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Invasive genetics of the mummichog (*Fundulus heteroclitus*): recent anthropogenic introduction in Iberia

Teófilo Morim¹, Grant R Bigg², Pedro M Madeira¹, Jorge Palma¹, David D Duvernell³, Enric Gisbert⁴, Regina L Cunha¹, Rita Castilho^{Corresp. 1}

¹ Center for Marine Sciences, University of Algarve, Faro, Portugal

² Department of Geography, University of Sheffield, Sheffield, United Kingdom

³ Department of Biological Sciences, Missouri University of Science and Technology, Rolla, Missouri, United States of America

⁴ RTA, Sant Carles de la Ràpita, Spain

Corresponding Author: Rita Castilho

Email address: rita.castil@gmail.com

Human activities such as trade and transport have increased considerably in the last decades, greatly facilitating the introduction and spread of non-native species at a global level. In the Iberian Peninsula, *Fundulus heteroclitus*, a small euryhaline coastal fish with short dispersal, was found for the first time in the mid-1970s. Since then, *F. heteroclitus* underwent range expansions, colonizing the southern region of Portugal, southwestern coast of Spain and the Ebro Delta in the Mediterranean Sea. Cytochrome *b* sequences were used to elucidate the species invasion pathway in Iberia. Three Iberian locations (Faro, Cádiz and Ebro Delta) and 13 other locations along the native range of *F. heteroclitus* in North America were sampled. Results revealed a single haplotype, common to all invasive populations, which can be traced to the northern region of the species' native range. We posit that the origin of the founder individuals is between New York and Nova Scotia. Additionally, the lack of genetic structure within Iberia is consistent with a recent invasion scenario and a strong founder effect. We suggest the most probable introduction vector is associated with the aquarium trade and we further discuss the hypothesis of a second human-mediated introduction responsible for the establishment of individuals in the Ebro Delta supported by the absence of adequate muddy habitats linking Cádiz and the Ebro Delta. Although the species has a high tolerance to salinity and temperature, benthic habitat constraints prevent along-shore colonisation suggesting that such expansions would need to be aided by human release.

1 **Invasive genetics of the mummichog (*Fundulus heteroclitus*): recent anthropogenic**
2 **introduction in Iberia**

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6 Gisbert⁴, Regina L. Cunha¹, and Rita Castilho*¹

7

8 ¹ CCMAR - Centre for Marine Sciences, University of Algarve, Campus de Gambelas, Faro
9 8005-139, Portugal.

10 ² Department of Geography, University of Sheffield, Winter Street, Sheffield S10 2TN, UK.

11 ³ Department of Biological Sciences, Missouri University of Science and Technology, Rolla,
12 Missouri 65409, USA

13 ⁴ RTA - Sant Carles de la Ràpita, Crta Poble Nou km 5.5, P.O. Box 200, 43540 Sant Carles de la
14 Ràpita, SPAIN

15

16

17 *Corresponding author:

18 Email: rcastil@ualg.pt

19 Telephone: +351 918397282

20

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28 **Abstract**

29 Human activities such as trade and transport have increased considerably in the last decades,
30 greatly facilitating the introduction and spread of non-native species at a global level. In the
31 Iberian Peninsula, *Fundulus heteroclitus*, a small euryhaline coastal fish with short dispersal, was
32 found for the first time in the mid-1970s. Since then, *F. heteroclitus* underwent range
33 expansions, colonizing the southern region of Portugal, southwestern coast of Spain and the Ebro
34 Delta in the Mediterranean Sea. Cytochrome *b* sequences were used to elucidate the species
35 invasion pathway in Iberia. Three Iberian locations (Faro, Cádiz and Ebro Delta) and 13 other
36 locations along the native range of *F. heteroclitus* in North America were sampled. Results
37 revealed a single haplotype, common to all invasive populations, which can be traced to the
38 northern region of the species' native range. We posit that the origin of the founder individuals is
39 between New York and Nova Scotia. Additionally, the lack of genetic structure within Iberia is
40 consistent with a recent invasion scenario and a strong founder effect. We suggest the most
41 probable introduction vector is associated with the aquarium trade and we further discuss the
42 hypothesis of a second human-mediated introduction responsible for the establishment of
43 individuals in the Ebro Delta supported by the absence of adequate muddy habitats linking Cádiz
44 and the Ebro Delta. Although the species has a high tolerance to salinity and temperature,
45 benthic habitat constraints prevent along-shore colonisation suggesting that such expansions
46 would need to be aided by human release.

47 Introduction

48 As a consequence of human activities involving large distance marine transportation and trade,
49 worldwide marine biological invasion rates have increased dramatically in the last 30 years (Bax
50 et al. 2003; Perrings et al. 2005; Meyerson and Mooney 2007; Hulme 2009). Along the European
51 coasts, there are over 850 invasive species, of which 237 occur along the western European
52 margins, 680 in the Mediterranean Sea and the remaining in the Baltic (Galil et al. 2014). Marine
53 invasive species pose a significant environmental threat as they are one of the major drivers of
54 biodiversity loss (Millennium Ecosystem Assessment 2005), altering ecosystems and their
55 dynamics, shifting the community structure and displacing endemic species (Bax et al. 2003;
56 Wallentinus and Nyberg 2007; Molnar et al. 2008). Negative impacts may also be registered at
57 the economic and social levels, affecting fisheries, aquacultures, tourism or human health (Bax et
58 al. 2003; Molnar et al. 2008). Invasive species spread and occupy new marine and coastal
59 ecosystems through several maritime introduction vectors such as ballast water, biofouling of
60 vessels, aquaculture escape or ornamental species trade (see Williams et al. 2013 and references
61 therein).

62 It is almost impossible to completely eradicate invasive non-native species after their
63 establishment (Katsanevakis and Crocetta 2014). Preventing the establishment of new invasive
64 species is, therefore, of extreme importance and probably the best way to avoid the negative
65 impacts associated with the spread of non-native species (Otero et al. 2013; Katsanevakis and
66 Crocetta 2014). Furthermore, a deep understanding of the invasion pathways and population
67 sources is relevant not only to prevent new invasions but also to aid the development of effective
68 conservation measures (Lawson Handley et al. 2011; Ghabooli et al. 2013; Galil et al. 2014). For
69 instance, information on the ecology of the invasive species within its native range may help in
70 choosing adequate biological control agents for containment measures (Roderick and Navajas
71 2003). Knowledge of the introduction vectors, population sources and their genetic
72 characteristics can be used to create risk maps, which provide useful insights into the potential
73 ecological impacts (Kulhanek et al. 2011), predicting the impacts of different routes and the
74 probability of new invasions occurring (Molnar et al. 2008; Hulme 2009).

