1 The evolution of red blood cell shape in a continental radiation of fishes

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15 Short running title: RBC shape in fishes

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17 Author contributions: BOM collected cell morphometric data assisted by MSS, LFB.

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28

29

30 Abstract

31 The size and shape of Red Blood Cells (RBC) can provide key information on life
 32 history strategies in vertebrates. However, little is known about how RBC shape
 33 evolved in response to environmental factors and the role of phylogenetic relationship.
 34 Here, we analyzed RBC morphometrics in a continental radiation of fishes testing the
 35 hypothesis that phylogenetic relationship determines species occupation of
 36 morphospace. We collected blood samples of five specimens of 15 freshwater fish
 37 species from six orders and used basic stereological methods to measure cell and
 38 nucleus area, perimeter, and diameter, cell and nucleus volume, nucleus:cytoplasm
 39 ratio, and shape factor of 50 cells per specimen. Then, we conducted a phylogenetic
 40 Principal Components Analysis using a dated phylogeny and built a phylomorphospace.
 41 To test if the phylogenetic relationship predicted the phenotypic similarity of species,
 42 we calculated multivariate phylogenetic signal. We also estimated the evolution rate of
 43 RBC shape for each node and tip using ridge regression. Finally, we tested if the
 44 position in the water column influenced RBC shape using a phylogenetic GLS. RBC
 45 shape seems to have evolved in a non-stationary way because the distribution pattern of
 46 species in the phylomorphospace is independent of the phylogeny. Accordingly, the rate
 47 of evolution for shape was highly heterogeneous, with an increase in the genus
 48 *Pygocentrus*. Water column position does not influence RBC shape. In conclusion,
 49 RBC shape seem to have evolved in response to multiple selective pressures
 50 independent of life history characters.

51 **Key-words:** phylomorphospace, erythrocytes, stereology, phylogenetic signal, rate of
 52 evolution.

53 54 Introduction 55

56 The shape, size, and number of Red Blood Cells (RBC) vary greatly in
57 vertebrates (Wintrobe 1934). Although the reasons for this variation are not well
58 understood, phylogenetic relationships can at least partially explain it, because early
59 diverging vertebrate lineages have larger but fewer RBC (Anderson et al., 2018). Early
60 diverging vertebrates also have round and flattened RBCs with a central nucleus, while
61 derived lineages have ellipsoid cells (Nikinmaa 1990; Glomski et al. 1992).

62 Both abiotic factors and life history traits can also affect the composition and
63 morphology of fish RBC (Dal'Bó et al., 2015; Kumar, 2016; Yakhnenko et al., 2016).
64 For example, RBC size is inversely related to swimming ability in teleosts (Lay and
65 Baldwin 1999; Witeska 2013), while size of RBCs is highest at intermediate altitude in
66 one lizard species (González-Morales et al. 2017). In addition, cell size has an inverse
67 relation with metabolic rate, suggesting lowest metabolic cost to maintain active
68 membrane transport in large cells (Szarki, 1970; Graham et al. 1985, Maciak et al,
69 2011). Deep-sea fish species have large RBC, compatible with their low metabolism
70 (Graham et al. 1985). Then, RBC size in teleost has an adaptative value and changes in
71 response to external factors (Lay and Baldwin 1999). RBC volume is another
72 morphological variable correlated with physiological state in fish. For example, cell
73 volume is negative correlated with hemoglobin concentration, while nuclear volume
74 explains only 17% of cell volume (Lay and Baldwin 1999).

75 The nucleus of RBC is strongly related to species adaptation to environmental
76 conditions. Nucleus size is positively correlated with genome size in vertebrates
77 (Gregory 2001). Accordingly, the amount of DNA regulates the size of cells as well as
78 the nucleus. Larger nuclei have more room for protein synthesis that are required in the
79 metabolism of larger cells (Gregory 2001). Variations in RBC DNA content are also
80 directly related to hematopoiesis, which in turn influence species adaptation. Genome

size seems also related to life-history traits. For example, genome size is positively related with the size and diameter of eggs, and negatively related with growth rate and standard size in fish (Hardie and Hebert, 2004; Smith and Gregory 2009).

