

1 The genome sizes of ostracod crustaceans correlate with body size and phylogeny

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3 Nicholas W. Jeffery^{1*}, Emily A. Ellis², Todd H. Oakley², T. Ryan Gregory¹

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5 *Present address: Fisheries and Oceans Canada, Bedford Institute of Oceanography, Dartmouth,
6 Nova Scotia

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8 ¹Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada. N1G 2W1

9 ²University of California Santa Barbara, Santa Barbara, California, USA. 93106

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11 Corresponding author email: nick.w.jeffery3@gmail.com

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46 **Abstract**

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48 Within animals a positive correlation between genome size and body size has been detected in
49 several taxa but not in others, such that it remains unknown how pervasive this pattern may be.
50 Here we provide another example of a positive relationship, in a group of crustaceans whose
51 genome sizes have not previously been investigated. We analyze genome size estimates for 46
52 species across Class Ostracoda, including 29 new estimates made using Feulgen image analysis
53 densitometry and flow cytometry. Genome sizes in this group range ~80-fold, a level of
54 variability that is otherwise not seen in crustaceans with the exception of some malacostracan
55 orders. We find a strong positive correlation between genome size and body size across all
56 species, including after phylogenetic correction. We additionally detect evidence of XX/XO sex
57 determination in all three species of myodocopids where male and female genome sizes were
58 estimated. On average, genome sizes are larger but less variable in myodocopids than in
59 podocopids, and marine ostracods have larger genomes than freshwater species, but this appears
60 to be explained by phylogenetic inertia. The relationship between phylogeny, genome size, body
61 size, and habitat is complex in this system, and will benefit from additional data collection across
62 various habitats and ostracod taxa.

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70 **Introduction**

71 Genome sizes (haploid nuclear DNA contents) have been estimated for more than 5,500
72 species of animals, revealing a greater than 7,000-fold range (Gregory 2017). Some important
73 patterns have emerged from comparative analyses of genome size, including well-established
74 links between genome size, nucleus size, cell size, and cell division rate (Gregory, 2002;
75 Gregory, 2005). However, the implications of these relationships at organismal and ecological
76 levels are more complex. Body size (Gregory, Hebert and Kolasa, 2000; Jeffery, Yampolsky and
77 Gregory, 2016; Wyngaard, *et al.*, 2005), metabolic rate (Gregory, 2002; Vinogradov, 1995),
78 developmental rate (Gregory, 2002; Gregory and Johnston, 2008), life history (Rees, *et al.*, 2007;
79 Reeves, *et al.*, 1998) , and geographic distribution (Bonnivard, *et al.*, 2009) have been found to
80 correlate with genome size in some animal groups, but these are far from universal, and depend
81 on the biology of the animals in question. Sorting out the broader patterns of genome size
82 diversity and its biological significance therefore requires studies of a wide variety of taxa.
83 However, many groups -- especially non-vertebrates -- remain to be studied from this
84 perspective.

85 Ostracod crustaceans represent a group of interest in this regard, due to their ecological
86 diversity and over 150-fold range in body size. Class Ostracoda contains an estimated 20,000
87 species divided into four orders, the two largest of which are Myodocopida and Podocopida.
88 About 10% of extant ostracod species are freshwater, while the remainder are marine (Horne,
89 Cohen and Martens, 2002). Myodocopids are strictly marine, but podocopids can be found in
90 marine, estuarine, and freshwater habitats, with three podocopid lineages having invaded non-
91 marine habitats independently at different times since the Devonian (Martens, *et al.*, 2008). The
92 ostracod clade has a rich fossil record (Foote and Sepkoski, 1999) dating to at least the early
93 Ordovician (Williams, *et al.*, 2008), but molecular evidence suggests an origin at least 500

94 MYA, while divergence of Myodocopida and Podocopida occurred approximately 480 MYA
95 (Oakley, *et al.*, 2013; Tinn and Oakley, 2008).

