Local parasite pressures and host genotype may modulate epigenetic diversity in a mixed-mating fish

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Running title: Parasites, genetics and epigenetics in a mixed-mating fish

Abstract

Parasite-mediated selection is one of the main drivers of genetic variation in natural populations. The persistence of asexual reproduction and self-fertilization, however, challenges the notion that low genetic variation and inbreeding compromise the host's ability to respond to pathogens. DNA methylation represents a potential mechanism for generating additional adaptive variation under low genetic diversity. We compared genetic diversity (microsatellites and AFLPs), variation in DNA methylation (MSAFLPs), and parasite loads in three populations of *Kryptolebias hermaphroditus*, a unique mixed-mating (partially self-fertilising) fish, to analyse the potential adaptive value of DNA methylation in relation to genetic diversity and parasite loads. We found strong genetic population structuring, as well as differences in parasite loads and methylation levels among sampling sites and selfing lineages. Globally, the interaction between parasites and inbreeding with selfing lineages influenced DNA methylation, but parasites seemed more important in determining methylation levels at the local scale.

Keywords: epigenetic variation; hermaphroditism; self-fertilisation; inbreeding; local adaptation; mangrove killifish

Introduction

Organisms with mixed-mating reproduction (alternating between self-fertilisation and outcrossing) can benefit from the advantages of both biparental and uniparental reproduction: outcrossing generates genetic variability and adaptability potential, while selfing ensures reproduction without partners (Jarne and Chalesworth 1993). Reproductive assurance (Darwin 1876) gives self-reproducing individuals an advantage when colonising new environments (Baker 1955) and ensures the genetic transmission of both sets of parental genes (Fisher 1941). The downside of selfing, however, is that the progeny can have reduced fitness compared to their outcrossed counterparts, and suffer from inbreeding depression (Charlesworth and Willis 2009). Thus, occasional outcrossing should be beneficial when inbreeding can impair offspring fitness (Damgaard et al. 1992; Maynard Smith 1978).

The Red Queen hypothesis (Van Valen 1973; Bell 1982) is often invoked to explain the occurrence of sexual reproduction in face of the advantages of asexual reproduction (Blirt and Bell 1987; Lively 1987; Lively and Morran 2014). According to this hypothesis, the more genetically diverse offspring of sexually reproducing individuals provide a “moving target” to parasites, making it more difficult for them to adapt compared to the “more static” offspring of asexual/uniparental individuals (Maynard Smith 1978; Hamilton 1980; Lively et al. 1990;). Yet, while sexual reproduction seems the general rule in animals (approximately 99%; Slowinski et al. 2016), asexual and self-fertilising lineages sometimes persist in a wide range of environments (Zhang et al. 2010), suggesting that their adaptation and long-term survival could be facilitated by other factors in addition to genetic variability (Verhoeven and Preite 2014).

Non-genetic factors (including epigenetic mechanisms) can play an important role in generating adaptive phenotypic variation (Bossdorf et al. 2008; Verhoeven et al. 2016; Bonduriansky and Day 2018), including resistance to parasites (Verhoeven et al. 2010;

Wenzel and Piertney 2014). Epigenetic mechanisms (e.g. histone modifications, microRNAs, DNA acetylation), can modulate changes in gene expression in response to environmental variation without involving changes in DNA sequence (Bossdorf et al. 2008; Richards et al. 2017). DNA methylation is the best characterized epigenetic modification (Lea et al. 2017), and has important roles on pre-transcriptional control in several biological processes, such as cell differentiation and genomic imprinting (Koch et al. 2016). Variation in DNA methylation is not completely independent from the genome, and epialelles can have different degrees of autonomy from the genotype (Richards 2006; Dubin et al. 2015). In addition, in some plants and animals, individuals with low levels of heterozygosity display high levels of genome-wide DNA methylation variation (Richards et al. 2012; Schrey et al. 2012; Liebl et al. 2013), sugesting that DNA methylation could contribute to the adaptation of asexual and inbred organims with limited genetic diversity to environmental change (Castonguay and Angers 2012; Schrey et al. 2012; Liebl et al. 2013; Verhoeven and Preite 2014; Douhovnikoff and Dodd 2015).

Increasing evidence suggests that epigenetic mechanisms, including genome-wide DNA methylation, are involved in host-pathogen interactions (Gómez-Díaz et al. 2012; Hu et al. 2018), but the mechanisms are better known in plants than in animals (Annacondia et al. 2018; Hewezi et al. 2018; Gómez-Díaz et al. 2012). Mixed-mating organisms represent ideal models to test the associations between genetic and epigenetic variation with pathogen pressures because selfed and outcrossed offspring naturally coexist, usually displaying very different levels of genetic diversity. Negative associations between genetic diversity and parasite loads have been previously observed in mixed-mating animals (Lively and Morran 2014; Ellison et al. 2011), with inbred individuals usually harbouring more parasites. The relationship between epigenetic variation, parasites and mixed-mating, however, has not been explored.

69 Here, we compared genetic diversity, variation in DNA methylation, and parasite
70 loads in three natural populations of the mixed-mating mangrove killifish *Kryptolebias*
71 *hermaphroditus* distributed along the Brazilian coast (Tatarenkov et al. 2017). The genus
72 *Kryptolebias* contains the only known mixed-mating vertebrate species (*K. marmoratus* and
73 *K. hermaphroditus*), characterised by variable rates of selfing and outcrossing (Tatarenkov et
74 al. 2017). Populations of both species consist mainly of self-fertilizing hermaphrodites and
75 varying levels of males at low frequencies (Tatarenkov et al. 2017; Berbel-Filho et al. 2016),
76 and exhibit high levels of homozygosity (Tatarenkov et al. 2009, 2017), suggesting that self-
77 fertilization is the most common mode of reproduction (Avise and Tatarenkov 2015).
78 We analysed microsatellites (previously shown to correlate with parasite loads in the
79 closely related *K. marmoratus*, see Ellison et al. 2011) and genome-wide methylation based
80 on identification of anonymous CpG by methylation-sensitive AFLP (MS-AFLPs, previously
81 used in non-model organisms) to identify epigenetic variation associated to parasite loads
82 (Wenzel and Piertney 2014). Based on the Red Queen Hypothesis and previous results in *K.*
83 *marmoratus*, we expected lower genetic diversity and higher parasite loads in inbred
84 compared to outbred individuals. We also predicted higher variation in DNA methylation in
85 relation to inbreeding and parasite loads, if methylation played an adaptive role, potentially
86 related to immunity, in *K. hermaphroditus*.
87

