Chapter 2

Genome Size Estimation and Quantitative Cytogenetics in Insects

J. Spencer Johnston, Angelina Bernardini, and Carl E. Hjelmen

Abstract

With care, it is possible using flow cytometry to create a precise and accurate estimate of the genome size of an insect that is useful for genomics, genetics, molecular/cell biology, or systematics. Genome size estimation is a useful first step in a complete genome sequencing project. The number of sequencing reads required to produce a given level of coverage depends directly upon the 1C amount of DNA per cell, while an even more critical need is an accurate 1C genome size estimate to compare against the final assembly. Here we present a detailed protocol to estimate genome size using flow cytometry. Published genome size estimates should be submitted to genomesize.com so that they are available to all.

Key words Flow cytometry, Chromatin structure, Endopolyploidy, Underreplication, Chromosomal sex determination, Sexual dimorphism, Ploidy, Cryptic species

1 Introduction

Accuracy and precision are important in genome size estimates. Genome size is expressed as 1C, or the amount of DNA in a gamete. As such, genome size estimation is a useful first step in a complete genome sequencing project. The number of sequencing reads required to produce a given level of coverage depends directly upon the 1C amount of DNA per cell, while an even more critical need is an accurate 1C genome size estimate to compare against the final assembly. As new technologies provide ever longer reads, the proportion of the genome assembled will approach the estimated genome size. However, large portions of the genome contain repeat elements, and these can vary in length. That variation means it will always be necessary to compare the assembled genome with the estimated size. While an accurate genome size estimate gauges the proportion of the genome assembled, an assembly that is greater than the estimate is a strong indication that structural variations, such as heterozygous inversions, have been assembled in

Susan J. Brown and Michael E. Pfrender (eds.), *Insect Genomics: Methods and Protocols*, Methods in Molecular Biology, vol. 1858, https://doi.org/10.1007/978-1-4939-8775-7_2,

tandem, rather than as alternative polymorphic sequences, and the tandem duplications have inflated the genome assembly.

Precision (estimates with small standard errors) is a necessity for intraspecific comparisons. Precise estimates of genome size by flow cytometry can help elucidate genome structure. Polyploidy, aneuploidy, and structural variants are revealed in comparisons within and between samples [1]. Cryptic species are often easily recognized by differences in DNA content [2]. Specialized DNA replication events, such as endopolyploidy [3, 4], chromatin diminution, or underreplication [5] can be discovered by comparing different tissues. Chromatin structure (euchromatin vs. heterochromatin) may be quantified by appropriate estimates of insect genome size [5, 6]. Differences between the genome size of males and females of a species provide feedback on the sex determination systems [7] and the role of endopolyploidy in sexual dimorphism [8]. The relative age of a "Y" (or w) sex chromosome is reflected by the extent of degeneration of sequences over time [9]. That degeneration is reflected by the difference in size of the genome of the homogametic and heterogametic sexes. A neo-X/Y is suggested when the genome size of heterogametic sex exceeds that of the homogametic sex. In a haplo/diplo species, a 2:1 ratio of genome size is seen in male/female comparisons. The presence of both a haploid peak and a second diploid peak in adult hymenopteran males revealed that acquired diploidy occurs in many, but not all adult males [10].

Intraspecific genome size variation exists and again the precision of estimates is an important consideration. Different strains and different inbred lines can differ significantly in genome size [11], and these differences may influence the choice of material for genome studies. Genome size variation between strains may also reflect life history differences [12, 13] that have important implications for genome size evolution.

