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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Material and methods

2.1 Animals

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

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

2.2 Cross-breeding experiments

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

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 ≥30, minimum
145 length ≥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 masspectrometer (Agilent, Böblingen, Germany). Briefly, after enzymatic
176 hydrolysis to nucleosides, the samples were spiked with 250 fmol [D_3]-5-methylcytosine as
177 internal standard. The mass transitions resulting from the loss of desoxyribose (5-

178 methylcytidine: 242 Th → 126 Th, [D_3]-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 *x 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 x* marbled crayfish
218 clutches and one *P. fallax x P. fallax* clutch developed into juveniles whereas the others
219 decayed during embryonic development. In the *P. fallax x 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 *x 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 3*a*),
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 3*b*), 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±0.15% (mean ± standard deviation) whereas the ten marbled
309 crayfish samples together had a level of only 2.40±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

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

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

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

4. Discussion

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

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

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

It is unknown whether marbled crayfish emerged in the natural range of *P. fallax* or in captivity. Scholtz [4], Faulkes [5] and Martin [46] summarized possible scenarios for the first 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

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

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

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

Chen *et al.* [88] reported that polyploidization is often accompanied or followed by intense rearrangements in the genome, which stabilize the new lineage. These rearrangements, which 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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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.

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

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

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822

823 **Table 1.** Crossbreeding experiments between marbled crayfish, *P. fallax* and *P. alleni*.
824

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

Specimens	Microsatellite loci	
	PclG-02	PclG-26
P. fallax 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

Figures and figure legends

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

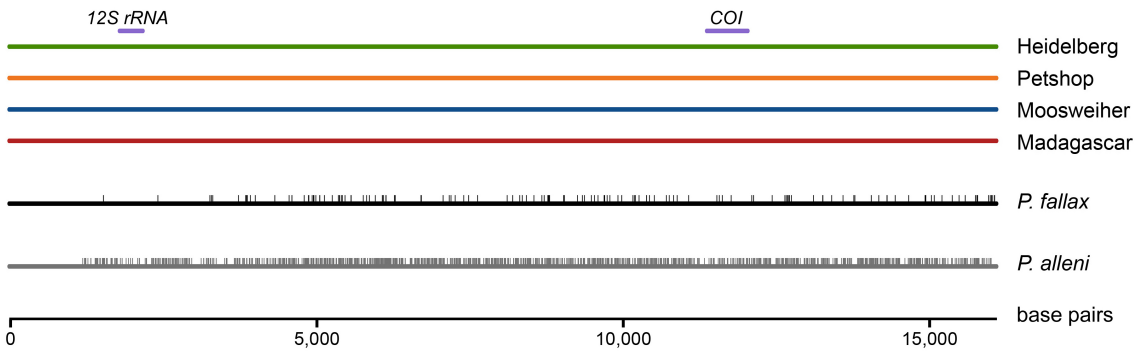
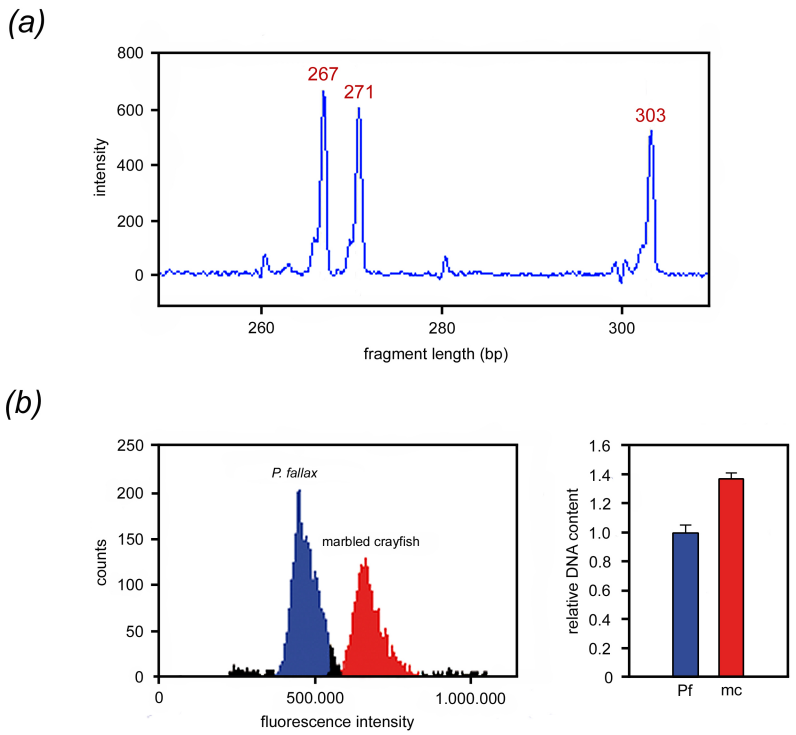


