C. R. Palevol 15 (2016) 77–85

Contents lists available at ScienceDirect

Comptes Rendus Palevol

www.sciencedirect.com

General Palaeontology, Systematics and Evolution (Vertebrate Palaeontology)

Early genome size increase in urodeles

Expansion précoce de la taille du génome chez les urodèles

Michel Laurin[a], Aurore Canoville[a,b,*], Mikayla Struble[c], Chris Organ[c,d], Vivian de Buffrénil[a]

[a] *UMR 7207, Centre de recherches sur la paléobiodiversité et les paléoenvironnements, Sorbonne Universités, CNRS/MNHN/UPMC, Muséum national d'Histoire naturelle, département « Histoire de la Terre », bâtiment de géologie, case postale 48, 57, rue Cuvier, 75231 Paris cedex 05, France*

[b] *Steinmann Institute for Geology, Mineralogy and Paleontology, University of Bonn, Nußallee 8, 53115 Bonn, Germany*

[c] *Department of Earth Sciences, Montana State University, Bozeman, MT 59717, USA*

[d] *Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717, USA*

ARTICLE INFO

Article history:
Received 22 October 2014
Accepted after revision 21 December 2014
Available online 26 March 2015

Handled by Jorge Cubo

Keywords:
Karauridae
Lissamphibians
Paleogenomics
Osteocyte lacunae
Inferences

ABSTRACT

Urodeles have the largest genomes among extant tetrapods, varying greatly between metamorphic and neotenic species, which have the smallest and the largest genomes of the group, respectively. The evolutionary tempo and mode of genome size expansion in urodeles are poorly documented, especially because genome size does not directly fossilize. Consequently, the ancestral state for genome size, and therefore, the polarity of its evolution in urodeles are uncertain. However, recent studies have demonstrated that osteocyte (lacuna) size is correlated with genome size. Below, we present histological data, on osteocyte lacuna size from one of the oldest known stem-urodeles, *Marmorerpeton*, from the Middle Jurassic (Bathonian, 166–168 Ma), as well as on five extant urodele species. Our analysis of these taxa, coupled with previously published data, suggests that stem-urodeles had already evolved large genomes, typical of extant urodeles by the Bathonian.

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Mots clés :
Karauridae
Lissamphibiens
Paléogénomique
Logettes ostéocytaires
Inférences

RÉSUMÉ

Les urodèles présentent les génomes les plus grands parmi les tétrapodes actuels, avec une différence entre les espèces métamorphiques et les espèces néoténiques, qui possèdent respectivement les plus petits et les plus grands génomes pour ce groupe. Le tempo évolutif et le mode d'expansion de la taille du génome chez les urodèles sont mal documentés, car la taille du génome ne se fossilise pas directement. L'état ancestral de la taille du génome, et par conséquent, le sens de son évolution chez les urodèles sont donc incertains. Cependant, des études récentes ont démontré que la taille des logettes ostéocytaires est corrélée avec la taille du génome. Ci-dessous, nous présentons des données histologiques, sur la taille de logettes ostéocytaires de *Marmorerpeton*, l'un des plus anciens urodèles basaux du Jurassique moyen (Bathonien, 166–168 Ma), ainsi que de cinq espèces actuelles d'urodèles.

* Corresponding author. UMR 7207, Centre de recherches sur la paléobiodiversité et les paléoenvironnements, Sorbonne Universités, CNRS/MNHN/UPMC, Muséum national d'histoire naturelle, département « Histoire de la Terre », bâtiment de géologie, case postale 48, 57, rue Cuvier, 75231 Paris cedex 05, France.

E-mail address: canoville.aurore08@gmail.com (A. Canoville).

http://dx.doi.org/10.1016/j.crpv.2014.12.006
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L'analyse de ces taxons et de données déjà publiées suggère que les urodèles basaux présentaient déjà, dès le Bathonien, de grands génomes typiques des espèces actuelles.
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1. Introduction

Genome size varies by several orders of magnitude, from less than 0.001 picogram (pg) in some bacteria to several hundred pg in some unicellular eukaryotes (Gregory, 2004). Among vertebrates, the largest genomes occur in dipnoans, followed closely by urodeles (Gregory, 2004). Among amniotes, the smallest genomes (about 0.91 pg) are found in hummingbirds (Gregory et al., 2009). In extant urodeles (salamanders and newts), genome size varies about twelve-fold between metamorphic taxa, which have the smallest genomes of the group, with a C-value around 10 pg in the plethodontid *Gyrinophilus porphyriticus* (Goin et al., 1968), and neotenic taxa (Gregory, 2002), which have the largest genomes of the group, with a C-value around 120 pg in the proteid *Necturus lewisi* (Olmo, 1973). The relationship between large genome size and neoteny in amphibians, first proposed decades ago (Commoner, 1964; Goin et al., 1968) is supported by recent studies (Gregory, 2002), and has interesting paleobiological implications, which we explore below. Numerous other phenotypic traits, such as metabolism or growth rate (Waltari and Edwards, 2002), developmental complexity (Gregory, 2002), and nucleotide substitution rates (Herrick and Sclavi, 2014), may also be correlated with genome size.

