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► To cite this version:

Francisco Oficialdegui, Miguel Clavero, Marta Sánchez, Andy J Green, Luz Boyero, et al.. Unravelling the global invasion routes of a worldwide invader, the red swamp crayfish (*Procambarus clarkii*). *Freshwater Biology*, 2019, 64 (8), pp.1382-1400. 10.1111/fwb.13312 . hal-02139508

HAL Id: hal-02139508

<https://amu.hal.science/hal-02139508v1>

Submitted on 31 Mar 2020

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1 **i. Title Page**

2 **Article Title**

3 Unravelling the global invasion routes of a worldwide invader, the red swamp crayfish (*Procambarus clarkii*)

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7

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26

27 **Keywords**

28 Admixture, invasion hubs, invasion process, mitochondrial DNA, propagule pressure,

29

30 **ii. Summary**

31 1. Understanding how introduced species succeed and become widely distributed within non-native areas is
32 critical to reduce the threats posed by them. Our goal was to reconstruct the main invasion routes and
33 invasion dynamics of a global freshwater invader, the red swamp crayfish, *Procambarus clarkii*, through
34 the analysis of its genetic variability in both native and invasive ranges.

- 35 2. We inferred invasion routes and population structure from the analysis of a fragment (608bp) of the
36 mitochondrial marker COI from 1,062 individuals of *P. clarkii* in addition to 354 GenBank sequences, for a
37 total of 122 populations (22 natives and 100 invaded). Genetic structure was assessed using analysis of
38 molecular variance and non-metric multidimensional scaling analyses. We analysed haplotype
39 frequencies for the genetic variability in each locality and region. The haplotype network was depicted by
40 using PopART software.
- 41 3. A high haplotype diversity was found in the native range (Hd: 0.90), but also in some non-native areas,
42 such as western United States (Hd: 0.80), areas of Mexico (Hd: 0.78) and some hotspots in Europe (e.g.,
43 southern Spain or Italy), suggesting a complex pattern of multiple introductions. We grouped all
44 localities in five differentiated groups according to biogeographic origin: the native area, West Americas,
45 East United States, Asia and Europe. Additionally, the identification of 15 haplotypes shared between at
46 least two localities, the phylogenetic network estimation and indices of genetic differentiation among
47 localities allowed us to identify a large genetic admixture in the native range; the two independent
48 invasion routes (i.e. westwards and eastwards) in US from the native range (Louisiana and Texas) with
49 translocations within each area; a stepping-stone introduction from US to Japan (involving few
50 individuals) themselves introduced to China afterwards; the entry of *P. clarkii* from Louisiana (US) into
51 southern Spain and their multiple secondary introductions over Europe as well as other possible
52 introductions in central Europe.
- 53 4. Our study emphasizes the need for unravelling the global invasion routes and the demographic processes
54 underlying the introduction of exotic species (i.e., admixture, bridgehead invasion effect and propagule
55 pressure) to control the spread of invasive species. Our findings highlight the value of genetic analyses to
56 identify the geographic origin of source populations as well as the variability of invaded areas in order to
57 reconstruct invasion dynamics and facilitate management of invasive species (e.g. through environmental
58 DNA monitoring).

59

60 **iii. Main Text**

61 **Introduction**

62 Humans have transported species across biogeographical barriers and introduced them to new territories for
63 millennia (Forcina et al., 2015), but large-distance movements of species have increased exponentially in
64 recent decades (Hulme, 2009; Lenda et al., 2014) driving to an homogenization of biotas that has involved the
65 break-down of long-established biogeographical barriers (Capinha et al., 2015). Those species that are
66 transported by humans, released into new environments, able to survive, establish self-sustained
67 populations, thrive, become abundant and spread geographically, are considered invasive species (Jeschke et
68 al., 2014). Biological invasions today are perceived as major components of global change, with severe
69 negative environmental (Simberloff et al., 2013; Blackburn et al., 2014; Jeschke et al., 2014) and socio-
70 economic impacts (Vilà et al., 2010). To manage invasive species, it is of vital importance to identify invasion

71 routes (De Kort et al., 2016). However, most knowledge about the transport routes of invasive species is
72 based on historical and observational data, which are usually scarce, confusing and sometimes inaccurate
73 (Roman, 2006; Haydar, 2012). Population genetic studies provide valuable tools to identify areas of
74 geographic origin of introductions, to detect single versus multiple introductions, and to describe expansion
75 patterns (Lejeusne et al., 2014; Cristescu, 2015; Blakeslee et al., 2017; O'Hanlon et al., 2018; Fang et al., 2018),
76 though caution must be used when interpreting demographic history over such short timescales (Fitzpatrick
77 et al., 2012). Such information can be useful for the management of invasive species and for the prevention of
78 future introductions (Estoup & Guillemaud, 2010), and phylogeographic studies have been proposed as an
79 integral tool of biodiversity conservation planning (van de Crommenacker et al., 2015).

80
81 Many invasive species have high economic value, which often results in their deliberate introduction by
82 humans into non-native areas where they can spread rapidly due to secondary introductions (see
83 Audzijonyte et al., 2017; Cao et al., 2017; Huang et al., 2017). Unlike accidental introductions that are
84 facilitated by humans, species that are deliberately introduced may have a higher chance of success because
85 humans take action to ensure such success (Pyšek et al., 2011). For example, deliberate introductions often
86 involve a high propagule pressure (i.e. number of introduction events and/or size of propagules; Lockwood
87 et al., 2005; Simberloff, 2009), the genetic admixture of introduced populations (i.e. the mixing of populations
88 from genetically distinct source populations; Dlugosch & Parker, 2008; Rius & Darling, 2014; Hufbauer,
89 2017), and the subsequent invasive bridgehead effect (i.e. a particularly successful invasive population
90 serves as a source for new introductions, Lombaert et al., 2010; Estoup & Guillemaud, 2010).

91
92 Due to the high intensity of human disturbances and the high connectivity among inland water systems,
93 freshwater environments are especially susceptible to invasions (Strayer, 2010; Havel et al., 2015; Tricarico et
94 al., 2016). Also, many freshwater species can be harvested from the wild and/or cultivated in farms for
95 commercial purposes, providing a high socioeconomic value (e.g., aquaculture; FAO, 2011). Among these,
96 several invertebrates are valued either for human consumption or as food for other cultured animals (e.g.
97 shrimp or prawn for fishes, crayfish for bullfrogs, etc.), which provides a high economic return (Resh &
98 Rosenberg, 2015). Freshwater crayfish are favoured for farming since they do not have a larval phase and are
99 polytrophic, they are relatively easy to rear compared with other cultured crustaceans (Holdich, 1993), and
100 their consumption has a long-lasting tradition in many regions worldwide (Gherardi, 2011). The red swamp
101 crayfish, *Procambarus clarkii* (Girard 1852), native to southern USA and northern Mexico, has been
102 successfully introduced into all continents except Australia and Antarctica (Loureiro et al., 2015) mainly due
103 to its economic value (Hobbs et al., 1989). Owing to its biological and ecological characteristics, this crayfish
104 is considered one of the worst invasive species worldwide, causing serious damage to biodiversity (e.g.,
105 other crayfish species, fish, amphibians, macroinvertebrates and macrophytes) and to human infrastructure
106 and ecosystem services (e.g., irrigation canals, water quality, rice crops, etc.) (Geiger et al., 2005;

107 Twardochleb et al., 2013). *Procambarus clarkii* is one of the most economically valuable aquatic species to be
108 farmed (Huner, 2002; Souty-Grosset et al., 2016), generating tens of billions of US dollars (USD) per year in
109 the world (http://www.fao.org/fishery/culturedspecies/Procambarus_clarkii/en#tcNA0064).

110

111 The first introductions of *P. clarkii* out of its native area took place in the early 20th century, when it was taken
112 to the Hawaiian Islands (1923), the Pacific drainages of USA (1924), Japan (1927) and China (1929), with
113 different motivations including aquaculture, fishing activities and food for cultured American bullfrogs
114 *Lithobates catesbeianus* (Holmes, 1924; Penn, 1954; Brasher et al., 2006). *Procambarus clarkii* was often able to
115 spread rapidly, occupying the rivers and lakes of non-native areas (e.g. Riegel, 1959, for California; Yue et al.,
116 2010, for China). In the mid-1960s, a batch of crayfish was sent to Uganda from Louisiana, then translocated
117 to Kenya, and later to other African countries (Huner, 1977; Lowery & Mendes, 1977). Concurrently, it
118 artificially spread out of its native area in Mexico, and then to Costa Rica, Puerto Rico, Venezuela, and the
119 Dominican Republic in the 1970s (Huner, 1977), eventually reaching Brazil in the mid-1980s (Huner, 1986). In
120 Europe, it was deliberately and legally introduced into Spain (Badajoz and Seville in 1973 and 1974,
121 respectively) from Louisiana (Habsburgo-Lorena, 1978; 1986). In only 45 years, *P. clarkii* has colonized many
122 countries in Europe, being widely established in Spain, Portugal, France, Italy, Belgium, Netherlands,
123 Germany and the United Kingdom (see Kouba et al., 2014 for the entire European distribution of this
124 species).

125

126 To date, most genetic studies of *P. clarkii* have focused on genetic variability at a regional scale (e.g.,
127 Barbaresi et al., 2007; Torres & Álvarez, 2012; Quan et al., 2014; Yi et al., 2018; Almerão et al., 2018). Of these,
128 very few studies have attempted to unveil the invasion routes, and when performed, they did so only at a
129 regional scale. Hence, almost nothing is known about the population genetics and invasion routes of *P.*
130 *clarkii* at a global scale. The general objective of this study is to provide a comprehensive overview of the
131 global invasion history of *P. clarkii*. We included not only most of the non-native range of this species in the
132 Northern Hemisphere, but also an exhaustive sampling of its native area in order to confirm the invasion
133 sources and routes previously in the literature and detect previously unreported ones. Hence, our specific
134 objectives were: 1) to describe the invasion dynamics of *P. clarkii* at continental and global scales, identifying
135 the main invasion routes; and 2) to examine the genetic variability and population structure of *P. clarkii* in
136 the native area and across the non-native range, with special focus on Europe, to reveal potentially
137 unreported introductions not cited in the literature.

