

1 **The marbled crayfish as a paradigm for saltational speciation by**  
2 **autopolyploidy and parthenogenesis in animals**

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22 **Abstract**

23 The parthenogenetic all-female marbled crayfish is a novel research model and potent invader  
24 of freshwater ecosystems. It is a triploid descendant of the sexually reproducing slough  
25 crayfish, *Procambarus fallax*, but its taxonomic status has remained unsettled. By cross-  
26 breeding experiments and parentage analysis we show here that marbled crayfish and *P. fallax*  
27 are reproductively separated. Both crayfish copulate readily, suggesting that the reproductive  
28 barrier is set at the cytogenetic rather than the behavioural level. Analysis of complete  
29 mitochondrial genomes of marbled crayfish from laboratory lineages and wild populations  
30 demonstrates genetic identity and indicates a single origin. Flow cytometric comparison of  
31 DNA contents of haemocytes and analysis of nuclear microsatellite loci confirm triploidy and  
32 suggest autopolyploidization as its cause. Global DNA methylation is significantly reduced in  
33 marbled crayfish implying the involvement of molecular epigenetic mechanisms in its  
34 origination. Morphologically, both crayfish are very similar but growth and fecundity are  
35 considerably larger in marbled crayfish, making it a different animal with superior fitness.  
36 These data and the high probability of a divergent future evolution of the marbled crayfish  
37 and *P. fallax* clusters suggest that marbled crayfish should be considered as an independent  
38 asexual species. Our findings also establish the *P. fallax*-marbled crayfish pair as a novel  
39 paradigm for rare chromosomal speciation by autopolyploidy and parthenogenesis in animals  
40 and for saltational evolution in general.

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42 **Key words:** marbled crayfish, autopolyploidy, parthenogenesis, epigenetics, chromosomal  
43 speciation, saltational evolution

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## 48 **1. Introduction**

49 In the last decade, the marbled crayfish (Marmorkrebs) has gained considerable attention in  
50 the scientific community and the public because of its obligatory parthenogenetic  
51 reproduction, its suitability as a research model and its high potential as an invasive species  
52 [1-9]. It was discovered in 1995 in the German aquarium trade [2] and has become a popular  
53 pet in Europe and other continents since then [10,11]. Thriving wild populations have  
54 meanwhile developed from releases in several European countries and Madagascar and are  
55 feared to threaten native crayfish species by competition and transmission of the crayfish  
56 plague [7-9,12,13].

57 By comparison of morphological traits and molecular markers, Martin and colleagues  
58 [14] have identified the sexually reproducing slough crayfish *Procambarus fallax* from  
59 Florida and southernmost Georgia as the mother species of marbled crayfish. However, its  
60 taxonomic position remained unsettled. Martin *et al.* [14] suggested the provisional name  
61 *Procambarus fallax* forma *virginalis*, being aware that forma is not a valid category in animal  
62 taxonomy. Meanwhile, several important characteristics of marbled crayfish have been  
63 described in detail, including morphology [12], embryonic development [15,16], life history  
64 [16-19], parthenogenetic reproduction [1,20,21] and a triploid karyotype [22].

65 Speciation in parthenogenetic lineages is a problematic issue because parthenogens do  
66 not fit into the classical concepts of speciation, as discussed in detail by Mayr [23], Coyne and  
67 Orr [24], Barraclough *et al.* [25], Birky and Barraclough [26] and Martin *et al.* [14]. However,  
68 Barraclough and colleagues emphasized the importance of understanding diversification and  
69 speciation in asexual organisms, not least to test theories about the evolutionary advantage of  
70 sex [25,26]. They provided a theory on speciation in asexuals, which they named  
71 Evolutionary Genetic Species Concept [26]. This theory focuses on the criterion that the  
72 individuals of the parent species and the neo-species form discrete clusters of very similar  
73 genotypes and phenotypes. The new cluster should be of a single origin and both clusters

74 must be separated from each other by reproductive or geographic isolation and a gap of  
75 genetic and phenotypic traits so that natural selection can ensure a divergent evolution over  
76 time [25-28].

77 Stimulated by the paper by Martin *et al.* [14] there is an ongoing discussion among  
78 marbled crayfish experts whether this animal should be treated as a parthenogenetic lineage of  
79 *P. fallax* or a species in its own right. In order to examine this issue in detail we have tested  
80 the above listed operational definitions for asexual species with several experimental and  
81 technical approaches. Cross-breeding experiments between marbled crayfish and slough  
82 crayfish and parentage analysis by microsatellite markers were performed to test for  
83 reproductive isolation. Complete mitochondrial genomes and nuclear microsatellite patterns  
84 of marbled crayfish from several laboratory lineages and wild populations were analysed to  
85 clarify single origin and to establish its genotypic characteristics. The DNA content of  
86 haemocytes, mitochondrial genome sequences and microsatellite patterns was compared  
87 between marbled crayfish, *P. fallax* and the closely related *Procambarus alleni* to obtain  
88 information about the mode of triploidization of the marbled crayfish. Global DNA  
89 methylation was determined to examine the involvement of epigenetic mechanisms in  
90 speciation. Finally, taxonomically relevant morphological characters and ecologically and  
91 evolutionarily important life history traits were compared to reveal phenotypic differences  
92 between the marbled crayfish and *P. fallax* clusters.

93

## 94 **2. Material and methods**

### 95 **2.1 Animals**

96 The following animals were used: (1) marbled crayfish *Procambarus fallax* (Hagen, 1870) f.  
97 *virginalis* from our laboratory lineages named "Heidelberg" and "Petshop" and from two wild  
98 populations in Germany and Madagascar, (2) *Procambarus fallax* (Hagen, 1870) from our  
99 laboratory population and the aquarium trade, (3) *Procambarus alleni* (Faxon, 1884) from the

100 aquarium trade, and 4) *Procambarus clarkii* (Girard, 1852) from an invasive Swiss  
101 population. The Heidelberg lineage was founded by G.V. in February 2003 from a single  
102 female, which originated from the oldest documented marbled crayfish aquarium population  
103 founded in 1995 by F. Steuerwald. The Petshop lineage was established by G.V. in February  
104 2004 from a single female purchased in a pet shop. The wild marbled crayfish were from  
105 Lake Moosweiher, Germany (provided by M. Pfeiffer), and a market in Antananarivo,  
106 Madagascar (provided by F. Glaw). Our *P. fallax* laboratory population was founded in  
107 February 2014 by a single pair obtained from the aquarium trade. All crayfish were raised  
108 under the same conditions. Animals were kept either individually or communally in plastic  
109 containers of 30x25x20 cm equipped with gravel and shelters. Tap water was used as the  
110 water source and replaced once a week. Water temperature was maintained at 20°C. All  
111 animals were fed with TetraWafer Mix pellets.

112

## 113 **2.2 Cross-breeding experiments**

114 For the 38 crossbreeding experiments we used three *P. fallax* males with total lengths (TL=tip  
115 of rostrum to end of telson) of 3.1-5.2 cm, five *P. fallax* females with TLs of 3.5-4.2 cm, 14  
116 marbled crayfish females with TLs of 4.0-6.3 cm and two *P. alleni* males with TLs of 5.1-5.3  
117 cm. All males were in the reproductively competent Form I as indicated by the presence of  
118 hooks on the ischia of the 3rd and 4th pereopods. Eight of the 14 marbled crayfish females  
119 and 4 of the 5 *P. fallax* females had well-developed glair glands on the underside of the pleon  
120 indicating ovarian maturity and receptiveness. The behavioural experiments were performed  
121 in aquaria with an area of 26x16 cm without shelter. Pairs were observed for 2 hours and  
122 copulation was regarded as successful when the partners remained in typical copulation  
123 position for more than 10 min. Parentage of the offspring was determined by microsatellite  
124 analysis.

