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Biological Invasions

DOI:
10.1007/s10530-015-0896-1

Published: 01/09/2015

Peer reviewed version

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--Manuscript Draft--

Manuscript Number:	BINV-D-13-00445R3
Full Title:	Invasion genetics of the Pacific oyster Crassostrea gigas in the British Isles inferred from microsatellite and mitochondrial markers
Article Type:	Research paper
Keywords:	invasion genetics, marine mollusc, microsatellites, mtDNA, Pacific oyster, Crassostrea gigas.
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Response to Reviewers:	The changes required by the reviewer have been made. The manuscript has been

	significantly reduced in length. The figures 2, 3 and 5 have been modified following the reviewer's feedback. Line 199, we have specified that marine geographic distance corresponds to the nearest waterway distance. The deltaK method of Evanno has been used to determine the number of genetic clusters (see line 211).

Invasion genetics of the Pacific oyster *Crassostrea gigas* in the British Isles inferred from microsatellite and mitochondrial markers.

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Abstract:

The Pacific oyster, *Crassostrea gigas*, native to northeast Asia, is one of the most important cultured shellfish species. In Europe, Pacific oysters first settled along the Atlantic coasts of France at the end of the 1960s but rapidly spread and are now widely established. Twenty-two sites in the United Kingdom (UK), Ireland, Denmark, France and Spain were sampled to assess genetic diversity and differentiation. Hatchery-propagated stocks from two hatcheries located in the UK also were included. Two main genetic clusters were identified from pairwise genetic differentiation indexes, Bayesian clustering methods or neighbour-joining analysis, based on 7 microsatellite loci: (1) a Northeast cluster (including feral samples from East England, Ireland and Denmark as well as UK hatchery stocks) and (2) a Southwest cluster (including samples from South Wales, South West England, France and Spain). The Southwest cluster had significantly higher allelic richness (*A*) and expected heterozygosity (H_e) (*A*: 45.68, H_e: 0.928) than in the Northeast (*A*: 26.58, H_e: 0.883); the two diverging by a small but significant F_{ST} value (F_{ST}=0.017, 95% CI: 0.014-0.021). A 739-bp fragment of the major noncoding region of the mitochondrial genome was sequenced in 248 oysters from 12 of the studied samples in Europe and in 25 oysters from Miyagi prefecture (Japan). A total of 81 haplotypes were found. Haplotype frequency analyses identified the same two clusters observed using microsatellites. This study highlights how the number and size of introduction events, aquaculture practices, genetic bottlenecks followed by genetic drift and natural dispersal can act concurrently to shape the genetic diversity and structure of introduced populations.

Keywords: invasion genetics, marine mollusc, microsatellites, mtDNA, Pacific oyster, *Crassostrea gigas*.

INTRODUCTION

Biological invasions are a major threat to coastal ecosystems and to global marine biodiversity. In addition to natural pathways of propagule dispersal (e.g. water currents), humans have caused an unprecedented redistribution of many taxa over recent centuries (Carlton 1989), by facilitating transport and introduction of species through a variety of activities such as shipping and aquaculture (Voisin et al. 2005; Lacoursière-Roussel et al. 2012). In order to successfully manage marine non-native species and to predict their potential range expansion, it is essential to identify or confirm the source locations, pathways and vectors (Grosholz 2002), along with the factors contributing to the success of an invasion.

Invasion genetics studies have been performed to determine the degree of population connectivity, the source of invasion and to assess the potential for spread of non-native species (Geller et al. 2010). Microsatellites (e.g. Astanei et al. 2005; Dupont et al. 2007; Rius et al. 2012) and mitochondrial DNA markers (e.g. Audzijonyte et al. 2008; Hayes et al. 2008; Kajita et al. 2012) have been widely used in such studies, including in combination (e.g. Neilson and Stepien 2011; Zhan et al. 2012).

The genetic diversity and differentiation of non-native populations are shaped by several factors, including those of the source populations and the demography of the invasion (Holland 2000). The number of individuals introduced, diversity and differentiation of source populations, and eventual selective processes determine the proportion of genetic diversity that is retained in the introduced range (Wilson et al. 2009). In particular, multiple introductions have been shown to facilitate the maintenance of high genetic diversity in introduced populations compared to source populations (Roman and Darling 2007; Dlugosch and Parker 2008; Gillis et al. 2009). Indeed, it has been shown that human-mediated dispersal may result in higher levels of within-population genetic diversity when compared to native populations (Voisin et al. 2005; Wilson et al. 2009).

The Pacific oyster, *Crassostrea gigas*, native to northeast Asia, has been introduced worldwide for the purpose of aquaculture and is among the most important cultured shellfish species in the world (FAO 2011). Naturalized oyster populations are well established in several European countries and can have important ecological impacts on coastal ecosystems (Troost 2010).

C. gigas has a complex history of introductions in Europe, which are relatively well documented (Figure 1). Significant quantities of juveniles were first imported from the native range (i.e., Japan) to British Columbia between 1926 and 1932 (Quayle 1988; Miossec et al. 2009). Massive introductions (562 tons of adult oysters from British Columbia, then 5 billion seed from the Miyagi prefecture in Japan) occurred in France in the 1970s to sustain oyster production, following severe

declines of the Portuguese oyster *Crassostrea angulata* (Grizel and Héral 1991). These rapidly became established in the wild. In the United Kingdom, several small introductions from British Columbia (50-76 adults at a time) were undertaken in the 1960s and 1970s and placed in quarantine facilities for hatchery propagation (Walne and Helm 1979; Utting and Spencer 1992; Spencer et al. 1994). Sporadic natural spatfalls (settlement and attachment of young oysters to the substrate) were first reported in Britain in 1990 (Spencer et al. 1994). In Ireland, *C. gigas* was first introduced in 1969 from the UK quarantined stocks (Shatkin et al. 1997). However, an open trade agreement in 1993 (Council Directive 91/67/EEC) meant that imports of seed to Ireland from France were permitted (Minchin et al. 1993). Since 1986, oyster seed produced in UK hatcheries were regularly imported into the northern area of the German Wadden Sea near and in the island of Sylt (Reise 1998). In Denmark, since the 1970s, large numbers were imported from the UK, The Netherlands and France (Nehring 2006). In 1999, natural dispersal from the German Wadden Sea was reported in the Danish Wadden Sea (Reise et al. 2005). In Northern Europe, *C. gigas* populations became established more recently and have been qualified as feral, since they commonly occur close to oyster farms (Troost 2010); they have been shown to be demographically independent and self-sustaining (Kochmann et al. 2012).

Invasion genetics of *C. gigas* have been studied in the Wadden Sea (Moehler et al. 2011), the South of France to the North of the Wadden Sea (Meistertzheim et al. 2013), and the South of France to Sweden (Rohfritsch et al. 2013). Two main genetic groups were identified, with a population break located in the Wadden Sea: (1) a high diversity southern group (including samples from France, The Netherlands and southern Wadden Sea, along with Japan (native range) and British Columbia (secondary source of introduction to various European countries) and (2) a low diversity northern group (including Germany, northern Wadden Sea, Denmark and Sweden). However, the spread of this species both into and from the British Isles, presumed to have been pivotal in the invasion of this species in Northern Europe, is unknown. In the British Isles, *C. gigas* has a patchy distribution, with some locally dense occurrences; however there are concerns that its range may expand further. A better understanding of population connectivity would help to predict such potential for further expansion.