138

139 **Methods**

140 *Sampling*

141 We collected 1,062 specimens of *P. clarkii* from 72 localities: 15 native (States of Louisiana and Texas, USA)
142 and 57 non-native localities distributed within the Northern Hemisphere (i.e., western US, eastern US,
143 Europe and Japan) (Table 1 and Fig. 1). Crayfish were individually preserved in 96% ethanol. Average
144 sample size per locality was 14.7 ± 6.6 individuals (mean \pm SE; range 2-21) (Table 1). We included in our
145 dataset the information for 354 additional individuals from 7 native and 43 non-native localities that we
146 obtained from data already published in previous studies (Genbank Accession numbers: AY701195;
147 JF438001- JF438004; JN000898- JN000908; JX120103- JX120108) available from Taylor & Knouft (2006),
148 Filipová et al. (2011), Torres & Álvarez (2012) and Li et al. (2012), respectively. Thus, a total of 1,416
149 individuals from 22 native and 100 introduced localities (Fig. 1a; Table 1) were used for this study. The
150 sequences recently published by Almerão et al. (2018) were not added into our global analyses of this study
151 because our sequences were larger than theirs. Even so, we compared their results in a subsequent analysis
152 (see Supplementary Material, Table S1 for synonymous haplotypes).

153

154 *DNA extraction and sequencing*

155 Genomic DNA was extracted from muscle tissue (gill tissue at LEN, FOR and PER localities; see Table 1 for
156 more details) using a modified DNA salt-extraction protocol (composition: NaCl 25 mM, Tris 12.5 mM (pH
157 8.0), EDTA 12.5 mM (pH 8.0), 31.5 μ L SDS 10%) and proteinase K (Aljanabi & Martinez, 1997). Logistical
158 support was provided by the Laboratorio de Ecología Molecular, Estación Biológica de Doñana, CSIC (LEM-
159 EBD). A fragment of the mitochondrial gene coding for the cytochrome *c* oxidase subunit I (COI) gene was
160 amplified using the primers LCO1490 and HCO2198 (Folmer et al., 1994). Amplifications were carried out in
161 a 20 μ l reaction volume, with 1-5 μ l of genomic DNA, 2 μ l of 10x buffer, 0.8 μ l of MgCl₂ (50 mM), 0.16 μ l
162 dNTP (100 mM), 0.5 μ l primer LCO 1490, 0.5 μ l primer HCO 2198 and 0.12 μ l TAQ polymerase (Bioline).
163 Polymerase chain reaction (PCR) consisted of an initial denaturation step at 94°C for 5 min, followed by 30
164 amplification cycles (94°C for 1 min, 47°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C
165 for 5 min. Sequencing was performed by Macrogen Europe Company.

166

167 *Genetic analyses*

168 Sequences were edited using the software Sequencher™ v4.9 (Gene Codes Corp., © 1991–2009, Ann Arbor,
169 MI 48108). Nucleotide sequences were aligned using the algorithm CLUSTAL W implemented in BioEdit
170 (Hall, 1999). No insertions nor deletions (indels) were found. A hierarchical series of tests based on the

171 Bayesian Information Criterion (BIC) was applied to identify the most appropriate nucleotide substitution
172 model among 88 models tested, as implemented in jModelTest 2 (Darriba et al., 2012). We used the nested
173 model Tamura & Nei (1993) with 133 parameters, 1382.91 $-\ln L$ for onwards analyses. DnaSP 6.0 software
174 was used to calculate the number of polymorphic sites (S), haplotype diversity (H_d), nucleotide diversity
175 (π), and total number of synonymous and non-synonymous mutations, for which nucleotide sequences were
176 translated into amino acid sequences using the *Drosophila* mitochondrial genetic code (Rozas et al., 2017). The
177 haplotype network was inferred by the TCS method (Clement et al., 2000) implemented in PopART software
178 (Leigh & Bryant, 2015).

179

180 Because of a smaller sampling size (1 or 2 individuals), ILL, LAf, FR1, FR2, FR3 and VAL localities were
181 excluded from downstream analyses. Pairwise ϕ_{ST} ($Phist$) and hierarchical analysis of molecular variance
182 (AMOVA) were calculated using Arlequin 3.5 (Tamura & Nei, 1993; Excoffier & Lischer, 2010). To examine
183 the genetic differentiation between any two of the populations, ϕ_{ST} calculations were calculated assuming
184 gamma-distributed substitution rates using the Tamura and Nei model (Tamura & Nei, 1993) to compute a
185 distance matrix and 10,000 bootstrap pseudo-replicates were used to estimate the standard error. The p -
186 *values* were corrected for multiple comparisons using the false discovery rate (FDR) control according to the
187 Benjamin and Hochberg (BH) correction method (Benjamin & Hochberg, 1995). To ascertain the genetic
188 structure of populations, AMOVAs were performed based on 10,000 random permutations. Due to the large
189 native range of *P. clarkii*, we classified native localities into five groups according to natural (e.g. river
190 catchments) and administrative (e.g. country or state frontiers) boundaries: Mexico, Texas, Louisiana east
191 (east of the Atchafalaya River), Louisiana west (west of the Atchafalaya River), and Mississippi River
192 upstream (upstream starting at Monroe, Memphis and north to Illinois). However, for all datasets, two
193 different *a priori* hypotheses were tested: (a) native versus introduced localities, and (b) population grouping
194 according to biogeographical distribution of this species into 5 zones: (1) native area, (2) West Americas
195 which included all samples from the USA west of Texas, including California, Oregon and Washington
196 State, plus all samples from Hawaii and invaded Central America, (3) East United States (from Louisiana to
197 the Atlantic Ocean and Chicago), (4) Asia, and (5) Europe. Another *a priori* hypothesis was analysed
198 exclusively for European populations to test whether there were one (i.e., the whole of Europe) or two
199 genetic clusters within Europe (i.e., southern and northern areas of *P. clarkii* distribution). A dissimilarity
200 matrix of Jost's D_{est} distances was also calculated with 10,000 replicates using SPADE (Jost, 2008; Chao &
201 Shen, 2012). Based on ϕ_{ST} and D_{est} estimates, two non-metric multidimensional scaling (NMDS) analyses
202 were used to graphically represent the differentiation among localities and their respective zones (described
203 above) using the *vegan* package in R (Oksanen, 2013).

204

205 **Results**

206 Among the 1,416 specimens of *P. clarkii* analysed, we obtained a matrix of 608 base pairs (bp) of the
207 cytochrome *c* oxidase subunit I (COI) with 54 polymorphic sites, yielding 65 haplotypes. Sequences of all
208 haplotypes were submitted to GenBank and assigned Accession Numbers: MK026671 - MK026735. Most of
209 the nucleotide substitutions were synonymous, but four non-synonymous changes were identified (2
210 substitutions at the 1st position corresponding to a change from an Isoleucine to a Valine, and from a
211 Methionine to a Valine, respectively; and 2 substitutions at the 2nd position corresponding to a change from a
212 Valine to an Alanine, and from a Threonine to a Methionine, respectively). Of these four non-synonymous
213 changes, one singleton was found in the ALB locality (Spain) and three parsimony sites were located at SMA
214 and COM localities in Texas (US) and in the DU locality in the invaded area of Mexico (see abbreviations in
215 Table 1 and marked in haplotype network in Fig. 2).

216 The overall haplotype diversity (Hd) and nucleotide diversity (π) were 0.76 and 0.0040, respectively.
217 The native area showed the highest haplotype and nucleotide diversity (0.90 and 0.0055, respectively), with
218 the highest figures being found in WOO, DES, MON and PIE localities (Table 1). For invaded areas,
219 haplotype and nucleotide diversities varied considerably between regions: Hd: 0.80 and π : 0.0048 in non-
220 native US, Hd: 0.78 and π : 0.0056 in non-native Mexico, Hd: 0.46 and π : 0.0023 in Asia and Hd: 0.58 and π :
221 0.0022 in Europe, respectively (for localities see Table 1). Of the entire dataset, a total of 15 haplotypes were
222 shared between at least two sampling localities (Fig. 1), of which Hap_04 was present at high frequency
223 almost worldwide, irrespective of the native (up to 13 localities) or non-native (up to 76 localities) status of
224 the populations. Other haplotypes were shared between continents, including coincidences between US and
225 Europe (Hap_01, Hap_03, Hap_05, Hap_09 and Hap_29) and North America and Asia (Hap_02 found in
226 invaded localities in Mexico, California, Japan and China, and Hap_40 shared between the native area and
227 Japan). Conversely, 50 haplotypes were restricted to one locality (i.e., private haplotypes), 29 of them found
228 in the native area and 21 being exclusive to one of the invaded localities (see Supplementary Material, Table
229 S2).

230 The statistical parsimony haplotype network showed a star-like structuring centered around the
231 Hap_04, which appeared in almost half of sampled crayfish (618 specimens) geographically widely
232 distributed over all zones (13 native and 62 invaded localities) (Fig. 2 and see Supplementary Material, Table
233 S2). Moreover, there were other smaller star-like structuring around three haplotypes (Hap_01, Hap_20 and
234 Hap_09). Hap_01 was mainly distributed in the US, both in its native (9 localities in Louisiana) and non-
235 native range (TOP and PIN in western US; and PER, LEN, FOR and CHI in Atlantic area), but also was
236 found in two Spanish localities (ECO and GIJ). Hap_20 was found in Louisiana and across the western US. In
237 addition, it closely joined (1 mutation) to Hap_28 which is widely found in Asia. Hap_09 was broadly
238 distributed among the native localities in Louisiana, as well as in three invaded North American localities,
239 and two Spanish and one French locality (AR4, CHO and FR1, respectively). In addition, this central
240 haplotype was connected by only one mutation with Hap_40 which was present in Japan. A thorough
241 analysis of the haplotype network in the native range (see Supplementary Material, Fig. S1) showed no clear

242 genetic structure except for Texas localities (Hap_15-19 and Hap_48-50), Mexico localities (Hap_61-62) and
243 the Hap_20 which was mainly found in southeastern Louisiana. Additionally, Hap_04, Hap_01 and Hap_09
244 were widely distributed over all localities in the native range as well as many localities grouped
245 evolutionarily differentiated haplotypes (e.g., MON, NAT or LO localities), indicating a large genetic
246 admixture in Louisiana.

247

248 Due to the large native area of *P. clarkii*, we tested whether the native area clustered into 5 groups (Mexico,
249 Texas, Mississippi River upstream, Louisiana east, and Louisiana west). As haplotype network showed,
250 AMOVA also revealed a slight genetic structure within the native area, where a small fraction of the total
251 variance was due to between-group variance (29.0%); nevertheless, most of the variance was explained by
252 variation within populations (61.6%) (Table 2). This may be due to the high proportion of private haplotypes
253 (Fig. 1b and Fig. 1c). For the whole dataset, native and invaded areas worldwide were not clustered into two
254 different genetic groups because only 14.3 % of total variation was due to differences between groups (Table
255 3), indicating the high variability of *P. clarkii* in the invaded range. However, after classifying the whole set
256 of localities according to their biogeographical ranges (see Methods), 39.6% of total variation was still
257 explained by differences within localities, but 36.0% of total variance was due to differences among these 5
258 established zones (Table 3). This result seems to indicate a slight genetic structure among these zones. In
259 Europe, a moderate genetic structure was detected between the northern and southern distribution areas of
260 *P. clarkii* (Fig. 1g), where Hap_04 was predominant in South Europe and Hap_11 in Central Europe.
261 AMOVA analyses showed that 40.7 % of explained variance was due to differences between both genetic
262 clusters (Table 4).