88 **Methods**

89 **Study system, field sampling and parasite screening**

90 A total of 128 specimens of *K. hermaphroditus* were collected using hand-nets from three
91 sampling sites on isolated mangroves on the North-eastern coast of Brazil between January
92 and September 2015: Ceará-Mirim river – Site 1; Curimataú river – Site 2; Ipojuca river -
93 Site 3 (Fig. 1). *K. hermaphroditus* is distributed along the Brazilian coast (Tatarenkov et al.
94 2017) and is typically found in shallow pools of high salinity levels (>30 ppt), clear waters
95 and muddy substrates, where there are few other sympatric fish (Lira et al. 2015; Berbel-
96 Filho et al. 2016). All specimens displayed the common hermaphrodite phenotype (dark
97 colour with well-defined ocellus on the caudal fin; Costa 2011). Fish were euthanized using
98 an overdose of tricaine methane-sulfonate (MS-222) following UK Home Office Schedule 1
99 (Hollands 1986), standard length was measured using a digital calliper (mm) and the whole
100 fish were preserved in 95% ethanol at -20 □ for parasite screening and DNA extraction.

101 In the laboratory, fish were dissected and screened for both external and internal parasite
102 infections using a dissecting microscope following the methods of Ellison et al. (2011). To
103 assess the reliability of parasite screening, a subsample of five fish was examined by a
104 different observer and the agreement was 100%. We defined parasite loads using a scaled
105 measure of parasite abundance, where for each parasite morphotype (i), the number of
106 parasites per individual (Ni) was divided by the maximum number found across all
107 individuals (Nimax). The final value of the scaled parasite load represents the sum of scaled
108 parasite loads across all parasite types. Given their uneven abundance (Table 1), this
109 approach minimizes bias when parasite loads are heavily influenced by a very abundant
110 parasite type (in our case bacterial cysts) (Bolnick and Stutz 2017).

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Genetic analysis

Genomic DNA from all 128 fish was extracted from gill tissue using a Nexttec extraction kit for blood and tissue samples (Nexttec, Leverkusen, Germany). Gills are an important physical and immunological barrier to pathogens in fish (Press and Evensen, 1999), and the organ where most parasites were found (Table 1). Twenty-seven microsatellite loci (Mackiewicz et al. 2006; Tatarenkov et al. 2017) were genotyped as in Ellison et al. (2011) and screened using GeneMapper v.4.0 (Applied Biosystems, Foster City, USA). Loci were tested for linkage disequilibrium and Hardy-Weinberg equilibrium using GENEPOP v. 4.5.1 (Rousset 2008). Mean number of alleles per locus (N_{ma}), observed heterozygosity (H_o) and expected heterozygosity (H_e) were estimated using GenALEX v. 6.5 (Peakall and Smouse 2012). The inbreeding coefficient (F_{IS}) was calculated in GENEPOP. Global heterozygosity for individual fish was estimated using the homozygosity by locus index (HL) implemented in the Excel macro Cernicalin v 1.3 (Aparicio et al. 2006).

We also used the Bayesian clustering algorithm INSTRUCT (Gao et al. 2007) to estimate the optimal number of selfing lineages (k) with four simultaneous chains of 2,000,000 MCMC runs, 10 as thinning, and 100,000 of burn-in period, resulting in 100,000 interactions for each chain. The potential number of k tested ranged from 2 to 12. We used the individual *q*-values (the likelihood of membership to a particular genetic cluster or selfing lineage) from INSTRUCT to classify individuals as either selfed or outcrossed (Vähä and Primmer 2006). A threshold of *q*-value ≥0.9 was used to classify selfed individuals, while <0.9 represented hybrids between two different selfing lineages, suggesting an outcrossing event (Ellison et al. 2011; Vähä and Primmer 2006). Pairwise F_{ST} values among sampling sites and selfing lineages were estimated with Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010) using 10,000 permutations. We used hierarchical analysis of molecular variance (AMOVA) to investigate population structuring among sampling sites and selfing lineages (according to individual *q*-

values) using 10,000 randomizations. Differences between selfed and outcrossed groups in the total number of parasites and homozygosity by locus (microsatellites) were analysed using median Mann-Whitney rank tests in R v. 3.3.

Epigenetic analysis

We used Methylation-Sensitive Amplified Fragment Length Polymorphisms (MS-AFLPs) to assess genome-wide DNA methylation patterns (Schrey et al. 2013). DNA extracted from gill filament tissue of 115 fish (33 classified as outcrossed and 82 as selfed according to the INSTRUCT *q*-values; 62, 36 and 17 from samplings sites 1, 2 and 3, respectively) was used for the MS-AFLPs analysis following Rodríguez López et al. (2012). A DNA aliquot of 100 ng per individual was split for digestion with two enzyme combinations: EcoRI/HpaII and EcoRI/MspI. The digested DNA was ligated to adaptors and a selective PCR was conducted using the primers ECORI-ACT: GACTGCGTACCAATTCACT and HPA-TAG: GATGAGTCTAGAACGGTAG following Ellison et al. (2015). The HpaII primer was end-labelled with 6-FAM. Fragments were run on an ABI PRISM 3100 (Applied Biosystems) and the resultant profiles were analysed using GENEMAPPER v. 4.0 (Applied Biosystems). To ensure reproducibility the following settings were applied: analysis range was 100-500 bp; minimum peak height was 100 relative fluorescence units; pass range for sizing quality: 0.75-1.0; maximum peak width: 1.5 bp. To confirm MS-AFLP reproducibility, 24 individuals (~20% of the total; eight from each sampling site) were reanalysed and compared using the same protocols.