The aims of the present study were to: i) assess the genetic diversity and differentiation of feral populations of *C. gigas* in the British Isles, since based on the history of introductions, the British Isles sites are expected to comprise a single genetically defined group; ii) compare patterns from microsatellite and mitochondrial DNA markers; iii) compare genetic diversity patterns with knowledge about the history of introductions and aquaculture practices.

METHODS

Sampling sites

Oysters were sampled between October 2009 and August 2011, in 22 sites (Table 1a, Table S1), in the UK, Ireland, France, Spain and Denmark (Figure 3b). These included 8 sites in East England (Kent: KB, KHB, KR; Essex: ESS, EWM, EBW, EB; the Wash: WGS), 4 in South West England (Devon: DS, DPS, DY; Cornwall: CF), 2 in South Wales (Milford Haven: MGP, MPP), 2 in Northern Ireland (Co Down: SLN, SLG), 2 in Ireland (Co Donegal: ILS; Co Galway: IGB), 2 in Spain (Galicia: GO, GC), 1 in Denmark (Nordjylland: DAN) and 1 in France (Brittany: FRB). Sites from France and Denmark were included to represent the higher diversity southern group and the lower diversity northern group, respectively, identified by Rohfritsch et al. (2013). The oysters were collected in a variety of habitats, including rocky shores, chalk reefs, flint boulders, groins, mussel beds, gravel, shingle, mud and muddy shingle. At Garron Pill (MGP, South Wales), we collected both loose oysters (presumed to be from a disused aquaculture facility) and oysters attached to the rock (Supplementary Figure S1). The number of oysters collected per site were 200 for ILS; 100 for KB, KR, KHB, SLN, SLG, WGS, MGP, DPS, DY, ESS, EBW, EB and DAN; 99 for FRB; 98 for EWM; 95 for IGS and DS; 75 for CF; 40 for GO; 38 for GC; 37 for MPP; totaling 2077 samples (Table 1a). Pieces of gill were preserved in 80% ethanol.

Three hatchery-propagated stocks also were analyzed (Table 1a) from 2 companies (Hatchery A: HA (year 2008); Hatchery B: HB1 (year 2005) and HB2 (year 2006)). Fifty oysters were analyzed for HA; 46 for HB1 and 49 for HB2.

DNA extraction and microsatellite genotyping

DNA was extracted from gill tissue with chloroform/isoamyl alcohol, followed by absolute ethanol and sodium acetate (3M pH 4.8) precipitation. The DNA pellets were resuspended in 100 µl ultrapure water.

Eight EST-SSRs (CGE007, CGE009, AMY, Cgsili46, Cgsili44, Cgsili39, Cgsili50 and Cgsili4) were amplified in three multiplex PCRs, following Li et al. (2010). Three anonymous microsatellites (Cg108, Cg49: Magoulas et al. 1998; L10: Huvet et al. 2000a) were amplified in a multiplex PCR reaction, following the same protocol, but with an annealing temperature of 55°C and a final concentration of 0.15 µM of each primer. PCR products were loaded in an ABI 3130*xl* Genetic Analyzer (Applied Biosystems) with GeneScan™ 500 ROX™ size standard. Fragment lengths were determined using GeneMapper v.4.0 (Applied Biosystems).

mtDNA amplification and sequencing

A 739-bp fragment of the Major Noncoding Region (MNR) of the mitochondrial genome was amplified from18-25 randomly picked individuals per sample, from 12 of the 25 samples analyzed, namely: ILS, SLG, EWM, KB, DS, DY, DAN, FRB, GO, HA, HB1 and HB2, representing the major regions plus hatchery stocks. Additionally, 25 individuals from JM (Miyagi Prefecture, Japan) were also analyzed. Since this population previously was genotyped for microsatellite markers (Rohfritsch et al. 2013) only its mtDNA was analyzed here. MNR sequence data from Moehler et al. (2011), including British Columbia (secondary source for the introductions in the UK) and the European Wadden Sea (location of the genetic break), were also analyzed. Primers developed by Aranishi and Okimoto(2005) were used in 25-µl PCR reactions containing 2 µl of 1:10 diluted DNA, 0.4 µM of each primer and 1X PCR Master Mix with 1.5 mM $MgCl_2$ (Thermo Scientific) and PCR amplification protocol of Moehler et al. (2011). Successful amplification was checked by electrophoresis on 2% agarose gels. PCR products were purified using Exo-TSAP (Promega) and sent to Macrogen Europe (Netherlands) for direct sequencing with the forward primer. Twenty-three singletons (sequences occurring once in the dataset) were re-amplified and re-sequenced to control for possible amplification or sequencing errors.

Microsatellite data analysis

Genotypic linkage disequilibrium was tested using the Markov chain exact probabilities from GENEPOP version 4.2 (Raymond and Rousset 1995; Rousset 2008), for each pair of markers per sample. Deviations from the Hardy Weinberg equilibrium (F_{IS}: estimator of fixation index) were evaluated using FSTAT v.2.9.3.2 (Goudet 2002) software for both loci and samples. Significance of any departure of F_{IS} values from 0 was assessed by randomizing alleles within samples, using 5,500 randomizations. Bonferroni corrections were applied to account for multiple comparisons.

Genetic diversity was assessed by computing number of alleles (N_a), allele frequencies, allelic richness (A) (El Mousadik and Petit 1996), observed (H_o) and unbiased expected (H_e) heterozygosities (Nei 1978) using FSTAT. The number of private alleles (unique to a single site) was calculated with GenAlEx v.6.41 (Peakall and Smouse 2006).

Genetic differentiation was assessed using different methods. Firstly, genetic differentiation was estimated using three methods. Theta (ϑ) (Weir and Cockerham 1984) was calculated with FSTAT, while G_{st_est} (Nei and Chesser 1983) and D_{est} (Jost 2008) were assessed using DEMEtics package (Gerlach et al. 2010). Pairwise genetic differentiation was plotted as heat maps using Pheatmap package (Kolde 2012). Secondly, neighbor-joining dendrograms based on pairwise Nei (D_a) genetic distances (Nei and Chesser 1983) were made with POPTREE2 (Takezaki et al. 2010). Confidence estimates of tree topology were calculated by bootstrap resampling of loci 1,000 times.

