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Population structure in marine macroparasites

1 The most vagile host as the main determinant of population
2 connectivity in marine macroparasites

3

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22

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26

Population structure in marine macroparasites

27 Abstract

28

29 Although molecular ecology of macroparasites is still in its infancy, general patterns are beginning to
30 emerge, e.g., that the most vagile host in a complex life cycle is the main determinant of population
31 genetic structure of their parasites. This insight stems from the observation that populations of parasites
32 with only freshwater hosts are more structured than those with terrestrial or airborne hosts. Until now, the
33 same has not yet been tested for marine systems where, in theory, a fully marine life cycle might sustain
34 high dispersal rates because of the absence of obvious physical barriers in the sea. Here, we tested
35 whether a marine trematode parasite that utilises migratory birds exhibited weaker population genetic
36 structure than those whose life cycle utilises marine fish as the vagile host. Part of the mitochondrial
37 cytochrome *c* oxidase 1 (COI) gene was sequenced from individual sporocysts from populations along the
38 Atlantic coast of Europe and North Africa.

39 Strong population structure ($\Phi_{st} = 0.25$, $P < 0.0001$) was found in the fully marine trematode *Bucephalus*
40 *minimus* (fish), while no significant structure ($\Phi_{st} = 0.015$, $P = 0.19257$) was detected in *Gymnophallus*
41 *choledochus* (birds). However, demographic models indicate recent colonisation rather than high dispersal
42 as an alternative explanation of the low levels of structure observed in *G. choledochus*.

43 Our study is the first to identify significant genetic population structure in a marine autogenic parasite,
44 suggesting that connectivity between populations of marine parasites can be limited despite the general
45 potential for high dispersal of their hosts in the marine environment.

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46 Introduction

47

48 Dispersal connects populations and has important effects on population dynamics, population genetic
49 structure, local adaptation and speciation (e.g., McPeck & Holt 1992; Avise 2000; Lenormand 2002;
50 Greischar & Koskella 2007). Dispersal also has practical implications for the management of natural
51 resources in the face of, e.g., exploitation or climate change (e.g., Walther et al. 2002; Luttikhuisen et al.
52 2003; Di Franco et al. 2012). Dispersal together with subsequent successful reproduction leads to gene
53 flow and counteracts the build-up of population genetic structure.

54

55 In parasites, dispersal and gene flow, though important for the spreading of diseases, are still relatively
56 poorly understood (Criscione et al. 2005; Webb et al. 2013; Blasco-Costa & Poulin 2013). Complex life
57 cycles involving multiple sequential host species are the norm for most macroparasites (Poulin 2007).
58 Furthermore, the scope for movement during the generally short free-living life cycle stages between hosts
59 is limited. This suggests that dispersal and gene flow and, hence, population structure in parasites are
60 mostly determined by the dispersal capacities of their hosts (Prugnolle et al. 2005; Criscione 2008). More
61 specifically, a parasite's dispersal should be shaped by the host's dispersal during the host's life stage that
62 harbours the parasite.

63

64 That host dispersal can be a determinant of parasite population structure has been demonstrated in
65 parasites with terrestrial, air-borne and aquatic hosts (e.g., Blouin et al. 1995; Criscione & Blouin 2004;
66 Blasco-Costa et al. 2012). Particular emphasis has been placed on whether all hosts of a parasite species
67 are aquatic ('autogenic') versus whether its hosts are a combination of species with aquatic and
68 terrestrial/air-borne dispersal ('allogenic'; Esch et al. 1988). The available data suggest that autogenic
69 parasites have more strongly structured populations than allogenic parasites, likely because freshwater-
70 bound dispersal by hosts is more limited due to the fragmented nature of the habitat (Blasco-Costa &

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71 Poulin 2013). The same has not yet been tested for marine parasites; the outcome is not immediately
72 obvious because dispersal by marine hosts such as fish may be expected to be considerable (Cowen et
73 al. 2000). Here, we take a comparative approach and test whether population genetic structure differs
74 between two marine trematode parasites that differ in their type of hosts, namely *Gymnophallus*
75 *choledochus* that uses birds and *Bucephalus minimus* that uses fish as their definitive hosts. Often cryptic
76 species are found in trematodes when molecular work is being done for the first time (e.g. Miura et al.
77 2005; Leung et al. 2009; Hayward 2010; Poulin 2011), which is why we have been alert for that.

78

79 In general, the life cycle of trematodes includes three hosts. In the first intermediate host, sporocysts
80 clonally multiply to and produce cercariae. Cercariae are shed into the water column and infect a second
81 intermediate host, in which they reside as metacercariae. When the second intermediate host is ingested
82 by the definitive host, the parasites reproduce sexually and eggs are subsequently released into the
83 environment. The cycle is completed when the larvae infect the first intermediate host. The two trematode
84 species studied here both use *Cerastoderma edule*, a bivalve mollusc that lives buried in soft intertidal
85 sediments of the northeast Atlantic, as their first intermediate host (Loos-Frank 1969a,b; Maillard 1975,
86 1976; Bartoli & Gibson 2007; Montaudouin et al. 2009; Pina et al. 2009) (Fig. 1).

87

88 For *Gymnophallus choledochus* Odhner, 1900, the cockle is also a facultative second intermediate host.
89 Though the cockle itself has high dispersal potential because of its pelagic larvae, it is unlikely to be an
90 important vector for dispersal of the parasites because: 1) only adult cockles, which are sedentary, carry
91 these parasites; 2) as a shellfish aquaculture product, cockles are rarely transferred among production
92 sites; and 3) cockles have an unexpectedly strong population genetic structure throughout their entire
93 distribution area, which points to very low effective dispersal in spite of having pelagic larvae (Krakau et al.
94 2012, Martinez et al. 2013).

95

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96 Adult polychaetes, in general the second intermediate hosts for *G. choledochus*, have a low dispersal
97 potential because they are bound to the sediment. They are therefore not likely to contribute much to the
98 parasite's dispersal over large distances. The limited capacity of polychaetes as a dispersal vector for
99 parasites is corroborated by genetic studies showing strong population structure even though they may
100 have free swimming larval stages (e.g., Kesaniemi et al. 2012; Zakas & Wares 2012; Chandarana et al.
101 2013).