The R package msap v. 1. 1. 9 (Pérez-Figueroa 2013) was used to analyse MS-AFLP data. To increase reproducibility of the genotyping, we used an error threshold of 5% as suggested by Herrera and Bazaga (2010). According to the binary band patterns, each locus was classified as either methylation susceptible loci (MSL; i.e. displaying a proportion of

HPA+/MSP- and/or HPA-/MSP+ sites which exceed the error threshold (5%) across all samples) or non-methylated loci (NML; if the same patterns did not exceed the error threshold) (Pérez-Figueroa 2013). MSL were used to assess epigenetic variation, while NML were used as a measure of AFLP genetic variation. Average group methylation percentages for inbreeding status were calculated using the different binary band patterns (hemimethylated pattern (HPA+/MSP-) + internal cytosine methylation pattern (HPA-/MSP+)/unmethylated pattern (HPA+/MSP+) + hypermethylation/absence of target (HPA/MSP-) *100) (Veerger et al. 2012).

Epigenetic (MSL) and genetic at AFLPs (NML) differentiation among sampling sites, selfing lineages and between outcrossed and selfed groups, was assessed by AMOVA with 10,000 randomizations. Epigenetic (MSL) and genetic (AFLP and microsatellites) differentiation among sampling sites, selfing lineages and inbreeding status was visualized by principal coordinates analysis (PCoA). Mantel tests based on distance matrices (Mantel 1967) were used to test for potential correlations between epigenetic and genetic data for MSL, NML and microsatellites using GENALEX v. 6.5 with and 10,000 permutations. To identify disproportionately differentiated methylation states, we used a F_{ST} outlier approach implemented in Bayescan 2.1 (Foll and Gaggiotti 2008; Perez-Figueroa et al. 2010), with $2x10^6$ iterations (thinning interval 20 after 20 pilot runs of 10^4 iterations each) and a burnin of $5x10^5$. We tested for outliers based on the MSL data generated on the comparisons among sampling sites, selfing lineages and between inbreeding status (inbred or outbred).

Statistical analyses

A Kruskal-Wallis test was used to examine the differences on scaled parasite load and bacterial cysts (the most prominent parasite) among selfing lineages. To test the relationship between genome-wide variation in DNA methylation and parasite loads, the proportion of

188 methylated loci per individual was calculated as the proportion of loci scored as methylated
189 over the total number of loci observed per individual ("0" for unmethylated and "1" for
190 methylated, excluding the missing data cells per individual). We then employed a generalized
191 linear model with a binomial link to model proportion of methylated loci as a function of
192 scaled parasite load, selfing lineage, sampling site and inbreeding status. We repeated the
193 analysis including only the most prominent parasite type (bacterial cysts).

194 Model selection was conducted using the multi-model averaging approach
195 implemented in the R package glmulti v 1.0.7 (Calcagno and de Mazancourt 2010). We chose
196 the minimal adequate models based on the lowest AICc values (Akaike Information Criterion
197 corrected for small sample size), Akaike weight (W_i) and evidence ratios (Burnham and
198 Anderson 2004). Models (within 2 AIC units) were also reported. Predictors were checked
199 for collinearity using *pair.panels* function in R package psych (Revelle et al. 2019). Model
200 residuals were checked and assumptions validated.

201 To disentangle potential confounding effects arising from the unequal distribution of
202 selfing lineages among sampling sites (i.e. five lineages are exclusive to a particular sampling
203 site, Table S1), we repeated the analyses (AMOVA, Mantel test, PCoA and GLMs) for both
204 genetic (microsatellites and AFLPs) and epigenetic (MSL) data using on individuals from
205 Site 1 (68 individuals for microsatellites and 62 for MS-AFLPs), as this was the only site
206 with more than two selfing lineages (Table S1).

207 **Results**

208 **Parasite screening**

209 Macroscopic parasite loads were generally low and we focused on the three most common
210 types of parasites identified. Bacterial cysts were present on the gills and consisted of white
211 to yellow spherical cysts circumscribed by a capsule, which resulted in hypertrophied gill
212 filaments. They were the most common type of pathogen appearing in 83.6% of the

213 individuals screened, with a prevalence ranging from 1 to 19 (mean=2.73, s.d.=2.99), and
214 were more prevalent in Site 1 (mean=3.16, s.d.=3.16), followed by Site 2 (mean=2.66,
215 s.d.=3.10) and Site 3 (mean=1.27, s.d.=0.80). The second most common macroscopic
216 parasites were protozoan cysts, which consisted of small dark oval cysts over the gills arch
217 and filaments. In total, 19.53% of the total number of individuals were infected with these
218 cysts, ranging from 1 to 6 (mean=0.54, s.d.=1.26). Protozoan cysts were absent in Site 1, but
219 present in Site 2 (mean 1.52, s.d.=1.6) and Site 3 (mean=0.33, s.d.=1.37). Finally, adult
220 nematodes were found in the gut of only eight individuals (6.25%), ranging from 1 to 3
221 (mean=0.09, s.d.=0.40). Nematodes were only detected in Sites 1 (mean=0.3, s.d.=1.37) and
222 2 (mean=0.02, s.d.=0.15) (Fig. S1; Tables 1 and S1). Only seven individuals (5.4%) were
223 uninfected with macroparasites. Significant differences were found on scaled parasite loads
224 (Chi square = 32.14, p = <0.001, df = 5) and bacterial cysts (Chi square = 12.98, p = 0.01, df
225 = 5) among selfing lineages.

226 **Genetic diversity and population structuring based on microsatellites**

227 No linkage disequilibrium was detected between any pair of microsatellite loci. As expected
228 from the high levels of self-fertilisation of the species, no loci were found to be in Hardy-
229 Weinberg equilibrium, and all 27 microsatellite loci showed an excess of homozygotes. The
230 global homozygosity index (HL) was very high (mean = 0.95), as well the estimated selfing
231 rates (Table 1). At the individual level, 93 individuals (72.6%) were homozygous across all
232 27 microsatellite loci. However, 17 individuals (13.28%), displayed intermediate to high
233 levels of heterozygosity (ranging from 0.13 to 0.69).

234 The clustering Bayesian algorithm INSTRUCT indicated that six was the most likely
235 number of selfing lineages (k). Selfing lineage 6 was shared between two different
236 mangroves (Site 1 with seven individuals and site 2 with one individual), separated by
237 approximately 100 km. The other five lineages were solely represented in one of the

mangroves (lineage 1 with 14 individuals, lineage 2 with 25 individuals and lineage 4 with 22 individuals in Site 1; lineage 3 with 41 individuals in Site 2; and lineage 5 with 18 individuals in Site 3) (Figs. 1 and 2; Table S1). High F_{ST} values were found both among sampling sites (mean = 0.28, s.d. = 0.02) and selfing lineages (mean=0.32, s.d. = 0.05). All pairwise comparisons were highly significant (Table S2).

