Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

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Tamaño genómico de especies acuáticas de importancia económica y biológica en el Estado de Baja California

Tesis

para cubrir parcialmente los requisitos necesarios para obtener el grado de Maestro en Ciencias

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Resumen de la tesis que presenta Constanza del Mar Ochoa Saloma como requisito parcial para la obtención del grado de Maestro en Ciencias en Acuicultura

Tamaño genómico de animales acuáticos de importancia económica y biológica en el Estado de Baja California

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El tamaño genómico o valor C, es toda la cantidad de DNA en un set genómico haploide, el cual es expresado en picogramos (pg). El tamaño genómico es una de las características celulares más analizadas que permite entender varias funciones y características como el estatus genético de las poblaciones silvestres, la identificación de poliploidía y alteraciones a nivel cromosómico, entre otros. En los últimos años, ha crecido un gran interés en el conocimiento del estatus genético de especies de importancia económica y biológica en Baja California. Sin embargo, la información sobre el tamaño genómico de especies acuáticas de Baja California es nula. Por lo tanto, la determinación del tamaño genómico ayudará a complementar los estudios genéticos que proporcionan una mejor comprensión para mejorar el uso sustentable de los recursos pesqueros y mejorar la acuicultura, teniendo un impacto positivo en la producción pesquera y acuícola de la región. De esta manera, los objetivos de este trabajo fueron: (1) Identificar los estándares de referencia apropiados para el estudio de estos organismos, (2) evaluar el tamaño genómico en espermatozoides, sangre y hemolinfa de los animales de estudio y por ultimo (3) establecer la metodología más apropiada para el análisis de los organismos estudiados. Muestras de sangre, hemolinfa y esperma de 23 especies fueron colectadas y el DNA teñido con yoduro de propidio. Las muestras fueron analizadas en un citómetro de flujo Attune® Flow Cytometer con una configuración láser Azul/Violeta (488 y 405 nm respectivamente). Aproximadamente, 10,000 núcleos por muestra fueron analizados por triplicado. El tamaño genómico se calculó con una desviación estándar de 0.001. El rango del tamaño genómico (diploide) de las especies analizadas fue de 1.21 pg a 8.83 pg. El más pequeño (1.21 pg) se encontró en bacalao negro (Anoplopoma fimbria) y el más grande (8.83 pg) para el tiburón cobrizo (Carcharhinus brachyurus). No se encontraron diferencias significativas entre el tamaño genómico de los individuos de la misma especie ($P > 0.5$). El tamaño genómico de 16 de las especies analizadas fueron comparadas con el tamaño genómico reportado en la Base de Datos Internacional de Tamaño Genómico Animal (International animal genome size database). La diferencia encontrada en aquellas especies cuyo tamaño genómico si se encontraba reportado en la base de datos fue debido al tamaño de la muestra y la precisión del análisis utilizado. La información obtenida en este trabajo permitirá í establecer la primera base de datos regional de tamaño genómico y la entrada a la base de datos de tamaño genómico internacional.

Palabras clave: Tamaño genómico, citometría de flujo, animales acuáticos.

Abstract of the thesis presented by **Constanza del Mar Ochoa Saloma** as a partial requirement to obtain the Master of Science degree in Aquaculture

Genome size of aquatic animal of economic and biological importance in Baja California

Abstract approved by:

Dra. Carmen Guadalupe Paniagua Chávez Codirectora de tesis	Dr. Jill Jenkins Codirectora de tesis

The genome size of a cell is the total amount of DNA in the nucleus. This amount is constant for organisms of the same species and it differs among species. This characteristic makes the genome size a crucial parameter for genetic research on endemic aquatic species and for genetic manipulations in aquaculture species. Genome size assessments for aquaculture species is the most precise tool for identifying hybrids, polyploidy, and identifying sex when sex chromosomes exist. No previous research on genome size has been performed in Baja California. The objectives of this research were to (1) determine the genome size of aquatic animals with biological and economical importance in Baja California, (2), to identify the appropriate reference standards, and (3) to establish standard operating procedures for these species. Blood, hemolymph or sperm samples from 23 species were collected and nuclear DNA was stained with propidium iodide solution. Genome size was determined by flow cytometry using ~10,000 nuclei per sample in triplicate. The genome size was calculated with a 0.001 standard deviation. Genome size from all species analyzed ranged from 1.21 pg to 8.83 pg, with the smallest genome size found in sablefish (*Anoploma fimbria*) and the largest (8.83 pg) found in copper shark (*Carcharhinus brachyurus*). No significant differences among individuals of the same species were found ($P < 0.05$). Genome size from 16 target organisms were compared with the genome size reported in the International animal genome size database. Differences found between studied species and database was due to a highest sample size and acute analysis used in this study. This is the first study of genome size determined for aquatic animals in Baja California. The data generated establishes the foundation for the country of Mexico by virtue of these species of regional importance, and the data will be submitted to the international animal genome size database.

Keywords: Genome size, Flow cytometry, aquatic animals, Baja California.

Dedicatoria

A mi madre, por darme la vida y el coraje para salir siempre adelante, por ser mi guía.

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...No cabe duda, ésta es mi casa,
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Chapter 1. Introduction

The genome size or C-value is defined as the total haploid content of nuclear DNA in the cell and is measured in picograms (pg). Genome size is also known as DNA content; consequently, in this work we use the term “genome size” as DNA content, as well. Genome size has become a critical characteristic of live organisms in the biological sciences over the last decade because it can identify chromatin modification in cells. Due to the consistency of genome size among the cells of organisms from the same species, modifications may indicate abnormalities in cell cycle or changes in chromosomal content. Studies of the genome characteristics or cellular status such as ploidy, apoptosis, cell cycle, sex classification, hybridization, or monosex organisms could been determined by flow cytometry. Other issues such as the effect of genotoxic compounds or diseases could be identified by measuring fragmentation of DNA with possible aneuploidy (Dallas and Evans, 1990; Birstein, Poletaev, and Goncharov, 1993; Zbigniew Darzynkiewicz, Halicka, and Zhao, 2010). Therefore, the measurement of the genome is an important tool in health science, evolution and ecology studies.

Despite genome size being useful in biomedical research, relatively little information is available on the genome size of aquatic species, and no data are available on aquatic animals of Baja California. Genome size for aquatic animals is useful in evolution, biosystematics and ecology studies (Kron, Suda, and Husband, 2007). Determination of the genome size for aquatic animals will complement and enhance research to improve aquaculture of important species in Baja California. The information obtained from this project begins the process of addressing questions related to ploidy, hybridization, monosex identification, among others.

The marine resources were very important since the prehispanic period in Baja California. In fact, one of the essential resources for the Indian Californians was the abalone (Saloma-Hernández, 1997). They used dried abalone as jerky meat as it helped them to walk long distances without access to any fresh food (Saloma-Hernández, 1997).

Until now, the majority of seafood production of Baja California derives from the fisheries, on the Pacific Coast of Mexico (Baja California, Baja California Sur, Sonora, Sinaloa). This represents 83.14% of the total national fisheries and aquaculture production (SAGARPA, 2013). The species with the highest production is the Pacific Sardine (*Sardinops sagax*) with a production of 733, 914 tons from the period of 2007-2013. In Baja California, the first records of the fishery industry appeared in 1920, when the first sardine packing was started in Ensenada. This fishery has taken on a very important role since then (Ochoa-Sánchez, 2003).

Moreover, Mexico is a country with a great biodiversity, and richness of wildlife, and the genome characteristics of the animals is not known. In Baja California, the fishery resources have always been important, influencing life style, culture and the economy. Most aquatic wildlife is endemic, some is endangered, others are migratory, and some others are still undiscovered. Trying to determine the genome size of all the aquatic animals with economical and biological importance in Baja California will be a titanic work that is why this project is just the beginning of a new field of investigation.

In my studies, I determined the genome size of 23 species. This is the first time that this kind of science has been performed in Mexico. Therefore, this thesis is the basis for others to continue with this work. The determination of the genome size is a first step of many new investigations.

Chapter 2. Background

The word “genome” was coined by Hans Winkler in 1920, as a combination of the words “gene” and “chromosome”. Genome refers to the complete set of chromosomes and genes in a cell (Gregory, 2002b). Genome size is defined as the total amount of DNA contained within a haploid chromosome set and can be measured in terms of mass (picograms of DNA per cell). Therefore, even without fully elucidating the molecular structure of DNA, studies on genome size, its evolution and the relationship with the characteristics of the organisms have been of great interest (Gregory, 2001b, 2002b).

The first measurement of DNA content was made by Boivin *et al*. (1948), before the discovery of the molecular structure of DNA by Watson and Crick. In the same year, constant DNA content per cell in each organism was confirmed by Vendrely and Vendrely (1948). Since then it has been well know that the genome size is unchanging per species and that spermatozoa contain half of the DNA content as somatic cells (Mirsky and Ris, 1951; Graur and Li, 1991).

After confirmation of the constant size of DNA content, it was hypothesized that a relationship between the genome size and the evolutionary position of organisms could exist (Hinegardner, 1968). However, no apparent relation was found between the evolutionary position of the organisms and the genome size. For example, the salamander *Amphiuma* sp has 26 times more DNA than humans and the *Euglena* has almost the same DNA content as humans (Mirsky and Ris, 1951). This finding was called the C-value paradox in the 70’s (Gregory, 2001b), where “C” refers to the constant value within any species (Graur and Li, 1991; Liu, 2008). The C-value is typically related such things as cell size, nuclear size, metabolic rate, longevity of the organisms, size of the eggs, environmental habit, as well as reproductive behavior,(Gregory, 2001a; Krishan *et al*., 2005; Smith and Gregory, 2009). Research on genome size is considered as a crucial aspect for integral programs for comparative genetic analysis (Gregory, 2005).

2.1. Genome size

In 1918, Hertwig described the first theory of the genome size, which indicates that the chromosomal content of all the nuclei in an organism is identical (Swift, 1950; Gregory, 2005). However, the analysis of the genome size in different tissues was not important until the late 1940's. Boivin and contributors compared the genome size of different tissues of pig and guinea pig, showing that the genome size of the diploid cells of different tissues is the same and twice that of spermatozoa of the same organisms (Boivin *et al*., 1948; Vendrely and Vendrely, 1948; Gregory, 2005). The first study on the determination of the genome size was performed in chicken (*Gallus gallus*) (Mirsky and Ris, 1951). In this study, it was also found that the spermatozoa contain half of the genome size of the somatic cells. After this study, new research was performed to determine if the genome size may change depending of the type of cells in the same organism. Results showed that the genome size among cells of the same organisms are not significantly different and that the spermatozoa contain half of the genome size (Mirsky and Ris, 1951).

Until 1950, the genome size determinations were performed by biochemistry reactions or Feulgen densitometry. In the 1980's, the flow cytometry allowed faster and more accurate analyses. In 1989, a catalog of the genome size of 25 animals was elaborated, including fish, amphibians, birds and mammals; these values could be used as future reference standards (Tiersch, Chandler, Wachtel, and Elias, 1989). In the 90's, the most extensive flow cytometry study obtained the genome size of 154 vertebrates (Vinogradov, 1998). Recently, a catalog of vertebrate genome size used trout and human blood as reference standards (Krishan *et al*., 2005). Five species were suggested as reference standards for analysis of genome size by flow cytometry: betta (*Betta splendens),* nematode *Caenorhabditis elegans,* domestic chicken *(Gallus gallus),* human *(Homo sapiens),* rainbow trout *(Oncorhynchus mykiss)* and leopard frog *(Rana (Lithobates[1]) pipiens*) (Suda and Leitch, 2010).

[1] Currently there are conflicts on the nomenclature of the monoplyletic group *Rana* (Hillis and Wilcox, 2005 Dubois, 2007; Hillis, 2007; Che *et al*., 2007; Boundy *et al*., 2012). Although some phylogenetic studies have been performed for this species (Che *et al.*, 2007), the American water frogs has been referred as *Rana* or as *Lithobates* (Boundy *et al*., 2012). Then, both genera *Rana (Lithobates*) will be used to refer this taxa.

Currently, there are several specialized databases on genome size for plants and animals. The database for animal genome size compiled by Gregory since 2001 is the most complete catalog of animal to date. This database includes approximately 4,000 species (2,500 vertebrates and 1,300 invertebrates) (www.genomesize.com) (Gregory, 2015). Nevertheless, this database is just a small part of the whole metazoan diversity. The purpose of this database is the standardization of protocols among others, to increase the reliability of future measurements (Gregory, 2005).

2.2. Genome size of aquatic species

In teleosts, studies on genome size were performed since the mid 1900's. In the 1950's, the relationship between ploidy level, genome size, and cell number was described for induced triploids of stickleback *Gasterosteus aculeatus* (Swarup, 1959). Next, the haploid genome size of two thousand teleosts was described, showing a range of 0.40 to 4.4 pg of DNA per cell (Hinegardner, 1968). In 1993, the genome size of 10 species of sturgeon (*Acipenser baeri, A.gueldenstaedti, A.medirostris, A.nudiventris, A.stellatus, Huso dauricus, H.huso, Huso huso X A. ruthenus, Pseudoscaphirhynchus kaufmanni, Polyodon spathula*) was determined by flow cytometry, using another sturgeon species (*Acipenser ruthenus*) as reference standard (Birstein *et al.*, 1993). The next important study reported the genome size of ~154 vertebrates, including 39 European and South America fishes (Vinogradov, 1998; Filipiak, Tylko, and Kilarski, 2012). Recently, the genome size of 20 species of Siluriformes of neotropical waters were described (Fenerich, Foresti, and Oliveira, 2004), In 2009, the relationship of the genome size of teleosts with some ecological and biological feature and their phylogenetic patterns within Order and Family (e.g. egg size, longevity, geographic distribution, etc.) were reported (Smith and Gregory, 2009).

Despite the great aquatic wildlife diversity of Baja California, the genome size has been only determined in abalone (Gallardo-Escárate, Alvares-Borrego, Von Brand-Skopnik, and del Río-Portilla, 2005; Gallardo-Escárate and del Río-Portilla, 2007). Moreover, no other work has been done regarding the genome size in any other aquatic species. The comparison of abalone with other species is a logical first approach.

Currently, the international genome size database (www.genomesize.com) lists haploid genome sizes of several aquatic animals (vertebrates and invertebrates) obtained by various technical methods included flow cytometry. The genome size (C-value) of aquatic animals ranges from a low of 0.4 pg in the Tetraodontidae family to a high of 133 pg in the marbled lungfish (*Protopterus aethiopicus*) (Gregory, 2005).