102

103 The adults of the parasite *G. choledochus* are found in the guts of several bird species, e.g., gulls, ducks
104 and waders (Loos-Frank et al. 1969a,b). In theory, birds could transport gut parasites between distant
105 locations by flying long distances and depositing faeces containing eggs. In particular, seasonally
106 migrating birds are the best candidates for dispersal of parasites over long distances. While in some of
107 these birds seasonal migration is limited within Europe, as e.g. in gulls *Larus argentatus* (Camphuysen et
108 al. 2011), shelducks *Tadorna tadorna* (Kear 2005) and eider ducks *Somateria mollissima* (Tiedemann et
109 al. 2004), other species undertake seasonal migration to varying degrees. These include, e.g., the
110 oystercatcher *Haematopus ostralegus* and the curlew *Numenius arquata*. Also highly migratory small
111 waders such as *Calidris* spp. are thought to serve as final hosts for *G. choledochus* (Loos-Frank et al.
112 1969a,b). Interestingly, species with strong seasonal migration can nonetheless show significant
113 population genetic structure due to homing behaviour. In the dunlin *Calidris alpina*, population structure
114 was even seen to persist in wintering areas (Lopes et al. 2008). It may well be possible that population
115 structure in parasites might then also be copied between breeding and wintering grounds in this species,
116 but probably not for a generalist parasite such as *G. choledochus*. In the case of this parasite we can
117 conclude that there is ample opportunity for long distance dispersal with its final host taxa.

118

119 For the other parasite species studied here, *Bucephalus minimus* (Stossich, 1887), small teleost fish
120 species (such as silversides, gobies, grey mullet, sea bream and flounder) have been described as

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121 second intermediate or paratenic hosts (e.g. Maillard 1975; Faliex & Biagiatti 1987; El-Darsh & Whitfield
122 1999). Long-range dispersal by the adults of some of these species has been studied using direct tagging.
123 The grey mullet *Mugil cephalus*, for example, has been observed to migrate for several hundreds of
124 kilometres, though in one elaborate study in fact more than 90% of the fishes were recaptured within 32
125 km (Whitfield et al. 2012). Similar leptokurtic dispersal has been verified using otolith chemistry in the
126 silverside *Menidia menidia* (Clarke et al. 2010). Genetic studies have shown that significant population
127 structure in small teleosts along the northeast Atlantic coastline indeed exists (Alarcon et al. 2004), as
128 may be expected when long-range dispersal is rare.

129

130 The adults of the parasite *B. minimus* live in the intestines of sea bass *Dicentrarchus labrax*. Though sea
131 bass are active swimmers that seasonally migrate offshore to spawning grounds (Pickett and Pawson
132 1994), significant population structure has been detected between its northeast Atlantic populations (Naciri
133 et al. 1999; Lemaire et al. 2005; Quéré et al. 2010). It has been suggested that homing behaviour may
134 play a role in shaping population genetic structure of sea bass (Bahri-Sfar et al. 2000). Tagging and
135 population dynamic studies indeed have identified stock structure even at the moderate spatial scale of
136 the waters around Great Britain and Ireland (Pawson et al. 2007a,b).

137

138 In summary, the entire complex life cycle of the fish parasite *B. minimus* holds little suggestion that long-
139 range dispersal is likely to occur regularly. Within the complex life cycle of *G. choledochus*, on the other
140 hand, there is opportunity for frequent and far dispersal during its adult life stage when it resides in birds.
141 This contrast leads to a clear and testable hypothesis: that there will be little or no population genetic
142 structure in the bird parasite *G. choledochus*, while relatively strong population structure may be present in
143 *B. minimus*. This hypothesis is tested through a comparison of partial cytochrome *c* oxidase 1 gene
144 sequences between *B. minimus* and *G. choledochus* from several different locations along the East
145 Atlantic coast of Europe and northern Africa.

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146 Materials and methods

147

148

149 Sampling

150

151 Cockles *Cerastoderma edule* were collected by hand or using a rake in the intertidal zone during low
152 water at six locations along the Atlantic coast of Europe and northern Africa between September 2010 and
153 March 2011 (Table 1, Fig. 2). Cockle body tissue was dissected in the lab and squeezed between two
154 glass plates under the dissection microscope in order to screen for macroparasites. Individual sporocysts
155 (clonally reproducing life stage of the trematode in the cockle as first intermediate host) were isolated and
156 stored separately in 100% ethanol.

157

158 DNA extraction, amplification and sequencing

159

160 Genomic DNA was extracted from the individual sporocysts using the GenElute™ Mammalian Genomic
161 DNA kit (SIGMA) according to the Mammalian Tissue protocol (partB), provided by the manufacturer.
162 Because it has been shown that, in general, most individuals within a first intermediate host are likely
163 belong to one clone (e.g. Rauch et al. 2005, Keeney et al. 2007), we sequenced only one individual per
164 host.

165

166 DNA concentration was measured on a Nanodrop to confirm DNA quality and quantity. A 527-basepair
167 (bp) (*Gymnophallus choledochus*) or 587-bp (*Bucephalus minimus*) fragment of the mitochondrial
168 cytochrome *c* oxidase 1 region (COI) was amplified using the primers MplatCOX1-dF (5'-
169 TTWCITTRGATCATAAG-3') and MplatCOX1-dR (5'-TGAAAYAAAYAIIGGATCICCACC-3') for *B. minimus*
170 (Moszczyńska et al. 2009) and a newly developed primer pair for *Gymnophallus choledochus*, namely

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171 FdigF (5'-TTIITTWCGTTRGATCATAAGC-3') and FdigR (5'-GAAAGMAGAAYCAAAATTACGATC-3').
172 Development of primers FdigF and FdigR was done using the program Primer3 (Rozen & Skaletsky 2000)
173 and based on COI sequences of *B. minimus* and eleven other digenean species (Genbank accession
174 numbers NC0111272, NC0121472, NC0025461, NC0025441, NC0023542, NC0096801, NC0025291,
175 NC0080741, NC0080671, NC0025451, EU8765281). PCR for *B. minimus* was performed in 25- μ l reaction
176 volumes containing 1 X PCR buffer, 0.25 mM of each dNTP, 0.5 μ M primer MplatCOX1-dF, 0.5 μ M primer
177 MplatCOX1-dR, 0.025 units Biotherm plus DNA polymerase and 1 μ l undiluted genomic DNA. Each 25- μ l
178 PCR reaction for *G. choledochus* contained 1 X buffer, 0.25 mM of each dNTP, 0.25 μ M primer FdigF,
179 0.25 μ M primer FdigR, 0.4 μ M BSA, 0.025 units Biotherm plus DNA polymerase and 2 μ l undiluted
180 genomic DNA extract. For the amplification, Doppio thermocyclers were used with the following
181 temperature cycling profile: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for
182 30 s (45°C for Fdig primers), and elongation at 72°C for 60 s. The final extension step was at 72°C for 10
183 min.