125

## 126 **2.3 Microsatellite analysis**

127 For microsatellite analysis, walking legs of specimens were fixed in 80% ethanol prior to  
128 extraction of nuclear DNA with the Blood & Cell Culture DNA Kit (Genomic Tips) from  
129 Qiagen (Hilden, Germany). A total of five microsatellite primer pairs were tested. Four of  
130 them were originally designed for *P. clarkii* (PclG-02, PclG-04, PclG-08, PclG-48) [29] and  
131 one pair (PclG-26) was designed for marbled crayfish based on the *P. clarkii* sequences [21].  
132 The same microsatellite loci were additionally investigated in *P. alleni* and *P. clarkii*. PCR  
133 was carried out using a Primus 96 Cycler (Peqlab Biotechnologie, Erlangen, Germany).  
134 Fragment analysis was performed on a Beckman Coulter CEQ 8000 eight capillary sequencer  
135 (Beckman Coulter, Krefeld, Germany) using the Beckman Coulter DNA Size Standard Kit  
136 400 bp. Loci were scored with GeneMarker, v.2.6 (SoftGenetics, State College, Pennsylvania,  
137 USA).

138

## 139 **2.4 Sequencing, assembly and comparison of mitochondrial genomes**

140 For comparison of complete mitochondrial genomes we used two cultured marbled crayfish  
141 from the Heidelberg and Petshop lineages, two wild marbled crayfish from Lake Moosweiher  
142 and Madagascar, one *P. fallax* female and one *P. alleni* female. DNA was isolated from  
143 hepatopancreases and abdominal musculature as described above and sequenced on an  
144 Illumina HiSeq platform. Read pairs were quality trimmed (quality value  $\geq 30$ , minimum  
145 length  $\geq 30$ ) and the mitochondrial genome of the Heidelberg animal was assembled by  
146 Velvet2.0 [30]. The sequences of the other specimens were established by mapping against  
147 the Heidelberg sequence using Bowtie2 [31]. For the identification of single nucleotide  
148 polymorphisms (SNPs) between the marbled crayfish populations, we used mpileup and  
149 bcftools from SAMtools [32], requiring a quality value  $> 30$  for SNP calling. Mitochondrial  
150 genome sequences of *P. fallax* and *P. alleni* were generated by MITObim1.6 [33] using  
151 published mitochondrial DNA fragments from *P. fallax* (FJ619800) and *P. alleni* (HQ171462,

152 FJ619802, HQ171451) as seed sequences. Mismatches in comparison to marbled crayfish  
153 sequences were identified by blastn alignments.

154

## 155 **2.5 Measurement of DNA content by flow cytometry**

156 Flow cytometry was used to determine the DNA content in haemocytes of *P. fallax* and  
157 marbled crayfish. Haemolymph was withdrawn through the articulating membrane between  
158 coxa and basis of the chelipeds, mixed 1:1 with crayfish anticoagulant buffer solution (100  
159 mM glucose, 34 mM trisodium citrate, 26 mM citric acid, 15.8 mM EDTA, pH 4.6) and  
160 centrifuged for 5 min at 1400 rpm. The pellet was washed and re-suspended with 100 µl PBS.  
161 Samples were either stored in 10% DMSO at -80°C or immediately used for analysis of the  
162 DNA content. For flow cytometry 4 µl RNase A (Sigma-Aldrich, Munich, Germany) stock  
163 solution (50 mg/ml) was added to the samples and incubated for 5 min at room temperature  
164 followed by an incubation for 60 min with 5 µl propidium iodide (Life Technologies,  
165 Darmstadt, Germany) stock solution (1 mg/ml). The samples were then mixed 1:1 with PBS  
166 and the DNA-related fluorescence intensities of single cells were measured on a BD Accuri  
167 C6 Cytometer (BD Sciences, Heidelberg, Germany) with blue laser 488 nm and detection  
168 filter FL2 585/40 nm.

169

## 170 **2.6 Measurement of global DNA methylation by mass spectrometry**

171 Global DNA methylation was determined in three whole juveniles and selected tissues  
172 (hepatopancreas, abdominal musculature and ovary) of three adults of marbled crayfish and *P.*  
173 *fallax*. Sample preparation and LC-MS/MS analyses were conducted as previously described  
174 [34] and were performed on an Agilent 1260 LC system connected to an Agilent 6460  
175 TripleQuad massspectrometer (Agilent, Böblingen, Germany). Briefly, after enzymatic  
176 hydrolysis to nucleosides, the samples were spiked with 250 fmol [D<sub>3</sub>]-5-methylcytosine as  
177 internal standard. The mass transitions resulting from the loss of desoxyribose (5-

178 methylcytidine: 242 Th → 126 Th, [D<sub>3</sub>]-5-methylcytidine: 245 Th → 129 Th) by collision  
179 induced dissociation (CID) were analysed in dynamic multiple reaction monitoring mode  
180 (DMRM). Calibration curves using a stable isotope labelled internal standard were established  
181 for quantification of 5-methylcytidine. The linear regressions resulting from the double  
182 logarithmic plots were used to correlate the respective signals from LC-MS/MS analysis to  
183 known amounts of substance. The yield of detected modification was normalized to  
184 guanosine content (as equivalent to cytidine content) because of better signal quality. To  
185 assess the amount of guanosine, the areas of the DAD results, gained during the LC analysis,  
186 were correlated to their respective amounts of substance in the same way as above.

187

## 188 **2.7 Investigation of morphological characters and life history traits**

189 For comparison of morphological characters between marbled crayfish and *P. fallax* we used  
190 marbled crayfish with TLs of 4.0-8.4 cm and body weights of 1.4-15.2 g and *P. fallax* females  
191 with TLs of 3.6-5.7 cm and weights of 1.1-4.5 g. We focussed on annulus ventralis (sperm  
192 receptacle), areola of the carapace, cheliped chelae and coloration, the taxonomically most  
193 relevant characters in female Cambaridae [35-37]. For comparison of life history traits we  
194 analysed growth, time of sexual maturity, body size and clutch size. Growth was determined  
195 in batches raised under the same conditions by measurement of carapace length (CL), total  
196 length (TL) and body weight. Sexual maturity was deduced from the presence of glair glands.  
197 Mean and maximum body and clutch sizes were taken from our laboratory animals and  
198 published data on wild marbled crayfish and *P. fallax*.