2.3. Genome size data for aquaculture species determined by flow cytometry

The determination of the genome size of several species of aquaculture importance by flow cytometry has been performed in several species, like sturgeon, to evaluate polyploidy (Birstein *et al.*, 1993), in pacific oysters (*Crassostrea gigas*) to evaluate and confirm polyploid larvae (Chaiton and Allen Jr., 1985), in rainbow trout (*Oncorhynchus mykiss*) to identified triploid organisms (Thorgaard *et al*., 1982) and in grass carp (*Mylopharyngodon piceus*) to identify diploid and triploids organisms (Jenkins and Thomas, 2007). In addition, flow cytometry has been use as a tool to quantify the defense mechanisms in aquaculture important animals such as oyster and fishes.

From all the genome size information recorded for aquatic animals currently, only 30 references are related to aquatic animals with economic and biological importance (Table1).

Table1. Comprehensive list of studies on DNA content in aquaculture species by using flow cytometry

Species	References
Phylum Mollusca	
Bivalve mollusk	Chaiton and Allen Jr., 1985
Abalone	Ieyama, Kameoka, Tan, and Yamasaki, 1994
	Rodríguez-Juíz, Torrado, and Méndez, 1996
	González-Tizón *et al*., 2000
	Goedken and De Guise, 2004
	Gallardo-Escárate *et al*., 2005
	Gallardo-Escárate and del Río-Portilla, 2007
	Anisimova, 2007
	Matt and Allen, 2014
Phylum Arthropoda	
Decapoda	Rheinsmith, Hinegardner, and Bachmann, 1974
	Libertini, Panozzo, and Scovacricchi, 1990
	Chow, Dougherty, and Sandifer, 1990
	Lécher, DeFaye, and Noel, 1995
	Deiana *et al*., 1999
Phylum Chordata	
Acipenseriformes	Knobloch, Vendrely, and Vendrely, 1957
Cypriniformes	Bachmann and Cowden, 1967
Perciformes	Beamish, Merrilees, and Crossman, 1971
Salmoniformes	Ebeling, Atkin, and Setzer, 1971
	Fontana, 1976
	Thorgaard *et al*., 1982
	Li, Li, and Zhou, 1983
	Allen Jr., 1983
	Majumdar and McAndrew, 1986
	Johnson, Utter, and Rabinovitch, 1987
	Arai, Suzuki, and Akai, 1988
	Gold, Ragland, and Schliesing, 1990
	Tiersch *et al*., 1990
	Cui, Ren, and Yu, 1991
	Grøsvik and Raae, 1992
	Lockwood and Derr, 1992
	Tiersch and Goudie, 1993
	Brenner *et al*., 1993
	Blacklidge and Bidwell, 1993
	Birstein *et al*., 1993
	Alfei *et al*., 1996
	Chilmonczyk and Monge, 1999
	Ciudad *et al*., 2002
	Fenerich *et al*., 2004
	Peruzzi, Chatain, and Menu, 2005

Chapter 3. Biotechnologies used to determine genome size

Over the years, many techniques have been used to calculate the genome size in animals, such as biochemistry, densitometry and fluorometry (Carvalho, Oliveira, Navarrete, Froehlich, and Foresti, 2002; Hardie, Gregory, and Hebert, 2002). The first relative quantification of nucleic acids was performed with densitometry by Caspersson in 1930 (Hardie *et al.*, 2002). Feulgen densitometry involves the fixation and staining of tissue samples for microscopic analyses. Otherwise, fluorometry is another technique to quantify the fluorescence of stained DNA (Hardie *et al*., 2002).

For decades, one of the major problems with the techniques to measure genome size, excepting flow cytometry, is that the results could not be compared among them (Vinogradov, 1998) (e.g. genome size results from a same species may be different depending on the technique and tissue used). Therefore, it is necessary to standardize methodologies for data comparison (Dolezel and Greilhuber, 2010). At present, the majority of analyses of fluorescence of DNA use the most accurate and dynamic process of the flow cytometry, with suspended nuclei (Hardie *et al*., 2002).

3.1. Feulgen Densitometry

In 1924, Feulgen and Rossenbeck developed a new research area called “Nucleareaktion” (Benedum and Meusch, 1999). They elaborated a chemistry method to produce an aldehyde-free DNA structure to detect DNA by colorimetry (Chieco and Derenzini, 1999; Hiraoka, 1973). This procedure is based on acid hydrolysis, which separates the sugar deoxyribose purine base, exposing free aldehyde groups and leaving the DNA backbone intact. After hydrolysis, the cells are exposed to Schiff reagent where the DNA apurinic sites turn to magenta color (Chieco and Derenzini, 1999). This procedure is known as the Feulgen reaction and is considered as the first stoichiometric DNA staining procedure (Chieco and Derenzini, 1999; Shapiro, 2003).

Feulgen densitometry or the Feulgen Reaction helped to establish the relationship between DNA content in nucleus and chromosome number (Vendrely and Vendrely, 1948). The first measurement of the DNA content of a chromosome was performed with

this technique (Chieco and Derenzini, 1999). Also several comparisons of genome size in different animals and plants has been performed with Feulgen densitometry (Atkin, Mattinson, Becak, and Ohno, 1965; Tiersch *et al*., 1989).

Feulgen densitometry has become one of the most used cytohistochemistry reactions in the health and biological sciences (Chieco and Derenzini, 1999). Despite modifications (Shapiro, 2003), Feulgen densitometry is still one of the most-used chemistry reactions for quantification of intracellular DNA, because this technique allows the analysis of fresh and fixed cells, as well as paraffin-embedded tissues (Chieco and Derenzini, 1999).

However, its reproducibility was questionable due to inconsistency of the data. Bedi and Goldstein (1976) concluded that the inconsistency came from the imprecision of the methodology (e.g. only a single by measuring the fluorescence of a single nucleus). Thus, several samples are needed to measure many density points to be compared to the standards (Gregory, 2005). Consequently, the Feulgen densitometry is considered a time-consuming and obsolete technique.

3.2. Feulgen image analysis densitometry

This technique was born from the fusion of flow cytometry and Feulgen densitometry, thanks to the advances in computers and photography. The principle consists of capturing digital images of stained nuclei. Each pixel on the image is assigned an individual density point, allowing a rapid and simultaneous analysis of large quantity of nuclei (Hardie *et al*., 2002; Gregory, 2005). This technique was applied in cancer research, being considered as accurate as flow cytometry and faster than the traditional densitometry methods (Hardie *et al.*, 2002). The disadvantage of this technique is the variations of the stain absorption among the different cell types (Gregory, 2005; Hardie *et al*., 2002).

In Feulgen image densitometry analysis, each pixel captured in the digital image is transformed to an absorbance value. Therefore, the measurement of 500 nucleic in less

than 5 minutes was possible, instead of the 50 nuclei in more than an hour with the old densitometry techniques (Hardie *et al.*, 2002).

The Feulgen image analysis densitometry has been adapted for the determination of the genome size in animals and plants. It is a very versatile technique that allows the analysis of different kinds of tissues, replacing Feulgen densitometry (Gregory, 2005).

3.3. Flow cytometry

Flow cytometry has become the most well-accepted technique for genome size research. This technique was utilized initially for cell counting. After that, it was adapted for the identification of anomalous DNA content in cancer cells (Gregory, 2005). The principle of the technique involves the measurement of the fluorescence intensity of the stained nuclei when they pass through a laser beam. This technique is fast and accurate, allowing the analysis of thousands of nuclei in a sample in seconds (Gregory, 2005). Consequently, flow cytometry has demonstrated to be the best method available for genome size determination (Gregory, 2002a).

Flow cytometric studies began in the 40's, but its use in scientific research increased two decades ago in areas such as evolutionary biology, ecology and biosystematics. In medical science, this technique has been widely used in the identification of carcinogenic cells or in the early diagnosis of chromosomal disorders such as trisomy of the chromosome 21, among many other studies (Kron *et al*., 2007; Smith and Gregory, 2009). Flow cytometry has been use in the study of genotoxicity effect in organisms or cells expose to cytogenetic agents (Lamb, Bickham, Whitfield Gibbons, Smolen, and McDowell, 1991; Tiersch and Wachtel, 1993). Also it is widely used in the evaluation of sperm viability (Garner, Johnson, Yue, Roth, and Haugland, 1994), and in marine aquaculture has been used to identify and quantify pathogenic bacteria (Endo, Nakayama, and Hayashi, 2000).

Flow cytometry is a high performance analytical tool that allows the simultaneous detection and quantification of the optical properties of cell such as size, shape and fluorescence (Kron *et al.*, 2007). It is a sensitive technique that has the capability to

analyze 3,000 cells per second, and allows highly reproducible and accurate results (Krishan *et al*., 2005). These features allow the analysis of a large quantity of fresh or preserved cells (Birstein *et al*., 1993; Krishan *et al*., 2005; Kron *et al*., 2007), providing the capability of analyzing cells in a high through put manner for different studies in the areas of genetics, taxonomy, reproductive biology, ecology, experimental evolution, biomedicine, among others (Robertson and Button, 1989; Kron *et al*., 2007).

The flow cytometer is composed of five main parts: (1) fluidic system, (2) light source, (3) optical system, (4) detectors and (5) electrical and computer systems (Rieseberg, Kasper, Reardon, and Scheper, 2001) (Figure 1). In the flow cytometer, a crucial point is where the cell and the laser intersect, being called “the interrogation point”. This is the point at which the cell (or nucleus) intersects the laser beam, thus the light properties emerge from the cells are captured by detectors and analyzed (Figure 2 a). When the cell passes through the laser beam, the emitting light provides information about the size, complexity and shape of the cell (Figure 2). The forward light scatter is the amount of light that is scattered in the forward direction (FWS) as the light strikes the cell, being proportional to the relative cell size (Figure 2 b). The complexity is measured when the cell passes through the laser beam and the light scatters. This side scattered (SSC) light is collected by detectors located 90 degrees from the laser beam (Figure 2 c). This SSC light reflects the cellular granularity and structural complexity. Finally, an often-used parameter for flow cytometry analysis is fluorescence. The fluorescent light emits from stained cells or components as they pass through the laser beam, which excites the fluorochrome (Figure 2 d). The emitted light travels along the side Scatter path and is directed through a series of filters, so that a particular wavelength range is delivered to the appropriate detectors that send the signal to the electrical and computer system to transform it into a digital data (Figure 3).

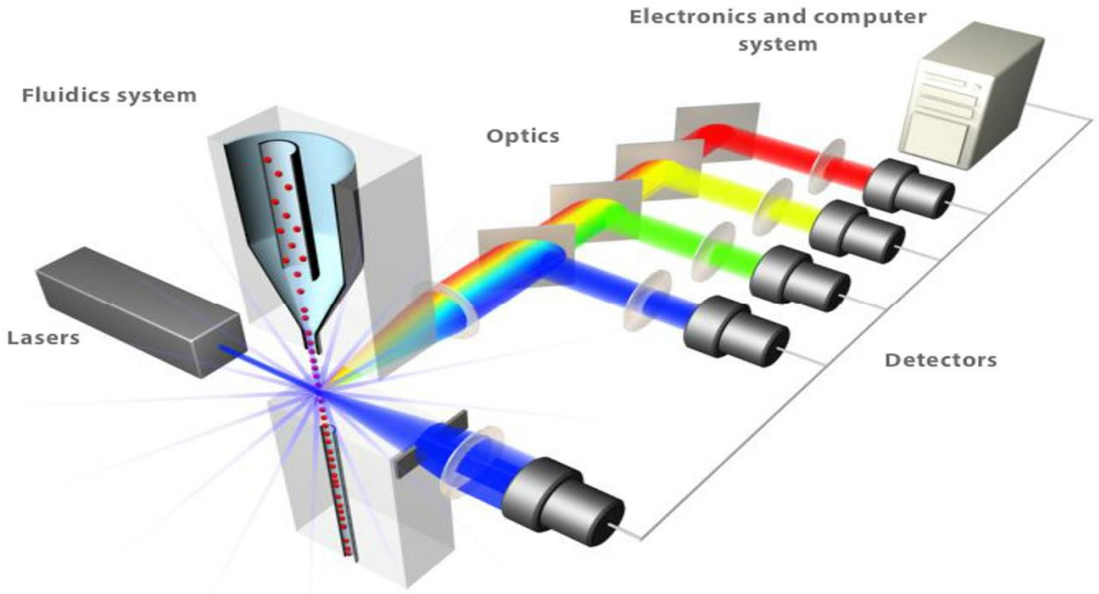

Figure 1. Flow cytometry components. The cells or nuclei are transported by the fluidic system, and aligned to pass through the laser light. The cell characteristics are collected by detectors. The fluorescence light passes through the optical system and is delivered to the appropriate detectors, which sends the fluorescence signal to electrical and computer system. These systems convert the signal to data expressed as cytograms. *(Imagen by Life Technologies www.lifetechnologies.com)*.

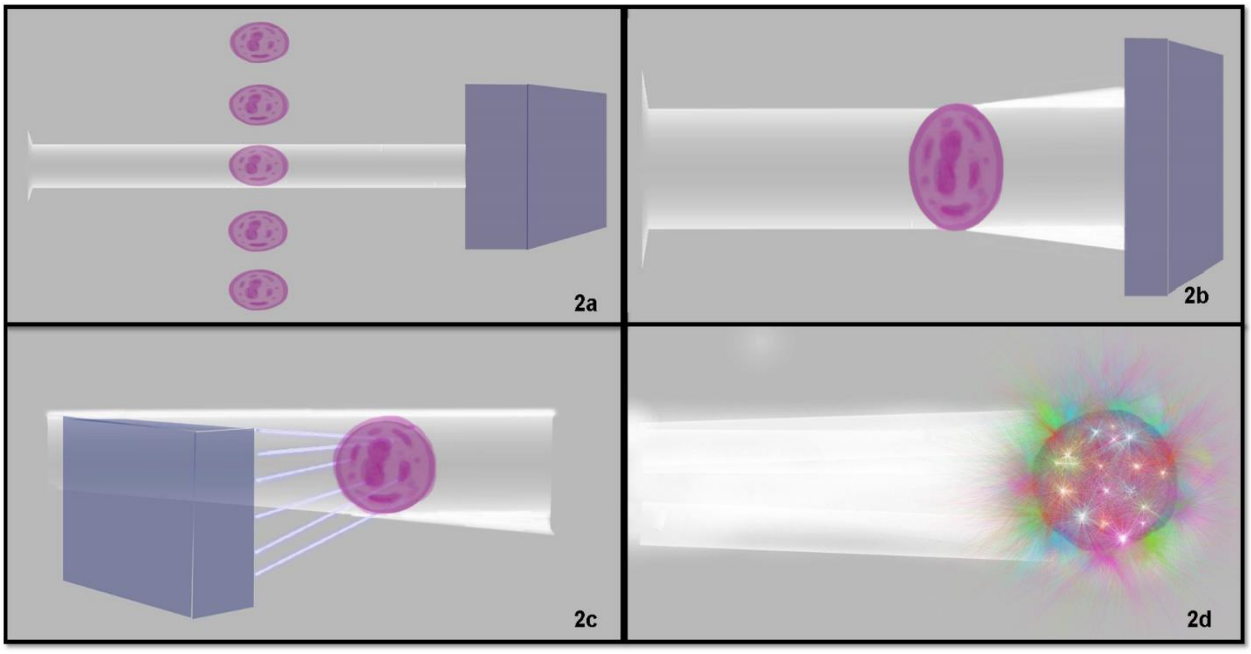

Figure 2 a-d. Optical properties of the cell. (a) Cells pass through the laser beam one by one. (b) Forward Scatter magnitude is proportional to the size of the cell. (c) Side Scatter provides cell complexity information. (d) Excitation of the fluorochrome by the laser beam.

