Flow cytometric analysis of genome size of *Oreochromis niloticus* and *O. aureus* and their interspecific hybrid

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Abstract. Srikandi tilapia, a hybrid resulting from an interspecific cross between female *Oreochromis niloticus* and male *O. aureus*, has shown advantages in farming performance. It shows higher productivity in high salinity aquaculture environment than that of both parental lines. From genetic point of view, organismal phenotypes, to some extent might be affected by its genome size or cellular DNA content. This study was aimed to identify the genome size of these two species along with their hybrid. 75 individuals representing three groups, namely two parental lines and its hybrid were sampled and measured for their DNA content by flowcytometry. The mean (±SD) DNA content of *O. aureus* (1.271 ± 0.0022 pg) was higher but was not statistically significantly different (P>0.0175) from that of the *O. niloticus* (1.261 ± 0.0022 pg). The mean (±SD) DNA content of the hybrid (1.263±0.001) was in between and was not significantly different (P>0.05) from both parental species. Individual variation of DNA content within species was lower than those between species. No significant difference was observed in DNA content between male and female within the respective groups.

1. Introduction

Genome size (GS) is a biodiversity trait which refers to the amount of DNA in cell nucleus [1]. It is frequently used interchangeably with C-value, which is the haploid genome size in picogram [2]. Knowledge of genome size is important for the purpose of genome studies such as genome structure, organisation and evolution. Additionally, it also has some practical reasons such as genome mapping, physical mapping and genome sequencing [2,3]. Genome projects, especially those focusing on obtaining DNA sequence of the whole genome, will be benefited from the availability of information of genome size.

Many methods are available for determining the cell DNA content. The Animal Genome Size database listed twelve different methods. They are Biochemical Analysis (BCA), Bulk Fluorometric Assay (BFA), Complete Genome Sequencing (CS), Feulgen Densitometry (FD), Static Cell Fluorometry (SCF), Feulgen Image Analysis Densitometry (FIA), Flow Cytometry (FC), Flow karyotyping (FK), Gallocyanin Chrom Alum Densitometry (GCA), and Methyl Green Densitometry, and ultraviolet microscopy (UM) [4]. Three out of these methods, namely FD, FIA, and FC have been the most widely applied methods [5, 6], in which combination of them accounted for 87% of genome size records deposited in the database. In the past, Feulgen staining methods have dominated the method of choice for genome size studies.
Presently, however, the trends have shifted toward the use of flow cytometer. While Feulgen staining methods have resulted in comparable precision to flow cytometer, the latter method has been considered more precise, sensitive and allows for more automation [2]. The Animal Genome Size Database, a primary online resource that is freely accessible, has been a repository for genome size record of 6222 animal species covering 3793 vertebrate and 2429 non-vertebrate [4]. Under group of Fishes, the database listed 2295 species including cartilaginous and ray-finned fishes [7], jawless fishes, and lobe-finned fishes. More recent studies on Genome sizes of commercially important fishes was carried out in China for eight species [8] and in India for several brackish water fishes and penaeid shrimps [9].

Genome sizes in Tilapiine species, including *O. niloticus* and *O. aureus* have been reported in a previous study using Feulgen staining methods [10]. Additionally, to the best of our knowledge, no study has been reported within these species regarding the possible significant variation in genome size as a result of a difference in sexual status. Likewise, no information of genome size is available as a result of interspecific hybridization of these two species. This study was aimed at 1) confirming the genome size of the two Tilapiine species using flow cytometric method, 2) exploring the possibility of the presence of intraspecific, namely among individuals and between-different sex, genome size variation, and 3) exploring DNA content in the interspecific hybrid resulting from the cross between male *O. aureus* and female *O. niloticus*.

