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

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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 the 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 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* (hosted by fish), while no significant structure (Φ_{st} = 0.015, p = 0.19257) was detected in *Gymnophallus choledochus* (hosted by 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.

KEY WORDS: Marine ecology · Population genetics · Parasite · Host parasite dynamics · Trematode · Invertebrates · *Gymnophallus choledochus* · *Bucephalus minimus*

INTRODUCTION

Dispersal connects populations and has important effects on population dynamics, population genetic structure, local adaptation and speciation (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

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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, Blasco-Costa & Poulin 2013, Webb et al. 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, 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.

The fact 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). In the present study, we take a comparative approach and test whether population genetic structure differs between 2 marine trematode parasites that differ in their type of hosts, namely *Gymnophallus choledochus* Odhner, 1900, that uses birds and *Bucephalus minimus* (Stossich, 1887) 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), and we have been alert for that possibility.

In general, the life cycle of trematodes includes 3 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 2 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, de Montaudouin et al. 2009, Pina et al. 2009) (Fig. 1).

For *Gymnophallus choledochus*, 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 *Gymnophallus 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 of polychaetes 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 *Gymnophallus choledochus* are found in the guts of several bird species, e.g. gulls, ducks and waders (Frank 1969, Loos-Frank 1969). 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* (Frank 1969, Loos-Frank 1969). Interestingly, species

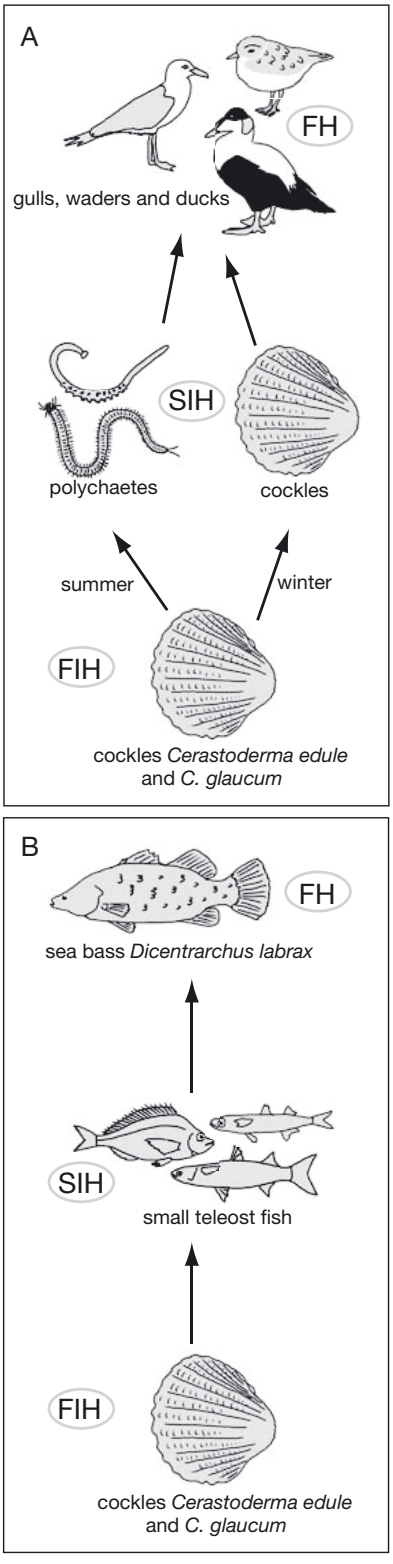

Fig. 1. Life cycle of trematode parasites (A) *Gymnophallus choledochus* and (B) *Bucephalus minimus*, after Loos-Frank (1969) and Maillard (1976). FIH: first intermediate host, hosting sporocysts; SIH: second intermediate host, hosting metacercariae; FH: final host, hosting adult trematodes