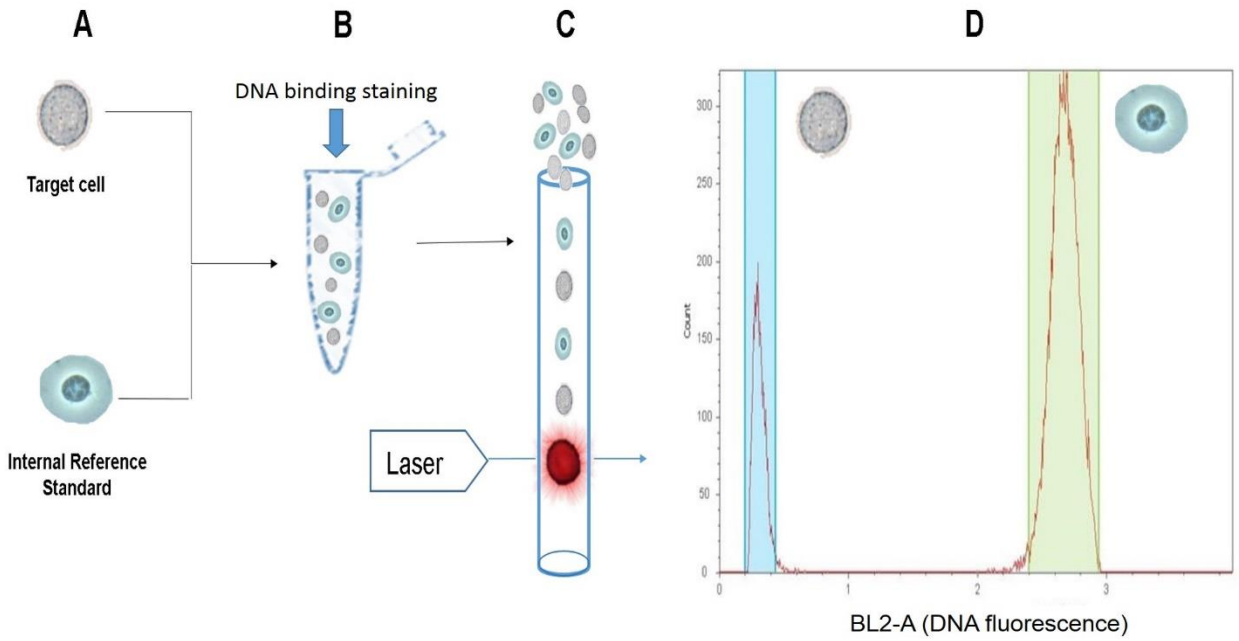

Figure 3. General flow cytometry procedure for DNA analysis. A) nuclei or cells are obtained from the reference standards and experimental target organisms, B) nuclei are mixed and stained together with DNA-specific fluorochrome, C) nucleus pass individually through the laser beam that excites the fluorochrome, and D) fluorescence light is acquired and displayed in a histogram to show relative DNA content..

3.4. Reference standard

Determination of genome size by flow cytometry is a relative quantification. Consequently, the use of reference standards are essential in determining the unknown experimental genome size (Jakobsen, 1983; Vindeløv, Christensen, and Nissen, 1983; Tiersch *et al*., 1989). Reference standards are cells of a species with a known genome size, relatively close to the size of the target cells (Tiersch *et al.,* 1989). When the target cells are analyzed together with the reference standard, the genome size of the target cells can then be directly measured (Tiersch and Chandler, 1989).

A constant issue for flow cytometry and genome size studies is finding the best, most appropriate reference standard that could even be used as a universal standard. However, this is not been possible because there is a wide range of genome sizes and ideal reference standards are close in size to the experimental target. An ideal reference standard would have a genome size determined by chemistry techniques or genetic sequencing where the results of both technique provides similar results (Suda and Leitch, 2010).

A very important characteristic that a reference organism could have is a uniform DNA content among somatic cell. Uniformity enables reproducibility, and the data interpretation among different analyses, techniques and among laboratories (Jakobsen, 1983; Dressler and Seamer, 1994). Also, considerations for an acceptable reference standard are those cells that could be easy to sample, stable with temperature changes, and within the close range of the genome size of the experimental target cells (Tiersch *et al.*, 1989).

Reference standards can be used as an: internal reference standard, when it is mixed and analyzed together in the same test tube as the target cells, or as an external reference standard that is analyzed individually (Tiersch *et al*., 1989; Dressler and Seamer, 1994). The external reference standards were the most-used for genome size determination and cell cycle analysis[2] by flow cytometry in the past, being used before and after target sample analysis. The main disadvantage is enhance of slight electronics shifts causing inaccurate data collection. To avoid errors, reference standards should be mixed with the target cells and analyzed together as internal standards. Consequently, the internal reference standard became the most accurate, and these option for genome size and cell cycle analysis (Vindeløv *et al*., 1983; Suda and Leitch, 2010). Since the target cells and internal reference standard are exposed to the same conditions, this yields more accurate results (Shackney, Erickson, and Skramstad, 1979; Jakobsen, 1983).

Nucleated blood cells, such as leukocytes in mammals or erythrocytes in non-mammalian, are the most used cell type for cell cycle analysis and genome size determination by flow cytometry. The advantage of using blood cells is that they are easy obtained from the animal and bleeding is considered non-invasive. Also, animals can be sampled repeatedly (Gregory, 2005). According to the literature, red blood cells (RBC) of chicken, trout and white blood cells (WBC) of humans are the most-used reference standards (Krishan *et al*., 2005) (Table 2).

[2] The cell cycle is the life history of each cell, where the genetic information passes through to two daughters cells from a single mother cell. Any alteration in the cell cycle could lead to chromosome abnormality such tumors, cancer, mutations, among others.

Table 2. Cells used as reference standards in flow cytometric analysis of genome size.

Reference standard	Scientific name	Reference
Sea urchin spermatozoa	*Stronglylocentrotus purpuratus*	(Hinegardner and Rosen, 1972)
Trout and human	*Oncorhynchus mikiss and Homo sapiens*	(Jakobsen, 1983)
Rainbow trout and chicken	*Oncorhynchus mikiss and Gallus gallus domesticus*	(Vindeløv *et al.*, 1983)
Rainbow trout and human	*Oncorhynchus mikiss and Homo sapiens*	(Iversen and Laerum, 1987)
Chicken and Atlantic salmon	*Gallus gallus domesticus and Salmo salar*	(Rens, Welch, and Johnson, 1999)
Mosquitofish, catfish, chicken, pig, human and bullfrog	*Gambusia affinis, Ictaluris punctatus, Gallus gallus domesticus, Sus scrofa, Homo sapiens, Rana catesbiana*	(Tiersch *et al.*, 1989)
Chicken	*Gallus gallus domesticus*	(Tiersch and Chandler, 1989)
Human	*Homo sapiens*	(Wheeless *et al.*, 1989)
Trout and sperm mussel	*Onchorhynchus mikiss and Mytilus spp.*	(Elston, Drum, and Allen, 1990)
Sterlet	*Acipenser ruthenus and Xenopus laevis*	(Birstein *et al.*, 1993)
Catfish and chicken	*Ictalurus punctatus and Gallus gallus domesticus*	(Tiersch and Wachtel, 1993)
Chicken	*Gallus gallus domesticus*	(Chang, Sang, Jan, and Chen, 1995)
European frog	*Rana temporaria*	(Vinogradov, 1998)
Chicken, common carp and rainbow trout	*Gallus gallus domesticus, Cyprinus carpio, Onchorhynchus mikiss*	(Carvalho *et al.*, 2002)
Chicken, common carp and rainbow trout	*Gallus gallus domesticus, Cyprinus carpio, Onchorhynchus mikiss*	(Fenerich *et al.*, 2004)
Trout, human	*Oncorhynchus mikiss and Homo sapiens*	(Krishan *et al*., 2005)

3.5. Identification of genome size using fluorescent dyes

In addition to the techniques for determining genome size, it has been necessary to find better fluorescent dyes that bind specifically to DNA, resulting in accurate quantifications of DNA content. Therefore, the ideal DNA fluorescent dye would be that which makes a better bond between the dye and molecule of DNA but not with RNA. Also, it could be a non-specific preference for bases or sequences and making a stoichiometric binding to DNA would be attributes of a preferred stain for genome size determination (Fried, Perez, and Clarkson, 1976; Shapiro, 2003).

Fluorescence depends, in part, on the binding affinity of the fluorochrome with DNA bases (Tiersch *et al*., 1989; Birstein *et al*., 1993; Kron *et al*., 2007). A wide variety of DNA or RNA fluorescent dyes is known exist, but just a few of them are DNA-specific dyes. The fluorochromes used to stain DNA are classified in two groups depending on their affinities: a) intercalating dyes that bind to a specific fraction of guanine-cytosine or adenine-thymine (Figure 4a). Examples include ethidium bromide/mithramycin and 4'-6-diamidino-2-phenylindole (DAPI). Or b) dyes that intercalate between base pairs within the phosphate backbone of double strained DNA or RNA, (Figure 4b) such ethidium bromide and propidium iodide, which do not have any sequence preference. (Taylor and Milthorpe, 1980; Martinez, Beck, Allsbrook, and Pantazis, 1990; Shapiro, 2003).

3.6. Propidium Iodide

Propidium iodide [3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide)] (PI), is a fluorescent dye that intercalates between the base pairs of the secondary helix of DNA (Figure 4 b) and is the most commonly used stain for genome size determination. Propidium iodide differs from ethidium iodide by the double charge of the isopropyl group that permits a better affinity for stained of DNA (Shapiro, 2003; Hawley and Hawley, 2004).

In the 1968, Hudson and contributors used, for first time, PI as analogue of ethidium bromide for circular DNA analysis. However, PI was officially used as a DNA dye for cell cycle analysis by flow cytometry until the 70's (Crissman and Steinkamp, 1973; Krishan,

1975; Fried *et al*., 1976). Since then, PI is considered as the best dye for genome size and cell cycle analysis by flow cytometry. It is excited at 535 nm, and when intercalate with DNA, the maximum fluorescence emitted is at 617 nm (Al-Rubeai and Emery, 1993). This dye also binds with RNA; then, RNA must be eliminated from the samples (Taylor and Milthorpe, 1980; Martinez *et al*., 1990), typically by an RNAse enzyme.

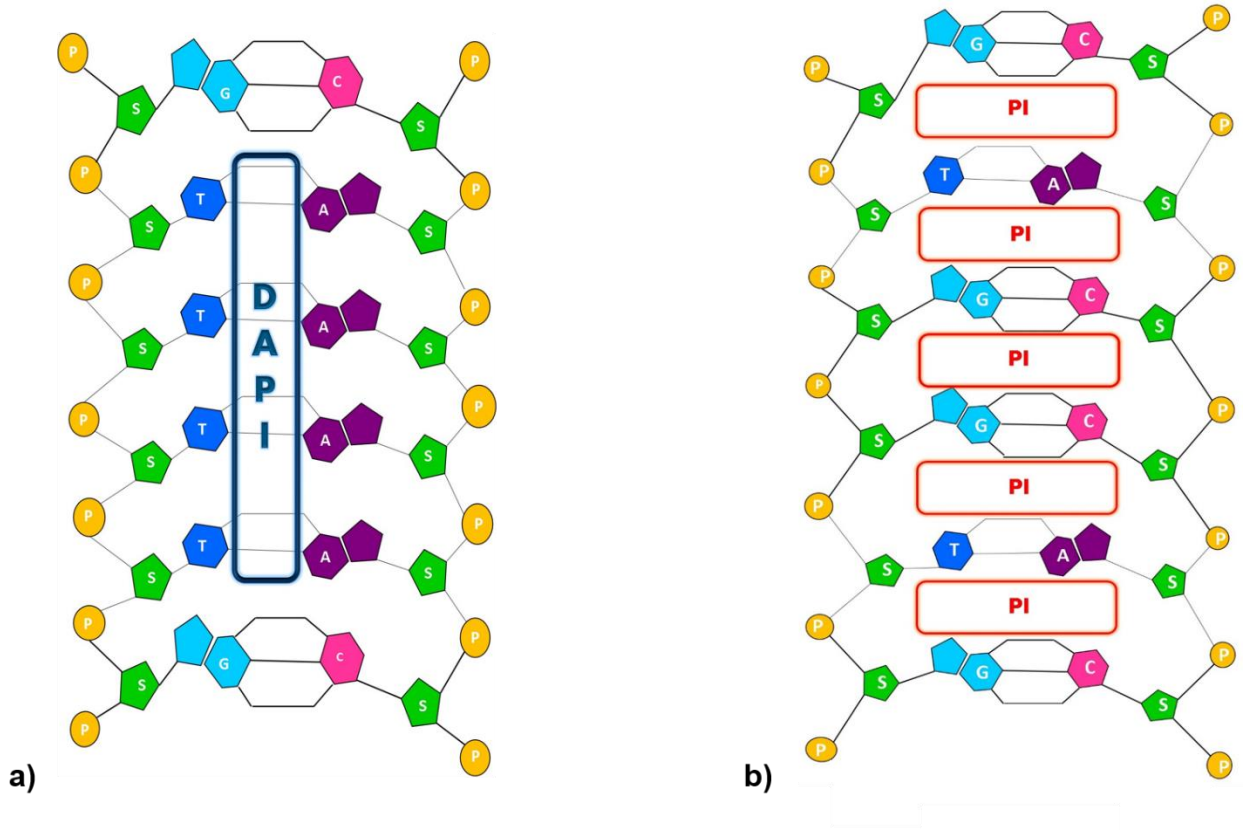

Figure 4. Binding affinity of the fluorochrome with DNA bases. a) DAPI interaction with DNA chain. b) Propidium Iodide (PI) interaction with DNA chain. Where the P is the phosphate group, (S) sugar group, and the base pairs (A) adenine, (C) cytosine, (G) guanine and (T) thymine.