The great variation in genome size of extant tetrapods leaves considerable uncertainty about the ancestral condition for amniotes, amphibians, and tetrapods (Organ et al., 2011). For instance, the taxonomic distribution of genome size in extant taxa led Mirsky and Ris (1951) to suggest that the ancestral tetrapod genome was large; a conclusion subsequently refuted using paleogenomic data (Thomson, 1972), which showed that the first dipnoans had relatively small genomes. Recent work has reached ambiguous conclusions about competing hypotheses of genome size evolution in tetrapods (Organ et al., 2011). For instance, Rho et al. (2009) suggested that the mammalian genome underwent multiple independent contractions following the K/Pg biological crisis, which occurred about 65.5 Ma ago. This suggestion is inconsistent with more recently obtained paleogenomic data that suggest a fairly small ancestral amniote genome size, which, at about 3.34 pg, was close to that of the mammalian average (Organ et al., 2011). Thus, paleogenomic data play an important role in constraining evolutionary scenarios about the evolution of genome size in vertebrates.

The suggestion (Vialli and Sacchi Vialli, 1969) and subsequent demonstration (Organ et al., 2007) that osteocyte lacuna volume is correlated with genome size have thus allowed considerable progress in our understanding of vertebrate genome size evolution. This has led to clarifications about genome size evolution in Paleozoic dipnoans (Thomson, 1972), Mesozoic dinosaurs (Organ et al., 2007, 2009), pterosaurs (Organ and Shedlock, 2009), and Paleozoic tetrapods (Organ et al., 2011).

Urodele genome size evolution is particularly challenging to infer because genome size is highly variable in this taxon and because genome size appears to have expanded apparently long ago, by the time crown-salamanders appeared (Sun and Mueller, 2014). Common methods used to infer ancestral character values are inappropriate when evolutionary trends are present, though accounting for path lengths in non-ultrametric trees can mitigate this problem (Pagel, 1999). Urodeles have been suggested to have ancestrally had a relatively small genome (i.e. closer in size to extant urodeles with the smallest genome; Roth et al., 1994), which is intuitive because most other vertebrates have smaller genomes (Gregory, 2004). However, this hypothesis has yet to be tested with paleogenomic data. Some preliminary steps in this direction were recently taken. Organ et al. (2011) reassessed the relationship between osteocyte lacuna volume and genome size using a dataset of 54 extant tetrapod species, including four urodele and six anuran extant terminal taxa. The most relevant paleontological data in that study were from three Paleozoic taxa, namely the lepospondyls *Brachydectes, Cardiocephalus*, and *Diplocaulus*, which have been suggested to be on the amphibian stem (Marjanović and Laurin, 2009, 2013), although others view these as stem-amniotes (Ruta and Coates, 2007). Mapping of observed genome size of extant taxa and inferred genome size of extinct taxa on a chronogram suggested that Paleozoic amphibians retained a mid-sized genome (C-value around 4 pg), that stem-batrachians had a greater genome (around 16 pg), and that early urodeles had a greater genome still (around 30 pg) (Organ et al., 2011). However, genome evolution in early lissamphibians was poorly documented in that study because it was based on ten extant amphibian species and no fossil data on lissamphibians. Thus, additional inferences on early urodele genome size should better constrain genome size evolution in urodeles.

Below, we present data about osteocyte lacuna volume and genome size from two specimens of the oldest known stem-urodeles, *Marmorerpeton* sp. (Evans et al., 1988). These fossils come from the Kirtlington locality and are interpreted as possible karaurids (Marjanović and Laurin, 2014). Given the Middle Jurassic age (Bathonian) of this locality (Evans et al., 1988), no older urodele remains are known (Averianov et al., 2008; Evans and Waldman, 1996; Skutschas, 2013). Finally, we also refined our inference model for urodeles (Organ et al., 2011) by adding three new extant urodele taxa for which the genome size is known, in order to yield more reliable paleogenomic estimates.

2. Materials and methods

2.1. Newly studied urodele material

We standardized our sampling of additional bones to cross-sections of stylopod (proximal) limb bones (humeri

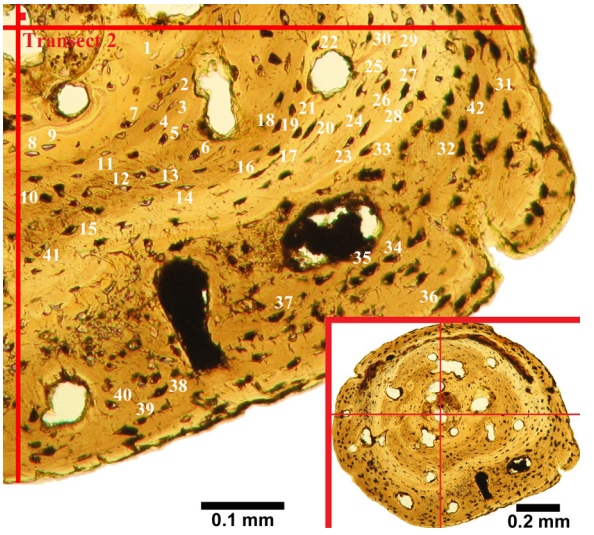

Fig. 1. (Color online.) One of the transects (2) of a section of humerus I of *Marmorerpeton* on which osteocyte lacunae were measured. The red dot close to the intersection between the perpendicular lines that delimit the transects was considered to be the ontogenetic center of the section and was thus used to measure the relative distance between the osteocyte and the section's center. Individual lacunae that were measured are identified by numbers. To facilitate viewing of these numbers in print, their size was increased, which in some cases may hamper identification of the designated lacuna. For better identification of these, see the version of this image with smaller numbers in the SOMs.