184
185 The ribosomal DNA (rDNA) internal transcribed spacer 2 (ITS2) was amplified for *G. choledochus* using
186 primers GITS2f (5'-ACTTTGAGCGGTGGATCACT-3') and GITS2r (5'-CCTGTTCCTCGCCGTTACT-3').
187 These primers prime on the flanking regions of ITS2 residing in the 5.8S and 28S rDNA, respectively. The
188 primers were developed using the program Primer3 (Rozen & Skaletsky 2000) based on an alignment of
189 published rDNA sequences for the trematodes *G. choledochus*, *G. australis*, *Meiogymnophallus minutus*
190 and *Bartolus pierrei* (Genbank accession numbers JN381027-JN381030; Pina et al., unpublished). PCR
191 was performed in 20- μ l reaction volumes containing 1 X PCR buffer, 0.25 mM of each dNTP, 1 μ M primer
192 GITS2f, 1 μ M primer GITS2r, 0.40 μ M BSA, 0.005 units Biotherm plus DNA polymerase and 2 μ l undiluted
193 genomic DNA extract. For the amplification, Doppio thermocyclers were used with the following
194 temperature cycling profile: 95°C for 5 min, followed by 35 cycles of 95°C for 60 s, annealing at 52°C for
195 60 s, and elongation at 72°C for 120 s. The final extension step was at 72°C for 5 min.

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196

197 Amplifications were checked with 2% agarose gel electrophoresis. PCR product purification and cycle
198 sequencing was performed at MacroGen Inc. (Seoul, South Korea) on ABI3730 automated sequencers.

199

200

201 Data analyses

202

203 Sequences were aligned by hand using the program BioEdit (Hall 1999). COI sequences of *B. minimus*
204 were cropped to 587 bp and those of *G. choledochus* to 527 bp. ITS2 sequences for *G. choledochus* were
205 cropped to 296 bp with flanking regions of 105 bp for the 5.8S gene downstream and 34 bp of the 28S
206 gene upstream. The taxon identity of the sequences was confirmed to be most similar to other digenean
207 trematodes based on a Genbank's BLAST (<http://blast.ncbi.nlm.nih.gov>). Amino acid translations were
208 examined on the basis of the echinoderm and flatworm mitochondrial genetic code. Minimum spanning
209 networks among haplotypes were estimated using Arlequin 3.5 (Excoffier et al. 2010).

210

211 Partitioning of molecular variance among alternative population groupings was estimated using analysis of
212 molecular variance (AMOVA) in Arlequin 3.5 (Excoffier et al. 2010). Pairwise population Φ_{st} values were
213 calculated also calculated in Arlequin 3.5 (Excoffier et al. 2010) with 10000 permutations. A Bonferroni
214 correction was applied.

215

216 Analyses were conducted with all locations and both species, as well as with the exclusion of some
217 locations and species. This was done to ensure that sites that did not have both species, or sites that did
218 not have similar sample sizes (i.e. Merja Zerga, Celtic Sea and Norsminde Fjord) could not artificially
219 influence the patterns observed in this study. Thus we considered all locations as well as a smaller subset

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220 consisting of the Wadden Sea, English Channel and Arcachon Bay. The latter was done for both
221 parasites.

222

223 To examine demographic population histories, mismatch distributions of the per population pooled data
224 were analysed as described by Rogers & Harpending (1992) and implemented in Arlequin 3.5. Fit to a
225 model of sudden expansion was evaluated based on the sum of squared deviation (SSD) and the
226 raggedness index (RI) statistics.

227

228 Demographic history was also examined by running an extended Bayesian skyline plot (EBSP) analysis
229 as implemented in Beast v1.7.5 (Drummond & Rambaut 2007; Heled & Drummond 2008). The
230 coalescent-based EBSP analyses use a set of DNA sequences sampled from a random mating population
231 to model population size through time (Drummond et al. 2005). This combines phylogenetic and
232 coalescent uncertainties in the same analysis. Neither generation time nor mutation rate is known for the
233 trematodes studied here and therefore the results can only be interpreted in a qualitative sense. A
234 standard molecular clock for mitochondrial DNA of 2% divergence per million years (e.g., Olson et al.
235 2009) was used and a generation time of one year was assumed. Fixed-rate analyses were run using a
236 strict molecular clock and maximum likelihood substitution model as determined using the software
237 MEGA5.2.2 (Tamura et al. 2011), for a chain length of 1×10^7 generations and a burnin time of 10%.
238 Graphical EBSP reconstructions were generated in Tracer v1.5 (Rambaut & Drummond 2007).

239

240

241

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242 Results

243

244 Sequence variation

245

246 From 18,059 cockles dissected, 166 *B. minimus* and 110 *G. choledochus* samples were taken. Infection
247 percentages in adult cockles ranged from 0.00% to 5.34% for *B. minimus* and from 0.05% to 10.1% for *G.*
248 *choledochus* (Table 1). Counting of infections ceased when 30 individuals were collected at a location. A
249 total of 135 *B. minimus* and 90 *G. choledochus* were successfully sequenced for cytochrome *c* oxidase 1,
250 resulting in the detection of 54 and 36 haplotypes, respectively (Genbank accession numbers KF880428 –
251 KF880481 and KF880482 – KF880517, Tables 2 and 3).

252

253 The minimum spanning network of *G. choledochus* COI haplotypes (shown in Fig. 3A) was relatively
254 simple with a central, very abundant, haplotype and most variants within a single mutational step. The
255 star-like structure of the network suggests recent population expansion. The five long branches involved
256 between 10 and >100 mutational steps are consistent with rare and highly diverged haplotypes that are
257 not in equilibrium. For *B. minimus*, the haplotype network is much more complex (Fig. 3B), consisting of
258 several closely related, relatively abundant haplotypes with associated mutational step haplotypes around
259 each one. This type is typically associated with stable demographics.

260

261 The divergent haplotypes in *G. choledochus* form a broad range in their level of divergence from the main
262 haplotype; *p*-distances range from 0.0019 to 0.30 (see also Fig. 3A). They contain no stop codons or any
263 other mutations suggestive of pseudogenes. However, many substitutions translate to amino acid
264 differences at the protein level. Haplotype GyA is the most extreme with 46 amino acid differences (among
265 a total of 175 amino acids examined) and 156 nucleotide differences (among a total of 527 nucleotides)
266 with respect to the most common haplotype GyAF; next are GyB (ten amino acid differences; 81

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267 nucleotide differences); GyX (nine amino acid differences; 17 nucleotide differences); GyG (eight amino
268 acid differences; 13 nucleotide differences); GyC; and GyO (six amino acid differences, and 16 and 15
269 nucleotide differences, respectively).