Thirdly, to detect hierarchical genetic structure among sites, Analyses of Molecular Variance (AMOVA) (Excoffier et al. 1992) were conducted in GenAlEx 6.41. Molecular variance was partitioned into three levels: among clusters, among populations within clusters and within populations. Significance was determined using 1,000 permutations. Fourthly, the pattern of isolation by distance (IBD) was assessed by plotting pairwise $F_{ST}/(1-F_{ST})$ values against the logarithm of the geographic distances (Euclidian distance and marine geographic distance i.e. nearest waterway distance) between all sample sites, as recommended for a two-dimensional model of IBD (Rousset 1997). The statistical significance of the correlation coefficient was tested using a Mantel test implemented in GENEPOP, with 10,000 permutations. The analyses were carried out at the global scale and within each cluster. The 3 hatchery samples were excluded from those analyses. Finally, we applied a non-spatial Bayesian model-based clustering algorithm, implemented in STRUCTURE v.2.3.3 (Pritchard et al. 2000), to further assess genetic structure. It infers the number of genetic clusters *K* and assigns individuals to clusters from the individual's genotypes dataset, while minimizing Hardy Weinberg disequilibrium and gametic phase disequilibrium between loci within groups. Ten independent runs were performed for each *K* using 1,000,000 iterations and a burn-in period of 100,000, with the model allowing for admixture and correlated allele frequencies between clusters (Falush et al. 2003), with and without sample group information (Hubisz et al. 2009). The number of clusters was estimated using the ΔK method (Evanno et al. 2005) as performed in STRUCTURE HARVESTER (Earl and von Holdt 2012). CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) was used to average the assignment scores over the 10 runs and results were visualized in DISTRUCT v.1.1 (Rosenberg 2004).

mtDNA data analysis

MNR sequences were checked visually and aligned using CodonCode Aligner software (CodonCode Corporation). Genetic diversity was assessed by calculating the numbers of haplotypes (H_t), singletons (H_u) and polymorphic sites (S); haplotype (H_d) and nucleotide (π) diversities (Nei 1987) using DnaSP v.5 (Rozas et al. 2003). Population differentiation was assessed by calculating pairwise genetic differentiation (F_{ST}) using Arlequin v.3.1 (Excoffier et al. 2005), with the pairwise difference distance method.

We performed Spatial Analysis of Molecular Variance (SAMOVA 1.0, Dupanloup et al. 2002, available at http://cmpg.unibe.ch/software/samova), which aims to define groups of populations that are geographically homogeneous, by maximizing F_{CT} value (i.e., the proportion of total genetic variance due to differences between groups of populations). Subdivisions were tested with a range of *K* values from two to eleven geographic groups using 100 simulated annealing processes.

In order to attempt to compare genetic patterns with the history of introductions, a haplotype distribution map was generated, covering the British Isles, Japan (overall source for the introductions in Europe), the European Wadden Sea (location of the genetic break in Europe) and British Columbia (secondary introduction to the UK). A median-joining network (Bandelt et al. 1999) was built using NETWORK v.4.6.1 to reconstruct the phylogenetic relationships among haplotypes.

RESULTS

Genetic diversity

Significant linkage disequilibrium was found in 138 out of 1375 pairwise comparisons among 11 loci for all populations. After Bonferroni correction, 47 tests remained significant. However, most of the genotypic linkage disequilibrium detected was due to the hatchery stocks (39 significant tests). Overall, there were significant heterozygote deficiencies for the 22 feral samples, and for 1 out of 3 hatchery batches, mainly due to loci Cg49, CGE007, Cgsili39 and Cg108. When those 4 loci were removed from the analyses, significant heterozygote deficiencies remained for only 2 out of the 22 feral samples, FRB (F_{IS} 0.051) and GO (F_{IS} 0.070) (Table 1a). Based on the remaining 7 microsatellites, there were heterozygote excesses in the three hatchery batches (F_{IS} between -0.081 and -0.052; Table 1a). As most analyses rely on Hardy Weinberg equilibrium assumptions, all further analyses were done with the 7 microsatellites conforming to Hardy Weinberg equilibrium expectations (CGE009, AMY, Cgsili46, Cgsili44, Cgsili50, Cgsili4 and L10).

Genetic diversity and heterozygosity per sample are given in Table S2, for each locus. Overall estimates, averaged over the 7 loci, are given in Table 1a. Mean allelic richness ranged from 13.68 (WGS)-22.34 (DY) for the 22 studied samples, and from 11.10-12.54 for the 3 hatchery batches. The mean number of private alleles ranged from 0 (KB, KR, KHB, SLG, WGS, ILS, ESS, EWM, EBW, EB, DAN)-1.71 (FRB) for the feral samples, and 0-0.14 for the hatchery batches. Mean expected heterozygosity (H_e) ranged from 0.880 (KB)-0.931 (GO) for the feral populations, and from 0.853-0.874 for the hatchery batches.

The MNR aligned dataset, derived from 273 individuals, consisted of 674 nucleotides with 109 (16.2%) variable positions, including 88 transitions, 7 transversions, and 15 indels. Of the 81 haplotypes identified, 19 were identical to those previously reported by Moehler et al. (2011). The 23 randomly-chosen singletons that were re-amplified and re-sequenced led to identical sequences. Two shared singletons (H10, H34) and 5 private singletons (H16, H28, H39, H45, H58) found in this study were identical to haplotypes found in Moehler et al. (2011). In the hatchery batches, 4-8 haplotypes were found, with no singletons. The samples DS, DY, FRB, GO, JM and SLG exhibited a

high number of haplotypes (12-18) and singletons (4-15). In contrast, DAN, EWM, KB and ILS exhibited a lower number of haplotypes (6-9) with just 1 or no singletons (Table 1b). Haplotype diversity ranged from 0.695 (Hatchery A) to 0.963 (DS), and nucleotide diversity from 0.00219 (GO) to 0.00552 (EWM and Hatchery B) (Table 1b).

Genetic differentiation

Based on the microsatellite markers, significant global genetic differentiation was detected among sites. ϑ and G_{st_est} values were very similar therefore only G_{st_est} values are reported in Table S3. Global ϑ was 0.014 (95% CI: 0.012-0.018), G_{st_est} was 0.017 (95% CI: 0.016-0.017) and global D_{est} was 0.156 (95% CI: 0.151-0.160). Genetic differentiation between each pair of samples is shown in Figure 2A as heat maps. Pairwise ϑ and D_{est} values are reported in Table S4, which showed very similar patterns of genetic differentiation. Two main groupings were observed; a Northeast cluster (sites from East England, Northern Ireland, Ireland, and Denmark) and a Southwest cluster (sites from South Wales, South West England, Spain and France). It was clear from the heat maps that the hatchery batches were more closely related to the Northeast cluster than to the Southwest cluster.

The neighbor-joining phylogram also revealed two clusters, Southwest and Northeast / UK hatcheries cluster (Figure 3A).

Bayesian clustering analysis further confirmed this pattern: two clear genetic clusters were identified (K=2) (Figure 3B-C). In the majority of cases, each sample was composed of individuals with a high probability of belonging to only one of the 2 clusters (Figure 3C). For the Irish (SLN, SLG, IGB, ILS) sites, there seemed to be a certain level of admixture of the two genetic clusters (Figure 3C): each individual was cross-assigned to both clusters. For Garron Pill sample (MGP), the genetic signature observed in the STRUCTURE analysis (Figure 3C) confirmed the sampled oysters were from two different origins (Figure S1).