Fig. 1. (Couleur en ligne.) Un des transects (2) de la section de l'humérus I de *Marmorerpeton*, sur lequel les logettes ostéocytaires ont été mesurées. Le point rouge, proche de l'intersection entre les lignes qui délimitent les transects est considéré comme étant le centre ontogénétique de la section osseuse et a été utilisé comme référence pour mesurer la distance relative entre chaque logette ostéocytaire et le centre de la section. Les lacunes qui ont été mesurées sont indiquées par des nombres.

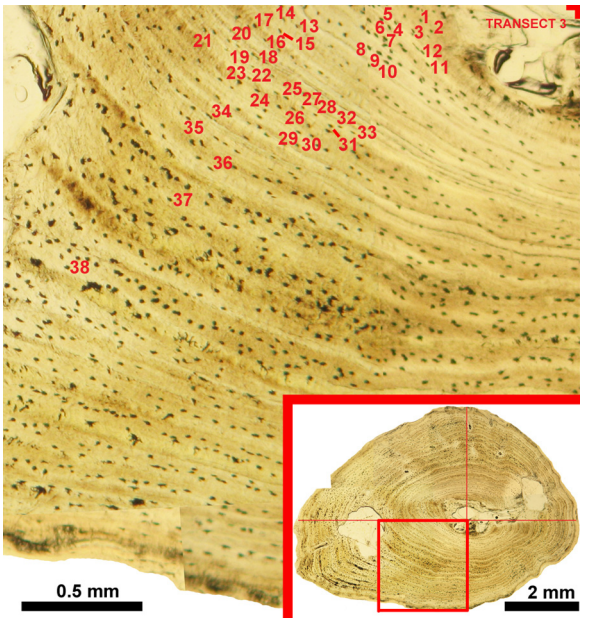

Fig. 2. (Color online.) One of the transects (3) of the *Andrias japonicus* femur on which osteocyte lacunae were measured. The intersection of the perpendicular lines delimiting the transects was considered to be the ontogenetic center of the section. In this case, the section is so large and the osteocyte lacunae so densely packed that the numbers indicate only the approximate location of the measured osteocytes. See the SOMs for more precise location data.

Fig. 2. (Couleur en ligne.) Un des transects (3) de la section fémorale d'*Andrias japonicus* sur lequel les logettes ostéocytaires ont été mesurées. L'intersection des lignes perpendiculaires qui délimitent les transects a été considérée comme étant le centre ontogénétique de la section. Dans ce cas, la section est tellement grande et la densité des logettes ostéocytaires tellement élevée, que les numéros n'indiquent que très approximativement la position des logettes mesurées. Voir les annexes pour des données de position plus précises.

for *Marmorerpeton* and femora for extant taxa) to minimize the loss of precision that bone identity or the section plane introduces into paleogenomic size estimates (D'Emic and Benson, 2013; Montanari et al., 2011; Stein and Prondvai, 2014). We could not use the same bone throughout the analysis because of limitations in the available material. We collected various measurements regarding osteocyte lacuna size and distance to the center of the section on two humeri of two post-metamorphic individuals of *Marmorerpeton*, namely those studied by Buffrénil et al. (2014). Section I is located at the mid-diaphysis of humerus I, whereas section II comes from the metaphysis of humerus II (Fig. 1). We measured 117 lacunae from section I, and 62 from section II. We also took osteocyte lacuna measurements for five extant urodele taxa: 30 from *Ambystoma andersoni*, 17 from *A. mexicanum*, 191 from *Andrias japonicus* (Fig. 2), 26 from *Cryptobranchus alleganiensis*, and 46 from *Desmognathus*. We thus measured a total of 489 lacunae (see the Supplementary On-line Material, SOM). Of these extant taxa, *A. mexicanum*, *A. japonicus*, and *C. alleganiensis* were not included in our previous inference model for urodeles (Organ et al., 2011), although their genome size is known. The model was therefore improved in the present study using these additional taxa to yield more reliable estimates.

2.2. Histomorphometric measurements and indices

Each measured osteocyte lacuna was identified by a number (and sometimes transect number) on a picture (SOM; see also Figs. 1 and 2). The recorded measurements are:

(i) lacuna dimensions (long axis and small axis of the lacuna, where both are orthogonal to each other, and the maximal length is the maximal dimension of the lacuna on a given plane);
(ii) estimated volume [assuming an ellipsoid and calculated as follow: $4/3 \times \pi \times (\text{small axis}/2)^2 \times (\text{long axis}/2)$];
(iii) bulk index (which corresponds to a ratio between the small axis and the long axis of the lacuna, and describes the overall shape of the latter; the lower the bulk index is, the more fusiform the osteocyte lacuna is; the highest possible bulk index, 1, corresponds to a circular outline of the osteocyte lacunae on the section plane);
(iv) relative distance to the center.

