Variation of osteocyte lacunae size within the tetrapod skeleton: implications for palaeogenomics

Shaena Montanari*, Stephen L. Brusatte, Wendy De Wolf and Mark A. Norell

American Museum of Natural History, Richard Gilder Graduate School, Central Park West at 79th Street, New York, NY 10024, USA

*Author for correspondence (smontanari@amnh.org).

Recent studies have emphasized the ability to reconstruct genome sizes (C-values) of extinct organisms such as dinosaurs, using correlations between known genome sizes and bone cell (osteocyte lacunae) volumes. Because of the established positive relationship between cell size and genome size in extant vertebrates, osteocyte lacunae volume is a viable proxy for reconstructing C-values in the absence of any viable genetic material. However, intra-skeletal osteocyte lacunae size variation, which could cause error in genome size estimation, has remained unexplored. Here, 11 skeletal elements of one individual from each of four major clades (Mammalia, Amphibia, Aves, Reptilia) were examined histologically. Skeletal elements in all four clades exhibit significant differences in the average sizes of their lacunae. This variation, however, generally does not cause a significant difference in the estimated genome size when common phylogenetic estimation methods are employed. On the other hand, the spread of the estimations illustrates that this method may not be precise. High variance in genome size estimations remains an outstanding problem. Additionally, a suite of new methods is introduced to further automate the measurement of bone cells and other microstructural features on histological thin sections.

Keywords: osteocyte lacunae; genome size; palaeogenomics; bone histology

1. INTRODUCTION

There is a well-established positive correlation between genome size (C-value) and the sizes of many cells in living vertebrates [1]. Osteocytes are the most abundant cell type in mature bone and are contained within small holes (lacunae) in the bone tissue [2]. In fossil organisms, even though the osteocytes are absent, their size and shape are preserved because the lacunae remain. Although red blood cell size is the most frequent proxy for genome size, it has been demonstrated that in their absence, the size of osteocyte lacunae can also serve as a valuable proxy [3]. This allows the estimation of genome size in extinct animals for which no preserved genetic material is available, and unlocks the once-intractable possibility of studying large-scale patterns of genome evolution over deep time [3–6].

The use of lacunae size to estimate genome size, and study genomic evolution, in long-extinct organisms is an emerging area of research. However, one potentially confounding problem has yet to be addressed. Published studies have been inconsistent in their choice of bones for lacunar measurements, and measurements from different types of bone shape (long, irregular, flat) have been included in the same datasets [3–6]. Previous work has hinted that there may be variation in the size of osteocyte lacunae across the skeleton of an individual [6], and if true, this may compromise the integrity of genome size estimates based on measurements from different types of bone. Differences in lacunae size among organisms could be owing to the uneven sampling of disparate bones, and not reflect true variation in genome size.

Here, we provide to our knowledge, the first rigorous examination of osteocyte lacunae size variation in living vertebrates, and show that, although there are significant differences in lacunae size among different bones, genome size estimates are mostly accurate in the face of this variation. With this being said, however, the overwhelming amount of variation present in measurements made at each step in the genome estimation process renders the method imprecise. Therefore, caution is recommended when estimating traits based on highly variable biological features, in this case osteocyte lacunae.

2. MATERIAL AND METHODS

Four representative taxa were chosen for thin sectioning: woodchuck (Marmota monax), Chinese alligator (Alligator sinensis), tiger salamander (Ambystoma tigrinum) and rock pigeon (Columba livia). For each individual specimen, 11 bones were transversely sectioned: tibia, fibula, rib, ulna, femur, thoracic vertebra, caudal vertebra, metatarsal, humerus, skull and radius. Long bones were transversely sectioned in the midshaft region. Histological thin sections were prepared according to established guidelines [7]. Bones were embedded in cold-setting resin, cut on a low-speed diamond saw, and subsequently ground and polished until desired optical clarity was reached. High-resolution micrographs were taken with a Zeiss Evo 60 environmental scanning electron microscope at the American Museum of Natural History. Osteocyte lacuna area was measured using the free NIH program ImageJ [8]. The image was cropped and segmented based on contrast-based thresholding. The ‘Analyze Particles’ feature was then used to automatically outline and calculate the area for all lacunae in the image area (figure 1). The average lacunae areas from different bones within the same taxon were statistically compared using ANOVA in Microsoft Excel 2008. Volumes were also calculated (see the electronic supplementary material), as published datasets have used volumes to estimate genome size [3–6]. For genome size estimation, the mean natural logarithm of osteocyte lacuna volume for each bone in each taxon was entered into a web-based program, sPlot, which uses a phylogeny and a maximum-likelihood framework to estimate missing parameters [9]. It was recently established that incorporating phylogenetic information in this way is far more accurate than non-phylogenetically informed methods [3]. Our data were added to the dataset and phylogeny of Organ et al. [4]. Estimated genome sizes for our four taxa were compared with measured C-values from the Animal Genome Size Database [10]. Because there is variability in the measured C-values for individual taxa in the database, we performed a sensitivity analysis using the minimum, maximum and average C-values for each species in order to determine the effect of variability on the genome size prediction (see the electronic supplementary material).

3. RESULTS

At p < 0.05, all four taxa have significantly different osteocyte areas and volumes across all bones sectioned...
Although it was previously shown that the size and shape of osteocyte lacunae vary between compact and cancellous bone in one studied taxon [11], variation in osteocyte measurements between numerous different bones of the skeleton had remained unexplored until the present study. Furthermore, the measurement method employed here should be more accurate, and less prone to human error, than the techniques of previous studies, as areas of all lacunae are automatically calculated using a computer program rather than by measuring the length and width by hand [3–6].

