Mitochondrial DNA phylogeography and mating compatibility reveal marked genetic structuring and speciation in the NE Atlantic bryozoan *Celleporella hyalina*

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Abstract

The marine bryozoan *Celleporella hyalina* is a species complex composed of many highly divergent and mostly allopatric genetic lineages that are reproductively isolated but share a remarkably similar morphology. One such lineage commonly encrusts macroalgae throughout the NE Atlantic coast. To explore the processes leading to geographical diversification, reproductive isolation and speciation in this taxon, we (i) investigated NE Atlantic *C. hyalina* mitochondrial DNA phylogeography, and (ii) used breeding trials between geographical isolates to ascertain reproductive isolation. We find that haplotype diversity is geographically variable and there is a strong population structure, with significant isolation by distance. NE Atlantic *C. hyalina* is structured into two main parapatric lineages that appear to have had independent Pleistocene histories. Range expansions have resulted in two contact zones in Spain and W Ireland. Lineage 1 is found from Ireland to Spain and has low haplotype diversity, with closely related haplotypes, suggesting a recent population expansion into the Irish Sea, S Ireland, S England and Spain. Lineage 2 is found from Iceland to Spain and has high haplotype diversity. Complete reproductive isolation was found between some geographical isolates representing both lineages, whereas it was incomplete or asymmetric between others, suggesting these latter phylogeographical groups probably represent incipient species. The phylogeographical distribution of NE Atlantic *C. hyalina* does not fall easily into a pattern of southern refugia, and we discuss likely differences between terrestrial and marine system responses to Pleistocene glacial cycles.

Keywords: Bryozoa, COI, marine phylogeography, mating trials, paternity testing, reproductive isolation

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Introduction

The strong climatic shifts of the Pleistocene had a dramatic impact on the geographical distribution of organisms (Lomolino *et al.* 2006). The effects of such population range fluctuations on the level and distribution of genetic diversity have allowed us to infer the position of population refugia during the colder climatic phases and the paths of interglacial range expansions and areas of secondary contact and hybrid zones (Hewitt 1999; Avise 2000). Since the pioneering work

of Saunders *et al.* (1986) on the horseshoe crab, *Limulus polyphemus*, showing a strong and unexpected genetic break in an otherwise continuously distributed marine species, the field of marine phylogeography has continued to gain momentum. Comparative phylogeography studies have indicated several regional genetic breaks in littoral marine organisms, often retracing well-known biogeographical barriers, such as the NE Pacific at the California Transition Zone (Dawson 2001), and in the W Atlantic in Florida, Cape Hatteras (Avise 2000), and Cape Cod (Wares 2002). In the NW Atlantic, phylogeographical research has given some support to the view that rocky shore communities became impoverished during glacial maxima and were subsequently repopulated from the NE Atlantic, although