We thus measured, for each lacuna, its distance to the ontogenetic center of the section (where growth seems to

have been initiated), the distance between that center and the bone surface (periphery of the section), and divided the first distance by the second. This yields a relative distance, bounded between 0 and 1, which expresses the position of the osteocyte lacuna along the radius of the bone section (Girondot and Laurin, 2003).

2.3. Statistical analyses

We first looked into one more possible factor (apart from section plane and bone identity) influencing osteocyte lacuna volume and shape, namely its position (central or peripheral) on the bone section. This procedure was prompted by our observation that peripheral lacunae of *Marmorerpeton* are very large, but they are also darker, probably because of infilling with minerals (permineralization). We were concerned that this might introduce a diagenetic artifact into the procedure, if peripheral osteocyte lacunae were used to assess genome size. To assess the biological significance of this possible pattern, we performed comparable analyses on cross-sections of extant urodeles. Osteocyte lacuna volume, and the bulk index were then regressed (as dependent variables) against the relative distance to the center (independent variable), using Statistica® (Statsoft France, 2003). We also regressed, for a subset of the data, lacuna estimated volume against bulk index, to assess a possible interaction between both variables.

To assess the potential impact of bone identity on our inferences, we performed a sensitivity analysis. For this, we removed all data points of the previously published dataset (Organ et al., 2011) derived from non-long bones in order to minimize the error induced by bone identity. We thus retained only sections from femora, humeri, and to a lesser extent tibia for extant species (anurans were thus absent in this dataset, with a total of 36 extant tetrapod species; the full data set contained 57 taxa). The phylogenetic framework follows Organ et al. (2011) with the additional species positioned according to Marjanović and Laurin (2007, 2014). We used the program BayesTraits V2.0 (http://www.evolution.rdg.ac.uk) to perform phylogenetic comparative analyses. The Markov Chain Monte Carlo (MCMC) option was used to produce posterior distributions of regression models while estimating phylogenetic signal (λ) of the data, given the tree. We used a binary dummy variable during the regression to separate urodeles from other tetrapods, due to their extremely large genome sizes. This resulted in a multiple linear regression model that had considerably more support than a simple linear regression model, as shown by their respective AIC (see Organ et al., 2011 for details on these models). Phylogenetically-informed predictions of genome size in *Marmorerpeton* were made by sampling regression models of genome size and osteocyte lacuna volume for extant taxa (both natural log transformed). These predictions (retrodictions) are adjusted by reference to the variance-covariance matrix derived from the tree (but scaled by λ) and are therefore informed by the extinct species' phylogenetic position (see Organ et al., 2007, 2011 for additional details). The MCMC settings were 2,001,000 iterations with a burn-in of 100,000 and a sample period of 1000. We used uninformed flat priors (−100.00 to 100.00). According to standard statistical protocol, outliers in our lacuna volume dataset were identified, taxon by taxon, in JMP and excluded from analysis. Standard phylogenetic regression assumes uniform rates of evolution across a phylogeny. We therefore tested our osteocyte lacuna data for variable rates of evolution in BayesTraits using the protocol of Venditti et al. (2011). The MCMC setting for variable rate tests were: 10,000,000 iterations, sampled every 1000 iterations, with a 1,000,000 burn-in, with flat priors as above. Ancestral values of genome size were inferred using a random walk model in BayesTraits using the time-calibrated tree or a tree in which the branch lengths have been transformed to represent variable rates of evolution. We evaluated hypotheses using Bayes factors where the marginal likelihoods were sampled using a stepping stone algorithm (Xie et al., 2010), or by differences in posterior probabilities from a null value (e.g., the posterior deviation of the slope parameter from the null value of 0).

Given that we performed several tests, we corrected for multiple tests using the False Discovery Rate (FDR below) (Benjamini and Hochberg, 1995), one of the best available methods, which retains more power than classical Bonferroni corrections (Curran-Everett, 2000).

3. Results

Simple linear regressions show that osteocyte lacuna volume in *Marmorerpton* increases with distance from the center (Table 1). This is true both on individual transects of both sections and when data for each section are merged. This reconfirms our initial suspicion, based on visual inspection (Fig. 1), as it is visually obvious on a bivariate plot of lacuna volume vs. distance from the center (Fig. 3). By contrast, none of the extant urodeles tested appears to display a similar pattern; marginally significant relationships were found for two of the three transects of the *A. japonicus* femur tested (Fig. 2), but these probabilities are no longer significant after corrections for multiple tests through FDR, which indicates that all probabilities greater than 0.01 are not significant. Furthermore, the polarity (sign of the slope) of the relationship in these two transects is reversed (Table 1), which also shows that these two results for *A. japonicus* are spurious.

The bulk index appears to show a slightly negative trend with distance from the center. However, significant results (even after corrections for multiple tests through FDR) were found only for humerus II of *Marmorerpeton*, when including all measured osteocytes (Table 2), and for one of the transects of the *A. japonicus* section. Thus, this point will need to be investigated using a larger sample.