Based on the mtDNA marker, pairwise F_{ST} values highlighted two groups: Southwest / Japan (GO, FRB, DY, DS, JM) and Northeast (KB, EWM, ILS, SLG, DAN) (Figure 2B). Within the Southwest / Japan cluster, F_{ST} values ranged from 0.0005-0.0075. Within the Northeast cluster, F_{ST} values ranged from 0.0130 to 0.0679. Between those two clusters, F_{ST} values were between 0.0187 (SLG-DY) and 0.1683 (ILS-FRB). The two hatchery stocks exhibited a higher genetic differentiation from the Southwest / Japan cluster than to the Northeast cluster. The F_{ST} value between the two hatchery stocks was 0.1072-0.1486 (Table S5). Among the 81 haplotypes identified, 13 were shared among sites. Haplotype frequencies are visualized on a geographic map in Figure 4A. DS, DY, FRB, GO and JM (Southwest / Japan cluster) were characterized by a high proportion of singletons (52 to 64%) and the dominance of haplotype H8 (20 to 39%). KB, EWM, ILS, SLG and DAN (Northeast cluster)

shared almost all their haplotypes with the hatchery stocks (e.g., H1, H2, H3, H4, H5), and had a more balanced haplotypes distribution. A median-joining network analysis revealed shallow divergence among the 81 haplotypes. Most haplotypes differed only by a single nucleotide substitution from the central haplotype H8, resulting in a star-like pattern (Figure 4B).

Genetic diversity and differentiation of the inferred clusters

The Wilcoxon paired sample test revealed a significantly higher allelic richness (*A*) and expected heterozygosity (H_e) in the Southwest cluster (*A*: 42.08, H_e: 0.928) than in the Northeast / Hatcheries cluster (*A*: 24.94, H_e: 0.891). Also, there were significantly more private alleles in the Southwest cluster (14.429 ± 1.850) than in the Northeast / Hatcheries cluster (1.000 ± 0.378). F_{IS} was 0.032 (p=0.0036) for the Southwest cluster and -0.003 (p=0.8107) for the Northeast / Hatcheries cluster. The F_{ST} between those two clusters was 0.017 (95% CI: 0.014-0.021).

After exclusion of the GP sample (mixture of individuals belonging to different clusters) from the analysis, hierarchical AMOVA attributed the majority of variation to intrapopulation differences (96-97.2%, p=0.001). However, the AMOVA revealed a significant genetic structuring among the two STRUCTURE identified groups (3.2%, p=0.001).

At the global scale, the correlation between $F_{ST}/(1-F_{ST})$ and the logarithm of geographic distance was significant using Euclidian distance (r=0.479, P<0.001) or using the marine geographic distance (r=0.432, P<0.001). Within each cluster, correlations were also significant for the Northeast cluster (Euclidian distance: r=0.665, P<0.001; marine distance: r=0.639, P<0.001), but not for the Southwest cluster (Euclidian distance: r=0.195, P=0.118; marine distance: r=0.206, P=0.099). Genetic (F_{ST}) and marine geographic distances were plotted within and between clusters. For a given geographic distance, genetic differentiation between pairs of samples from different clusters was larger than that between pairs within the cluster (Figure 5). Therefore, genetic differentiation between the Northeast and Southwest clusters was not solely attributable to geographic distance.

Based on the mtDNA marker, there was a much higher number of haplotypes and haplotype diversity in the Southwest / Japan cluster (H_t: 69, H_d: 0.879 ± 0.031) than in the Northeast / Hatchery B cluster (H_t: 18, H_d: 0.851 ± 0.017) and Hatchery A (H_t: 4, H_d: 0.695 ± 0.079). Pairwise F_{ST} values were 0.118 between Northeast / Hatchery B and Southwest / Japan clusters (*P*<0.001), 0.066 between Northeast / Hatchery B cluster and Hatchery A (*P*=0.009), and 0.148 between Southwest / Japan cluster and Hatchery A (*P*<0.001). The SAMOVA analysis led to an optimal number of four geographic groups: ILS-KB-HB1-HB2 (Ireland / East England / Hatchery B), HA (Hatchery A), DAN-EWM (Denmark / East England) and DS-DY-FRB-GO-JM-SLG (Southwest / Japan / North Ireland) (F_{CT}=0.12232,

P<0.001). Finally, 12.23% of the total genetic variation was explained by variation among groups of samples, whereas variation within samples accounted for 87.77%.

DISCUSSION

There is a need for a better integrated understanding of how genetic diversity can be reshuffled by anthropogenic activities during the invasion process (Keller et al. 2012). Elucidating the factors that determine the invasive capacity of species is important not only to help predict the likeliness of an invasion, but also to propose management strategies and mitigation measures to minimize ecological impact. The introduction of *C. gigas* in Europe is relatively well-documented (Figure 1) and therefore constitutes a rare opportunity to confirm (or invalidate) by genetic analysis its introduction history and genetic signature and to document the relationships between propagule pressure, aquaculture practices and genetic diversity.

Genetic diversity and differentiation of *C. gigas* in the British Isles

In our study, we combined two types of markers, nuclear (microsatellites) and mitochondrial (Major Noncoding Region: MNR). In our study, results from both types of markers were highly correlated. Based on the history of introductions (Figure 1), we hypothesized that there should be genetic homogeneity among sites in the British Isles. However, the fact that two clear genetic clusters were observed in the UK (Figures 3 and 4) suggests that the source of *C. gigas* in Southwest England and South Wales was not solely UK hatcheries as initially presumed. Possible explanations include natural dispersal from North Brittany, importation of seed from natural recruitment from France for cultivation purposes, unintentional introduction by hull fouling or release of larvae from ship ballast water. However, we do not have evidence to favor one possibility over the others.

The lower genetic diversity in the Northeast / Hatcheries cluster (Table 1) was however expected as this has frequently been reported in hatchery shellfish seed (e.g. Gaffney et al. 1992; Taris et al. 2006; Hara and Sekino 2007; Lind et al. 2009). The presence of two genetic clusters poses the question of the implications of the level of genetic diversity on their adaptive potential (i.e., invasion success). It is generally accepted that introduction events can be accompanied by founder events and that associated loss of genetic diversity may result in inbreeding depression, which could reduce ability of the invasive species to adapt to its new environment. However, there have been several cases of introduced populations with low genetic diversity (genetic bottlenecks) that have been successful (ant *Linepithema humile*: Tsutsui et al. 2000, Yang et al. 2012; mosquitofish *Gambusia affinis*: Purcell et al. 2012). The “genetic paradox” concept in invasion biology questions

how newly founded populations can overcome low genetic diversity and associated constraints on evolutionary potential outside their native range (Roman and Darling 2007). However, in the majority of successful aquatic invasions, introduced populations are characterized by no observed reduction in genetic diversity compared with the native range and, on some occasions, exhibit even higher genetic diversity due to multiple introductions from various sources (Roman and Darling 2007; Dlugosch and Parker 2008). The role of multiple introductions in facilitating biological invasions is now well recognized (Geller et al. 2010): multiple introductions from various sources can increase genetic diversity in invaders, hence avoiding the potential negative impacts of genetic bottlenecks (Roman and Darling 2007; Dlugosch and Parker 2008); also the conversion of among-population genetic diversity in the native range to within-population genetic diversity in the introduced range can promote range expansion (European green crab *Carcinus maenas*: Roman 2006; nassariid gastropod *Cyclope neritea*: Simon-Bouhet et al. 2006) and create novel allelic combinations which can drive phenotypic diversification (Cuban lizard *Anolis sagrei*: Kolbe et al. 2007). Therefore, the relationship between level of genetic diversity and invasion success is not straightforward and requires further investigation in *C. gigas*.