199

## 200 **3. Results**

### 201 **3.1 Crossbreeding experiments and parentage analysis**

202 Crossbreeding experiments were performed to investigate whether marbled crayfish and *P.*  
203 *fallax* can interbreed and produce viable offspring. Behavioural observations revealed that

204 marbled crayfish females and *P. fallax* males recognize each other as sexual partners.  
205 Courtship and mating behaviour included frontal approach, tearing with the chelipeds, intense  
206 sweeping with the antennae, sudden turning of the female and mounting by the male (figure  
207 1). This courtship behaviour is also typical of other *Procambarus* species [38]. *P. fallax* males  
208 copulated with marbled crayfish females in 15 of 21 trials (71%) and with *P. fallax* females in  
209 6 of 8 trials (86%) (table 1). In the marbled crayfish  $\times$  *P. fallax* pairs, the first contact was  
210 often initiated by the marbled crayfish females. Some matings lasted for more than 1 hour. *P.*  
211 *fallax* males can turn significantly larger marbled crayfish females on the back but are not  
212 long enough to simultaneously fix the female's chelipeds and insert the gonopods into the  
213 annulus ventralis. *P. alleni* males copulated neither with *P. fallax* nor with marbled crayfish  
214 females (table 1) suggesting that they did not recognize them as sexual partners.

215 We obtained a total of ten clutches from the crossbreeding experiments, eight from  
216 crosses of three *P. fallax* males with eight marbled crayfish females and two from crosses of  
217 two *P. fallax* males with two *P. fallax* females. Four of the *P. fallax  $\times$  marbled crayfish  
218 clutches and one *P. fallax  $\times$  *P. fallax* clutch developed into juveniles whereas the others  
219 decayed during embryonic development. In the *P. fallax  $\times$  *P. fallax* clutch we counted 10  
220 females and 9 males at juvenile stage 7, reflecting the typical 1:1 sex ratio of sexually  
221 reproducing crayfish [39]. In contrast, in the four marbled crayfish  $\times$  *P. fallax* batches the 6,  
222 12, 61 and 93 analysed stage 7 offspring were all females indicating reproduction by  
223 parthenogenesis.***

224 The progeny of our crossbreeding experiments were also investigated by microsatellite  
225 analysis to further clarify parentage. Microsatellite analysis is an established approach to  
226 assess parentage and geographic structuring in crayfish populations and to identify clonal  
227 lineages, triploids and hybrids [40-43]. Of the five primer pairs tested, three revealed PCR  
228 products that could be used for fragment length determination in marbled crayfish and *P.*  
229 *fallax*, namely PclG-02, PclG-04 and PclG-26. PclG-02 and PclG-26 were polymorphic and

230 thus suitable for parentage testing. The microsatellite allele combinations in the analysed  
231 family groups of marbled crayfish females 1-4 x *P. fallax* male 1 were identical between  
232 mothers and offspring, namely 267 bp/ 271 bp/303 bp at locus PclG-02 and 189 bp/191 bp at  
233 locus PclG-26, but differed from the allele combination of the male that was 255 bp/267 bp  
234 and 185 bp/207 bp, respectively (table 2). All measurements were repeated at least twice, and  
235 in the case of the unusual PclG-02 up to five times per specimen. Our data indicate that the  
236 male did not contribute to the genome of the offspring and that the progeny is the product of  
237 apomictic parthenogenesis. The microsatellite patterns were not only identical between  
238 mother and offspring but also between the four batches (table 2) demonstrating clonality of all  
239 marbled crayfish from our laboratory.

240 The *P. fallax* male 1 x *P. fallax* female 1 family was used as a positive control. Analysis  
241 of locus PclG-26 revealed the allele combinations 185 bp/207 bp in the father, 179 bp/185 bp  
242 in the mother and 179 bp/185 bp (2x), 179 bp/207 bp (4x), 185 bp/185 bp (4x) and 185  
243 bp/207 bp (4x) in the 14 offspring. These data indicate Mendelian distribution and  
244 demonstrate that both parents contributed equally to the genome of the offspring, as is  
245 expected for sexually reproducing species.

246

### 247 **3.2 Single origin and clonality of marbled crayfish populations**

248 For a more detailed genetic analysis of marbled crayfish, we established complete  
249 mitochondrial genome sequences of specimens from our Heidelberg and Petshop lineages and  
250 from wild populations of Lake Moosweiher (Germany) and Madagascar by high-coverage  
251 shotgun sequencing and sequence mapping. Remarkably, these mitochondrial genome  
252 sequences were completely identical (figure 2), thus confirming the clonal nature of the tested  
253 populations and their single origin. Comparison of our sequences with the mitochondrial  
254 genome sequence of marbled crayfish published earlier by Shen *et al.* [44] revealed 6  
255 scattered mismatches and major differences in one fragment ranging from position 4600 to

256 5500. These differences are probably related to technical issues because Shen and colleagues  
257 used PCR-based methods and primer walking single/double strands sequencing [44] whereas  
258 we used next-generation sequencing with a sequencing coverage per nucleotide of >100x.

259 We also established complete mitochondrial genome sequences for *P. fallax* and *P.*  
260 *alleni*. Analysis of the mitochondrial *12S rRNA*, *16S rRNA* and *cytochrome oxidase subunit I*  
261 genes have earlier indicated a close relationship between marbled crayfish and these species  
262 [1,7,14]. *P. alleni* occurs sympatrically with *P. fallax* in many locations in Florida [45] and  
263 was therefore regarded as a candidate that might have contributed to the origination of  
264 marbled crayfish by hybridization with *P. fallax* [46]. Sequence comparison revealed 144  
265 single nucleotide polymorphisms (SNPs) between marbled crayfish and *P. fallax* but 1165  
266 SNPs between marbled crayfish and *P. alleni* (figure 2). Interestingly, these SNPs were not  
267 evenly distributed over the mitochondrial genome, which explains why in the study by Martin  
268 *et al.* [14] small genetic differences between marbled crayfish and *P. fallax* were detected in  
269 the *cytochrome oxidase subunit I* gene but not in the *12S rRNA* gene. Our results confirm the  
270 close genetic relationship between marbled crayfish and *P. fallax* and a greater distance  
271 towards *P. alleni*.

272 The single origin and clonality of marbled crayfish from the laboratory and the wild was  
273 further confirmed by the analysis of microsatellite loci PclG-02, PclG-04 and PclG-26 in 24  
274 specimens from our laboratory lineages (see parentage analysis), six specimens from a stable  
275 wild population in Lake Moosweiher [47] and one specimen from Madagascar [7]. All these  
276 marbled crayfish showed the same microsatellite patterns, namely the allele associations 267  
277 bp/271 bp/303 bp at locus PclG-02, 159 bp at PclG-04 and 189 bp/191 bp at PclG-26. The  
278 fragment lengths of the alleles of locus PclG-02 overlapped in marbled crayfish (267-303 bp)  
279 and *P. fallax* (239-267 bp) but were longer in *P. alleni* (329-384 bp) and shorter in *P. clarkii*  
280 (211-228 bp). Marbled crayfish shared two of six alleles with *P. fallax*, namely 267 bp at

281 locus PclG-02 and 159 bp at locus PclG-04, but none with the other species thus confirming  
282 the particularly close relationship between *P. fallax* and marbled crayfish.

283

### 284 **3.3 Ploidy status of marbled crayfish**

285 Martin *et al.* [22] recently used karyological analysis to demonstrate that marbled crayfish has  
286 a triploid genome. Our microsatellite analysis confirms this finding. Marbled crayfish  
287 generally have the allele association 267 bp/271 bp/303 bp at locus PclG-02 (figure 3a),  
288 whereas *P. fallax*, *P. alleni* and *P. clarkii* have one or two alleles at this locus, which is  
289 consistent with diploid and sexually reproducing species. In an earlier paper, Martin *et al.*  
290 [20] have also analysed locus PclG-02 and reported only two alleles of 267 bp and 271 bp.  
291 However, a recent re-examination of their material confirmed the presence of the third 303 bp  
292 allele (G. Scholtz, personal communication).