263

264 In a NMDS plot based on D_{est} distances (Fig. 3), most localities remained within the 95% confidence intervals
265 (CI) of the native area group, except some localities from Japan, China and western North America, in which
266 Hap_28 was present at high frequency. This is due to the fact that we did not find this haplotype in the
267 native area, despite the exhaustive sampling done there. All localities from eastern US were closely grouped
268 within the range of the native area. However, the localities from western North America were more different
269 from each other, for instance PIN was closer to European localities, whereas VEN and CH were more similar
270 to Asian locations. In addition, the other locality from western North America not only had a clear proximity
271 to each other but also to localities from within the native area (COM, SMA, COC and NL), indicating a
272 similarity among them. The result of AMOVA analysis for European populations, where two genetic clusters
273 were found between localities from the northern and southern European distributions, was also reinforced
274 by NMDS plot. LON, HOL, ECA, BIO and BRI localities (depicted by a green triangle in the NMDS plot)
275 were situated all together outside the 95% CIs of the European group, having greater proximity to Texas and
276 Mexico localities from the native area and those from western America than to south European localities.

277

278 **Discussion**

279 The high haplotype diversity of *P. clarkii* found in some invaded localities suggests that its global invasion,
280 driven mostly by human-mediated introductions, may have involved admixture in the native range, an
281 invasive bridgehead effect, and high propagule pressure. However, we also detected low levels of genetic
282 diversity in some non-native areas (e.g. Asia), attributable to potential bottlenecks or founder effects. Our
283 results allow the identification of the likely geographic origin and main routes of invasion, helping us to
284 understand how the invasion has happened over a long time scale (Fig. 4).

285

286 *The native range of the red swamp crayfish*

287 Admixture has been proposed to be a causal mechanism triggering the invasiveness of some introduced
288 species (Kolbe et al., 2007; van Boheemen et al., 2017; Fischer et al., 2017; Wagner et al., 2017; but see also, Rius
289 & Darling, 2014) by enhancing genetic variability, thus improving population growth, decreasing the risk of
290 extinction, and favouring adaptation to novel environments. In the present study, we found the highest
291 haplotype diversity in the native area. The vast majority of haplotypes found in invaded areas also appeared
292 in Louisiana but not in other native populations of US or Mexico. This pattern is arguably related to the
293 commercial exploitation of this species in Louisiana, where *P. clarkii* has been reared, harvested and sold
294 globally by food-industry companies for a long time (Gary, 1975; Alford et al., 2017). Although some genetic
295 clusters seem to be differentiated between east and west Louisiana (Hap_01, Hap_04 and Hap_20
296 predominated in Louisiana east while Hap_03 and Hap_08 predominated in Louisiana west), Texas and
297 Mexico localities, most of the genetic variation in those areas occurred within localities. The lack of a clear
298 genetic structure in the native area might imply a pattern of admixture owing to farming activities in
299 Louisiana, in which crayfish are often exchanged and translocated from wild to captive populations (Huner,
300 2002). Similar genetic patterns of native admixed populations have been identified in other species related to
301 aquaculture such as the topmouth gudgeon, *Pseudorasbora parva*, when they were translocated together with
302 Chinese carp species (Hardouin et al., 2018). The exchanges found in the Louisiana populations do not seem
303 to have occurred in Texas, since eight private haplotypes were found in two populations and almost all
304 haplotypes were grouped together (see Hap_15-19 and Hap_48-50). Admixed source populations, like for *P.*
305 *clarkii* in Louisiana, can lead to high genetic variability in invasive populations, thus allowing the invasive
306 species to face novel environments and to thereby increase invasion success in the introduced range. But this
307 assumption should be interpreted with caution since some species are able to show high invasiveness
308 despite low genetic variability, such as *Procambarus virginialis*, a potentially highly invasive parthenogenetic
309 crayfish that is able to establish wild populations from a single released individual (Feria & Faulkes, 2011).

310

311 *The invasion dynamics of the red swamp crayfish*

312 According to the literature, the first invasion of *P. clarkii* took place in Hawaiian streams in 1923 (Brasher et
313 al., 2006) and in California in 1924 (Holmes, 1924). However, the geographical origins of both invasions
314 remain unclear. In 1934, another event of introduction occurred in the island of Oahu, Hawaii, from Santa
315 Barbara, California, from which subsequently *P. clarkii* apparently spread over the rest of the Hawaii
316 archipelago (Penn, 1954). The Hap_27 found in each of these US states (California and Hawaii) may confirm
317 this second introduction event of *P. clarkii* from California, but this result should be treated with caution
318 since only four crayfish were sampled from Hawaii. In the continental USA, the California introduction was
319 followed by later introductions to Oregon in the early 1980s (Larson & Olden, 2011) and to Washington State
320 in the 2000s (Mueller, 2001). Theoretically, we might expect higher genetic variability in California, with a
321 decrease of variability from the place of first introduction (California) northwards (Washington, Oregon) due
322 to secondary bottlenecks and/or founder effects. Our results seem to indicate a more complex pattern of
323 invasion (Fig. 4), in which shared haplotypes between populations in the western US confirm the
324 connectivity among localities (Hap_01, Hap_09, Hap_20 or Hap_27). The development of the crayfish
325 industry in California, where *P. clarkii* has been cultured and traded for many years, seems to have
326 contributed to the dispersal of the crayfish along the West Coast of the US (Comeaux, 1978; Mueller, 2001).
327 Moreover, this scenario might have been favoured by the large number of biological supply companies in
328 this area, and also by the use of live animals for classroom observations, some of which were given to
329 students after school-years to take home and were probably released in the wild later on (Larson & Olden,
330 2008).

331 Regarding the origin of California populations, we were not able to identify the precise geographic
332 origin of this invasion because though low ϕ_{ST} values were found among California and native localities, and
333 these native localities were not close to each other (see Supplementary Material, Table S3). The haplotypes
334 found in Topanga Creek (TOP) suggest that the origin of this invasion came from southeastern Louisiana,
335 but the presence of the Hap_08 (mainly distributed in western Louisiana; and considerably distinct from
336 ancestral haplotype), would contradict this idea. For the other two California localities (VEN and SYZ), we
337 were also unable to unveil their origins accurately because their haplotypes were not shared with the native
338 area; however, their private haplotypes were more evolutionarily related to Hap_04 and Hap_20, which
339 would indicate again a possible origin from southeastern Louisiana. Given that crayfish populations in
340 Topanga Creek were recently established (around 2001, Garcia et al., 2015), this population could come from
341 previous established populations in California, having undergone a possible bottleneck. If so, we could be
342 underestimating the haplotype diversity in the area and more haplotypes would be present in California.
343 This latter surmise is reinforced by the fact that *P. clarkii* has long been considered a pest in southern
344 California (Riegel, 1959), the higher haplotype diversity in population WAV (Oregon) and the presence of
345 Hap_04 at a high frequency only in PIN (Washington State). This suggests that (1) more genetic variability is
346 to be expected in California, acting as an admixed or bridgehead zone because of its anteriority in

347 introduction and the numerous biological supply companies which can move live crayfish, or (2) other
348 distinct introduction events may have occurred in northern states from the native area, which seem unlikely
349 given the great demand for the crayfish industry in California (Comeaux, 1978) and since the northwest US
350 is the native range of another commercially and culturally important crayfish species (i.e., signal crayfish,
351 *Pacifastacus leniusculus*) (Holdich, 1993).

352 Localities from the eastern US showed a different haplotype frequency (Hap_01 was the most common
353 haplotype) from those of the western part of the country, suggesting another independent route of invasion
354 to North Carolina and north of Illinois with subsequent secondary events (Fig. 4). This pattern is congruent
355 with the native area located in the middle of the US, making it easier to move crayfish in two independent
356 directions than from coast to coast, as well as the presence of one of the biggest biological supply companies
357 in North Carolina (US) which was supplying most of the eastern US invaded areas. Although the eastern US
358 is a suitable area for *P. clarkii* (Larson & Olden, 2011), the low haplotype diversity found in eastern localities
359 of the US (from Hd = 0.20 in LEN to Hd = 0.50 in FOR) suggests a low propagule pressure from the native
360 area or from shipments of the biological supply company in North Carolina.

361
362 In Asia, according to the literature (Penn, 1954), 100 specimens of *P. clarkii* were carried from New Orleans to
363 Japan in 1927, of which only 20 specimens arrived alive to a pond near Tokyo (Penn, 1954; Kawai &
364 Kobayashi, 2005); two years later, *P. clarkii* from Japan were translocated to Nanjing, in China (Li et al., 2007).
365 This historical report perfectly matches the genetic pattern (i.e., founder effect and strong bottleneck) found
366 in Japanese and, overall, Chinese populations of *P. clarkii* (Yue et al., 2010; Li et al., 2012; Zhu et al., 2013, this
367 study), in which a smaller batch was introduced to Japan and subsequent invasions came from the Japan
368 population with few founders (Fig. 4). The lack of ectoparasites of the order Branchiobdellida is often
369 attributed to long shipments in poor conditions (Gelder & Williams, 2015; Clavero et al., 2016). Kawai &
370 Kobayashi (2005) found no Branchiobdellida on Japanese specimens of *P. clarkii*, a pattern that could support
371 the hypothesis that all specimens of *P. clarkii* in Japan (and thus, in China) descend from the initial
372 introduction at the end of 1920s. Our results show low haplotype diversity in Japanese and Chinese
373 populations (Hd = 0.48 and 0.35, respectively) with only four haplotypes appearing in the extensive area
374 sampled, only two of them at high frequency (Hap_04 and Hap_28), as similarly found by Li et al. (2012).
375 Apart from Asian populations, Hap_28 was only found in few individuals of the CH population (Mexico)
376 and VEN (California) but not in the native area. Surprisingly, our genetic results seem to contradict previous
377 literature because neither Hap_28 nor Hap_40 appeared in localities sampled around the native locations of
378 New Orleans, Louisiana. The finding of Hap_28 in California and the similar date of both introductions (i.e.,
379 California in 1924 and Japan in 1927) suggests that a route of invasion from California to Japan is more
380 plausible (Fig. 4). Additionally, as Hap_28 was a rare haplotype in our sampling (only 5 of the 988 non-Asian
381 individuals carrying this haplotype), another old introduction into Asia seems unlikely because a different

382 haplotype frequency would be expected. This strong genetic bottleneck did not prevent *P. clarkii* from
383 invading successfully (Estoup et al., 2016) and becoming a pest across Japanese and Chinese territories
384 (Penn, 1954; Kawai, 2017). A similar pattern has been recorded for the parthenogenetic crayfish, *P. virginalis*,
385 in other areas (Feria & Faulkes, 2011). Finally, the presence of the Hap_40 in TOK (Japan) and NAT
386 (northwest Louisiana) led to two possible hypotheses: (1) this haplotype was present in the initial
387 translocated batch but has been lost in subsequent secondary invasions by genetic drift or bottleneck, or (2)
388 one new introduction event has recently occurred from the native area but has not been spread yet (nor been
389 reported). Of both hypotheses, the first one seems more plausible, but we are not able to resolve them.

