Life-history traits display strong associations to genome size in annelids

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Abstract

Genome size, known also as the C-value, has been proposed as an important determinant of life-history variation in numerous animal taxa. We assessed the relationships between genome size and fitness related life-history traits in six species of interstitial marine annelids of the genus

Ophryotrocha. Life-history traits and genome-size data obtained from 18 additional annelid species was included in our analyses to have a broader phylogenetic scope. Unexpectedly, genome sizes assessed here by flow cytometry in four *Ophryotrocha* species were three times larger than previously reported values obtained using Feulgen densitometry. This has implications for the hypothesis that harsh interstitial habitats select for small genomes in meiofaunal annelids. Within the genus *Ophryotrocha*, significant and positive relationships were found between genome size and nucleus size, and between genome size, age at first egg mass deposition, body size, and lifespan. These relationships held up in the broader phylogenetic comparison. Our study provides evidence to the important role played by genome size in the evolution of life-history traits in annelids.

Keywords: *C-value; flow cytometry; Ophryotrocha; body size; developmental rate; lifespan*

Introduction

One longstanding and unresolved puzzle in evolutionary biology is the tremendous variation in genome size among eukaryotes. Genome size, here defined as the haploid nuclear content (or the C-value in pg DNA cell $^{-1}$), varies some 7000-fold among animals (0.02 – 132.83) (Gregory, 2020) with no apparent relationship with neither organismal complexity nor number of genes (Cavalier–Smith, 1985). Instead, genome size is known to correlate to non-coding DNA, more specifically transposable elements (Lynch & Conery, 2003). The C-value enigma (Gregory, 2005) refers to unresolved questions regarding the origin of the non-coding DNA, the phenotypic effects of non-coding DNA, and how it varies so greatly among taxa. The sheer amount of DNA in a genome can affect organismal phenotype through its nucleotypic effects. Several life-history traits, such as body size in species with determinate growth, have been found to correlate with genome size through the associated effects of nuclear DNA content on cell size (Hessen & Persson, 2009; Dufresne & Jeffery, 2011). Similarly, significant associations between genome size and life-history traits and developmental rate (Wyngaard et al., 2005) suggest that genome size could co-evolve with life history. These genome size – life-history traits relationships suggest that certain environments and lifestyles may be associated with larger genomes (Leiva et al., 2019). However, opposing evidence exists regarding the impact of deep-sea environment on genome size selection in amphipods (Ritchie et al., 2017). Non-adaptive theories suggest that mutations and genetic drift are the major drivers of genome size variation (Lynch & Conery, 2003). The *Mutational Hazard hypothesis* stipulates that larger genomes evolve in lineages with smaller long-term effective population size because this allows mildly deleterious insertions of non-coding DNA to accumulate by drift, rather than being eliminated by purifying selection (Lynch & Conery 2003). This has recently been shown in subterranean isopods (Lefébure et al., 2017). Hence under this hypothesis, the evolution of genome size is controlled by the opposing forces of mutations generating large scale insertions and their removal by selection or their fixation by drift.

Annelids are significantly underrepresented in the existing genome size database, and show a remarkable range of genome size (0.06 - 7.64 pg) (Gregory, 2020). Interstitial species, those that live among grains of sediment, are reported to have particularly small genomes relative to macrobenthic epifaunal species (Gambi et al., 1997). This is potentially a result of the evolution of their ecological strategies, notably their small body size and *r* reproductive strategy (Gambi et

al., 1997). Among interstitial annelid species, those belonging to the *Ophryotrocha* genus (Dorvilleidae, Annelida) are particularly known in the literature, thanks to the easy with which some of them have been cultured in the laboratory for a wide array of biological, eco-toxicological, and eco-evolutionary investigations (e.g. Thornhill et al., 2009; Prevedelli et al., 2006). The genus *Ophryotrocha* is a widely distributed group of benthic annelids occupying diverse habitats and including more than 70 known species (Thornhill et al., 2009), ten of which have recorded genome size showing a threefold variation (Sella et al. 1993). Moreover, information on life history traits of many *Ophryotrocha* species is available, making them ideal models to explore genome size – life-history traits relationships.