270

271 The presence of long branches in *G. choledochus* could point to cryptic species. To examine the
272 possibility of cryptic species, we sequenced a representative set of 14 individuals for the ITS2 (internal
273 transcribed spacer 2) and portions of the flanking regions of the ribosomal genes 5.8S and 28S (Genbank
274 accession numbers KF880518 - KF880531). The length of ITS2 was 296 bp in all haplotypes; the portion
275 of 5.8 S sequenced was 105 bp and the portion of 28 S was 34 bp. All sequence portions for 5.8S and
276 28S were identical to the *G. choledochus* rDNA entry in Genbank (JN381029, sampled in Portugal from
277 *Cerastoderma edule*) as well as to the *G. australis* entry (JN381028, sampled in Argentina from
278 *Perumytilus purpuratus*) (Pina, Cremonte & Rodrigues, unpublished). The minimum spanning network
279 among the six ITS2 haplotypes detected is shown in Fig. 4. The most common ITS2 haplotype was
280 identical to the Genbank entry for *G. choledochus* from Portugal, while there were five gaps plus a 21%
281 sequence difference with respect to ITS2 in *G. australis* from Argentina. Comparative COI and ITS2
282 divergences among trematode species pairs were investigated by Vilas et al. (2005). They found
283 differences of as little as 6.3% for COI and 0.3% for ITS2. Given our much higher divergences of 15.3%
284 and 0.68%, respectively, it is probable that at least this haplotype may represent a cryptic species.

285

286 Because pseudogenes (although unlikely) and cryptic speciation may underlie the long branches in *G.*
287 *choledochus* COI, we carried out all population and species level comparisons both with and without a
288 long branch subset. The long branch subset consisted of all haplotypes six or more replacement
289 substitutions away from the main haplotype: GyA, GyB, GyX, GyG, GyC and GyO. This decreased the
290 number of individuals in the *G. choledochus* COI data set by N=11.

291

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292 Population comparisons

293

294 Haplotype diversity for *G. choledochus* ranged from 0.63 in Arcachon Bay to 0.86 in the Celtic Sea (Table
295 2); when excluding the long branch subset, gene diversity ranged from 0.51 in Arcachon Bay to 0.83 in the
296 Celtic Sea. For *B. minimus*, gene diversity ranged from 0.38 in Merja Zerga to 0.93 in Arcachon Bay.

297 Nucleotide diversity for *G. choledochus* ranged from 0.0070 in the English Channel to 0.042 in Arcachon
298 Bay (Table 2), and this reduced to between 0.0016 in Arcachon Bay and 0.0045 in the English Channel. In
299 *B. minimus*, nucleotide diversity ranged from 0.00068 in Merja Zerga to 0.0064 in Arcachon Bay (Table 3).

300

301 For *B. minimus*, the overall level of population differentiation was estimated at $\Phi_{st} = 0.25$ ($P < 0.0001$); for
302 *G. choledochus*, $\Phi_{st} = 0.015$ ($P = 0.192$) (Table 4). The latter analysis is based on a five-sample
303 comparison, *i.e.*, excluding Merja Zerga, which had only one sequence. For comparison, we also analysed
304 the *B. minimus* data without Merja Zerga. This decreased the Φ_{st} value to 0.053 ($P < 0.0001$) (Table 4).

305

306 Pairwise population comparisons were never significant for *G. choledochus* (Table S1-S6).

307

308 For *B. minimus*, pairwise population comparisons (shown in Table S7-9) were significantly different in all
309 but two cases: the Wadden Sea sample did not differ from the English Channel sample, nor did English
310 Channel differ from Arcachon Bay. A small but significant difference, in contrast, was estimated for the
311 Wadden Sea versus Arcachon Bay comparison ($\Phi_{st} = 0.0792$, $P = 0.00059$). The same pattern was visible
312 for pairwise population comparisons between only Wadden Sea, English Channel and Arcachon Bay. In
313 other words, *G. choledochus* shows no structure; *B. minimus* shows structure, only when Arcachon Bay
314 was compared with the Wadden Sea.

315

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316 The demographic analyses were done with groupings based on the AMOVA and pairwise comparison
317 results. Hence, all samples for *G. choledochus* were grouped and analysed together, and all samples for
318 *B. minimus* were analysed separately. Mismatch distributions are shown in Figure S1. The RI statistic
319 rejected a model of sudden population expansion in none of the seven data sets analysed (Table 5). The
320 SSD statistic rejected the null hypothesis of significant population expansion model in two out of seven
321 cases. In *G. choledochus*, a model of expansion was rejected only in case the 'replacement set' of
322 haplotypes was included in the analysis ($P < 0.005$, Table 5). In *B. minimus*, the expansion model was
323 rejected for the sample originating from the Celtic Sea ($P < 0.05$, Table 5).

324

325 Extended Bayesian skyline plots (EBSP) are shown in Figure S2. Five out of seven data sets fitted best to
326 the HKY model of nucleotide substitution, the other two to an HKY+gamma model (Table 5). In all cases,
327 the coalescent analyses supported a model of population change; one population size change had the
328 highest posterior probability in all cases except that of *G. choledochus* with the replacement set of
329 haplotypes included, in which case two population size changes had the highest posterior probability. The
330 95% highest posterior density (HPD) interval included 'zero changes' only for the *B. minimus* Merja Zerga
331 sample, in all other cases 'zero changes' fell outside of the 95% HPD interval (Table 5). Furthermore, all
332 inferred population size changes were expansions, except in the case of *G. choledochus* with the
333 replacement set of haplotypes included, where population size was inferred to have decreased (Fig. S2).
334 Under the (highly uncertain) assumptions of one generation per year and a divergence rate of 2% per
335 million years, all population size increases would be inferred to have taken place between 100,000 and
336 200,000 years ago, both for all *B. minimus* samples and for the *G. choledochus* analysis excluding the
337 replacement set of haplotypes. In the case of population dropout, i.e., when *G. choledochus* was analysed
338 with the replacement set, the decrease was inferred to be extremely recent (Fig. S2).

339

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340 Discussion

341

342 The hypothesis that population genetic structure in trematode parasites is influenced by dispersiveness of
343 the most vagile host - typically the final host - is corroborated by the data presented here. As predicted,
344 the fish parasite *Bucephalus minimus* shows significant population structure along the northeast Atlantic
345 coastline, and the bird parasite *Gymnophallus choledochus* does not. This suggests that populations of *B.*
346 *minimus* are isolated through limited interpopulation dispersal of the trematode throughout its life, and,
347 hence, limited dispersal of the life stages of the hosts it infects. We tentatively draw the conclusion that the
348 general notion that autogenic parasites have more strongly structured populations than allogenic parasites
349 also holds true for the marine realm.