2 Materials

1. Galbraith's buffer (*see* **Note 1**): 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.10% v/v Triton X, pH 7.0. Place a stir bar in a 1 L beaker on a stir plate. Add 450 mL distilled H_2O, and while stirring slowly, add 4.4 g sodium citrate ($Na_2C_6H_6O_7{\cdot}2H_2O$), 2.1 g MOPS (3-[*N*-morpholino]-propane sulfonic acid), 2.13 g $MgCl_2{\cdot}6H_2O$, 0.5 mL Triton X-100, and 50 μL 10 mg/mL RNAse A (*see* **Note 2**). Bring the total volume up to 500 mL with dH_2O. Slowly add HCl solution to lower the pH to 7.2 (*see* **Note 3**). Filter to sterilize and store in a sterilized container.
2. Propidium iodide (PI) (*see* **Note 4**) 1 mg/mL stock solution: Add 1 mg PI to 1 mL of Galbraith's buffer, or, if purchasing

100 mg quantities of PI make 100 mL and store in smaller aliquots at −80 °C.

3. 2 mL Dounce tissue grinder set with (required) type A (loose fitting) and (rarely required) B (tight fitting) pestles.
4. Pipet filter: Cut the ends off 2 1 mL pipet tips. Place 41 μm nylon mesh between pipet tips.

3 Methods

3.1 Tissue Preparation

1. Dissect live or fresh frozen tissue to be analyzed (*see* **Note 5**). Standards and unknowns should be prepared, stained, and measured together in the same tube (*see* **Note 6**).
2. Place tissue in 1 mL of ice-cold Galbraith's buffer in a 2 mL Dounce tissue grinder. Keep on crushed ice prior to grinding.
3. Grind the tissue with 15 gentle strokes using the A (loose) pestle (~3 strokes per 2 s) (*see* **Note 7**).
4. Filter through pipet tips containing nylon mesh into a micro-centrifuge ("bullet") tube. Store on crushed ice.

3.2 Staining

1. Add Galbraith's buffer as needed to bring samples to 1 mL, add 25 μL PI stock and mix by inverting and gently shaking the tubes.
2. Store samples in the dark on ice or refrigerate for at least 20 min before running on the flow cytometer. Because the sample and standard may saturate at different rates, precision increases if all samples are run after the same period of staining; accuracy requires that saturation rates be compared by running the sample and standards over increasing stain time, working out the stain time when genome size reaches a plateau (Fig. 1). Some material may only plateau after samples stain for up to 24 h (*see* **Note 8**).

3.3 Running the Samples

1. Set cytometer to activate (trigger) on fluorescence, not scatter. Even with fluorescence activation, a tighter cleaner peak will be observed using a gate that excludes anything with high scatter (Fig. 2a–c). This gate will limit the output to fluorescence of clean intact nuclei. Because the goal is to determine the mean fluorescence from tight, symmetric G1 peaks from the sample and standard, a linear scale is essential (*see* **Note 9**). Log scales do not work for genome size estimation.
2. Set the flow rate to the slowest possible setting. This ensures that the nuclei are aligned in the center of the activating laser, minimizing the width of the fluorescence peaks. The slow rate will mean that fluorescence can be monitored over time. A second gate, based on red fluorescence over time, should be

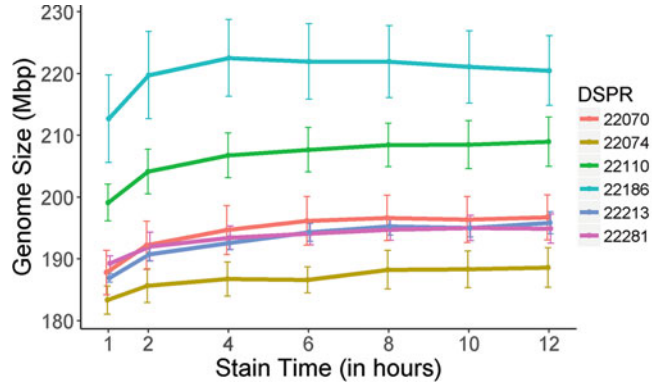

Fig. 1 Estimated genome size as a function of stain time. The estimated genome size will plateau at the most accurate value when the sample and standard reach the same levels of saturation. As shown here, optimal stain time can vary not only between species, but also among strains. Here, sample (DSPR strains of *Drosophila melanogaster*) and standard (*D. virilis* 1C = 328 Mbp) reach equal saturation levels after 4–8 h of stain time

used to select only the period when fluorescence output is constant (Fig. 2d). A tight, narrow peak is the best indication that the preparations are free of cytological tags from broken nuclei and other cell debris, and that the nuclei are aligned directly in the path of the exciting light. A tight peak is also critical to reduce bias due to overlap of peaks from the sample and standard (*see* **Note 9**).