We used flow cytometry to measure the genome size (C-value) and nucleus size (forward light scatter) to explore their relationships to key life-history traits in six *Ophryotrocha* species *Ophryotrocha robusta* Paxton & Åkesson, 2010, *Ophryotrocha labronica* La Greca & Bacci, 1962, *Ophrotrocha diadema* Åkesson, 1976, *Ophryotrocha puerilis* Claparède & Mecznikow, 1869, *Ophryotrocha adherens* Paavo, Bailey-Brock & Åkesson, 2000, *Ophryotrocha japonica* Paxton & Åkesson, 2010. We report that the *de-novo* genome size measured in the six *Ophryotrocha* species has been greatly underestimated in the past, and that these interstitial species in fact have large genomes. We show that body size, lifespan, and age at first deposition (a proxy for developmental rate) increase with genome size in the interstitial annelid assemblage investigated. The relationships of genome size to life-history traits were then tested on a broader phylogenetic scale, with regressions run using an additional 18 annelid species for which body size, age at first deposition, lifespan and/or fecundity could be found in the literature. We show that genome size – life-history relationships remain significantly positive for body size, age at first deposition and lifespan at this broader phylogenetic scale.

Material and Methods

Ophryotrocha species rearing and genome size determination

Specimens of the six *Ophryotrocha* species investigated in our study came from laboratory strains established from individuals collected in Italy (La Spezia, 44°06'N; 09°49'E, and Porto Empedocle 37°18'N; 13°32'E) and kept under control laboratory conditions (salinity: 32-35; temperature: 22-24 °C; pH_{NBS} = 8.1; photoperiod L:D of 12:12 h) for approx. 20 to 60 generations prior to genome size estimation. Thirty mature individuals were ground in 1 mL of

Galbraith buffer (Galbraith et al., 1983) in each flow cytometry run. Three to seven runs were performed for each species. *Daphnia pulex* Leydig 1869 was used as standard for analyses (Vergilino et al., 2009). The mixture of nuclei (*Ophryotrocha* - *Daphnia*) was co-stained using 20 µL of propidium iodide (1.0 mg mL^{-1}) for 45 min. and analyzed on a CytoFLEX flow cytometer (see Supplementary Figure 1). Nuclear DNA content of all annelid species was calculated using the following equation: nuclear DNA = *Ophryotrocha* fluorescence / (*Daphnia* fluorescence x 0.45 pg), where the nuclear DNA content is pg DNA and 0.45 pg corresponds to the nuclear DNA content of *D. pulex* (Vergilino et al., 2009).

Flow cytometry data also yields information on particle size through forward light scatter. In general, forward light scatter correlates closely with particle size (e.g., Figure 3 in Belzile and Gosselin 2015). Forward light scatter has been used previously as an index of nucleus size in *Daphnia* (Jalal et al., 2013) and will be henceforth referred to as such. Mean forward scatter of the *nuclei* was thus recorded in order to assess the relationship between this measure and genome size, and data was analyzed using CytExpert Software v.2.3 (Beckman Coulter).

Life-history traits and species selection

Life-history traits for the six laboratory *Ophryotrocha* species were obtained from studies that used comparable rearing conditions (Simonini and Prevedelli 2003; Grandi 2009; Martino, 2012; Paxton and Åkesson 2010) : body size (mm), growth rate (chaetigers . day $^{-1}$), age at first deposition (d), egg size (µm), fecundity (eggs . clutch $^{-1}$), lifetime fecundity (eggs . individual $^{-1}$), and lifespan (d). Age at first deposition is considered here as a developmental proxy. Body size and fecundity were measured as the maximum body length (mm) recorded in the species and the average number of eggs laid per clutch, respectively. Growth rates were measured as number of chaetigers (segments bearing bristles, Massamba-N'Siala et al., 2011) added daily until reaching the maximum body size (measured as number of chaetigers). Lifetime fecundity referred to the total amount of eggs produced by an individual during its lifetime. Finally, egg size was measured as the arithmetic mean between the longer and the shorter axes (Simonini and Prevedelli, 2003). Life-history data of the additional annelid species was obtained from the literature (see Supplementary Tables I & II). Four life-history traits were considered for all species: body size (mm), fecundity (eggs . clutch $^{-1}$), lifespan (d), and age at first deposition (d). Growth rates, lifetime fecundity, and egg size were traits available only for the six *Ophryotrocha* species.