350

351 The data for the fish parasite *B. minimus* clearly show population structure consistent with isolation,
352 although the widely known potential confounding factors related to studying only mitochondrial DNA apply
353 (Ballard & Whitlock 2004). These confounding factors include mitochondrial parental leakage, which has
354 been reported for the trematode *Schistosoma mansoni* (Jannotti-Passos et al. 2001) and could, in
355 principle, account for the extreme haplotypes in *G. choledochus*; introgression of the genome of *G.*
356 *choledochus* by the mitochondria of another species, **which would lead to treating two separate**
357 **species as a single one**; studying a single locus; and non-neutral effects, amongst others selective
358 sweeps and local adaptation (Ballard & Whitlock 2004; Dowling et al. 2008). If selective sweeps had
359 occurred in *B. minimus*, the actual level of isolation would be stronger rather than weaker than we
360 observed and hence, not challenge our conclusions. Local adaptation, however, could be a non-neutral
361 effect to counter our conclusion: if the haplotypes did not establish locally by chance but as a result of
362 locally varying selection pressures on linked nucleotides, there might be no isolation even though
363 differentiation is present.

364

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365 The data for the bird parasite *G. choledochus*, however, are less clear. While the lack of population
366 structure shown by these data may be indicative of population connectivity, they are also consistent with
367 recent colonisation. In the latter case, not enough time would have elapsed since colonisation to allow
368 significant population differentiation to develop. Note that time must be interpreted in a population genetic
369 sense, i.e., as a composite of generation time, effective population size and time in years. In short, the fact
370 that *G. choledochus* is less differentiated could be due to 1) larger effective population size; 2) longer
371 generation time; 3) a more recent (re)colonisation of the study area, as compared to *B. minimus*; and/or 4)
372 more effective gene flow in *G. choledochus*, or a combination of several of these factors.

373

374 The difference observed between the two parasite species is not caused solely by sampling effects. The
375 most strongly differentiating sample in *B. minimus* is from Merja Zerga in Morocco, where we only
376 encountered two infections with *G. choledochus* among a total of 4021 cockles screened (infection level of
377 0.05%). Of these two, only one was successfully sequenced, which gives only anecdotal insight into the
378 genetic variation present at that location: that is of the most frequent haplotype found. When we compare
379 the two trematode species without Merja Zerga, however, the difference in population structure remains
380 present; populations of *B. minimus* are still highly significantly differentiated among the four more northern
381 samples, albeit with a reduced Φ_{ST} value (Table 4).

382

383 Management considerations

384

385 The results presented here are not only adding to our understanding of parasite population structure but
386 are also relevant for fisheries biology and the management of marine resources. Because populations of
387 the parasite *B. minimus* are significantly isolated, all of its hosts must therefore also be isolated. In
388 fisheries biology terms this is referred to as significant and non-transient stock structure (Begg & Waldman
389 1999). Isolation must be demonstrated for both intermediate and definitive hosts, and any paratenic hosts

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390 must either be isolated or are effectively not important as hosts. For some of the hosts involved the
391 existence of population structure has indeed been found (Naciri et al. 1999; Bahri-Sfar et al. 2000; Alarcon
392 et al. 2004; Lemaire et al. 2005; Quéré et al. 2010) but our results suggest that they also should exist for
393 other associated hosts. As many of these hosts are fish, some of which are commercially exploited, it
394 would be interesting to know whether isolation is due to extrinsic factors such as physical barriers caused
395 by oceanic current patterns, or by intrinsic factors related to the biology of the fish. Dispersal can be
396 addressed directly using tagging or otolith chemistry studies (e.g., Clarke et al. 2010; Whitfield et al. 2012)
397 and indirectly with genetic data in which isolation by distance (IBD) may be positively correlated with
398 genetic distance (e.g., Chevolut et al. 2006; Cuveliers et al. 2012; Varela et al. 2013). Here, a pattern
399 suggestive of isolation by distance (IBD) was observed in *B. minimus* for three locations that are situated
400 along the North-South coastline of the European mainland. While no difference was detected between the
401 Wadden Sea sample (North) and the English Channel sample (intermediate), nor between the latter and
402 the Arcachon Bay sample (South), the Wadden Sea sample and the Arcachon Bay sample did reveal a
403 difference (Table S7-9). The density of samples analysed here was, however, not high enough to allow a
404 proper IBD test such as the Mantel test. In general, the use of parasite population genetic surveys to infer
405 population structure of their hosts may be a promising additional tool for fish stock identification in
406 combination with existing approaches that evaluate differences in parasite communities among fish stocks
407 (MacKenzie & Abounza 1998; MacKenzie 2002).

408

409 Demographic history of all populations found in both parasite species was characterised by population
410 expansion. This is most likely related to postglacial colonisation in the more northern locations, while for
411 the southern sites an ephemeral nature of local populations may be the cause of this. For the Celtic Sea
412 sample of *B. minimus*, the significant SSD value may result from mild admixture effects, or perhaps a
413 lingering effect due to refuge populations during the last glacial maximum. However, the EBSP analysis
414 was consistent with population expansion and therefore no conclusions can be drawn on this.

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415

416 Using the complete dataset, **i.e., including the long branches we found**, recent population decline of *G.*
417 *choledochus* was not supported. **Excluding the long branches from these analyses yielded the same**
418 **conclusion of population expansion not being supported (Fig. S1; Table 5).** On the basis of the
419 nuclear sequences, **we may tentatively conclude that the long branches found for this species are**
420 **most likely the result of either non-neutral effects or represent cryptic species. Further research is**
421 **needed to determine whether cryptic species of this parasite truly occur in cockle hosts, and as a**
422 **feasible addition to COI and ITS2 in this non-model taxon we suggest to study variation at other**
423 **nuclear loci using EPIC primers ('exon priming intron crossing', see e.g. Chenuil et al. 2010; Frade**
424 **et al. 2010; Aurelle et al. 2011; Gostel & Weeks 2014).**

425

426 The Bayesian Skyline Plot for *B. minimus* in Merja Zerga, Morocco, shows a very wide 95% HPD interval
427 (Fig. S2), which is probably due to the shallowness of the gene tree in that sample. This means that a
428 larger sample size would be needed from that location to increase certainty about demographic history.