We did not find any relationship between osteocyte lacuna volume and bulk index (Table 3), though we investigated this only in *Marmorerpeton*, because only in this taxon did we find good evidence for a pattern of change in lacuna volume against distance to the center.

For our long bones-only data set we find a strong relationship between genome size and osteocyte lacunae volume ($n = 36$, average posterior slope for urodeles = 0.67 (99% pp support), average posterior slope for non-urodeles = 0.25 (96% pp support), average posterior

Table 1
Pattern of osteocyte lacuna volume as a function of distance from the center of the section. This was tested by a linear regression of lacuna volume (dependent variable) against relative distance (standardized to an interval of 0 to 1) between lacuna and ontogenetic center on the section. The null hypothesis is that the slope is 0. As shown by the probabilities in the last column, in *Marmorerpeton*, there is a positive relationship between lacuna volume and distance from the center (peripheral lacunae are larger), but this does not apply to extant urodeles tested. For some taxa, the section had been divided into transects, and the relationship was tested both within individual transects and over the whole section, by merging data on individual transects. For *Marmorerpeton*, we tested both with, and without the darkest, most peripheral osteocytes, whose size seems less reliable, both for individual transects (when applicable; not all transects displayed these) and for entire sections. Legend: significant probabilities in bold type; those that are no longer significant after correction for multiple tests through FDR are in bold, italics type; *n*, number of osteocyte lacunae measured.

Tableau 1
Volume des logettes ostéocytaires en fonction de la distance au centre de la section. Testé par une régression linéaire entre le volume des logettes (variable dépendante) et la distance relative (standardisée à un intervalle compris entre 0 et 1) entre les lacunes ostéocytaires et le centre ontogénétique de la section. L'hypothèse nulle est que la pente est égale à 0. Les probabilités de la dernière colonne montrent que, chez *Marmorerpeton*, il y a une relation positive entre le volume des logettes ostéocytaires et la distance au centre (les logettes périphériques sont plus grandes), mais cette relation n'est pas vérifiée pour les espèces d'urodèles actuelles testées dans cette étude. Pour certains taxons, la section a été subdivisée en transects, et la relation a été testée pour chaque transect individuellement et pour la section complète, en rassemblant les données des différents transects. Pour *Marmorerpeton*, les tests ont été faits avec et sans les logettes périphériques sombres, pour lesquelles la taille semble moins fiable, et ce, pour chaque transect individuellement (quand ceci est applicable; tous les transects ne présentent pas de telles logettes) et pour les sections complètes. Légende : les probabilités significatives sont en gras ; celles qui ne sont plus significatives après correction pour tests multiples avec FDR sont en italique et en gras ; *n*, nombre de logettes ostéocytaires mesurées.

Taxon	Bone	Transect	*n*	Slope	*p* (slope = 0)
Marmorerpeton	Humerus II	1	35	3027.07	**0.000014**
		1	30	2004.92	**0.001229**
		2	27	4946.22	**0.00053**
		2	21	3176.82	***0.033836***
		1–2	62	3713.81	**$<10^{-6}$**
			51	2306.22	**0.000555**
	Humerus I	1	35	1677.39	**0.000077**
		1	33	956.99	**0.006274**
		2	42	1083.33	**0.000393**
		2	41	881.24	**0.00099**
		3	24	1128.40	**0.000001**
		3	19	859.41	**0.000368**
		4	16	621.64	***0.043575***
		1–4	117	1080.15	**$<10^{-6}$**
		1–4	109	835.04	**$<10^{-6}$**
Ambystoma andersoni	Femur	NA	30	–95.23	0.908353
Ambystoma mexicanum	Femur	NA	17	469.17	0.699369
Andrias japonicus	Femur	1	54	1158.677	***0.012879***
		3	38	15759.79	0.450095
		4	99	–978.39	***0.028658***
		1, 3, 4	191	–787.09	0.597378
Cryptobranchus alleganiensis	Femur	NA	26	–138.34	0.68539
Desmognathus	Femur	1	25	–844.82	0.139347
		2	21	237.034	0.69748
		1–2	46	–387.32	0.342486

$r^2 = 0.69$, and average posterior $\lambda = 0.65$). These results suggest that statistical models relating genome size to cell size are robust, despite various sources of error. The results reported below use all bone samples in our database.

For our full data set regression model ($n = 57$) the posterior slope parameters for the urodele group and non-urodele group deviate from 0 (99% and 92% respectively). The mean of the posterior distribution for $r^2 = 0.6$. We also find moderate levels of phylogenetic signal (mean $\lambda = 0.76$) and no evidence for variable rates of evolution in osteocyte lacuna volume (Bayes factor = 1.4). Interestingly, when variable rates are tested using extant species alone, we find more, but still weak, support for variable rates of evolution (Bayes factor = 4.2). Phylogenetically-informed predictions of genome size for *Marmorerpeton* (Fig. 4) (by considering it an urodele through the binary dummy variable) suggest that it had a large genome (ln *C*-value = 3.6, $\sigma = 0.19$; about 36.7 pg, with σ encompassing values ranging from 30.2 pg to 44.2 pg), which is consistent with its fairly large osteocyte lacunae (Table 4). We estimate that the most recent common ancestor of *Marmorerpeton* and extant urodeles had a genome size of 3.5 ln *C*-value, $\sigma = 0.2$ (about 33.1 pg, with σ encompassing values ranging from 27.2 to 40.3 pg).