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*, small teleost fish species (such as silversides, gobies, grey mullet, sea bream and flounder) have been described as second intermediate or paratenic hosts (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 >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 *Bucephalus minimus* live in the intestines of sea bass *Dicentrarchus labrax*. Though sea bass are active swimmers that seasonally migrate offshore to spawning grounds (Pickett & Pawson 1994), significant population structure has been detected between northeast Atlantic sea bass 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 *Bucephalus minimus* holds little suggestion that long-range dispersal is likely to occur regularly. Within the complex life cycle of *Gymnophallus choledochus*, in contrast, 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 6 locations along the Atlantic coast of Europe and northern Africa between September 2010 and March 2011 (Table 1, see Fig. 2). Cockle body tissue was dissected in the lab and squeezed between 2 glass plates under the dissection microscope 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 1 clone (e.g. Rauch et al. 2005, Keeney et al. 2007), we sequenced only 1 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′-TTW CIT TRG ATC ATA AG-3′) and MplatCOX1-dR (5′-TGA AAY AAY AII GGA TCI CCA CC-3′) for *B. minimus* (Moszczynska et al. 2009) and a newly developed primer pair for *G. choledochus*, namely FdigF (5′-TTI ITT WCG TTR GAT CAT AAG C-3′) and FdigR (5′-GAA AGM AGA AYC AAA ATT ACG ATC-3′). The primers FdigF and FdigR were developed using the program Primer3 (Rozen & Skaletsky 2000) and based on COI sequences of *B. minimus* and 11 other digenean species (GenBank accession numbers NC0111272, NC0121472, NC0025461, NC0025441, NC0023542, NC0096801, NC0025291, NC0080741, NC0080671, NC0025451 and EU8765281). Polymerase chain reaction (PCR) for *B. minimus* was performed in 25 µl reaction volumes containing 1X 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 1X 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 *Gymnophallus choledochus* using primers GITS2f (5′-ACT TTG AGC GGT GGA TCA CT-3′) and GITS2r (5′-CCT GTT CAC TCG CCG TTA CT-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 to JN381030; S. Pina, F. Cremonte, P. Rodrigues pers. comm.). PCR was performed in 20 µl reaction volumes containing 1X 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 (Seoul, South Korea) on ABI3730 automated sequencers.

Data analyses

Sequences were aligned by hand using the program BioEdit (Hall 1999). COI sequences of *Bucephalus minimus* were cropped to 587 bp and those of *Gymnophallus choledochus* to 527 bp. ITS2 sequen-

ces 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 & Lischer 2010).

Partitioning of molecular variance among alternative population groupings was estimated using analysis of molecular variance (AMOVA) in Arlequin 3.5 (Excoffier & Lischer 2010). Pairwise population Φ_{st} values were also calculated in Arlequin 3.5 (Excoffier & Lischer 2010) with 10 000 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 of the 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. The 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 approach 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 (Olson et al. 2009) was used, and a generation time of 1 yr 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 burn-in time of 10%. Graphical EBSP reconstructions were generated in Tracer v1.5 (Rambaut & Drummond 2007).

RESULTS

Sequence variation

From 18 059 cockles dissected, 166 *Bucephalus minimus* and 110 *Gymnophallus 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 COI (Fig. 2), resulting in the detection of 54 and 36 haplotypes, respectively (GenBank accession numbers KF880428 to KF880481 and KF880482 to KF880517; Tables 2 & 3).

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 1. Only 1 sequence was obtained per individual host

Location	Coordinates	Collection date	Infection (%)			$N_{parasites}$	
			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	30	25
Arcachon Bay, France	44° 35′ N 01° 14′ W	3, 4 November 2010	5.34	0.43	4701	24	16
Merja Zerga, Morocco	34° 52′ N 06° 16′ W	10, 11, 14, 19 January 2011	0.88	0.05	4021	26	1