293 We further corroborated triploidy in marbled crayfish by flow cytometric measurement of  
294 the DNA content of haemocytes in marbled crayfish and *P. fallax*. Haemocytes are  
295 particularly suitable for this purpose because they are devoid of somatic polyploidization [48].  
296 Our results showed a significant 1.4-fold higher DNA content in the blood cells of marbled  
297 crayfish (figure 3b), which is consistent with triploidy.

298

### 299 **3.4 Comparison of DNA methylation between marbled crayfish and *Procambarus fallax***

300 In order to test if the marbled crayfish and *P. fallax* clusters also differ with respect to  
301 epigenetic markers we determined global DNA methylation by mass spectrometry in  
302 identically raised and age and size-matched representatives of both crayfish. DNA  
303 methylation represents a widely conserved epigenetic mark that is often associated with  
304 polyphenism and adaptive phenotypic changes [49,50]. Comparison of three juveniles and  
305 selected organs (hepatopancreas, abdominal musculature and ovary) of three adults revealed a  
306 consistently and highly significantly reduced level of DNA methylation in marbled crayfish

307 when compared to *P. fallax* (figure 4). The ten *P. fallax* samples together had a DNA  
308 methylation level of  $2.93 \pm 0.15\%$  (mean  $\pm$  standard deviation) whereas the ten marbled  
309 crayfish samples together had a level of only  $2.40 \pm 0.08\%$ . These results suggest that marbled  
310 crayfish have a considerably different DNA methylation pattern.

311

### 312 **3.5 Comparison of morphological characters between marbled crayfish and *P. fallax***

313 Comparison of the most relevant taxonomic characters of cambarid females [35-37] between  
314 marbled crayfish and *P. fallax* corroborated the high degree of morphological similarity  
315 between the two crayfish as previously established by Kawai *et al.* [12] and Martin *et al.* [14].  
316 The diagnostically most meaningful trait in females of the genus *Procambarus* is the annulus  
317 ventralis, which is bell-shaped with a tilted S-shaped sinus in both marbled crayfish and *P.*  
318 *fallax* (figure 5*a,b*). This typical form is not found in other *Procambarus* species [37] as best  
319 exemplified by the differently shaped sperm receptacle of the closely related *P. alleni* (figure  
320 5*c*). The areola, an unpaired structure on the dorsal midline of the carapace, is also very  
321 similar in marbled crayfish and *P. fallax* with respect to shape and length-to-width proportion  
322 (figure 5*d,e*). The same holds for the cheliped chelae, which closely resemble each other in  
323 both crayfish in shape, dentation and setation (figure 5*f,g*), and the coloration pattern, which  
324 consists of distinct marmorated spots and dark dorsolateral stripes on carapace and pleon  
325 (figure 5*h,i*). Size, form and coloration of the marmoration spots are highly variable not only  
326 in the sexually reproducing *P. fallax* but also in the genetically uniform marbled crayfish as a  
327 result of stochastic developmental variation [21,51].

328

### 329 **3.6 Comparison of life history traits between marbled crayfish and *P. fallax***

330 In contrast to the morphological characters, life history features like growth and fecundity are  
331 markedly different between marbled crayfish and *P. fallax*. Figure 6 gives an example for  
332 differences in the speed of growth between identically raised laboratory populations of the

333 same age. At day 250 after hatching, when the first females in both crayfish had reached  
334 sexual maturity, mean body weight was almost twice as large in marbled crayfish as in *P.*  
335 *fallax* females.

336 Maximum body and clutch sizes were also markedly higher in marbled crayfish. The  
337 largest specimen in our laboratory had a carapace length of 4.9 cm, a total length of 10.3 cm  
338 and a body weight of 30.1 g (figure 7a). In the wild, the largest of the 1084 marbled crayfish  
339 measured [7,12,47, M. Pfeiffer and C. Chucholl, personal communication] was found in Lake  
340 Moosweiher and had a CL of 4.9 cm and a weight of 32.0 g [47]. In contrast, the largest of the  
341 4710 wild *P. fallax* examined [36,52-54] had a CL of only 3.4 cm, corresponding to a TL of  
342 7.4 cm and a weight of approximately 11.5 g. The largest clutches of marbled crayfish in the  
343 laboratory and the wild consisted of 731 eggs (figure 7b) and 724 eggs [47], respectively,  
344 which is 5.6 fold higher than the largest clutch of 130 eggs reported for *P. fallax* in literature  
345 [53]. The analysis of life history features of the slough crayfish by van der Heiden [54]  
346 corroborated that *P. fallax* reaches only rarely a size of more than 6.5 cm TL.

347 The differences in growth and fecundity between marbled crayfish and *P. fallax* were  
348 also confirmed by the analysis of published data for egg-carrying females from comparable  
349 climatic regions. Ovigerous marbled crayfish from Madagascar had a mean CL of 3.5 cm, a  
350 mean TL of 7.4 cm and a mean clutch size of 300 eggs [7], whereas ovigerous *P. fallax* from  
351 the Everglades National Park in Florida had a mean CL of 1.8 cm, a mean TL of 3.8 cm and a  
352 mean clutch size of 41 eggs only [53], indicating that body size and fecundity is significantly  
353 increased in marbled crayfish (figure 7c,d). These findings identify important phenotypic  
354 differences between marbled crayfish and *P. fallax* that have not been recognized previously.

355

#### 356 **4. Discussion**

357 Our results demonstrate that marbled crayfish meets all criteria for asexual speciation [25-28].  
358 It is separated from the mother species, *P. fallax*, by reproductive isolation, significant

359 genomic and epigenetic differences and superior life history traits. Our data further support a  
360 single origin. In addition, all populations known to date live outside the natural range of *P.*  
361 *fallax*, suggesting geographical isolation. They are unified in one cluster by common  
362 phenotypic, genetic and epigenetic characteristics, despite their broad geographical  
363 distribution. These commonalities and differences towards *P. fallax* make it very likely that  
364 the marbled crayfish and slough crayfish clusters will evolve differently, which is the main  
365 criterion for erecting an asexual species [26]. Martin *et al.* [14] have previously suggested that  
366 marbled crayfish should be considered as an independent species when a single origin and/or  
367 regional populations in the wild have been established. Our findings clarify the former issue  
368 and provide additional evidence for cytogenetic, genetic and phenotypic differences between  
369 marbled crayfish and *P. fallax*. As such, marbled crayfish should now be named *Procambarus*  
370 *virginalis*, as suggested previously [14]. The formal description of marbled crayfish as a new  
371 species will be detailed in a separate publication.

372 Marbled crayfish appeared first in 1995 in the German aquarium trade. Thereafter,  
373 aquarists have propagated it in captivity, and since about 2003, releases have resulted in the  
374 establishment of thriving wild populations in Central Europe and Madagascar [5,7-9,12,47].  
375 The "mega-population" [46] in innumerable aquarium tanks on various continents and the  
376 known wild populations are apparently all descendants of the single clone or single individual  
377 that was introduced in Germany in 1995. Our results confirm this single origin by the identity  
378 of the mitochondrial genomes and microsatellite patterns in samples of captive and wild  
379 populations. One of the samples analysed in our study, the Heidelberg specimen, can be  
380 directly traced back to the year 1995 and to the oldest marbled crayfish for which written  
381 records exist (F. Steuerwald, personal communication).