The shape of RBC also changes according to cell maturation stage. While immature RBC (erythroblasts) are usually rounded, older cells are fusiform (Fijan 2002, Passantino et al. 2004). However, the degree of ellipticalness seems to vary among species (Dal'Bó et al., 2015). However, little is known about the physiological implications of the variation in the fusiform shape for species. Similarly, little is known about the factors involved in the variation of RBC volume and its implications (Lay and Bladwin 1999) but increasing temperature and body mass increase RBC volume in tetrapods (Gillooly and Zenil-Ferguson 2014). Also, RBC volume is related to polyploidy in fish (Gregory 2001). However, little is known about the relative role of phylogenetic relationships and abiotic factors in determining RBC shape.

No study has yet analysed how RBC shape have evolved in fish using modern phylogenetic comparative methods. As shape is multivariate, here we used a set of first- and second-order stereological variables to describe the shape of RBC. Also, we tested if the position in the water column, as a proxy for partial oxygen pressure, species are usually found affect RBC shape. Our hypothesis is that neustonic species (that live closer to the surface) would have smaller RBC due to higher surface area to volume ratio. We also built the phylomorphospace of RBC shape and tested for phylogenetic signal. We expect species with similar habitat use would be close together in the morphospace. This is the first study combining common stereological analysis and phylogenetic comparative method to understand how fish RBC evolved. Our results can help understand how fish species evolved to occupy habitats with different oxygen

concentration or toxicity, besides understanding how metabolic rate vary among species.

107

108 **Methods**

109 **Specimen sampling**

110 We analyzed five individuals of 15 species belonging to 15 genera and 2
111 superorders: *Pygocentrus nattereri*, *Gymnotus inaequilabiatus*, *Astyanax lineatus*,
112 *Bujurquina vittata*, *Cichlasoma dimerus*, *Metynnis maculatus*, *Prochilodus lineatus*,
113 *Piaractus mesopotamicus*, *Rhamdia quelen*, *Poecilia reticulata*, *Hypostomus*
114 *boulengeri*, *Pseudoplatystoma corruscans*, *Synbranchus marmoratus*, *Brycon hilarii*,
115 and *Hyphessobrycon anisitsi* (Figure 1). We obtained adult fish specimens from both
116 sexes between 2017 and 2018 from a number of sources: *Pygocentrus nattereri* was
117 fished in a river the Pantanal field station (Corumbá, Mato Grosso do Sul, central
118 Brazil); *Gymnotus inaequilabiatus*, *Hyphessobrycon anisitsi*, and *Synbranchus*
119 *marmoratus* were bought from a fish store; *Astyanax lineatus* and *Bujurquina vittata*
120 were collected in an urban lake in Campo Grande; *Cichlasoma dimerus*, *Metynnis*
121 *maculatus*, *Prochilodus lineatus*, *Piaractus mesopotamicus* were donated by the city
122 aquarium; *Pseudoplatystoma corruscans*, *Brycon hilarii*, and *Hypostomus boulengeri*
123 were obtained from the university Aquaculture Research ponds; *Poecilia reticulata* was
124 collected in an urban stream in Campo Grande. These species were selected because
125 they are common in our region, use distinct habitats, and belong to distant lineages.

126 Fish were anaesthetized with eugenol solution (50 mg L⁻¹) before blood
127 sampling. Blood was collected by caudal vein or cardiac puncture with syringe with 3%
128 EDTA or by decapitation in small species (Ranzani-Paiva et al., 2013). Blood smears
129 were stained with May-Grunwald-Giemsa-Wright for erythrocyte morphometry

(Tavares-Dias and Moraes 2002). Detailed protocol is available at Rodrigues et al. (2020; see also Figure S1).

132

133 **Red blood cell stereology**

134 Design-based stereological methods have been used since the 1970s to quantify
135 shape and size changes in cells and histological structures (West 2012). This approach
136 uses linear (first-order) measurements to derive tridimensional (second-order) variables
137 that describe the shape of cells in tissues. To take measurements, we used a digital
138 camera (Nikon D3500) coupled in a light microscopy (Zeiss Primo Star®). Digital
139 images in 1000x magnification were used to measure the following first-order variables:
140 area (μm^2), perimeter, and largest and smallest diameter (μm) of cells and area,
141 perimeter, and diameter of the nucleus in 50 randomly selected cells per animal (see
142 Supporting material), i.e., 250 cells per species (50 cells * 5 animals). From these linear
143 measurements, we calculated the following second-order stereological measurements:
144 To calculate length, we took the mean of the largest and smallest diameters. Then, to
145 calculate radius, we simply divided length by 2. Cell volume (μm^3) was calculated using
146 the formula:

$$\frac{4}{3}\pi r^3$$

147 , where r is radius (see West 2012); nucleus volume (μm^3) was calculated by the
148 formula:

$$\frac{CV * N:C}{100}$$

149 , where CV is cell volume and N:C is nucleus:cytoplasm ratio. Cell and nucleus
150 circularity (or shape factor) were estimated by the formula:

$$\frac{4\pi A}{P^2}$$

151 , where A is cell area and P cell perimeter (Russ and Dehoff 2000). Circularity ranges
152 from 0 (elliptical shape) to 1 (circular shape). All measurements were made in Motic
153 Images Plus 2.0 in a double-blind fashion (see Fig. S2).

154 We calculated the mean of each variable for each species (Table S1), which were
155 used as phenotypic traits in further analysis. To visualize the correlation pattern between
156 variables, we built a correlogram considering the average of each variable (Fig. S3)
157 using the *corrplot* package (Wei and Simko, 2017) of R software.

158

159 **Phylogenetic comparative methods**

160 To carry out the comparative analyzes, we first obtained a phylogeny with
161 branch length in millions of years representing the relationship of the 15 species for
162 which we have phenotypic data (Figure 1). This phylogeny was obtained from a fully-
163 sampled topology for Actinopterygii recently published (Rabosky et al., 2018). Analysis
164 was done in the *fisher* package (Chang et al., 2019).

165 Then, we performed a phylogenetic Principal Components Analysis (pPCA) on
166 the matrix of phenotypic data with the eight variables considering the phylogeny
167 (Revell, 2009). The pPCA was performed using the correlation matrix of variables and
168 assuming that traits have evolved according to a Brownian Motion (BM) model of
169 evolution. The pPCA uses a **C** matrix that describes the variance-covariance structure
170 between species given by the phylogeny and the evolutionary model (BM in this case)
171 to obtain the Principal Components and species scores (see also Adams & Collyer
172 2019). Analysis was performed using the `phyl.pca` function of the *phytools* package
173 (Revell, 2012).

174 From this exploratory analysis, we built a morphospace for all 15 species
175 showing their similarity in relation to RBC shape. The analysis estimates the node

176 values using a Maximum Likelihood method. Finally, we built the phylomorphospace
177 by projecting the phylogeny onto the morphospace built with the first two Principal
178 Components, which allows us to explore how lineages occupied the morphospace as
179 their traits evolved (Adams and Collier 2018, 2019). Analysis was performed using the
180 phylomorphospace function of the phytools package.

181 Finally, we calculated a phylogenetic signal measure for multivariate data called
182 K_{mult} (Adams, 2014) using all pPCs to avoid missing variation (Uyeda et al., 2015). This
183 method allows calculating a statistic that measures how similar closely related species
184 are in relation to their phenotypic measurements. Blomberg's K (Blomberg et al., 2003)
185 is a measure widely used to measure phylogenetic signal for univariate data and ranges
186 from 0 <K> 1. High phylogenetic signal ($K > 1$) indicates that closely related species
187 tend to be similar in relation to phenotypic attributes (Münkemüller et al., 2012). This
188 method has been adapted for multivariate data recently (Adams, 2014), along with a
189 Monte Carlo randomization procedure that allows testing the significance of the
190 statistic. Analysis was performed with the physignal function of the geomorph
191 package (Adams et al. 2020).

192 To test the effect of the position in the water column species usually occupy on
193 RBC shape, we gathered data on depth range from FishBase (Froese and Pauly 2019)
194 and treated it as a categorical variable, henceforth referred to as habitat. Thus, species
195 were classified as demersal (n=4), pelagic (n=2), and benthopelagic (n=9). Afterwards,
196 we compared the fit of evolutionary models to the eight pPCs. Specifically, we fit
197 Pagel's lambda transformation, Brownian Motion, and Ornstein-Uhlenbeck models to
198 data using the leave one out cross-validation of the penalized log-likelihood, as
199 implemented in the mvglms function (Clavel and Morlon 2020) of the package
200 mvMORPH (Clavel et al. 2015) including habitat as a predictor variable. Then, we

201 compared the model fit using the Extended Information Criterion (EIC), which showed
202 that the best model was Brownian Motion (BM; Table S2). We built a phylogenetic
203 Generalized Least Squares (Clavel and Morlon 2020) using BM to test the effect of
204 habitat on RBC shape, with the Pillai's trace as test statistic and Type I Sum of Squares.