Based on the *q*-values from the INSTRUCT lineages, 92 fish (71%; 46 from Site 1, 30 from Site 2, 16 from Site 3) were classified as selfed (with *q*-values ≥0.9) and 36 (29%; 22 from Site 1, 12 from Site 2 and two in Site 3) as outcrossed (with *q*-values <0.9) (Fig. 2; Table S1). Overall, outcrossed individuals had significantly lower homozygosity by locus values (at microsatellites) and total parasite loads than selfed individuals (Table 2).

Overall, AMOVA analyses using microsatellites indicated strong and significant differentiation among sampling sites (F_{ST} = 0.28, P = 0.001) and selfing lineages (F_{ST} = 0.32, P = 0.001), with the latter explaining more of the genetic variance than the former (Table 3). Although significant, very low genetic differentiation was found between selfed and outcrossed individuals (F_{ST} = 0.01, P = 0.002) (Table 3; Fig. S2). These patterns were also seen on PCoA analysis, with individuals generally clustering by selfing lineages in the microsatellites data (25.84% of overall variation), with individuals from lineage 4 being the most differentiated from the other lineages on Site 1. In this site, substantial overlap was found among selfing lineages and between selfed and outcrossed, despite its significant differences (F_{ST} = 0.03, P = 0.001) (Table S4; Fig. S3).

Genetic and epigenetic variability and population structuring based on MS-AFLPs

The epigenetic analysis identified 381 MS-AFLP loci, of which 267 (70.07%) were methylation-susceptible loci (MSL) and 106 (27.82%) non-methylated loci (NML). Of the MSL loci, 236 (88.3%) were polymorphic and therefore were used for the variability

analysis. Reproducibility comparisons between original and replicated genotypes for 24 individuals revealed 262 loci with an average of 10% differences (sum of number of differences between first and second set of genotypes divided by number of individuals). AMOVA analysis for reproducibility revealed no significant differences between methylation and AFLP variation patterns between original and replicated set of individuals (Table S3). Average methylation ranged from 47.51% on lineage 2 to 38.17 % on lineage 5, and was 44.82% for inbred and 45.77% for outbred individuals.

AMOVA revealed very low but significant differentiation among sampling sites, for both genetic (AFLPs: Φ_{ST} = 0.02, P = 0.001) and epigenetic loci (Φ_{ST} = 0.02, P < 0.001). Significant differentiation among selfing lineages was also found on genetic (AFLPs: Φ_{ST} = 0.02, P = 0.004) and epigenetic loci (Φ_{ST} = 0.02, P = 0.001). Overall, higher genetic and epigenetic variance was found within than between groups (Table 3). As with microsatellites, no clear genetic (at AFLPs) or epigenetic differentiation was found between selfed and outcrossed individuals (Fig. S2). There was, however, a significant positive association between epigenetic (MSL) and genetic diversity, both using AFLPs (Mantel test, r = 0.11; P = 0.002) and microsatellites (r =0.09; P= 0.001). No MSL epiloci were identified as an F_{ST} outlier in any of the comparisons.

No significant differences between selfing lineages were found among lineages for individuals from Site 1 for AFLPs genetic data (selfing lineages: Φ_{ST}=0.008, P = 0.12) or MSL epigenetic data (selfing lineages: Φ_{ST}=0.006, P = 0.20) (Table S4). In the PCoA, substantial overlap was found among selfing lineages and between selfed and outcrossed individuals (Fig. S3). Mantel tests between genetic and epigenetic data indicated a significant positive association between AFLPs and MSL data (r = 0.21; P <0.001), but not between microsatellites and MSL (r = -0.005; P =0.45).

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287

288 **Parasite loads, genetic and epigenetic variation**

289 According to a multi-model testing approach, the most plausible model for the proportion of
290 methylated DNA included selfing lineage, scaled parasite load, inbreeding status and the
291 interactions between selfing lineage and scaled parasite load and inbreeding. The proportion
292 of methylated loci significantly varied among selfing lineages (estimate = 0.51, S. E.= 0.13, P
293 < 0.001) and was affected by parasite loads and inbreeding status through its interactions with
294 selfing lineage (parasite loads and selfing lineage: estimate = -0.55, S.E.=0.46, P = 0.005;
295 inbreeding and selfing lineage interaction: estimate = -1.64, S.E.=0.14, P = 0.04) (Fig 3b-c;
296 Tables 4 and S7). The second most likely model (ΔAICc=1.00) included only selfing lineage
297 (estimate = -0.43, S. E.=0.08, P < 0.001) and the interactions between inbreeding and selfing
298 lineage (estimate = -1.10, S. E.=0.12, P = 0.04) as significant predictors. However, this model
299 explained substantially less of the overall variation compared to the first model (weight: 0.17
300 vs. 0.28). and was 1.39 times less likely than the first one (Table S5).

301 Overall, the results of the single-taxa models (using number bacterial cysts) were very
302 similar to those for scaled parasite loads. The best model to explain the proportion of
303 methylated loci included selfing lineage, and the interactions between selfing lineage and
304 bacterial cysts, and selfing lineage and inbreeding (Table S6).

305 When using only individuals from Site 1 (to remove any potential confounding effect
306 between sampling site and selfing lineages) for the proportion of methylated loci the model
307 with the lowest AIC indicated that selfing lineage, inbreeding and the interactions between
308 inbreeding and selfing lineage and inbreeding and scaled parasite loads were all significant

309 predictors (Table S7). However, the second best-fitting model (ΔAICc = 0.02) explained the
310 same amount of variation (weight=0.39) and the evidence ratio (-0.66) suggested that it was
311 more likely (evidence ratio of 1.50) than the first model. This second model indicated that
312 overall, the proportion of methylated DNA significantly increased with scaled parasite loads
313 (estimate = 0.43, S. E.= 0.11, P = 0.03) and that DNA methylation levels were also affected
314 by the interaction between scaled parasite loads and inbreeding (estimate = -1.29, S. E.=0.38,
315 P <0.001), with inbred individuals having increased methylation levels with increased
316 parasite loads, while outbred individuals had decreased methylation levels with increased
317 parasite loads (Fig. 3d; Table 4).