382 It is unknown whether marbled crayfish emerged in the natural range of *P. fallax* or in  
383 captivity. Scholtz [4], Faulkes [5] and Martin [46] summarized possible scenarios for the first  
384 alternative including hybridization with coexisting *Procambarus* species and geographic

385 parthenogenesis. These authors and Chucholl [9] also stressed that in captivity there were  
386 many more candidates for hybridization than the naturally coexisting six *Procambarus*  
387 species [36,52] because crayfish were popular pets already in the 1990s. Faulkes [5]  
388 emphasized that all surveys on *P. fallax* in Florida and Southern Georgia revealed males and  
389 females arguing against the presence of pure marbled crayfish populations in the natural range  
390 of *P. fallax*. Moreover, none of the articles on wild *P. fallax* [36,45,52-54] mentioned  
391 specimens above 7.4 cm TL, which would again support the absence of primary populations  
392 of marbled crayfish. In sympatric populations, small and medium-sized marbled crayfish and  
393 *P. fallax* females would be indistinguishable by morphological criteria alone. However, by the  
394 use of genetic markers marbled crayfish could now be identified. Particularly useful is the  
395 highly specific tri-allelic microsatellite locus PclG-02, which could be assayed in large  
396 samples with reasonable expenditure. However, time for the detection of primary populations  
397 may be limited because marbled crayfish are already widespread in American aquaria [11]  
398 and their release into the natural range of *P. fallax* would render the search for primary  
399 populations of marbled crayfish impossible.

400 Our crossbreeding experiments with marbled crayfish, *P. fallax* and *P. alleni* revealed that  
401 marbled crayfish and *P. fallax* still recognize each other as sexual partners but not marbled  
402 crayfish and *P. alleni*. Recognition of sexual partners in crayfish is mainly based on chemical  
403 signatures of the urine but may also include visual and tactile cues [38,39]. Marbled crayfish  
404 and *P. fallax* copulate readily with each other. However, the progeny of such pairings are pure  
405 marbled crayfish resulting from parthenogenesis. These findings demonstrate reproductive  
406 isolation and suggest that the reproductive barrier is set at the cytogenetic rather than the  
407 behavioural level. Mechanical barriers can be largely excluded because the sperm receptacles  
408 are structurally very similar in marbled crayfish and *P. fallax* females and because we have  
409 repeatedly observed insertion of the male gonopods into the annulus ventralis of marbled  
410 crayfish. We attempted to directly prove sperm transfer by analysing moulted sperm

411 receptacles of females that had successfully produced offspring. However, we did not find  
412 any sperm remnants neither in marbled crayfish nor *P. fallax* females.

413 The morphological features and microsatellite patterns strongly suggest that marbled  
414 crayfish originated by autopolyploidization and not by hybridization with a closely related  
415 species, which is by far the most frequent cause of triploidy in animals [55-58]. Typically,  
416 hybrids between two crayfish species are clearly recognizable because of their intermediate  
417 morphological characters [59,60]. However, marbled crayfish do not show such hybrid  
418 features [12,14, this study]. Conversely, autopolyploids are usually morphologically similar to  
419 their diploid progenitors [61] ], and the morphological similarity between marbled crayfish  
420 and *P. fallax* is therefore consistent with autopolyploidization. There is also no evidence for  
421 hybridization on the genetic level and no strong bias towards heterozygosity in the  
422 microsatellite pattern, which would be typical for hybrids [62,63]. Of the seven microsatellite  
423 loci that were investigated in marbled crayfish so far, three were homozygous and four were  
424 heterozygous [20,21, this study], thus largely excluding allopolyploidization for marbled  
425 crayfish. Furthermore, Martin and colleagues have recently shown that the nuclear elongation  
426 factor 2 (EF-2) gene is identical in marbled crayfish and *P. fallax* but differs from other  
427 *Procambarus* species like *P. alleni*, *P. clarkii*, *P. acutus* and *P. liberorum* [22]. These  
428 findings provide additional support for the origin of marbled crayfish by autopolyploidization.

429 We admit that the presence of three alleles, as observed in locus PclG-02 in marbled  
430 crayfish, can be interpreted to reflect an origin by hybridization. However, such a pattern can  
431 also occur in autopolyploids, namely when an unreduced diploid egg is fertilized by a sperm  
432 from the same species, or alternatively, by simultaneous fertilization of a haploid egg by two  
433 sperms with different alleles. In shrimp, fish and bivalve aquaculture, autopolyploid triploids  
434 with tri-allelic loci are artificially produced by the prevention of polar body I extrusion in  
435 fertilized eggs either by temperature shock or chemicals like 6-dimethylaminopurine [64,65].

436 Marbled crayfish may thus have arisen by a heat or cold shock in the sensitive phase of egg  
437 development in a captive *P. fallax* female, possibly during transportation.

438 The origin of parthenogenesis in marbled crayfish is probably a by-product of  
439 polyploidization but the causal relationship of polyploidy and parthenogenesis is not yet  
440 understood [46]. Infectious parthenogenesis by the feminizing bacterium *Wolbachia*, which is  
441 widespread in crustaceans [66], was excluded by the use of molecular probes for the parasite  
442 [2]. In plants, it was shown that polyploidy per se can have an immediate impact on the  
443 reproductive biology of a species [67]. In animals, however, obligate parthenogenesis is  
444 relatively rare. It has been described in some asexual invertebrate families and a few  
445 vertebrate hybrids [26,68-71] and is mostly associated with allopolyploidy. Autopolyploidy is  
446 much less common and is usually not associated with parthenogenesis, perhaps with the  
447 exception of some high arctic ostracods and polyploid populations of the brine shrimp  
448 *Artemia parthenogenetica* [72,73]. Artificially induced autopolyploid shrimp and fish are  
449 usually sterile [74], making the combination of autopolyploidy and parthenogenesis in  
450 marbled crayfish rather unique.

451 Polyploids often have life history traits that are different from those of the parent species.  
452 Growth, number of offspring and other quantitative traits can either be decreased or increased  
453 when compared to the diploid ancestors [75-77]. In marbled crayfish, growth, maximum body  
454 size and fecundity were significantly increased when compared to *P. fallax*, whereas the time  
455 of sexual maturity was similar (7,36,47,54, this study). Longevity may also be increased in  
456 marbled crayfish. Maximum age so far recorded is 1610 days in marbled crayfish [19] and  
457 980 days in *P. fallax* (Z. Faulkes, personal communication). These superior fitness traits,  
458 together with parthenogenetic reproduction, are probably causative for the remarkable success  
459 of marbled crayfish as an invasive species in Central Europe and Madagascar [7-9,47].  
460 Chucholl [9] calculated an almost double FI-ISK (Freshwater Invertebrate Invasiveness  
461 Scoring Kit) score for marbled crayfish when compared to *P. fallax*, making it a high risk

462 species for Central Europe. Moreover, Feria and Faulkes [78] predicted with climate and  
463 habitat based Species Distribution Models that marbled crayfish could inhabit a larger  
464 geographical area than its mother species *P. fallax* when released in the southern states of the  
465 USA, thus illustrating the ecological superiority of marbled crayfish.