96 Prior to this study, there were only two published ostracod genome size estimates, with
97 an additional 15 unpublished values included in the Animal Genome Size Database and (P.D.N.
98 Hebert unpublished). Genome size estimates are sparse for crustaceans in general, and obtaining
99 a greater number of estimates for ostracods will provide data for potential genome sequencing
100 projects and provide a better understanding of how a basic organismal property such as genome
101 size can influence the overall life history of these crustaceans.

102 Here, we report new estimates for 29 species of ostracods from two orders -
103 Myodocopida and Podocopida - and analyze them in combination with publicly available
104 estimates. We also constructed a genus-level phylogeny for the taxa with genome size estimates
105 using 18S rRNA sequences to examine genome size diversity across the ostracod phylogeny.
106 This allowed us to test possible relationships between genome size and body size within a
107 phylogenetic context, and to examine patterns of genome size distribution across taxa and
108 habitats.

109 **Methods**

110 *Specimen collection and biological/ecological trait data*

111 We collected sediment samples from various locations in Canada, the United States, and
112 Australia (Table 1, Supplementary Table S1). To collect marine and freshwater species, we used
113 a 150 μ m plankton net to horizontally collect the top half-inch layer of sediment from the
114 benthos. The resulting sediment from marine samples was further sorted using a 500 μ m sieve
115 and a 100 μ m sieve, and sorted by eye using a dissection microscope. We collected some species
116 at night – as many are known to be nocturnally active in search of mates (Speiser, *et al.*, 2013) –

117 by using a light trap and vertical plankton tows. In many cases, we were able to identify taxa to
118 the species level using published generic and specific dichotomous keys. We measured total
119 body size (length of carapace) using an ocular micrometer.

120 *Genome size estimation*

121 Ostracods were dissected from their valves in 40%(v/v) acetic acid on microscope slides
122 using insect pins, flattened with a coverslip which was held onto the slide with a clothespin, and
123 then frozen on dry ice. The coverslip was then removed with a razor blade and the slides were
124 immersed in 100% ethanol and dried at room temperature.

125 All prepared slides were fixed in 85:10:5 methanol:formalin:acetic acid overnight. The
126 slides underwent hydrolysis in 5N HCl at 20°C for 120min. The slides were stained in prepared
127 Schiff reagent for 120min, followed by 3 rinses in bisulfite solution and repeated rinses in
128 deionized water (Hardie et al. 2002). Each set of slides was co-stained with slides of domestic
129 chicken blood (1C=1.25pg) and rainbow trout blood (1C=2.60pg) for use as internal standards.
130 Individual nucleus densities were measured using a Leica DM LS compound microscope with a
131 mounted Optronics DEI-750 CE CCD camera and Bioquant Life Science software.

132 We estimated the genome size of some species by flow cytometry (Table 1). Specimens
133 were crushed in 500µl cold LB01 buffer (Dolezel, Binarova and Lucretti, 1989) and co-stained
134 with chicken blood or crushed *Daphnia pulex* (1C=0.23pg) after adding 12µl propidium iodide
135 (24µg/ml) and 2µl RNase (4µg/ml) to each sample. These were stained in the dark for 1 hour and
136 analyzed on an FC500 flow cytometer (Beckman-Coulter). All coefficients of variation (CVs)
137 were <8% and a minimum of 1000 nuclei per ostracod were analyzed.

138 We included 17 estimates from the Animal Genome Size Database (www.genomesize.com)
139 (Hebert, unpublished). These estimates used epithelial tissue dissected from ostracods, but the
140 standard used for estimation is unknown.