Species for which genome size was available in the literature were selected for the final analysis based on the availability of COI and 16S sequences and the reliability of genome size measures (species reported by Sella et al. 1993 were omitted due to considerable discrepancies between their study and ours). In addition, deep-sea and vent species were removed due to signs of gigantism (> 1000 mm body lengths seen in *Tevnia jerichonana* and *Riftia pachyptila*, for example). Finally, the catworm *Nephtys incisa* was not included in fecundity analysis because of its disproportionate higher reproductive output (250 000 eggs size . clutch $^{-1}$) compared to the other annelid species (1 – 2000 eggs . clutch $^{-1}$).

Maximum likelihood phylogenies

Two maximum likelihood (ML) phylogenies were constructed with COI and 16S sequences. The first phylogenetic tree was comprised exclusively of sequences obtained from laboratory specimens of the six *Ophryotrocha* species (Tempestini *et al.*, in press.). The second phylogenetic tree was constructed by adding the sequences of 18 annelid species collected from GenBank to the original six *Opryotrocha* species. The marine nemertean worm *Cerebratulus lacteus* served as outgroup for both phylogenies (Struck et al., 2011). Accession numbers are provided below (section *Data availability*). Multiple sequence alignments were performed with MUSCLE (Edgar 2004) using the software MEGAX (Kumar et al., 2018) with default parameters and concatenated in MEGAX. The alignments were run through RAxML-HPC2 (Stamatakis, 2014) using default parameters as well. In R (R v3.4.2 and RStudio v1.1.383), packages *ape* and *phytools* were used to import and transform the resulting tree as well as the phenotypic data. Final phylogenetic trees were produced using FigTree v1.4.4 (Figures 1 and 2).

Statistical analyses

A one-way analysis of variance (ANOVA) test with species as fixed factor and flow cytometry runs as replication units was first performed to determine if the six *Ophryotrocha* species differed in genome size. Pairwise comparisons were subsequently performed using Tukey's HSD test. Linear regressions models were conducted to test for significant relationships between genome size and single life-history traits in six species of *Ophrytrocha*. Furthermore, the relationships between genome size and four life-history traits was tested in the expanded data set containing 18 additional annelid species. Life-history traits and genome size values were corrected for phylogenetic relatedness using phylogenetically independent contrasts (*pic* function in *ape*) for both phylogenies, and these analyses were run through the origin. Significant and

marginally significant relationships were plotted in R for both phylogenetically-corrected and non corrected data. Body size was tested as a covariate alongside other life history traits in all linear models, before being removed from the model once deemed non-significant. Normality of residuals, tested with a Shapiro-Wilks test, was rejected for the ANOVA test, which was corrected with a $\log_{10}$ transformation of genome size data. Normality of residuals was also rejected in four instances for the regression models: the relationship between phylogenetically corrected genome size and nucleus size in the six *Ophryotrocha* species, the relationship between raw and phylogenetically corrected genome size and fecundity in the enlarged dataset, and the relationship between raw genome size and lifespan in the enlarged dataset. In all cases except the first one, a logarithmic transformation of the raw values was sufficient to meet the assumption of normality.

Statistical analyses were conducted using R (R v3.4.2).

Results

The mean genome size was 1.47, 1.23, 1.04, 1.45, 0.80 and 1.40 pg for *O. robusta*, *O. labronica*, *O. diadema*, *O. puerilis* , *O. adherens* and *O. japonica* respectively. Significant differences in $\log_{10}$ transformed mean genome size were found among species ($F_{(5, 20)} = 76.2$; $P = 2.51 \cdot 10^{-12}$). *Ophryotrocha japonica* and *O. puerilis* had the largest genome sizes that differed significantly from the ones of *O. adherens* and *O. diadema*. The genome size of *O. labronica* was significantly smaller than that of *O. puerilis* and significantly larger one than that of *O. adherens* (Supplemental Table 1). All life-history trait regression results for *Ophryotrocha* are summarized in Table I. Body size (mm), age at first deposition (d), fecundity (eggs . clutch $^{-1}$) and lifespan (d) regression results for the expanded annelid dataset are summarized in Table 2.