466 In allopolyploids, the increase of life history traits is usually explained as the result of  
467 heterozygosity, which is well known as heterosis effect or hybrid vigor [79,80]. However, this  
468 explanation is not applicable for autopolyploids because autopolyploidization enhances only  
469 the copy number of already existing genes. However, novel traits do not necessarily require  
470 new genes or new developmental pathways to come into being but can instead arise from  
471 recruitment of already existing developmental processes into new contexts [81,82]. Thus, trait  
472 alteration in marbled crayfish may have been caused by altered gene dosage, the  
473 rearrangement of gene-networks and the modulation of gene expression by changes in  
474 epigenetic regulation.

475 Changes in epigenetic regulation can be deduced from the significantly reduced level of  
476 global DNA methylation in marbled crayfish when compared to *P. fallax*. DNA methylation  
477 is an epigenetic mechanism that considerably affects plant and animal phenotypes [49,50,83].  
478 It is responsive to environmental and genomic stresses including polyploidization [50] and  
479 might thus contribute to speciation in polyploids. In plants, the increase or reduction of global  
480 DNA methylation after autopolyploidization is well known [61,84]. It is also well established  
481 that DNA methylation and other epigenetic mechanisms contribute to the establishment of  
482 reproductive barriers [85,86] and the expression of hybrid vigor in allopolyploid plants [87].  
483 In marbled crayfish, epigenetic mechanisms may thus have been involved in the acquisition of  
484 novel fitness traits.

485 Chen *et al.* [88] reported that polyploidization is often accompanied or followed by intense  
486 rearrangements in the genome, which stabilize the new lineage. These rearrangements, which  
487 are associated with epigenetic changes, can include loss of DNA. For example, in synthetic

488 autopolyploids of annual phlox, *Phlox drummondii*, an immediate loss of 17% of total DNA  
489 has been observed with a further reduction of up to 25% upon the third generation [89]. Such  
490 mechanisms may also have operated during transition from *P. fallax* to marbled crayfish and  
491 might explain why triploid marbled crayfish have only a 1.4-fold rather than a 1.5-fold  
492 increased DNA content when compared with its diploid mother species.

493 Speciation by autopolyploidization is a special case of chromosomal speciation that is  
494 well-known in plants [61] but virtually unknown in animals. Chromosomal speciation is a  
495 complementary concept to the better known speciation by changes in allele frequency  
496 distribution and can result in the almost instantaneous production of new species and  
497 phenotypic novelty within one generation [90-92]. This "saltational speciation" or "saltational  
498 evolution" [93-95] has largely been ignored by gradualism-based Modern Synthesis, which  
499 may be due to its rarity in animals, the lack of mechanistic understanding and the dearth of  
500 suitable models. Marbled crayfish represents a contemporary animal example of  
501 autopolyploid speciation, which likely started about 20-30 generations ago. Comparative  
502 genome and epigenome sequencing approaches will be required to fully understand the  
503 genetic and epigenetic differences between both species.

504

## 505 **5. Conclusion**

506 Marbled crayfish can be regarded as a new species that originated from *P. fallax* by  
507 triploidization and concomitant epigenetic alterations, as shown by our combined  
508 morphological, behavioural, genetic and epigenetic analysis. Marbled crayfish is  
509 morphologically very similar to its mother species but has superior fitness traits. Genetic data  
510 suggest an instantaneous speciation by autopolyploidization and parallel change of the mode  
511 of reproduction from gonochorism to parthenogenesis. The young evolutionary age of  
512 marbled crayfish, which is possibly three decades or less, may offer the possibility to identify  
513 key events for this type of speciation. The combination of autopolyploidy and obligate

514 parthenogenesis is common in plants but very rare in animals. Thus, the *P. fallax*-marbled  
515 crayfish pair provides an interesting new model system to study asexual speciation and  
516 saltational evolution in animals and to determine how much genetic and epigenetic change is  
517 necessary to create a new species.

518

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534

535 **Authors' contributions.** G.V. conceived of the study, participated in the design of the study,  
536 sampled the tissues, performed the cross-breeding experiments and analysed the  
537 morphological and life history data; C.F. carried out the assembly and analysis of  
538 mitochondrial genome sequences and the determination of DNA contents by flow cytometry;  
539 K.H. maintained laboratory crayfish cultures and prepared DNA samples; A.S., J.P. and R.S.

540 performed the analysis of the microsatellite markers; K.S and M.H. carried out the mass  
541 spectrometric measurement of DNA methylation; F.L. participated in the design of the study  
542 and coordinated the study. G.V. and F.L. wrote the manuscript. All authors revised the  
543 manuscript and gave final approval for publication.

544 **Data accessibility:** The mitochondrial DNA sequences have been deposited in GenBank  
545 under the accession numbers KT074363, KT074364 and KT074365.

546 **Ethics statement:** All crayfish experiments were performed by approval of the institutional  
547 animal welfare committee, in compliance with local standards and guidelines.

548 **Competing interests:** We have no competing interests.

549

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- 821

822

823 **Table 1.** Crossbreeding experiments between marbled crayfish, *P. fallax* and *P. alleni*.

824

Males	Marbled crayfish females														<i>P. fallax</i> females				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	P1	P2	P3	P4	P5
<i>P. fallax</i> 1	x	x	x	x	xx		x	xo	o	x		x		o	x	x			x
<i>P. fallax</i> 2						o	x		o	x	o	x					o	x	
<i>P. fallax</i> 3								x					x			x			x
<i>P. alleni</i> 1					o		o	oo											o
<i>P. alleni</i> 2							oo	oo											o

825 x: mating; o: no mating; two letters: results of two trials.

826

827

828 **Table 2.** Parentage analysis in crossbreeds of marbled crayfish  $\times$  *P. fallax*.

Specimens	Microsatellite loci	
	PclG-02	PclG-26
<i>P. fallax</i> father 1	255/267	185/207
Marbled crayfish mothers 1-4	267/271/303	189/191
Offspring of mother 1 (n=6)	267/271/303	189/191
Offspring of mother 2 (n=5)	267/271/303	189/191
Offspring of mother 3 (n=6)	267/271/303	189/191
Offspring of mother 4 (n=3)	267/271/303	189/191

829 Values indicate fragment lengths in base pairs.

830

831

832 **Figures and figure legends**

833



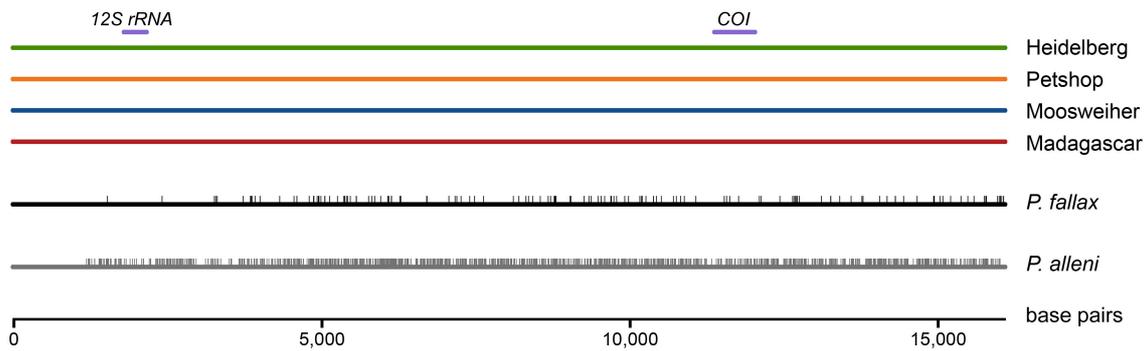
834

835 **Figure 1.** Mating of marbled crayfish female with *P. fallax* male. The male (top) holds the  
836 female firmly with the chelipeds and ischial hooks and his gonopods are plugged into the  
837 female's spermatheca.