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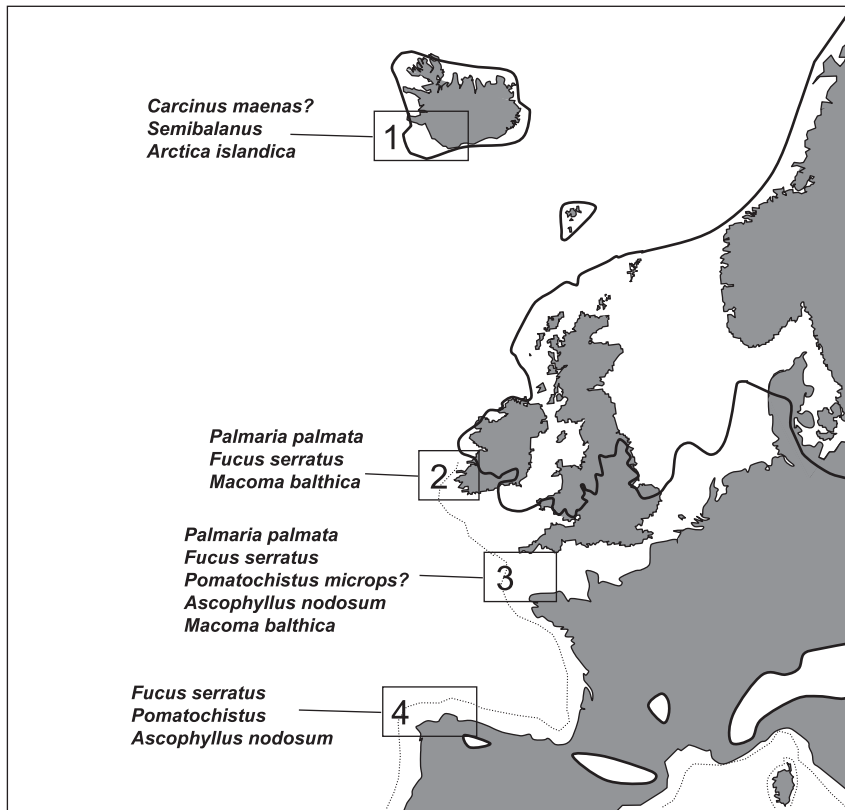
Fig. 1 Map of the NE Atlantic showing the position of proposed glacial refugia in numbered boxes and the organisms supporting each one (see text for references), 1, W Iceland, 2, SW Ireland, 3, the English Channel, 4, Iberian Peninsula. The thick line in the map encloses areas covered by ice sheets, and the stippled line offshore indicates the approximate position of the shoreline during the last glacial maximum (*c*. 20 000 years ago) following Dawson (1992) and Ménot *et al*. (2006) with modifications.

there are exceptions, with NW Atlantic refugia found in several species (Wares & Cunningham 2001). Recent studies on eastern Atlantic fauna and flora are starting to clarify patterns of phylogeographical history, revealing marked breaks between Mediterranean and Atlantic basins, the distinctive history of the Baltic sea, or sharp genetic discontinuities between the North Sea and the Atlantic, often as a result of the presence of cryptic species (Wilke & Davis 2000; Luttikhuizen *et al*. 2003; Gysels *et al*. 2004a; Peijnenburg *et al*. 2004; Roman & Palumbi 2004; Bargelloni *et al*. 2005; Jolly *et al*. 2005; Papadopoulos *et al*. 2005; Johannesson & Andre 2006). Nevertheless, given the long history of Atlantic marine research, our understanding of marine phylogeography for the eastern Atlantic lags surprisingly far behind that achieved for the western Atlantic (Avise 2000; Provan *et al*. 2005). Since there are well-established contrasts in the geological and climatic history, and associated patterns of extinction and colonization of the eastern and western Atlantic fringes (Cunningham & Collins 1998), additional data from the former provides a rare opportunity for comparison.

The NE Atlantic underwent dramatic climatic and sea level changes during the Pleistocene. Vast marine areas and their associated coastlines (North Sea, Baltic Sea, Irish Sea and English Channel) disappeared completely during glacial maxima, either under the ice caps or due to the much lower sea level (Dawson 1992). In the last glacial maximum

(LGM, 20 000 BP) the marine pack ice and terrestrial ice caps reached as far south as the British Isles (Dawson 1992) (see Fig. 1). Consequently, the littoral marine communities currently found in such areas must have been established since the last glacial maximum. Refugial populations are therefore expected to be located south of the permanent ice, and potentially in smaller northerly refugia that might have been free of ice even during the LGM (W Scotland, W Ireland), given that summer ice probably did not reach south of 60°N (Dawson 1992).

A particularly exciting topic of phylogeographical research is the inference of speciation patterns in their temporal and geographical settings (Hewitt 2001). Indeed, phylogeographical analyses have added surprising complexity and detail to previously simplistic views of speciation (Feder *et al*. 2003; Hoskin *et al*. 2005) and have also supported the key role of Pleistocene climatic changes on population differentiation, range shifts and speciation (Klicka & Zink 1997; Avise & Walker 1998; Avise *et al*. 1998; Johnson & Cicero 2004; Zink & Klicka 2006). A challenge for the study of speciation is integrating our understanding of the geographical differentiation of genetic lineages with other biological features of populations, and in particular the evolution of reproductive isolation (Palumbi 1994). To address some of these issues, we combine a phylogeographical study with investigation of reproductive isolation in the marine bryozoan *Celleporella hyalina*.