141 *Statistical analyses and phylogenetic correction*

142 We first applied a general linear model of log-transformed genome size, log-transformed
143 body size, and habitat type as a categorical variable (0=freshwater, 1=marine) to test for
144 significant relationships between variables without phylogenetic correction. To correct for
145 phylogenetic non-independence of our data, we used software implemented in the publically
146 available Galaxy computing platform Osiris (Oakley, *et al.*, 2014), to estimate a genus-level
147 phylogeny from publicly available 18S ribosomal RNA sequences (Supplementary Table S2).
148 These sequences were aligned using MUSCLE v3.8 (Edgar, 2004). We performed maximum-
149 likelihood and bootstrap statistical analyses in Garli v2.0 (Gutell and Jansen, 2006) using a
150 GTR+G model with 8 gamma categories. For tips in the phylogeny for which we had multiple
151 species per representative genus in the phylogeny, we took the average genome size. The
152 resulting phylogeny had 16 genera of ostracods and relationships were consistent with previously
153 published phylogenies (Oakley, *et al.*, 2013; Tinn and Oakley, 2008) (Figure 1). We used the
154 package *phytools* (Revell, 2012) in R v3.2.3 to map genome size onto our phylogeny and
155 conduct a test for phylogenetic signal using Pagel's lambda. We then conducted a phylogenetic
156 generalized least squares model of genome size, body size, and habitat, using lambda as the
157 correlation structure, and implemented in the R packages *ape* (Paradis, Claude and Strimmer,
158 2004) and *nlme* (Pinheiro, *et al.*, 2014).

159 **Results**

160 Genome size estimates for all analyzed ostracods range approximately 80-fold, from
161 0.11 ± 0.003 pg (mean \pm s.e.) in the podocopid *Xestoleberis* sp. to 8.98 ± 0.14 pg in the myodocopid
162 *Asteropterygion climax* (Figure 1). Freshwater ostracods, found entirely in the order Podocopida,
163 had an average genome size of 0.86 ± 0.09 pg, while marine ostracods from both Podocopida and
164 Myodocopida had an average genome size of 2.88 ± 0.46 pg. The single estuarine species in this
165 study, *C. beaconensis*, had a small genome size of 0.28 ± 0.02 pg. Larger genomes are found
166 within Myodocopida on average (3.81 ± 0.51 pg) versus the Podocopida (0.82 ± 0.11 pg); however,
167 the Podocopida exhibit a higher coefficient of variation in genome size (73.6%) relative to
168 Myodocopida (49.8%). For three species within Myodocopida, we estimated genome size for
169 both males and females, and found that males have lower DNA content by an average of 6.3%
170 (Table 1).

171 A linear regression of non-phylogenetically corrected log-transformed genome size
172 versus body size showed a significant positive relationship across all of the species for which
173 genome sizes were estimated in this study ($r^2=0.47$, $p<0.0005$, $n=41$) (Figure 2), and a general
174 linear model of genome size, body size, and habitat revealed that both body size and habitat were
175 significantly associated with genome size ($t=6.351$ and $p<0.0001$, $t=3.076$ and $p=0.004$
176 respectively). When splitting ostracods by order, both myodocopids and podocopids showed a
177 significant relationship between transformed genome size and body size ($r^2=0.53$, $p=0.002$ and
178 $r^2=0.31$, $p=0.001$ respectively) (Figure 2). To examine this relationship within a phylogenetic
179 context, it was necessary to conduct the analysis at the genus level ($n=15$ genera). After
180 controlling for phylogeny, body size remained significantly associated with genome size ($t=3.63$,
181 $p=0.004$) but habitat did not ($t=-0.45$, $p=0.66$). Overall, genome size showed moderate but
182 significant phylogenetic signal for the genera examined here ($\lambda=0.60$, $p=0.027$).

183 **Discussion**

184 *Genome size in ostracods*

185 We revealed an 80-fold range in genome size within ostracods, a large range which has
186 not been observed in other crustaceans with the exception of Isopoda and Amphipoda (Jeffery,
187 2015). Our genus-level phylogeny is largely consistent with previously published phylogenies
188 (e.g. Yamaguchi and Endo, 2003) and we find moderate but significant phylogenetic signal for
189 genome size across the phylogeny. This suggests that genome size evolution deviates from a
190 pure Brownian motion model, which would be expected if $\lambda=1.0$, and rather that genome size
191 correlates modestly among closely related species and differs between more deeply divergent
192 clades. This could be consistent with evidence for potential polyploidy or quantum leaps in
193 genome size across this phylogeny, such as the case between the sister-genera *Tetraleberis* and
194 *Asteropterygion*, where the genome of *Asteropterygion* is nearly double that of *Tetraleberis*.
195 Discontinuous patterns of genome size have been reported in other crustaceans, including
196 copepods (e.g. Gregory, *et al.*, 2000).