3.4 Calculating Genome Size

Genome size determination is based on the relative position of the G1 (unreplicated) peaks of the sample and standard. It is critical that the ploidy of the first real (G1) fluorescent peak be correctly determined. To ensure this is so, the voltage to the red fluorescence photomultiplier tubes (PMT) must be adjusted with care. Genome size can vary between individuals by several orders of magnitude, and even within a single tissue preparation, highly polyploid nuclei may exist that produce a clean peak that is 2, 4, 8, or even 1200 times greater than that of the first G1 (diploid or haploid) nuclei (Fig. 3). In order to ensure the G1 peak is being estimated, the channel position of the red fluorescence should be adjusted upward until counts accumulate in the lowest channels, indicating unstained debris. Then the voltage should be decreased until the first clean tight peak is observed. This is the G1 peak that needs to be estimated. The red fluorescent peak position can then be adjusted for accurate estimation (channels 200–800 on many instruments). With a new sample, it is often a good idea to set up the flow cytometer with an easily prepared standard, such as *D. melanogaster*, and follow that with a sample (if sufficient material is available) of the unknown by itself. This allows you to determine

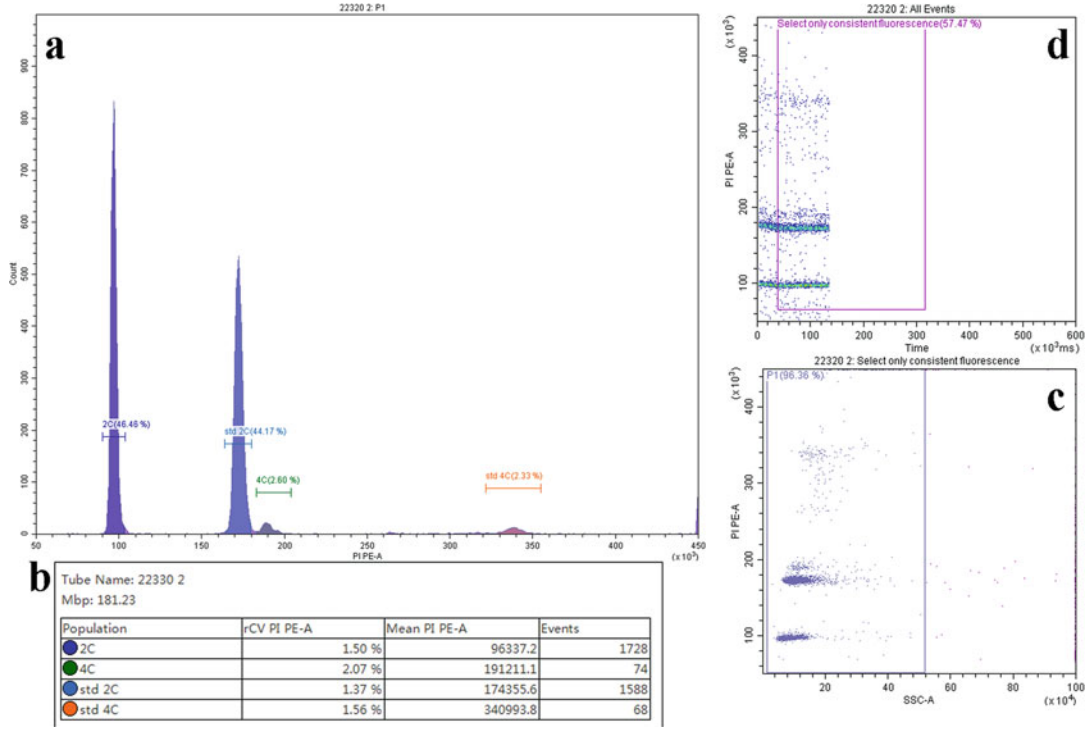

Fig. 2 Real-time flow cytometric histogram and cytograms. The G1 peaks of the sample and standard based on relative red propidium iodide fluorescence from PI-stained intact G1 nuclei is displayed as a histogram (**a**). The mean channel number of the G1 peaks, the number of nuclei scored under each peak, and the coefficient of variation for each peak is output (**b**) by internal flow cytometry software based on statistical gates set by the operator. A scatter gate (**c**) is set to exclude material with high scatter based on a cytogram showing red fluorescence versus side or forward scatter. A second time gate includes only unchanging fluorescence levels (**d**). To ensure that the histogram peaks are based on clean isolated nuclei free of cytological tags and free of fluorescence variation over time, the histogram is built from fluorescence output that meets criteria set by both scatter and time gates