318 **Discussion**

319 Overall, our results did not indicate significant differences in genome-wide DNA methylation
320 variation between selfed and outcrossed individuals, and our models only identified
321 inbreeding significantly related to DNA methylation via its interaction with selfing lineage
322 (all sampling sites) and parasites (at the local scale in Site 1). Higher variation in DNA
323 methylation has been reported for clonal and inbred individuals (Nakamura and Hosaka 2010;
324 Massicotte and Angers 2012; Richards et al. 2012; Liebl et al. 2013; Veerger et al. 2012), and
325 has been interpreted as an adaptive mechanism to compensate for low genetic variarion
326 (Schrey et al. 2012), or as a potential consequence of inbreeding (as in Vergeer et al. 2012)
327 responsible, at least in part, for inbreeding depression (Nakamura and Hosaka 2010; Vergeer
328 et al. 2012). Yet, our results suggest that, at least in this species, either inbreeding does not
329 affect genome-wide DNA methylation variation or it does in a gene-specific manner (Venney
330 et al. 2016), although further research would be needed to address this question.

331 We found that that the different selfing lineages of *Kryptolebias hermaphroditus*
332 distributed in three sampling sites of north-eastern Brazil differed significantly in parasite
333 loads and genetic composition, which might indicate specific interactions between host

334 genotypes and parasites (Dybdhal and Lively 1998; Ebert 2008). Previous studies on
335 mangrove killifishes had identified extensive genetic structuring both between (Tatarenkov et
336 al. 2015; 2017) and within mangrove systems even at close geographical proximity
337 (Tatarenkov et al. 2007; 2012; Ellison et al. 2012), as a consequence of the self-fertilising
338 nature of these fish. We found strong evidence of genetic structuring between sampling sites
339 and selfing lineages using microsatellites, but lower differentiation for AFLP genetic markers
340 (likely due to the different mutation rate of the markers) and epigenetic markers (MS-
341 AFLPs). Overall, inbred individuals (with lower heterozygosity) harboured higher parasite
342 loads than their outcrossed counterparts, supporting the prediction that low genetic diversity
343 due to self-fertilisation may reduce fitness (considering parasite loads as a proxy for pathogen
344 pressure), as for other mixed-mating species (King et al. 2011; Ellison et al. 2011; Lively and
345 Morran 2014). Extensive periods of self-fertilisation can reduce offspring fitness due to the
346 accumulation of deleterious alleles and inbreeding depression (Charlesworth et al. 1993).
347 Species with mixed-mating seem to overcome these problems through occasional outcrossing
348 (Ellison et al. 2011; Morran et al. 2011), which can generate genetic diversity to face natural
349 enemies, such as parasites (Lively 2014). Here, the relationship between parasites and
350 inbreeding status (selfed or outcrossed) suggests that outcrossing might confer a fitness
351 advantage (in terms of parasite loads), even when it occurs at very low frequencies (Ellison et
352 al. 2011). However, despite the adaptive potential of outcrossing, the main reproductive
353 strategy of *K. hermaphroditus* seems to be self-fertilisation (Tatarenkov et al. 2017). This
354 suggests that other evolutionary mechanisms may be balancing the harmful effects of parasite
355 infections, or that parasite selection is of low (Lively 2014), as theory predicts that low
356 selection levels imposed by natural enemies are consistent with the maintenance of asexual
357 reproduction (Ladle et al. 1993; Judson 1997). For example, in the mixed-mating
358 *Potamopyrgus* snails, the oldest asexual lineages are restricted to populations where parasites

359 are rare (Neiman et al. 2005). Thus, the low number of parasites found in *K. hermaphroditus*
360 (i.e. mean of 3.38 parasites per individual compared to 22.41 of *K. marmoratus* in Belize;
361 Ellison et al. 2011), may explain the high prevalence of selfing in *K. hermaphroditus*. The
362 long-term persistence of self-fertilising organisms suggests that non-genetic mechanisms may
363 play a role in generating adaptive responses to environmental change and compensate for low
364 genetic variation (Shrey et al. 2012; Liebl et al. 2013; Douhovnikoff and Dodd 2015; Hu et
365 al. 2018). Using data from all sampling sites, we found that genome-wide DNA methylation
366 was strongly influenced by selfing lineage and only at a smaller scale by inbreeding through
367 its interaction with selfing lineage (Bell et al. 2011; Bjornsson et al. 2008; Gertz et al. 2011;
368 Dubin et al. 2015). Strong epigenetic differences between selfing lines had been identified
369 previously in *K. marmoratus* (see Ellison et al. 2015), indicating an important role of the
370 genetic background in the epigenetic variation of these species. In addition, we also found a
371 significant correlation between DNA methylation and genetic variation (at both AFLP and
372 microsatellites data), suggesting that autonomous variation in DNA methylation may be
373 limited in this study system (Dubin et al. 2015).

374 DNA methylation can interact with genotypes in a genotype-by-environment manner
375 to generate plastic responses (Herman and Sultan 2016). Several abiotic and biotic factors,
376 including parasites (Norouzitallab et al. 2014; Hu et al. 2018), are known to influence DNA
377 methylation, however information on how DNA methylation varies across different genetic
378 backgrounds is still scarce. Our results showed that genome-wide DNA methylation levels
379 for all sampling sites were significantly influenced by parasite loads through the interaction
380 with selfing lineage, suggesting a potential genotype-by-environment interaction on parasites
381 responses. Yet, as most of the selfing lineages were exclusive to specific sampling sites, we
382 could completely discard confounding effects between both variables. In fact, selfing lineage
383 did not affect genome-wide DNA methylation levels in Site 1, but only parasites and their

interaction with inbreeding status. The anonymous nature of our genetic and epigenetic markers is a limiting factor to infer the potential adaptive/functional role of the DNA methylation variation in response to parasites. Further analyses, ideally under controlled experimental conditions and using higher resolution sequencing methods (i.e. whole-genome bisulfite sequencing, RNAseq), should help to clarify how reduced DNA methylation may affect immune responses in mixed-mating *Kryptolebias* species.