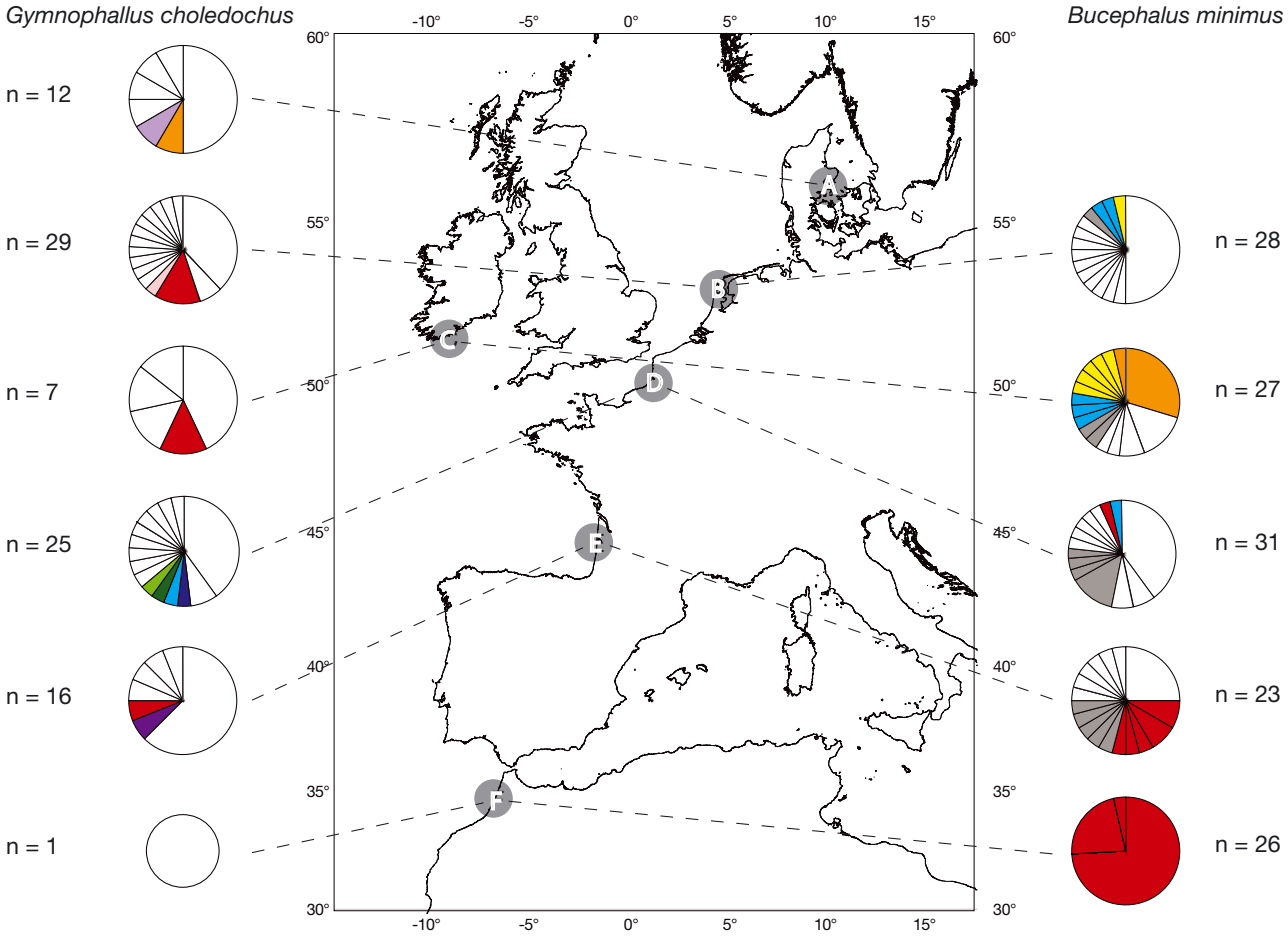

Fig. 2. Map of sampling locations for cockles *Cerastoderma edule*, showing pie charts of cytochrome *c* oxidase 1 haplotype frequencies in parasite species *Gymnophallus choledochus* and *Bucephalus minimus*. A: Norsminde Fjord, Denmark; B: Wadden Sea, the Netherlands; C: Celtic Sea, Ireland; D: English Channel, France; E: Arcachon Bay, France; F: Merja Zerga, Morocco. Colours represent different clusters of haplotypes and correspond to those in Fig. 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 5 long branches involved between 10 and >100 mutational steps are consistent with rare and highly diverged haplotypes that are not in equilibrium. For *Bucephalus 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 *Gymnophallus 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 (10 amino acid differences; 81 nucleotide differences), GyX (9 amino acid differences; 17 nucleotide differences), GyG (8 amino acid differences; 13 nucleotide differences), GyC and GyO (6 amino acid differences, and 16 and 15 nucleotide differences, respectively).