the instrument settings for the sample, and allows you to choose the appropriate standard (discussed in the following paragraph). While the positions of separately run sample and standard peaks are important for selection of a standard and instrument settings, they should not be used for estimation of genome size.

3.5 Statistical Analysis

Methods have been devised to compensate for inhibitors and non-linear responses. Among the most complete of these is described in [14]. These methods are most useful for plant material where multiple compounds, anthocyanin, caffeine, and tannins inhibit staining. Except for gall forming wasps that develop in the heavy tannins of oak galls, the methods are excessive for insect estimates. In general, the statistical gates built into the cytometer software are adequate to find the mean or peak position. Ideally the peaks are symmetric and the peak average channel number is the parameter of

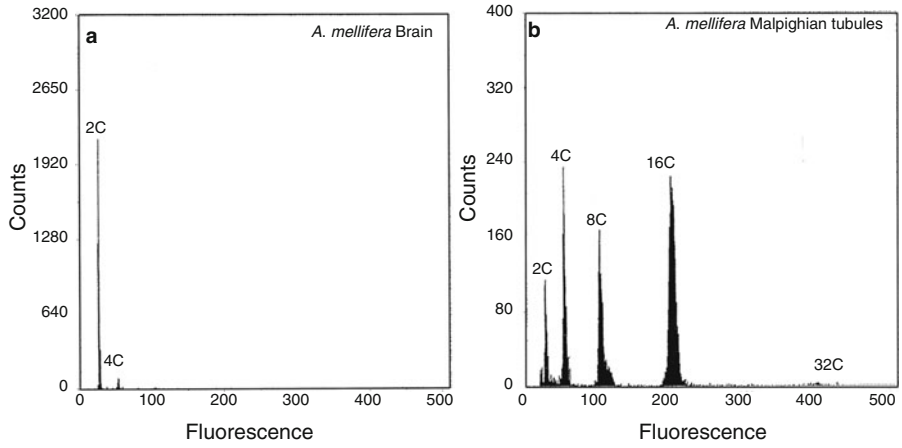

Fig. 3 Peaks produced by differing ploidy levels. Peaks produced by nuclei from G1, G2, and higher levels of endopolyploidy in the brain and Malpighian tubules of the worker honey bee, *Apis melifera*. Tissue suitable for genome size estimation will have a single strong symmetric G1 peak (**a**). Identification of the G1 peak may be problematic for tissues with nuclei at high ploidy levels (**b**)

choice to produce the lowest overall standard error about the mean. If the peak is skewed, the statistical gates can be set tighter around the peak, or the peak position itself may be used to provide a lower standard error. Second party software is available to fit a normal distribution about peaks. However best practice cytometry is preferred to methods that recover values from skewed or kurtotic peaks. Once the G1 peaks for the sample and standard have been identified and the mean or peak position scored, the amount of DNA in the unknown is a simple ratio of the average peak position of the unknown divided by the peak position of the standard times the 1C amount of DNA in the standard.