75

76 Reconstructing the invasion pathways and identifying the putative source populations
77 with historical and contemporary vector records is a difficult task (Estoup and Guillemaud 2010;

78 Lawson Handley et al. 2011). In this context, molecular genetic data is a powerful tool to
79 reconstruct the invasive history by identifying putative source populations and genetic
80 bottlenecks (Estoup and Guillemaud 2010; Lawson Handley et al. 2011; Bock et al. 2015;
81 Cristescu 2015). Although genetic data does not always allow for successful identification of
82 these processes, there are three conditions which increase the probability of accurate
83 reconstruction of the invasive pathway, all fulfilled for *Fundulus heteroclitus*: (1) extensive
84 sampling along the native range to ensure all the putative source populations are known, (2) the
85 presence of genetic structure in the native range to narrow down the putative source regions and
86 (3) a short amount of time passed since the invasion so that processes such as genetic drift do not
87 increase the genetic differentiation between native and invasive populations (Geller et al. 2010).

88 The mummichog, *F. heteroclitus* (Linnaeus, 1766), is a small teleost naturally occurring
89 almost continuously in saltmarshes of the North American east coast, from Newfoundland to
90 Florida (Hardy Jr 1978). This species is extremely resistant to a wide range of salinities and
91 temperatures, and can be found in freshwater, brackish or saltwater, inhabiting sheltered coastal
92 areas such as saltmarshes, tidal creeks, estuaries or bays all year-round (Bigelow and Schroeder
93 1953; Hardy Jr 1978). *F. heteroclitus* is one of the most stationary marine species (Bigelow and
94 Schroeder 1953), with short dispersal distances (1-2 km, Fritz et al. 1975; Lotrich 1975), high
95 site fidelity closely related to the saltmarshes (Kneib 1984) and short home ranges (36-38 m,
96 Lotrich 1975).

97 Due to its biology and ecological patterns, *F. heteroclitus* is an important model for the
98 study of adaptive genetic clines, exhibiting distinct and extensive latitudinal clinal variation in
99 relation to morphological, physiological and genetic traits along the North American Atlantic
100 coast, which have been thoroughly studied for a long time (e.g., Powers et al. 1986).
101 Geographical analysis of morphological traits (Morin and Able 1983; Able and Felley 1986),
102 enzyme-coding loci (Powers and Place 1978; Powers et al. 1986; Ropson et al. 1990),
103 mitochondrial DNA (González-Vilaseñor and Powers 1990; Bernardi et al. 1993; Smith et al.
104 1998), putative neutral nuclear microsatellite loci (Adams et al. 2006; Duvernell et al. 2008), and
105 a variety of nuclear single-nucleotide polymorphisms (Williams et al. 2010; Strand et al. 2012;
106 McKenzie et al. 2015; McKenzie et al. 2016) demonstrated concordance of clinal patterns, where
107 a break is placed between the meridians 40° and 41° N, along the coast of New Jersey. These
108 studies concur with Morin and Able (1983), supporting the division into two subspecies: *F.*

109 *heteroclitus heteroclitus* (Linnaeus, 1766) to the north of the cline and *F. heteroclitus*
110 *macrolepidotus* (Walbaum, 1792) to the south of the cline.

111 In the Iberian Peninsula, *F. heteroclitus* was first detected in the 1970s, in the
112 Guadalquivir and Guadiana saltmarshes (southwestern coast of Spain) (Hernando 1975) and in
113 the Guadiana Delta (Coelho et al. 1976). More recently, its presence was also reported in the
114 Ebro Delta, northeastern coast of Spain (Gisbert and López 2007) and in the Ria Formosa, south
115 of Portugal (e.g., Catry et al. 2006; Paiva et al. 2006b). The species was probably introduced
116 between 1970 and 1973 in the Spanish saltmarshes (Fernández-Delgado 1989) either
117 involuntarily via aquarium trade (Hernando and Soriguer 1992; Bernardi et al. 1995; Elvira and
118 Almodóvar 2001), ballast water (García-Revilla and Fernández-Delgado 2009; Fernández-
119 Delgado 2010), or intentionally for purposes of biological control (Gozlan 2010). The Ebro Delta
120 individuals were probably caught in the Spanish saltmarshes to be used in aquaculture and the
121 aquarium trade (Gisbert and López 2007) and were later released from captivity. Another
122 possibility for the Ebro Delta introduction is an accidental escape from a research centre nearby,
123 where this species had already been used as a scientific model (Gisbert and López 2007). Two
124 previous studies have analysed the origin of the southwestern Spanish populations using
125 mitochondrial DNA (mtDNA), concluding the founder individuals were original from the
126 northern native region between Maine and Nova Scotia where the northern subspecies *F. h.*
127 *macrolepidotus* is distributed (Bernardi et al. 1995; Fernández-Pedrosa et al. 1996).

128 In the present study, we aim to build on previously published studies on the invasive
129 range of *F. heteroclitus* (Bernardi et al. 1995; Fernández-Pedrosa et al. 1996) by using (1) more
130 sampling locations (one in the Mediterranean Sea and two locations in the eastern Atlantic, and
131 thirteen native locations), (2) a significantly larger number of individuals (248 in total), and (3) a
132 three times larger fragment of the mitochondrial DNA cytochrome *b* gene; and to evaluate the
133 genetic diversity and invasion pathways. Given that the species was recently reported in the
134 Iberian Peninsula and has a limited adult dispersal capability, we tested the hypothesis of a
135 human-mediated single Iberian introduction followed by dispersal promoted along the main
136 oceanographic currents. This hypothesis leads to the expectation of an Iberian invasion based on
137 a few founder individuals, with consequent lower genetic diversity than the putative identified
138 source population. Also, the Iberian populations are expected to show no evident genetic
139 structure given the short time since invasion.

140 **Materials and methods**

141 **Sampling.** A total of 248 *Fundulus heteroclitus* individuals from 16 locations: 13 sites in the
142 western Atlantic, one in the Mediterranean Sea and two locations in the eastern Atlantic (Table 1
143 and Fig. 1) were obtained and stored in 96% ethanol and kept at -20 °C. The populations from
144 the western Atlantic sampled above 40° N are hereafter referred to as northern locations (ID 1-8),
145 while samples collected below that latitude are referred to as southern locations (ID 9-13).
146 Samples collected from the Mediterranean and eastern Atlantic are referred to as Iberian (ID 14-
147 16).

148
149 **DNA extraction, PCR amplification and sequencing.** Total genomic DNA was extracted from
150 caudal fin tissue following a standard Chelex 100 protocol (Walsh et al. 1991). Extraction results
151 were checked by electrophoresis in 0.8% agarose gel stained with GelRed. Polymerase Chain
152 Reactions (PCR) were conducted in a total volume of 25 µL, with 1X buffer, 10 mM dNTPs,
153 10mM of each primer, 1U Taq Advantage 2 Polymerase mix DNA polymerase (CLONTECH-
154 TaKaRa), 2 µL of DNA and Milli-Q water to the final volume. A fragment of the cytochrome *b*
155 (*cyt b*) gene (1000 base pairs) was amplified with the forward primer GludG-L14724 (5'-
156 TGACTTGAARAACCA YCGTTG-3') (Palumbi et al. 1991) and the reverse primer cb6b.h (5'-
157 GGAATTCACCTCTCCGTTTACAAGAC-3') (Martin and Bermingham 1998). PCR
158 amplification consists of an initial 4 min denaturation step at 95 °C, followed by 40 cycles of 1
159 min at 94 °C (denaturation), 1 min at 50 °C (annealing) and 1.5 min at 72 °C (extension), and a 5
160 min final extension step. When amplification was not successful the following profile was used:
161 initial 3 min denaturation step at 95 °C, followed by 32 cycles of denaturation for 30 s at 95 °C,
162 annealing for 30 s at 54 °C and extension for 1 min at 68 °C, and a final extension step for 4 min
163 at 68 °C. PCR products were checked afterwards by electrophoresis in a 1% agarose gel stained
164 with GelRed. Mitochondrial DNA was purified by ethanol/sodium acetate precipitation
165 (Sambrook and Russel 2001). Its purity and quantity were analysed using a NanoDrop1000
166 spectrophotometer (Thermo Fisher Scientific, USA). Sequencing was performed on an ABI
167 3130xl capillary sequencer (Applied Biosystems – CCMAR, Portugal) using the forward primer
168 from the PCR amplification (GludG-L14724).