838

839

840



841

842 **Figure 2.** Comparison of complete mitochondrial genomes of marbled crayfish, *P. fallax* and

843 *P. alleni*. The sequences of marbled crayfish from two laboratory populations (Heidelberg,

844 Petshop) and two wild populations (Moosweiher, Madagascar) are completely identical. In

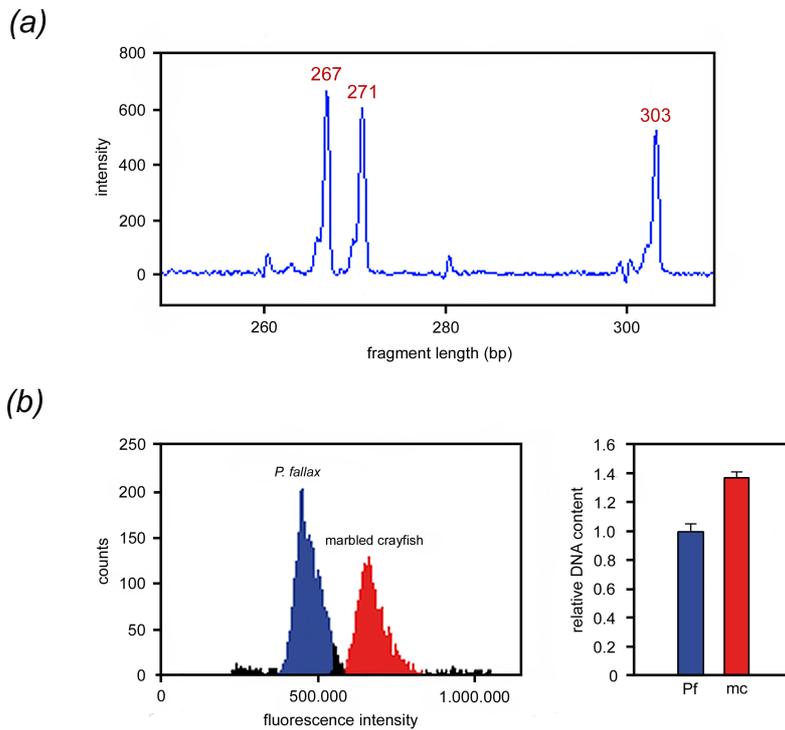
845 contrast, the sequences of *P. fallax* and *P. alleni* differ in 144 and 1165 SNPs (vertical lines)

846 from marbled crayfish, respectively. Purple bars indicate positions of *12S rRNA* and *cyto-*

847 *chrome oxidase subunit I* (COI) genes that were earlier used for phylogenetic analysis [14].

848

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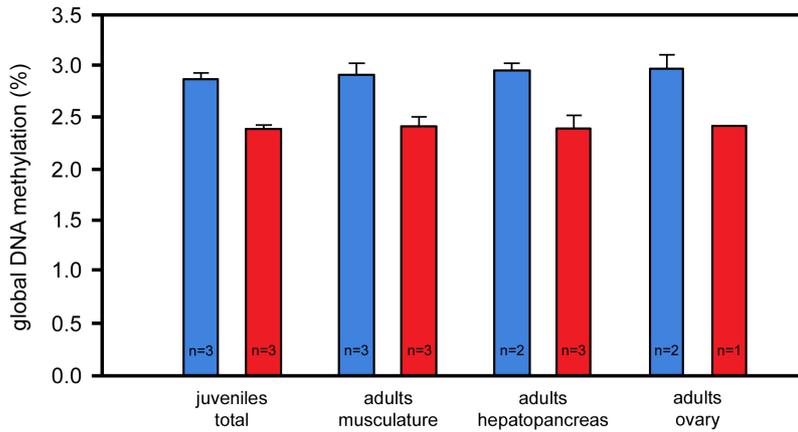


850

851 **Figure 3.** Ploidy status of the marbled crayfish genome. (a) Microsatellite locus PclG-02 in  
852 marbled crayfish showing a combination of three alleles of 267 bp, 271 bp and 303 bp  
853 fragment length. (b) Flow cytometry of haemocytes of *P. fallax* (Pf) and marbled crayfish  
854 (mc) revealing an approximately 1.4 fold increased DNA content in marbled crayfish. The  
855 right panel shows the means and standard deviations of two biological and three technical  
856 replicates. Differences are highly significant ( $p=1.33 \times 10^{-7}$ , Welsh two-sided t-test).

857

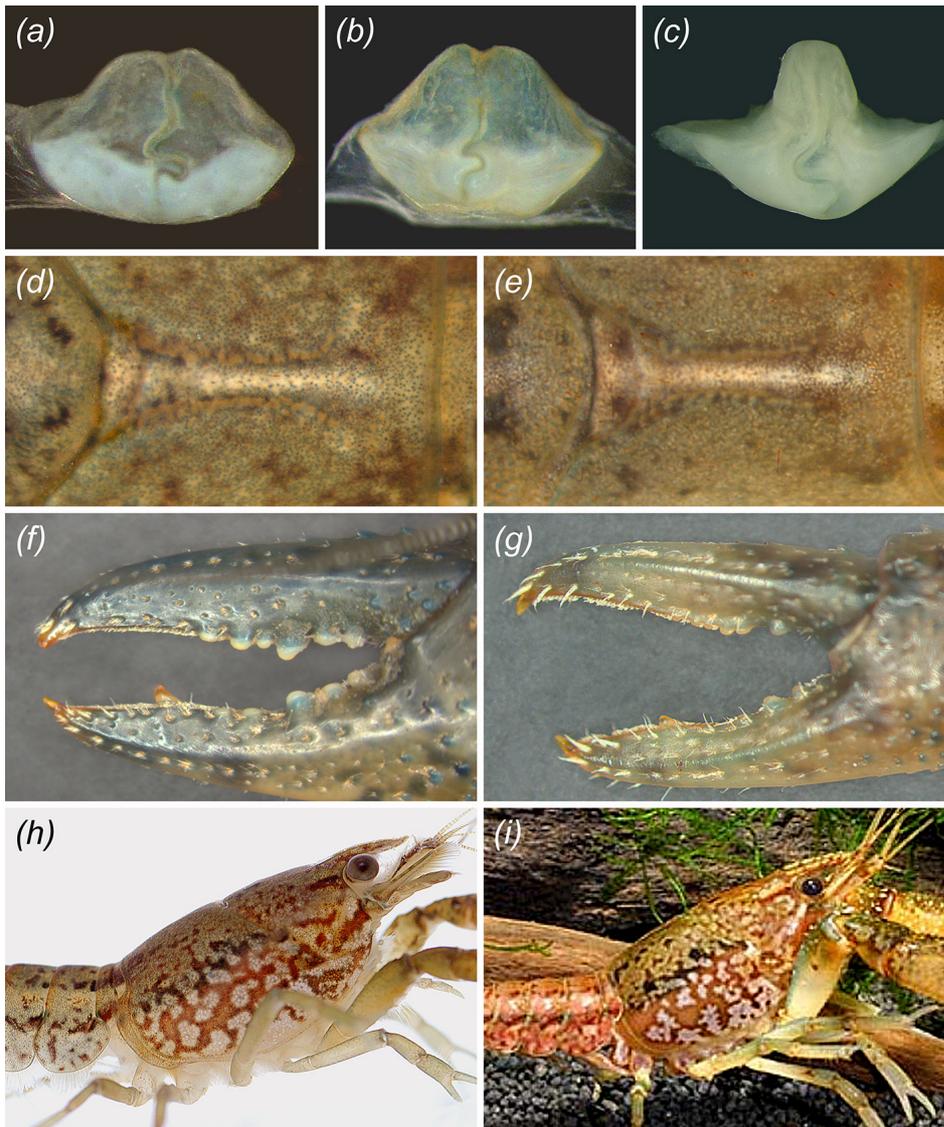
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859