4. Discussion

Recently, the relationship between osteocyte lacuna volume and genome size has been shown to be more complex than previously thought. D'Emic and Benson (2013) and Montanari et al. (2011) showed that osteocyte lacuna volume varies not only interspecifically, but also between various bones of each species. The plane at which bones are sectioned also influences estimates of osteocytic lacuna volume (Stein and Prondvai, 2014). However, given that we have used only cross-sections of bones, and that most bones that we used are from the stylopod (humeri and femora), these sources of errors (which could generate bias) have been minimized to the extent that availability of the material allowed it. We nevertheless find a strong relationship between genome size and osteocyte lacuna volume. These results suggest that statistical models relating genome size to cell volume are robust, despite various sources of error.

Table 2
Pattern of the bulk index as a function of distance from the center of the section. For methods and legend, see Table 1.
Tableau 2
Indice de forme en fonction de la distance au centre de la section. Pour la méthode et les légendes, se référer à la légende du Tableau 1.

Taxon	Bone	Transect	*n*	Slope	*p* (slope = 0)
Marmorerpeton	Humerus II	1	35	−0.40156	***0.021538***
		1	30	−0.35998	0.068272
		2	27	−0.49943	0.055164
		2	21	−0.40435	0.266299
		1–2	62	−0.44481	**0.002268**
		1–2	51	−0.38846	***0.029373***
	Humerus I	1	35	−0.07015	0.67762
		1	33	−0.07629	0.697485
		2	42	0.07571	0.308563
		2	41	0.08395	0.275145
		3	24	−0.05310	0.575209
		3	19	−0.14849	0.291421
		4	16	−0.3275	0.031311
		1–4	117	−0.02628	0.643726
		1–4	109	−0.03377	0.599329
Ambystoma andersoni	Femur	NA	30	0.07498	0.761401
Ambystoma mexicanum	Femur	NA	17	0.19605	0.709492
Andrias japonicus	Femur	1	54	0.13923	***0.04924***
		3	38	0.84796	0.459147
		4	99	−0.24305	**0.000036**
		1, 3, 4	191	−0.18521	***0.036319***
Cryptobranchus alleganiensis	Femur	NA	26	0.03227	0.728149
Desmognathus	Femur	1	25	−0.54728	0.174548
		2	21	−0.26943	0.539477
		1–2	46	−0.39495	0.173356

Table 3
Relationship between osteocyte lacuna volume and bulk index. This was investigated only on the sections where the strongest indication of a relationship between lacuna volume and distance to the center had been found (hence, only in *Marmorerpeton*). For methods and legend, see Table 1.
Tableau 3
Relation entre le volume des logettes ostéocytaires et l'indice de forme. Cette relation n'a été testée que pour les sections pour lesquelles une forte relation entre le volume des logettes ostéocytaires et la distance au centre a été préalablement trouvée (et donc seulement chez *Marmorerpeton*). Pour la méthode et les légendes, se référer à la légende du Tableau 1.

Taxon	Bone	Transect	*n*	Slope	*p* (slope = 0)
Marmorerpeton	Humerus II	1–2	62	−1335.39	***0.049546***
		1–2	51	−681.04	0.218322
	Humerus I	1–4	117	−14.34	0.960826
		1–4	109	−1.63	0.994579

Notwithstanding the variations in genome size obtained from the humerus and femur (Montanari et al., 2011), the magnitude of our estimate of genome size in *Marmorerpeton* falls within the range of extant urodeles.

One potential remaining artefact that might affect our paleobiological inferences concerns the pattern of osteocyte lacuna volume change along the radius of the section in *Marmorerpeton*. This pattern is difficult to interpret because we could not detect it in extant urodeles. This could possibly be an artifact caused by a greater permineralization of superficial (in the outer cortex) lacunae than deep ones, which might make the lacunae more visible, perhaps to the point of introducing a bias in size measurement. This effect might be especially worrisome if the permineralization extended deep into the canaliculi of the osteocytes, thus, greatly inflating their apparent volume.

Table 4
Osteocyte lacuna measurements gathered in the present study and used for paleogenomic analysis. Only extant urodele species for which the genome size was known have been used in this analysis and added to the dataset previously published in Organ et al. (2011) to infer the genome size of *Marmorerpeton*. Outliers in the dataset were excluded prior to analysis; hence, not all measured lacunae were used. This explains the discrepancy between numbers in this table and the SOM.
Tableau 4
Mesures des logettes ostéocytaires collectées dans cette étude et utilisées pour l'analyse paléogénomique. Seules les espèces d'urodèles actuelles pour lesquelles la taille du génome est connue ont été utilisées dans cette analyse, en supplément du jeu de données déjà publié dans Organ et al. (2011), pour inférer la taille du génome chez *Marmorerpeton*. Les valeurs aberrantes ont été éliminées avant analyse ; par conséquent, certaines logettes ostéocytaires n'ont pas été prises en compte. Cela explique les différences de valeurs entre ce tableau et les SOM.