169

170 **Genetic analysis.** Cyt *b* sequences were aligned and manually checked using the software
171 Geneious v4.8.2 (Biomatters, Ltd., Auckland, New Zealand). The number of haplotypes (n),
172 number of private haplotypes (n_p), and the haplotype (h) (Nei and Tajima 1981) and nucleotide
173 diversities (π) (Nei 1987) were calculated for each location using the DNAsP v5.10.1 (Librado
174 and Rozas 2009). Sequences were uploaded to GenBank (MH809691 - MH809938). To
175 represent the phylogeographic relationships among haplotypes, a haplotype network was
176 constructed using the Median Joining algorithm implemented in NETWORK v5.0 (Bandelt et al.
177 1999, fluxus-engineering.com).

178 We used two approaches to infer the most probable source area of the introduced Iberian
179 populations within the native range of *F. heteroclitus*: (1) the geographical distribution of
180 haplotypes in native populations, and (2) the phylogeographical relationships among haplotypes.
181

182 **Ecological niche modelling.** To explore the potential spread of *F. heteroclitus* within European
183 waters beyond its currently known locations we used an ecological niche model. We selected the
184 interactive AquaMaps system in FISHBASE (www.fishbase.org). Using observed locations of
185 adults to construct a range of acceptable environmental conditions within which a species can
186 exist, this model uses a c-squares distribution modelling approach (Rees 2003) to predict the
187 probability of occurrence of the adult fish for a particular location, possessing specific
188 environmental parameters. While this is normally computed just within the native range of a
189 species it can be extended within the interactive system to consider all possible locations, and the
190 associated probability of occurrence linked to the location's mean environmental state if the
191 species was able to reach that region. The basic statistical approach is given in Kaschner et al.
192 (2006) and the AquaMaps version is described in Kesner-Reyes et al. (2012). Another ecological
193 niche model, based on maximum entropy principles (MAXENT, Phillips and Dudik 2008), was
194 also tested using environmental parameters of sea surface temperature (SST) and sea surface
195 salinity (SSS), but the specialist ecological needs of *F. heteroclitus* led to poor solutions for
196 range prediction. This latter approach is therefore not considered further here.

197 A set of favourable and extreme environmental conditions compatible with the native
198 occurrence of *F. heteroclitus* is automatically specified by AquaMaps, relying heavily on Page
199 and Burr (2011). It includes parameter ranges for water depth, SST, SSS, primary production
200 rates and sea-ice cover, leading to probabilities of occurrence exceeding 0.6 along the whole

201 eastern seaboard of North America from South Carolina to the Canadian Maritime Provinces
202 south to the Gulf of St. Lawrence. This matches well to the known range (Page and Burr 2011).
203 However, the long-term survival of *F. heteroclitus* in two Iberian estuaries has implications for
204 extending the extreme salinity level that this species can tolerate beyond that automatically
205 specified. The extreme salinity tolerance has therefore been raised to 39.6 psu, compared to the
206 automatic 36.47 psu, consistent with the values found for the Ria Formosa (Cristina et al. 2016).
207 In addition, the sea-ice variable has been excluded from constraining the AquaMaps solution, as
208 this is not relevant to the area being considered in this study, and also the primary production
209 variable, as coastal environments satisfy the automatic requirement almost uniformly. The
210 environmental variables used for the AquaMaps simulation shown here are given in Table 2.

211 AquaMaps was re-run with these environmental constraints and using the combined
212 occurrence data from the native range, along the North American eastern seaboard, and the two
213 Iberian sites of Ria Formosa and the Ebro River Delta. There is no change to the North American
214 predictions, so here we concentrate only on those for European waters. The occurrence of *F.*
215 *heteroclitus* was constrained to coastal zones with the muddy benthic habitats in which *F.*
216 *heteroclitus* is found. These are defined as the coastal fine muds to muddy sands benthic
217 environments of the EMODnet Seabed Habitats project (www.emodnet-seabedhabitats.eu).
218 These environments are formally defined by Long (2006), but basically include sediments with
219 more than a 10% mud component.

220

221

222 Results

223 Data analysis on the sampled specimens resulted in *cyt b* sequences with a final length of 700
224 base pairs (bp), which comprised 77 (11%) polymorphic sites and 32 (41.6%) parsimony-
225 informative sites. These polymorphisms defined 70 haplotypes, of which 62 (88.6%) are private
226 haplotypes (present in one location only) and 55 (78.6%) are singletons (present in one
227 individual only). Overall haplotype diversity was high ($0.74 \pm \text{SD } 0.03$), ranging from null in
228 Iberia (ID 14-16) to 0.93 in Tuckerton (ID 9) and Roanoke Island (ID 12), whereas mean
229 nucleotide diversity was low ($0.26\% \pm \text{SD } 0.06\%$) ranging from null diversity in Iberia to 0.91%
230 in Woods Hole (ID 4) (Table 1 and Fig. 2).

231 The most abundant haplotype in North American locations is shared by 50.4% ($N = 125$)
232 of the individuals and is present in all northern group locations, in one southern location and in
233 all Iberian locations (Fig. 1c). This is the only haplotype detected in the invasive range of the
234 species (Faro, Cádiz and Ebro Delta). The second most frequent haplotype in North American
235 locations is shared by 9.7% ($N = 24$) individuals in six locations, although in higher frequency in
236 the southern group locations. Two other haplotypes were found in 3.2% ($N = 8$) and in 2.4% ($N =$
237 6) of the individuals from two locations. All other haplotypes were present in five or less
238 individuals and in less than three locations (Fig. 1d).

239 The *cyt b*-based haplotype network (Fig. 3) displays two haplogroups separated by 9
240 mutational steps. Haplogroup A is constituted by all eight northern group locations (Bridgewater
241 to Red Bank, ID 1-8), two individuals from the southern group (Suffolk, ID 11) and by the
242 Iberian locations (Faro to Ebro Delta, ID 14-16). Haplogroup B is formed by all the other
243 individuals from all southern locations (Tuckerton to Skidaway Island, ID 9-13) and includes
244 eight individuals from northern locations: one from Bridgewater (ID 1) and seven from Woods
245 Hole (ID 4). Overall, both haplogroups display star-like configurations with different levels of
246 complexity. The northern haplogroup (A) is simpler with 90% of the haplotypes separated by a
247 single mutation, while the southern haplogroup (B) is more complex, with 3 mini-stars
248 interconnected by one mutation each, and haplotypes separated by up to five mutations. The
249 haplotype found in the Iberian Peninsula belongs to the northern haplogroup.