Care must be taken to interpret the genome size expressed as 1C. For the homogametic sex (X/X or Z/Z) the 1C value represents the amount of DNA in a gamete. For the heterogametic sex (X/O, X/Y, Z/O, Z/Z, Xi/Xj/Y, . . .) the 1C is the average of the two gametes produced. To find the difference in genome size associated with the sex chromosome, it is necessary to double the estimate then subtract. For example, to find the difference in size of the X and Y, where A represents the autosomal chromosome size common to both sexes, X – Y = 2A + XX – 2A + XY. Similarly, the size of the X can be found in an X/O system as 2A + XX - 2A + X/O = X.

The CV (determined as the width at half the peak height or mean/variance) is useful to report the quality of the preparation and cytometry, but not useful for statistics. Calculation of average and standard errors using the PIVOT Table in MS Excel, or similar program is encouraged, as it is easy to recalculate estimates as new data are added, and easy to identify outlier values that inflate the

SE. Replication has the advantage that it allows reporting of the mean and the standard error of the mean (*see* **Note 10**). An average also solves another problem. A single genome estimate is a ratio of the peak position of the G1 nuclei from the sample and the standard. As such, it is a Cauchy distribution with no defined mean and variance. The central limit theorem ensures us that the average will be more normally distributed, thereby reducing problems with assumption underlying statistical tests.

The methods described here are useful for flow cytometric genome size estimates based on live or fresh frozen insects. Alternative methods that have been used to estimate genome size have been discussed elsewhere [15, 16]. The method described here was first described by Hare and Johnston 2011 [17]. The stain amount has been adjusted, additional information is provided for the different steps, and suggestions are added in an effort to provide greater accuracy as needed to determine the percent completion of assembled genomes.

4 Notes

1. Galbraith's buffer has the advantage that samples are stable over time, and cytometry permitting, allows technical replicates over time. Other buffers that have been recommended for problematic plant material [18] work well for insects and may produce excellent results with shorter stain times.
2. RNAse A can be purchased ready-to-use or as powder. If purchased as powder, it is important to boil the final stock solution 10 min to eliminate any DNase that might contaminate the product.
3. A pH slightly above 7.2 is ok, but not below 7.2. If the pH is too low, add NaOH to raise it to 7.2.
4. Propidium iodide is recommended as the stain, even though it stains both DNA and RNA and is actively pumped from living cells. Propidium iodide is recommended because it is stochastic (increases linearly with DNA amount), relatively insensitive to methylation and compaction of DNA, and produces an unbiased estimate, provided RNA has been removed by RNase. Other stains are available, such as DAPI and Geimsa that are specific for DNA and stain the nuclear DNA in whole cells. However, when used alone these stains produce biased estimates. The stains are sequence specific, which means estimates based on fluorescence reflect not only the actual genome size but also the AT/GC ratio. These stains also require shorter or longer wavelength excitation that is often not available on basic instruments. When instruments are available to score both PI and one or more of the sequence-specific stains, the

comparison of the estimates may be of interest, as an indication of the AT/GC ratio associated with specific repeats [19]. That information has been used to quantify intraspecific variation associated with variation in the repeats in sex chromosomes, telomere/centromere repeats, and highly repetitive sequences in supernumerary or "B" chromosomes.