390
391 Of all invaded areas, the European invasion by *P. clarkii* has perhaps been the best reported, with the first
392 two events of introduction from Louisiana to Spain (Halsburgo-Lorena, 1978) and later into other European
393 countries (i.e., in Spain Gutiérrez-Yurrita, et al., 1999; in France Changeux, 2003 and Laurent, 1997; in Italy
394 Gherardi et al., 1999) (Fig. 4). The invasion routes through European countries and connectivity between
395 European populations are poorly understood, possibly because they are due to multiple and uncontrolled
396 deliberate introductions by private citizens (Clavero, 2016; this likely also occurred with signal crayfish, *P.*
397 *leniusculus*, see Petrusek et al., 2017). In European populations, we found a moderately high overall
398 haplotype diversity ($H_d = 0.58$; i.e. lower than for invasive US populations, but higher than in Asia). The
399 European invasion has probably not been based on as many introduction events as invasive American
400 populations (e.g., California) given the differences in proximity to the native area (a possible cause of the
401 higher haplotype diversity found on the American continent); however, the large number of *P. clarkii*
402 imported to Spain (100 kg, around 6,500 crayfish) probably also included high genetic variability from the
403 native area compared to the Asian introduction. According to our results, a clear decrease in haplotype
404 diversity was found from the initial sites of introduction ($H_d = 0.66$ in rice fields near Seville, and $H_d = 0.72$
405 in Doñana National Park) northwards, excepted for TOS in Italy ($H_d = 0.72$), which could be explained by
406 intensive farming activities on Lake Massaciuccoli (Gherardi et al., 1999) or a second introduction.

407
408 The most surprising result was the finding of two independent genetic groups in Europe. The Hap_04 was
409 widely distributed over the Iberian Peninsula, South France and Italy, while the Hap_11 predominated in
410 Northern France and Italy, Belgium, the Netherlands and United Kingdom, but was not found in the Iberian
411 Peninsula. Two possible scenarios could explain this result: (1) we did not capture all haplotypes from the
412 first introduction in southern Europe, and northern populations have undergone a strong bottleneck; or (2)
413 another unreported introduction from outside Europe has occurred, independently from those reported
414 from southern Spain (Fig. 4). The first scenario is unlikely, due to the extensive sampling effort on both the
415 Iberian Peninsula and the native area. In such a scenario, the Hap_11 should have appeared in the Iberian
416 Peninsula because of other high frequencies in North Europe. Moreover, Almerão et al. (2018) found nine

417 haplotypes in Central France, four of which seem to match with our database but not Hap_11. On the other
418 hand, unreported introductions of *P. clarkii* could be a consequence of the sales in pet shops which are
419 common and one of the primary introduction pathways in Central Europe (Chucholl, 2015; Faulkes, 2015).
420 These results, however, also support previous historical reports (Laurent, 1990; Holdich, 2002) suggesting
421 how live *P. clarkii* may have been brought from Kenya to Europe in the 1970s. Both hypotheses could explain
422 the presence of this haplotype across the northern European range of *P. clarkii*. To clarify our results, samples
423 from pet shops or African samples of *P. clarkii* should be obtained in order to resolve the likely second
424 invasion route. The second scenario therefore seems the most plausible (as Barbaresi et al., 2007, also
425 suggested), with a plausible introduction from Kenya to Central Europe.

426
427 National and international translocations have occurred within Europe (Fig. 4). On the one hand, we found
428 Hap_04 and Hap_05 to be highly frequent all over the Iberian Peninsula to South France, which perfectly
429 matches with the literature signaling where live specimens having been translocated from South Spain
430 (Laurent, 1997). On the other hand, the presence of the Hap_06 at higher frequencies in southern Portugal
431 and dated reports of introduction events across Portugal seem to confirm the spread of *P. clarkii* from south
432 (near the first introduction site in 1973; Cruz & Rebelo, 2007) to north Portugal (Gutiérrez-Yurrita et al.,
433 1999). In addition, Hap_06 was also found in MAD (Spain) and LAZ (Italy), suggesting a connection among
434 these invaded areas as well as POR in Portugal and LEZ in the Ebro Basin, Northern Spain (Fig. 4). Another
435 possible connection was between TOS in Italy and southern Spain, with most haplotypes shared, suggesting
436 another possible invasion route. Continuous exchanges and secondary translocations of *P. clarkii* through
437 invaded areas have produced a very complex invasion process, which could accelerate the invasiveness of
438 this kind of species (Wagner et al., 2017).

439
440 Our results provide a clear example of how different features of introduction events and invasion processes
441 (e.g. genetic admixture, propagule pressure or secondary introductions) can generate contrasting genetic
442 diversity patterns across non-native populations of a global invader (Roman & Darling, 2007). For example,
443 Asian populations of *P. clarkii* underwent a strong bottleneck as a consequence of the introduction of few
444 individuals in a single introduction event, which arguably originated from an already introduced population
445 (probably in western US) that might have already gone through previous bottlenecks. Genetic diversity was
446 notably higher in the *P. clarkii* populations in western US, probably due to the existence of numerous
447 introduction events (e.g. facilitated by vicinity to the native range and the development of biological supply
448 companies), involving large batches of individuals with high genetic admixture. The European case is
449 apparently intermediate, with numerous individuals imported from an admixed native range to SW Spain,
450 from which the species expanded across the continent through multiple secondary introductions, involving
451 a clear loss in haplotype diversity. However, higher genetic variabilities were found in European (Petrušek

452 et al., 2017) and Japanese populations of *P. leniusculus* (Usio et al., 2016) in comparison to that reported by us
453 for *P. clarkii*, arguably due to the combination of several introduction events involving large batches of
454 individuals and coming from a variety of origins in the US, including native and non-native populations. A
455 striking pattern deriving from our results is that the invasiveness of *P. clarkii* does not seem to depend, at
456 least in the short- and mid-term, on the genetic diversity of introduced populations. Although genetic
457 diversity can fuel invasiveness by allowing the efficient adaptation of introduced populations to spatial and
458 temporal variability in the recipient ecosystems, the relationship between those two features is obscure
459 (Estoup et al., 2016). There is growing evidence that the loss of genetic diversity in introduced populations
460 can be compensated through epigenetic processes (Estoup et al., 2016). The most extreme example of high
461 invasiveness with low genetic variability is the clonal species *P. virginalis* (Feria & Faulkes, 2011), which is
462 able to thrive in a wide variability of environmental conditions (Andriantsoa et al., 2019).

463
464 Apart from informing about invasion routes, our results might also be relevant for new approaches for the
465 detection and surveillance of invasive species. Environmental DNA (eDNA) is a rapidly emerging
466 monitoring tool for freshwater invasive species based on the persistence of DNA fragments in the
467 environment (Ficetola et al., 2008; Mauvisseau et al., 2018). Large-scale phylogeographic studies provide
468 accurate datasets for improving invasive species detection protocols based on eDNA (Ficetola et al., 2008;
469 Larson et al., 2017). Admixture in both native and invasive ranges, as well as the bridgehead invasive effect,
470 has led to large intraspecific genetic variability within and among invaded areas, which may reduce the
471 efficacy of eDNA protocols (Wilcox et al., 2015). In fact, the spatial gradients in genetic variability and the
472 presence of different genetic clusters in Europe reported here, probably led to the failure of eDNA probes in
473 detecting French populations of *P. clarkii* (Tréguier et al., 2014; Mauvisseau et al., 2018), which had worked
474 well with the less variable Chinese populations (Cai et al., 2017). Our study may thus be useful for the
475 development of better site-specific eDNA-based protocols to detect *P. clarkii* (Manfrin et al., 2019).

476 477 *Conclusions*

478 Our results illustrate extensive admixture of *P. clarkii* in its native area, report two independent invasion
479 routes in the US (i.e., westwards and eastwards), and support the historical reports of a single introduction
480 event into Asia involving few individuals. They also suggest that Europe may have received *P. clarkii*
481 through more introduction routes than the frequently reported imports into Spain. To find other likely
482 introduction routes, more effort should be put on sampling in previously unstudied sites (e.g., Texas, pet
483 shops, biological supply trade and/or Southern Hemisphere countries where other introduced populations
484 might act as sources of invasion, for example, African or South American populations).

485

486 We have traced the complex scheme of invasion of *P. clarkii* (Fig. 4), with a key role for human-mediated
487 dispersal. The economic value of *P. clarkii* and the ease with which it is transported have favoured the spread
488 of the species worldwide (largely for aquaculture, the aquarium trade and other forms of human
489 exploitation as food) as the consequence of multiple subsequent introduction events. Genetic admixture,
490 invasive bridgehead effects, extensive genetic variation in the native area and high propagule pressure are
491 apparent drivers of genetic variability across its broad geographic distribution. Such extensive genetic
492 variability in invaded areas should be taken into account to improve management measures based on
493 mtDNA for environmental detection of this invasive species. Overall, invasive species, and invasive crayfish
494 in particular, continue to be artificially introduced into more countries through the aquarium trade (e.g. fish
495 species, Strecker et al., 2011; crayfish species, Patoka et al., 2014 and particularly *P. virginalis*, Faulkes 2015).
496 The example of the successful worldwide invasion of *P. clarkii* highlights the high spread potential of
497 intentionally introduced freshwater species, especially those species also involved in aquaculture (Naylor et
498 al., 2001). Once a species has been introduced in a new territory, management strategies aimed at reducing
499 the spread and impacts of invasive species should focus on avoiding secondary introductions and would
500 benefit from the early detection of potential invasion hubs.

501

502 **iv. Acknowledgements**

503 We thank R. López, M. A. Bravo, and Á. Vidal for field and lab assistance, as well as the many colleagues
504 who kindly provided samples from Spain [J. Molero, J. L. Grau, E. Carnerero, A. Pradilla (CCEDCV - EL
505 PALMAR), J. Rucio & M. Alcántara (Aragón Government), L.M. Álvarez (Asturias Government), Parque
506 Martioda & J. Carreras (Alava Provincial Council), J. L. Moreno, N. Franch (Parc Natural del Delta de l'Ebre),
507 Centro de Acuicultura Vegas del Guadiana (Extremadura Government), L. Valls, F. J. Galindo (Andalusia
508 Government), F. Unzu & L. Lopo (La Rioja Government), J. Bosch, Presi, Kike, F. J. Oliva, I. Vedia, A. Mestre
509 and F. Mesquita], Europe: [F. Ribeiro, A. F. Filipe, I. Roessink, D. Ghia, E. Tricarico, R. Cammaerts, P. Fossat,
510 D. Nicolas, E. Petit, O. Adrien and J. Cucherousset] and the United States [R. Fagundo & B. Williams (North
511 Carolina Museum of Natural Sciences), T. García, J. Olden & L. Kuehne, E. R. Larson, C. Gabor and R.
512 Keller]. We also thank both Laboratorio de Ecología Molecular (LEM-EBD) and Laboratorio de SIG y
513 Teledetección (LAST-EBD) at Estación Biológica de Doñana (CSIC) for providing logistical support. In
514 addition, we thank Eric R. Larson and another anonymous reviewer who improved the final version of this
515 manuscript. F.J.O. was supported by an Andalusia Government grant. This study was funded by the
516 Andalusia Government (RNM-936) and the bilateral CNRS-CSIC PICS programs (PICS07360 and
517 PIC2015FR4).