250 Conditions for the spread of *F. heteroclitus* are fundamentally favourable
251 (probability > 0.5), or at least acceptable (probability > 0.25), along much of the Atlantic coastline
252 of Europe and around the Alboran Sea in the western Mediterranean (Fig. 4).

253

254

255 Discussion

256 Results revealed the presence of a single haplotype common to all individuals in the Iberian
257 Peninsula. This haplotype is the most abundant in the northern group of the native distribution,
258 indicating the most probable origin of the invasion. We posit as the most likely introduction
259 vector the aquarium trade and propose that the Ebro Delta colonization results from an
260 independent human-mediated secondary introduction. Before addressing the main interpretations
261 and conclusions of these results, one main caveat must be addressed. Successful understanding

262 of the invasion pathway relies on (1) comparable genetic data retrieved from an adequate number
263 of sampled individuals throughout the entire native range, (2) presence of genetic clines within
264 the native populations and (3) the use of adequate molecular markers (Geller et al. 2010). While
265 the first two points are fulfilled for *F. heteroclitus*, the use of a single mitochondrial DNA
266 marker constitutes the main caveat of this study. Mitochondrial DNA has been a widely used
267 molecular marker in population genetics studies (Ballard and Whitlock 2004). Nevertheless, the
268 use of high variable nuclear markers, such as microsatellites, provides an opportunity to perform
269 assignment tests based on their multiple-locus genotypes, to test for recent reductions in
270 population sizes and to estimate effective population sizes. However, previously published
271 studies show relatively low microsatellite genetic variation in the northern group (Duvernell et
272 al. 2008). Single nucleotide polymorphisms (SNPs, Morin et al. 2004), extend the previously
273 referred analytical possibilities improving their statistical power because of the sheer number of
274 existing loci genome wide.

275

276 **Genetic diversity**

277 The presence of a single haplotype common to all *F. heteroclitus* sampled in the Iberian
278 Peninsula lends support to the hypothesis of an extremely recent introduction of the species
279 which has not allowed the accumulation of mutations at the mtDNA level, and with a single
280 introduction event composed by a very small number of individuals (Roman and Darling 2007).
281 Theoretically, invasive species are expected to suffer loss of genetic variation since the new
282 established populations are often based on a few individuals, which by definition, have lower
283 genetic diversity than the native source populations (Nei et al. 1975; Allendorf and Lundquist
284 2003; Dlugosch and Parker 2008). The single-haplotype characteristic can be found in other
285 invasive species, such as *Equulites elongatus*, the slender pony fish (Sakinan et al. 2017);
286 *Cercopagis pengoi*, the fishhook waterflea, a planktonic cladoceran crustacean (Cristescu et al.
287 2001); *Corbicula fluminea*, the Asian clam (Gomes et al. 2016) and *Didemnum perlucidum*, a sea
288 squirt (Dias et al. 2016). However, many successful invasive species do not display significant
289 erosion of genetic diversity (Roman and Darling 2007; Dlugosch and Parker 2008; Rius et al.
290 2014). For example, in a recent review of the literature on European seas invasion genetics, in
291 54% of studies that compared the genetic diversity between introduced species and their native
292 range, 74% reported comparable levels of diversity between them, while only 23% displayed a

293 reduction in the genetic diversity of introduced species, and the remaining 2% showed an
294 increase in diversity (Rius et al. 2014).

295

296 **Population sources**

297 We identified a single Iberian haplotype present in all northern populations in high frequency
298 (between 47 and 93%) and in two individuals in Suffolk, one of the southern locations.

299 According to our results, the northern group is the most probable source of the founder

300 individuals, which corresponds to the natural range of the subspecies *F. h. macrolepidotus*.

301 However, we cannot definitely exclude Suffolk as a presumptive population source. The absence

302 of genetic diversity in the Iberian Peninsula populations prevents the precise determination of the

303 putative source population. The low spatial resolution of our data arises from the lack of genetic

304 variability in the invasive range, rather than insufficient sampling of *F. heteroclitus* individuals

305 in the Iberian Peninsula or in its native range (Muirhead et al. 2008).

306 Nevertheless, our findings are consistent with two previous studies on the origin of invasive

307 individuals found in the Guadalquivir. First, based on mtDNA restriction fragment length

308 polymorphisms (RFLP) (Fernández-Pedrosa et al. 1996) reported the presence of two

309 haplotypes: the most abundant corresponding to the northern haplotype 1, dominant between

310 Maine and Nova Scotia in North America; the other haplotype did not match any of the sampled

311 native haplotypes and we found no evidence of its presence in the present study, using a larger

312 number of individuals. It was previously suggested to be either a native unsampled haplotype or

313 an endemic haplotype from Iberia, which is rather unlikely due to its recent invasion

314 (Fernández-Pedrosa et al. 1996) and total absence of records in the area. Although there are no

315 reports of hybridization between *F. heteroclitus* and any of the Spanish endemic species, the

316 presence of a new haplotype could nonetheless be due to hybridization (Rius et al. 2014).

317 Secondly, a study based on *cyt b* sequences (Bernardi et al. 1995) concluded the individuals from

318 the Guadalquivir originated in the region between Maine and Nova Scotia. Although our results

319 are congruent with this proposed origin, we show that source populations could come from lower

320 latitudes (40° N), even if only the northern group is considered.

321

322

323

324 Introduction vector

325 Since it was first recorded in Iberian saltmarshes, several studies linked *F. heteroclitus*
326 introduction to different vectors. The aquarium trade has been suggested as the most important
327 vector responsible for the introduction of this species (Hernando and Soriguer 1992; Elvira and
328 Almodóvar 2001; García-Llorente et al. 2008; Ribeiro et al. 2008a; Gozlan 2010), followed by
329 ballast water (García-Revilla and Fernández-Delgado 2009; Fernández-Delgado 2010),
330 biological control (Gozlan 2010) and unknown origins (Fernández-Delgado 1989; Gutiérrez-
331 Estrada et al. 1998). We posit that the introduction via the aquarium trade is the most probable
332 scenario responsible for the establishment of the first individuals in Iberia, given the genetic
333 results obtained.

334 It is well known that vessels can transport large numbers of organisms from several
335 species at the same time in their ballast water (Carlton 2001; Gollasch 2007). Since there are
336 countless vessels active around the world (e.g., Kaluza et al. 2010), ballast water-mediated
337 transport allows the possibility of multiple introduction events, each with large groups of
338 individuals (Hulme 2009). Considering such large groups usually comprise higher genetic
339 diversity than fewer individuals alone, the assemblages transported are likely to display levels of
340 genetic diversity similar to the levels found within their native range (Roman 2006; Dlugosch
341 and Parker 2008; Wilson et al. 2009). Our findings, however, suggest that this was not the
342 introduction vector responsible for the spread of the species; in contrast to the expectation of
343 similar levels of genetic diversity between the native and invasive range, the Iberian locations
344 display a strong founder effect, with all samples sharing a single haplotype.

