Identification of sex in chickens by flow cytometry

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In chickens, the difference in DNA content between the Z and W sex chromosomes is measurable as a 2% greater amount of DNA in cells of males (ZZ) vs. females (ZW). High-resolution measurement of cellular DNA content (genome size) is possible by use of flow cytometry. In this technique, nuclei are stained with a nucleic-acid specific fluorochrome, and are passed single-file through a high-intensity light source such as a laser beam. The fluorescence emitted from each nucleus is directly proportional to the amount of DNA present, and can be used to quantify genome size on a per-cell basis. This technique is rapid (two or three samples per minute after a 10-minute staining period), accurate, and straightforward. Commercially available clinical and research instruments are generally expensive (US$80,000 to 400,000), but it may be possible to couple the principles of flow cytometry with existing egg handling equipment to enable rapid throughput for identification of sex in chicks before hatching.

Keywords: sex identification; flow cytometry; genome size; sex chromosomes

Introduction

Early sex identification is often desired for culling of agricultural animals. Many techniques have been developed to address this problem, yet satisfactory solutions are often lacking for specific applications. There are a number of techniques available for identifying the sex of chickens after hatching, but few are available for use before hatch. Techniques suitable for analysis of cell or fluid samples from eggs include molecular genetic procedures (*e.g.* polymerase chain reaction), immunochemical assays (*e.g.* colorimetry), and measurement of cellular DNA content (genome size). This last approach is based on the difference in DNA mass of the Z and W sex chromosomes (approximately 2% of the cellular DNA content). The purpose of this article is to overview the technical and biological considerations involved in identification of sex in chickens by the use of flow cytometry to measure genome size.

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Measurement of cellular DNA content

METHODS

A variety of techniques with different levels of precision and accuracy have been used to estimate genome size since work began in this area in the 1940's. These include DNA reassociation kinetics, fluorimetric methods, Feulgen staining, and flow cytometry. Analysis of the rate of DNA strand reassociation (Britten and Davidson, 1971) provides physical-chemical estimates of genome size, but offers low precision. Fluorometric determination of the total mass of DNA standardized to a known number of cells has been used to estimate genome size in hundreds of species with a measurement error of about 5% (Hinegardner, 1976). Microdensitometry of Feulgen-stained nuclei has also been used for the estimation of DNA content in phylogenetic surveys of genome size (*e.g.* Bachmann *et al.*, 1972), and can yield estimates precise enough to differentiate male and female in birds, based on ZZ-ZW sex chromosome heteromorphism (Rasch, 1976). Despite some discrepancies in the data reported by different authors, the values obtained with this technique agree with those obtained with other methods including flow cytometry (Rasch, 1985).

Flow cytometry can be used to obtain high-resolution estimates of nuclear DNA content (Hoehn *et al.*, 1977; Vindelov *et al.*, 1983; Elias *et al.*, 1988). In this method, the cells are lysed and stained with a fluorochrome specific for nucleic acid, such as propidium iodide. Cell lysis is assisted by use of a detergent (*e.g.* Triton X-100) and a hypotonic staining solution. Often RNase is added to remove extraneous RNA, which can bind the fluorochrome. By use of a laser (or other high-intensity light source) the nuclei are analysed individually for fluorescence, which is directly proportional to the amount of DNA that they contain (*Figure 1*). Several thousand nuclei can be analysed in a few seconds. In older instruments, the fluorescence of each nucleus is converted to an analog signal, and the signal is transmitted to a computer where it is digitised to generate pulse-height histograms. The newest generation of instruments can directly collect digitised data.

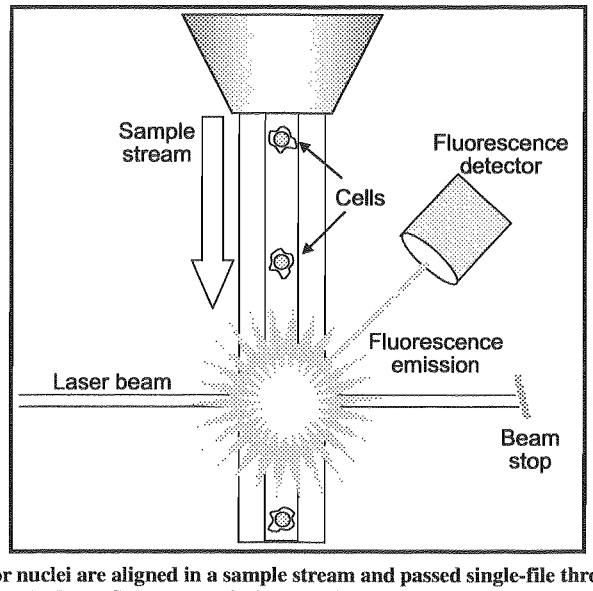

Figure 1 Lysed cells or nuclei are aligned in a sample stream and passed single-file through a laser beam. Following excitation by the laser, fluorescence is detected from the stained nuclei in direct proportion to the quantity of DNA present in each nucleus.