The cheilostome bryozoan *Celleporella hyalina* (Linnaeus, 1767) inhabits the low intertidal–shallow subtidal zone of cold-temperate to polar oceans circumglobally. The sessile modular colonies live epiphytically, typically on macroalgal fronds, often in large numbers. *Celleporella hyalina* is most often found in relatively sheltered areas, such as coastal bays, inlets, straits, rías and fjords. Colonies grow actively during summer and autumn, when sexual zooids and larvae are produced, but they are mainly quiescent during the winter months, when mortality can be high. Although *Celleporella* is a simultaneous hermaphrodite, colonies are usually self-incompatible (Hoare *et al.* 1999; Hoare & Hughes 2001; Hughes *et al.* 2001). Autozooids capture sperm during filter-feeding, and eggs, presumably after fertilization, pass from the female zooids into special chambers called ovicells, where the embryos undergo placental brooding until released as free-swimming larvae. Many of these features make *C. hyalina* an excellent laboratory system in which to study the nature and evolution of reproductive isolation since acceptance of sperm is chemically, not behaviourally, determined and fertilization success can be easily determined by scoring embryo production within the colony.

As *C. hyalina* colonies are sessile, dispersal is assumed to occur through the nonfeeding, yolk-rich larvae in a short planktonic phase. Larval settlement happens usually within 4 h, with most larvae settling in around 1 h if suitable substrata are available (Cancino & Hughes 1987; Cancino *et al.* 1991; Orellana & Cancino 1991). Dispersal through sperm seems to be equally limited, as the half-life of released spermatozoa is about 1 h (Manríquez *et al.* 2001). Therefore, the restricted movement of larvae and gametes could indicate a high potential for population differentiation as populations should be highly sensitive to even transient geographical, ecological or hydrographical barriers to gene flow (Hellberg 1998; Goldson *et al.* 2001). On the other hand, the nature of *C. hyalina's* common substratum (macroalgae) makes it likely that rafting on detached floating fronds could be a significant means of dispersal, enabling colonization of new and distant habitats. Accordingly, the wide geographical distributions of many bryozoan species have been linked to rafting ability (Watts *et al.* 1998; Thiel & Gutow 2005). Finally, dispersal due to human activities such as shipping, fisheries, aquaculture, discard of floating debris, etc. (Watts *et al.* 1998; Ruiz *et al.* 2000; Barnes 2002) could also have affected the recent distribution of genetic variation in *Celleporella*. Indeed, *Celleporella* species have been described as nonindigenous recent invaders in the Venice lagoon (Italy), Port Philip Bay (Australia) and Humboldt Bay (California, USA) (Occhipinti Ambrogi & d'Hondt 1996; Hewitt *et al.* 1999; Boyd *et al.* 2002).

Celleporella hyalina is a cryptic species complex composed of numerous genetic, reproductively isolated lineages that occupy mostly allopatric regions around the globe, yet are morphologically very similar (Gómez *et al.* 2006). The large genetic distances (8–20% in mitochondrial DNA) between these lineages make it difficult to draw inferences on the development of reproductive barriers and speciation in this taxon. Here, we focus on one of these lineages, the most commonly found in the Northeast Atlantic coastal region from Iceland to Spain and from Ireland to Shetland. We investigated the mtDNA phylogeography of *C. hyalina* in the NE Atlantic to assess the relative role of ongoing gene flow and historical processes in structuring its genetic diversity. In addition, we carried out cross-breeding experiments (including backcrossing and F_1 compatibility) between geographical isolates to assess the development of reproductive barriers and speciation.