Taxon	*n*	Average lacuna volume (μm^3)	σ Lacuna volume
Ambystoma mexicanum	17	465.4	226.4
Andrias japonicus	184	1421.86	699.15
Cryptobranchus alleganiensis	26	641.9	384.3
Marmorerpeton	179	614.3	331.0

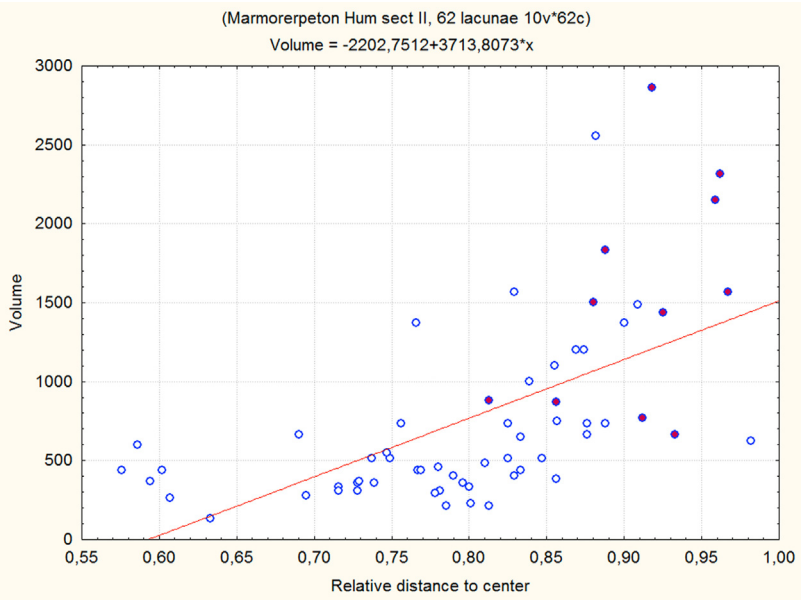

Fig. 3. (Color online.) Bivariate plot showing the relationship between osteocyte lacuna volume and distance to the center. Based on a humeral cross-section of specimen II of *Marmorerpeton*, including (in red) the peripheral, dark osteocyte lacunae whose size may have been overestimated because of the dark permineralization.
Fig. 3. (Couleur en ligne.) Analyse bivariée montrant la relation entre le volume des logettes ostéocytaires et la distance au centre. Cette analyse est basée sur la section de l'humérus II de *Marmorerpeton*, et inclut (en rouge) des logettes ostéocytaires périphériques sombres pour lesquelles la taille pourrait être surestimée à cause du phénomène de perminéralisation.

If so, paleobiological inferences based on this character would need to take this possible artifact into account. However, the relationship between lacuna volume and distance to the center holds even when the suspicious peripheral, darker osteocytes are removed from the analyses (Table 1). This leads us to prefer the alternative hypothesis, that the relationship is genuine, in *Marmorerpeton*. This hypothesis is supported by the suspicious osteocytes, which do not clearly form outliers on the bivariate plot (Fig. 3). In fact, three out of eleven fall below the regression line, three more fall just above the regression line and well within the dot cloud, and of the others, only one extends beyond

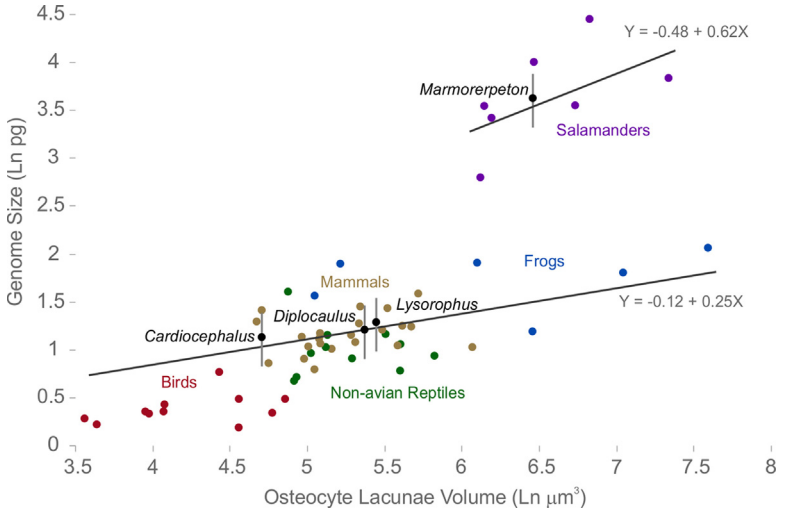

Fig. 4. (Color online.) Relationship between osteocyte lacuna volume and genome size in extant and extinct tetrapods. The regression model is phylogenetically controlled. See text for details. Phylogenetically-informed predictions of genome size for extinct amphibians are black dots with their 95% credibility intervals denoted by gray lines. Note that the estimate for *Marmorerpeton* falls well within the range of extant urodeles.
Fig. 4. (Couleur en ligne.) Relation entre le volume des logettes ostéocytaires et la taille du génome chez les tétrapodes actuels et éteints. Le modèle de régression prend en compte la phylogénie. Voir le texte pour plus de détails. Les prédictions de la taille du génome des espèces d'amphibiens éteints prennent en compte leur position systématique et sont représentées par des points noirs avec leurs intervalles de crédibilité en gris. Notez que la valeur estimée pour *Marmorerpeton* se trouve parmi les valeurs des urodèles actuels.