518

519 **Conflicts of Interest**

520 The authors declare no conflict of interest.

521

522 **v. References**

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785 **vi. Tables**

786 Table 1. Genetic diversity parameters based on the COI gene for each *Procambarus clarkii* locality. Note that
787 localities are grouped in biogeographical zones (see Methods). Sequences retrieved from GenBank are

788 shown in italics (see references in Materials and Methods section). *: 0.05 > p > 0.01; **: 0.01 > p > 0.001; ***: p
 789 < 0.001

| Country | Locality | Code | Lon | Lat | N | <i>h</i> | Hd | π | R2 | Tajima | Fs |
|----------------------------|---------------------|-------------|---------------|-----------------|-----------|----------|--------------|----------------|--------|--------|----------|
| NATIVE RANGE | | | | | 179 | 39 | 0.902 | 0.00549 | | | |
| East Louisiana | | | | | | | | | | | |
| | Poison | LA2 | 30.220 | -91.614 | 20 | 5 | 0.795 | 0.00349 | 0.172 | 0.810 | 0.854 |
| | Haha Bay | LA4 | 30.147 | -91.628 | 20 | 8 | 0.775 | 0.00395 | 0.104 | -0.519 | -1.573 |
| | Baton Rouge | BAT | 30.370 | -91.189 | 4 | 3 | 0.833 | 0.00302 | 0.265 | 1.090 | 0.006 |
| | Morgan City | MOR | 29.767 | -91.127 | 10 | 6 | 0.867 | 0.00428 | 0.157 | 0.215 | -1.164 |
| | Pierre Part | PIE | 29.950 | -91.283 | 10 | 8 | 0.956 | 0.00450 | 0.135 | -0.144 | -3.882** |
| | Des Allemands | DES | 29.798 | -90.505 | 4 | 4 | 1.000 | 0.00630 | 0.114* | 0.039 | -0.884 |
| | Jean Lafitte | JEA | 29.732 | -90.075 | 9 | 4 | 0.694 | 0.00265 | 0.172 | -0.526 | -0.061 |
| | <i>New Orleans</i> | <i>L Af</i> | <i>29.950</i> | <i>-90.083</i> | <i>1</i> | <i>1</i> | | | | | |
| West Louisiana | | | | | | | | | | | |
| | Abbeville | ABB | 29.911 | -92.200 | 4 | 3 | 0.833 | 0.00247 | 0.276 | -0.754 | -0.288 |
| | Alexandria | ALE | 31.097 | -92.493 | 4 | 3 | 0.833 | 0.00877 | 0.327 | -0.222 | 1.606 |
| | Woodworth | WOO | 31.186 | -92.467 | 4 | 4 | 1.000 | 0.00356 | 0.223 | -0.065 | -1.741* |
| | Natchitoches | NAT | 31.740 | -93.077 | 17 | 6 | 0.765 | 0.00816 | 0.199 | 1.495 | 2.133 |
| | <i>Kaplan</i> | <i>L At</i> | <i>29.991</i> | <i>-92.260</i> | <i>5</i> | <i>2</i> | <i>0.600</i> | <i>0.00197</i> | 0.300 | 1.459 | 1.688 |
| | <i>Calcasieu L.</i> | <i>LO</i> | <i>29.870</i> | <i>-93.260</i> | <i>10</i> | <i>4</i> | <i>0.533</i> | <i>0.00643</i> | 0.143 | -0.352 | 2.256 |
| Upstream Mississippi River | | | | | | | | | | | |
| | Monroe | MON | 32.497 | -91.669 | 5 | 4 | 0.900 | 0.00724 | 0.218 | 0.132 | 0.286 |
| | Memphis | MEM | 35.366 | -90.033 | 5 | 2 | 0.400 | 0.00066 | 0.400 | -0.817 | 0.090 |
| | <i>Horseshoe</i> | <i>ILL</i> | <i>37.138</i> | <i>-89.343</i> | <i>1</i> | <i>1</i> | | | | | |
| Texas | | | | | | | | | | | |
| | Comal | COM | 29.711 | -98.134 | 18 | 6 | 0.686 | 0.00170 | 0.103 | -0.917 | -2.350* |
| | San Marcos | SMA | 29.882 | -97.934 | 14 | 3 | 0.560 | 0.00103 | 0.157 | -0.011 | -0.072 |
| Mexico | | | | | | | | | | | |
| | <i>Sabinas</i> | <i>NL</i> | <i>26.483</i> | <i>-100.221</i> | <i>4</i> | <i>1</i> | | | | | |
| | <i>Hidalgo</i> | | | | | | | | | | |
| | <i>Río Jiménez</i> | CON | 29.154 | -100.764 | 5 | 2 | 0.400 | 0.00066 | 0.400 | -0.817 | 0.090 |
| | <i>Río Sabinas</i> | COC | 27.969 | -101.582 | 5 | 1 | | | | | |

WEST AMERICAS

Non-native US

| | | | | | | | | | | |
|------------|-----|--------|----------|----|---|-------|---------|-------|----------|-------|
| Santa Ynez | SYZ | 34.557 | -119.881 | 10 | 3 | 0.378 | 0.00190 | 0.187 | -1.388'' | 0.762 |
| Topanga | TOP | 34.064 | -118.587 | 20 | 5 | 0.679 | 0.00859 | 0.194 | 1.541 | 4.185 |
| Ventura | VEN | 34.345 | -119.299 | 4 | 2 | 0.667 | 0.00439 | 0.333 | 2.080 | 2.719 |
| Pine | PIN | 47.587 | -122.044 | 21 | 3 | 0.581 | 0.00183 | 0.186 | 0.883 | 1.537 |
| Waverly | WAV | 44.640 | -123.069 | 20 | 7 | 0.832 | 0.00501 | 0.121 | -0.356 | 0.057 |
| Waiiau | HW | 19.713 | -155.149 | 3 | 1 | | | | | |

Non-native Mexico

| | | | | | | | | | | |
|------------------|-------------|---------------|-----------------|----------|----------|--------------|----------------|--------------|--------------|--------------|
| | | | | 13 | 4 | 0.782 | 0.00557 | | | |
| <i>Teopisca</i> | <i>CHIS</i> | <i>16.554</i> | <i>-92.476</i> | <i>5</i> | <i>1</i> | | | | | |
| <i>El Arenal</i> | <i>DU</i> | <i>24.043</i> | <i>-104.428</i> | <i>5</i> | <i>2</i> | <i>0.600</i> | <i>0.00493</i> | <i>0.300</i> | <i>1.686</i> | <i>3.526</i> |
| <i>Las Varas</i> | <i>CHt</i> | <i>29.797</i> | <i>-106.693</i> | <i>3</i> | <i>1</i> | | | | | |

Costa Rica

| | | | | | | | | | | |
|------------------|-----------|--------------|----------------|----------|----------|--------------|----------------|--------------|---------------|--------------|
| <i>Cachí Dam</i> | <i>CR</i> | <i>9.825</i> | <i>-83.821</i> | <i>4</i> | <i>2</i> | <i>0.500</i> | <i>0.00082</i> | <i>0.433</i> | <i>-0.612</i> | <i>0.172</i> |
|------------------|-----------|--------------|----------------|----------|----------|--------------|----------------|--------------|---------------|--------------|

EAST USA

| | | | | | | | | | | |
|-------------|-----|--------|---------|----|---|-------|---------|-------|---------|---------|
| Pee Dee | FOR | 36.150 | -80.291 | 4 | 2 | 0.500 | 0.00082 | 0.433 | -0.612 | 0.172 |
| Pamplico | LEN | 35.244 | -77.559 | 10 | 2 | 0.200 | 0.00033 | 0.300 | -1.112 | -0.339 |
| Albemarle | PER | 36.268 | -76.378 | 21 | 3 | 0.338 | 0.00100 | 0.111 | -0.707 | 0.204 |
| North Shore | CHI | 42.032 | -87.710 | 20 | 5 | 0.442 | 0.00113 | 0.105 | -1.888* | -2.091* |

EUROPE

Spain

| | | | | | | | | | | |
|------------|-----|--------|--------|-----|----|-------|---------|-------|---------|--------|
| | | | | 355 | 13 | 0.469 | 0.00239 | | | |
| Balboa | EXT | 38.883 | -6.871 | 20 | 2 | 0.395 | 0.00065 | 0.197 | 0.723 | 0.976 |
| Manecorro | MAN | 37.124 | -6.489 | 20 | 5 | 0.716 | 0.00443 | 0.143 | 0.214 | 1.571 |
| Cantaritas | AR4 | 37.046 | -6.213 | 20 | 5 | 0.663 | 0.00447 | 0.140 | 0.243 | 1.596 |
| Colomera | GRA | 37.384 | -3.719 | 20 | 3 | 0.468 | 0.00300 | 0.154 | 0.255 | 2.904 |
| Hueznar | HUE | 37.933 | -5.697 | 20 | 2 | 0.100 | 0.00066 | 0.218 | -1.868* | 0.998 |
| Arreo | ALA | 42.778 | -2.991 | 20 | 2 | 0.479 | 0.00315 | 0.239 | 2.024 | 5.159 |
| Elorz | EL | 42.798 | -1.667 | 19 | 4 | 0.585 | 0.00275 | 0.105 | -0.921 | 1.200 |
| Expo | EXP | 41.671 | -0.909 | 13 | 3 | 0.615 | 0.00405 | 0.200 | 1.009 | 3.086 |
| Gijón | GIJ | 43.536 | -5.640 | 15 | 3 | 0.257 | 0.00085 | 0.121 | -1.317 | -0.379 |
| Jiloca | JIL | 40.544 | -1.293 | 15 | 2 | 0.419 | 0.00207 | 0.210 | 1.078 | 3.248 |
| Leza | LEZ | 42.441 | -2.311 | 20 | 2 | 0.268 | 0.00177 | 0.134 | -0.138 | 3.143 |
| Almenara | ALM | 39.761 | -0.183 | 20 | 1 | | | | | |