205 Finally, to test the hypothesis if a change in evolution rate is the mechanism
206 allowing species to occupy different positions in the morphospace, we estimated tip-
207 level evolution rate of RBC shape using phylogenetic ridge regression, as implemented
208 in the RRphylo package (Castiglione et al. 2018). For this analysis we used all eight
209 pPCs. This analysis also automatically finds nodes in which there was a rate shift. The
210 R markdown dynamic document describing how analyses were conducted and
211 associated data are available at FigShare (Martins et al. 2020).

212

213 **Results**

214

215 The shape of Red Blood Cells (RBC) of fish species studied varied from oval to
216 ellipsoid and a rounded nucleus (Figure 2). Cell size varied by an order of magnitude,
217 from the largest cell of *Synbranchus* to the smallest in *Poecilia*. The same phenomenon
218 happened to cell volume (Fig. 3). Nucleus area varied from $7.45 \mu\text{m}^2$ (± 1.2 SD) in
219 *Poecilia* to $27.30 \mu\text{m}^2$ (± 4.9 SD) in *Synbranchus*. Additionally, the range of
220 intraspecific variation in some measurements varied among species. For example, cell
221 volume had the highest standard deviation, while shape factor, nucleus area and cell
222 perimeter seem to vary less within species (Table S1).

223 The pPC1 retained 66.1%, while pPC2 retained 21.4% of the variation in RBC
224 shape. Cell and nucleus area, cell and nucleus perimeter, cell and nucleus volume were
225 highly negatively correlated (> 0.84) with pPC1, whereas the nucleus:cytoplasm ratio
226 was highly (-0.87) correlated with pPC2 (Table S2). Species positively correlated with

pPC1 had higher shape factor, whereas those negatively correlated had higher cell and nucleus area, nuclear and cell perimeter, cell and nucleus volume, and nucleus:cytoplasm ratio (Table S2). Species positively correlated with pPC2 had higher cell perimeter, cell and nuclear area, whereas those negatively correlated had higher nucleus:cytoplasm ratio, nucleus and cell volume, and nucleus area (Fig. S4).

The distribution of species in the phylomorphospace suggest that closely related species do not have RBC with similar shape (Fig. 4). Accordingly, RBC shape does not exhibit phylogenetic signal ($K_{\text{mult}} = 0.6317$; Effect size = 0.7863; $P = 0.159$). The low effect size suggest phylogenetic signal is not concentrated in a few dimensions. To test if only a subset of dimensions displays phylogenetic signal (see Adams & Collyer 2019), we re-run the analysis with only the first two pPCs. Results did not change (see Martins et al. 2020). However, the morphospace suggest a non-stationary pattern in the evolution of RBC shape, since most closely related species occupy distant positions, but the position of siluriformes (*Pseudoplatistoma*, *Rhamdia*, and *Hypostomus*) mirror their phylogenetic relationship in the upper part of the morphospace. Interestingly, the Acanthopterygii included in our sampling: *Synbranchus*, *Poecilia*, *Bujurquina*, and *Cichlosoma* had the most divergent pattern, occupying extreme positions in the phylomorphospace, whereas species of Ostariophysi were more packed together. There is a high divergence in RBC shape within Characiformes. Therefore, there seems to be a variation in the pattern of RBC shape evolution at the level of higher order groups (Superorders), instead of a homogeneous pattern throughout the whole phylogeny.