The relationship between parasite loads and outcrossing seems to be common to several mixed-mating species (Steets et al. 2007; Ellison et al. 2011; King et al. 2011) in addition to *K. hermaphroditus*, suggesting that the influence of parasites in the regulation of mixed-mating could be generalised. The extent of this relationship, however, may depend on the severity of the selection imposed by coevolving parasites (Lively and Morran 2014). Our results indicate that genotype composition (and its interaction with inbreeding) may be important in DNA methylation responses to environmental variation in wild populations, and that, if DNA methylation responded in a genotypic-specific manner to parasites pressures, it could contribute to local adaptation (Foust et al. 2016; Smith et al. 2016). The mangrove killifish, with its naturally inbred populations and marked methylation differences between populations and genotypes, represents an ideal model to analyse the relative roles of genetic and epigenetic diversity in modulating local adaptation.

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409

410 **Compliance with ethical standards**

411 All the experiments in this study have been conducted following Home Office regulations,
412 approved by Swansea, Cardiff and UFRN (CEUA) Universities Animal Ethics Committees,
413 and under sampling permit number 30532-1/2011 issued by ICMBio/SISBIO. The authors
414 declare they have no conflict of interest.

415

416 **Data availability**

417 Data available from Dryad Digital Repository: https://doi.org/XXX/XXXX

418 **Authors contributions**

419 SC, WMB-F & CGL conceived the work; SMQL planned the field work and conducted the
420 sampling together with WMB-F, CGL & SC; WMB-F did the microsatellite and parasite
421 screening, with contributions from JC; WMB-F and PM performed the MS-AFLP analyses.
422 WMB-F analysed the data with the contribution of SC, CGL and PM. WMB-F and SC wrote
423 the paper with contributions from all authors.

424

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Tables

Table 1 Genetic diversity (at 27 microsatellite loci), mean parasites number (standard deviation in brackets) and parasite prevalence in *Kryptolebias hermaphroditus* at sampling sites in North-eastern Brazil. N= sampling size; N_a = mean number of alleles of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; F_{IS} = inbreeding coefficient; HL = homozygosity by locus; S = selfing rates.

	Site 1	**Site 2**	**Site 3**	**All sites**
Genetic diversity				
N	68	42	18	128
N_a	3.03	3.44	3.14	3.21
H_e	0.28	0.26	0.33	0.295
H_o	0.025	0.015	0.043	0.028
F_{IS}	0.91	0.94	0.87	0.93
HL	0.95	0.97	0.93	0.95
S	0.92	0.93	0.87	0.90
Parasite loads				
Bacterial gills cysts	3.16 (3.16)	2.66 (3.10)	1.27 (0.80)	2.73 (2.99)
Protozoan gills cysts	0	1.52 (1.60)	0.33 (1.37)	0.54 (1.26)
Nematodes	0.16 (0.53)	0.02 (0.15)	0	0.09 (0.40)
Total parasite load	3.33 (3.27)	4.21 (3.17)	1.61 (1.73)	3.38 (3.17)
Parasite prevalence (% of fish with infection)				
Bacterial gills cysts	91.17	71.42	83.33	83.59
Protozoan gills cysts	0	57.14	5.55	19.53
Nematodes	10.29	2.38	0	6.25

718 **Table 2** Homozygosity by locus (HL) (at 27 microsatellite loci), mean parasites loads
719 (standard error in brackets) and parasite prevalence in *Kryptolebias hermaphroditus* classed as
720 either selfed or outcrossed based on q-values from selfing lineages structure estimated using
721 INSTRUCT. P and z-values extracted from a two median Mann-Whitney test.

	Selfed	**Outcrossed**	**z**	***P* value**
Genetic diversity				
N	92	36		
HL	0.98	0.88	-4.76	**<0.001**
Parasite loads				
Bacterial gills cysts	3.25 (2.99)	1.69 (2.59)		
Protozoan gills cysts	0.57 (1.26)	0.47 (1.28)		
Nematodes	0.1 (0.4)	0.05 (0.42)		
Total parasite load	3.82 (3.47)	2.25 (1.94)	-2.84	**0.004**
Parasite prevalence (% of fish with infection)				
Bacterial gills cysts	89.13	69.44		
Protozoan gills cysts	18.47	22.22		
Nematodes	7.6	2.77		

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Table 3 Hierarchical analysis of molecular variance (AMOVA) for microsatellites and MS-AFLPs data among **a** sampling sites **b** selfing lineages and **c** between selfed and outcrossed individuals and **b** among sampling sites in *Kryptolebias hermaphroditus*. df= degrees of freedom; SSD= sum of squared deviations; Mol. var. (%) = molecular variance percentages from variance components sigma 2; Φ_{ST} = Phi statistics for population differentiation. *P* value derived from 10,000 permutations.

	Microsatellites				**NML**				**MSL**			
	df	Mol. var. (%)	F_{ST}	*P* value	df	Mol. var. (%)	Φ_{ST}	*P* value	df	Mol. var. (%)	Φ_{ST}	*P* value
a Sampling sites												
Among sites	2	28.46	0.28	**0.001**	2	2.20	0.02	**0.001**	2	2.96	0.02	**<0.001**
Within sites	227	71.54			112	97.80			112	97.05		
b Selfing lineages												
Among lineages	5	32.40	0.32	**0.001**	5	2.00	0.02	**0.004**	5	2.15	0.02	**0.001**

Within lineages	250	67.60			109	98.00			109	97.85		
c Inbreeding status												
Between selfed and outcrossed		1.28	0.01	**0.002**	1	0.15	0.02	0.32	1	0.82	0.02	0.06
Within selfed and outcrossed		98.72			113	99.85			113	99.18		

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733 **Table 4** Results of the best-fitting generalized linear models for proportion of methylated loci
734 (binomial distribution) in *Kryptolebias hermaphroditus*, using the multi-model averaging
735 approach (see appendix for the full model comparisons). df= degrees of freedom; Coeff =
736 mean coefficient estimates.
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Independent variable	**df**	**Coeff**	***z***	***P*-value**
Proportion of methylated loci				
Selfing lineage	5	-0.51	-4.50	**<0.001**
Scaled parasite load	1	-0.02	-0.02	0.83
Inbreeding	1	-0.50	1.73	0.15
Selfing lineage x parasite scaled	5	-0.55	-3.90	**0.005**
Selfing lineage x inbreeding	4	-1.64	-1.64	**0.04**
Proportion of methylated loci for site 1				
Scaled parasite load	1	-0.23	-11.49	**0.03**
Inbreeding	1	-0.31	-10.64	0.09
Inbreeding x scaled parasite load	1	-1.87	-17.93	**<0.001**