860 **Figure 4.** Differences in global DNA methylation between marbled crayfish (red) and *P.*  
861 *fallax* (blue). Analysed were three complete juveniles and major organs of three adult females  
862 in each crayfish. Note consistently and significantly greater methylation levels in *P. fallax*  
863 ( $p=1.48 \times 10^{-7}$  for the sum of all samples, Welsh two-sided t-test). Error bars: standard  
864 deviations.

865



866

867 **Figure 5.** Comparison of morphological characters between marbled crayfish and *P. fallax*.

868 (a) Annulus ventralis from exuvia of marbled crayfish. (b) Annulus ventralis of *P. fallax*. (c)

869 Annulus ventralis of *P. alleni*. Note striking structural difference to sperm receptacles of

870 marbled crayfish and *P. fallax*. (d) Areola of marbled crayfish. (e) Areola of *P. fallax*. (f) Left

871 cheliped of marbled crayfish of 8.4 cm TL. (g) Left cheliped of *P. fallax* female of 4.7 cm TL.

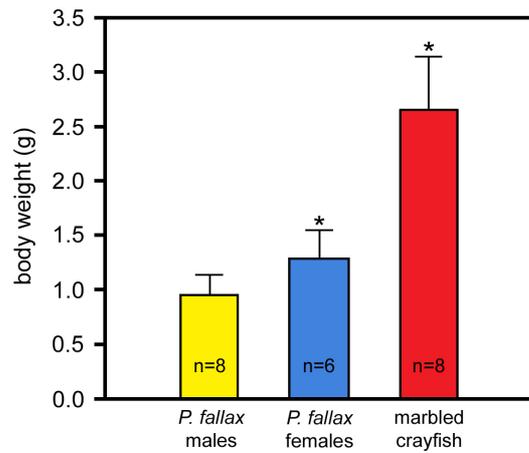
872 Form, dentation and setation of the chelae are very similar in both species. (h) Coloration of

873 cephalothorax in marbled crayfish. (i) Coloration of cephalothorax in *P. fallax* male (photo:

874 C. Lukhaup).

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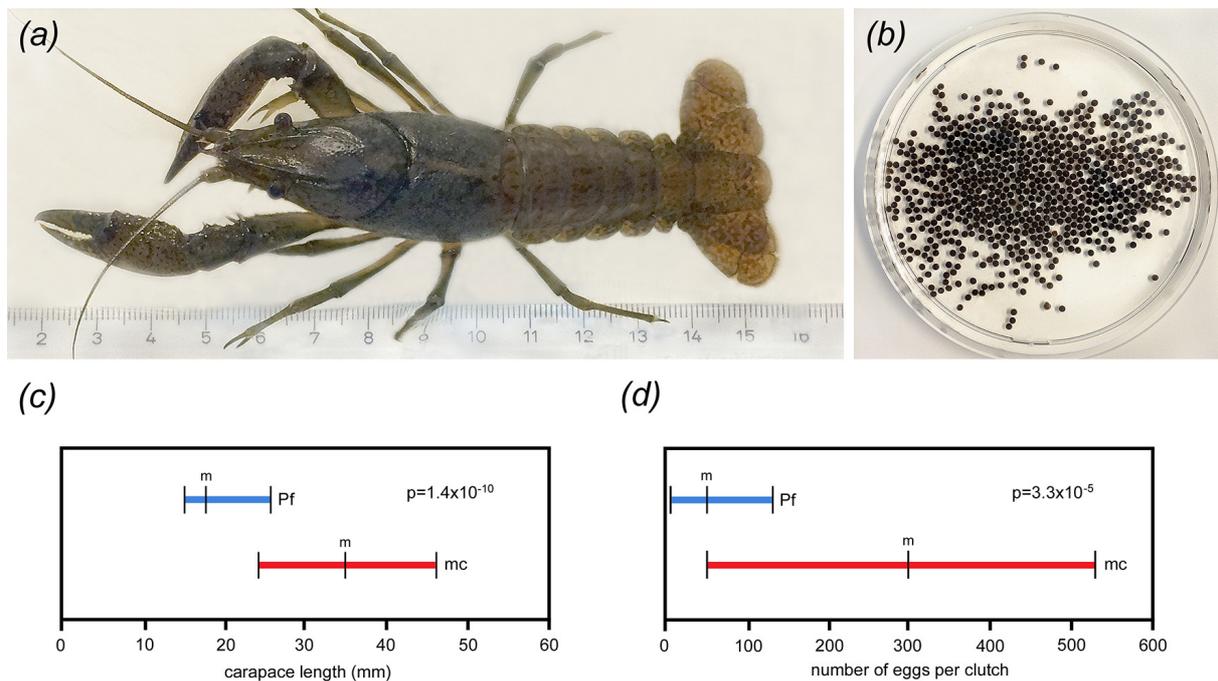


877

878 **Figure 6.** Comparison of growth between marbled crayfish and *P. fallax*. The three groups  
879 were reared for 250 days at 20°C under identical conditions and fed with the same food *ad*  
880 *libitum*. The differences between marbled crayfish and *P. fallax* females are highly significant  
881 (asterisks;  $p=2.06 \times 10^{-5}$ ; Welch two-sided t-test). Error bars: standard deviations.

882

883



884

885 **Figure 7.** Comparison of body size and fecundity between marbled crayfish and *P. fallax*. (a)

886 Largest marbled crayfish from our laboratory having a total length of 10.3 cm. (b) Clutch of

887 same specimen consisting of 731 eggs. (c) Differences in carapace length between

888 populations of ovigerous marbled crayfish (mc) and *P. fallax* females (PF) from comparable

889 climatic regions. Data for marbled crayfish (n=57) was obtained in Madagascar [7] and data

890 for *P. fallax* (n=27) was obtained in Florida [53]. Horizontal bars indicate ranges and vertical

891 lines indicate mean values (m) and lower and upper range limits. The difference between

892 marbled crayfish and *P. fallax* females is highly significant as indicated by the p-value. (d)

893 Differences in clutch size between the same populations as in (c). The difference is highly

894 significant as indicated by the p-value. For statistical calculations, the standard deviation was

895 taken as half the range, and a Bonferroni adjustment for multiplicity was applied.