REFERENCE STANDARDS

Typically the DNA content of target cells is established relative to a standard DNA content in cells from a reference species (Vindelov *et al.*, 1983; Jakobsen, 1983; Tiersch *et al.*, 1989b). Standards are best used as internal references, when target cells and reference cells are mixed together and assayed simultaneously. The standard can be used for several calculations. The standard can be assigned a known DNA mass, against which nuclear DNA from target cells can be estimated directly as picogram quantities (10^{-12} gram) (Johnson *et al.*, 1987). Alternatively, a standard can be used as an internal reference in the analysis of individual samples, following which the reference is cancelled during estimation of a DNA Index (Elias *et al.*, 1988). An internal reference would also be cancelled during the calculation of picogram (pg) quantities of nuclear DNA relative to a separate standard that has a known DNA mass (and a known relationship to the internal reference). These last two calculations do not require knowledge of the precise DNA mass of the internal reference (Tiersch *et al.*, 1989a).

It is often necessary to normalize values in relation to a particular standard when relating one study to another (Tiersch *et al.*, 1989b). The chicken has been especially valuable as a reference species (Rasch *et al.*, 1971) and has an accepted value of 2.5 pg (Tiersch and Wachtel, 1991). Because the use of reference standards provides a relative measure of DNA content, it is crucial that the reference cells and the target cells have a similar DNA content to minimize possible technical variation such as zero shift error (Vindelov *et al.*, 1983), and in the case of an internal reference standard, that the values for the reference and target cells do not overlap.

Genome size

VERTEBRATES

Genome size is currently generally accepted to refer to the diploid DNA content of interphase cells, such as blood cells. The earliest studies were based on study of sperm and thus early literature will refer to haploid values for genome size. The biological significance of genome size has been a subject of interest since differences in the DNA content of cells from diverse species were first reported (Mirsky and Ris, 1951). Genome size was initially called the DNA "C-value" to reflect the assumed constancy observed among individuals of a given species. Subsequent work has shown that genome size is not a constant feature for each species, and variation within populations can be considerable (discussed below).

Differences in DNA content among taxonomic groups do not reflect evolutionary relationships. For example, DNA mass does not increase from primitive to advanced organisms, nor does it increase with genetic complexity (Cavalier-Smith, 1985). In fact, genome size has not steadily increased or decreased throughout evolutionary history, and increases and decreases each seem to have occurred within various groups. The bony fishes, for example, possess the largest (>200 pg) and smallest (<1 pg) values for genome size reported for vertebrates (Wachtel and Tiersch, 1993).

It is also well established that the quantity of genomic DNA greatly exceeds the quantity of DNA that is expressed — even in the smallest of vertebrate genomes. The variable and seemingly unpredictable nature of genome size has been perplexing and characterized as "the C-value paradox." Although the explanation for variation in genome mass remains obscure (*e.g.* Vinogradov, 1995; Vinogradov, 1997; Monaghan and Metcalfe, 2000), it can be evaluated at a number of levels (Wachtel and Tiersch, 1993) ranging across localized DNA duplications, chromosome polymorphisms (such as heterochromatic blocks or extra arms), sex chromosome differences, chromosomal aneuploidy (such as trisomy) (Elias *et*

al., 1988), and polyploidy (such as tetraploidy) produced through genome duplication. These mechanisms can work in combination and there can be losses as well as gains in DNA content.

BIRDS

Genome size has been studied in 135 species of birds representing 17 different orders by normalizing several data sets in relation to a standard diploid value of 2.5 pg DNA for the domestic chicken (Tiersch and Wachtel, 1991). The overall mean value for genome size was 2.82 pg, and the range of values was from 2.0 pg to 3.8 pg. These and other observations (Cavalier-Smith, 1978) show that birds have the smallest amount of among-species variation of any vertebrate class. The variation within avian species was also minimal. For example, the mean DNA mass in 49 Japanese quail *(Coturnix coturnix)* was 2.81 ± 0.02 pg (mean ± SD), with a range of 2.78 to 2.85 pg, and a variation of 2.5% (Tiersch and Wachtel, 1991).

The domestic chicken has exhibited a wide array of heteroploid forms including haploid-diploid, haploid-triploid, haploid-diploid-triploid, diploid-triploid and diploid-tetraploid (Fechheimer and Jaap, 1978; Thorne *et al.*, 1987). These combinations can involve complex origins, as in the case of the haploid-diploid chicken embryo derived from three paternal haploid genomes (Fechheimer and Jaap, 1978).