Materials and methods

Sample collection and DNA extractions

Macroalgal samples were collected in the intertidal or subtidal zone manually or by SCUBA diving. Sixteen sites encompassing the known geographical range of *Celleporella hyalina* were sampled during the summers of the years 2000–2003 (Table 1 and Fig. 2). Individual colonies were removed from the fronds, cutting the algae around the colony. If possible, 20 mature, healthy-looking colonies from each site were collected and preserved in 100% ethanol in cool, dark conditions until required. Note, however, that in the NE Atlantic range focus of this study, *C. hyalina* is formed by several cryptic species (Gómez *et al.* 2006), and this led to reduced sample sizes for the NE Atlantic lineage in some locations (RON, OBA, DOR, see Table 1). Live colonies were also collected, and transported to the laboratory between wet tissues in a thermos flask or plastic boxes in order to produce bryozoan cultures to be used in mating trials. In one site, Ría de Ferrol (Spain), two types of colonies can be recognized morphologically ('typical' and 'reticulata' form) (Fernández-Pulpeiro & Reverter-Gil 1992; Wright 2004) and they were treated as separate populations in our analyses.

A method for total DNA extraction from small invertebrates (Gómez & Carvalho 2000) was used with minor modifications. In brief, a small fragment of an individual colony (5–10 zooids) or, preferentially, an embryo dissected from its ovicell, or a single ancestrula, were isolated under a stereoscope, rinsed in distilled water and transferred to 45 μL of 6% Instagene Matrix (©Bio-Rad). The sampled material was then crushed using a sterile pipette tip, and the mixture briefly shaken and boiled for 10 min. From the supernatant, 2 μL was used as DNA template for polymerase chain reaction (PCR). Special care was taken not to include any potentially PCR-inhibiting exudates from algal substratum in the DNA extraction. Because of the occurrence of a wide variety of organisms as epibionts on wild colonies, which readily amplify using 'universal' primers, the use of

Table 1 Sampling locations and summary statistics of population genetic diversity NE Atlantic *Celleporella hyalina*. Location codes, and geographical coordinates. Geographical samples used in the mating trials are marked with an asterisk (*) after the code

Location	Coordinates	Code	N	Lineage	h	S	H_d	π
Alfsnes, Reykjavik, Iceland	64°09′N, 21°58′W	ICE*	4	2	3	5	0.83	0.0045
South Voe-Lang Sound, between W and E Burra, Shetland	60°04′41″N, 1°20′03″W	SHE	4	2	2	8	0.67	0.0090
St. Peter's Pool, Deerness, Orkney	58°55′02″N, 2°47′19″W	DEE	5	2	4	7	0.90	0.0047
Oyce of Herston, Widewall Bay, S Ronaldsay, Orkney	58°48′22″N, 2°59′51″W	RON	2	2	2	14	—	—
Port nan Cuilc, Gallanach Harbour, Oban, Scotland	56°22′46″N, 5°31′34″W	OBA	1	2	1	0	—	—
The Dorn, Strangford Lough, Northern Ireland	54°26′03″N, 05°33′02″W	DOR	2	1	1	0	—	—
Achill Sound, Corraun, Ireland	53°52′11″N, 09°56′32″W	ACH	7	1,2	4	20	0.81	0.0108
Amlwch Harbour, Wales	53°25′08″N, 04°20′10″W	AML*	9	1	1	0	0	0.0000
Dublin Harbour, Ireland	53°20′40″N, 06°11′22″W	DUP	5	1	2	1	0.40	0.0007
Holyhead Harbour, Wales	53°18′44″N, 04°38′48″W	HOLY	5	1	2	1	0.60	0.0010
Dun Laoghaire Harbour, Ireland	53°17′48″N, 06°08′21″W	DUN	5	1	2	1	0.40	0.0007
Ardfry Rapids, Oranmore, Ireland	53°16′01″N, 08°58′05″W	ARD	9	2	5	19	0.81	0.0129
Church Island, Menai Bridge, Wales	53°13′27″N, 04°09′50″W	CHI, MB	10	1	1	0	0	0.0000
The Rapids, Lough Hyne, Ireland	51°28′43″N, 09°18′15″W	IRL*	9	1	2	1	0.22	0.0004
Plymouth, England	50°15′N, 04°10′W	PLY*	10	1	2	2	0.20	0.0007
Ría de Ferrol, San Felipe, La Coruña, Spain	43°28′06″N, 8°16′09″W	SPA*, RET*	12	1,2	3	21	0.68	0.0150

N, number of sequenced individuals; lineage, mtDNA lineage found in this study; S, number of segregating sites; h, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity.

embryos obtained from ripe colonies was favoured for DNA extractions from field-collected material.