197 *Genome size, body size, habitat, and sex*

198 Larger genomes are observed in the order Myodocopida, which have larger body sizes
199 than the Podocopida. Moreover, the positive relationship between genome size and body size
200 remained even after correcting for phylogeny. This positive relationship between body size and
201 genome size has only been observed in few other crustaceans, including copepods (Gregory, *et*
202 *al.*, 2000; Wyngaard and Rasch, 2000), amphipods, and some branchiopods (Hessen and
203 Persson, 2009). This correlation suggests that body size is a good predictor of genome size in
204 ostracods, though this is by no means an exhaustive examination of this trend. It remains to be
205 seen how general the body size correlation is within and among crustacean groups (and other

206 animals) more broadly. It is likely that this relationship will be most evident when cell numbers
207 contribute less to body size diversity than individual cell sizes.

208 Habitat initially was revealed to be significantly correlated with genome size, as larger
209 genomes appear to be found in marine habitats. However after correcting for phylogeny, habitat
210 remained no longer significant. This is because Myodocopida are entirely marine and have larger
211 genome sizes than the predominantly freshwater Podocopida, but within Podocopida the marine
212 species we examined had smaller genomes than freshwater species. Denser taxon sampling could
213 certainly change these conclusions.

214 We revealed that in the three myodocopid species for which we had individuals of each
215 sex, males had smaller genome sizes than their female counterparts. This is consistent with an
216 $XX_{\text{♀}}/XO_{\text{♂}}$ sex determination system in which males lack one copy of a sex chromosome that is
217 present in two copies among females (Moguilevsky, 1990). The extent to which this sex
218 determination system occurs across ostracods is unknown, but we detected evidence in Families
219 Cypridinidae and Philomedidae, and previous research has found evidence in each of these
220 families (Moguilevsky, 1985; Moguilevsky, 1990; Rivera and Oakley, 2009). This suggests that
221 the common ancestor of all myodocopids had XX/XO sex determination (Figure 1).

222 *Alternative hypotheses of genome size evolution*

223 We observe here that genome size is highly variable among ostracods and that genome
224 size correlates with body size, but are unable to determine if these patterns are the result of
225 ecological pressures leading to rapid genome size change in extant species. Podocopids possess
226 much smaller genomes than myodocopids on average, suggesting that they are subject to
227 additional constraints on genome size, perhaps relating to body size or other ecological pressures
228 that impact cell size and/or cell division rate. Indeed, the myodocopids tend to be physically

229 larger in size than podocopids. It is also noteworthy that the Myodocopida are strictly marine,
230 whereas the Podocopida inhabit a much broader range of habitats (leaf litter, freshwater,
231 brackish, marine) (Martens, *et al.*, 2008). This could help to explain why, despite small absolute
232 genome sizes, the Podocopida exhibit higher relative variability in genome size due to
233 differential selective pressures in different environments, as reflected by coefficient of variation.
234 While on average we find larger genomes in marine species, a simple marine versus freshwater
235 distinction is not likely to account for genome size differences across ostracods, however, given
236 that within the Podocopida, marine species tend to have smaller genomes than freshwater
237 species. Within Ostracoda, genome size may thus be driven by differential selective pressures for
238 changes in body size within lineages and across habitat types.

239 *Concluding remarks*

240 The present study has highlighted some interesting patterns in genome size diversity among
241 the previously overlooked Ostracoda. We provided new genome size estimates for 29 species of
242 ostracods relative to only 17 estimates contained within the Animal Genome Size Database.
243 Even with a comparatively small sampling of overall ostracod diversity, a more than 80-fold
244 range in genome size was observed. This, combined with the relationships between genome size
245 and body size, and potential differences in selective pressures from different habitats indicate
246 that this is an excellent group to target for further study.