It is the small but measurable difference in DNA content of the Z and W sex chromosomes in birds that is relevant to sex identification in chickens. Thus, the small size and uniformity of the avian genome assist in the measurement of the sex-associated DNA content difference which is a minor component in the overall variation possible in genome size.

SOURCES OF BIOLOGICAL VARIATION

The DNA content observed in cells of a given species may vary, and within-species variation in DNA mass can be large, in some cases exceeding the variation observed between species (Gold and Amemiya, 1987). For example, within-population variation of 35% has been reported in pocket gophers (Sherwood and Patton, 1982). The organisms within a species can have different chromosomal morphologies and accordingly, variations in nuclear DNA content. For example, the human chromosomes 1, 9, 16 and Y were found to be most variable, followed by chromosomes 13, 14, 15, 21 and 22 (Harris *et al.*, 1986). The difference between the largest chromosome 1 and the smallest chromosome 1 was 21%, or almost 1% of the total genome (a mass greater than that of chromosome 21). The Y chromosome was especially variable with a difference of 86% found between the largest Y and the smallest. In another study, microspectrophotometric analysis of banded Y chromosomes revealed variation of 70%, attributed to the number of discrete blocks of heterochromatin present (Wall and Butler, 1989). Corresponding variation could be associated within particular groups for the avian W chromosome.

DNA aneuploidy, meaning an abnormal quantity of DNA in cells, is often associated with malignancy (Dressler *et al.*, 1988; Murphy *et al.*, 1986). For that reason flow cytometric evaluation of DNA aneuploidy is widely used in cancer diagnosis. In addition, mixtures of various cell types can cause a shift in DNA values above those of blood (Tiersch and Wachtel, 1993). For example, analysis of feather pulp yielded values higher than those of blood cells collected from the same bird (Tiersch and Mumme, 1993).

Strains produced by artificial selection, via breeding practices utilized in species of commercial importance, may differ in nuclear DNA content. Hybridisation, whether naturally occurring or caused by humans, may also affect DNA content. These and other factors could contribute genomic variation sufficient to mask the measurement of the Z-W difference. In other words, detailed study with specific lines or strains will be necessary to

define the ratio of signal (Z-W difference) to noise (other genomic variation) in determination of sex in chickens. It is likely that it will not be possible to unambiguously identify sex in every bird that is analysed. In previous work with Florida scrub jays, *Aphelocoma c. coerulescens*, there was a zone of overlap between the DNA values of the two sexes (Tiersch and Mumme, 1993) that produced a "gray zone" in sex identification (*Figure 2*).

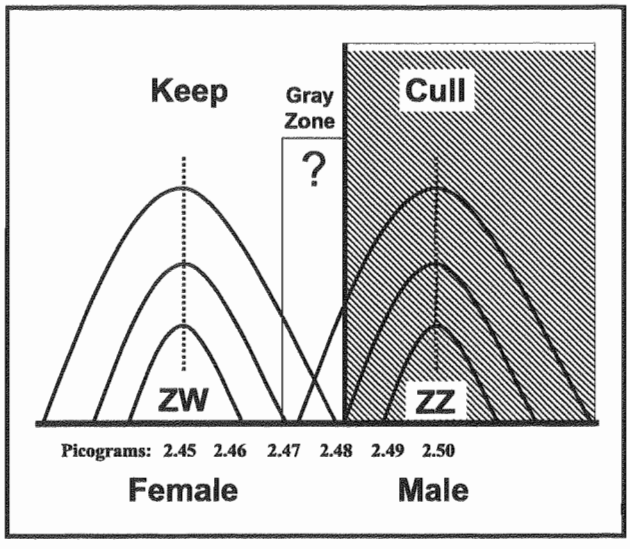

Figure 2 Male chickens (ZZ) possess around 2% more DNA in their cells than do females (ZW). Variation in DNA content of other chromosomes could obscure the sex difference and create uncertainty (a "gray zone") in identification of sex and culling of chicks. Thus a practical threshold for culling would have to be established. In this example, consider a population of laying chickens with a mean value of 2.45 pg for females and 2.50 pg for males. Depending on the level of variation around the means there could be overlap in DNA values for the two sexes (gra°y zone). A conservative cut-off value for culling could be established above 2.48 pg.