345 Similarly to ballast water, the aquarium and ornamentals trade transport many species at a
346 global scale (Padilla and Williams 2004). For instance, at least 19% of the invasive fishes found
347 in the Iberian Peninsula were introduced via the aquarium trade (Maceda-Veiga et al. 2013).
348 However, there are two main differences between introductions that follow ballast water or the
349 aquarium trade. First, each introduction event after aquarium release is likely to comprise a small
350 number of individuals (Duggan et al. 2006; Roman and Darling 2007). Thus, even though this
351 vector may be responsible for the establishment of several non-native species (Padilla and
352 Williams 2004), multiple introductions would be necessary for an invasive species to display
353 high genetic diversity (Facon et al. 2003; Roman and Darling 2007). Secondly, the individuals
354 released by aquarists are usually adults of higher fitness, which makes them better adapted to

355 survive in a natural environment (Padilla and Williams 2004). Thus, not only are the *F.*
356 *heteroclitus* invasive genetic diversity and structure consistent with an introduction of a low
357 number of individuals via the aquarium trade, but it is also plausible that a few resistant
358 individuals would manage to survive, reproduce and colonize the environment in which they
359 were released.

360

361 **Human mediated introduction in the Ebro Delta**

362 Although the absence of genetic structure within Iberia limits possible insights into the invasion
363 pathway, our data support the hypothesis of a human-mediated introduction episode responsible
364 for the establishment of *F. heteroclitus* in the Ebro Delta, as previously suggested by Gisbert and
365 López (2007) based on taxonomic identification. The hypothesis of a long-distance colonization
366 via natural dispersal is quite unlikely.

367 According to a review of the geographical distribution of Cyprinodontiformes along the
368 northeastern coast of Spain by (García-Berthou and Moreno-Amich 1991), no *F. heteroclitus*
369 individuals were found at the Ebro Delta in 1989; the first record of the species was only
370 registered 16 years later by Gisbert and López (2007). Thus, we estimate the date of
371 establishment in the Ebro Delta ranges between 12 and 28 years ago. Assuming this estimate is
372 correct, a natural colonization hypothesis implies individuals would have taken roughly two
373 decades to travel more than 1000 km from their southernmost limit located in the Guadalquivir
374 saltmarshes (Gutiérrez-Estrada et al. 1998) to the Ebro Delta. However, when compared with the
375 natural colonization of the Ria Formosa, this hypothesis seems quite improbable. While *F.*
376 *heteroclitus* was never collected during sampling events that happened in the Ria Formosa
377 between 1980 and 2006 (Ribeiro et al. 2006; Ribeiro et al. 2008b; França et al. 2009), analysis of
378 prey remains left by Little Terns (*Sterna albifrons*) in the salt-pans and barrier islands revealed
379 this prey species was present in the salt-pans and adjacent channels at least since 2002 (Catry et
380 al. 2006; Paiva et al. 2006a). Although this may sound contradictory, *F. heteroclitus* could in fact
381 have been present in the Ria Formosa in specific unsampled locations or at extremely low
382 densities, avoiding capture. Nonetheless, assuming that colonization happened around 2002, it
383 seems that *F. heteroclitus* took no more than ca. 20 years to travel around 50 km from the
384 Guadiana Delta, where it was first detected in 1976 (Coelho et al. 1976). This estimate indicates
385 that if the Ebro colonization happened via natural dispersal, it must have happened 20 times

386 faster than the natural colonization of the Ria Formosa. Given that *F. heteroclitus* has very low
387 dispersal abilities (e.g., Fritz et al. 1975; Lotrich 1975), the natural colonization scenario for the
388 Ebro seems unlikely.

389 Furthermore, if establishment in the Ebro Delta followed a natural range expansion one
390 would expect to find several established populations between the Barbate marshes in the
391 Guadalquivir, its southernmost location (Gutiérrez-Estrada et al. 1998), and the Ebro Delta.
392 However, we know that no invasive individuals can be found at the Mar Menor (personal
393 communication), a coastal lagoon with suitable conditions for successful establishment
394 (Gutiérrez-Estrada et al. 1998; Pérez-Ruzafa et al. 2006), located midway between the
395 Guadalquivir and the Ebro Delta.

396 Finally, the Strait of Gibraltar would represent a barrier to dispersal and gene flow,
397 limiting *F. heteroclitus* natural range expansion towards the Mediterranean Sea (Fernández-
398 Delgado et al. 1986; Moreno-Amich et al. 1999; Doadrio et al. 2002) because of the strong
399 currents prevailing in the area and the absence of suitable habitats. Although uncertain for *F.*
400 *heteroclitus*, this has already been documented for two other Iberian toothcarps, where restricted
401 gene flow in this region led to speciation of the *Aphanius iberus* in the Mediterranean Iberian
402 coast and *Aphanius baeticus* in the southwestern Atlantic Spanish coast (Perdices et al. 2001;
403 Doadrio et al. 2002).

404

405 **Ecological niche modelling**

406 Conditions for the spread of *F. heteroclitus* are limited by the existence of benthic muddy
407 saltmarsh environments compatible with the species' ecological requirements. These habitats are
408 only found near major estuaries or lagoons areas along the Atlantic and western Mediterranean
409 coastlines. The exception to this is along the North African coast, where such environments are
410 more continuous, although other environmental aspects are less favourable there, as suggested by
411 the rapid decline in occurrence probability eastwards. The consequence of this benthic habitat
412 constraint is to make along-shore colonisation unlikely in most areas, suggesting that any such
413 sudden expansion would need to be aided by human release.

414

415

416 **Conclusions**

417 *Fundulus heteroclitus* invasive Iberian populations revealed the presence of a single *cyt b*
418 haplotype common to all individuals. This haplotype is predominant in the northern group of the
419 native distribution, and although we cannot determine which exact location was at the origin of
420 the introduced individuals, one can identify the northern group as the source of the introduction.
421 The lack of genetic diversity is consistent with a strong founder effect at the origin of *F.*
422 *heteroclitus* in Iberia. Although there is no direct evidence, we infer that the most likely vector
423 was the aquarium trade, and that the Ebro Delta colonization results from a human-mediated
424 secondary introduction isolated from the rest of Iberia. Considering the tolerance of the species
425 to high salinity, its temperature range, and the significant amount of colonized area in the Iberian
426 southern region, we predict that *F. heteroclitus* will most likely keep on expanding its invasive
427 range until it faces unfavourable environmental conditions.

428

429

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443

444

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Figure 1(on next page)

Distribution map of localities and haplotypes

Distribution map of localities along the coast of (a) North America, and (b) Iberian Peninsula of *Fundulus heteroclitus*. Locations and sample details can be found in Table 1. (c) Coloured circles display the distribution of the relative proportions of the cytochrome b haplotypes from each location: the four most common haplotypes, the private haplotypes, and all the other shared, but less frequent haplotypes.