Our analysis indicates that *Ophryotrocha* species possessing larger genome size displayed larger nucleus sizes estimated through forward scatter; a significant positive relationship was found between these two traits after phylogenetic correction ($R^2 = 0.764$; $F_{(1,4)} = 12.97$; $P = 0.023$; Figure 3). Species with larger genome sizes were found to have larger body sizes and nucleus sizes. These traits show a significant positive relationship in *Ophryotrocha* after phylogenetic correction (Figures 4A). Similarly, there was a significant increase in age at first deposition (d) in *Ophryotrocha* species with larger genome sizes after phylogenetic correction (Figures 4B). Fecundity did not differ significantly in *Ophryotrocha* species with small and large genomes

(Figures 4C). The relationship between lifespan and genome size was significant in *Ophryotrocha* after phylogenetic correction (Figure 4D). No significant relationships were detected between genome size and growth rate, genome size and egg size, and genome size and lifetime fecundity in the *Ophryotrocha* group (Table 1).

Further analysis of genome size and life-history on a broader phylogenetic scale revealed similar patterns for three of the four significant traits mentioned above. Annelid species with larger genome sizes displayed a significantly larger body size (Figure 4E), a later age at first deposition (Figure 4F) and an increased lifespan (Figure 4H) after phylogenetic correction. There was no significant relationship between fecundity and genome size in extant annelid species (Figure 4G).

Discussion

Our study reveals that a number of important life history traits positively correlate to genome size in a set of species from the marine annelid *Ophryotrocha*. Age at first deposition, body size, and lifespan were positively associated to genome size whereas no significant associations were found for egg size, fecundity, and growth rate. Those patterns held up on a broader phylogenetic scale using additional annelid species for which genome size and life-history data were available. We also report that genome size estimates measured here in flow cytometry contradict previous estimates using Feulgen densitometry, with implications for downstream genomic applications.

The six *Ophryotrocha* species investigated here exhibit a three-fold difference in body size, which significantly increases with genome size. The relationship remained significant among the additional annelid species tested here with body sizes varying ten-fold. It was initially suggested by Gambi *et al.* (1997) that harsh interstitial habitats select for small genomes in meiofaunal annelids *via* the genome size - body size relationship. This was apparent when considering the reported genome size range of 0.07 to 1.16 pg in interstitial species and 0.4 to 7.2 pg in macrobenthic species. However, this hypothesis does not appear to hold for *Ophryptrocha* species, as the genome sizes in this group are considerably large (0.80 - 1.47), while they possess fairly small body sizes (2 to 7 mm). Positive relationships between body size and genome size have been reported in numerous invertebrates (Hessen & Persson, 2009; Jeffery et al., 2017; Lefébure et al., 2017) but are not ubiquitous. These relationships are most often found in species

where growth occurs largely as a result of increase in cell volumes, rather than by increasing cell numbers. The strong relationship between genome size and nucleus size found in *Ophryotrocha* potentially contributes to the positive relationship between genome size and body size, which suggests that cell volume influences whole-organism body size in this genus.