Sex identification by flow cytometry

Cellular DNA content is heritable in fishes (Tiersch and Goudie, 1993) and birds (Tiersch *et al.*, 1991), and is stable during ontogeny, allowing measurements to be made during early development, including before hatching in chicks. Flow cytometry has been used to distinguish differences in the sex chromosome complement of leukocytes of humans (Elias *et al.*, 1988), horses (Kent *et al.*, 1988), rattlesnakes (Tiersch, unpublished), and birds (Nakamura *et al.*, 1990). The differences observed are relatively consistent (around 2%) regardless of the sex determining mechanism or genome size (*Table 1*), and in general correlate with the degree of heteromorphism present between the sex chromosomes (Cavallo *et al.*, 1997). Within birds, for example, parrots and their relatives of the order Psittaciformes possess a relatively large (around 3%) sex-related DNA content difference (*Table 2*) and a correspondingly high degree of differentiation between the Z and W chromosomes (Nakamura *et al.*, 1990). On the other hand, members of the order Casuariiformes, such as the emu, *Dromiceius novaehollandiae*, have a small or non-existent DNA content difference (~0.6%), and as is characteristic of this order, do not possess morphologically distinct sex chromosomes.

Table 1 Representatives of three vertebrate classes (with the number of species studied in parentheses), mechanism of sex determination, approximate genome size, and actual and percent difference of cellular DNA content for males and females with heteromorphic sex chromosomes.

Class (species)	Sex-determining mechanism	Genome size (pg)	Male-female difference* Actual (pg)	Percent
Mammals (5)	XX-XY	7.0	0.14	2.0%
Reptiles (3)	ZZ-ZW	3.5	0.07	1.9%
Birds (30)	ZZ-ZW	2.8	0.03	2.3%

*Data compiled from: Nakamura *et al.*,1990; Elias *et al.*, 1988; Kent *et al.*, 1988, and Tiersch, unpublished.

Table 2 Male-female differences in genome size have been identified for representatives of several orders of birds. The DNA content differences correspond to the magnitude of size difference between the Z and W sex chromosomes (Nakamura *et al.*, 1990; Cavallo *et al.*, 1997).

Order (number of species)	Examples (common names)	Percent difference
Sphenisciformes (1)	Penguin	3.5%
Psittaciformes (13)	Parrots	3.2 + 2.0%
Anseriformes (6)	Ducks, geese	2.3 + 0.6%
Galliformes (4)	Chickens, pheasants	2.0 + 1.0%
Gruiformes (2)	Cranes	1.8 + 0.8%
Piciformes (1)	Barbet	1.6%
Falconiformes (2)	Eagles	1.5 + 1.0%
Casuariformes (1)	Emu	0.6%

Domestic chickens have a distinct Z and W, and roosters consistently possess about 2% more cellular DNA than do hens (*Table 3*). It is possible that different strains or lines of domestic chickens contain different mean values for DNA content, and different amounts of Z-W differentiation (*Table 3*). It would be informative in this regard to sample large numbers of chickens of both sexes across a wide variety of lines to quantify the range and types of genome size variation in this species.

Table 3 Genome size estimates and percent difference for male and female of four strains of domestic chickens (from Nakamura *et al.*, 1990).

Strain	Male	Female	Percent difference
Rhode Island Red x Barred Rock	2.50	2.44	2.7%
500 New Hampshire	2.50	2.45	2.3%
SC White Leghorn	2.45	2.40	1.9%
Rhode Island Red	2.44	2.40	1.5%
Mean ± SD	2.47 ± 0.03	2.42 ± 0.03	2.1 ± 0.5%

Considerations for commercial application

Standard flow cytometers such as those produced by Becton Dickinson Immunocytometry Systems (www.bdfacs.com) or Beckman Coulter Inc. (www.beckmancoulter.com) are typically configured as clinical or research instruments. For these, a minimum analysis time for high-resolution measurement of genome size (suitable for W-Z discrimination) would be 20-30 seconds per sample. Accordingly, this would yield estimates of 120-180

(~150) samples per hour and 960-1,440 (~1,200) samples in an 8-hour day. Throughput would also include a 10-minute period before analysis for sample staining.

The advantages of flow cytometry are that it is fast (compared to molecular genetic or immunochemical techniques), accurate, precise, and requires a small sample volume (*e.g.* less than 1 microlitre of blood). The technology can be standardized and provides research opportunities. The disadvantages are that flow cytometers are expensive, with clinical instruments starting around US$80,000 and research models costing as much as US$400,000. These types of instruments require trained personnel to operate and maintain them. The analysis is labour intensive, requires a tissue sample (blood cells), and can be susceptible to biological variation (the gray zone described above).

All told, conventionally configured flow cytometers would likely not be a good match for commercial-scale sexing of chicks. However, the principles of flow cytometry would still be applicable. Small bench-top instruments that use a mercury arc lamp instead of a laser, such as those produced by Partec, Inc. (www.partec-cytomics.com) are less expensive and can be used for sex identification in birds (De Vita *et al.*, 1994; Cavallo *et al.*, 1997). The coupling of genome size measurements with sampling modules designed for rapid throughput analysis of allantoic cells from eggs, such as those described by P. Phelps of Embrex, Inc. (www.embrex.com) during this conference, could enable rapid screening of sex in chicks before hatching.

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