The presence of long branches in *Gymnophallus choledochus* could point to cryptic species. To examine the possibility of cryptic species, we sequenced a representative set of 14 individuals for the ITS2 and portions of the flanking regions of the ribosomal genes 5.8S and 28S (GenBank accession numbers KF880518 to KF880531). The length of ITS2 was 296

Table 2. Haplotype frequencies for 527 bp fragment of cytochrome *c* oxidase 1 in *Gymnophallus choledochus*; π: nucleotide diversity with standard deviation; *h*: haplotype diversity; standard deviations (SD) between brackets

Haplo-type	Norsminde Fjord, Denmark	Wadden Sea, The Netherlands	Celtic Sea, Ireland	English Channel, France	Arcachon Bay, France	Merja Zerga, Morocco	Sum
GyA					1		**1**
GyB	1						**1**
GyC		4	1		1		**6**
GyD	1			1			**2**
GyE	1						**1**
GyF		1					**1**
GyG	1						**1**
GyH	1						**1**
GyI	1	1		2			**4**
GyJ		1					**1**
GyK		1					**1**
GyL		1					**1**
GyM		1					**1**
GyN				1			**1**
GyO		1					**1**
GyP					1		**1**
GyQ		1					**1**
GyR		1		1			**2**
GyS		2					**2**
GyT		1					**1**
GyU		1		1			**2**
GyV		1		1			**2**
GyW					1		**1**
GyX				1			**1**
GyY				1			**1**
GyZ				1			**1**
GyAA				1			**1**
GyAB			1				**1**
GyAC					1		**1**
GyAD			1		1		**2**
GyAE			1				**1**
GyAF	6	11	3	10	10	1	**41**
GyAG				1			**1**
GyAH				1			**1**
GyAI				1			**1**
GyAJ				1			**1**
Sum	12	29	7	25	16	1	**90**
π (SD)	0.0314 (0.0170)	0.0108 (0.00594)	0.0106 (0.00662)	0.00701 (0.00408)	0.0418 (0.0218)	0.0000 (0.0000)	
***h* (SD)**	0.773 (0.128)	0.847 (0.0607)	0.857 (0.137)	0.847 (0.0718)	0.625 (0.139)	1.00 (0.0000)	

bp in all the 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*) (S. Pina, F. Cremonte, P. Rodrigues pers. comm.). The minimum spanning network among the 6 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 5 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). These authors 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 *Gymnophallus 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 ≥6 replacement substitutions away from the main haplotype: GyA,

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 (SD) between brackets

Haplo-type	Wadden Sea, The Netherlands	Celtic Sea, Ireland	English Channel, France	Arcachon Bay, France	Merja Zerga, Morocco	Sum
LaA	1					**1**
LaB				2	20	**22**
LaC				1		**1**
LaD				1		**1**
LaE			1	2		**3**
LaF		1				**1**
LaG			1			**1**
LaH	1					**1**
LaI					5	**5**
LaJ		8				**8**
LaK		1				**1**
LaL					1	**1**
LaM		1				**1**
LaN		1				**1**
LaO				1		**1**
LaP	1					**1**
LaQ	1					**1**
LaR	1	1	1			**3**
LaS		1				**1**
LaT		1				**1**
LaU	1					**1**
LaV		2				**2**
LaW				1		**1**
LaX			1			**1**
LaY		1				**1**
LaZ		1				**1**
LaAA				1		**1**
LaAB				1		**1**
LaAC				1		**1**
LaAD	1					**1**
LaAE				1		**1**
LaAF		1	4			**5**
LaAG		1				**1**
LaAH		1				**1**
LaAI			1			**1**
LaAJ	1					**1**
LaAK	1					**1**
LaAL	1		1			**2**
LaAM		1				**1**
LaAN			2	1		**3**
LaAO	1					**1**
LaAP	1					**1**
LaAQ	14	4	12	6		**36**
LaAR			1			**1**
LaAS				1		**1**
LaAT	1		1			**2**
LaAV	1					**1**
LaAW				1		**1**
LaAX			1			**1**
LaAY				1		**1**
LaAZ				1		**1**
LaBA			1			**1**
LaBB			2			**2**
LaBC				1		**1**
Sum	28	27	30	24	26	**135**
π (SD)	0.003569 (0.002287)	0.005387 (0.003204)	0.003004 (0.001991)	0.006437 (0.003753)	0.000681 (0.000728)	
***h* (SD)**	0.7593 (0.0891)	0.9003 (0.0461)	0.8366 (0.0592)	0.9328 (0.0424)	0.3846 (0.1017)	