429

430 In general, our finding of a lack of population structure in the bird parasite *G. choledochus* is in line with
431 the only other existing population genetic study on marine allogenic parasites. Populations of the two
432 trematodes *Maritrema novaezealandensis* and *Philophthalmus* sp. in New Zealand failed to show
433 significant genetic population structure. Both species infect the intertidal snail *Zeacumantus subcarinatus*
434 as the first intermediate host and use birds as their definitive hosts, suggesting that bird dispersal secures
435 high connectivity among coastal populations of the two allogenic parasites (Keeney et al. 2008, 2009). In
436 contrast, comparable studies on the genetic population structure in autogenic parasites (using fish as
437 definitive hosts) have been lacking so far. Vilas et al. (2004) reported allozyme data from three autogenic
438 trematodes in fish hosts from the Portuguese coast but the study covered only a small spatial scale
439 (maximum geodesic distance between populations approximately 800 km compared to 2700 km in our

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440 study) and later investigations suggest the existence of cryptic species (Criscione et al. 2011), which
441 renders the study unsuitable to estimate large-scale population structure in these parasite species. Hence,
442 our study is the first to identify significant genetic population structure in a marine autogenic parasite,
443 suggesting that connectivity between populations of marine parasites can be limited despite the general
444 potential for high dispersal of their hosts in the marine environment (Cowen et al. 2000). A dispersal
445 limitation of autogenic parasites is also suggested by comparative macroecological studies. For example,
446 the similarity of trematode parasite communities between two host populations decreases at a faster rate
447 with their environmental distance in autogenic than in allogenic parasites (Thieltges et al. 2009). The
448 difference in the “distance decay” is attributed to a better long-distance dispersal capacity of bird
449 compared to fish hosts (Thieltges et al. 2009) and thought to underly the presence of a positive
450 abundance-occupancy relationship in allogenic trematodes in snail first intermediate host populations
451 while absent in autogenic parasites (Thieltges et al. 2013). In line with these macroecological studies, our
452 data suggest that the allogenic-autogenic divide known from freshwater and terrestrial systems (Esch et
453 al. 1988; Blouin et al. 1995; Criscione & Blouin 2004; Blasco-Costa et al. 2012) also holds true in the
454 marine realm, i.e. fish disperse their parasites less broadly than avian hosts do. However, further analyses
455 on the population genetics of marine parasites with differential hosts use, ultimately allowing for
456 comparative analyses, will be needed to verify the generality of this rule. We consider our study to be an
457 important first step into this direction.

458

459

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468

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658

659 Data Accessibility

660

661 DNA sequences: Genbank accessions KF880428 – KF880531.

662 ESBP figures: Supplementary information.

663

664

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665 Author Contributions

666

667 Designed research: MEF, PCL, DWT and JLO. Data collection and performed research: MEF, DWT, PCL,
668 XM, KTJ, HB and SCC. Analysed data: MEF and PCL. Wrote the paper: MEF, PCL, DWT and JLO. This
669 study was part of MEF's Master thesis, supervised by PCL, DWT and JLO.

670

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671 Figure captions

672

673 Fig. 1 - Schematic representation of life cycle of trematode parasites *Gymnophallus choledochus* (A) and
674 *Bucephalus minimus* (B), after Loos-Frank (1969) and Maillard (1976); FIH = first intermediate host,
675 hosting sporocysts; SIH = second intermediate host, hosting metacercariae; FH = final host, hosting adult
676 trematodes.

677

678 Fig. 2 - Map of sampling locations for cockles *Cerastoderma edule*, showing pie charts of cytochrome *c*
679 oxidase 1 haplotype frequencies in parasite species *Gymnophallus choledochus* and *Bucephalus*
680 *minimus*. A = Norsminde Fjord, Denmark; B = Wadden Sea, the Netherlands; C = Celtic Sea, Ireland; D =
681 Somme, France; E = Arcachon Bay, France; F = Merja Zerga, Morocco. Colours correspond to those in
682 Fig. 3.

683

684 Fig. 3 - Minimum spanning networks for cytochrome *c* oxidase 1 haplotypes (circles) in *Gymnophallus*
685 *choledochus* (A) and *Bucephalus minimus* (B). Number of nucleotide differences is proportional to branch
686 length (shortest branches represent one nucleotide difference), unless indicated otherwise. Frequency of
687 observation is proportional to circle area. Colours represent different clusters of haplotypes.

688

689 Fig. 4 - Minimum spanning network for internal transcribed spacer haplotypes in *Gymnophallus* spp.
690 Colours correspond to those in Fig. 3. The *G. choledochus* haplotypes that are one or two steps removed
691 are GyC from the Wadden Sea and Arcachon Bay; GyB from Norsminde Fjord; and GyAG and GyY from
692 the English Channel. The other GyC are all from the Wadden Sea. GyAF has two sequences from
693 Arcachon Bay and one from Wadden Sea, English Channel, Celtic Sea and Merja Zerga.

694

695

Population structure in marine macroparasites

696 Fig. S1 - Mismatch distributions on the basis of observed data (broken lines) and under a model of
697 population expansion (continuous lines) for *Bucephalus minimus* and *Gymnophallus choledochus*.
698 Mismatch value equals the number of nucleotide differences between haplotypes.

699

700 Fig. S2 - Extended Bayesian Skyline Plots for *Bucephalus minimus* and *Gymnophallus choledochus*. For
701 all plots, x-axis indicates time in millions of years ago, y-axis is effective population size N_e . Continuous
702 line equals the median estimate and broken lines are upper and lower limit of the 95% HPD (Highest
703 Posterior Density) estimate.

Population structure in marine macroparasites

704 Table 1 - Trematode parasites *Bucephalus minimus* (*B*) and *Gymnophallus choledochus* (*G*) collected
 705 from cockles as first intermediate host; N_{cockles} = total number of cockles dissected; N_{parasites} = number of
 706 parasites successfully sequenced for cytochrome c oxidase I. Only one sequence was obtained per
 707 individual host.

708

Location	Coordinates	Collection date	infection (%)		N _{cockles}	N _{parasites}	
			<i>B</i>	<i>G</i>		<i>B</i>	<i>G</i>
Norsminde Fjord, Denmark	56°01'N 10°15'E	1, 14 March 2011	0.00	10.1	187	0	12
Wadden Sea, The Netherlands	53°03'N 04°45'E	20 September; 4, 7, 12, 22 October; 6, 8 December 2010	1.92	1.51	1992	28	29
Celtic Sea, Ireland	51°38'N 08°41'W	7, 14, 18 February 2011	1.26	0.21	4295	27	7
English Channel, France	50°14'N 01°35'E	26, 27 February 2011	4.00	1.05	2863	31	25
Arcachon Bay, France	44°35'N 01°14'W	3, 4 November 2010	5.34	0.43	4701	23	16
Merja Zerga, Morocco	34°52'N 06°16'W	10, 11, 14, 19 January 2011	0.88	0.05	4021	26	1

709

710

711 Table 2 - Haplotype frequencies for 527 bp fragment of cytochrome *c* oxidase 1 in *Gymnophallus choledochus*; π = nucleotide diversity with standard deviation; *h*
 712 = haplotype diversity; standard deviations between brackets.