5. The choice of tissue is often an easy one. The adult head of an adult insect usually has one strong peak of G1 nuclei (Fig. 3a). That peak is primarily produced by nuclei isolated from the soft, neural tissue. There are exceptions however, such as the highly polyploid tissues of the Malpighian tubules (Fig. 3b). Some biting insects, such as sandflies, have potent DNase in the salivary glands that will degrade the DNA in the co-prepared nuclei of the standard and the unknown. Careful dissection of neural tissue is required to create good peaks. Dung beetles may also have DNase that degrades DNA. Thorough washing and careful dissection of tissue without contact with the cuticle may help there. In a few insects, such as the cicada, stained nuclei isolated from the brain do not produce a tight peak. Fortunately, a single cicada eye is adequate to produce nuclei for a strong clean G1 peak. Two other examples where the head contained no suitable tissues for genome size estimates are pseudoscorpion (a chelicerate) and shrimp (a crustacean). The pseudoscorpion produces a clean G1 peak from the pedipalps and not from any other tissue. For shrimp, a small segment of a leg produces a strong G1 peak; other tissues fail to do so. Given that a peak of high quality and a suitable quantity of G1 nuclei cannot be produced with many tissues, it is always wise to try several tissues and identify not only ones that produce a strong tight peak, but also ones that produce a single strong peak from G1 nuclei.
6. Selection of the species to be used as the standard is very critical for co-preparation with the unknown. The standard should have a well-established genome size and produce one strong peak, with little background and few secondary peaks that may overlap the peak from the unknown. Ideally, the standard should fall between the 2C and 4C peaks of the unknown. More often, one must settle for a standard that is between ½X and 2X the size of the unknown. A standard chosen for its phylogenetic position relative to the unknown is likely to lead to bias, unless the genome size of that standard has been determined by a large number of replicates against another well-established standard from a model organism. The estimated genome size of the standard is one of the largest sources of bias in genome size estimates. Model insect species are recommended for standards for most comparisons [20]. The male of the seed beetle, *Callosobruchus maculatus* 1.23 pg.

(1C = 1205 Mbp), available from Carolina Biological Supply is an excellent alternative to chicken red blood cells (CRBCs) that are commonly used for estimates of genome size in that range. CRBCs will not saturate at the same rate as an insect cell, so a saturation curve must be run with the co-prepared sample and CRBC. Care must be taken as well to find the correct genome size of the CRBCs that are used. The literature includes values (2C) of 2.54 pg. (1C = 1240 Mbp), 2.78 pg. (1C =1360 pg.), and 2.33 pg. (1C = 1140 Mbp) for CRBCs. If the CRBC's are from a hen, these same values can be 0.04 pg. (1C = 20 Mbp) less. Bennett et al. found that 2.33 pg. [21], as originally estimated by Galbraith for nuclei extracted from a white leghorn rooster [22], provides values that agree well with the estimated values for model insect species.

7. Tissue grinding is an art, but one that is easily mastered. The goal of grinding is to release clean nuclei in numbers needed, without excessive debris. Grinding too fast or using too many strokes produces background from DNA released from and caught within broken nuclei. Too few strokes or grindingly too slowly releases few nuclei with subsequent long run times to accumulate 1000+ nuclei under G1 peaks. While fifteen strokes at a rate of three strokes every 2 s is generally a good starting point, soft bodied insects such as aphids may need fewer strokes, while hard bodied ones, including many of the beetles, may require slower grinding speeds.
8. The recommendation of 20 min of stain time is a minimum and assumes that the rate of stain uptake is the same for the sample and standard, which is not always true. In mosquitos with relatively large genomes, such as *Aedes*, the chromatin saturates slowly. Estimates taken after 20 min, 1 h, and 4 h will show the genome of the mosquito increasing by 10% or more. To ensure saturation of stain in the sample and standard, it is best to score the co-preparation after they have stained for different periods of time. The estimated genome size will eventually stabilize; it may be necessary to stain overnight to ensure equal stain saturation in the sample and standard.
9. The most challenging step for an experienced flow cytometer technician is the unavoidable variation in sample preparation for genome size estimates. Genome size estimation preparations are relatively crude and even the most careful grinding and filtering will result in broken nuclei, nuclei with adhering bits of DNA from broken nuclei and debris from the cell. All of these will scatter light and the very low scatter of a cleanly isolated nucleus is the best selection criteria for use in genome size estimates. The cytometer itself is a very important issue in genome size estimates. The cytometer should have a single excitation source in blue (488 nm) or green (514–533 nm)