| | | | | | | | | | | |
|----------------------|------------|---------------|--------------|----------|----------|-------|---------|-------|----------|----------|
| Brugent | BRU | 42.006 | 2.607 | 20 | 2 | 0.337 | 0.00222 | 0.168 | 0.565 | 3.843 |
| Ecomuseu | ECO | 40.724 | 0.722 | 20 | 3 | 0.195 | 0.00099 | 0.159 | -2.056** | 0.136 |
| Alpedrete | MAD | 40.667 | -4.016 | 20 | 2 | 0.442 | 0.00073 | 0.221 | 1.026 | 1.169 |
| Júcar | ALB | 39.148 | -1.809 | 11 | 3 | 0.618 | 0.00114 | 0.192 | 0.036 | -0.113 |
| Mundo | MUN | 38.458 | -1.761 | 20 | 1 | | | | | |
| Sa Pobra | SAP | 39.791 | 3.063 | 5 | 2 | 0.400 | 0.00263 | 0.400 | -1.094'' | 2.202 |
| Soller | SOL | 39.787 | 2.794 | 5 | 3 | 0.700 | 0.00428 | 0.205 | 0.562 | 1.090 |
| Carucedo | CAR | 42.488 | -6.784 | 5 | 2 | 0.400 | 0.00132 | 0.400 | -0.973 | 1.040 |
| Chozas | CHO | 42.518 | -5.714 | 4 | 2 | 0.500 | 0.00247 | 0.433 | -0.754 | 1.716 |
| Valparaiso | VAL | 41.995 | -6.288 | 2 | 2 | 1.000 | 0.01151 | 0.500 | 0.000 | 1.946 |
| Pisuerga | VLB | 41.801 | -4.588 | 21 | 3 | 0.343 | 0.00172 | 0.105 | -0.742 | 1.384 |
| Portugal | | | | 114 | 4 | 0.399 | 0.00118 | | | |
| Aboboda | ABO | 38.736 | -9.319 | 15 | 2 | 0.343 | 0.00169 | 0.171 | 0.342 | 2.710 |
| Lousal | LOU | 38.027 | -8.431 | 20 | 2 | 0.479 | 0.00079 | 0.239 | 1.262 | 1.311 |
| Alpiarça | POR | 39.245 | -8.594 | 20 | 4 | 0.642 | 0.00291 | 0.123 | -0.727 | 1.429 |
| R. de Monsaraz | REG | 38.478 | -7.522 | 20 | 2 | 0.505 | 0.00083 | 0.253 | 1.430 | 1.409 |
| Requeixo | REQ | 40.592 | -8.526 | 20 | 2 | 0.100 | 0.00016 | 0.218 | -1.164 | -0.879'' |
| Vila-Rica | VILA | 41.229 | -7.096 | 19 | 1 | | | | | |
| France | | | | 84 | 5 | 0.561 | 0.00218 | | | |
| Marais Bruges | BOR | 44.903 | 0.596 | 20 | 1 | | | | | |
| Briere | BRI | 47.343 | -2.246 | 20 | 1 | | | | | |
| Tour du Valat | CAM | 43.508 | 4.668 | 20 | 2 | 0.526 | 0.00346 | 0.263 | 2.511 | 5.567 |
| Lamartine | TOU | 43.506 | 1.341 | 21 | 2 | 0.095 | 0.00016 | 0.213 | -1.164 | -0.919'' |
| <i>Rochechevreux</i> | <i>FR1</i> | <i>45.467</i> | <i>1.217</i> | <i>1</i> | <i>1</i> | | | | | |
| <i>Rochechevreux</i> | <i>FR2</i> | <i>46.467</i> | <i>1.217</i> | <i>1</i> | <i>1</i> | | | | | |
| <i>Givrezac</i> | <i>FR3</i> | <i>45.403</i> | <i>0.216</i> | <i>1</i> | <i>1</i> | | | | | |
| Italy | | | | 60 | 6 | 0.731 | 0.00266 | | | |
| Bernate | BER | 45.485 | 8.795 | 20 | 2 | 0.479 | 0.00079 | 0.239 | 1.262 | 1.311 |
| Monterotondo | LAZ | 42.052 | 12.547 | 20 | 2 | 0.505 | 0.00083 | 0.253 | 1.430 | 1.409 |
| Fucecchio | TOS | 43.810 | 10.794 | 20 | 5 | 0.716 | 0.00448 | 0.150 | 0.257 | 1.608 |

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|----------------|-------------------|-----|--------|---------|-----|---|-------|---------|-------|----------|-------|--|
| Holland | Hardinxveld-Giess | HOL | 51.817 | 4.836 | 20 | 1 | | | | | | |
| Belgium | | | | | 19 | 2 | 0.409 | 0.00067 | | | | |
| | Bioul | BIO | 50.339 | 4.809 | 12 | 1 | | | | | | |
| | Ecaussinnes | ECA | 50.576 | 4.139 | 7 | 2 | 0.476 | 0.00078 | 0.238 | 0.559 | 0.589 | |
| United Kingdom | Hampstead-Heath | LON | 51.561 | -0.162 | 20 | 1 | | | | | | |
| ASIA | | | | | | | | | | | | |
| Japan | | | | | 122 | 4 | 0.476 | 0.00237 | | | | |
| | Wakamatsu | FUK | 33.911 | 130.782 | 20 | 1 | | | | | | |
| | Hourai | HOK | 42.939 | 143.224 | 20 | 1 | | | | | | |
| | Waga | IWA | 39.436 | 140.776 | 9 | 2 | 0.556 | 0.00274 | 0.278 | 1.948 | 3.276 | |
| | Ohfuna | KAN | 35.353 | 139.529 | 20 | 2 | 0.521 | 0.00257 | 0.261 | 2.266 | 4.362 | |
| | Rakusho | OKA | 34.714 | 133.933 | 20 | 3 | 0.279 | 0.00106 | 0.108 | -0.626 | 0.286 | |
| | Ohtsu | SHI | 35.013 | 135.865 | 10 | 2 | 0.533 | 0.00263 | 0.267 | 1.831 | 3.338 | |
| | Kanda | TOK | 35.685 | 139.774 | 13 | 3 | 0.410 | 0.00240 | 0.164 | -1.335'' | 1.625 | |
| | Saitama | SA | 35.850 | 139.650 | 10 | 2 | 0.533 | 0.00263 | 0.267 | 1.831 | 3.338 | |
| China | | | | | 293 | 2 | 0.350 | 0.00173 | | | | |
| | Shanghai | SH | 31.030 | 121.230 | 8 | 2 | 0.571 | 0.00282 | 0.286 | 1.982 | 3.149 | |
| | Jiaxing | JX | 30.750 | 120.770 | 10 | 2 | 0.356 | 0.00175 | 0.178 | 0.021 | 2.334 | |
| | Binhu. Wuxi | WXB | 31.520 | 120.280 | 7 | 2 | 0.571 | 0.00282 | 0.286 | 1.811 | 2.920 | |
| | Nantong | NT | 32.020 | 120.870 | 7 | 2 | 0.286 | 0.00141 | 0.350 | -1.358'' | 1.514 | |
| | Xiaba village | XB | 32.200 | 118.870 | 8 | 2 | 0.571 | 0.00282 | 0.286 | 1.981 | 3.149 | |
| | Wuxi | WX | 31.570 | 120.300 | 8 | 2 | 0.536 | 0.00264 | 0.268 | 1.601 | 2.988 | |
| | Wangjiang | WJ | 30.120 | 116.700 | 8 | 1 | | | | | | |
| | Maanshan | MAS | 31.550 | 118.500 | 10 | 2 | 0.356 | 0.00175 | 0.178 | 0.021 | 2.338 | |
| | Chaohu | CH | 31.620 | 117.870 | 8 | 2 | 0.429 | 0.00211 | 0.214 | 0.458 | 2.469 | |
| | Hefei | HF | 31.820 | 117.230 | 7 | 2 | 0.286 | 0.00141 | 0.350 | -1.358'' | 1.514 | |
| | Dingyuan | DY | 32.280 | 117.830 | 10 | 2 | 0.556 | 0.00274 | 0.278 | 2.057 | 3.451 | |
| | Nanbei Port | NBP | 29.720 | 116.170 | 8 | 2 | 0.250 | 0.00123 | 0.331 | -1.448'' | 1.415 | |
| | Zhongxian | ZX | 30.280 | 108.030 | 10 | 2 | 0.533 | 0.00263 | 0.267 | 1.831 | 3.338 | |

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|---------------------------|-------------|--------|---------|----|---|-------|---------|-------|-------|-------|
| <i>Jiayang</i> | <i>JY</i> | 30.380 | 104.550 | 10 | 2 | 0.467 | 0.00230 | 0.233 | 1.152 | 2.985 |
| <i>Chongqing</i> | <i>CQS</i> | 29.550 | 106.530 | 6 | 1 | | | | | |
| <i>Ningbo</i> | <i>NB</i> | 29.880 | 121.550 | 7 | 1 | | | | | |
| <i>Xuyi-culture</i> | <i>XYC</i> | 33.000 | 118.500 | 7 | 1 | | | | | |
| <i>Xuyi-wild</i> | <i>XYW</i> | 33.030 | 118.420 | 10 | 1 | | | | | |
| <i>Xiaguan district</i> | <i>XG</i> | 32.080 | 118.750 | 10 | 1 | | | | | |
| <i>Baguazhou township</i> | <i>BGT</i> | 32.170 | 118.820 | 8 | 1 | | | | | |
| <i>Guangfengwei</i> | <i>CJR</i> | 30.120 | 116.870 | 8 | 1 | | | | | |
| <i>Sanli township</i> | <i>SLT</i> | 29.750 | 116.220 | 8 | 1 | | | | | |
| <i>Poyang lake</i> | <i>PYL</i> | 28.870 | 116.430 | 10 | 1 | | | | | |
| <i>Youlan.</i> | <i>NCYL</i> | 28.520 | 116.120 | 6 | 1 | | | | | |
| <i>Nanchang</i> | | | | | | | | | | |
| <i>Nanhu lake</i> | <i>NHL</i> | 30.020 | 114.030 | 8 | 1 | | | | | |
| <i>Yuni Lake</i> | <i>YNL</i> | 30.000 | 112.200 | 7 | 1 | | | | | |
| <i>Xiantao</i> | <i>XT</i> | 30.300 | 113.400 | 8 | 1 | | | | | |
| <i>Qianjiang</i> | <i>QJ</i> | 30.400 | 112.600 | 10 | 1 | | | | | |
| <i>Liangzi lake</i> | <i>LZL</i> | 30.000 | 114.000 | 10 | 1 | | | | | |
| <i>Honghu lake</i> | <i>HLL</i> | 29.700 | 113.400 | 8 | 1 | | | | | |
| <i>Changhu lake</i> | <i>CHL</i> | 30.300 | 112.100 | 6 | 1 | | | | | |
| <i>Yuanjiang</i> | <i>YJ</i> | 28.850 | 112.370 | 10 | 1 | | | | | |
| <i>Ningxiang</i> | <i>NX</i> | 28.280 | 112.550 | 8 | 1 | | | | | |
| <i>Dongting lake</i> | <i>DTL</i> | 29.300 | 113.020 | 10 | 1 | | | | | |
| <i>Dongting Lakeside</i> | <i>DTLs</i> | 29.350 | 113.130 | 9 | 1 | | | | | |

