A Comparison of Two Methods for Determining the Genome Size of Taimen

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Abstract. To compare the two methods for determining the genome size of Taimenes (Hucho taimen). The present study determined genome size (or C-values) of 30 individuals of Taimenes by the method of PI (propidium iodide) and DAPI (4',6-diamidino-2-phenylindole), through the flow cytometry, using chicken erythrocyte DNA content as a standard. The result showed that (1) With the method of PI and DAPI through the flow cytometry, DNA content in blood erythrocyte was 5.65±0.25 pg*2C-1 and 5.04±0.12 pg*2C-1(Fig. 1 and Fig. 2), and genome size (Gb) were 2.70 ± 0.12 Gb and 2.41 ± 0.06 Gb. By Independent-Samples T Test, the genome size (Gb) of blood erythrocyte determined by PI was significantly greater than that determined by DAPI(Table 1). (2)Several of the main support salmonids were clustered by furthest neighbor of any two C-values with the measure of euclidean distance. From the Fig. 3, we found that taimen and fine scale (Brachymystax lenok)firstly clustered into the same subgroup 1, brook trout (Salvelinus fontinalis) and Atlantic salmon (Salmo salar) clustered into the same sub-group 2, rainbow trout (Oncorhynchus mykiss) and pelyad (Coregonus peled) clustered into the same sub-group 3, Subgroup 1 and subgroup 2 firstly clustered into the same team, then come together with subgroup 3. The result indicates that (1) PI method can more accurately reflect the distribution of DNA content in blood erythrocyte of taimen, and more accurate prediction genome size of taimen.(2)Cluster analysis of C-values of several salmons may be related to their respective origin distribution and the filter feeding characteristics of Pelyad.

1. Introduction

Genomic size (also known as C-value) refers to the DNA content of a haploid nucleus in a species. The unit of measurement for genome size is usually pg (10-12g) or the number of nucleotide base pairs expressed in millions, written as Mb or Mbp, and 1 pg is approximately 978 Mb [1]. In eukaryotes, the total amount of haploid genomic DNA in each organism is highly constant, so each organism has its own specific C-value, which can be used as a specific parameter for each species [2]. At present, with the advantages of convenient sample preparation, rapid analysis, relatively accurate data, and large data collection, flow cytometry (FCM) has become increasingly popular in the fields of genome size determination, ploidy screening, and cell cycle analysis, [3]
Previous studies on Hucho taimen have focused on germplasm resources \[3, 4\], breeding \[5-7\], nutrition \[8\], and molecular biology \[9, 10\]. However, there is a lack of related research on genome size and DNA content. It is only found that the DNA content of taimen somatic cells was determined by DAPI method \[11\], 4',6-diamidino-2-phenylindole (DAPI) is a specific fluorescent dye that labels cellular DNA. The specific binding to the A-T base pair on the DNA strand is non-embedded. The emission is blue, excited by 355 nm excitation light, and the emission spectrum is 400 to 500 nm. When DAPI binds to double-stranded DNA, the fluorescence intensity is enhanced by 20-fold, while binding to single-stranded DNA has no fluorescence enhancement, and binding to RNA does not form strong fluorescence, which is significantly weaker than DNA \[12\]. The advantage of DAPI is simple operation, no need to use RNase to process cells, and is suitable for rapid detection of DNA content. Propidium iodide (PI) can be selectively inserted into the double-stranded base pair of nucleic acid, and one fluorescent molecule is inserted every 5 bases, and the binding to nucleic acid uniformly and stably \[13\]. After RNase treatment, PI can specifically bind to intracellular DNA and be excited by a 488 nm laser on a flow cytometer to emit fluorescence at a peak of 610 to 620 nm. The advantage of PI is that the binding with nucleic acid uniformly and stably, and can accurately reflect the distribution of DNA content in the cell population, and is suitable for quantitative DNA detection \[13\]. This study is based on PI and DAPI staining to determine the genome size of taimen blood cells, which provides indispensable basic data for genome sequencing of this species. It is instructive for the pre-sampling budget assessment and is also important for enriching the cytogenetic data of taimen.

2. Materials and Methods

2.1. Instruments and Reagents

Partec: Cyflow® Cube flow cytometer; PI kit (CyStain PI absolute T) Product content: (1) extraction buffer, (2) staining buffer, (3) propidium iodide stock solution, (4) RNase A, (5) extraction buffer reagent. DAPI kit (CyStain UV Ploidy) product content: Staining Solution.