Figure 2. Comparison of complete mitochondrial genomes of marbled crayfish, *P. fallax* and *P. alleni*. The sequences of marbled crayfish from two laboratory populations (Heidelberg, Petshop) and two wild populations (Moosweiher, Madagascar) are completely identical. In contrast, the sequences of *P. fallax* and *P. alleni* differ in 144 and 1165 SNPs (vertical lines) from marbled crayfish, respectively. Purple bars indicate positions of *12S rRNA* and *cyto-chrome oxidase subunit I* (COI) genes that were earlier used for phylogenetic analysis [14].

849

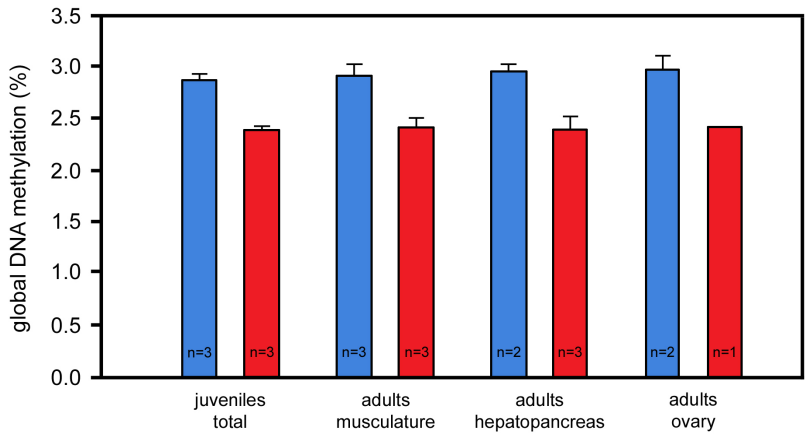


850

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 ($p=1.33x10^{-7}$, Welsh two-sided t-test).

857

858



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 ($p=1.48x10^{-7}$ for the sum of all samples, Welsh two-sided t-test). Error bars: standard
864 deviations.

