignificant (Taylor et al., 2010; Grueber et al., 2011; Herdegen et al., 2014). This was probably due to high variation at genomic level, whereas in the present study, the AY and NA1 strains were purposely produced to be inbred, thus a MLH correlation had a higher chance of showing significant correlation between fitness traits due to reduction in variation. Other possibilities such as marker sensitivity would also be an important aspect in detecting correlations between MLH (Chistiakov et al., 2006). Screening for selection markers is important before conducting experiments. One of the main problems of producing inbred fish is the increase of null alleles and although the occurrence is low, some markers might show high null alleles compared to others (Chybicki and Burczyk, 2009). In this study, markers selection was done to reduce the probability of null alleles and this would also help to increase the sensitivity of MLH methods.

The SH and IR are two useful tools to help detect inbreeding levels in guppies and probably with other species as well. We demonstrated a correlation between body length with SH and IR and this correlation can be a base reference for detecting inbreeding. The advantage using SH is that it gave more weight to the number of heterozygote alleles (Colman et al., 1999), while IR gave more weight to rare alleles (Amos et al., 2001). These methods have proved to be more reliable than IH. The MLH was correlated with better growth performance; this study indicates that individuals could be screened for heterozygosity levels prior to inclusion in culture programs.

References