2.2. Test materials

The test fish was from the Yudushan Cold Water Fish Base of the Beijing Fisheries Research Institute. 30 taimens of 2 years old was taken, using electronic balance (OHAUS EP6102C, Ohaus Instruments (Shanghai) Co., Ltd., 6100 g, 0.01 g) to weigh the body weight before blood collection. The average weight is 162.08±12.42g, the water temperature during sampling It is 8 to 10 °C. Ten healthy cocks (Gallus domesticus) of 2 year old were taken with an average body weight of 852.83 ± 118.65 g.

2.3. Blood Cell Preparation

The commonly used material for measuring the size of fish genome by flow cytometry is blood. Because it is a single cell suspension with a large number of cells, simple experimental treatment and reliable measurement results \[14\]. Therefore, the blood sample is taken in this experiment. In the fish tail (or the base of the cock's wings), use a syringe to collect 1 ml of blood, dilute to 2 ml with physiological saline, add 3 ml of lymphocyte separation solution to a 10 ml centrifuge tube, and slowly add blood to the surface of the separation solution to avoid mixing, maintaining a clear layered state. After centrifugation at 2 500 r/min for 30 min at room temperature. It can be seen that the blood in the centrifuge tube is clearly divided into 4 layers, the lowermost is the red blood cell layer, and the uppermost is the plasma layer, and the white blood cell layer is underneath. After absorbing the upper three layers of liquid, the red blood cell layer was retained, and the red blood cells were equally divided into 3 portions and stored in a refrigerator at 4 °C.

2.4. Dyeing

Before PI staining, red blood cells were washed twice with PBS buffer, centrifuged at 3000 r/min for 5 min at 4 °C, and 2 to 4 uL per cell after centrifugation. Add 0.5 mL of cell extract (25 mL extraction buffer + 0.525 g extraction). Buffer reagent), shake in the shaker, place for 3min, then add the prepared staining solution (2mL staining buffer +12uL PI stock solution +6uL Rnase stock solution), shake in the shaker, incubate at room temperature for 30min. After 30um screen filtering, wait for the machine to detect. Before DAPI staining, red blood cells were washed twice with PBS buffer, centrifuged at 3000 r/min for 5 min at 4 °C, and 1 to 2 uL per cell after centrifugation. Add 2 mL of DAPI staining solution, shake in a
shaker, incubate at room temperature for 2 min, a 30um filter screen a relatively pure red blood cell suspension 2.0 mL were screened by 30um filter screen for testing.

2.5. Sample Test and Result Calculation
Red blood cell samples prepared by PI staining or DAPI staining were measured on a Partec flow cytometer (CyFlow Cube). Using the red blood cells of the cock as an external standard, the instrument was first calibrated and then used as an internal standard to determine the DNA content of the eschar. PI stained samples were first excited with 488 nm blue light, then the emission fluorescence of PI was detected with an emission wavelength of 610 nm; DAPI-stained samples were used to detect the emission fluorescence of DAPI with ultraviolet wavelength. By adjusting the voltage to display the main cell population of blood cells in the EV histogram, set the gate (EV) to circle the main group cells, and display the cell population in the EV gate in the FL3 or FL4 histogram. The test results were saved after each testing.

The amount of embedding or binding of the fluorescent dye PI or DAPI during the coloring process is proportional to the amount of DNA, so the available fluorescence intensity can indicate the relative content of DNA. The proportional relationship between the DNA content of the sample to be tested and the DNA content of the standard sample can be determined by measuring the fluorescence intensity of the PI or DAPI emission of the sample to be tested and the standard sample. In this test, chicken blood cells are used as standard cells, and the DNA content thereof is known, so that the DNA content of the sample to be tested can be calculated. Chicken blood cells are an internationally accepted control standard [15], and their absolute content is calculated as 2.30 pg•2C⁻¹. In this experiment, chicken red blood cells were used as external and internal standards, and the genome size of taimen was calculated by comparing the relationship between the peak of chicken red blood cells and that of taimen red blood cells.

2.6. Statistical Processing
Statistical analysis was performed using SPSS16.0 software. The data were expressed as (x±s). The results were compared using independent sample t test. P<0.05 was considered statistically significant. Combining several species of DNA with the determined DNA content, cluster analysis of the longest distance method was carried out by SPSS16.0 with the Euclidean distance of C-value between any two varieties.