Genome size was strongly correlated with age at first deposition in *Ophryotrocha* as well as in the larger annelid dataset. This relationship has been described in different groups, with genome size impacting different proxies for developmental rate/time, such as voltinism in Lepidoptera (Miller, 2014), maturation rates in copepods (Wyngaard et al., 2005), embryonic development in salamanders (Jockusch, 1997), and age at sexual maturity and hatching time in birds (Yu et al., 2020). The relationship is overall apparent in pancrustaceans (i.e. insects and crustaceans), where species possessing smaller genomes show a faster development (Alfsnes et al., 2017). Since *Ophryotrocha* species have a direct development, we hypothesize that genome size could be less constraining in this group than in taxa possessing complex life-history strategies with multiple larval stages. In contrast to age at first deposition that is a proxy of growth, growth rate did not show an association with genome size. We expected that *Ophryotrocha* species with smaller genomes would have a higher growth rate due to their potentially faster cell divisions. It could be that our proxy for growth rate 'number of chaetigers deposited per day' is not precise enough in this small dataset. Surprisingly, lifespan was positively associated with genome size both in *Ophryotrocha* and in the annelid dataset. Genome size is not known to be correlated to lifespan (or longevity) in reptiles (Olmo, 2003), birds (Gregory, 2002; Yu et al., 2020) nor in fish species (Gregory 2004; Hickey & Clements, 2005). This positive relationship between genome size and longevity in annelids may be mediated by age at first deposition and warrants further studies.
Genome size increase in *Ophryotrocha* was not significantly associated with fecundity nor with egg size. The relationship between egg size and genome size depends on the group investigated. For example, egg size is positively associated with genome size in fish (Hardie & Hebert, 2011) and in rotifer (Stelzer et al., 2011) but not in in salamanders (Jockusch, 1997).
In addition, we show here that the genome size of *O. robusta* (0.47 instead of 0.37 pg), *O. puerilis* (1.45 instead of 0.46 pg) *O. labronica* (1.23 instead of 0,44 pg), and *O. diadema* (1.04 instead of 0.44 pg), are 2.4 to 4 times larger than previously reported (Sella et al. 1993; Soldi et al. 1994). These previous estimates were assessed through Feulgen densitometry and are

compared to those measured in flow cytometry (Supplementary Figure 1). Artefacts associated with Feulgen technique such as sample size limitation, staining issues (comparison of different cell types with different levels of DNA compaction and stain uptake), conditions of slide fixation may have biased these past estimates (Hardie et al., 2002). *Ophryotrocha labronica* has historically been used for the investigation of life-history traits ecology and evolution (Simonini & Prevedelli, 2003; Prevedelli et al., 2006; Rodríguez-Romero et al., 2016) and is emerging as a model organism for the investigation of transgenerational responses of marine invertebrates to global change drivers (Chakravarti et al., 2016; Rodríguez-Romero et al., 2016; Gibbin et al., 2017a, 2017b; Jarrold et al., 2019). As it will be part of a foreseeable sequencing endeavour for the development of –omics approaches, it would have been misleading to assume that its genome size was 2.5-fold smaller than expected (1.04 *vs.* 0.40 pg). Considering that nearly 20 % of annelid genome size in the database reference these two studies, it is likely that inferences based on this data should be reconsidered.

In conclusion, our study provides strong evidence of the determinant role played by genome size in the evolution of life-history traits, validated at both the genus and phylum level. Annelids being characterised by an overwhelming biodiversity in marine environments represent a very promising group to delve deeper into the c-value paradox.

Declarations

Compliance with Ethical Standards

All applicable international, national, and/or institutional ethics guidelines for sampling, care and experimental use of organisms have been followed in this study.

Data Availability

We have deposited the primary data underlying these analyses as follows:

Sampling locations, morphological data, and microsatellite genotypes: Dryad

DNA sequences: Genbank accessions *Branchiura sowerbyi* (LN810299.1, KY636792.1), *Cerebratulus lacteus* (KC698905.1, KX261740.1), *Erpobdella obscura* (AF003273.1, JQ821464.1), *Hirudo medicinalis* (EF446704.1, AF315058.1), *Laeonereis culveri* (MH235843, MH264663.1), *Limnodrilus hoffmeisteri* (LN810304.1, AY885613.1), *Limnodrilus udekemianus* (LN810320.1, KY636789.1), *Lumbriculus variegatus* (FJ639308.1, AY521550.1), *Myxicola infundibulum* (HQ024104.1, HM800977.1), *Neanthes acuminata* (KJ539071.1, KJ538996.1), *Nephtys incisa* (KT307667.1, GU179356.1), *Ophidonais serpentina* (LN810257.1,

DQ459939.1), *Ophryotrocha adherens* (MK933737, MT737363.1), *Ophryotrocha japonica* (MK933739, MT737362.1), *Ophrytrocha diadema* (MK933738, MT737364.1), *Ophrytrocha labronica* (MK933740, MT737361.1), *Ophrytrocha puerilis* (MK933741, MT737365.1), *Ophrytrocha robusta* (MK933742, MT737360.1), *Platynereis dumerilii* (KP127954.1, KP640622.1), *Polygordius appendiculatus* (KF808170.1, MG603472.1), *Scalibregma inflatum* (GU672569.1, KF511816.1), *Spirosperma ferox* (KY636947.1, KY636799.1), *Syllis prolifera* (JF903780.1, JF903739.1), *Tubifex tubifex* (HM138034.1, AF326005.1).