Genetic diversity patterns are consistent with history of introductions and aquaculture practices

Combining our results with previous studies centered on the Wadden Sea (Moehler et al. 2011) or performed at a larger European scale (Meistertzheim et al. 2013; Rohfritsch et al. 2013), two distinct European genetic clusters were identified. Our results suggest that genetic diversity patterns are generally consistent with the history of introductions (Figure 1). However, the genetic pattern observed does not reflect some reported introduction events, namely the introduction of seed from France and the Netherlands to Denmark (Nehring 2006), suggesting that those importations did not result in considerable spatfall.

Part of the genetic diversity patterns observed in the present study can be explained by aquaculture practices. In France, large scale introductions took place resulting in rapid establishment (Robert and Gérard 1999). The fact that populations from Brittany and Galicia clustered with those from Miyagi and British Columbia (Figure 4A-C) shows that the high genetic diversity present in the native range was maintained during the introductions, as previously proposed by Huvet et al. (2000b). In contrast, in the UK, having three small introductions of adult oysters, founder effects and resulting genetic bottlenecks occurred. Subsequently, the breeding practices performed in the quarantine facilities of MAFF (Utting, pers. com.) might have led to further shifts in allele and haplotype frequencies, resulting from the high variance in reproductive success known to occur in this species (Boudry et al. 2002).

Overall, our results show that its genetic diversity patterns are mostly consistent with its known history of introductions. This study highlights how the number and size of introduction events, aquaculture practices (natural recruitment versus hatchery-produced seed), genetic drift and natural dispersal can act concurrently to shape the genetic diversity and differentiation of introduced populations. The elucidation of pathways of introduction and dissemination of *C. gigas* in Europe contributes to the establishment of future management strategies that might also be relevant for other invasive marine species.

Acknowledgements

This research was partially financed by the European Union Atlantic Area Transnational Programme (2007-2013), project SEAFARE, grant agreement n°2009-1/123. The authors thank José Luis Gomez and Lasse Fast Jensen for providing oysters samples from Galicia and Limfjord. The authors are very grateful to Mike Camplin, Roger Covey, Claire Guy, Philip Hollyman, Ron Jessop, Judith Kochmann, Sven Laming, Hywel Lloyd, Willie McKnight, Nigel Mortimer, Hanna Nuuttila, Francis O'Beirn, Jack Sewell, Romy Wild and Nadescha Zwerschke for help during the field work. The Bayesian clustering analysis (STRUCTURE) was conducted on the University of Oslo Bioportal. Finally, the authors are grateful to Mathias Wegner for sharing the haplotype sequences and frequency table of the Wadden Sea samples. We thank associate editor Carol Stepien for correcting the English.

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Table 1. a) Genetic diversity indices based on7microsatellite markers. Sites locations and their abbreviations (Abbr.), sample size (*N*), habitat in which the oysters were collected, as well as key summary genetic parameters: allelic richness (*A*), number of private alleles (N_p), expected heterozygosity (H_e) with standard deviations (SD) and F_{IS} values. Significant F_{IS} values are in bold (5,500 randomizations). b) Genetic diversity indices of *C. gigas* MNR mitochondrial DNA sequences. *N*: number of samples successfully sequenced. H_t: number of all haplotypes. H_u: number of singletons (haplotypes found only once at a single site). *S*: number of polymorphic sites. H_d: haplotype diversity. π: nucleotide diversity. SD: standard deviation. Northeast cluster includes KB, EWM, SLG, ILS and DAN. Southwest cluster includes DS, DY, FRB and GO.

a)

	Abbr.	**Geographic region**	**Location**	***N***	**Habitat/substrate**	***A* ± SD**	**N_p ± SD**	**H_e ± SD**	**F_{IS}**
Feral	KB	Kent (E England)	Birchington	100	Chalk reef	13.82 ± 3.99	0.00 ± 0.00	0.880 ± 0.03	-0.010
	KR	Kent (E England)	Ramsgate	100	Groyne	13.89 ± 4.14	0.00 ± 0.00	0.881 ± 0.03	-0.002
	KHB	Kent (E England)	Herne Bay	100	Flint boulders	13.93 ± 3.91	0.00 ± 0.00	0.888 ± 0.03	0.007
	SLN	Co Down (N Ireland)	Strangford Lough	100	Gravel and shingle	15.67 ± 4.02	0.14 ± 0.14	0.897 ± 0.04	-0.008
	SLG	Co Down (N Ireland)	Strangford Lough	100	Gravel and shingle	16.35 ± 4.96	0.00 ± 0.00	0.897 ± 0.04	-0.007
	WGS	The Wash (E England)	Gat Sand	100	Mussel bed	13.68 ± 4.54	0.00 ± 0.00	0.882 ± 0.05	-0.002
	ILS	Co Donegal (Ireland)	LoughSwilly	200	Mussel bed	15.37 ± 3.67	0.00 ± 0.00	0.894 ± 0.03	-0.016
	IGB	Co Galway (Ireland)	Galway Bay	95	Harbour wall	15.55 ± 4.45	0.14 ± 0.14	0.895 ± 0.04	-0.005
	MGP	Milford Haven (Wales)	GarronPill	100	Mud and shingle	20.28 ± 7.03	0.14 ± 0.14	0.915 ± 0.04	0.021
	MPP	Milford Haven (Wales)	Pennar Point	37	Rock	21.19 ± 7.42	0.29 ± 0.18	0.928 ± 0.04	0.014
	DS	Devon (SW England)	Salcombeestuary	95	Rock	21.62 ± 6.78	1.00 ± 0.44	0.930 ± 0.04	0.024
	DPS	Devon (SW England)	Plymouth Sound	100	Rock	21.39 ± 7.60	1.00 ± 0.31	0.925 ± 0.04	0.015
	DY	Devon (SW England)	Yealmestuary	100	Rock	22.34 ± 7.56	0.86 ± 0.34	0.930 ± 0.04	0.034
	CF	Cornwall (SW England)	Falestuary	75	Rock	21.30 ± 6.13	0.71 ± 0.18	0.925 ± 0.03	0.013
	ESS	Essex (E England)	Southend on Sea	100	Gravel and shingle	14.06 ± 4.21	0.00 ± 0.00	0.888 ± 0.04	0.001
	EWM	Essex (E England)	West Mersea	98	Mud and shingle	14.31 ± 4.24	0.00 ± 0.00	0.891 ± 0.03	-0.016
	EBW	Essex (E England)	Blackwater	100	Mud and shingle	13.86 ± 4.38	0.00 ± 0.00	0.885 ± 0.04	0.004
	EB	Essex (E England)	Brightlingsea	100	Mud and shingle	14.42 ± 4.26	0.00 ± 0.00	0.888 ± 0.04	0.013

	FRB	Brittany (France)	Rade de Brest	99	Rock	22.10 ± 8.48	1.71 ± 0.52	0.928 ± 0.04	**0.051**
	DAN	Nordjylland (Denmark)	Limfjord	100	Mussel bed	15.01 ± 4.63	0.00 ± 0.00	0.887 ± 0.05	0.027
	GO	Galicia (Spain)	Ria de Ortigueira	40	Rock	22.26 ± 6.76	0.57 ± 0.20	0.931 ± 0.04	**0.070**
	GC	Galicia (Spain)	Ria de Celeiro	38	Rock	21.31 ± 7.01	0.57 ± 0.20	0.920 ± 0.04	0.045
Hatchery	HA	United Kingdom	A – year 2008	50	n/a	11.57 ± 3.47	0.14 ± 0.14	0.853 ± 0.04	-0.058
	HB1	United Kingdom	B – year 2005	46	n/a	11.10 ± 2.21	0.00 ± 0.00	0.854 ± 0.04	**-0.081**
	HB2	United Kingdom	B – year 2006	49	n/a	12.54 ± 3.10	0.00 ± 0.00	0.874 ± 0.04	-0.052

b)