DNA amplification and sequencing

Mitochondrial DNA sequences were obtained by cycle sequencing of PCR-amplified DNA. PCR amplifications were performed in 10-μL final volume containing 2 μL template DNA, 1.5 mM MgCl$_2$, 200 μM of each nucleotide, 2.5 pmol of each primer, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), 0.01% Tween 20 buffer, and 0.125 U *Taq* DNA polymerase. Amplifications were performed using the following cycling conditions: 3 min denaturing at 93 °C; (15 s at 92 °C, 20 s at 45–55 °C, 1 min at 70 °C) × 40, 3-min extension at 72 °C. A 710-bp region of the cytochrome *c* oxidase gene subunit I (COI or *cox1*) was amplified with primers LCO1490 and HCO2198 (Folmer *et al*. 1994). End-labelled (Cy5) versions of the primers were used for sequencing the PCR products using the Thermo Sequenase Primer Cycle Sequencing kit (Amersham Pharmacia Biotech). Sequencing runs were performed on an ALFexpress (Amersham Pharmacia Biotech) automated sequencer. Forward and reverse sequences were aligned by eye and polymorphic sites were double-checked manually. All sequences and alignments (as popsets) obtained in this study were deposited in GenBank (accession nos EF371553–EF371616). The mtDNA data set included previously published sequence data from populations of Iceland, Oban, Achill, Amlwch, Spain and The Dorn (accession nos DQ999714–18, DQ999720–723, DQ999740–750).

Phylogenetic reconstructions

A phylogeny of the mtDNA haplotypes was inferred using neighbour joining (NJ), maximum likelihood (ML) and Bayesian inference (BI). We used the program MODEL-GENERATOR version 6.0 (Keane *et al*. 2006) to find the most appropriate model of evolution for ML. The program phyml (Guindon & Gasquel 2003) was used to implement the ML analyses. MEGA 3.1 (Kumar *et al*. 2004) was used to produce an NJ tree. Confidence in established phylogenetic relationships was assessed by 1000 nonparametric bootstrap pseudoreplicates. MRBAYES version 3.1.1. (Ronquist & Huelsenbeck 2003) was used to perform a partition-likelihood Bayesian search using the codon positions in the partition and a 4-by-4 nucleotide model for each codon position with six nucleotide substitution rates. The default priors in MRBAYES were used except for ratepr which was set to variable = dirichlet. Two simultaneous Metropolis-coupled Markov chain Monte Carlo analysis were run each with four chains (3 'heated') for 2 000 000 generations, sampling trees and parameters every 100 generations. We discarded the first 250 000 generations (2500 trees) on each run as 'burn-in' after confirming chain stationarity from plots of likelihood against generation. The consensus tree was obtained from the 35 000 trees from both runs sampled after the initial burn-in period.