2. Methods

Determination of fish genome size using flow cytometry method was carried out by comparing the DNA content of the unknown samples against that of a known standard. DNA content of a cell was allowed to measure by staining the cell nuclei with fluorescent dye, followed by measuring the light emitted by the nuclei when it was stimulated with a specific light source. The absolute genome size, indicated by the most common fluorescent level, was compared against that of a known standard [2]. In this study, red blood cell (RBC) of chicken (*Gallus domesticus*) which have been widely used in genome size determination [11] was used as a standard. The use of chicken RBC as a standard was based on several reasons: it is easy to assess, it has been used in many animal studies, and it is an amenable material. A thorough discussion on the use of CRBC as well as other materials as standards for genome size measurement can be found in Benneth and Leitch [6].

2.1. Fish

Three groups, consisted of two species of tilapia, namely *Oreochromis niloticus*, *O. aureus*, and the hybrid of male *O. aureus* and female *O. niloticus*, were sampled. Each group was represented by 25 fish. The *O. niloticus* used in this study represent the ninth generation of strain Nirwana 3, a selectively bred tilapia for a better growth performance developed by Institute for Development of Aquaculture and Stock Enhancement of Tilapia and Common Carp, Wanayasa (West Java, Indonesia). The *O. aureus* used representing the fourth generation of blue tilapia that has been managed by the Research Institute for Fish Breeding, Subang (West Java, Indonesia). The latter species were being used in breeding program for salinity tolerance improvement. The hybrids were the offspring produced from crossing between the male *O. aureus* and female *O. niloticus*. All the samples represent live collection owned by the Research Institute. The age of fish of the respective group were four months for two parental species and six months for the hybrid. The size ranges, in standard length and total body weight of individuals belonging to the respective groups, were 11.20 ± 0.81 cm and 25.07 ± 4.78 g for *O. niloticus*, 11.21 ± 0.76 cm and 24.71 ± 0.81 g for *O. aureus*, and 16.77 ± 1.80 cm and 103.70 ± 40.14 g for the hybrids.

2.2. Laboratory analyses

One mL of fish blood sample was drawn from each individual using a 1 mL syringe that has been filled with 0.1 mL Acid Citrate Dextrose (ACD). The blood was put in a 1.5 mL micro tube and was centrifuged in a refrigerated spinner (4°C) at 500 xg for 10 minutes that two layers were formed. These parameters of centrifugation condition which included temperature, gravity and duration, were the same.
for the subsequent steps were applied. The supernatant (upper layer) was discarded and 0.5M of 0.9% NaCl was added prior to the second centrifugation. The supernatant after the second centrifugation was discarded and 0.5 mL of 1M PBS was added followed by the third centrifugation. Three cycles of centrifugation using PBS solution were applied to discard the thrombocytes and leucocytes. Erythrocyte density was determined using a haemocytometer and was diluted to a final concentration of 10^9 cell/mL. As nuclear DNA content measurement using flow cytometry method by comparing the size of sample’s genome size relative to that of the reference, the same procedures were applied to the chicken red blood cell.

A DAPI stain solution, a fluorescent stain that binds strongly to adenine-thymine rich region of dsDNA was prepared by mixing 2 µL of 4,6-diamidino-2-phenylindole (DAPI), 9.9 mL nuclease free water, 10 µL Triton X-100, and 0.1 mL D-PBS. Four hundred µL of this solution was then thoroughly mixed with 2 µL of the previously prepared red blood sample of both fish and chicken in 1.5 mL micro tube and was incubated in a dark room at room temperature for 20 min. before loading it into the Attune acoustic focusing cytometer (Applied Biosystem) for measurement. The configuration setting of the instrument, particularly with respect to the threshold and voltage, were 100000 and 1700 mV, respectively. These values were obtained during optimization stages conducted in preliminary experiments. Based on the fluorescence values generated by the instrument, the nuclear DNA content of the cell (C-value) was determined by a formula C-value (in picogram)= 1.25 X/H, where X= fluorescence value of the sample (fish) and H= the fluorescence values of the reference genome (chicken).

2.3. Data analysis

A series of analyses were applied on genome size data, expressed in C-value (pg) as well as in mega base pair (Mb), in order to shed a light over the proposed question. The possibility of significant variation in genome size between a pair of samples among was explored by applying t-test with alpha level at 0.05. The sample pairs to be compared were 1) between sexes within the respective groups, 2) multiple comparisons between groups. For the latter comparisons, a Bonferroni correction for multiple comparisons was applied [12]. Comparative analyses of Tilapiine genome size generated from this study against other fish taxa were carried out descriptively by comparing them against those deposited in the Animal Genome Databases [13].