Population	***N***	H_t	H_u	***S***	H_d **(SD)**	π **(SD)**
DS	20	16	11	20	0.963 (0.033)	0.00330 (0.00043)
DY	22	17	14	29	0.935 (0.047)	0.00424 (0.00065)
FRB	21	14	11	14	0.786 (0.096)	0.00227 (0.00050)
GO	18	12	10	13	0.817 (0.095)	0.00219 (0.00047)
JM	25	18	15	24	0.880 (0.064)	0.00335 (0.00056)
DAN	21	6	0	11	0.862 (0.032)	0.00484 (0.00050)
EWM	19	7	1	13	0.830 (0.054)	0.00552 (0.00044)
KB	21	9	1	14	0.843 (0.057)	0.00509 (0.00038)
SLG	21	13	4	19	0.943 (0.031)	0.00436 (0.00054)
ILS	24	6	1	11	0.790 (0.047)	0.00475 (0.00034)
HA	20	4	0	8	0.695 (0.079)	0.00414 (0.00066)
HB1	20	8	0	11	0.821 (0.072)	0.00500 (0.00033)
HB2	21	7	0	12	0.748 (0.084)	0.00552 (0.00044)
Northeast / Hatchery B	147	18	7	24	0.851 (0.017)	0.00515 (0.00012)
Southwest / Japan	106	69	61	80	0.879 (0.031)	0.00311 (0.00027)
Overall	273	81	68	94	0.923 (0.008)	0.00462 (0.00016)

Figure 1.History of introductions of the Pacific oyster *Crassostrea gigas* in Europe.

Figure 2. Pairwise genetic differentiation heat maps. (A) Based on 7 microsatellite loci. Above diagonal: θ, estimator of F_{ST} (Weir and Cockerham 1984). Below diagonal: D_{est}, bias-corrected differentiation index (Jost 2008). (B) Based on mtDNA MNR marker. Estimator of F_{ST}.

Figure 3.Between samples genetic structure based on 7 microsatellite markers. (A) Neighbor-joining tree based on Nei (D_a) genetic distances among the 25 samples of *Crassostrea giga*s. Nodes supported by bootstrap values > 50% in 1,000 pseudoreplicates are indicated with filled circles. Branches are color-coded according to clusters identified in STRUCTURE analysis. (B) Map showing the distribution of sampling sites of *Crassostrea gigas*, with colored pie charts showing the contribution of the two main genetic clusters identified by STRUCTURE analysis. (C) Bayesian individual clustering of *Crassostrea gigas* performed in STRUCTURE, using the admixture model with correlated allele frequencies, and using sample group information (Hubisz et al. 2009). Each individual is indicated by a thin vertical line, with coloured bars representing proportions of membership to each cluster. Bold vertical lines separate sampling sites, with site abbreviations shown below the plot (see Table 1 for full names). Results shown are for K=2.

Figure 4.Between sample genetic structure based on the Major Noncoding Region (MNR) of the mitochondrial genome. The ten most common shared haplotypes are color-coded. (A, C) Maps of MNR haplotype frequencies. At each site, singletons have been pooled for graphical representation (pSingl: private singletons; sSingl: singletons shared between samples). The numbers of private singletons are written on the pie charts. (A) Samples analyzed in this study (see Table 1 for nomenclature). (C) Samples analyzed by Moehler et al. (2011). (B) Median-joining network for the 81 MNR haplotypes of *Crassostrea gigas*. Connecting lines represent single mutation change and black dots represent inferred missing haplotypes. The size of the circles is proportional to the number of individuals observed for each haplotype.

Figure 5. Pairwise F_{ST} versus marine geographic distance within and between clusters identified with STRUCTURE, for 22 feral samples of *Crassostrea gigas*, based on 7 microsatellites.

Figure 1
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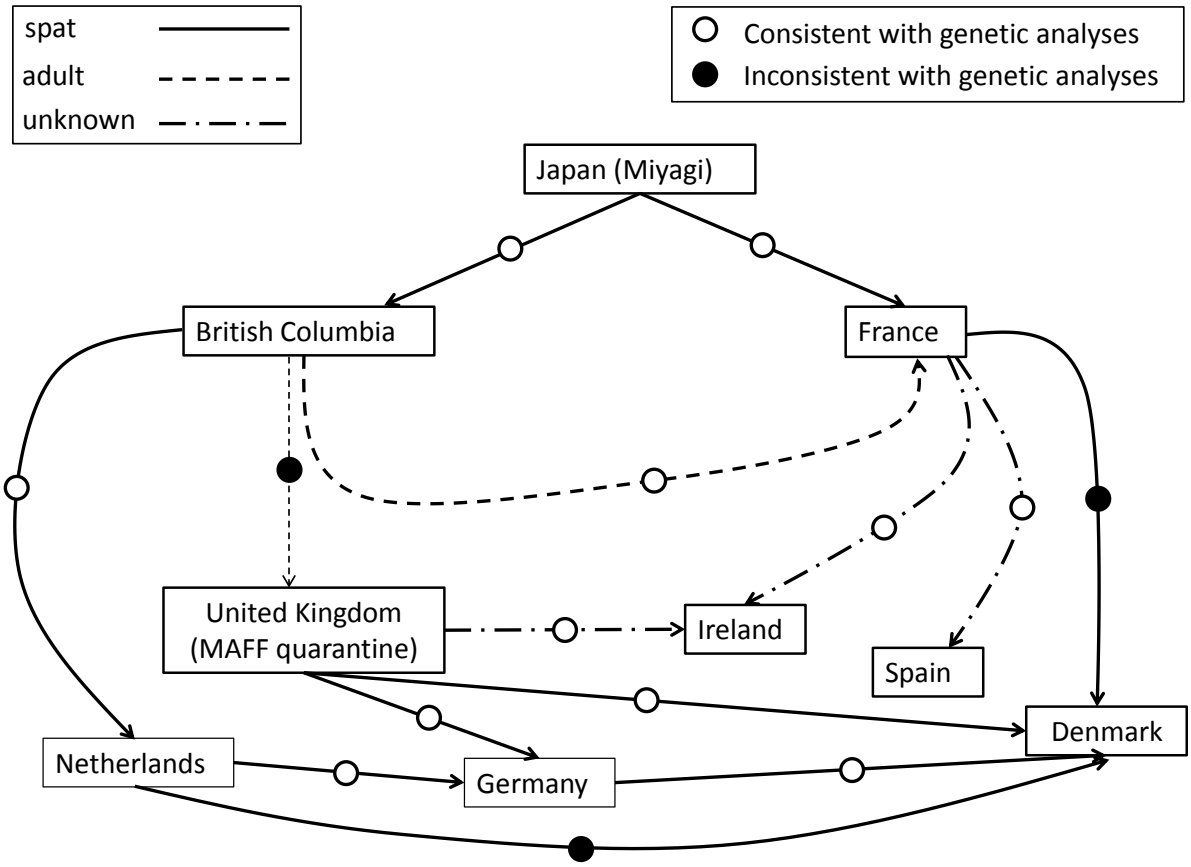