Population structure and phylogeographical analyses

Number of segregating sites, haplotype diversity, nucleotide diversity per population, pairwise and global population
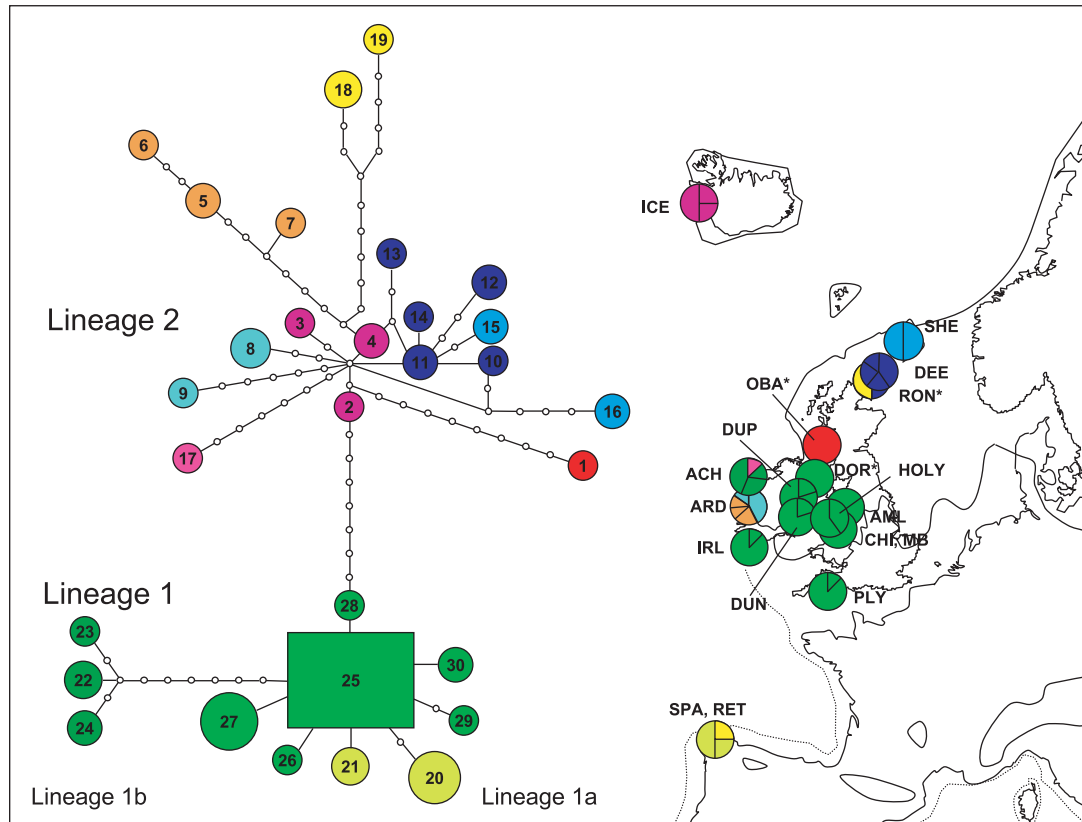
Fig. 2 Haplotype network and sampling locations of NE Atlantic *Celleporella hyalina*. The network on the left shows the relationships of the 30 haplotypes found, the numbers inside each circle is that assigned to each haplotype, and the size of each circle is proportional to its frequency (except for haplotype 25 represented as a square for scale reasons). The map on the right shows the sampling sites and sample composition (the pie charts indicate the relative frequency of the haplotypes found in each sampling point (same colour codes as in the network). The asterisks after the population code indicates that sample sizes were below 4. The thick line in the map encloses areas covered by ice caps, and the stippled line offshore indicates the approximate position of the shoreline during the last glacial maximum (*c*. 20 000 years ago) modified from Dawson (1992).

F_{ST} *sensu* Hudson *et al*. (1992), and neutrality tests were computed with DNASP version 4.00 (Rozas *et al*. 2003) in the 13 populations of sample size of four or more. To examine the data set for evidence of isolation by distance (Wright 1943), we regressed pairwise population genetic distance, defined as $F_{ST}/(1 - F_{ST})$, on the logarithm of geographical distance between populations, as suggested by Rousset (1997) for bidimensional distributions. We then evaluated the relative roles of gene flow and random genetic drift using the pattern revealed by the scatter plot (Hutchison & Templeton 1999). The Pearson product–moment correlation coefficient between the genetic and geographical distance matrices was assessed using Mantel tests (Mantel 1967; Mantel & Valand 1970; Manly 1997), as implemented in POPTOOLS version 2.6 (Hood 2005). *P* values were obtained through 10 000 permutations. Given that bryozoans are unlikely to disperse over land, we calculated shortest distances between sites across seawater (accounting for landmasses) using GOOGLEEARTH.

We used nested clade analysis (NCA) (Templeton 1998) to test for associations between genealogy and geography in the NE Atlantic *C. hyalina* data set, and to discriminate between current patterns of gene flow and past events of population subdivision or range expansions. A statistical parsimony unrooted network of the mtDNA haplotypes was constructed using TCS version 1.18 (Clement *et al* 2000). We followed rules described in Templeton *et al*. (1992) and Crandall (1996) to construct a nested design based on the network. The program GEODIS 2.4 (Posada *et al*. 2000) was used to calculate the distance measures and their statistical significance. A matrix with the shortest distance across water between populations (see above) was used in the GEODIS input file. The statistical distribution of the distance measures was determined recalculating all distances after 1000 random permutations of clades against sampling locality. The latest GEODIS key was used to infer the causes of significant geographical associations (November 2005, available in http://darwin.uvigo.es/download/geodisKey_11Nov05.pdf).