247

248 **Authors' Contributions**

249 NWJ and TRG conceived of the study. NWJ, EAE, and THO collected and identified specimens.
250 NWJ and EAE prepared the samples and EAE constructed the phylogeny. NWJ and EAE
251 analyzed the samples and results. NWJ, EAE, THO, and TRG wrote the paper. All authors edited
252 and approved of the paper.

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258 size data. We are also grateful to Alexandra Wen and Abigail Chua for their assistance in
259 collecting and preparing slides for genome size analysis. We thank the Australian Museum (loan
260 number MI2013059) for loaning us prepared slides and Paul Valentich-Scott for assistance in
261 accessioning slides (accession numbers x-x) at the Santa Barbara Museum of Natural History.

262

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268 Celia K.C. Churchill.

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271 **Data Availability**

272 Sampling locations and species identifications are available in Table 1. All 18S sequence
273 Genbank accession numbers are provided in Supplementary Table S1. All genome size estimates
274 will be publicly available in the Animal Genome Size Database prior to final publication.

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356 **Figure captions**

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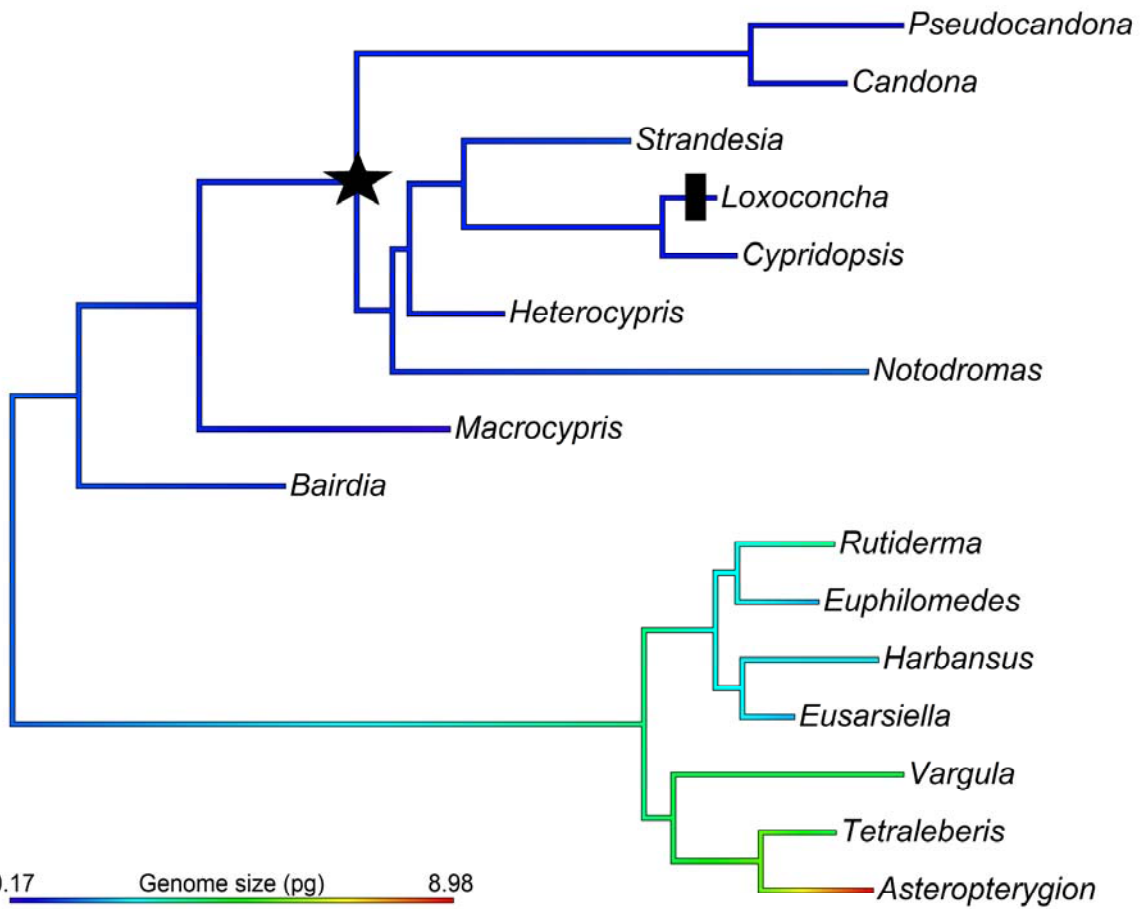
358 **Figure 1.** Genus-level 18S phylogeny showing podocopid and myodocopid clades with genome
359 size mapped onto the branches. The transition to freshwater is marked with a star, and the
360 reversal to marine is marked with a black bar.