Figure 1

Figure 2
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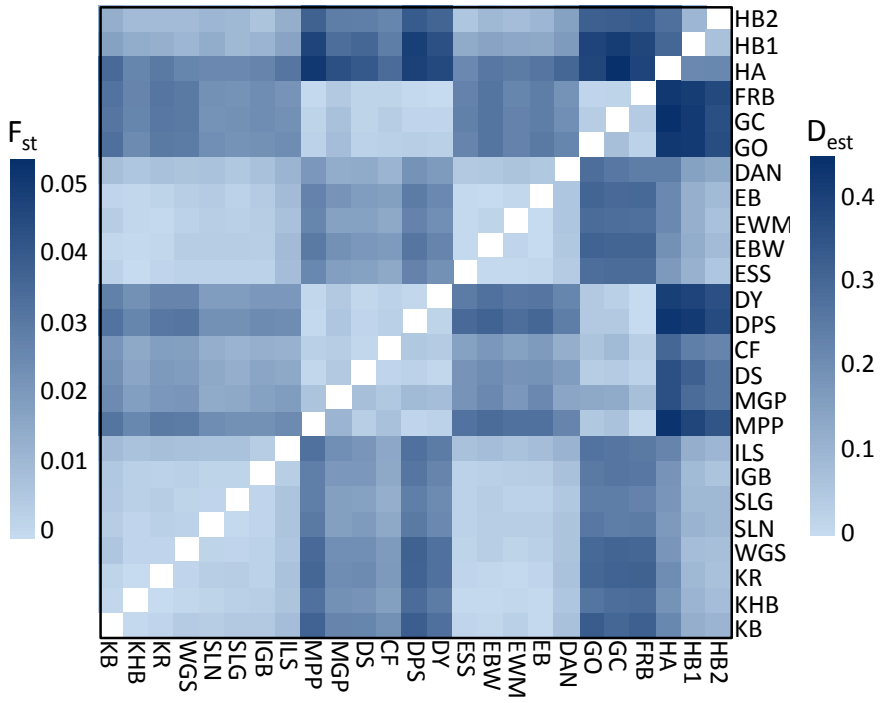
(a)



(b)

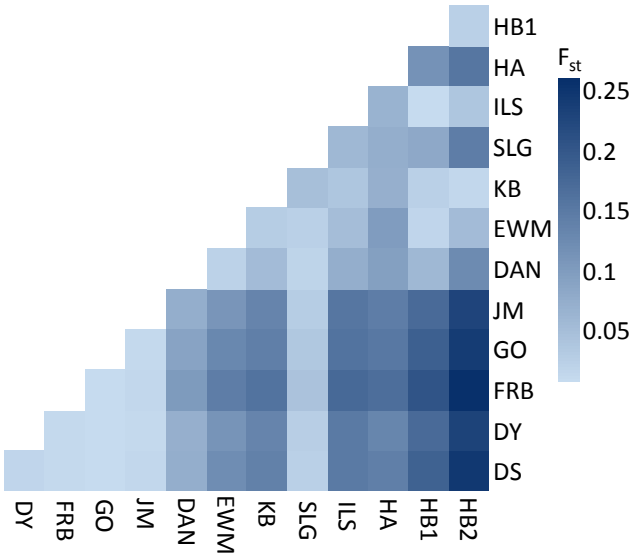


Figure 2

Figure 3
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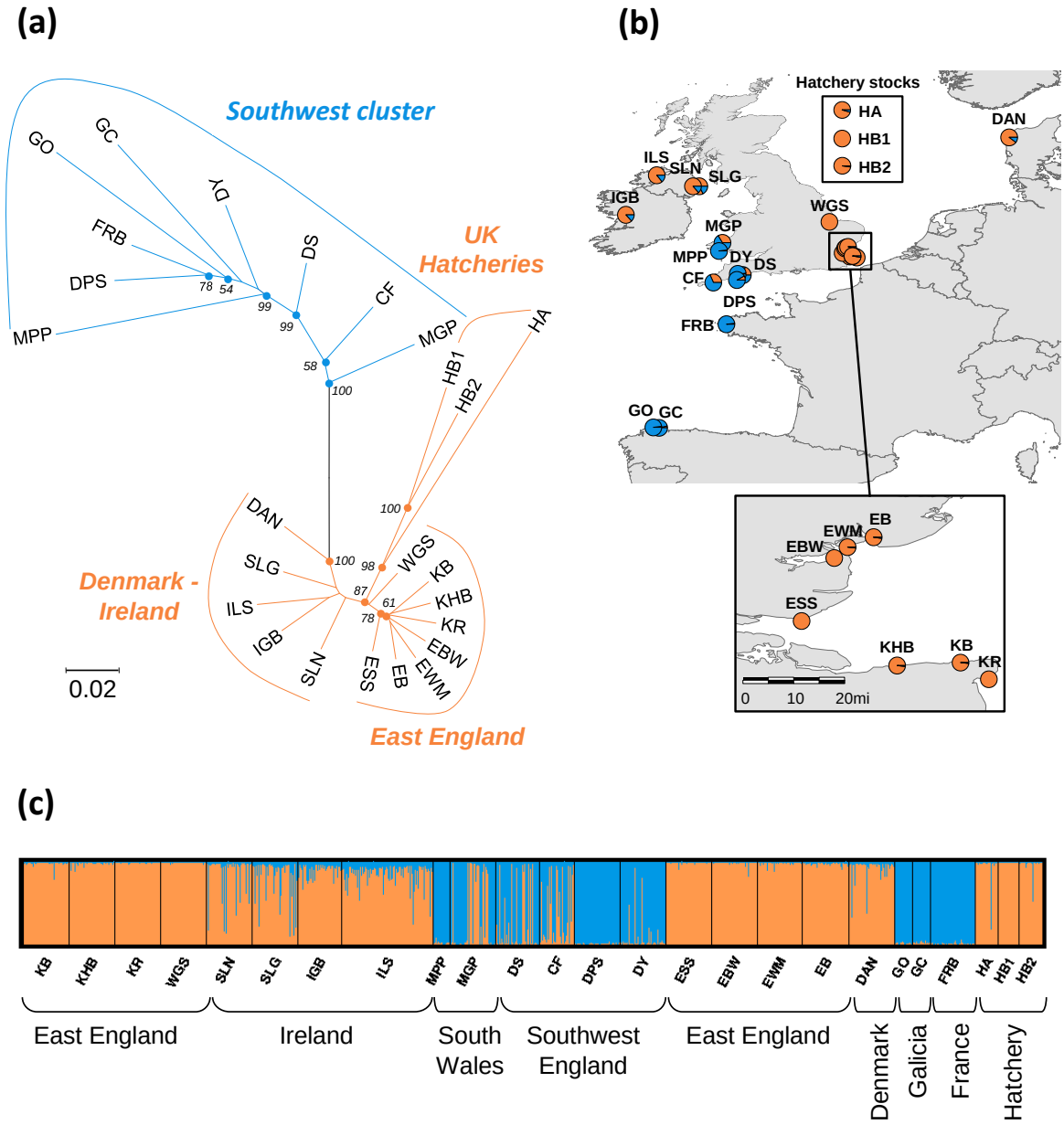