and two parameter output, for red fluorescence and forward or side scatter. Most importantly, the cytometer must be capable of producing a peak from a stained nuclear preparation from a *Drosophila melanogaster* yellow body, white eye strain (y/w) that is symmetric with a coefficient of variation (CV) of 2.0 or less. Overlap of sample and standard peaks, which is inevitable with broad peaks, will bias the estimate. The red fluorescent output from the cytometer must also be linear. All manufacturers claim their cytometers are linear, yet nearly all have an offset from zero in order to produce a symmetric peak for unstained nuclei. The best way to demonstrate linearity is to run sufficient numbers of nuclei to create reliable 2C and 4C peaks. If the 4C is not twice the 2C, linearity is a problem. Running samples and standards between channel 200 and 800 will reduce, but not entirely eliminate bias due to nonlinearity. Running samples and standards that are close to one another, but not overlapping, also helps. Clean tight peaks are even more critical in tests for small intraspecific differences. The critical test for real differences is to co-prepare samples that were scored as different when prepared individually. If the differences are artifacts of preparation [23], the co-preparation will produce a single peak with a CV that is almost as low as that of either sample alone (Fig. 2b). In contrast, real genome size differences, whether between sexes or between individuals of the same sex reared in different locations and different environmental conditions, will be revealed by the presence of two side-by-side florescence peaks.

10. The offset built into the cytometer is not always the same each day and that can produce a significant day effect. The only easy solution is to run replicates of an unknown on different days. Lacking that, it is possible to produce an estimate that is very precise, but not accurate. Two types of replicates are generally possible and always desirable with cytometry. The sample may be split into two or three aliquots and each run as a technical replicate. These are most useful to determine the optimal stain time, but will also provide feedback on machine (run-time) error. Different preparations from different tissues will provide feedback on errors due to preparation and tissue type and will help determine the optimal tissue for use in in biological replicates. The more important replication is biological. Whenever possible, co-preparations of five samples plus the standard should be prepared and run on different days. Biological replication has the advantage that it allows reporting of the average genome size and the standard error.