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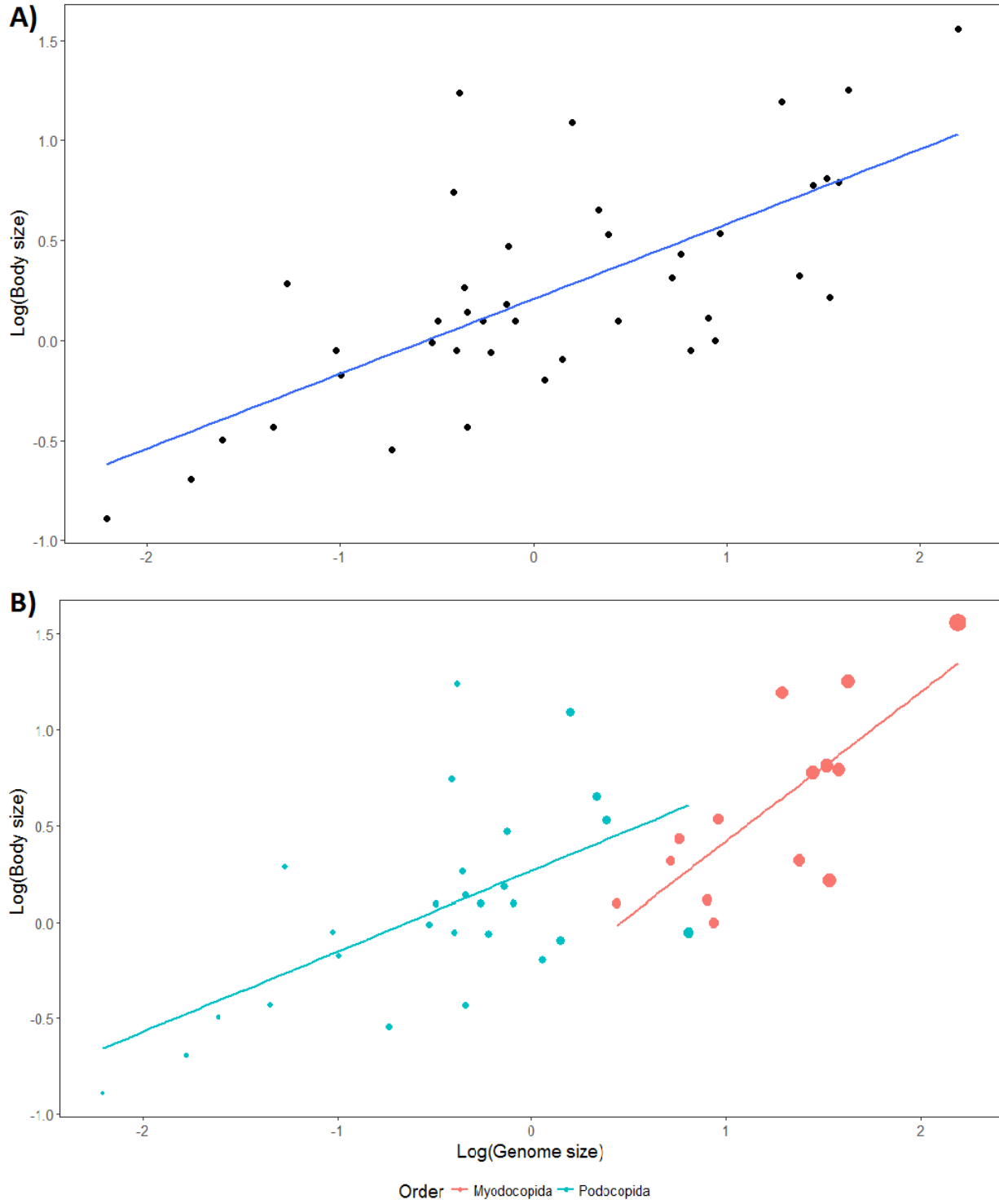
363 **Figure 2.** Log-transformed body size versus log-transformed genome size in (A) 41 species of
364 ostracods showing a significant positive relationship ($r^2=0.47$, $p<0.0005$) and (B) splitting the
365 relationship by order reveals a significant relationship within both Myodocopida and Podocopida
366 ($r^2=0.53$, $p=0.002$ and $r^2=0.31$, $p=0.001$ respectively). Dot size is proportional to genome size in
367 panel B) to show the larger genomes in Myodocopida overall.