Figure 3

Figure 4
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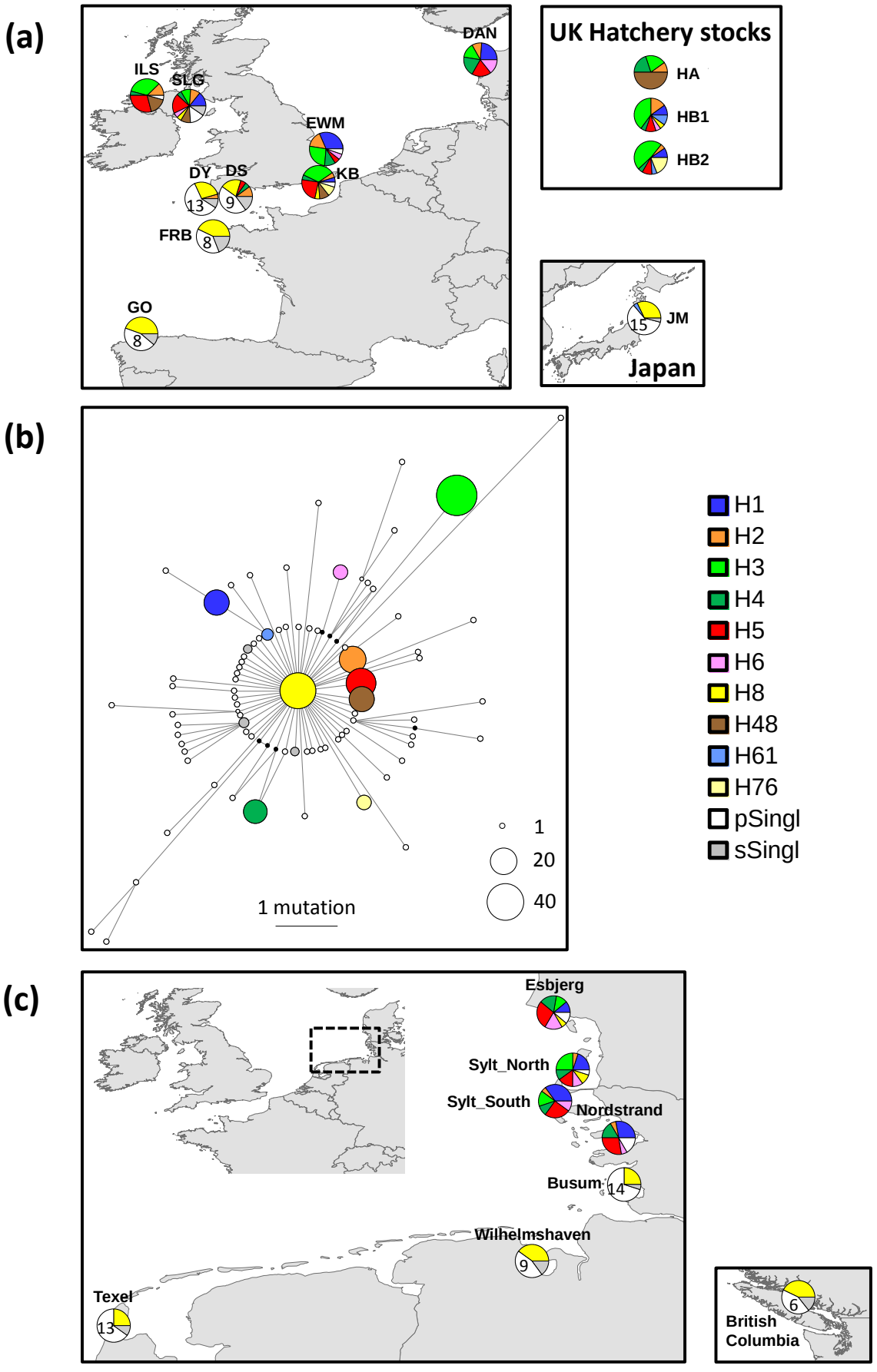

Figure 4

Figure 5
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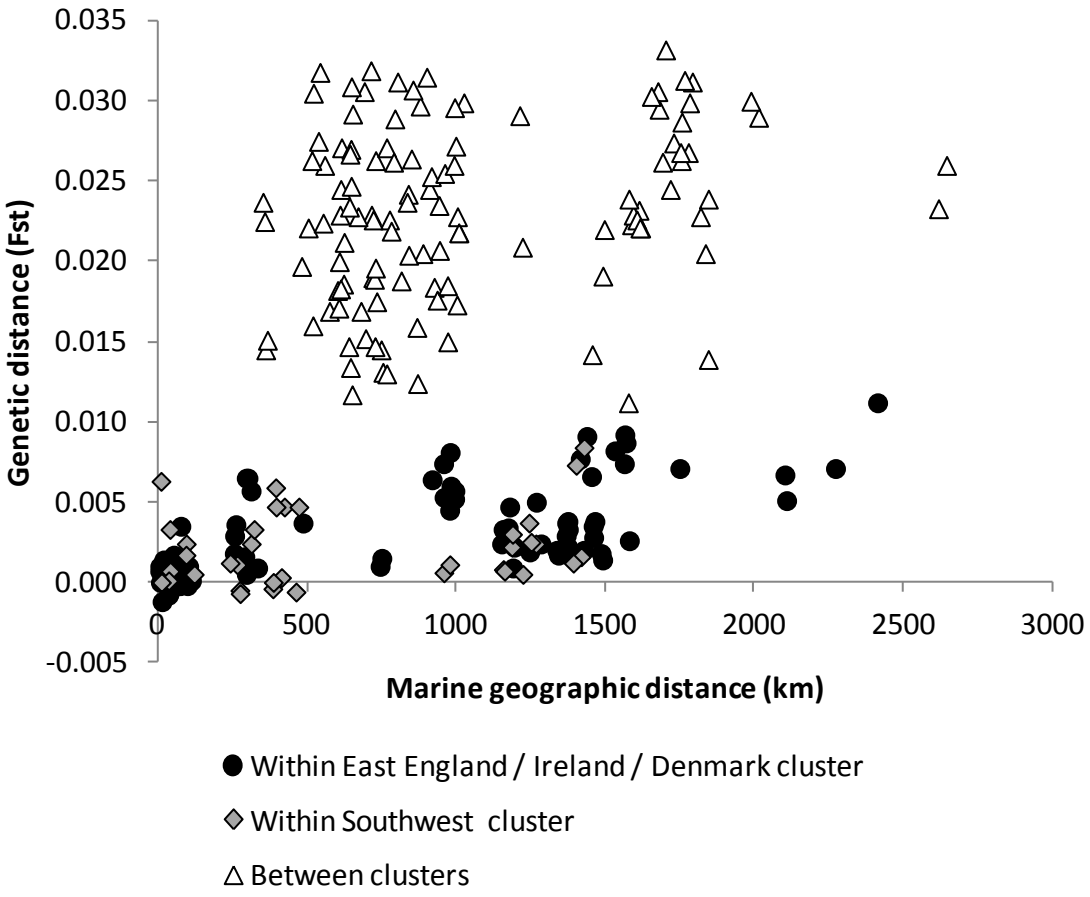

Figure 5

Supplementary Material

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