Data citation

The main dataset has been assembled and presented here as supplementary material.

Author contributions

The experimental design and work have been conceived and planned by NB, GMN, PC and FD. Life-history data was extracted from the literature by NB and GMN. NB conducted genome size measurements under the supervision of CB and FD. NB conducted statistical analyses and results interpretation supervised by GMC, PC and FD. NB wrote the first draft of this manuscript supported by PC and FD. All authors contributed to the final version of this manuscript.

Competing interests

We have no competing interests.

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Figure Captions

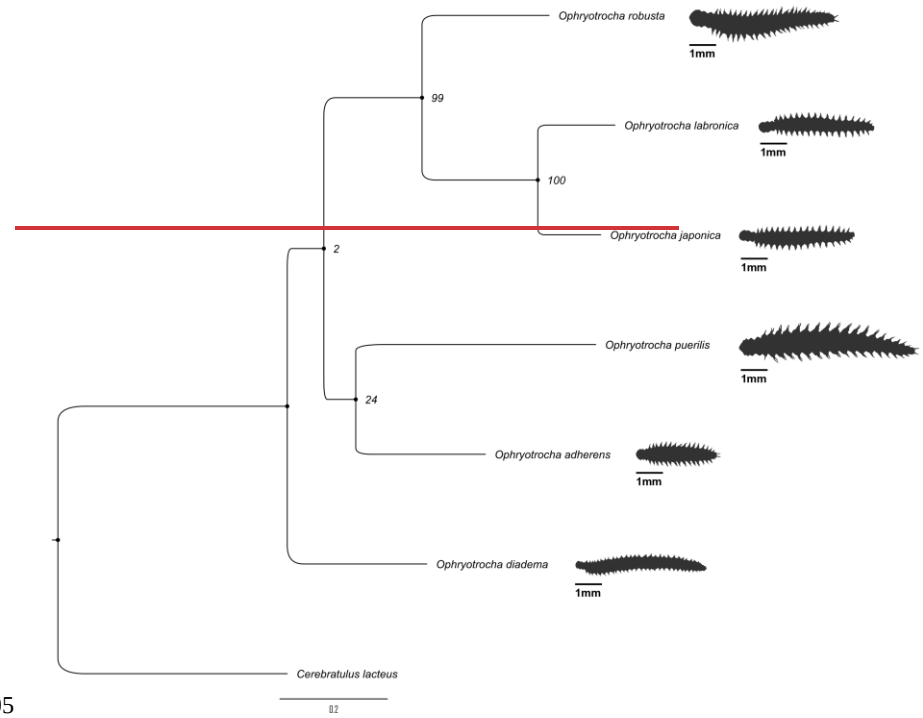
Figure 1: Maximum likelihood phylogenies of cytochrome oxidase I and 16S sequences in six *Ophryotrocha* species with outgroup *Cerebratulus lacteus,* produced using RAxML-HPC2 and plotted in FigTree v1.4.4.

Figure 2: Maximum likelihood phylogenies of cytochrome oxidase I and 16S sequences in 23 annelid species (B) with outgroup *Cerebratulus lacteus,* produced using RAxML-HPC2 and plotted in FigTree v1.4.4.