Table 2 Reproductive isolation in *Celleporella hyalina* in the NE Atlantic. Column and row headers indicate the population origin of the colonies used in the mating trials. The three numbers in each cell represent (i) fraction of genotypes releasing ≥ 100 larvae in each trial. All crosses were made of four replicates except for the ones involving Spain RET, which had three replicates; (ii) percentage of the first 100 settled larvae reaching sexual maturity (averaged across replicates); and (iii) fraction of the F_1 genotypes releasing ≥ 100 larvae pooled between the $F_1 \times F_1$ and the F_1 backcross trials. 0 indicates that no larvae settled or none was produced. — indicates test not performed. Populations belonging to lineage 1 are in normal font; those belonging to lineage 2 are shown in bold. Diagonal entries show the average number of larvae (up to the first 100) released by each genotype in controls for reproductive competence. Results of controls for self-fertilization are presented in the text

| | Father | | | | | |
Mother	**Iceland**	**Spain 'RET'**	Ireland	Plymouth	Spain 'typical'	Amlwch
Iceland	50, 64, 28.5, 89	0	0	—	0	0
Spain 'RET'	0	100, 100, 100	—	—	3/3; 100; 1/2	2/3; 100; –
Ireland	0	—	100, 100, 100, 100	4/4; 100; 8/8	4/4; 100; 7/8	4/4; 100; 8/8
Plymouth	—	—	3/4; 99; 5/8	100, 100, 100, 100	3/4; 100; 4/8	3/4; 99; 8/8
Spain 'typical'	0	1/3; 70; —	1/4; 100; 3/5	4/4; 99; 5/8	100, 100, 100, 100	3/4; 100; 8/8
Amlwch	0	0/3; —; —	2/4; 100; 6/8	1/4; 100; 8/8	3/4; 100; 7/7	100, 100, 100, 100

Production of ramets and laboratory culture

Algal fronds bearing colonies were placed in 2-L plastic bottles, each containing 1 L of 2 μm-filtered, U.V.-irradiated seawater (FSW), to which 100 mL of *Rhinomonas reticulata* culture were added daily at a concentration of approximately 700 cells/μL. Light aeration via an aquarium airstone was provided. An acetate sheet, which had been conditioned in running seawater for 1 week, provided a substratum for larval settlement. Newly formed ancestrulae were excised from the settlement sheet by cutting out a surrounding 10 × 10 mm square of acetate, which was then glued with cyanoacrylate onto another piece of acetate 38 × 75 mm. This procedure was carried out before first feeding to prevent uptake and storage of allosperm (Hughes *et al.* 2002). Each newly excised ancestrula was placed in isolation in a 300-mL glass jar containing 225 mL FSW, to which 20 mL of algal culture was added daily. Each jar was fitted with a lid and aeration and food ports designed to reduce the risk of accidental allosperm transfer (Manríquez *et al.* 2001). After *c.* 3 months, each colony had reached a size (2–3 cm diameter) suitable for cloning to produce ramets. The resulting ramets were placed in slide racks and housed in 2-L plastic drinks bottles furnished and maintained as described above. Each genotype was maintained in reproductive isolation after cloning, ensuring that all ramets used in the mating trials were virgin. Colonies were maintained at 15 °C, within 5 °C of mean summer sea-surface temperature maxima at all native localities.

Mating trials and paternity analyses

Six populations of NE Atlantic *C. hyalina* were used (Table 2). For each trial, potential partners were placed together in a 300-mL glass jar set up and maintained as above for 2 weeks. Each colony was therefore able to act as both a male (sperm donor) and a female (sperm recipient) in each mating. Partners were checked weekly for the presence of embryos under a microscope, and those found to be brooding embryos were removed to a separate glass jar fitted with a conditioned acetate sheet, in which they were cultured in isolation for a further 2–3 months. Separating the partners before the release of larvae enabled maternal genotypes to be ascertained for each larva released. To estimate the fecundity of each cross, we counted the number of larvae released by each ramet (to maintain a manageable workload we ignored larvae beyond the first 100 released and recorded the results as > 100).