References

1. Jacobson AL, Johnston JS, Rotenberg D, Whitfield AE, Booth W, Vargo EL, Kennedy GG (2013) Genome size and ploidy of thysanoptera. Insect Mol Biol 22(1):12–17. https://doi.org/10.1111/j.1365-2583.2012.01165.x
2. Lower SS, Spencer Johnston J, Stanger-Hall K, Hjelmen CE, Hanrahan SJ, Korunes K, Hall D (2017) Genome size in North American fireflies: substantial variation likely driven by neutral processes. Genome Biol Evol 9 (6):1499–1512. https://doi.org/10.1093/gbe/evx097
3. Rangel J, Strauss K, Seedorf K, Hjelmen C, Johnston J (2015) Endopolyploidy changes with age-related polyethism in the honey bee, *Apis mellifera*. PLoS One 10(4):e0122208. https://doi.org/10.1371/journal.pone.0122208
4. Scholes DR, Suarez AV, Paige KN (2013) Can endopolyploidy explain body size variation within and between castes in ants? Ecol Evol 3 (7):2128–2137
5. Johnston JS, Schoener M, McMahon DP (2013) DNA underreplication in the majority of nuclei in the *Drosophila melanogaster* thorax: evidence from Suur and flow cytometry. J Mol Biol Res 3(1):47
6. Bosco G, Campbell P, Leiva-Neto JT, Markow TA (2007) Analysis of *Drosophila* species genome size and satellite DNA content reveals significant differences among strains as well as between species. Genetics 177(3):1277–1290
7. Barcenas N, Thompson N, Gomez-Tovar V, Morales-Ramos J, Johnston J (2008) Sex determination and genome size in Catolaccus grandis (Burks, 1954) (Hymenoptera: Pteromalidae). J Hymenopt Res 17:201–209
8. Johnston J, Ross L, Beani L, Hughes D, Kathirithamby J (2004) Tiny genomes and endoreduplication in Strepsiptera. Insect Mol Biol 13(6):581–585
9. Bachtrog D (2013) Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. Nat Rev Genet 14(2):113–124
10. Aron S, de Menten L, Van Bockstaele DR, Blank SM, Roisin Y (2005) When hymenopteran males reinvented diploidy. Curr Biol 15 (9):824–827
11. Huang W, Massouras A, Inoue Y, Peiffer J, Ramia M, Tarone AM, Turlapati L, Zichner T, Zhu D, Lyman RF, Magwire MM, Blankenburg K, Carbone MA, Chang K, Ellis LL, Fernandez S, Han Y, Highnam G, Hjelmen CE, Jack JR, Javaid M, Jayaseelan J, Kalra D, Lee S, Lewis L, Munidasa M, Ongeri F, Patel S, Perales L, Perez A, Pu L, Rollmann SM, Ruth R, Saada N, Warner C, Williams A, Wu YQ, Yamamoto A, Zhang Y, Zhu Y, Anholt RRH, Korbel JO, Mittelman D, Muzny DM, Gibbs RA, Barbadilla A, Johnston JS, Stone EA, Richards S, Deplancke B, Mackay TFC (2014) Natural variation in genome architecture among 205 *Drosophila melanogaster* genetic reference panel lines. Genome Res 24 (7):1193–1208. https://doi.org/10.1101/gr.171546.113
12. Arnqvist G, Sayadi A, Immonen E, Hotzy C, Rankin D, Tuda M, Hjelmen CE, Johnston JS (2015) Genome size correlates with reproductive fitness in seed beetles. Proc R Soc B 282 (1815):20151421
13. Ellis LL, Huang W, Quinn AM, Ahuja A, Alfrejd B, Gomez FE, Hjelmen CE, Moore KL, Mackay TFC, Johnston JS, Tarone AM (2014) Intrapopulation genome size in *D. melanogaster* reflects life history variation and plasticity. PLoS Genet 10(7):e1004522. https://doi.org/10.1371/journal.pgen.1004522
14. Barre P, Noirot M, Louarn J, Duperray C, Hamon S (1996) Reliable flow cytometric estimation of nuclear DNA content in coffee trees. Cytometry 24(1):32–38
15. Desalle R, Gregory TR, Johnston JS (2005) Preparation of samples for comparative studies of arthropod chromosomes: visualization, in situ hybridization, and genome size estimation. Methods Enzymol 395:460–488
16. Gregory TR, Nathwani P, Bonnett TR, Huber DP (2013) Sizing up arthropod genomes: an evaluation of the impact of environmental variation on genome size estimates by flow cytometry and the use of qPCR as a method of estimation. Genome 56(9):505–510
17. Hare EE, Johnston JS (2011) Genome size determination using flow cytometry of propidium iodide-stained nuclei. In: Orgogozo V, Rockman MV (eds) Molecular Methods for Evolutionary Genetics, vol 772. Humana Press, New York, NY, pp 3–12. https://doi.org/10.1007/978-1-61779-228-1_1
18. Loureiro J, Rodriguez E, Doležel J, Santos C (2006) Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. Ann Bot 98 (3):515–527
19. Šmarda P, Bureš P, Šmerda J, Horová L (2012) Measurements of genomic GC content in plant

genomes with flow cytometry: a test for reliability. New Phytol 193(2):513–521

20. Hanrahan SJ, Johnston JS (2011) New genome size estimates of 134 species of arthropods. Chromosom Res 19:809–823. https://doi.org/10.1007/s10577-011-9231-6
21. Bennett MD, Leitch IJ, Price HJ, Johnston JS (2003) Comparisons with Caenorhabditis (~100 Mb) and Drosophila (~175 Mb) using flow cytometry show genome size in Arabidopsis to be ~157 Mb and thus ~25% larger than the Arabidopsis genome initiative estimate of ~125 Mb. Ann Bot 91(5):547–557
22. Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220(4601):1049–1051
23. Bennett MD, Price HJ, Johnston JS (2007) Anthocyanin inhibits propidium iodide DNA fluorescence in Euphorbia pulcherrima: implications for genome size variation and flow cytometry. Ann Bot 101(6):777–790