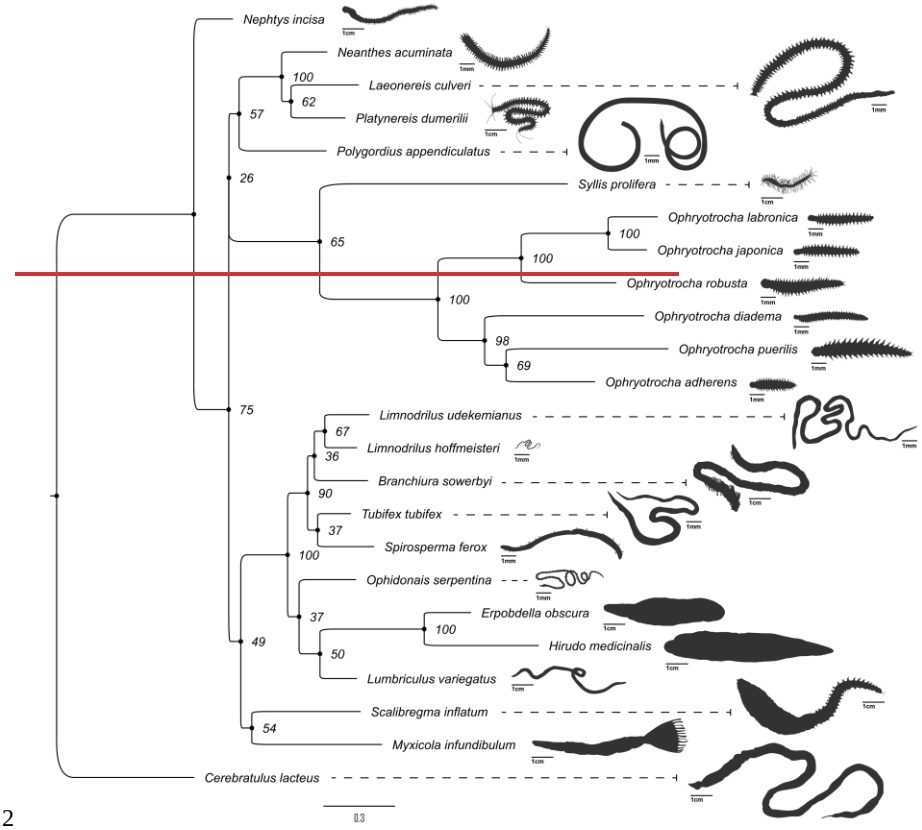
Figure 3: Relationship between genome size (C-value expressed in pg) and nucleus size estimated from forward light scatter in flow cytometry. Forward light scatter correlates closely to particle size and has previously been used as an index of nucleus size in Daphnia. The data points were corrected by phylogenetically independent contrasts applied using a cytochrome oxidase I and 16S maximum likelihood (RAxML-HPC2) phylogeny detailed in the present paper.

Figure 4: Relationships between genome size (C-value expressed in pg) and (A) body size (mm), (B) age at first deposition (d), (C) fecundity (eggs . clutch -1) and (D) lifespan (d) for the six laboratory Ophryotrocha species. The same relationships were plotted for (E) body size (mm) in 16 total species, for (F) age at first deposition (d) in 12 total species, for (G) log 10 transformed fecundity (eggs . clutch -1) in 13 total species and for (H) lifespan (d) in 13 total species. The data points were corrected by phylogenetically independent contrasts applied using two cytochrome oxidase I and 16S maximum likelihood (RAxML-HPC2) phylogenies detailed in the present paper.

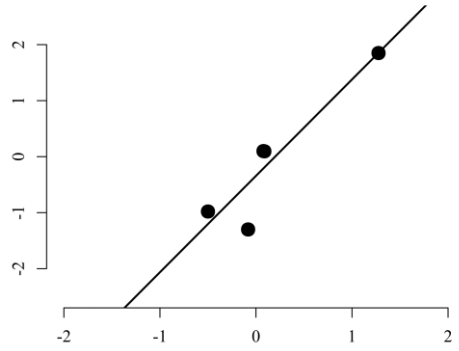
Figure S1: Frequency histograms of isolated nuclei propidium iodide fluorescence; (A) *O. diadema* and (B) *O. labronica*. *Daphnia pulex* nuclei peaks are blue and *Ophryotrocha* nuclei peaks in red. The black arrow indicates where the supposed *Ophryotrocha* peaks would be found according to the genome size values reported by Sella *et al.*, (1993).


Ophryotrocha robusta
1mm
99
Ophryotrocha labronica
1mm
100
Ophryotrocha japonica
1mm
2
Ophryotrocha puerilis
1mm
24
Ophryotrocha adherens
1mm
Ophryotrocha diadema
1mm
Cerebratulus lacteus
0.2

~~Figure 1~~



~~Figure 2~~



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