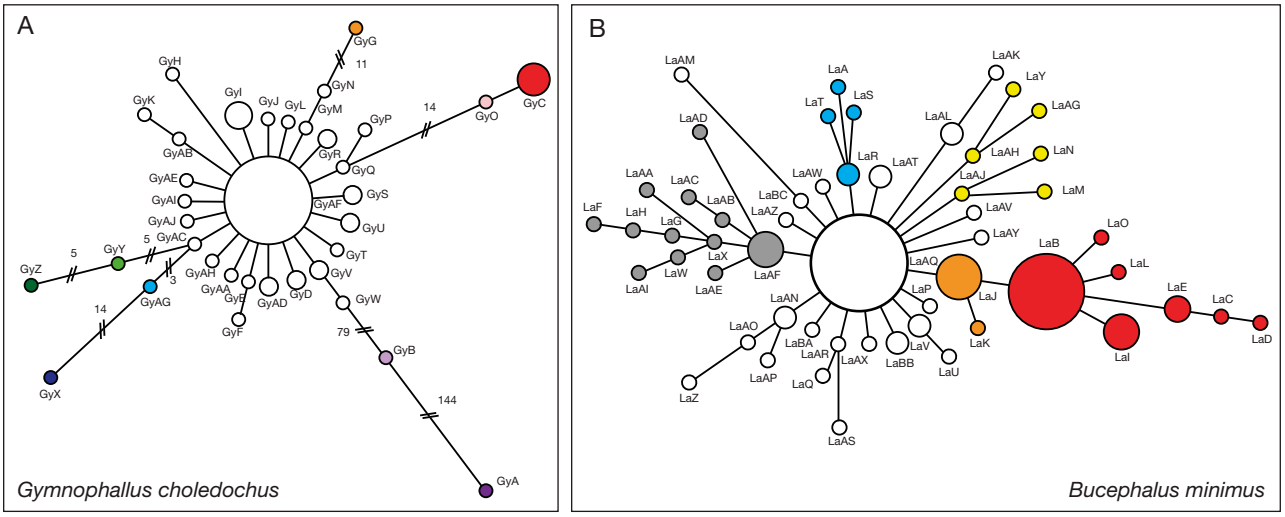

Fig. 3. Minimum spanning networks for cytochrome *c* oxidase 1 haplotypes (circles) in (A) *Gymnophallus choledochus* and (B) *Bucephalus minimus*. Number of nucleotide differences is proportional to branch length (shortest branches represent one nucleotide difference), unless indicated otherwise. Frequency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

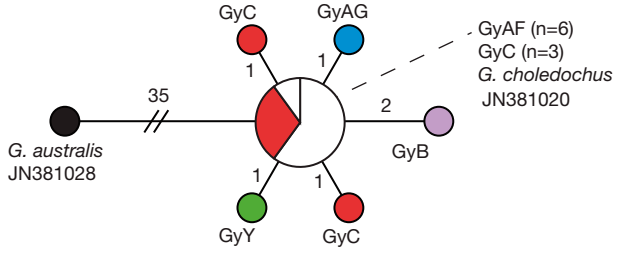

Fig. 4. Minimum spanning network for internal transcribed spacer haplotypes in *Gymnophallus* spp. Colours correspond to those in Fig. 3. The *G. choledochus* haplotypes that are 1 or 2 steps removed are GyC from the Wadden Sea and Arcachon Bay; GyB from Norsminde Fjord; and GyAG and GyY from the English Channel. The other GyC are all from the Wadden Sea. GyAF has 2 sequences from Arcachon Bay and 1 from Wadden Sea, English Channel, Celtic Sea and Merja Zerga

GyB, GyX, GyG, GyC and GyO. This decreased the number of individuals in the *G. choledochus* COI data set by N = 11.