Chapter 4. Relevance of Genome Studies in Mexico

Mexico is a megadiverse country harboring the majority of the Earth's species, and thus designated as megadiverse country (Galindo Leal *et al*., 2015). Baja California is one of the biggest peninsulas of the world, it is surrounded by the cold waters from the Pacific Ocean and by the warm waters of the Gulf of California, also called the aquarium of the world. Today, most species there are endangered due to overfishing (Galindo Leal *et al*., 2015). Therefore, several plans for resource conservation, as national management plans and the national plan of action for the conservation of the aquatic genetic resources have been developed. However, one of the biggest problems to develop appropriate genetic resources for conservation action is the lack of information about the aquatic resources in Baja California and their aquaculture potential.

Currently, molecular genetic techniques and genetic engineering are revolutionizing studies on aquatic animals, ecosystems, fishery biology, also have been used worldwide to enhance aquaculture. Studies on genome size along with genetic analysis have contributed greatly in many areas of natural sciences (Kron *et al*., 2007). Studies on genome size have been used to assist aquaculture and plan managements for aquatic resources in other countries . However, this has not yet been applied in Mexico. The application of the genome size analysis in aquatic animals to assist aquaculture is thus very important because it allows the confirmation and identification of polyploidy, hybrids organisms, and sex differentiation (sex chromosome), among other features. In addition, genome analyses allows the identification of genetic alterations such as chromosomal abnormalities like trisomy and some diseases like cancer, which could be a great benefit and application in Mexican fisheries and aquaculture.

Despite the knowledge of the genome size as a useful tool in the chromosomal manipulation of cultured organisms; studies on this topic for Mexican species are sparse/absence. Therefore, the determination of the genome size of species with economical and biological value of Baja California will help in the development of a wide diversity of studies in diverse science areas. Finally, it will contribute to enhance

the information for massive sequence analysis. In addition, it will contribute to reach some objectives established in the National Plan of Action for Aquatic Genetic Resources of Mexico, in the conservation programs, development and sustainable use of the aquatic genetic resource, among others. In addition, the development of a catalog containing the genome size of species for Mexico will provide important information to be included in the government fisheries and aquaculture fact sheets.

Chapter 5. Objectives and Hypothesis

5.1. General objective

Determine the DNA content of aquatic animals with biological and economical importance to Baja California, Mexico to establish the first national database of high quality for inclusion in the International animal genome size database.

5.2. Specific objectives

1. Identify appropriate reference standards for the studied organisms.

2. Assess DNA content of spermatozoa, whole blood or hemolymph from different aquatic animals.

3. Establish standard operating procedures for the aquatic animals studied.

5.2. Hypothesis

- The genome size among organisms of the same species is not significantly different.

- The DNA content in blood cells is twice that in spermatozoa of the same organism.

Chapter 6. Materials and Methods

6.1. Collection of blood samples used as reference standards.— The reference standards used in this study were blood cells from chicken (*Gallus gallus*) 2.54 pg DNA content, red-eared turtle (*Trachemys scripta elegans*) 5.30 pg DNA content, and bullfrog (*Rana catesbiana*) 15.00 pg DNA content (Tiersch *et al.*, 1989).

6.1.1. Collection of blood samples of chicken.— A chicken from La Nueva Granja pet shop in Ensenada, Baja California was immobilized for the procedure. The blood samples were obtained with an insulin syringe (27 G x 13 mm) by puncturing the branchial vein of the right wing. The samples were placed in a 1.5 mL microcentrifuge tubes containing Acid Citrate Dextrose (ACD) anticoagulant (Appendix A) and 10% DMSO. Then, the samples were placed in a cooler and transported to the Germplasm Bank for Aquatic Species (GBAS) of the National Subsystem for Aquatic Genetic Resource of Mexico (SUBNARGENA in Spanish) in CICESE. Once in the laboratory, the blood was distributed in 0.2-mL PCR posteriorly were plunged into liquid nitrogen (-196°C) until use.

6.1.2 Collection of red-eared turtle blood samples.— A turtle (*Trachemys scripta elegans)* was obtained from La Nueva Granja pet shop and transported to the GBAS. The blood was obtained by heart puncture with an insulin syringe. The blood was handled and stored as above.

6.1.3 Collection of bullfrog blood samples.— Two bullfrog males where obtained from La Paz farm in Estado de Mexico, Mexico. The bullfrogs were kept in captivity until the dissection. The blood was obtained by heart puncture. The samples were placed (1ACD:9Blood) in 2.0 mL microcentrifuge tubes containing ACD anticoagulant. Next, the samples were placed in a cooler and shipped to the GBAS at CICESE, where the samples were handled and stored as above.

6.2. Collection of blood and hemolymph samples from target species.— The technique for obtaining blood or hemolymph depended on the type of organism (see

Appendix C). Overall, all the organisms were donated from different companies or collected from specific places (see Table 4).

6.2.1 Hemolymph collection from target organisms.— Ten organisms from Pacific white shrimp (*Penaeus (Litopenaeus) vannamei*) were sampled in Gez Acuicola, Mazatlán. Hemolymph collected with an insulin syringe (27 G x 13 mm) from the lymphatic sinus, each sample was placed in 1.5 mL microcentrifuge tubes and transported to National Subsystem for Aquatic Genetic Resource of Mexico (SUBNARGENA in Spanish) in CICESE. The samples were placed in a 1.5 mL microcentrifuge tubes containing anticoagulant (AASH) (See Appendix A) and 10% DMSO and were plunged into liquid nitrogen (-196°C) until use.

6.2.2. Blood collection from target organisms.— Organisms from each species were obtained from different places (Table 4) and blood was obtained by tail vain puncture. The samples were placed in a 1.5 mL microcentrifuge tubes containing Acid Citrate Dextrose (ACD) anticoagulant and 10% DMSO and placed in a cooler, transported to GBAS and cryopreserved in liquid nitrogen (-196°C) until use.

6.2.3.Milt collection.— Sperm samples were collected from the Germplasm Bank for Aquatic Species (GBAS) of the National Subsystem for Aquatic Genetic Resource of Mexico (SUBNARGENA in Spanish) in CICESE. Samples were thawed in a water bath for 10 seconds or until ice crystals melt. Then, samples were placed in conical tubes and prepared for staining according to Appendix B. Sperm from barracuda was obtained by hand stripping method placed in conical tubes and kept cold until use.

Table3. List of fish obtained from different institutions or companies

Scientific name	Common name	Company or institution
Anoplopoma fimbria	Sablefish	CICESE
Atractoscion nobilis	White seabass	Mendez seafood store
Caulolatilus princeps	Ocean whitefish	Mendez seafood store
Carcharhinus brachyurus	Copper shark	Artisanal fishery, Guerrero Negro, Baja California Sur
Chirostoma humboldtianum	Shortfin silverside	Universidad Autónoma de México (UAM)
Lutjanus novemfasciatus	Black snapper	Mendez seafood store
Morone saxatilis	Striped bass	Pacific aquaculture S.A de C. V
Narcine entemedor	Giant electric ray	Artisanal fishery, Guerrero Negro, Baja California Sur
Oncorhynchus mykiss nelsoni	San Pedro Martir trout	CICESE
Paralabrax clathratus	Kelp bass	Mendez seafood store
Paralichthys californicus	California Halibut	CICESE
Rhinobatos productus	Northern Guitarfish	Artisanal fishery, Guerrero Negro, Baja California Sur
Sarda chiliensis	Pacific bonito	Mendez seafood store
Sardinops sagax	Pacific sardine	CICESE
Sebastes caurinus	Copper rockfish	Mendez seafood store
Sebastes constellatus	Starry rockfish	Mendez seafood store
Sebastes rubrivinctus	Flag rockfish	Medez seafood store
Semicossyphus pulcher	California sheephead	Mendez seafood store
Seriola lalandi (captive)	Yellowtail	CICESE
Seriola lalandi (wild)	Yellowtail	Mendez seafood store
Sphyraena argentea	Pacific barracuda	Mendez seafood store
Sphyrna zygaena	Smooth hammerhead	Artisanal fishery, Guerrero Negro, Baja California Sur
Totoaba macdonaldi	Totoaba	CICESE

6.3. Flow cytometry configuration

The equipment used to determine the genome size in this project was an *Attune*® *Focusing Flow Cytometry* (Foster City, CA). This flow cytometer has the capability of aligning the cells using ultrasonic waves. The flow cytometer is equipped with violet and

blue lasers configuration (405 and 488 nm, light intensity of 50 and 20 mW respectively). This configuration allows data collection in six fluorescent channels (Table 5) in addition to the forward and side scatter information. The flow cytometer can detect particles with a size ranges of 1 to 50 microns, and data acquisition up to 20 thousand events per second. The software used was Attune® Cytometric Software 2.1.

Table 4. Optic configuration of *Attune® Focusing Cytometer*

Excitation laser (nm)	Fluorescence channel	Default filter (nm)	Filter range (nm)
Violet 405	Blue	450/40	430-470
	Green	522/31	507-537
	Orange	603/48	579-627
Blue 488	Green	530/30	515-545
	Orange	574/26	561-587
	Red	640 LP	>640

6.3.1. Flow cytometry standardization

To ensure the laser performance, the flow cytometer was calibrated with calibration beads before analysis (Attune™ Performance Tracking Beads). Frozen cells from chicken, turtle or frog were thawed and analyzed individually or combined to calibrate the flow cytometer parameters. Aliquots of the frozen cells were placed in 0.5-mL black microcentrifuge tubes with the propidium iodide (PI) lysis-staining buffer (Tiersch *et al.*, 1989) (See Appendix A), and incubated for 15 minutes at 24°C before flow cytometric analysis. The voltage was adjusted to set the standard in a relatively consistent channel and that the threshold was set on BL2-A to allow only fluorescently stained particles to be analyzed.

6.4. Identification of appropriate reference standards for the target species

The chicken, red-eared turtle and bullfrog were placed individually in 0.5 mL black microcentrifuge tubes with the lysis-staining buffer described above. Using one parameter BL2 area (BL2-A) histogram and two parameters channel dot plot BL2 width (BL2-W) versus BL2-A created with Attune ® cytometric software 2.1, the nuclei population of each standard were established. Once each standard nuclei was found, standard mixtures were made: (1) chicken + turtle, (2) chicken + bullfrog, (3) turtle + bullfrog and (4) chicken + turtle + bullfrog (Figure 5).

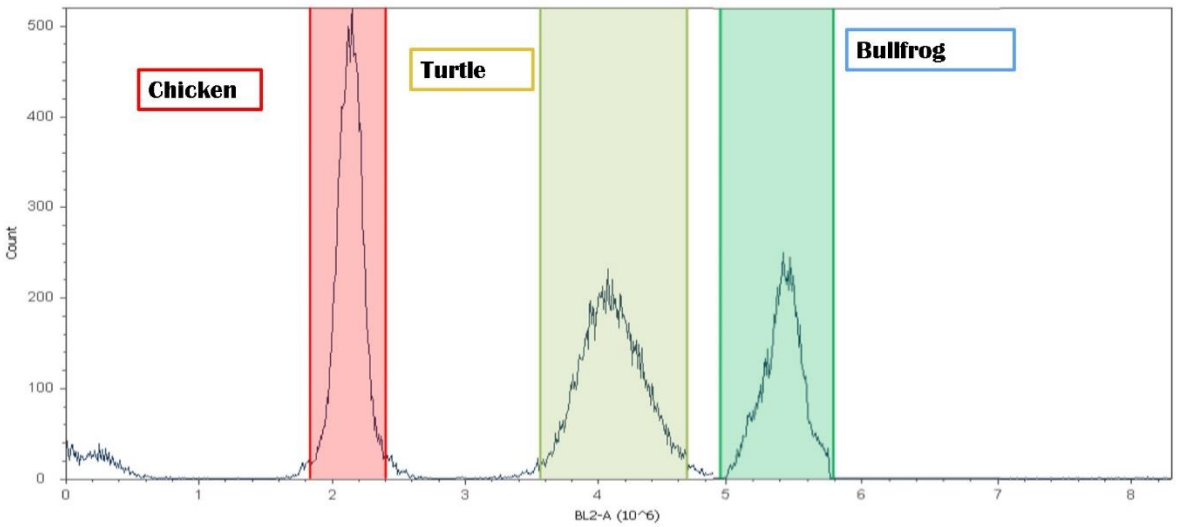

Figure 5. Representative flow cytometric histogram with fluorescence of DNA (BL2-A) and nuclei count of chicken, red-eared turtle and bullfrog.

6.5. Determination of genome size from target species

Once the voltage and threshold parameters for the reference standards were established, the target cells were analyzed individually with the same settings as the external reference standards. The target cells were placed in 0.5-mL black microcentrifuge tubes with the lysis-staining buffer described above. Approximately 10,000 nuclei per sample were acquired for each organism at 25uL/min. All the analyses were performed by triplicate.

One parameter BL2 area (BL2A) histogram and two parameters channel dot plot BL2 width [BL2W] versus BL2A; BL2A versus FSC; FSC versus SSC were created with

Attune® Cytometric Software 2.1.; Statistical information of peak channel values and coefficient of variation (CV; the flow cytometric analysis of measurement of dispersion) were generated.

The genome size per organism was estimated relative to the genome size per nuclei of the internal reference standard, according to the Formula (1)

(1)

$$\frac{\text{Mean peak of reference standard}}{\text{Genome size (pg) of the reference standard}} = \frac{\text{Mean peak of target cell}}{\text{unknown pg}}$$

In the case where spermatozoa samples were obtained, the genome size was determined as with blood and hemolymph samples.

6.6. Establishing Standard Operation Procedures

All the standard operation procedures for the determination of genome size of the aquatic animal studied of Baja California are presented in the appendix section.

6.7. Data Analysis

Statistical analysis was performed using SAS software for Windows® (SAS Institute, Cary, North Carolina). A complete random design with subsamples was used to analyze differences among replicates or organisms of each species. Specific differences among treatment groups were identified by a Tukey test. The level of significance was $\alpha < 0.05$ was chosen as the level of significance.

Chapter 7. Results

7.1. Identification of appropriate reference standard

The appropriate reference standard for spermatozoa samples was chicken blood in all cases. The best reference standard for all the species, was chicken except for the California sardine, where blood red-eared turtle was used (Table 6).