Habitat did not influence RBC shape (Pillai's trace=0.6936; $P=0.461$). The results did not change if we use a multivariate PGLS (Table S4). We also found that evolution rate of RBC shape was very heterogeneous at the tip level (Fig. 5). The species with the highest rate was *Pygocentrus* (0.741) and the lowest was *Bujurquina*

(0.087). There was a tendency to increase the rate in the common ancestor of *Pygocentrus*, *Metynnis*, and *Piaractus* in relation to the background rate, but the rate shift was not significant (rate difference = 0.392; $P > 0.005$). Interestingly, the species occupying the most extreme positions in the phylomorphospace, such as *Synbranchus* (0.514), *Astyanax* (0.555), and *Gymnotus* (0.17) were not the ones with the highest rates of evolution. Therefore, our results suggest that an increase in the rate of evolution does not necessarily produces morphological specialization and rate disparity is not the main pattern involved in the formation of RBC morphospace.

Discussion

We found that the shape of Red Blood Cells (RBC) varies greatly among and within freshwater fish species. Contrarily to our initial hypothesis, we did not find a correlation between water column position (proxy for oxygen availability) and RBC shape. Accordingly, we did not find phylogenetic signal in RBC shape, suggesting closely related species do not have similar RBC shape. There does not seem to be a single mechanism driving the evolution of RBC shape, since the phylomorphospace shows a non-stationary pattern in the species similarity.

Small RBC allow species to occupy environments with low dissolved oxygen, since they usually have a higher number of cells, making them more efficient in oxygen uptake and transport (Silkin et al., 2019; Hawkey et al., 1991). Species with RBCs that have large surface area and volume have low metabolic rate, since the cell surface:volume ratio decreases the metabolic cost of gas exchange between membranes (Jones 1979, Lay and Baldwin, 1999). *Synbranchus* had higher perimeter (i.e., higher surface) and higher area. Thus, our results suggest that this species could have a lower metabolic rate than *Poecilia*, because the later has a smaller cell area and perimeter.

Most species occupied the center of the morphospace, independent of the phylogenetic relationship. This clustering of distantly related species suggests that convergence, apparently not caused by water column position, could be a mechanism influencing RBS shape evolution (Adams and Collyer 2019). Clustering in morphospace can also suggest a developmental constraint (Jablonski 2020) that impedes species to change their traits after speciation (Pie and Weitz 2005). Also, a few species were in extreme positions. Even though water column position did not explain RBC shape, the extreme position of *Synbranchus*, *Astyanax*, and *Poecilia* in the morphospace could be explained by differences in their life history strategies. For example, *Synbranchus* displays facultative pulmonary respiration, usually occurs in habitats with hypoxia, and burry itself during the dry season (Froese and Pauly, 2019). *Astyanax* also occurs in habitats with hypoxia (Froese and Pauly, 2019). The pattern we found points to clade disparity differences (Adams & Collyer 2019), with Acanthopterygii having higher dispersion in the morphospace than Ostariophysi (Fig. S6). These two orders have different ecological and biogeographic characteristics. Species from Ostariophysi are essentially from freshwater, while Acanthopterygii has marine origins, with secondary freshwater introgressions. One interesting pattern that emerges from the morphospace is that apparently there is no constraints in the morphospace, because almost all regions have one species. Siluriformes seem to have similar RBC shape, given their position in the morphospace, and similar evolution rate. This lineage occupies the upper position of the morphospace, along with *Piaractus* and *Hyphessobrycon*, and had high cell area and perimeter, and low nucleus:cytoplasm ratio, nucleus volume, and circularity. Exception for *Rhamdia* and *Hyphessobrycon*, all those species are demersal (benthic). Together these results suggest that the evolution of

302 RBC shape was driven by fluctuating selection, although this result should be taken
303 with care, since our sample size is not large.

304 We found a high heterogeneity in evolution rate of RBC shape. This high rate
305 heterogeneity helps explain why we did not detect a significant phylogenetic signal, i.e.,
306 the change in shape seems to vary strongly at the tip level, instead of large clades. This
307 is consistent with the model selection procedure that pointed Brownian Motion (BM) as
308 best fit model to the data. BM can be caused by genetic drift, when selection randomly
309 varies in direction through time, or when selection is weak compared to the time scale
310 analysed (Felsenstein 1988). The large intraspecific variation in most variables (Table
311 S1) may suggest that variables describing RBC shape are under weak selection pressure
312 (Nikinmaa 2019). Interestingly, the position extreme positions of *Synbranchus* (0.514),
313 *Astyanax* (0.555), and *Pygocentrus* (0.741) could be explained at least partially by an
314 increase in evolution rate, which allowed them to diverge from their sister species,
315 remaining *Acanthopterygii*, *Hyphessobrycon*, and *Metynnis* respectively. However,
316 given our sample size we cannot rule out that RBC shape of these species are evolving
317 under directional selection. Conversely, if lineages followed a random walk (akin of
318 BM) one would expect species to diverge and occupy positions in the morphospace
319 irrespective of their habitat or phylogenetic position (Pie and Weitz 2005).