713

Location	Gy A	Gy B	GyC	GyD	GyE	GyF	G y G	GyH	GyI	GyJ	GyK	GyL	GyM	GyN	GyO	GyP	GyQ	GyR	GyS	GyT
Norsminde, Denmark		1		1	1		1	1	1											
Wadden Sea, Texel			4			1			1	1	1	1	1		1		1	1	2	1
Celtic Sea, Ireland			1																	
English Channel, France				1					2					1				1		
Arcachon Bay, France	1		1													1				
Merja Zerga, Morocco																				
sum	1	1	6	2	1	1	1	1	4	1	1	1	1	1	1	1	1	2	2	1

(continued)	GyU	G y V	GyW	Gy X	GyY	G y Z	GyAA	GyAB	GyA C	G y D	GyAE	G y F	GyAG	Gy AH	GyAI	GyA J	Sum	π (stdev)	<i>h</i> (stdev)
Norsminde, Denmark													6				12	0.0314 (0.0170)	0.773 (0.128)
Wadden Sea, Texel	1	1											11				29	0.0108 (0.00594)	0.847 (0.0607)
Celtic Sea, Ireland								1		1	1		3				7	0.0106 (0.00662)	0.857 (0.137)
English Channel, France	1	1		1	1	1	1						10	1	1	1	25	0.00701 (0.00408)	0.847 (0.0718)

Arcachon Bay, France			1							1	1							16	0.0418 (0.0218)	0.625 (0.139)			
Merja Zerga, Morocco																		1	0.0000 (0.0000)	1.00 (0.0000)			
sum	2	2	1		1	1	1	1	1	1	2	1						41	1	1	1	1	90

714

715 Table 3 - Haplotype frequencies for 587 bp fragment of cytochrome *c* oxidase 1 in *Bucephalus minimus*; π = nucleotide diversity with standard deviation; h =
716 haplotype diversity; standard deviations between brackets.

717

Location	LaA	LaB	LaC	LaD	LaE	LaF	LaG	LaH	LaI	LaJ	LaK	LaL	LaM	LaN	LaO	LaP	LaQ	LaR	LaS	LaT
Wadden Sea, Texel	1							1								1	1	1		
Celtic Sea, Ireland						1				8	1		1	1				1	1	1
English Channel, France					1		1											1		
Arcachon Bay, France		2	1	1	2										1					
Merja Zerga, Morocco		20							5		1									
sum	1	22	1	1	3	1	1	1	5	8	1	1	1	1	1	1	1	3	1	1

(continued)

	LaU	LaV	LaW	LaX	LaY	LaZ	LaAA	LaAB	LaAC	LaAD	LaAE	LaAF	LaAG	LaAH	LaAI	LaAJ	LaAK	LaAL	LaAM	LaAN
--	-----	-----	-----	-----	-----	-----	------	------	------	------	------	------	------	------	------	------	------	------	------	------

Wadden Sea, Texel	1								1							1	1	1		
Celtic Sea,		2			1	1						1	1	1					1	

Ireland																					
English Channel, France			1								4				1			1			2
Arcachon Bay, France			1				1	1	1		1										1
Merja Zerga, Morocco																					
sum	1	2	1	1	1	1	1	1	1	1	1	5	1	1	1	1	1	2	1	3	

(continued)	LaAO	LaAP	LaAQ	LaAR	LaAS	LaAT	LaAV	LaAW	LaAX	LaAY	LaAZ	LaBA	LaBB	LaBC	LaC	sum	π (stdev)	h (stdev)
Wadden Sea, Texel	1	1	14			1	1									28	0.003569 (0.002287)	0.7593 (0.0891)
Celtic Sea, Ireland			4													27	0.005387 (0.003204)	0.9003 (0.0461)
English Channel, France			12	1		1			1			1		2		31	0.003004 (0.001991)	0.8366 (0.0592)
Arcachon Bay, France			6		1			1		2	1			1		23	0.006437 (0.003753)	0.9328 (0.0424)
Merja Zerga, Morocco																26	0.000681 (0.000728)	0.3846 (0.1017)
sum	1	1	36	1	1	2	1	1	1	2	1	1	2	1	135			

718
719

720 Table 4 - Results from Analyses of Molecular Variance (AMOVA) on partial cytochrome *c* oxidase 1
721 sequences in *Bucephalus minimus* ('*B*') and *Gymnophallus choledochus* ('*G*'). Samples are indicated as:
722 N = Norsminde, Denmark; W = Wadden Sea, the Netherlands; C = Celtic Sea, Ireland; E = English
723 Channel, France; A = Arcachon Bay, France; M = Merja Zerga, Morocco. 'Full' data set indicates no data
724 were omitted from the analysis; 'without repl.' means the 'replacement set' of lineages, i.e. with an excess
725 of replacement mutations (see main text for details), were omitted from the analysis.

species	samples	data set	Φ_{st}	<i>P</i>
<i>G</i>	NWCEA	full	0.0150	0.192
<i>G</i>	NWEA	full	0.0262	0.0590
<i>G</i>	WEA	full	0.0341	0.0284
<i>G</i>	NWCEA	without repl.	-0.0077	0.721
<i>G</i>	NWEA	without repl.	-0.0047	0.653
<i>G</i>	WEA	without repl.	-0.0025	0.553
<i>B</i>	WCEAM	full	0.250	<0.00001
<i>B</i>	WCEA	full	0.0532	<0.00001
<i>B</i>	WEA	full	0.0434	0.00178

726

727

728 Table 5 - Results of demographic analyses for *Gymnophallus choledochus* and *Bucephalus minimus*
729 based on partial cytochrome *c* oxidase 1 sequences. RI = raggedness index of mismatch distribution; SSD
730 = sum of squared deviation between observed and expected mismatch distributions. *G* = *G. choledochus*;
731 *B* = *B. minimus*; N = Norsminde Fjord, Denmark; W = Wadden Sea, the Netherlands; C = Celtic Sea,
732 Ireland; E = English Channel, France; A = Arcachon Bay, France; M = Merja Zerga, Morocco; 'with/without
733 repl.' indicates whether the 'replacement set' of lineages, i.e. with an excess of replacement mutations
734 (see main text for details), were omitted from analyses; 'subst. model' = substitution model with best fit to
735 data; N_{changes} = 95% highest posterior density interval of number of population size changes supported by
736 the data and coalescent model with mode in bold.