7.2. DNA Content for spermatozoa, whole blood or hemolymph

The genome size of each organisms by species was determined using the cytograms proportionated by the Attune cytometer software 2.1. (Appendix E). No significant differences among individuals of the same species were found ($P < 0.05$). In addition, for the species which the spermatozoa were obtained, the haploid genome size of the spermatozoa was half of the diploid genome size of the blood or hemocytes of the target species. No significant differences ($P < 0.05$) among individuals were found for yellowtail in captivity; but there was a difference between the captive organisms and organisms of wild population ($P > 0.05$) (Table 6).

7.3. Establishment of the best standard operation procedures

The standard operation procedures elaborated are showed in Appendixes.

Tabla 5. Genome size of different target organisms. Ch, chicken; T, turtle.

Scientific name	Common name	Genome size (pg)		Internal Reference Standard	Animals sampled (n)
		Haploid	Diploid		
Anoplopoma fimbria	Sablefish	0.6098 ± 0.01	-	Ch	3
Atractoscion nobilis	White seabass	-	1.5515± 0.09	Ch	2
Caulolatilus princeps	Ocean whitefish	-	1.9670	Ch	1
Carcharhinus brachyurus	Copper shark	-	8.8353 ± 0.1	Ch	2
Chirostoma humboldtianum	Shortfin silverside	-	1.5180± 0.05	Ch	8
Penaeus (Litopenaeus) vannamei	Pacific white shrimp	-	5.1784± 0.03	Ch	8
Lutjanus novemfasciatus	Black snapper	-	2.0880±0.006	Ch	3
Morone saxatilis	Striped bass	0.6305	-	Ch	1
Narcine entemedor	Giant electric ray	-	7.1596 ± 0.1	Ch	2
Oncorhynchus mykiss nelsoni	Baja California rainbow trout	-	5.5226	Ch	1
Paralabrax clathratus	Kelp bass	-	2.0035	Ch	1
Paralichthys californicus	California Halibut	0.6234± 0.49	-	Ch	3
Rhinobatos productus	Northern guitarfish	-	7.7640	Ch	1
Sarda chiliensis	Pacific bonito	-	1.7620±0.007	Ch	2
Sardinops sagax	California sardine	-	2.1717 ± 0.1	T	10
Sebastes caurinus	Cooper Rockfish	-	1.9411	Ch	1
Sebastes constellatus	Starry Rockfish	-	2.0093±0.01	Ch	3
Sebastes rubrivinctus	Flag Rockfish	-	1.9959± 0.08	Ch	3
Semicossyphus nebulifer	California Sheephead	-	1.8558	Ch	1
Seriola lalandi	Yellowtail (captive)	-	1.5872± 0.01	Ch	10
Seriola lalandi	Yellowtail (wild)	0.7298	♀ 1.6460	Ch	2
Sphyraena argentea	Pacific barracuda	0.6456	1.4250± 0.01	Ch	2
Sphyrna zygaena	Smooth hammerhead	-	5.2989±0.007	Ch	2

Chapter 8. Discussion

In Baja California, one of the most important economic activities are fishery and aquaculture. Despite the extensive research performed for aquatic animals of this region, little information on genetics characteristics has been reported. Genome size data will provide important information for the development of more studies.

This project has generated new information about the genome size of the aquatic species of Baja California. Also, it allowed us to corroborate the genome size reported in the animal genome size database, and provide an update using more accurate technology.

In the animal genome size database, the genome sizes are reported in picograms of haploid DNA content. In some cases, the genome size reported in the papers is reported as diploid DNA content. This means that for some species the haploid DNA content was calculated from the diploid DNA content originally reported. In this thesis, the genome size is reported as haploid or diploid form or both, because in some cases it was possible to collect samples of haploid and diploid cells. Therefore, in this work, the genome size is reported from the type of cells collected.

As the technologies advance, more efficient methods have been developed, and flow cytometry is currently the most accurate and most used technology. Historically, the genome size has been determined with about 12 different methods. In the international animal genome size database (www.genomesize.com), we can find the genome size of a lot of animal species. Specifically for around 5685 species (3,731 vertebrates and 1,904 invertebrates) (Gregory, 2015).

8.1. General findings

In this thesis, the genome sizes of 23 different species were determined by flow cytometry, in 12 of them, the genome size has not been published in the international animal genome size database or it was unknown. This is the case for shortfin silverside fish, San Pedro Martir rainbow trout and totoaba, which are important endemic species

of Mexico. The genome size obtained in this work was contrasted with the know genome size reported in the database, first the haploid genome size (pg), following for the diploid genome size (pg/cell).

Almost all the species that were analyzed in this thesis are endangered due to overfishing, lack of national or binational management plans, human consumption demand, among others factors. Molecular and genetic techniques have become more important to understand biology and oceanic systems. The fusion of these techniques with other biology studies is needed to the better understood of the aquatic world.

Genome size studies could help in the identification of population, as in the case of the yellow tail and California sardine, also in the species identification as in the case of the rockfishes. In addition, it could help to identify stocks relevant to health status organisms such as the Pacific white shrimp and abalone. Finally, identification of hybrid and polyploid organisms it would be for great help to assist aquaculture production and research like in abalone hybrid studies.

8.2. Invertebrates

8.2.1. Shrimp

The Pacific white shrimp (*Penaeus (Litopenaeus) vannamei*) is the second most important cultivated species in Mexico, and worldwide. It is distributed on the Eastern Pacific, from Sonora, Mexico to northern Peru (Valles-Jimenez, Cruz, and Perez-Enriquez, 2004). Mexico is considered the sixth largest producer of shrimp (CONAPESCA, 2013), and together with totoaba and shark fishery, the shrimp fishery is one of the “oldest” fisheries of the region. In the middle 1930’s, the fishery of the totoaba started to decline, while the fishery of shrimp started to rise (Cudney and Turk, 1998). Since then, the shrimp fishery is one of the most important activities in Mexico.

Most of the genomic studies on shrimp are focused on breeding programs for increasing disease resistance or tolerance to disease. However, only one study has been performed on genome size. The genome size of four species of the genus *Penaeus*

(*Litopenaeus)* were determined. Up to date, only one report about genome size of the Pacific white shrimp has been registered (Chow *et al.,* 1990), being 2.50 pg (5.0 pg/cell).

The genome size found in this thesis was 5.18 pg. The difference between the genome size reported by Chow *et al*. (1990) and this thesis could be due to the calculation formula used to determine the genome size. As I describe before, one of the biggest problems on the genome size determination was the reference standard, the methods and the formulas used to calculate the amount of DNA. Chow *et al.* (1990) reported the genome size as percent of the genome size of the reference standard (human blood cells, 7.3 pg/cell). While in this thesis, chicken, red-eared turtle and bullfrog were used as reference standards, and the genome size was reported as mass unit (pg). In addition, the genome size used as reference for Chow *et al*. (1990) was 7.3 pg when the genome size reported as standard for humans is 7.0 pg. Therefore, the genome size reported in this thesis is likely more accurate than the genome size reported by Chow *et al*. (1990).

Another interesting finding in this thesis was that an additional genome size was found in some samples of shrimps that did not correspond to either to shrimp or the standard reference. The additional genome size was 0.0514 pg (diploid), smaller than shrimp genome size, corresponding probably, to bacterium. Identification of additional genome sizes found in the samples was beyond of the objectives of this thesis. However, future work related to diagnosis of shrimp health using hemolymph samples without killing the organism will be of great interest for shrimp industry.

8.3. Vertebrates

8.3.1. Sablefish

The sablefish (*Anoplopoma fimbria*) has an economical importance and aquacultural potential in Mexico. Nevertheless, little information about this species has been recorded. Sablefish are distributed on the Northern of Pacific Ocean, from the Bering Sea, USA to Baja California, Mexico, and are found at depth from 305 m to 2,740 m (Allen and Smith, 1988).

Two genome size data are reported in the animal genome size database for sablefish. The first indicated that the sablefish has a genome size of 0.84 pg (1.68 pg/cell), and was determined using the bulk fluorometry (Hinegardner and Rosen, 1972). The second report indicated that the genome size was 0.71 pg (1.43 pg/cell) and was determined by Feulgen image analysis densitometry, with a sample size of two organisms (Hardie and Hebert, 2004). In this thesis, the genome size found in cryopreserved spermatozoa was 0.60 pg (1.20 pg/cell). The difference on the genome size reported by Hinegardner and Rosen (1972) and this thesis may be due that flow cytometry is more accurate than the bulk fluorometry. In the case of the genome size reported by Hardie and Hebert (2004), they use a sample size of two organisms from the Alaska ground fish observer program and found a genome size of 0.71 pg. This difference could be also due to the methodology used, the sample size or the capture zone.

In 2012, Tripp-Valdez *et al*. suggested the possibility that sablefish contains 2 subpopulations: the North subpopulations (Bering Sea and Golf of Alaska) and Southern subpopulations (Oregon, USA and San Quintin, Baja California, Mexico). These results came from a geometric morphometric analysis of these four populations, but in the genetic analysis the results did not show a strong differentiation. Two years later, differences in some characteristics, such as the reproductive season among the Northern populations and Mexico population were determined (Sánchez-Serrano, 2014). Because, the genome size found for the organisms caught in Baja California Coast is different for the reported from the organisms caught in Alaska, we can confirmed base on the genome size the existence of two subpopulations.

8.3.2. White seabass

The white seabass (*Atractoscion nobilis*) is the largest member of the croaker family (Scianidae) in California. It is distributed from Juneau, Alaska to Magdalena Bay, in Baja California, and is also found in the northern Gulf of California.

The white seabass has been target of commercial and recreational fishery. Before 1982, most of the commercial catch was extracted from Mexican waters by US fisherman. After this year, the Mexican government denied access to US fisherman

(California Department of Fish and Game Marine Region, 2008). Despite of this governmental decision, almost all the fish seabass capture is exported to USA. Now, most of the research are focus on repopulation programs, and genetic diversity of wild populations. However, genome size determination has not been performed before.

For the Sciaenidae family, genome size of some species has been determined. The genome size ranges from 0.62 pg (1.24 pg/cell) for *Micropogonias furnieri* to 0.98pg (1.96 pg/cell) for *Pogonias cromis*. Genome size 0.68 pg (1.36 pg/cell) for another species of the genus *Atractoscion* (*A. aequidens)* was determined. The genome size found in this thesis for *Atractoscion nobilis* was 1.04 pg (2.08 pg/cell). Another species of the Sciaenidae family analyzed in this study, *Totoaba macdonaldi*, has a genome size of 0.80 pg (1.60 pg/cell). Comparing this information with the data obtained in this thesis, we can suppose that the white seabass has the largest genome size for the Sciaenidae family.

8.3.3. Totoaba

The Totoaba (*Totoaba macdonaldi*) is one of the most important biological species for Baja California and Mexico. This species is endangered and endemic for the California Gulf. Along with abalone, sharks and sardine, the totoaba were one of the first fisheries developed in Baja California. Since 1940, the totoaba is protected species due to overfishing. In 1975, the fishery was totally banned and in 1991 totoaba was declared endangered (Pedrin-Osuna, Cordova-Murueta, and Delgado-Marchena, 2001). Researches from the Autonomous University of Baja California (UABC) they have been working with this species since 1990's. Today, the university has developed the biotechnology to culture this species and also a management plan has been implemented. The UABC is the principal fry producer of totoaba to be used in repopulation programs.

Due to its ecological and economical importance, several genetic, reproduction and management studies have been performed. However, its genome size has not been reported prior to this thesis. The genome size of this species was 0.80 pg (1.60 pg/cell). It is important to point out that genome size of any other species taxonomically near to

totoaba has not been determined. Therefore, comparison with some closer species was not possible because the totoaba is the only species of the genus, and the nearest is the white seabass (*Atractoscion nobilis)* whose genome size was also determined in this study.

8.3.4. Ocean whitefish

The ocean whitefish, *Caulolatilus princeps* is a malacanthid with a distribution from Vancouver Island, Canada to the Gulf of California, Mexico. It is a very important fishery species in Mexico, and is assumed that at least two population exist in Baja California, in the Pacific Ocean of Baja California and in the Gulf of California (Elorduy-Garay, Ruiz-Córdova, and Díaz-Uribe, 2005).

In 1972, Hinegardner and Rosen reported the genome size of 275 species of teleost fishes by bulk Fluorometry. In this list, the genome size for the Ocean whitefish (*Caulolatilus princeps*) was reported as 0.98 pg (1.96 pg/cell).

In this thesis, the genome size for the species was 0.98 pg (1.97 pg/cell). These results agree with the reported by Hinegardner and Rosen. From all species analyzed in this thesis, this is the only genome size determined by bulk Fluorometry and flow cytometry that show very similar results.

8.3.5. Shortfin silverside

The shortfin silverside (*Chirostoma humboldtianum*) is an endemic species from Mexico. It is distributed in the Lerma-Chapala-Santiago plateau in the center of Mexico. This species has a big economical, biological, cultural and social importance for Mexico. This fish has been part of the Mexican diet since the prehispanic time and many families depend on the fishery of this species. Nowadays, the population has been dismissed due to habitat pollution, overfishing and habitat destruction (Elías Fernández, Navarrete Salgado, and Rodríguez Robles, 2008; Hernández-Batista, Ramírez-Torrez, Azaola-Espinosa, Mayorga-Reyes, and Monroy-Dosta, 2015).

One of the principal problems is the identification of the species, due the presence of hybrids in the natural environment, and also because morphometric and meristic similarities. The identification of the species by traditional taxonomic methods is not an option and for this reason searching for molecular and genetic methods for identification is a priority (Perez-Ramirez, 2003). At present only, the alloenzyme, RFLP, and karyotyping (Autonomous University of Mexico (UAM) in Mexico City) has been used to identify the species (Perez-Ramirez, 2003).

Until now, the genome size from the shortfin silverside was unknown, and no data about other members of the family Atherinopsidae exist. The genome size found in this thesis was 0.75 pg (1.51 pg/cell), and this is the first time that the genome size of the *C. humboldtianum* has been determined.

More genome size analysis should be done to determine the genome size for each species, for a better identification of organisms; also, some organisms are catalogue as tetraploids by karyotyping. In this case, the genome size analysis could be an additional analysis for identify and confirmed ploidy in the shortfin silverside.

8.3.6. Black snapper

The black snapper (*Lutjanus novemfasciatus*) is one of the biggest snappers and it is distribute from northern Mexico to Panama. Most of the studies have been focused on the red snapper (*Lutjanus campechanus*), the principal snapper fishery on the Gulf of Mexico. However, little information about the black snapper (*L. novemfasciatus*) is known (Allen, 1985; Ibarra-Zatarain, 2003).