3. Results

3.1. Determination of Genome Size
Using the DNA content of cock red blood cells (2.30 pg•2C⁻¹) as the standard, the DNA content of the taimen red blood cells determined by PI method and DAPI method was respectively 5.65±0.25 pg•2C⁻¹ and 5.04±0.12 pg•2C⁻¹, the results of the flow cytometry measurement are shown in Figures 1 and Figures 2. According to the conversion formula of 1 pg= 978 Mb [11], the genome size of taimen was 2.70±0.12 Gb by PI method, and the genome size of taimen was 2.41±0.06 Gb by DAPI method.
Figure 1. The histogram of Gallus domesticus (left) and Hucho taimen (right) sample by PI Note: Abscissa, DNA content; Ordinate, the number of cells

Figure 2. The histogram of Gallus domesticus (left) and Hucho taimen (right) sample by DAPI Note: Abscissa, DNA content; Ordinate, the number of cells

3.2. Comparison of Two Methods for Determining Genome Size

Table 1. Independent samples test of difference measurements on genome size (Gb)

|                      | Levene's test for equality of variances | T-test for equality of means |
|----------------------|----------------------------------------|-----------------------------|
|                      | F       | Sig.   | t       | df  | Sig. |
| Difference measurements on genome size (Gb) | Equal variances assumed | 18.815   | .000   | -13.194 | 58     | .000  |
|                      | Equal variances not assumed             | -13.194 | 46.70   | 1   | .000  |

The two independent samples corresponding to the two methods are normally distributed, so the independent sample t test can be used for analysis. It can be seen from Table 1 that the Levene's variance homogeneity test results show that the two population variances are not equal (F=18.815, P=0.000), and the t test results (t=-13.194, P=0.000) when the variance is homogeneous indicate that α is Level of =0.05, does not accept H0. It is considered that the difference between the two samples is
extremely significant, that is, the PI method and the DAPI method are extremely different between the genome size (Gb) of the blood cells of taimen.

3.3. Comparison with Other C-values (Gb) of Salmonids
At present, the size of several major salmons family fishes in China is shown in Table 2. Since different cell types and different assay methods may affect the assay results to varying degrees, the same reference standard chicken red blood cells make the C-values of these species of salmon have certain comparability. The clustering of the longest distance method was carried out based on the Euclidean distance of the C-values between any two species (see Figure 3). It was found that the taimen and thin scale's salmon (Brachymystax lenok) were first clustered into the same subgroup 1, brook trout (Salvelinus fontinalis) and Atlantic salmon (Salmo salar) were clustered into the same subgroup 2, and rainbow trout (Oncorhynchus mykiss) and pelyad (Coregonus peled) were clustered into the same subgroup 3. Subgroup 1 and subgroup 2 were firstly clustered into the same group, and then to be together with the subgroup 3.

Table 2. Comparison of C-value (Gb) of some different salmons

| Genus    | Species                | Cell Type            | Method       | DNA content of diploid cells (pg/2C) | C-value (Gb) |
|----------|------------------------|----------------------|--------------|-------------------------------------|--------------|
| Oncorhynchus | Oncorhynchus mykiss    | Blood erythrocyte    | FCM(PI)      | 3.47                                | 1.66         |
| Coregonus | Coregonus peled        | Blood erythrocyte    | FCM(DAPI)    | 4.39                                | 2.10         |
| Hucho     | Hucho taimen           | Blood erythrocyte    | FCM(DAPI)    | 5.04                                | 2.41         |
| Hucho     | Hucho taimen           | Blood erythrocyte    | FCM(PI)      | 5.65                                | 2.70         |
| Brachymystax | Brachymystax lenok   | Caudal fin somatic cells | FCM(DAPI)    | 5.24                                | 2.51         |
| Salvelinus | Salvelinus fontinalis  | Blood erythrocyte    | FCM(DAPI)    | 5.47                                | 2.61         |
| Salmo     | Salmo salar            | Blood erythrocyte    | FCM(DAPI)    | 5.70                                | 2.73         |

Figure 3. Cluster analysis of C-values of several salmons

4. Discussion

4.1. Principle Analysis of Two Methods
The selection of different fluorescent probes is essential for the detection of nuclear DNA content in animal cells. The fluorescent dye commonly used in the detection of DNA content by flow cytometry is 4',6-diamidino-2-phenylindole (4'), 6-diamidino-2-phenylindole, DAPI, propidium iodide (PI), mithramycin (MI), and ethidium bromide (EB). In this study, the PI method was used to determine the genome size of taimen was 2.70±0.12 Gb, which was significantly greater than the genome size determined by DAPI method by 2.41±0.06 Gb (P<0.01), which was related to the specificity of dye-base binding. Both DAPI and MI are specific marker DNA fluorescent dyes and are non-embedded stains. DAPI mainly binds to the AT base of DNA, ignoring the DNA and many GC bases, which will cause the detection genome to be small [16]; MI mainly binds to the GC base of DNA, which will cause the detection genome to be too large [17]. PI is a non-specific labeling dye that can be
selectively inserted into the double-stranded base pair of nucleic acid, inserting one fluorescent molecule every 5 bases, and binding to DNA without base specificity [18], binding with nucleic acid uniformly and stably.