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Figure 1.



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Figure 2.

375 **Table 1.** Genome size estimates in picograms for 46 species of ostracods from the orders Myodocopida and Podocopida collected
 376 from Canada, Australia and the USA. When known, the sex is also listed after the species name. The data includes published values
 377 for two species as well as unpublished data by P. Hebert.

Family	Species	Genome size \pm SE (pg)	n=	Habitat	Locality
Myodocopida					
Cylindroleberididae	<i>Tetraleberis pix</i>	5.10	1	Marine	Lizard Island, Aus.
Cylindroleberididae	<i>Tetraleberis triplex</i>	3.62 \pm 0.09	2	Marine	Lizard Island, Aus.
Cylindroleberididae	Unknown	4.85	1	Marine	Lizard Island, Aus.
Cylindroleberididae	<i>Asteropterygion climax</i>	8.98 \pm 0.14	4	Marine	Lizard Island, Aus.
Cypridinidae	<i>Pterocypridina</i> sp.	4.57 \pm 0.09	3	Marine	Lizard Island, Aus.
Cypridinidae	<i>Vargula tsuji</i> (female)	4.46 \pm 0.08	3	Marine	Santa Barbara, CA
Cypridinidae	<i>Vargula tsuji</i> (male)	4.05	1	Marine	Santa Barbara, CA
Philomedidae	<i>Euphilomedes</i> sp. (female)	2.65 \pm 0.04	2	Marine	Lizard Island, Aus.
Philomedidae	<i>Euphilomedes</i> sp. (male)	2.58 \pm 0.06	2	Marine	Lizard Island, Aus.
Philomedidae	<i>Euphilomedes morini</i> (female)	1.59 \pm 0.03	5	Marine	Santa Barbara, CA
Philomedidae	<i>Euphilomedes morini</i> (male)	1.49 \pm 0.06	5	Marine	Santa Barbara, CA
Philomedidae	<i>Harbansus slatteryi</i>	2.56	1	Marine	Lizard Island, Aus.
Rutidermatidae	<i>Alternochelata lizardensis</i>	3.95 \pm 0.14	4	Marine	Lizard Island, Aus.
Rutidermatidae	<i>Rutiderma dux</i>	2.47	1	Marine	Lizard Island, Aus.
Rutidermatidae	<i>Rutiderma rotunda</i>	4.64 \pm 0.19	4	Marine	Santa Barbara, CA
Sarsiellidae	<i>Eusarsiella asciformis</i> (female)	2.14 \pm 0.06	4	Marine	Lizard Island, Aus.
Sarsiellidae	<i>Eusarsiella springthorpei</i> (male)	2.05 \pm 0.1	2	Marine	Lizard Island, Aus.