Therefore, in the genome size database has been reported the genome size for other species of the genus *Lutjanus*, the genome size for the genus range from 0.80 pg (1.60 pg/cell) in the *L. sebae*, to 1.40 pg (2.8 pg/cell) in the *L. campechanus.* In this thesis, It was found a genome size of 1.04 pg (2.08 pg/cell) for *L. novemfasciatus* was in the range of *Lutjanus* species; this is the first report of genome size for the black snapper.

8.3.7. Striped bass

The striped bass (*Morone saxatilis*) is native to Atlantic Slope drainages, from St. Lawrence River, Canada, south to the St. Johns River, Florida and Gulf slope drainages from western Florida to Lake Pontchartrain, Louisiana, USA. The striped bass has been introduced widely in different landing areas of the USA and on the Pacific Coast from British Columbia (Fuller and Neilson, 2015), to Baja California where is an important cultured species.

Two genome sizes have been reported for striped bass. The first study was performed with bulk Fluorometry assay by Hinegardner and Rosen (1972) and the genome size reported for this species was 0.89 pg (1.78 pg/cell). The other genome size reported was 0.95 pg (1.90 pg/cell) using the Feulgen image analysis densitometry (Hardie and Hebert, 2003, 2004). These genome sizes are bigger to those reported in this thesis 0.63 pg (1.26 pg/cell). The differences could be due to the accurate analysis of the flow cytometer. In this work 30 thousand nuclei were analyzed instead of few nuclei (50) analyzed by bulk fluorometry or 3 hundred nuclei analyzed by Feulgen image analysis densitometry.

8.3.8. San Pedro Martir trout

Mexico has a great quantity of endemic trout species (Abadía-Cardoso, Garza, Mayden, and García-de-León, 2015). One of them is the San Pedro Martir trout or Baja California rainbow trout (*Oncorhynchus mykiss nelsoni*). This trout lives in water bodies or streams of sierra San Pedro Martir in Baja California, Mexico. The altitude of this sierra ranges between the 600 and 2,000 m. In addition, this trout is considered the southernmost population of North America (Valles-Ríos and Ruiz-Campos, 1997; Aguilar-Juárez, Ruiz-Campos, and Paniagua-Chávez, 2011). This subspecies is considered endangered because its confined distribution, low abundance and environmental changes (Ruiz-Campos and Cota-Serrano, 1992).

In the international animal genome size database, a total of 24 reports of genome size of rainbow trout (*O. mykiss*) are published, with 5 different methods (flow cytometry,

feulgen imagen densitometry analysis, biochemical analysis, feulgen densitometry and ultraviolet microscopy), with 10 different reference standards (human, chicken, frogs, betta, mouse, among others). The range of genome size for the rainbow trout is from 1.87 pg (3.74 pg/cell) to 2.92 pg (5.84 pg/cell). In addition, some genome size reported as rainbow trout in the database are not for the rainbow trout are for species of genus *Salmo*. However, information on genome size is not available yet. For this reason, the comparison with the genome size of the *O. mykiss* was not possible. The genome size found in this thesis for the San Pedro Martir trout was 2.76 pg (5.52 pg/cell), this genome size is in the range of the rainbow trout (*O. mykiss*), and this is the first time that the genome size was determined for this subspecies.

8.3.9. Kelp bass

The Serranidae family is one of the most diverse families of teleost, the *Paralabrax* genus have nine species divided into groups depending on the geographical distribution (the North America group and the Center and South America group). The species *P. clathratus* belongs to the North American group (Martínez-Brown, Medel-Narváez, Hernández-Ibarra, and Ortiz-Galindo, 2012).

The kelp bass is distributed from the Columbia River to Baja California, with some populations on Guadalupe and San Benitos Island. Some studies evaluated if there is a genetic structure through these populations. In 1983, Beckwitt found that there is no evidence of population subdivision in the *P. clathratus* species.

However, no information about the genome size is known, in this study the genome size found for kelp bass was 1.00 pg (2.00 pg/cell). In the international genome size database the genome size of barred sand bass (*P. nebufiler*) is recorded with a genome size of 1.30 pg (2.60 pg/cell) that is bigger than the genome size found for the kelp bass.

8.3.10. California halibut

The California halibut (*Paralichthys californicus*) is an important commercial fishery resource in Baja California. It is distributed from the Quillayute River, Washington to Magdalena Bay, Baja California Sur (Haugen, 1990).

Currently, only one study about the genome size has been reported. In 1972, Hinegardner and Rosen, reported that the genome size of California halibut was 0.80 pg (1.6 pg/cell). However, in this work, I found a genome size of 0.62 pg (1.24 pg/cell). This difference could be attributed to the accuracy of flow cytometry.

8.3.11. Pacific bonito

The genus *Sarda* has 4 species, the Atlantic bonito (*Sarda sarda*), the Australian bonito (*Sarda australis*), the striped bonito (*Sarda orientalis*) and the Pacific bonito (*Sarda chiliensis*) (Viñas, Alvarado Bremer, and Pla, 2010). The pacific bonito is restricted to the eastern Pacific Ocean (Collette and Nauen, 1983) and this geographical ranges includes a northern and southern subspecies separated by a tropical species (*S. orientalis*). The northern subspecies of Pacific bonito (*Sarda chiliensis lineolata*) is distributed from Alaska to Cabo San Lucas at Baja California and in the Revillagigedo Island, Mexico (Collette and Nauen, 1983).

The genome size of the genus *Sarda* has been determined for the Atlantic bonito 0.81 pg (1.62 pg/cell), Australian bonito 0.92 pg (1.84 pg/cell) and striped bonito 0.92 pg (1.84 pg/cell), but not information about the genome size of the Pacific bonito has been reported. The genome size found in this thesis for the Pacific bonito (*Sarda chiliensis lineolata*) was 0.88 pg (1.76 pg/cell) that is in the ranges of the genus.

For the Pacific bonito the scientific name is a little controversial. Based on the geographical distribution of this species, the classical morphological studies, and behaviors studies, has been considered that the *Sarda chiliensis* had two subspecies *Sarda chiliensis lineolata* on the North Pacific and *S. chiliensis chiliensis* on the South Pacific.

Consequently, several authors have been searching molecular and genetic evidences that could help in the classification of the species. Based in molecular data and mtDNA data, the existence of the subspecies of *Sarda chiliensis* are invalid (Orrell, Collette, and Johnson, 2006; Viñas *et al*., 2010).

Considering the identification and classification of the species need the morphological, molecular and genetics support, the genome size of this species could be helpfully to elucidate the existence or not of the subspecies.

8.3.12. California sardine

The California sardine (*Sardinops sagax*) is an important commercial fishery for Baja California, and is distributed from Alaska, USA to Baja California and Gulf of California, Mexico (García-Rodríguez, García-Gasca, Cruz-Agüero, and Cota-Gómez, 2011).

Two studies were made about the genome size of *Sardinops sagax*. The first was reported in 1991 and the technique used was Feulgen densitometry (Ida, Oka, and Hayashigaki, 1991). The genome size found for this species using Feulgen densitometry was 1.35 pg (2.69 pg/cell) and the organisms were obtained from Japan. The second work was reported by Hardie and Herbert (2004). In their study, the Feulgen imagen analysis densitometry was used and the genome size found for the sardine obtained from Australia was 1.01 pg (2.03 pg/cell). Differences in genome size between both works are notable. In this thesis, the genome size reported for the sardine caught in the Baja California coast and maintained in captivity for more than 2 years was 1.08 pg (2.17 pg/cell). These differences could be due to the use of different methodologies or sampling zones indicating that subpopulations of sardine have different genome sizes.

Currently, the scientific name for the sardine has been *Sardinops sagax caureleus* or *Sardinops caureleus,* referring to California or Pacific sardine. Nevertheless, these scientific names are invalid and are synonymous. However, the correct and current name for the California sardine is *Sardinops sagax* (Page *et al.*, 2013).

In 1952, Svetovidov proposed to use the species *Sardinops sagax* for all the forms of Sardinops, and designated regional forms as subspecies (Grant, Clark, and Bowen, 1998). In 1998, restriction fragment length polymorphism (RFLP) and nucleotide sequence analysis of mitochondrial DNA (mtDNA) analyses resolved these discrepancies. In this work, they recommended the designation of three subspecies: *Sardinops sagax ocellatus* for Southern Africa, Australia and New Zealand, *S. sagax sagax* for Chile, Peru, Ecuador, Mexico, United States and Canada, and *S. sagax melanostictus* for China, Korea, Japan and Russia (Grant *et al.*, 1998)

The animal genome size database uses only the species *Sardinops sagax* to refer to "South American pilchard". Consequently, the sampling place is crucial for designating subpopulations from which genome sizes are reported. Therefore, in the work of Ida *et al*. (1991), the organisms were caught in the Japanese Coast, so the genome size reported was from the Japanese sardine population. Also, Hardie and Hebert, (2004) reported the genome size of 5 species of the Clupeidae subfamily, and the only species of the genus *Sardinops* was *S. sagax neopilchardus*, sampled in Sydney, Australia.

The differences found between the genome sizes for the sardine caught in different places around the world suggest that maybe exist a notable subpopulation distribution, and that the nomenclature use to name the sardine must be considered. Thus, the genome size analysis could be useful for validate the scientific name and subpopulation of the species.

8.3.13. Rockfish

More than 100 species of Rockfish are known worldwide and most of them are limited to the North Pacific and Gulf of California (about 96 species). A greatest diversity of rockfish (56 species) are found within Southern California (Love, Yoklavich, and Thorsteinson, 2002). The communities of Rockfish of the Pacific Coast of Baja California are little known. The number of species of the northern Baja California is similar to the species found in California, USA.

Some studies of genome size have been done in some species of *Sebastes*, but none for species of the Pacific Coast of Baja California. The genome size of few species has been analyzed and reported in the animal genome size database. Only 7 species has been reported from the 96 that we can found in the North Pacific to the Gulf of California. The genome size of those species range from 0.95 pg (1.90 pg/cell) in the Pacific Ocean perch (*Sebastes alutus*) to 1.10 pg (2.20 pg/cell) cells in the calico rockfish (*S. dallii*), with an average of 0.99 pg (1.98 pg/cell). The genome size found for the three species of rockfish of analyzed in this thesis are in the average of the genus, where the copper rockfish (*Sebastes caurinus*) had a genome size of 0.97 pg (1.94 pg/cell), the flag rockfish (*S. rubrivinctus*) 0.99 pg (1.99 pg/cell), and the starry rockfish (*S. constellatus*) with 1.00 pg (2.00 pg/cell).

Some DNA studies suggest that the rockfishes are monophyletic and closely related. Consequently, the identification of most of them is difficult. Some of the characteristics that all the rockfishes of the genus *Sebastes* share an internal fertilization and the developing of the embryos (matrotrophic viviparity), high fecundity and spines all over the body (Love *et al*., 2002). Due to differentiation among species being complicated, determining the genome size along with karyotyping will be a useful tool.

8.3.14. California sheephead

The California sheephead (*Semicossyphus pulcher*) is a protogynous hermaphrodite wrasse, distributed from Point Conception, California, USA to Cabo San Lucas, Baja California, Mexico and they can be found in the northern Gulf of California, including the California Channel Island and the Guadalupe Island, Mexico (Cornish and Dormeier, 2006; Poortvliet, Olsen, Selkoe, Coyer, and Bernardi, 2009; Poortvliet *et al*., 2013).

Until now, the genome size for California sheephead was unknown, and no data about relative species exist. The genome size found in this thesis was 0.92 pg (1.85 pg/cell). This is the first time that the genome size of *Semicossyphus pulcher* has been determined.

In the case of the California sheephead, a phylogeography study was done with organisms of different regions. The results indicated that it was not a population structure differentiation among regions (Poortvliet *et al.*, 2013). This suggest that the genome size found in this thesis for the California sheephead from the Pacific Coast of Baja California (1.85 pg/cell) could be similar for the USA population and Gulf of California population.

8.3.15. Yellowtail

Yellow tail (*Seriola lalandi*) is distributed from British Columbia, Canada, to Chile. The Yellowtail is a very important fishery species to Baja California. Almost all the fishery production is for local and national market and the aquaculture production is exported to the USA market.

Only one report of the genome size of this species has been registered. In that study the genome size 0.70 pg (1.4 pg/cell) was analyzed by Feulgen image densitometry (Hardie and Hebert, 2004). The sample size used in that work was from two organisms from the Sydney fish market.

In this thesis, the genome size of yellowtail organisms of wild population and culture population was possible, and the genome size of the culture population was similar 0.79 pg (1.58 pg/cell), however, the genome size of the wild population 0.82 pg (1.64 pg/cell) was different of the culture population.

Because the *Seriola* species do not have a heteromorphic sex chromosome (Caputo, Marchegiani, and Olmo, 1996; Sola *et al*., 1997), the genome size between males and females inside the groups (wild and culture population) was similar. The reason for the difference between populations, could be for the origin zone of the culture organisms, this because the hatchery import the fry from different places like Chile and Hawaii.

For many years, the yellowtail *Seriola lalandi* has been considered as a cosmopolitan species, even when depending on the geographic distribution different names are use for yellowtail population around the world. However, new studies have been done to

evaluate the genetic structure of the population of *Seriola* species. Martinez-Takeshita *et al*. (2015), demonstrated the existence of three distinct genetic lineages for the *Seriola lalandi*, in the North West Pacific, North East Pacific and the Southern Hemisphere. In addition, Purcell *et al*. (2015) found the presence of four population the NE Pacific, NW Pacific, South Pacific and South Atlantic.

Considering the new information about different species of *Seriola lalandi* and the difference in the genome size found between the wild population and the culture population in this study, it could be related with the new differentiation of yellowtail species. The genome size found in this study for the wild population corresponds to the NE Pacific population (from California, USA, to Baja California and Gulf of California, Mexico). Because the culture population fry came from Chile, the genome size found 0.79 pg (1.58 pg/cell) correspond for the Chile yellowtail population.

8.3.16. Pacific barracuda

The pacific barracuda (*Sphyraena argentea*) is a voracious predator that it is distributed from Alaska, USA to Cabo San Lucas, Baja California Sur, Mexico (Berdegué, 1956; Sommer, 1995). This species is a very important fishery resource in Baja California since 1920. Reports indicate that the capture was > 3,700 ton per year in the first half of the 20's. After that, the fishery showed a decrease in the capture due to overfishing. In the 70's a capture of only 6 tons per year was reported for pacific barracuda and was considered almost an extinct resource (Schultze, 1983). Moreover, between 2005 and 2010 it has been reported a fishery of 69 annual tons showing an increasing of the population.

