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

Marieke E. Feis [1,2,7*], David W. Thieltges [1], Jeanine L. Olsen [2], Xavier de Montaudouin [3], K. Thomas Jensen [4], Hocein Bazaïri [5], Sarah C. Culloty [6], and Pieternella C. Luttikhuizen [1].

[1] NIOZ Royal Netherlands Institute for Sea Research, P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands

[2] Marine Benthic Ecology and Evolution Group, Centre for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

[3] Station Marine d'Arcachon, UMR EPOC 5805 Université de Bordeaux CNRS, 2 rue du Pr Jolyet, F-33120 Arcachon, France

[4] Aquatic Biology, Department of Bioscience, University of Aarhus, Ole Worms Allé 1, Bld. 1135, DK-8000 Aarhus C, Denmark

[5] Faculté des Sciences, Université Mohammed V-Agdal, Avenue Ibn Battouta, B.P. 1014 RP, 10106 Rabat, Morocco

[6] School of Biological, Earth and Environmental Sciences, University College Cork, Enterprise Centre, Distillery Fields, North Mall, Cork, Ireland

[7] Corresponding author, email: marieke.feis@awi.de

* Current address: Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research, Wadden Sea Station Sylt, Hafenstraße 43, 25992 List/Sylt, Germany, Fax: +494651956200

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Abstract

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

Sampling

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

DNA extraction, amplification and sequencing

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

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

FdigF (5’-TTIITTWCGTTRGATCATAAGC-3’) and FdigR (5’-GAAAGMAGAAYCAAAATTACGATC-3’). Development of primers FdigF and FdigR was done using the program Primer3 (Rozen & Skaletsky 2000) and based on COI sequences of *B. minimus* and eleven other digenean species (Genbank accession numbers NC0111272, NC0121472, NC0025461, NC0025441, NC0023542, NC0096801, NC0025291, NC0080741, NC0080671, NC0025451, EU8765281). PCR for *B. minimus* was performed in 25-µl reaction volumes containing 1 X PCR buffer, 0.25 mM of each dNTP, 0.5 µM primer MplatCOX1-dF, 0.5 µM primer MplatCOX1-dR, 0.025 units Biotherm plus DNA polymerase and 1 µl undiluted genomic DNA. Each 25-µl PCR reaction for *G. choledochus* contained 1 X buffer, 0.25 mM of each dNTP, 0.25 µM primer FdigF, 0.25 µM primer FdigR, 0.4 µM BSA, 0.025 units Biotherm plus DNA polymerase and 2 µl undiluted genomic DNA extract. For the amplification, Doppio thermocyclers were used with the following temperature cycling profile: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 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 min.

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

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

Data analyses

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

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

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

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

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

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

Results

Sequence variation

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

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

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

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

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

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

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 Φ_{st} = 0.25 (*P*<0.0001); for
302 *G. choledochus*, Φ_{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 (Φ_{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

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

Discussion

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

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

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

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

Management considerations

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

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

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

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

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

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

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

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468

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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

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

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

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

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

			infection (%)			$N_{parasites}$	
Location	Coordinates	Collection date	*B*	*G*	$N_{cockles}$	*B*	*G*
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

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	**GyA**	**GyB**	**GyC**	**GyD**	**GyE**	**GyF**	**GyG**	**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**	**GyV**	**GyW**	**GyX**	**GyY**	**GyZ**	**GyAA**	**GyAB**	**GyAC**	**GyAD**	**GyAE**	**GyAF**	**GyAG**	**GyAH**	**GyAI**	**GyAJ**	**Sum**	***π* (stdev)**	***h* (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	1	25	0.00701 (0.00408)	0.847 (0.0718)

Arcachon Bay, France			1						1	1		10					16	0.0418 (0.0218)	0.625 (0.139)
Merja Zerga, Morocco												1					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		

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

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**	**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		

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}	*P*
G	NWCEA	full	0.0150	0.192
G	NWEA	full	0.0262	0.0590
G	WEA	full	0.0341	0.0284
G	NWCEA	without repl.	-0.0077	0.721
G	NWEA	without repl.	-0.0047	0.653
G	WEA	without repl.	-0.0025	0.553
B	WCEAM	full	0.250	<0.00001
B	WCEA	full	0.0532	<0.00001
B	WEA	full	0.0434	0.00178

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

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

* *P*<0.05; ** *P*<0.005

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

	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	-

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

	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	-

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

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

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

	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	-

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

	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	-

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

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

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

	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**	-

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

	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	-

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

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