3. Results and discussion

3.1. Histogram of flow cytometry between the samples and a standard

Profile of peak of DAPI-stained nuclei of the three groups of Tilapiine fishes is shown in Figure 1. In general, the known standard, the RBC of G. domesticus with genome size of 1.25 pg, was lower in its reflectance value than those of the unknown samples. These are indicated by its position which is constantly located in the left of the unknown samples. However the difference was so small that in some cases both peaks appear to be overlapped. Despite subtle differences, the figures clearly show the different peaks between the known standard, namely the CRBC and the unknown samples, which are O. niloticus, O. aureus, and the Hybrid. In order for the peak differences to be seen clearly, further studies involving Tilapiine species may use a different and more suitable standard. The present study used the RBC of G. domesticus as genome size standard for measurement of the unknown samples mainly due to their advantageous features. Its size has been known, the nuclei suspension is homogenous, and is relatively tolerant to long term storage at low temperature [14]. These characteristics have made it a size standard of choice in many genome size studies [11]. In fact, among 86 species reported to be used as size standards in genome size studies, it has been the most frequently used. Out of over 11.000 records of genome sizes, 27% were generated using G. domesticus as size standard [4]. Despite its popularity, there are situations that other size standards may be more suitable. The situation occurred in the present study may exemplify one of them. As mentioned previously, the difference in peak profiles between the standard and the samples were so small that only well trained personnel can recognize them. Therefore, further studies involving the measurement of tilapia genomes, or other species’ genomes which their
expected genome sizes resemble to that of the *G. domesticus*, need to consider of using other species as size standards. This to ensure that peak sizes, can be scored accurately without any doubt.

![Figure 1](image)

**Figure 1.** The Peaks of DAPI-stained nuclei of the known standard (chicken RBC) and the unknown sample of *O. niloticus* (A), *O. aureus* (B) and the hybrids (C). The vertical axis indicates the number DAPI-stained cells that passed through the light detector while the VL1-A in horizontal axis indicates the light intensity of the violet laser reflected by the cells passing through the detector.

3.2. Intraspecific variation of genome size within three groups of Tilapiine species

The profile of genome sizes between different sexes within the respective species/group is presented in Table 1. The table shows at least two important features. Firstly, within-sex variations in genome size as indicated by coefficient of variation values (CV) in all species/groups were low. In male group, within-sex variations in genome size ranged from 0.3% (hybrids Srikandi) to 1.8% (*O. aureus*). In female group, they ranged from 0.5% (*O. niloticus*) to 1.6% (*O. aureus*). The ranges of variation in genome size observed in this study were within the ranges of previously reported for European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*), thinlip mullet (*Liza ramada*), and European eel (*Anguilla anguilla*). Intraspecific genome size variation within those species was reported to be 0-9% with the average of 4%. Secondly, between-sex variation in genome size within the respective species were also low, proven by the lack of genome size differences between male and female. The relatively homogenous genome size between the two sexes found in this study was in agreement to that found in Ictalurid catfish [15] European seabass and thinlip mullet [16]. However, it is in contrast to the finding in other studies in which genome size differences were observed between male and female. Although the idea of intraspecific variation in genome size has gained fewer acceptances, several studies have shown compelling evidences. In studies with *Canabis sativa*, 2.5% genome size differences were observed [17], while in a study with *Drosophila melanogaster* [18, 19], 10% between-sex genome size differences, were reported. Other study with drosophila [18] found that genome size differences occurred not only between species but also among strains.