Five species of the genus *Sphyraena* have been reported in the animal genome size database. The genome size reported for the Pacific barracuda using bulk Fluorometry was 0.83 pg (1.66 pg/cell) (Hinegardner and Rosen, 1972). The genome size determined in this thesis was 0.64 pg (1.42 pg/cell). These results did not agree with those of Hinegardner and Rosen, (1972). Differences may be due to methodology accuracy and sample size used in this thesis.

8.3.17. Chondrichthyes

In this study the genome size of four species of Chondrichthyes was analyzed, all were sampled in the Baja California waters. The smooth hammerhead (*Sphyrna zygaena*), giant electric ray (*Narcine entemedor*) and northern guitarfish (*Rhinobatos productus*) are distributed in tropical and template waters. The stocks of the Eastern Pacific are found from California, USA to Baja California Sur, Gulf of California, Mexico (Casper *et al*., 2005; Garayzar, 2015; Márquez, Smith, and Bizzarro, 2006). In the case of the copper shark (*Carcharhinus brachyurus*) is distributed in anti-tropical waters and in the Eastern Pacific are found from California, USA to Baja California Sur, Mexico (Benavides *et al.*, 2011).

The genome size of elasmobranch is considered as the largest genome size among vertebrates, except for lungfishes. The genome size is 4-5 times largest than teleosts. They cover ranges of genome size from 3 to 34 pg/cell (Stingo, Du Buit, and Odierna, 1980). For the species that were analyze in this study, only the copper shark had a genome size reported in the international animal genome size database.

The genome size found for the smooth hammerhead (*Sphyrna zygaena*) was 2.64 pg (5.29 pg/cell), for giant electric ray (*Narcine entemedor*) was 3.57 pg (7.15 pg/cell) and for northern guitarfish (*Rhinobatos productus*) was 3.88 pg (7.76 pg/cell). The genome size has not been recorded in the international animal genome size database and so this is the first time that the genome size for those species was determined.

In 2004, Hardie and Herbert analyzed the genome size of several organisms; one of them was the copper shark (*Carcharhinus brachyurus*) with a genome size of 2.86 pg (5.72 pg/cell). This genome size differs to the genome size found in this study 4.41pg (8.83 pg/cell). The genome size determined in this study is larger than the genome size reported. This could be due for several reasons, from the technique used to determinate the genome size (Feulgen Imagen Densitometry Analysis) up to the capture zone of the organisms. Hardie and Herbert captured the organisms from the Australian waters and the copper shark analyzed in this study was caught in the Gulf of California, Baja California Sur, Mexico.

Benavides *et al*. (2011), found that there are at least three distinct population groups for the species: the Australian-New Zealand, South Africa-Namibia and Peruvian. The genome size found in this study could be have a relationship with the genetic differentiation of copper shark populations found in 2011 by Benavides *et al*.

Chapter 9. Conclusions

- No significant differences were found in the genome size among the organisms of the same species.

- The genome size of the spermatozoa was near to the half of the genome size of the blood or hemolymph cells of the organisms analyzed.

- The evaluation of additional samples per species will allow a more accurate and representative genome size determination for the species.

- The analysis of the spermatozoa and blood cell of the same species allows an accurate genome size determination.

- The use of internal reference standards facilitates the determination of the genome size, and the use of different reference standards allows the confirmation of the genome size for the species.

- Reporting all the flow cytometry parameters used and sample handing procedures, allows these genome size analyses.

- This catalog support the development of further genetic studies for the benefit the aquaculture, and fishery and management plans for Mexico and around the world.

Chapter 10. Recommendations

- More analysis should be done to evaluate if the geographic distribution of the species as sablefish, yellowtail and sardine, are associate with the genome size.

- In the case of yellowtail, more analysis should be done to evaluate if exist a difference between the genome size of the wild population and the captive population.

- More analysis should be done to assess the presences of other cell populations like bacteria in the Pacific white shrimp samples.

- In this thesis the analysis of hemolymph of red abalone (*Haliotis rufescens*) was made. An extra population was found by flow cytometry analysis with a small genome size; these organisms were confirmed sick by histology and PCR analysis, for this reason, more analysis should be performed to evaluate the use of the flow cytometry as a tool in the diseases diagnosis.

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Appendixes

Appendix A. Preparation of anticoagulants and fixatives

Anticoagulant Acid Citrate Dextrose (ACD)

The reagents (Table 8) were weighed and dissolved in 60 mL of distilled water. Then, dissolution was made up to 100 mL with distilled water. Dissolution was stored in a refrigerator (4°C) until use.

Table 8. Preparation of ACD.

Reagent	Formula	Concentration (g per 100 mL)	Molarity (mM)
Citric acid	$C_6 H_8 O_7$	0.8	41.6
Sodium Citrate	$C_6 H_5 Na_3 O_7$	2.26	76.8
D-Glucose	$C_6 H_{12} O_6$	2.2	122.1

*All the reagents are SIGMA-ALDRICH

Anticoagulant (AASH) of hemolymph

The reagents (Table 9) were weighed and dissolved in 60 mL of distilled water. Then, dissolution was made up to 100 mL with distilled water. The solution was stored in a refrigerator (4°C) until use.

Table 9. Preparation of AASH

Reagent	Formula	Concentration (g per 100 mL)	Molarity (mM)
Sodium Chloride	NaCl	0.625	106.9
EDTA	$C_{10} H_{16} N_2 O_8$	2.26	77.3

*All the reagents are SIGMA-ALDRICH

Fixative solution for hemolymph

The fixative solution was prepared with 3% formalin in filtered sterile seawater. A proportion 1:1 was used to fix hemolymph.

Appendix B. Preparation of staining solutions

DNA staining solutions

Stock Propidium Iodide Solution (SPIS) For the stock PI solution, 0.56 g Sodium citrate and 25 mg propidium iodide were mixed on 50mL distilled water and stirred for 30 min in dark. The solution was stored at 4°C until use.

Working Propidium Iodide Solution (WPIS)

For the working PI solution, 5 mL of stock PI solution, 50 µL Triton and 75 µL RNAase were mixed on 45 mL distilled water and stirred for 30 min in dark. The solution was stored at 4°C until use.

RNAase A Solution (RNAase-A)

A 1.58g of Tris was dissolved in 10 mL distilled water. Then 100 mg pancreatic RNAase was dissolve in 8.4 mL (0.01 M) of sodium citrate and mixed. This mixture was placed into a heated water bath at 100°C for 15 min after boiling started. The solution was cooled to room temperature and pH was adjusted by adding 0.1 volumes 1M Tris until the RNAase solution reached pH 7.4. The solution was stored in aliquots at -20°C until use.

Appendix C. Sample collection

Hemolymph collection

For mollusks, an insulin syringe was used to extract ~1.0-1.5mL of hemolymph. For abalone, the needle was inserted in the angle between the foot and the head. The hemolymph was collected from the cephalic arterial sinus. The hemolymph was placed in tubes and mixed with anticoagulant (AASH) at ratio (1:1). Samples were kept cold (4°C) until use. In the case of shrimp, the hemolymph was taken from the ventral sinus located at the base of the first abdominal segment.

Fish blood collection

Ten juvenile yellowtail and sardines kept in captivity were collected from the Marine Fish Laboratory in CICESE. The organisms were caught with a net and placed individually in a bucket containing 2-phenoxyethanol (0.3mL/L) to sedate the fish. When the organisms lost balance, they were placed on a sponge and the body was cover with a wet towel. Only the posterior body was uncovered. Blood was collected with an insulin syringe (needle size 30G X 13”). The puncture was made in the tail vein. When the needle touched the fish vertebra, the needle was pulled back, just a little, to obtain the blood.

The other species of fishes were collected from wild population, from Camalú, Baja California (30°50’16” N, 116°03’26” W) and where transported to Mendez seafood store in Ensenada, Baja California. The blood was collected by tail vein puncture before eviscerate. A 3-mL syringe (needle size 21 G X 32mm) was inserted near the anal fin. When the needle touched the fish vertebra, the needle was pulled back, just a little, to obtain the blood. Blood was placed in conic tubes with anticoagulant ACD (1:9) and transported cold (4°C) to the laboratory.

Sperm collection

Cryopreserved sperm samples of sablefish, striped bass, halibut, were collected from the Germplasm Bank for Aquatic Species (GBAS) of the National Subsystem for Aquatic Genetic Resource of Mexico (SUBNARGENA in Spanish) in CICESE. All the

samples were in matrix with specie-specific cryoprotectant and Richardson's solution (300 mOsmol/Kg) was used as buffer.

Samples were thawed in a water bath for 10 seconds or until ice crystals melt.

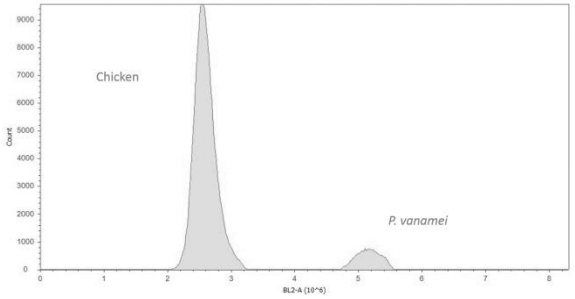
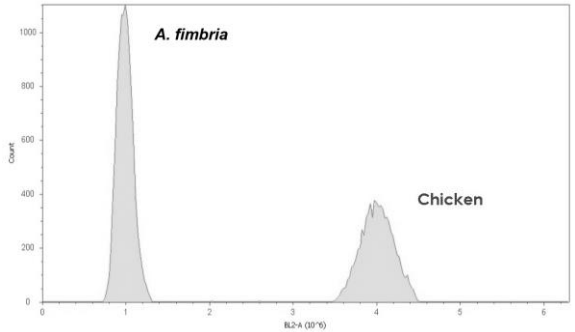
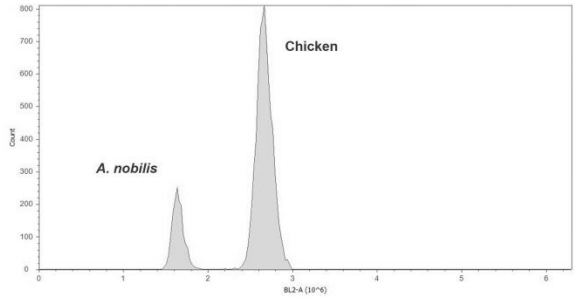
Milt from barracuda was obtained by hand stripping method, the spermatozoa was placed in conical tubes and diluted with filtered marine water as buffer and stored at 4°C until use.

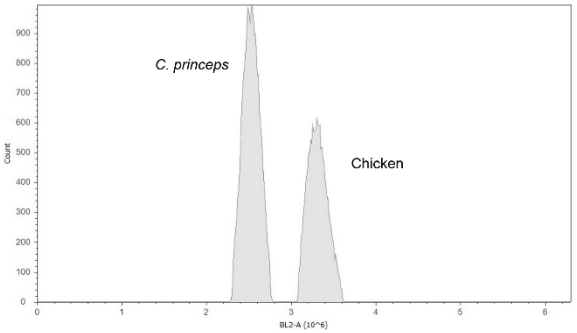
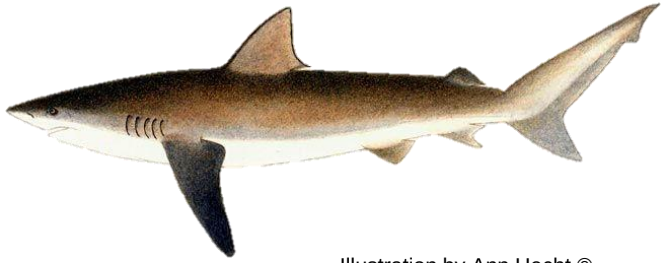
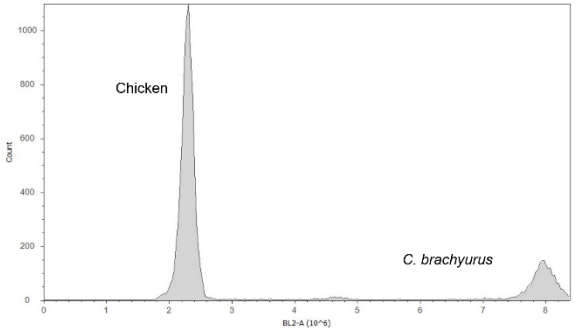
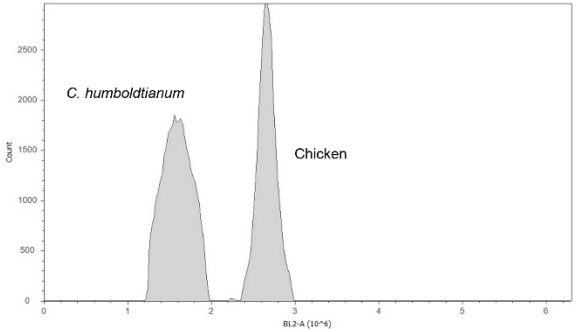
Sample storage and cell preparation

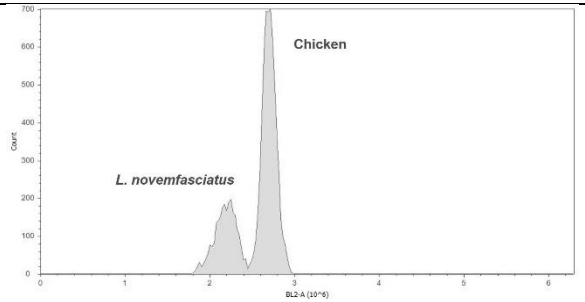
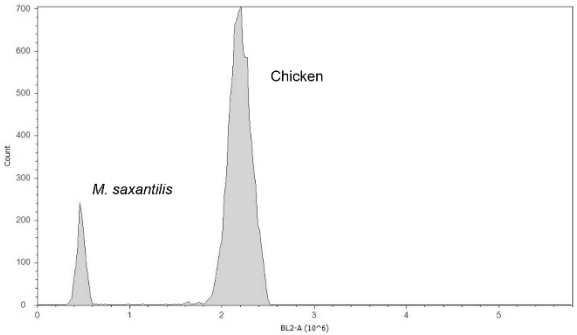
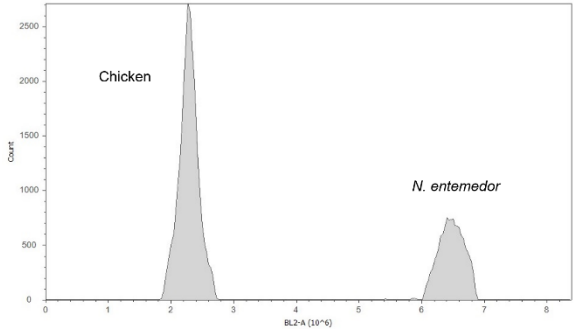
Sample storage was performed according to Jenkins and Thomas (2007) with a modification. The blood was mixed with ACD anticoagulant and 10% DMSO, placed in conical tubes and plunged into liquid nitrogen (-196°C). Samples were maintained at -196°C in liquid nitrogen until use.