Our results unmistakably show that there is statistically significant variation in osteocyte lacuna size across the skeleton of extant vertebrates. With this established, the important question is: to what degree does this variation affect genome size estimates? Are C-value estimates relatively robust to variation in lacuna size among different bones, or is predicting genome size based on bone cell size doomed to intractable error? This is critical, because genome size is a correlate of many attributes of organismal physiology, such as metabolic rate, that are notoriously difficult to assess in fossil organisms [12].

Although there is demonstrable variation in the volume and area of osteocyte lacunae in the four vertebrates sampled, genome size estimates are often accurate despite this variability. The tiger salamander and rock pigeon measurements performed the best, with the vast majority of skeletal elements, including all bones in the rock pigeon, predicting the measured C-value within 2 s.e.m. The genome size estimations for woodchuck and Chinese alligator, on the other hand, were not as accurate, as the majority of skeletal elements incorrectly predicted the genome size. This illustrates that genome size estimates based on osteocyte size may not always be accurate on a taxon-by-taxon basis, which raises serious doubts about the reliability of genome size reconstruction. This is not surprising, however, given that Organ et al. [3] found an overall significant correlation between osteocyte lacuna size and genome size in living vertebrates based on a large dataset of 26 species, but several cases of individual taxa in which this relationship does not hold. As a result, their regression analysis found that osteocyte size predicted only 32 per cent of the variation in genome size, meaning most of the variation is not described by this relationship. It is important to note, however, that phylogenetic regressions still offer a vast improvement in the

Table 1. ANOVA for (a) osteocyte lacuna areas (bold p-value indicates significance at a level of 0.05) and (b) osteocyte lacuna volumes.

<table>
<thead>
<tr>
<th>taxon</th>
<th>d.f.</th>
<th>F</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tigrinum</td>
<td>10</td>
<td>4.799</td>
<td>2.97E–05</td>
</tr>
<tr>
<td>M. monax</td>
<td>10</td>
<td>6.457</td>
<td>2.30E–09</td>
</tr>
<tr>
<td>C. livia</td>
<td>10</td>
<td>2.031</td>
<td>0.04</td>
</tr>
<tr>
<td>A. sinensis</td>
<td>10</td>
<td>9.708</td>
<td>1.99E–13</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. tigrinum</td>
<td>10</td>
<td>3.95</td>
<td>0.00026</td>
</tr>
<tr>
<td>M. monax</td>
<td>10</td>
<td>8.92</td>
<td>1.72E–13</td>
</tr>
<tr>
<td>C. livia</td>
<td>10</td>
<td>3.13</td>
<td>0.0015</td>
</tr>
<tr>
<td>A. sinensis</td>
<td>10</td>
<td>7.08</td>
<td>1.16E–09</td>
</tr>
</tbody>
</table>

Figure 1. An illustration of the automated contrast-based thresholding measurement method. (a) The raw photograph of a woodchuck ulna obtained from scanning electron microscopy. (b) The same image cropped with the threshold adjusted so that the lacunae are highlighted. (c) The image once the lacunae have been outlined and measured by the ‘Analyze Particles’ feature in ImageJ. Certain cells with other features outline, such as canaliculi seen in the bottom right of the image, can be manually excluded because each outlined cell is numbered on the image and in the resulting measurement output.
accuracy of genome size estimations over simple linear regressions [3,6].

Variability in lacunar size is also a vexing problem. First, there is no systematic pattern in osteocyte lacuna size across the skeleton of all vertebrates. In other words, there is not one bone or bone type that always results in the smallest or largest osteocyte measurements in the four taxa sampled. Instead, it seems as if osteocyte size is essentially randomly variable across the vertebrate skeleton. Therefore, consistently measuring osteocytes from the same bone would not be expected to improve resolution, standardize data collection or reduce measurement error.

In addition, even within bones, osteocyte size can be highly variable: each histological slide contains abundant osteocyte lacunae, so there will always be a standard error of measurement. Problematically, the commonly used phylogenetic estimation methods do not take this variability into account, because only one value for ‘measured osteocyte size’ can be entered. This is clearly a quandary for genome estimations, because the standard errors of the lacunae volume calculations are disproportionally large, often more than 50 per cent of the average calculated volume for that bone. Therefore, using only an average measure to estimate the genome size effectively fails to propagate uncertainty by not taking standard errors into account.

We do find that average osteocyte size in many bones, despite their variability, still accurately predicts genome size, at least in the sense that the 95 per cent confidence interval of the estimation includes the measured value in the Animal Genome Size Database. Accuracy, however, must not be mistaken for precision, and this should be remembered when estimating genome size based on highly variable osteocyte sizes.

Figure 2. A visual representation of the estimated genome size measurements for all bones in each of the four taxa sampled. The filled circles represent the mean estimated C-value in picograms (pg) and the lines extending from them represent a spread of 2 s.e. around the mean. The horizontal dashed lines represent the average measured C-value for each taxon from the Animal Genome Size Database. (a) Woodchuck; (b) pigeon; (c) tiger salamander; and (d) Chinese alligator.
In summary, considerable variability in osteocyte lacunae measurements across the skeleton shows that lacunae size are an imperfect, but in some cases still useful, proxy for genome size estimation. We have found three important results: (i) the size of osteocyte lacunae in compact bone vary within an individual; (ii) lacunae size measured on most bones are often an accurate method for predicting genome size, but this is not true for each taxon; and (iii) variability in osteocyte size is often large, meaning that genome size estimations are imprecise. Together, these qualms mean that genome size estimates may not always be accurate, and are usually far from precise, but in the absence of genetic material in fossil organisms, they still prove our only means to trace the large-scale trends of genomic macroevolution in deep time. Coarse questions, such as whether Mesozoic theropod dinosaurs had relatively small genomes on par with those of avians [3], should be tractable with this method, but genome size estimates for individual taxa should not be considered accurate or precise.

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