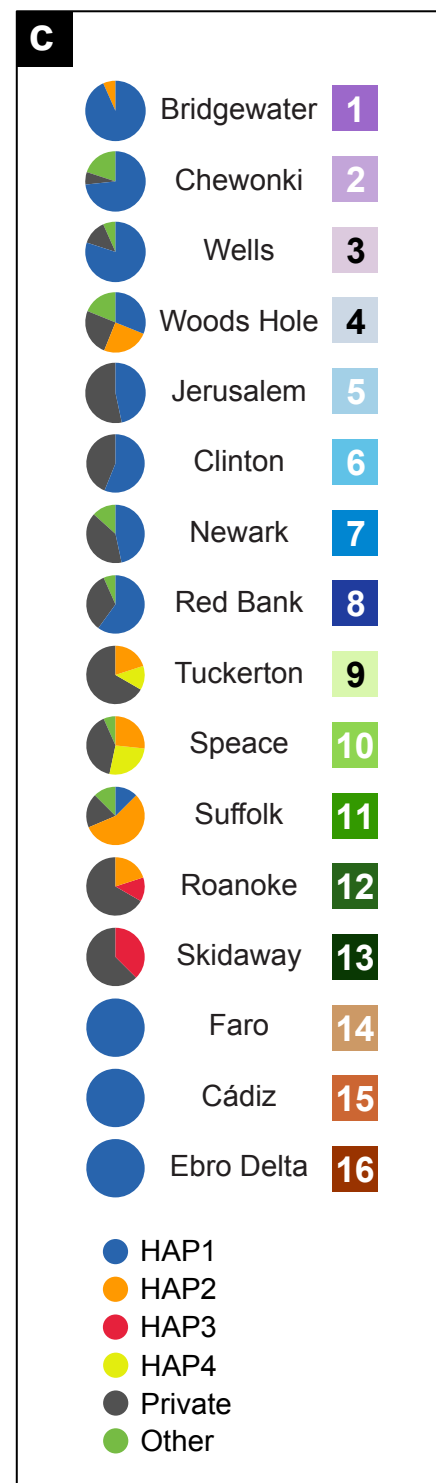
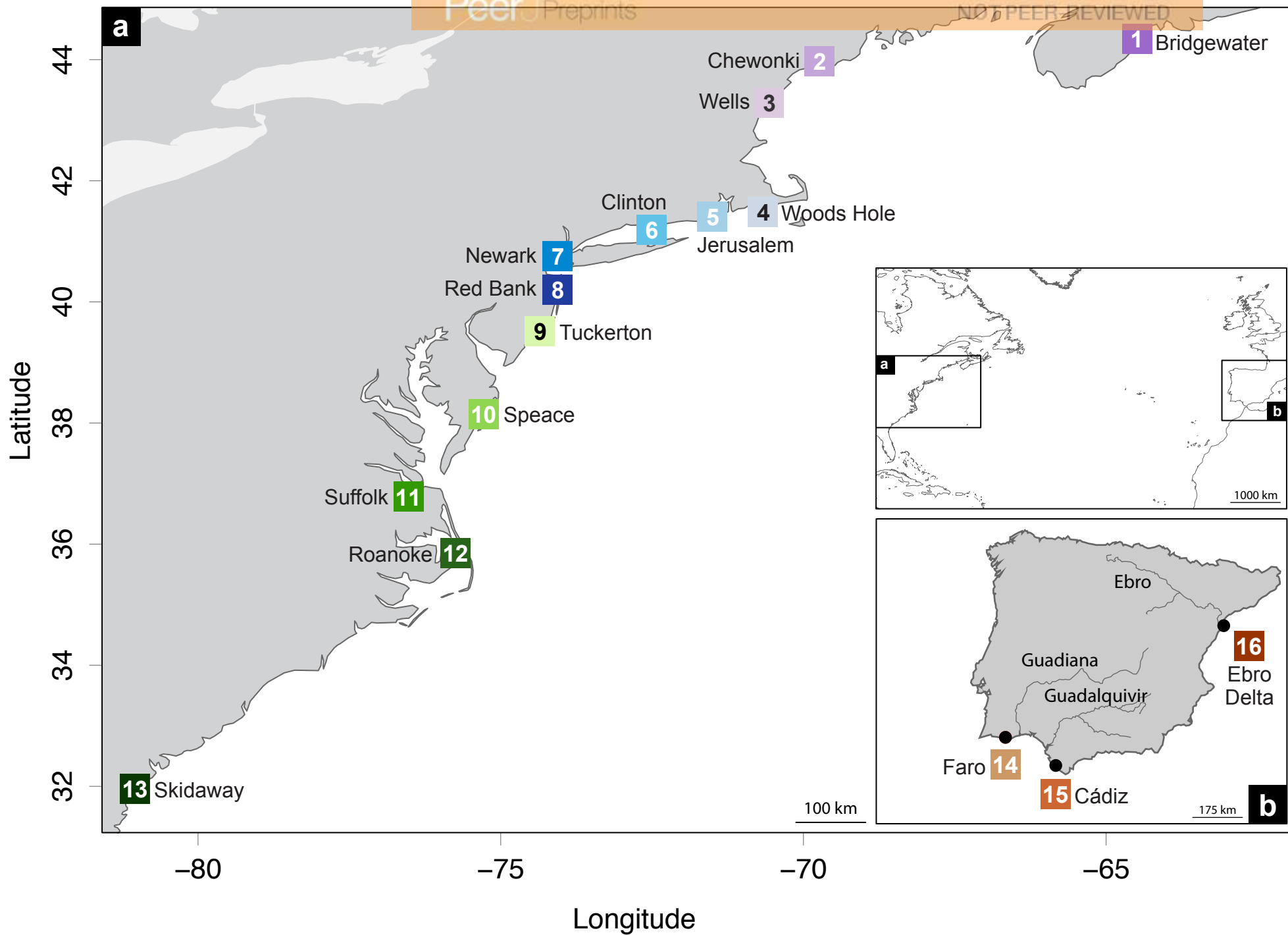


Figure 2 (on next page)

Haplotype and nucleotide diversity

Haplotype and nucleotide diversity of *Fundulus heteroclitus* from all sampled locations.

Abbreviations in the legend are defined in Table 1, and colours are the same as in Fig. 1.