320 Water column position did not explain RBC shape in this freshwater fish species
321 radiation. This result suggest that RBC shape may not be entirely determined by partial
322 pressure of dissolved oxygen in water. It also suggests that water column position does
323 not represent a main constraint in the evolution of these traits. A recent study (Minias
324 2020) also found that life history traits did not explain hemoglobin and hematocrit
325 evolution in birds. Differently from mammals, fish RBC are nucleated cells and have
326 many cytoplasmic organelles, such as mitochondria, Golgi complex, centrioles,

327 ribosomes, microtubules, rough and smooth endoplasmic reticulum (Sekhon and Beams
328 1969), as well as a range of cytoplasmatic enzymes involved in the anaerobic glycolysis
329 (Sephton et al. 1991). These organelles and enzymes allow the functions of RBC to be
330 finely regulated and somewhat independent from other components of the
331 hematopoietic system, enabling fish to occupy a wide range of environments (Nikinmaa
332 et al. 2019). Therefore, species may respond rapidly to changes in oxygen availability
333 (Witeska, 2013), which indicates that it is not constrained by inheritance (Arnold 1992)
334 or phylogenetic inertia, at least to a certain degree.

335 In conclusion, our study provides a novel macroevolutionary perspective on fish
336 RBC shape by combining commonly used design-based stereological techniques with
337 modern phylogenetic comparative methods. We found that the RBC of the two main
338 orders of Neotropical freshwater fish vary greatly in shape, but that their water column
339 alone does not explain it. Future studies should use quantitative genetic approaches to
340 explore the underlying genetic basis for RBC morphology, possibly using geometric
341 morphometrics and experimental approaches motivated by ecological data.

342 **Acknowledgements**

343 This study was supported by Universidade Federal de Mato Grosso do Sul –
344 UFMS/MEC – Brasil. This study was funded in part by the Coordenação de
345 Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.
346 F. R. Carvalho and K. Tondato provided insightful comments as members of BOM
347 bachelor's honors thesis examining committee.

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- 489
490 Figure Legends
491
492 Figure 1. Dated phylogenetic tree with species to which we have phenotypic data used
493 in the analysis, pruned from Rabosky et al. (2018).
494
495 Figure 2. Shape and size of Red Blood Cells of the 15 fish species analysed. Staining:
496 MMGW. Scale bars = 10 µm.
497
498 Figure 3. Diagram showing the mean of each stereological variable with the
499 phylogenetic tree of the species sampled. Data on standard deviation for each variable is
500 available at Table S1.
501
502 Figure 4. Phylomorphospace constructed with phylogenetic Principal Components 1
503 and 2 showing species distribution in the reduced space. Red Blood Cell (RBC) images
504 are all in the same magnification and belong to species occupying the extreme positions
505 along each pPC. The RBC associated positively with pPC1 is from *Poecilia*, which has
506 a high shape factor, while the RBC associated negatively with pPC1 is from
507 *Synbranchus* that has high cell area and perimeter, and high nucleus area and perimeter.
508 The RBC associated positively with pPC2 belongs to *Pseudoplatystoma*, which has high
509 cell perimeter. The RBC image associated negatively with pPC2 is from *Astyanax* and
510 has a high shape factor, high nucleus:cytoplasm ratio, and high nucleus volume. Colours
511 represent depth in the water column species are usually found, green = Pelagic; black =
512 Benthopelagic; red = Demersal.

513
 514 Figure 5. Phylogenetic tree showing the results of the tip-level evolution rate estimated
 515 using ridge regression for the stereological variables describing the Red Blood Cell
 516 shape. Analysis was conducted in RRphylo.
 517
 518 Data accessibility statement
 519
 520 All the data and R code used to run the analysis will be deposited in FigShare upon
 521 acceptance.

Acanthopterygii

Ostariophysi