all other points (but not by much). The second-largest measured osteocyte is not among these suspicious ones (Fig. 1). The fact that we could not find this relationship in extant urodeles may result from the generally lower number of measured osteocytes (except in *A. japonicus*), but more than likely it results from the fact that the *Marmorerpeton* specimens are probably ontogenetically younger (under two years old; Buffrénil et al., 2015) than most extant specimens sectioned and that ontogenetically young osteocytes, which are located in freshly-deposited bone, are often located in large lacunae (Alcobendas et al., 1991; Boyde, 1980). We do not have precise data on the ontogenetic age of the extant taxa, but for at least some of them (*A. japonicus, C. alleganiensis*), the specimens are obviously several years old. In these, the superficial osteocyte lacunae may not be visibly enlarged because the bone already grew more slowly, and this phenomenon (of initially large lacunae) may not be observable as a result. This is a question that would deserve greater scrutiny, but that falls beyond the scope of our study. In any case, the decreasing bulk index in early ontogeny of *Marmorerpeton* (Table 2) might simply indicate that its growth slowed down during the first couple of years, which would naturally lead to more spindle-shaped lacunae in the superficial cortex.

The relatively large inferred genome size of *Marmorerpeton* is compatible with the previous suggestion (Buffrénil et al., 2015) that this possible karaurid was neotenic, although it gives little additional support for this claim. A neotenic lifestyle would not be surprising because the fossil record has a strong positive bias in favor of aquatic taxa (Shipman, 1981), and in urodeles, neoteny is typically associated with an aquatic lifestyle for the whole life cycle. Moreover, an inferred aquatic lifestyle is congruent with geological and paleontological data suggesting that the Kirtlington fauna was deposited in a shallow aquatic environment (Evans and Milner, 1994).

However, extant neotenic urodeles have a variable genome size. For example, *A. mexicanum* and *Pleurodeles waltl*, which are facultatively neotenic taxa (Laurin, 2014), have C-values of only 21.40 pg (Capriglione et al., 1987) and 19.5 pg (Licht and Lowcock, 1991) respectively (data also checked on the www.genomesize.com), well below our estimate for *Marmorerpeton*. Obligatory neotenic taxa normally have a genome size (C-value) of at least 45 pg (Gregory et al., 2007; Herrick and Sclavi, 2014; www.genomesize.com). Most non-neotenic urodeles have smaller genomes than facultative and obligatory neotenic urodeles, but some non-neotenic species exhibit very large genomes, such as *Aneides ferreus* (42.4 pg) and *Speleomantes italicus* (76.2 pg) (Sessions and Larson, 1987). Thus, it is unclear if our findings document an earlier association between neoteny, identified in *Marmorerpeton* based on morphological (Evans et al., 1988) and histological data (Buffrénil et al., 2015), and a rather large genome size. Given that the genome size inferred for *Marmorerpeton* would be moderate for a neotenic urodele, it is tempting to hypothesize that *Marmorerpeton* was rather facultatively neotenic. Gregory (2002) had inferred that "the association between obligate neoteny and large genome size appears to have evolved independently three times among salamanders (in the Sirenidae, Amphiumidae, and Proteidae)". We cannot compare the genome size of *Marmorerpeton* with that of other mid-Jurassic, metamorphic urodeles to adequately test the hypothesis that genome size and neoteny were associated in early urodeles. Unfortunately, most early known urodeles appear to have been aquatic, neotenic taxa (Wang and Rose, 2005), so resolving this question will be difficult. The association of large genome size and neoteny in stem-urodeles would help reconstruct the developmental biology of very early urodeles because neoteny could then be inferred from osteocyte size data. *Marmorerpeton* is thus the geologically oldest well-documented case of neoteny in urodeles because sirenids, amphiumids, and proteids appeared later, in the Cenomanian (basal Late Cretaceous) for sirenids, Maastrichtian (Latest Cretaceous) for amphiumids, and Thanetian (Latest Paleocene) for proteids, according to the fossil record (Marjanović and Laurin, 2014). Of course, molecular ages for all these clades are somewhat older (Pyron, 2011; San Mauro, 2010; Zhang and Wake, 2009) but they are unknown for karaurids, so our basis for comparison of these ages must necessarily be paleontological.

Acknowledgments

We thank Susan Evans (University College London) for providing the *Marmorerpeton* specimens, Francis Renoult and Daniel Robineau for the specimens from the Comparative Anatomy collections of the MNHN (*Andrias japonicus, Cryptobranchus alleganiensis, Ambystoma mexicanum*), Hélène Francillon-Vieillot (University Paris 7) for the *Desmognathus* specimen, and Victor Hugo Reynoso Rosales (Universidad Nacional Autonoma de México) for the loan of the *Ambystoma andersoni* specimen. We thank Jean-Claude Rage and two anonymous reviewers for comments that improved this draft.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crpv.2014.12.006.

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