790 Number of sequences (N), number of haplotypes (*h*), haplotype diversity (Hd), nucleotide diversity (π).

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797 **Table 2.** Analysis of Molecular Variance (AMOVA) within the native area of *Procambarus clarkii*, giving corresponding
 798 values for F_{CT} (difference among groups), F_{SC} (differences among localities within groups), and F_{ST} (differences among all
 799 localities). Five groups were considered: the native localities in Mexico, Texas, east Louisiana, west Louisiana and
 800 upstream Mississippi River.

| Source of Variation | d.f. | Sum of squares | Variance components | Percentage of variation |
|--|------|----------------|---------------------|---|
| México - Texas – E. Louisiana – W. Louisiana – Upstream Mississippi River | | | | |
| Among groups | 4 | 77.228 | 0.53574 | 29.04 ($F_{CT} = 0.290$, $p = 0.000$) |
| Among localities within groups | 15 | 39.135 | 0.17294 | 9.38 ($F_{SC} = 0.132$, $p = 0.002$) |
| Within localities | 157 | 178.350 | 1.13598 | 61.58 ($F_{ST} = 0.384$, $p = 0.000$) |
| Total | 176 | 294.712 | 1.84466 | |

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802 **Table 3.** Analysis of Molecular Variance (AMOVA) among the native and introduced localities of *Procambarus clarkii*
 803 worldwide and among the five zones (native range, west Americas, east USA, Europe and Asia), listing the
 804 corresponding values for F_{CT} (difference among groups), F_{SC} (differences among localities within groups), and F_{ST}
 805 (differences among all localities)

| Source of Variation | d.f. | Sum of squares | Variance components | Percentage of variation |
|---|------|----------------|---------------------|---|
| Native Area – Invaded Area | | | | |
| Among groups | 1 | 69.842 | 0.19760 | 14.35 ($F_{CT} = 0.143$, $p = 0.000$) |
| Among localities within groups | 114 | 932.781 | 0.63021 | 45.76 ($F_{SC} = 0.534$, $p = 0.000$) |
| Within localities | 1293 | 710.207 | 0.54927 | 39.89 ($F_{ST} = 0.601$, $p = 0.000$) |
| Total | 1408 | 1712.829 | 1.37708 | |
| Native Area – West Americas – East USA – Europe – Asia | | | | |
| Among groups | 4 | 491.791 | 0.49942 | 36.04 ($F_{CT} = 0.360$, $p = 0.000$) |
| Among localities within groups | 111 | 510.831 | 0.33716 | 24.33 ($F_{SC} = 0.380$, $p = 0.000$) |
| Within localities | 1293 | 710.207 | 0.54927 | 39.63 ($F_{ST} = 0.604$, $p = 0.000$) |
| Total | 1408 | 1712.829 | 1.38585 | |

806 **Table 4.** Analysis of Molecular Variance (AMOVA) within Europe between northern and southern distribution of
 807 *Procambarus clarkii*, listing the corresponding values for F_{CT} (difference among groups), F_{SC} (differences among localities
 808 within groups), and F_{ST} (differences among all localities).

| Source of Variation | d.f. | Sum of squares | Variance components | Percentage of variation |
|---|------|----------------|---------------------|---|
| North (UK, HOL, BIO, ECA, BRI) – South European distribution (rest of European localities) | | | | |
| Among groups | 1 | 59.646 | 0.40773 | 40.74 ($F_{CT} = 0.407$, $p = 0.000$) |
| Among localities within groups | 37 | 101.955 | 0.13481 | 13.47 ($F_{SC} = 0.227$, $p = 0.000$) |
| Within localities | 628 | 287.734 | 0.45817 | 45.78 ($F_{ST} = 0.542$, $p = 0.000$) |
| Total | 666 | 449.334 | 1.00071 | |

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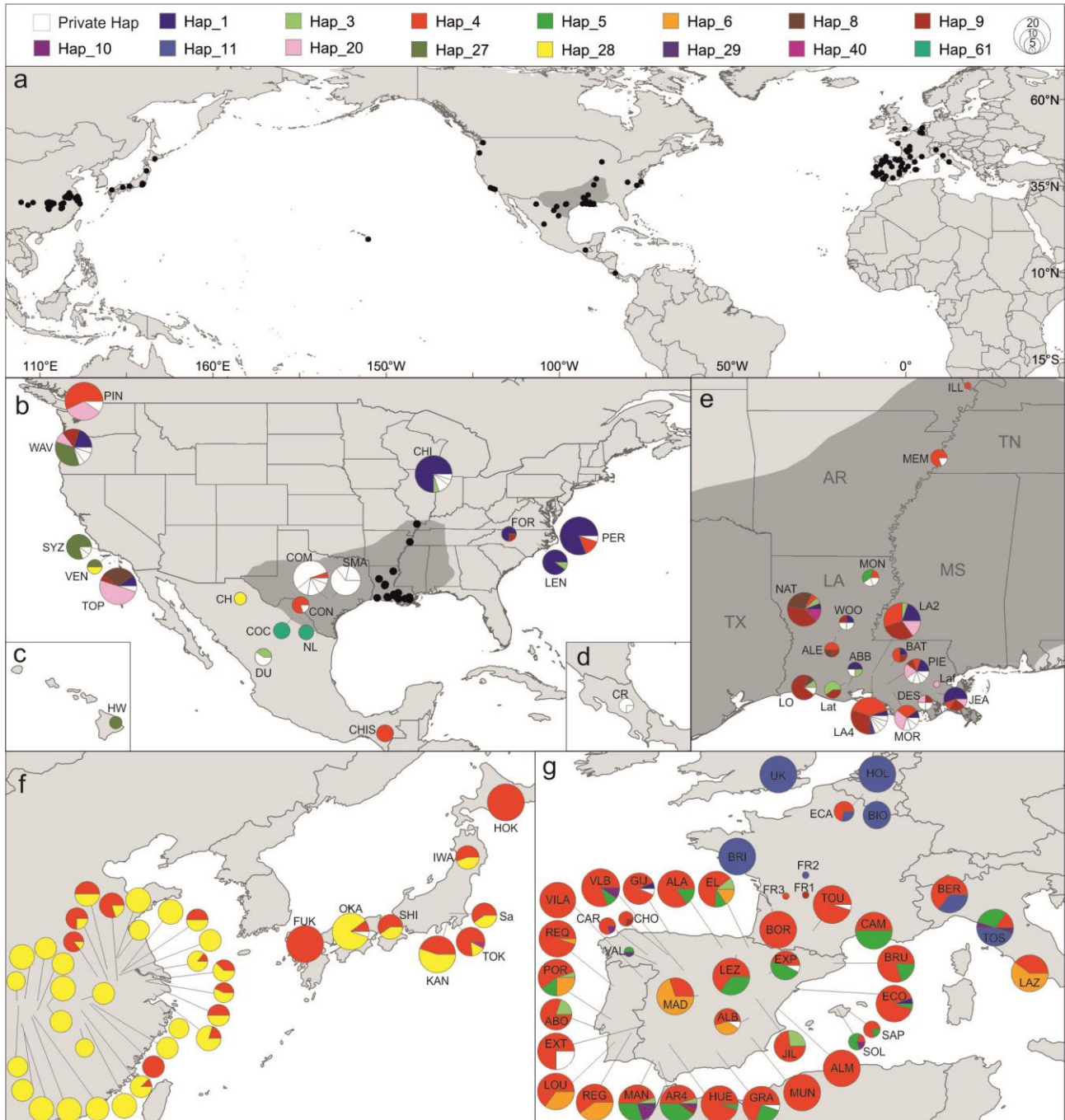
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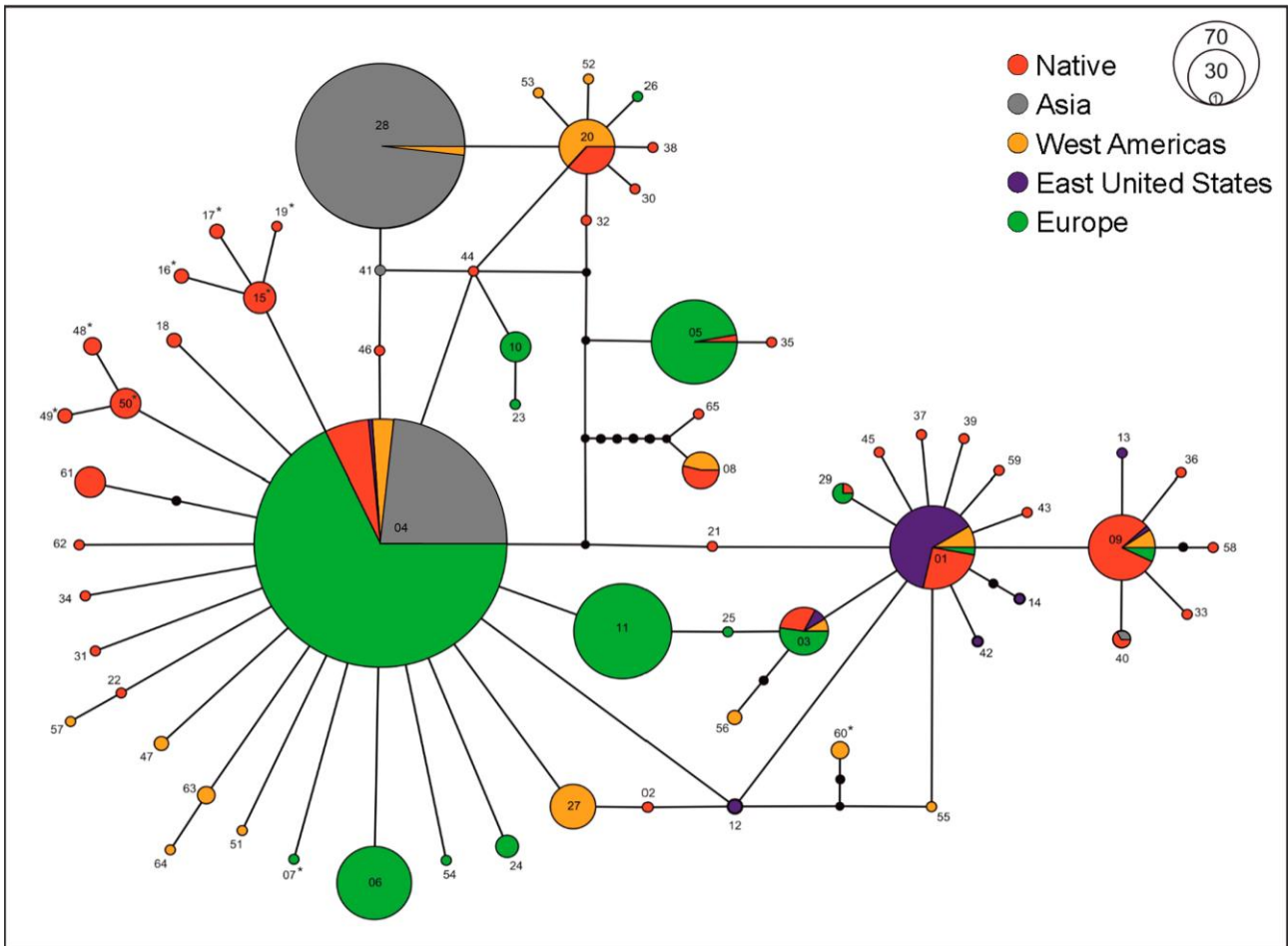
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827 **Figure 1.** Haplotype frequencies of *Procambarus clarkii* in the 122 localities distributed worldwide. The size of pie charts is
 828 proportional to the sample size. Haplotypes restricted to one sampling locality (i.e., private haplotypes) are coloured in
 829 white within pie charts, while haplotypes shared between localities are shaded using colours. Black spots show each one
 830 of the 122 localities used, and dark grey areas represent the native range of *Procambarus clarkii*. a) Global map; b) United
 831 States and Mexico; c) Hawaiian Islands; d) Costa Rica; e) close-up of Louisiana (US) within its native range; f) East Asia
 832 (China and Japan) and; g) Europe.