865

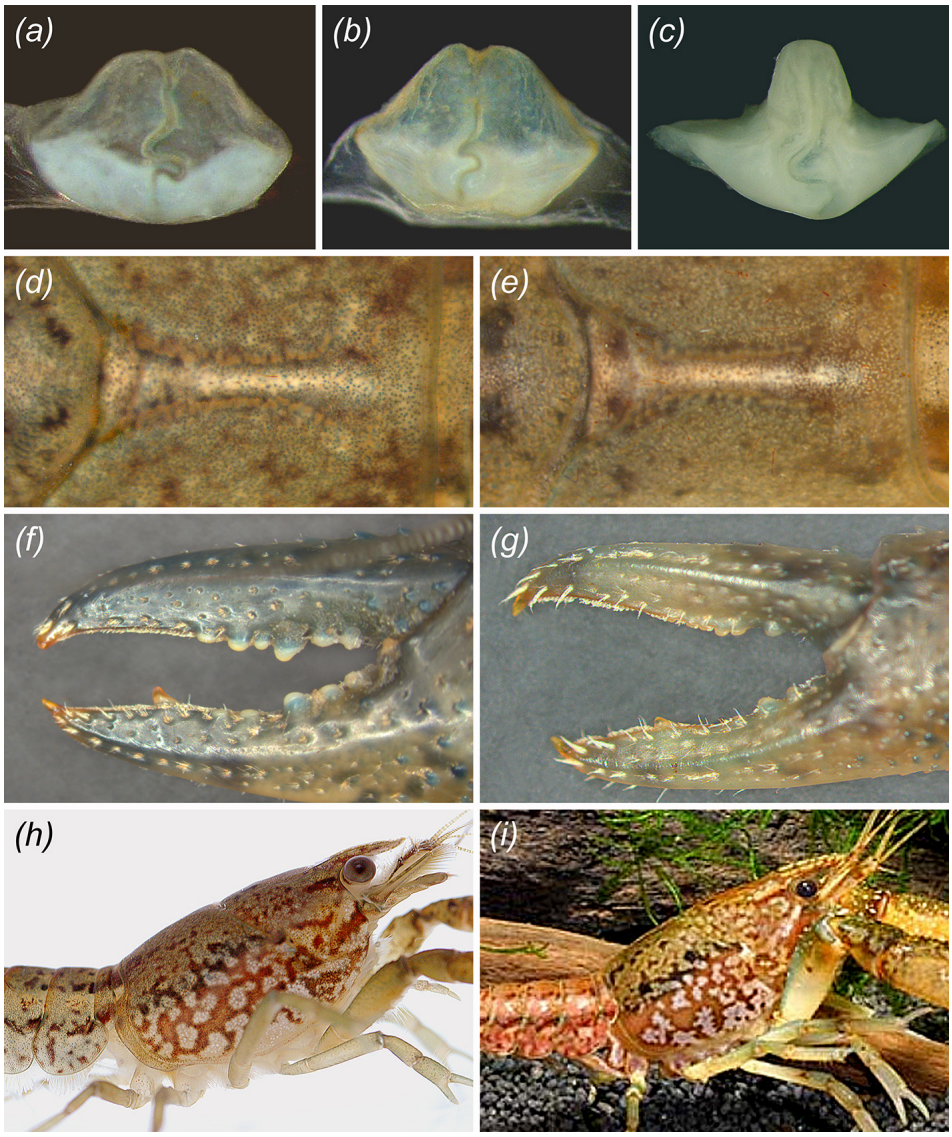


Figure 5. Comparison of morphological characters between marbled crayfish and *P. fallax*. *(a)* Annulus ventralis from exuvia of marbled crayfish. *(b)* Annulus ventralis of *P. fallax*. *(c)* Annulus ventralis of *P. alleni*. Note striking structural difference to sperm receptacles of marbled crayfish and *P. fallax*. *(d)* Areola of marbled crayfish. *(e)* Areola of *P. fallax*. *(f)* Left cheliped of marbled crayfish of 8.4 cm TL. *(g)* Left cheliped of *P. fallax* female of 4.7 cm TL. Form, dentation and setation of the chelae are very similar in both species. *(h)* Coloration of cephalothorax in marbled crayfish. *(i)* Coloration of cephalothorax in *P. fallax* male (photo: C. Lukhaup).

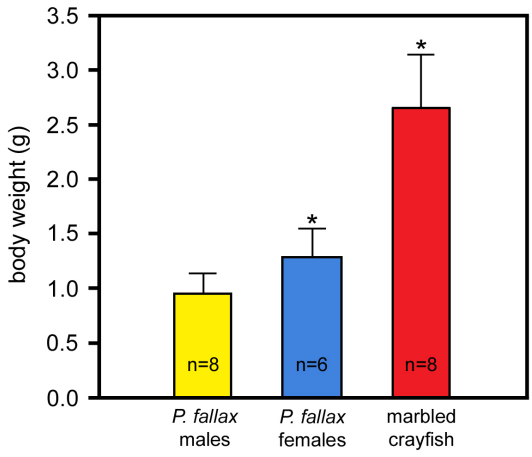


Figure 6. Comparison of growth between marbled crayfish and *P. fallax*. The three groups were reared for 250 days at 20°C under identical conditions and fed with the same food *ad libitum*. The differences between marbled crayfish and *P. fallax* females are highly significant (asterisks; $p=2.06 \times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