4.2. Comparison of Measured Values
In this study, the genome size of Taimen determined by PI method was 2.70±0.12 Gb, which is close to the genome size of 2.60 Gb determined by high-throughput sequencing in our laboratory. Therefore, PI method can more accurately reflect DNA content in Taimen red blood cells, more accurately predict the genome size of Taimen, which was consistent with the results of Xiaoxue Xu et al [13]. The red blood cell DNA content measured by the DAPI method in this study was 5.04 pg•2C⁻¹, which was slightly smaller than the 5.12 pg•2C⁻¹ determined by Shuqun Xue et al. [11], which may be related to the environmental factor latitude [19]. The sampling location of this experiment is Beijing, and its latitude is lower than the latitude of Heilongjiang (sampling location of Shuqun Xue). Whether this result is consistent with the environmental complex holiday [20], and it needs further verification.

4.3. C-value Based Clustering Analysis
The total amount of DNA contained in the haploid genome of an organism is called the C-value. C-values are useful analytical tools and evidence for population evolution, species classification, and ecological studies [21]. The C-values of closely related species are very similar. The feature information of genome size obtained by C-value can be used to construct the phylogenetic tree of species and analyze the genetic relationship between species [22]. In this study, Taimen and thin scale's salmon were first clustered into the same subgroup 1, and brook trout and Atlantic salmon were clustered into the same subgroup 2, and the rainbow trout and the pelyad were clustered into the same subgroup 3, which is consistent with their origin distribution. Taimen and thin scale's salmon were native species in Chinese, and they were from the Heilongjiang River Basin [6, 23, 24]. The brook trout is native [25-27] in the Labrador area of eastern Canada; the Atlantic salmon is native to the northern part of the Atlantic [28], and the Labrador area connected to the northern Atlantic Ocean. Pelyad is native to the lakes of the Mejin River to the Korema River in Russia north of 50° north latitude [11, 29], especially in the Ob River Basin in Siberia, Russia; the rainbow trout is native to the Pacific coast of North America [30], and the Siberian region and the Pacific coast of North America are also connected by land and sea. Subgroup 1 and subgroup 2 clustered firstly into the same group, and then clustered with subgroup 3, which may be related to the characteristics of Pelyad filter plankton [31].

5. References
[1] Vinogradov A E 1998 Genome size and GC-percent in vertebrates as determined by flow cytometry: the triangular elationship. Cytometry 31, 100-109.
[2] Jin L, Xiaojun Z, Lin S, et al 2012 Genome size determination of sea cucumber (Apostichopus japonicas). Journal of Fisheries of China 36, 686-695.
[3] Guangxiang T, Youyi K, Jiasheng Y 2009 AFLP analysis of genetic diversity of taimen (Hucho taimen) in wild populations. Journal of Fishery Sciences of China 16, 833-841.
[4] Youyi K, Guangxiang T, Wei X, et al 2010 Analysis on population genetic structure of taimen (Hucho taimen) in the Heilongjiang River. Journal of Fisheries Sciences of China 17, 1208-1217.
[5] Wei X, Huiwu S, Haihong G, et al 2007 Growth development and reproduction of reared Hucho taimen. Journal of Fisheries Sciences of China 14, 896-902.
[6] Wenxue G, Guangxiang T, Yongquan Z, et al 2015 Study on large-scale breeding on hybrids of Hucho taimen (♀) × Brachymystax lenok (♂) and its growth characters. Freshwater Fisheries 45, 89-93.
[7] Guiqiang Y, Zhanquan Y, Yuan D, et al 2015 A Comparative Study of the Anesthetic Effects of Three Anesthetics on Taimen (Hucho taimen, Pallas). Acta Agriculturae Universitatis Jiangxiensis (Natural Sciences Edition) 37, 898-902.
[8] Meiyan Z, Changan W, Qiyou X, et al 2015 Effects of Myo-Inositol on Antioxidant Capacity and Histopathological Observation of Hucho taimen. Chinese Journal of Animal Nutrition 27,
631-637.

[9] Guangxiang T, Youyi K, Lingxue X, et al 2011 Mathematical Analysis of Effects of Morphometric Attributes on Body Weight in Taimen Hucho taimen. Chinese Journal of Fisheries 24, 31-36.