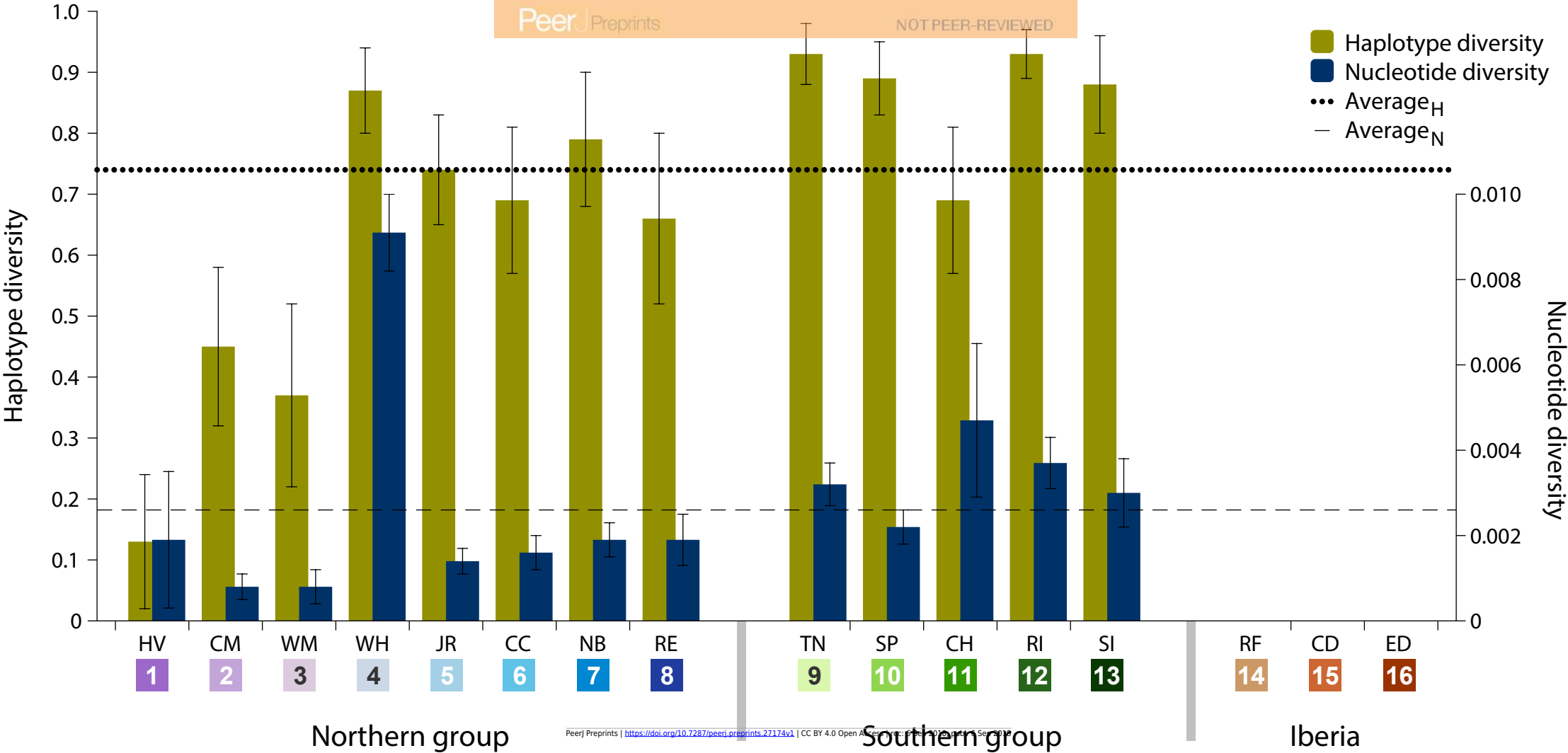
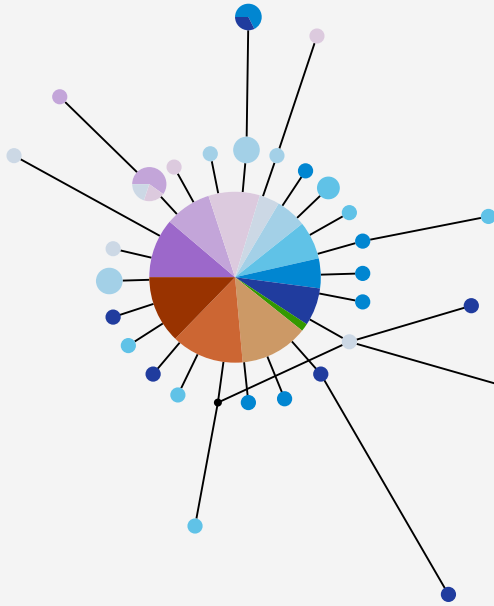


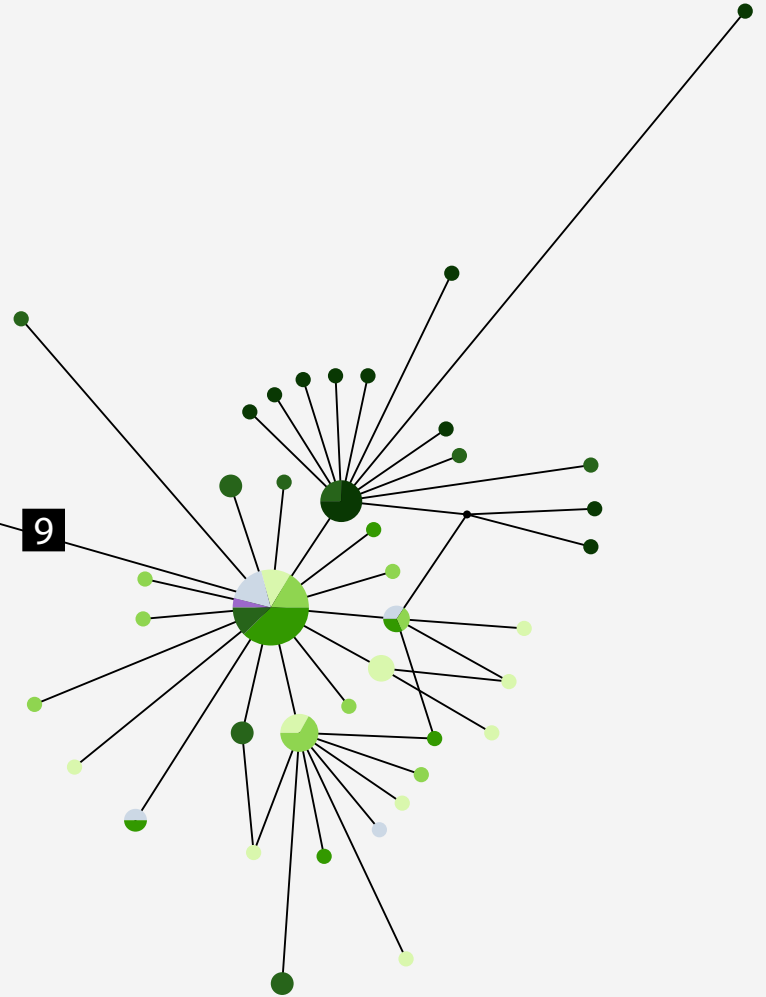
Figure 3(on next page)

Median-joining cytochrome *b* haplotype network for *Fundulus heteroclitus*

Median-joining cytochrome *b* haplotype network for *Fundulus heteroclitus*. Each circle represents a different haplotype with size proportional to the frequency of the haplotype within the sample. Line length is proportional to the number of mutations between haplotypes. Each colour corresponds to a different location. Colours are the same as in Figure 1. Locations details can be found in Table 1. The two black dots represent putative unsampled haplotypes, and the number in black square shows the number of mutations separating haplogroup A from the haplogroup B.