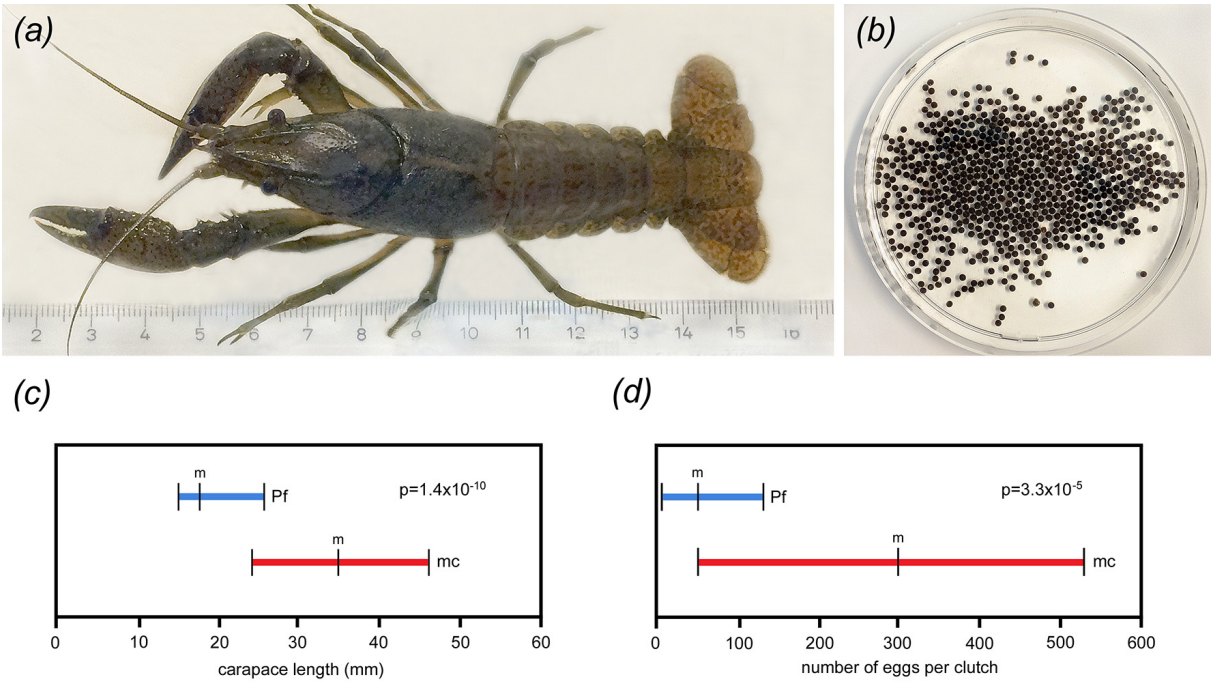


Figure 7. Comparison of body size and fecundity between marbled crayfish and *P. fallax*. *(a)* Largest marbled crayfish from our laboratory having a total length of 10.3 cm. *(b)* Clutch of same specimen consisting of 731 eggs. (*c)* Differences in carapace length between populations of ovigerous marbled crayfish (mc) and *P. fallax* females (PF) from comparable climatic regions. Data for marbled crayfish (n=57) was obtained in Madagascar [7] and data for *P. fallax* (n=27) was obtained in Florida [53]. Horizontal bars indicate ranges and vertical lines indicate mean values (m) and lower and upper range limits. The difference between marbled crayfish and *P. fallax* females is highly significant as indicated by the p-value. (*d*) Differences in clutch size between the same populations as in (*c*). The difference is highly significant as indicated by the p-value. For statistical calculations, the standard deviation was taken as half the range, and a Bonferroni adjustment for multiplicity was applied.