Podocopida					
Bairdiidae	<i>Bairdia</i> sp.	0.91±0.04	3	Marine	Lizard Island, Aus.
Candonidae	<i>Candona candida</i> ^c	0.59	1	Freshwater	Circle, AK
Candonidae	<i>Candona rectangulata</i> ^a	0.46	N/A	Freshwater	Unknown
Candonidae	<i>Candocyprinotus ovatus</i> ^a	0.80	N/A	Freshwater	Unknown
Candonidae	<i>Candocyprinotus</i> sp.	0.70±0.01	9	Freshwater	Twentynine Palms, USA
Candonidae	<i>Pseudocandona</i> sp. ^c	0.61	1	Freshwater	Circle, AK
Cyprididae	<i>Bradleystrandesia reticulata</i> ^a	1.06	N/A	Freshwater	Unknown
Cyprididae	<i>Bradleystrandesia splendida</i> ^a	3.13	N/A	Freshwater	Unknown
Cyprididae	<i>Cypricercini</i> sp. ^a	0.70	N/A	Freshwater	Unknown
Cyprididae	<i>Cypriconcha alba</i> ^a	1.22	N/A	Freshwater	Unknown
Cyprididae	<i>Cypridopsis vidua</i> ^c	0.53±0.01	4	Freshwater	Guelph, ON
Cyprididae	<i>Cypridopsis vidua</i> ^a	0.71	N/A	Freshwater	Unknown
Cyprididae	<i>Cypris pubera</i> ^a	0.88	N/A	Freshwater	Unknown
Cyprididae	<i>Heterocypris glaucus</i> ^b	0.71	N/A	Freshwater	Churchill, MB
Cyprididae	<i>Heterocypris (Cyprinotus) incongruens</i> ^c	0.77±0.01	4	Freshwater	Guelph, ON
Cyprididae	<i>Heterocypris (Cyprinotus) incongruens</i> ^b	0.82	N/A	Freshwater	Canada & USA
Cyprididae	<i>Heterocypris punctata</i>	1.40±0.05	4	Freshwater	Twentynine Palms, CA
Cyprididae	<i>Sarscypridopsis aculeata</i> ^a	0.48	N/A	Freshwater	Unknown
Cyprididae	<i>Spirocypris horridus</i> ^a	1.16	N/A	Freshwater	Unknown
Cyproidinae	<i>Cyprois marginata</i> ^a	1.47	N/A	Freshwater	Unknown

Cyroidinae	<i>Cypris occidentalis</i> ^a	2.25	N/A	Freshwater	Unknown
Cytherideidae	<i>Cyprideis beaconnensis</i>	0.28±0.02	2	Estuarine	Santa Barbara, CA
Limnocytheridae	<i>Cytheridella (ilosveayi)</i>	0.36±0.03	4	Freshwater	Santa Barbara, CA
Loxoconchidae	<i>Loxoconcha</i> sp. ^a	0.70	N/A	Marine	Unknown
Macrocyprididae	<i>Macrocyprina barbara</i>	0.17±0.003	4	Marine	Santa Barbara, CA
Notodromadidae	<i>Notodromas monacha</i> ^a	0.87	N/A	Freshwater	Unknown
Xestoleberidae	<i>Xestoleberis</i> sp.	0.11±0.003	3	Marine	Santa Barbara, CA
Xestoleberidae	<i>Xestoleberis hopkinsi</i>	0.20±0.004	5	Marine	Santa Barbara, CA
Unknown	Unknown ^a	1.03	N/A	Unknown	Unknown
Unknown	Unknown ^c	0.66±0.005	5	Freshwater	Guelph, ON
Unknown	Unknown	0.68	1	Freshwater	Fox Lake, YT
Unknown	Unknown	0.67	1	Freshwater	Dixie Springs, NV
Unknown	“Micropodo”	0.26±0.014	4	Marine	Lizard Island, Aus.
Unknown	“Aquarium”	0.37±0.013	4	Freshwater	Freshwater Aquarium

^aUnpublished data by Dr. Paul Hebert

^b(Turgeon and Hebert, 1994)

^cGenome size estimated by flow cytometry