Population comparisons

Haplotype diversity for *Gymnophallus choledochus* ranged from 0.63 in Arcachon Bay to 0.86 in the Celtic Sea (Table 2); when excluding the long branch subset, gene diversity ranged from 0.51 in Arcachon Bay to 0.83 in the Celtic Sea. For *Bucephalus minimus*, gene diversity ranged from 0.38 in Merja Zerga to 0.93 in Arcachon Bay. Nucleotide diversity for *G. choledochus* ranged from 0.0070 in the English Channel to 0.042 in Arcachon Bay (Table 2), and this reduced to between 0.0016 in Arcachon Bay and 0.0045 in the English Channel. In *B. minimus*, nucleotide diversity ranged from 0.00068 in Merja Zerga to 0.0064 in Arcachon Bay (Table 3).

For *Bucephlus minimus*, the overall level of population differentiation was estimated at Φ_{st} = 0.25 ($p < 0.0001$); for *Gymnophallus choledochus*, Φ_{st} = 0.015 ($p = 0.192$) (Table 4). The latter analysis is based on a 5-sample comparison, i.e. excluding Merja Zerga, which had only 1 sequence. For comparison, we also analysed the *B. minimus* data without Merja Zerga. This decreased the Φ_{st} value to 0.053 ($p < 0.0001$) (Table 4).

Pairwise population comparisons were never significant for *Gymnophallus choledochus* (Tables S1–S6 in the Supplement at www.int-res.com/articles/suppl/m520p085_supp.pdf).

For *Bucephalus minimus*, pairwise population comparisons (shown in Tables S7–S9 in the Supplement) were significantly different in all but 2 cases: the Wadden Sea sample did not differ from the English Channel sample, nor did the English Channel sample differ from Arcachon Bay. A small but significant difference, in contrast, was estimated for the Wadden Sea versus Arcachon Bay comparison (Φ_{st} = 0.0792, p = 0.00059). The same pattern was visible for pairwise population comparisons between only Wadden Sea, English Channel and Arcachon Bay. In other words, *Gymnophallus choledochus* shows no

Table 4. Results from analyses of molecular variance (AMOVA) on partial cytochrome *c* oxidase 1 sequences in *Bucephalus minimus* and *Gymnophallus choledochus*. N: Norsminde, Denmark; W: Wadden Sea, the Netherlands; C: Celtic Sea, Ireland; E: English Channel, France; A: Arcachon Bay, France; M: Merja Zerga, Morocco. Full: no data were omitted from the analysis; Without repl.: 'replacement set' of lineages, i.e. with an excess of replacement mutations (see main text for details), were omitted from the analysis. Φ_{st}: population differentiation

Samples	Data set	Φ_{st}	p
Gymnophallus choledochus			
NWCEA	Full	0.0150	0.192
NWEA	Full	0.0262	0.0590
WEA	Full	0.0341	0.0284
NWCEA	Without repl.	−0.0077	0.721
NWEA	Without repl.	−0.0047	0.653
WEA	Without repl.	−0.0025	0.553
Bucephalus minimus			
WCEAM	Full	0.250	<0.00001
WCEA	Full	0.0532	<0.00001
WEA	Full	0.0434	0.00178

structure; *B. minimus* shows structure only when Arcachon Bay was compared with the Wadden Sea.