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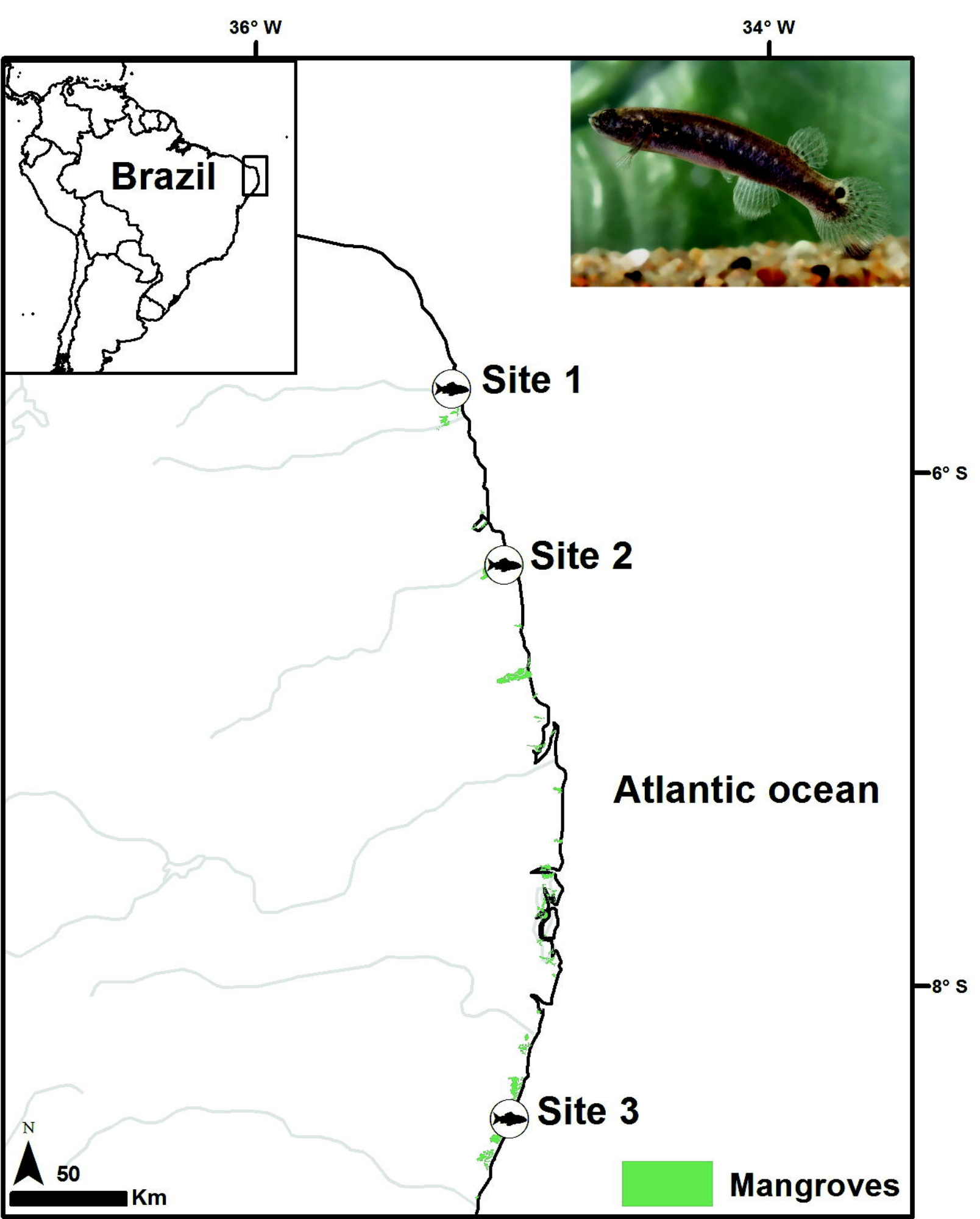
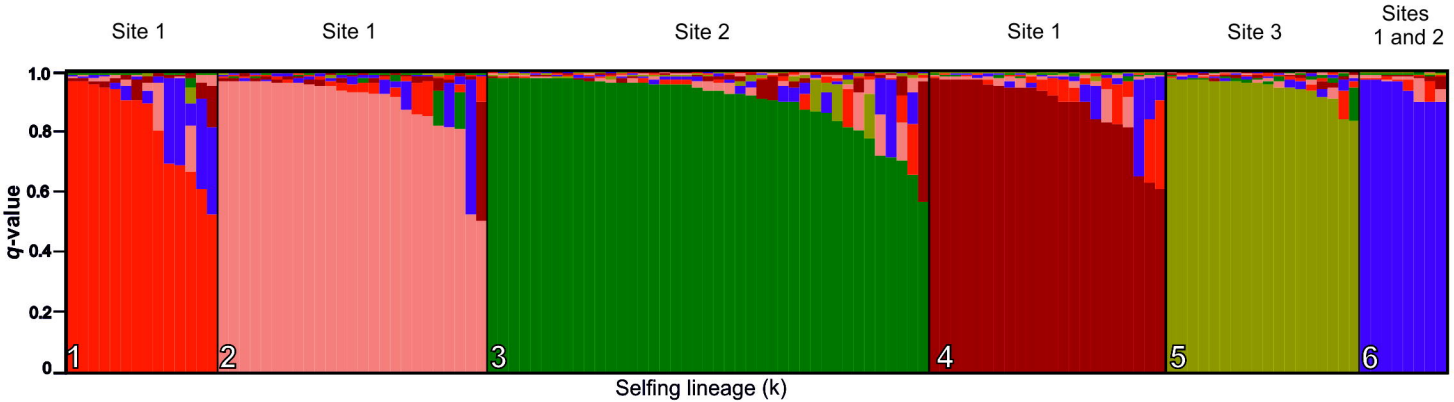
747 **Figure legends**