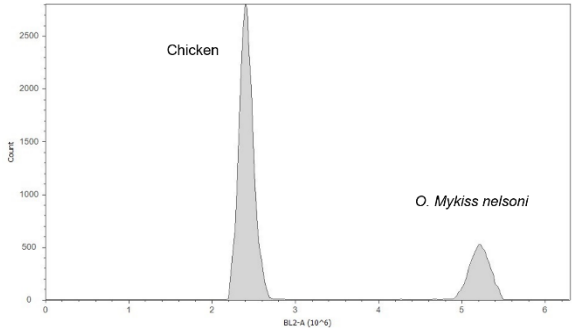
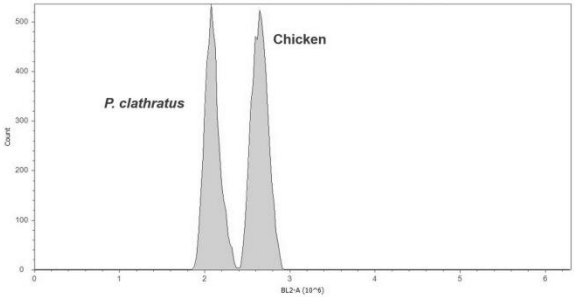
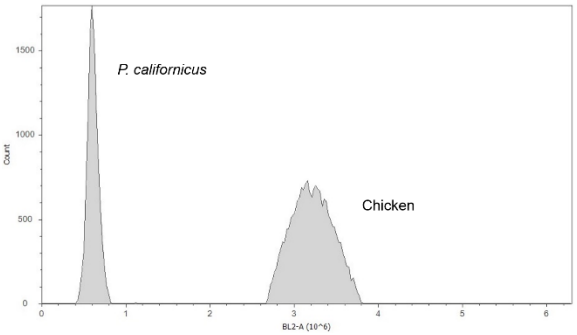
Blood, hemolymph and sperm aliquots were mixed with 500 µL of working DNA staining solution for 15 min at room temperature in black conical tubes. Then, samples were analyzed in the cytometer according to procedures showed before.

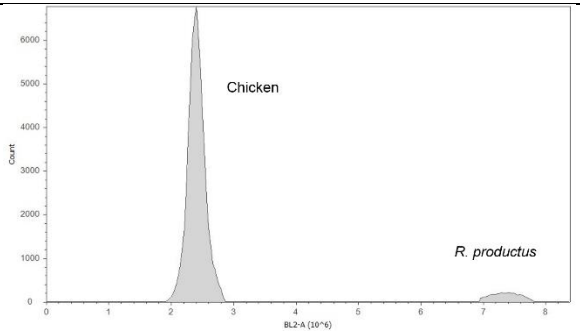
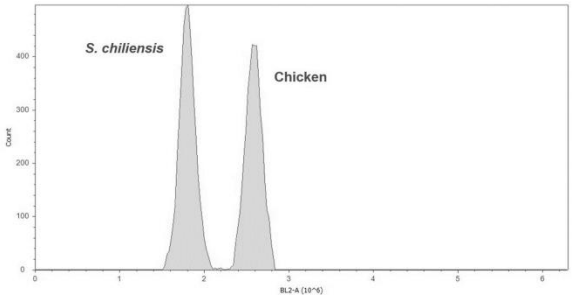
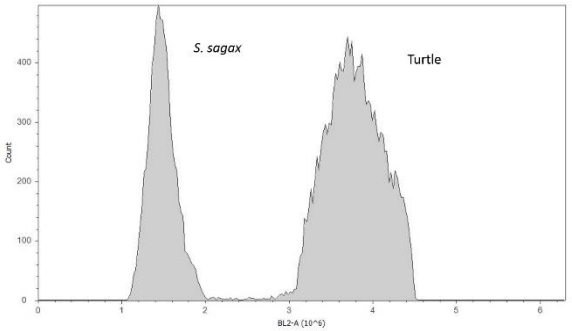
Appendix E. Examples cytogram of animals studied

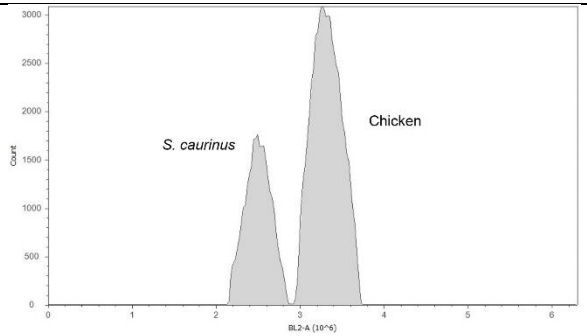
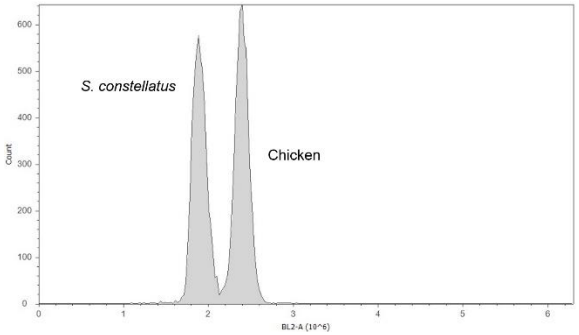
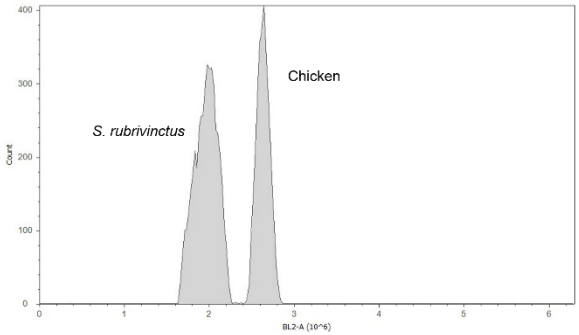
Species	Picture	Cytogram
Pacific white shrimp *Penaeus (Litopenaeus) vannamei*	 Illustration by A. M. Arias 2005	
Sablefish *(Anoplopoma fimbria)*		
White seabass *(Atractoscion nobilis)*		

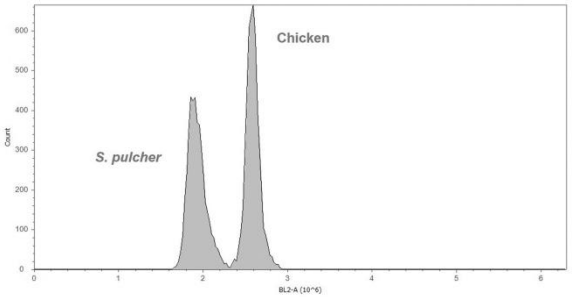
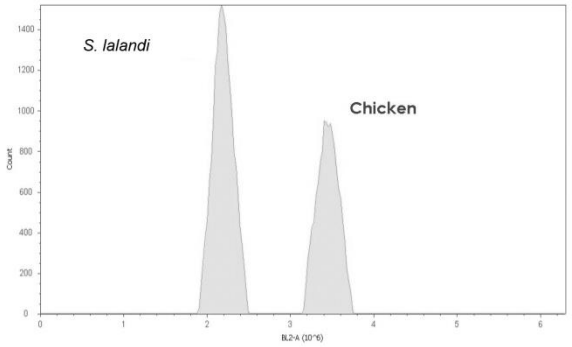
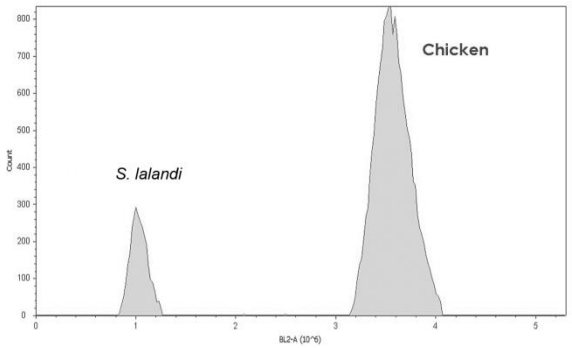
Species	Picture	Cytogram
Ocean whitefish *(Caulolatilus princeps)*		
Copper shark *(Carcharhinus brachyurus)*	 Illustration by Ann Hecht ©	
Shortfin silverside *(Chirostoma humboldtianum)*	 Illustration by Irma Urbina	

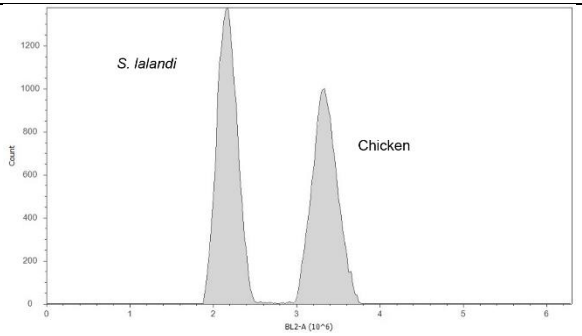
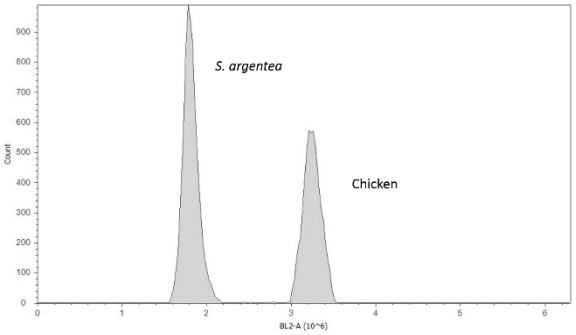
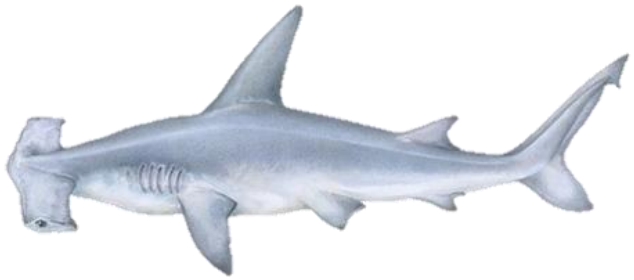
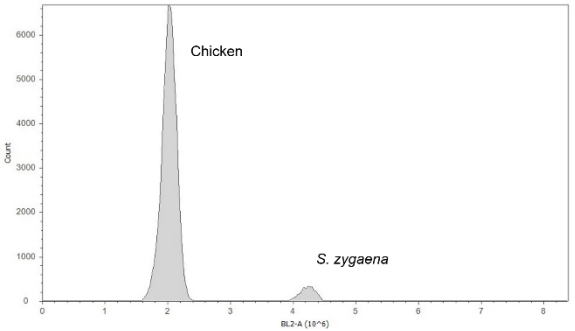
Species	Picture	Cytogram
Black snapper *(Lutjanus novemfasciatus)*		
Striped bass *(Morone saxatilis)*	 © Joseph Tomelleri	
Giant electric ray *(Narcine entemedor)*	 CIAD Mazatlán	

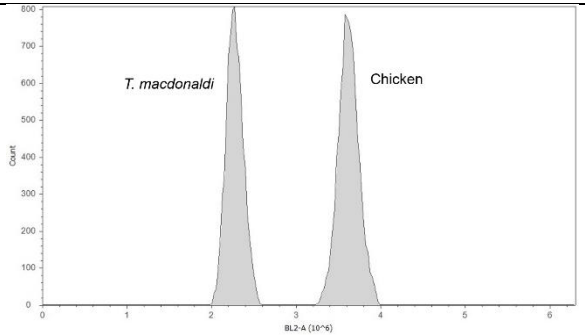
Species	Picture	Cytogram
Baja California rainbow trout *(Oncorhynchus mykiss nelsoni)*		
Kelp bass *(Paralabrax clathratus)*		
California halibut *(Paralichthys californicus)*		

Species	Picture	Cytogram
Northern guitarfish *(Rhinobatos productus)*		
Pacific bonito *(Sarda chiliensis)*		
California sardine *(Sardinops sagax)*		

Species	Picture	Cytogram
Copper rockfish *(Sebastes caurinus)*		
Starry rockfish *(Sebastes constellatus)*		
Flag rockfish *(Sebastes rubrivinctus)*		

Species	Picture	Cytogram
California sheephead *(Semicossyphus pulcher)*		
Yellowtail *(Seriola lalandi)* Captive population		
Yellowtail *(Seriola lalandi)* Wild population (sperm)		

Species	Picture	Cytogram
Yellowtail *(Seriola lalandi)* Wild population		
Pacific barracuda *(Sphyraena argentea)*		
Smooth hammerhead *(Sphyrna zygaena)*		

Species	Picture	Cytogram
Totoaba *(Totoaba macdonaldi)*		

Appendix F. Genome size found in this thesis and the genome size reported previously

Species	Genome size found in this study (pg/cell)	Cited by		First time reported
		Hinegardner and Rosen 1972	Hardie and Hebert 2004	
Anoplopoma fimbria	1.20	1.68	1.43	-
Atractoscion nobilis	2.08	-	-	✓
Caulolatilus princeps	1.97	1.96	-	-
Carcharhinus brachyurus	8.83	-	5.72	-
Chirostoma humboldtianum	1.51	-	-	✓
Lutjanus novemfasciatus	2.08	-	-	✓
Morone saxatilis	1.26	1.78	1.90	-
Narcine entemedor	7.15	-	-	✓
Oncorhynchus mykiss nelsoni	5.52	-	-	✓
Paralabrax clathratus	2.00	-	-	✓
Paralichthys californicus	1.24	1.60	-	-
Penaeus (Litopenaeus) vannamei	5.18	5.00 (Chow *et al*., 1990)	-	-
Rhinobatos productus	7.76	-	-	✓
Sarda chiliensis	1.76	-	-	✓
Sardinops sagax	2.17	2.69 (Ida *et al*., 1991)	2.03	-
Sebastes caurinus	1.94	-	-	✓
Sebastes constellatus	2.00	-	-	✓
Sebastes rubrivinctus	1.99	-	-	✓

Species	Genome size found in this study (pg/cell)	Cited by		First time reported
		Hinegardner and Rosen 1972	Hardie and Hebert 2004	
Semicossyphus pulcher	1.85	-	-	✓
Seriola lalandi (captive)	1.58	-	1.40	-
Seriola lalandi (wild)	1.64			
Sphyraena argentea	1.42	1.66	-	-
Sphyrna zygaena	5.29		-	✓
Totoaba macdonaldi	1.60	-	-	✓