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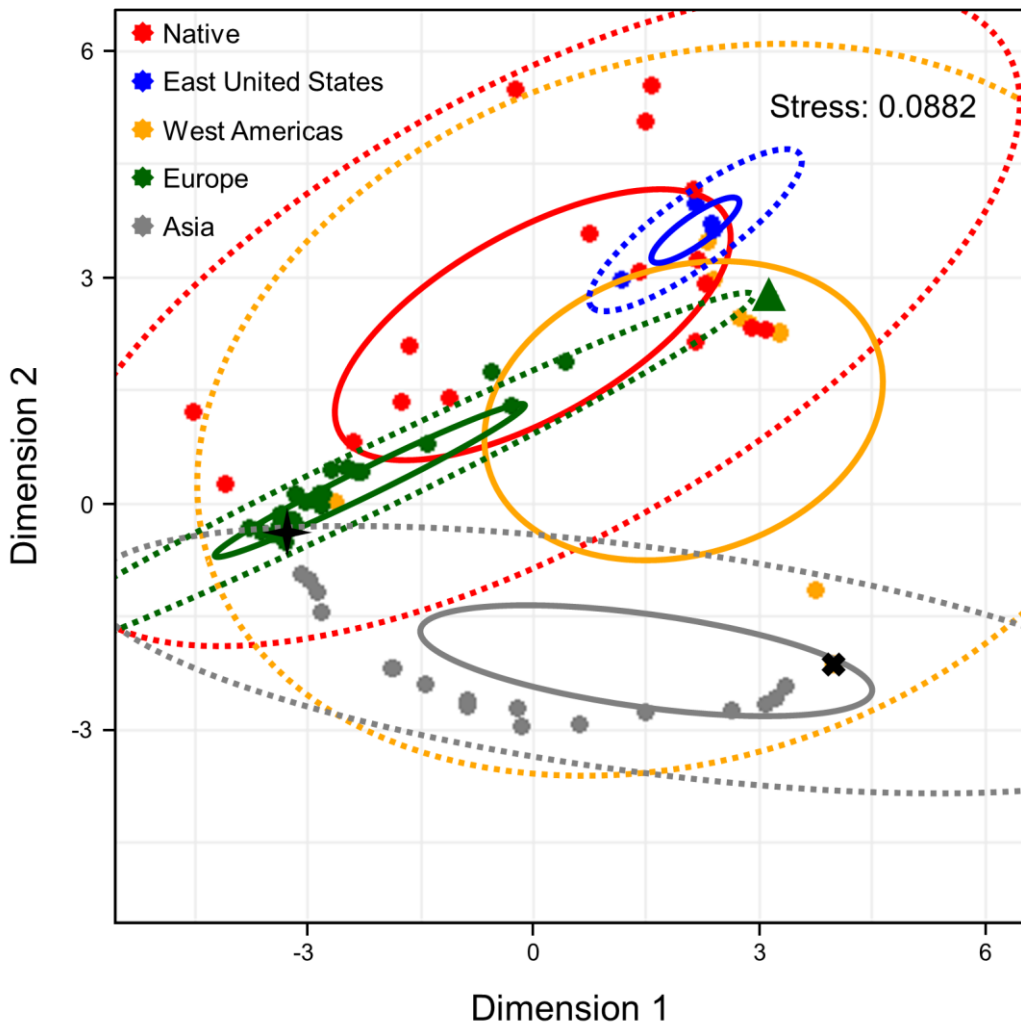
837 **Figure 2.** Haplotype network (statistical parsimony-based) for cytochrome c oxidase subunit I (COI) sequences of the red
 838 swamp crayfish, *Procambarus clarkii*. Each circle represents one haplotype and its size is proportional to the haplotype
 839 frequency. Within the network, each line between haplotypes represents a mutational change and small black dots show
 840 unsampled haplotypes inferred from the data. Localities from the same geographical region share the same colour.
 841 Haplotypes with non-synonymous changes are indicated by *.



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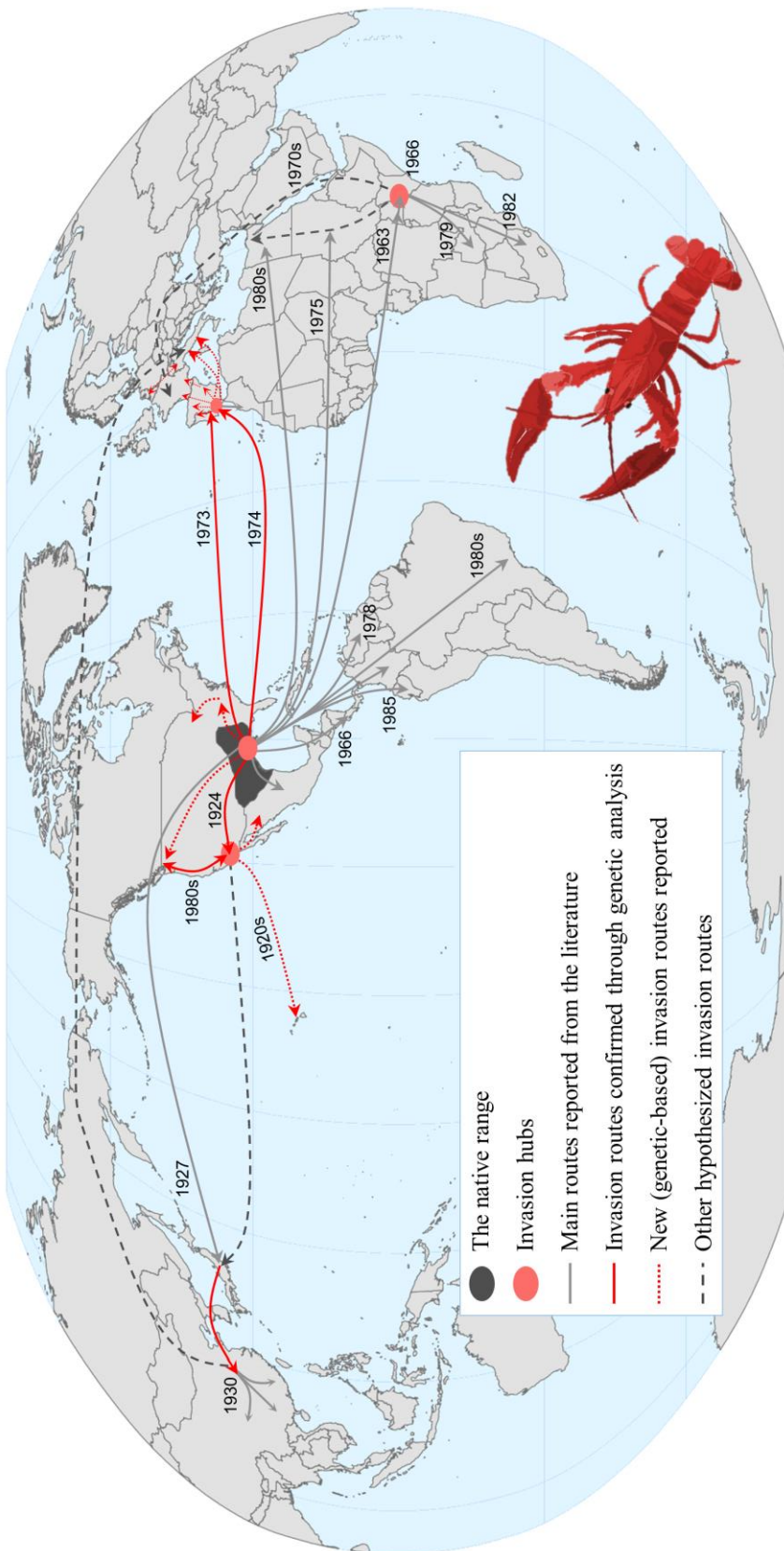
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844 **Figure 3.** NMDS analysis on D_{est} Jost distances. The graph depicts the pairwise dissimilarity between localities in a low-
 845 dimensional space where each point represents one population, ellipses depict established groups and dashed ellipses
 846 their 95% confidence intervals (CI). For a better interpretation, a green triangle indicates overlapping Central European
 847 localities (BIO, LON, BRI and HOL), a black "X" indicates the Mexican and Chinese overlapping localities (CHt, NB,
 848 XYc, XYw, XG, BGt, CJr, SLt, PYL, NCyL, NHL, YNL, XT, QJ, LZL, HLL, CHL, YJ, NX, DTL, DTLs) and a black star
 849 indicates the European, Asian and Mexican overlapping localities (BOR, VILA, MUN, ALM, WJ, CQs, HOK, FUK,
 850 CHIS).



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852 **Figure 4.** The global invasion routes of the red swamp crayfish, *Procambarus clarkii*, native from southern US and
853 northeastern Mexico, based on mtDNA (present study) and reports from the literature. Main and secondary introduction
854 routes are confirmed, described and hypothesized. Relevant invasion hubs, which usually act as recipients and sources
855 of new invasions, are shown as red circles: Louisiana (in the native range), California, Kenya and Spain.



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