The demographic analyses were done with groupings based on the AMOVA and pairwise comparison results. Hence, all samples for *Gymnophallus choledochus* were grouped and analysed together, and all samples for *Bucephalus minimus* were analysed separately. Mismatch distributions are shown in Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m520p085_supp.pdf. The RI statistic rejected a model of sudden population expansion in none of the 7 data sets analysed (Table 5). The SSD statistic rejected the null hypothesis of significant population expansion model in 2 of 7 cases. In *G. choledochus*, a model of expansion was rejected only in case the 'replacement set' of haplotypes was included in the analysis ($p < 0.005$, Table 5). In *B. minimus*, the expansion model was rejected for the sample originating from the Celtic Sea ($p < 0.05$, Table 5).

EBSP are shown in Fig. S2 in the Supplement. Five of 7 data sets fitted best to the HKY model of nucleotide substitution, and the other 2 fitted best to an HKY+gamma model (Table 5). In all cases, the coalescent analyses supported a model of population change; one population size change had the highest posterior probability in all cases except that of *Gymnophallus choledochus* with the replacement set of haplotypes included, in which case, 2 population size changes had the highest posterior probability. The 95% highest posterior density (HPD) interval included 'zero changes' only for the *Bucephalus minimus* Merja Zerga sample; in all other cases, 'zero changes' fell outside of the 95% HPD interval (Table 5). Furthermore, all inferred population size changes were expansions, except in the case of *G. choledochus* with the replacement set of haplotypes included, where population size was inferred to have decreased (Fig. S2). Under the (highly uncertain) assumptions of 1 generation per year and a divergence rate of 2% per million years, all population size increases would be inferred to have taken place between 100 000 and 200 000 yr ago, both for all *B. minimus* samples and for the *G. choledochus* analysis excluding the replacement set of haplotypes. In the case of population dropout, i.e. when *G. choledochus* was analysed with the replacement set, the decrease was inferred to be extremely recent (Fig. S2).

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; 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**. $^{*}p < 0.05$; $^{**}p < 0.005$

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

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 *Bucephalus 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 *Gymnophallus choledochus*; introgression of the genome of *G. choledochus* by the mitochondria of another species, which would lead to treating 2 separate species as a single one; studying a single locus; and non-neutral effects, including, 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 *Gymnophallus choledochus*, however, are less clear. While the lack of population structure shown by these data may be indicative of population connectivity, the data 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 2 parasite species is not caused solely by sampling effects. The most strongly differentiating sample in *Bucephalus minimus* is from Merja Zerga in Morocco, where we only encountered 2 infections with *Gymnophallus 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 one is of the most frequent haplotype found. When we compare the 2 trematode species without Merja Zerga, however, the difference in population structure remains present; populations of *B. minimus* are still highly significantly differentiated among the 4 more northern samples, albeit with a reduced Φ_{ST} value (Table 4).

Management considerations

The results presented here not only add 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 *Bucephalus minimus* are significantly isolated, all of its hosts must 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 population structure also should exist for other associated hosts. Because 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 IBD was observed in *B. minimus* for 3 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 Arca-

chon Bay sample (South), the Wadden Sea sample and the Arcachon Bay sample did reveal a difference (Tables S7–S9 in the Supplement). 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 the 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 post-glacial colonisation in the more northern locations, while for the southern sites, an ephemeral nature of local populations may be the cause of the observed pattern. For the Celtic Sea sample of *Bucephalus 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 data set, i.e. including the long branches we found, recent population decline of *Gymnophallus 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 EBSP for *Bucephalus 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 *Gymnophallus choledochus* is in line with the only other existing population genetic study on marine allogenic parasites. Populations of the 2 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 2 allogenic parasites (Keeney et al. 2008, 2009). In contrast, comparable studies of the genetic population structure in autogenic parasites (using fish as definitive hosts) have been lacking to date. Vilas et al. (2004) reported allozyme data from 3 autogenic trematodes in fish hosts from the Portuguese coast, but the study covered only a small spatial scale (maximum geodesic distance between populations of 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 for estimating 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 2 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 hosts compared to fish hosts (Thieltges et al. 2009) and is thought to underly the presence of a positive abundance-occupancy relationship in allogenic trematodes in snail first intermediate host populations, while such a relationship is 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 of the population genetics of marine parasites with differential host 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.

Data archive. DNA sequences: GenBank accession numbers KF880428 to KF880531.

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