Several mechanisms have been proposed to explain the presence of intraspecific genome size variation. These included polymorphism in X chromosome [20], aneuploidy in which individual gain or loss one or a few chromosome [5], problem with delineating taxonomic boundary, as was the case with Arctic ciscoes, *Coregonus autumnalis*, where the population was a mixture of fish from at least two genetically divergent populations [21], and variation in chromosomes size [22].

| No | Species/Group | Sex | n | Genome size | CV (%) |
|----|---------------|-----|---|-------------|-------|
| 1  | *O. niloticus* | M   |   |             |       |
| 2  | *O. aureus*   | M   |   |             |       |
| 3  | *O. niloticus*| F   |   |             |       |
| 4  | *O. aureus*   | F   |   |             |       |

Table 1. Genome sizes, expressed in picogram (pg) and mega base pair (Mb), of male (M) and female (F) within the *O. niloticus*, *O. aureus* and the hybrids. The n indicates number of sample and CV indicates coefficient of variation.
3.3. Genome sizes of parental species and their hybrids

The mean (±SD) DNA content of *O. aureus* (1.271 ± 0.0022 pg) was higher but was not statistically significantly different (P>0.017) from that of the *O. niloticus* (1.261 ± 0.0022 pg) (Figure 2). Actually, the initial testing for significant differences between these two parental species resulted in P-value of 0.03, which was statistically significantly different as P<0.05. However, following a correction with a Bonferroni correction due to multiple pairwise comparisons [12], P-value threshold reduced to 0.017 leading to a conclusion that both species were not statistically significantly different as the corrected P-value became larger than the threshold (0.017).

![Figure 2. Genome size of three groups of Tilapiine species, O. niloticus, O. aureus, the hybrid, expressed in picogram (left) and in mega base pair (Mb). The same letters above the bar indicate the absence statistically significant differences (P>0.05)](image)

Apart from the discussion of significant differences in statistical perspective, the genome sizes of both Tilapiine species observed in this study were really close to each other. Roughly, only two percent genome size differences were observed. The relatively close genome size *O. aureus* and *O. niloticus* observed in this study were in contrast to those found in previous study [10]. With respect to *O. aureus*, both studies found a similar figure of C-value, which is 1.2 pg. A big difference was found for genome size of *O. niloticus*. While the present study found C-value of 1.2 pg, the previous study found a much lower value of genome size namely 0.95, resulting in around 20% differences. The even bigger differences were observed when these figures were compared to that produced by genome sequencing method. While genome size of *O. niloticus* in this study estimated to be 1.23 Gb, that obtained by genome sequencing method tuned out to be 1.75 Gb [23]. It needs further investigation whether this phenomenon has association with different methods of genome size measurement or it is associated with other aspects.
Genome size of the hybrid between female *O. niloticus* and male *O. aureus* was 1.263 ± 0.010 pg or 1.235 ± 9.47 Mb (Figure 2). These figures suggested that genome size of the hybrid was laid between those of its parental species, a pattern that is actually expected and was in line with other previous results. This phenomenon suggested that nuclear DNA of parental species segregates as a function of haploid DNA content and is stable within interspecific hybrids. The intermediate position of genome size of the hybrids have been reported for plant and animal taxa [24, 25]. A study with plant genomes of the genus cucurbita found that despite a uniform number of chromosomes, genome size differences among congeneric species were quite big. Additionally, the relative genome size in interspecific hybrids was intermediate and differed significantly from those determined in parental species [25]. A similar pattern was also observed in a study with an animal taxon, namely catfish of the family Ictaluridae. The genome size of the hybrid between different genera of family Ictaluridae was exactly intermediate to the genome size of parental stocks, making it possible to be used as a method to identify intergeneric catfish hybrid in natural populations [24]. In addition to the intermediate position, the genome size of the hybrid may also be bigger than those of parental stocks. This is particularly the case when ploidy level of the hybrids is bigger than that of the parental stocks as exemplified by a study with Asteracea [26].

4. Conclusion

The Flow cytometry method has successfully confirmed the Feulgen densitometry method in determination of genome size of *O. aureus*, but not with *O. niloticus*. Interspecific genome size of *O. aureus*, *O. niloticus* and their interspecific hybrids were not significantly different. Likewise, a similar pattern was observed for intraspecific genome size variation, which represented both between sex and among-individual variations. Genome size of the hybrids was in between those of the parental species confirming the segregation of haploid genome according to Mendelian manner.

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