9



- | Northern group | Southern group | Iberia |
|---|---|---|
| ■ Bridgewater | ■ Tuckerton | ■ Faro |
| ■ Chewonki | ■ Speace | ■ Cádiz |
| ■ Wells | ■ Suffolk | ■ Ebro Delta |
| ■ Woods Hole | ■ Roanoke | |
| ■ Jerusalem | ■ Skidaway | |
| ■ Clinton | | |
| ■ Newark | | |
| ■ Red Bank | | |

Figure 4(on next page)

Western European and Mediterranean coastal environments showing AquaMap probabilities of occurrence of *F. heteroclitus*.

Western European and Mediterranean coastal environments showing AquaMap probabilities of occurrence of *F. heteroclitus*. Those areas in bold show coastal seabed habitats with a mud content > 10%.

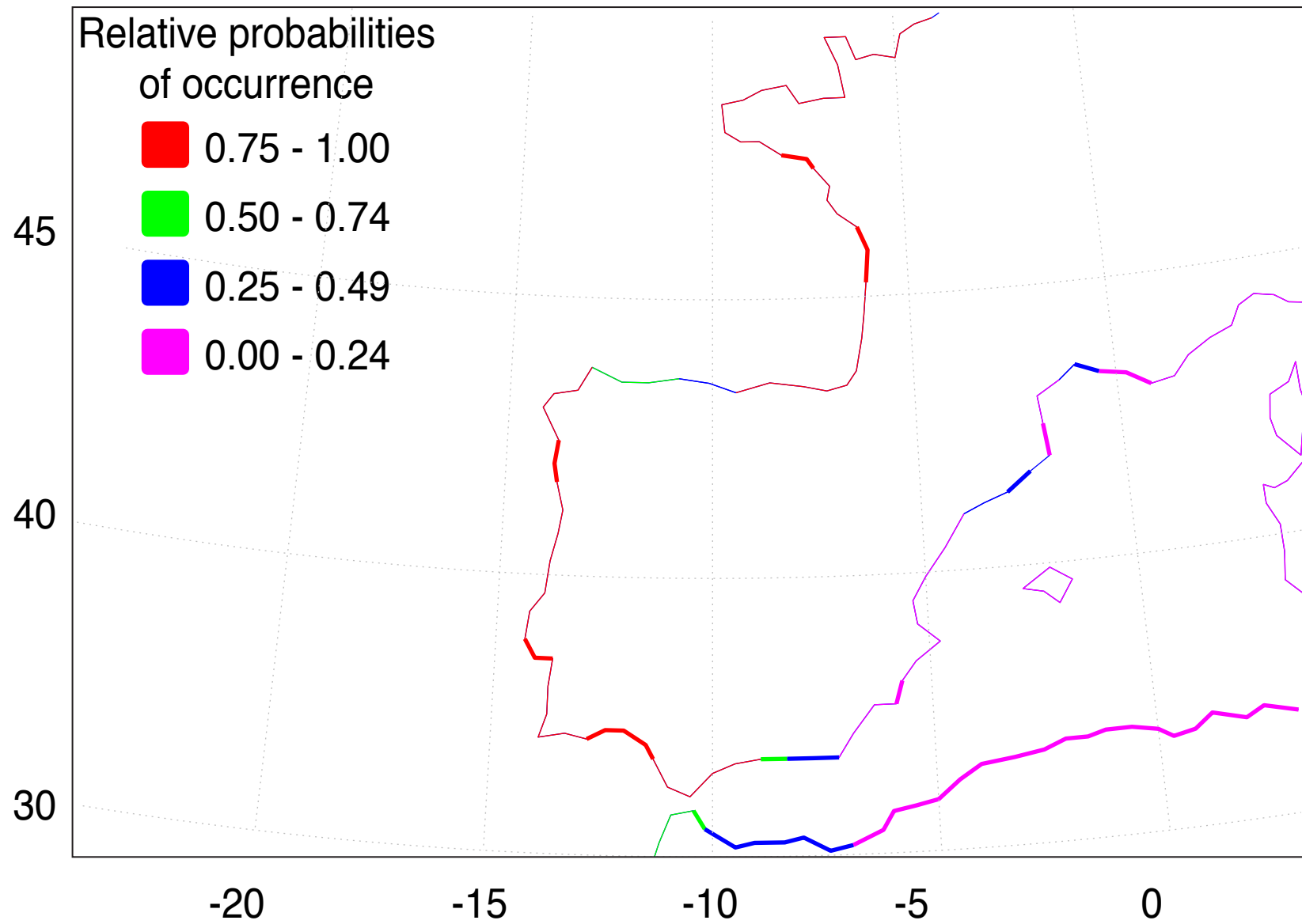


Table 1 (on next page)

Sample details

Sample location, sample abbreviations and summary statistics for a cytochrome *b* sequence fragment from *Fundulus heteroclitus*. ID refers to numbers in Fig 1

1 **Table 1** Sample location, sample abbreviations and summary statistics for a cytochrome *b*
 2 sequence fragment from *Fundulus heteroclitus*. ID refers to numbers in Fig 1

3

Location	ID	Code	Latitude / longitude	n	n_h	n_p
Bridgewater	1	HV	44°22.0'N / 64°31.0'W	15	2	0
Chewonki	2	CM	43°57.3'N / 69°43.2'W	15	3	1
Wells	3	WM	43°19.2'N / 70°34.2'W	15	4	2
Woods Hole	4	WH	41°31.5'N / 70°40.4'W	16	9	4
Jerusalem	5	JR	41°23.1'N / 71°31.5'W	15	5	4
Clinton	6	CC	41°15.3'N / 72°32.8'W	16	7	6
Newark Bay	7	NB	40°41.2'N / 74°06.7'W	15	8	6
Red Bank	8	RE	40°20.9'N / 74°05.0'W	15	7	5
Tuckerton	9	TN	39°32.2'N / 74°19.4'W	15	10	8
Speace	10	SP	38°09.1'N / 75°17.2'W	15	9	6
Suffolk	11	CH	36°51.8'N / 76°28.7'W	16	7	3
Roanoke Island	12	RI	35°53.8'N / 75°36.9'W	15	9	7
Skidaway Island	13	SI	31°56.8'N / 81°04.2'W	16	11	1
Faro	14	RF	37°00.3'N / 07°58.0'W	16	1	0
Cádiz	15	CD	36°31.4'N / 06°11.4'W	17	1	0
Ebro Delta	16	ED	40°37.38'N / 0°39.44'E	16	1	0

4 n , number of individuals; n_h , number of haplotypes; n_p , number of private haplotypes

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Table 2 (on next page)

Aquamaps environmental envelope

Aquamaps default environmental envelope for *F. heteroclitus*

1 **Table 2** Aquamaps default environmental envelope for *F. heteroclitus*

Variable	Absolute	Preferred	Preferred	Absolute
Depth [m]	0	0	3	5
Sea Surface Temperature	4.76	6.41	23.53	26.1
Sea Surface Salinity [SSS;	28.96	29.94	35.74	39.6

2

3