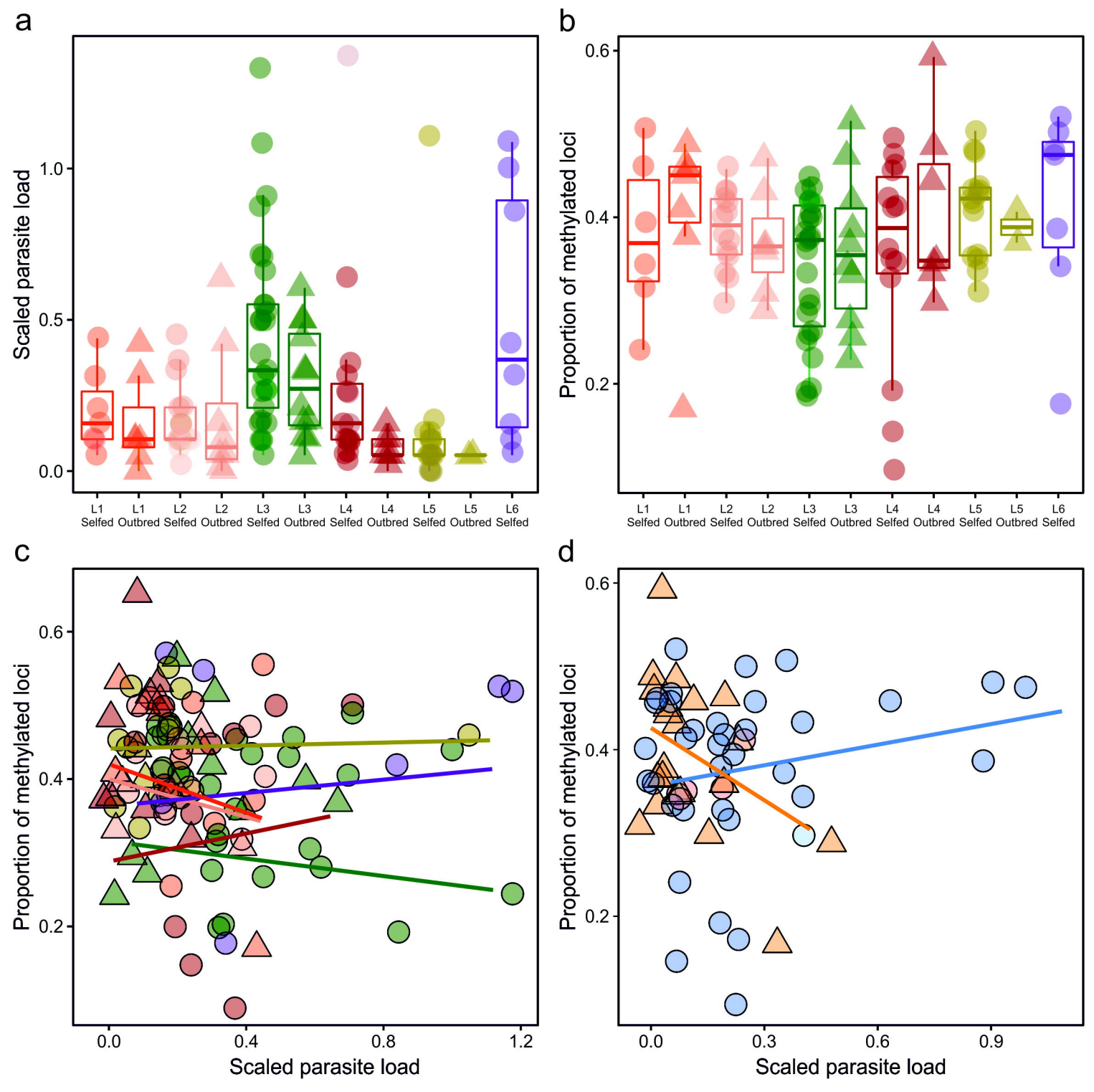
748 **Fig. 1** Sampling locations for *Kryptolebias hermaphroditus* (picture of a live individual on
749 the top-right corner) in North-eastern Brazil. Ceará-Mirim river – Site 1; Curimataú river –
750 Site 2; Ipojuca river - Site 3.

751 **Fig. 2** Genetic assignment of *Kryptolebias hermaphroditus* to six selfing lineages using
752 INSTRUCT. Each individual is represented by a bar, which represents the likelihood of the
753 individual to belong to a specific genetic cluster (colour).

754 **Fig. 3** Relationships between **a** scaled parasite load across selfing lineages and inbreeding
755 status **b** proportion of methylated loci across selfing lineage and inbreeding status (selfed or
756 outcrossed) **c** Proportion of methylated loci and selfing lineages and scaled parasite loads **d**
757 proportion of methylated loci across inbreeding status for sampling site 1 individuals. Circles
758 for selfed, triangles for outcrossed individuals. Red = selfing lineage 1 (site 1); salmon =
759 selfing lineage 2 (site 1); green = selfing lineage 3 (site 2); brown = selfing lineage 4 (site 1);
760 yellow = selfing lineage 5 (site 3); purple = selfing lineage 6 (sites 1 and 2); orange =
761 outcrossed individuals; blue = selfed individuals.


36° W
34° W
Brazil
Site 1
Site 2
Site 3
6° S
8° S
Atlantic ocean
N
50
Km
Mangroves


Site 1
Site 1
Site 2
Site 1
Site 3
Sites 1 and 2
1.0
0.8
0.6
0.4
0.2
0
q-value
1
2
3
4
5
6
Selfing lineage (k)


a
Scaled parasite load
0.0
0.5
1.0
L1 Selfed
L1 Outbred
L2 Selfed
L2 Outbred
L3 Selfed
L3 Outbred
L4 Selfed
L4 Outbred
L5 Selfed
L5 Outbred
L6 Selfed
b
Proportion of methylated loci
0.2
0.4
0.6
L1 Selfed
L1 Outbred
L2 Selfed
L2 Outbred
L3 Selfed
L3 Outbred
L4 Selfed
L4 Outbred
L5 Selfed
L5 Outbred
L6 Selfed
c
Proportion of methylated loci
0.2
0.4
0.6
0.0
0.4
0.8
1.2
Scaled parasite load
d
Proportion of methylated loci
0.2
0.4
0.6
0.0
0.3
0.6
0.9
Scaled parasite load