737

species	data set	RI	SSD	subst. model	N _{changes}
<i>G</i>	NWCEA with repl.	0.0424	0.169**	HKY+gamma	[2,3,4]
<i>G</i>	NWCEA without repl.	0.0627	0.00333	HKY	[1,2,3]
<i>B</i>	W	0.0653	0.0174	HKY	[1,2,3]
<i>B</i>	C	0.0208	0.124*	HKY+gamma	[1,2,3]
<i>B</i>	E	0.0830	0.00648	HKY	[1,2,3]
<i>B</i>	A	0.0167	0.00198	HKY	[1,2,3]
<i>B</i>	M	0.186	0.00808	HKY	[0,1,2,3]

738 * $P < 0.05$; ** $P < 0.005$

739

740

741 Table S1 - Pairwise population comparisons for *Gymnophallus choledochus* with replacement set based
 742 on partial cytochrome *c* oxidase 1 sequences. Values in bold are significantly different from 0 after
 743 Bonferroni correction (corrected *P*-level 0.005). *P*-values are above diagonal.

744

	Norsminde Fjord	Wadden Sea	Celtic Sea	English Channel	Arcachon Bay
Norsminde Fjord	-	0.05990	0.65003	0.09405	0.85289
Wadden Sea	0.04927	-	0.91793	0.01901	0.27572
Celtic Sea	-0.03396	-0.07857	-	0.20958	0.81269
English Channel	0.04072	0.07996	0.03715	-	0.10850
Arcachon Bay	-0.02852	0.01461	-0.06384	0.02420	-

745

746

747 Table S2 - Pairwise population comparisons for *Gymnophallus choledochus* with replacement set, but
 748 without the Celtic Sea sample, based on partial cytochrome *c* oxidase 1 sequences. Values in bold are
 749 significantly different from 0 after Bonferroni correction (corrected *P*-level 0.00833). *P*-values are above
 750 diagonal.

751

	Norsminde Fjord	Wadden Sea	English Channel	Arcachon Bay
Norsminde Fjord	-	0.06009	0.08356	0.85625
Wadden Sea	0.04927	-	0.01950	0.27522
English Channel	0.04072	0.07996	-	0.10732
Arcachon Bay	-0.02852	0.01461	0.02420	-

752

753

754 Table S3 - Pairwise population comparisons for *Gymnophallus choledochus* with replacement set, but
 755 without the Celtic Sea and Norsminde Fjord samples, based on partial cytochrome *c* oxidase 1
 756 sequences. Values in bold are significantly different from 0 after Bonferroni correction (corrected *P*-level
 757 0.0167). *P*-values are above diagonal.

758

	Wadden Sea	English Channel	Arcachon Bay
Wadden Sea	-	0.02228	0.27770
English Channel	0.07996	-	0.10989
Arcachon Bay	0.01461	0.02420	-

759

760 Table S4 - Pairwise population comparisons for *Gymnophallus choledochus* without replacement set
 761 based on partial cytochrome *c* oxidase 1 sequences. Values in bold are significantly different from 0 after
 762 Bonferroni correction (corrected *P*-level 0.005). *P*-values are above diagonal.

763

	Norsminde Fjord	Wadden Sea	Celtic Sea	English Channel	Arcachon Bay
Norsminde Fjord	-	0.70597	0.31561	0.69805	0.14850
Wadden Sea	-0.01160	-	0.69369	0.40610	0.91229
Celtic Sea	0.01333	-0.01817	-	0.88407	0.50965
English Channel	-0.01460	0.00270	-0.03579	-	0.51737
Arcachon Bay	0.01583	-0.01354	0.00013	-0.00449	-

764

765

766 Table S5 - Pairwise population comparisons for *Gymnophallus choledochus* without replacement set, but
 767 without the Celtic Sea sample, based on partial cytochrome *c* oxidase 1 sequences. Values in bold are
 768 significantly different from 0 after Bonferroni correction (corrected *P*-level 0.00833). *P*-values are above
 769 diagonal.

770

	Norsminde Fjord	Wadden Sea	English Channel	Arcachon Bay
Norsminde Fjord	-	0.71062	0.69221	0.14791
Wadden Sea	-0.01160	-	0.41382	0.90912
English Channel	-0.01460	0.00270	-	0.53054
Arcachon Bay	0.01583	-0.01354	-0.00449	-

771

772

773 Table S6 - Pairwise population comparisons for *Gymnophallus choledochus* without replacement set, but
 774 without the Celtic Sea and Norsminde Fjord samples, based on partial cytochrome *c* oxidase 1
 775 sequences. Values in bold are significantly different from 0 after Bonferroni correction (corrected *P*-level
 776 0.0167). *P*-values are above diagonal.

777

	Wadden Sea	English Channel	Arcachon Bay
Wadden Sea	-	0.40996	0.91377
English Channel	0.00270	-	0.52391
Arcachon Bay	-0.01354	-0.00449	-

778

779 Table S7 - Pairwise population comparisons for *Bucephalus minimus* based on partial cytochrome *c*
 780 oxidase 1 sequences. Values in bold are significantly different from 0 after Bonferroni correction (corrected
 781 *P*-level 0.005). *P*-values are above diagonal.

782

	Wadden Sea	Celtic Sea	English Channel	Arcachon Bay	Merja Zerga
Wadden Sea	-	0.00000	0.50787	0.00059	0.00000
Celtic Sea	0.05946	-	0.00020	0.00307	0.00000
English Channel	-0.00130	0.06758	-	0.03109	0.00000
Arcachon Bay	0.07916	0.05913	0.04671	-	0.00000
Merja Zerga	0.60575	0.43821	0.62259	0.37205	-

783

784

785 Table S8 - Pairwise population comparisons for *Bucephalus minimus* without Merja Zerga sample based
 786 on partial cytochrome *c* oxidase 1 sequences. Values in bold are significantly different from 0 after
 787 Bonferroni correction (corrected *P*-level 0.00833). *P*-values are above diagonal.

788

	Wadden Sea	Celtic Sea	English Channel	Arcachon Bay
Wadden Sea	-	0.00059	0.49025	0.00030
Celtic Sea	0.05946	-	0.00010	0.00347
English Channel	-0.00130	0.06758	-	0.02980
Arcachon Bay	0.07916	0.05913	0.04671	-

789

790

791 Table S9 - Pairwise population comparisons for *Bucephalus minimus* without Merja Zerga and Celtic Sea
 792 samples, based on partial cytochrome *c* oxidase 1 sequences. Values in bold are significantly different
 793 from 0 after Bonferroni correction (corrected *P*-level 0.0167). *P*-values are above diagonal.

794

	Wadden Sea	English Channel	Arcachon Bay
Wadden Sea	-	0.49886	0.00079
English Channel	-0.00130	-	0.02604
Arcachon Bay	0.07916	0.04671	-

795

796