[10] Xiumei W, Liming X, Jingshi Z, et al 2015 Prokaryotic expression and bioactivity analysis of Hucho taimen insulin-like growth factor-II Journal of Fisheries Sciences of China 22, 243-249.

[11] Shuqun X, Zhongwu S, Hongbin Y 2011 Cytogenetic analysis of coregonus Peled. Acta Hydrobiologica Sinica 35, 869-873.

[12] Xijuan L, Huirong D, Hong Z 2010 A methodology study on flow cytometric analysis of cell DNA stained with DAPI and Hoechst 33342. Journal of Peking University (Health sciences) 42, 480-484.

[13] Xiaoxue X, Naili H, Lina S, et al 2014 Comparison of two propidium iodide stained DNA quantitative methods for detection of apoptosis in HL-60 cells. Journal of Capital Medical University 35, 823-826.

[14] Kun Y, Dongmei Z, Xinbei Z, et al 2012 Analysis of DNA Contents and Cell Cycle of Different Tissues of Pseudorasbora parva. Chinese Agricultural Science Bulletin 28, 113-118.

[15] Birstein V J, Poletaev A I 1993 Goncharov B F. DNA content in Eurasian sturgeon species determined by flow cytometry. Cytomery 14, 377-383.

[16] Yan W, Yuan X, Wei L, et al 2015 Operation Skills of Flow Cytometer for Detecting Nuclear DNA Contents in Higher Plant Cells. Plant Science Journal 33, 126-131.

[17] Johnson OW, Utter FM, Rabinovitch PS 1987 Interspecies differences in salmonid cellular DNA identified by flow cytometry. Copeia 4, 1001-1009.

[18] Gray J W, Langlois R G 1986 Chromosome classification and purification using flow cytometry and sorting. Annu Rev Biophys Biophys Chem 15, 195-235.

[19] Bennett M D 1976 DNA amount, latitude, and crop plant distribution. Environmental and Experimental Botany 16, 93-108.

[20] Mergen F, Thiélges B A 1967 Intraspecific variation in nuclear volume in four conifers. Evolution 21, 720-724.

[21] Loureiro J, Trávníček P, Rauchová J, et al 2010 The use of flow cytometry in the biosystematics, ecology and population biology of homoploid plants. Preslia 82, 3-21.

[22] Rhein M, Walter M, Mertens C, et al 2004 Evolution of genome size in pines (Pinus) and its life-history correlates. Supertree Analyses Evolution 58, 1705-1729.

[23] Zhenyu X, Shangwu Y, Rongyuan Y 1959 Hucho taimen and Brachymystax lenok and their hybrids in the Heilongjiang River Basin. Acta Hydrobiologica Sinica 58, 215-220.

[24] Gefeng X, Jiasheng Y, Yang L, et al 2010 A preliminary study on technique of artificial reproduction between Hucho taimen(♀) and Brachymystax lenok(♂). Journal of Shanghai Ocean University 25, 178-184.

[25] Nadir B 2004 The early development of brook trout Salvelinus fontinalis: survival and growth rates of alevins. Turkish Journal of Veterinary and Animal Sciences 28, 297-301.

[26] Perry G M L, Tarte P, Croisetiere S, et al 2004 Genetic variance and covariance for 0° brook charr (Salvelinus fontinalis) weight and survival time of furonculosis (Aeromonas salmonicida) exposure. Aquaculture 235, 263-271.

[27] Ohlund G, Nordwall F, Degerman E, et al 2008 Life history and large-scale habitat use of brown trout (Salmo trutta) and brook trout (Salvelinus fontinalis) — implications for species replacement patterns. Canadian Journal of Fisheries and Aquatic Sciences 65, 633-635.

[28] Denggao Q, Shihong X, Ying L, et al 2015 Effects of different types of environment light on the growth perfor-mance and feeding of Atlantic salmon (Salmo salar) in recirculating aquaculture systems. Journal of Fishery Sciences of China 22, 68-78.

[29] Sumei W, Tianxi S, Lina M, et al 2015 Artificial breeding of Peled and large-scale water release and proliferation technology. Modern Agriculture 474, 82-83.

[30] Neal A G J, Edward S R, Doran M M 2007 Diet, feeding rate, growth, mortality, and production of juvenile Steelhead in a Lake Michigan Tributary. North American Journal of Fisheries
Management 27, 578-592.

[31] Huaiming L, Zhenbo M, Yongfa L, et al. 1991 Artificial breeding of Peled and large-scale water release and proliferation technology. Chinese Journal of Fisheries 4, 20-25.