To estimate F_1 survival, the first 100 larvae released were grown to sexual maturity (when both male and female zooids are found within the colony) and the percentage of the first 100 settled larvae reaching sexual maturity was calculated. In order to monitor the growth of the first 100 settled larvae, any subsequently released larvae were scraped off the acetate sheet. Four replicates were carried out for each mating trial, using four different genotypes from each experimental population. However, only three Spanish genotypes of the *reticulata* form survived for use in mating trials.

Each mating trial was complemented by a set of controls to ensure that (i) all of the genotypes being used in the trial were reproductively active at the time that the trial took place, and (ii) self-fertilization was not occurring. To assess reproductive activity, a ramet of every genotype from each of the two populations used in a mating trial was placed with another genotype from the same population in a 300-mL glass jar, and the test was performed and evaluated as above. In the self-fertilization controls, ramets of each genotype were maintained in isolation in stock bottles. These ramets were checked weekly for production of embryos or larvae throughout each mating trial.

As a further assessment of reproductive compatibility between geographical isolates, we estimated F_1 fecundity in $F_1 \times F_1$ trials and backcrosses to genotypes from the maternal population as follows. For each successful mating trial, one newly settled ancestrula (F_1) released by each maternal colony was removed from the settlement jar by excision of the acetate substratum, glued onto another piece of acetate, and placed in isolation as described above. The resulting colony was grown to a size sufficient to produce two ramets. One ramet was then used to perform a backcross mating to a genotype from the maternal population (not the maternal genotype) and the other ramet was paired with another F_1 genotype released by a colony from the paternal population, but not the paternal genotype. During the cloning procedure, a small part of each F_1 colony was preserved in 100% ethanol for paternity analysis.

To confirm outcrossing of colonies, we amplified the highly polymorphic microsatellite locus CHY1 in the putative hybrid offspring obtained in successful breeding tests using the primers and PCR conditions described in Hoare *et al.* (1998) with the reverse primer end-labelled with Cy5DNA. Extractions were performed on individual parental and offspring (F_1) colonies as described above. Ten F_1 colonies were screened from each parental colony used in each reciprocal population cross and included those to be used in the $F_1 \times F_1$ and backcross trials. The PCR products were resolved on 6% acrylamide gels using an automatic sequencer (ALFexpress, Amersham Pharmacia Biotech), along with appropriate internal size markers (Van Oppen *et al.* 1997). Allele sizes were scored using the program FRAGMENT MANAGER (Amersham Pharmacia Biotech).

Results

Genetic diversity, phylogenetic relationships and population structure

We found a total of 30 different haplotypes in the 592-bp fragment obtained from 99 individual *Celleporella hyalina* colonies. There were 74 polymorphic sites, 48 of them parsimony informative, and six replacement changes that occurred scattered through the data set. Of the 30 haplotypes found, 29 were restricted to single sample sites (private haplotypes). Just one haplotype (H25) was found in multiple sampling sites (eight sites from Wales, England and E and S Ireland).

All phylogenetic methods retrieved similar topologies (Fig. 3). Two lineages showed marked geographical orientation. Lineage 1 was a strongly supported branch including all individuals from England, Wales, E and S Ireland, six out of seven individuals from Achill (W Ireland) and nine out of 12 from Spain. This group had relatively low diversity, with a common widespread haplotype (H25), but high bootstrap support indicated subdivision into two

sublineages, one of them formed by some Achill individuals (lineage 1b) and the other for the rest of the lineage (lineage 1a). Lineage 1a has low haplotype diversity and closely related haplotypes. All populations in lineage 1a, except that from Spain, were found in areas either covered by ice or situated inland (due to lower sea levels) at the time of the LGM (see Fig. 1). This group had relatively low diversity and was star-shaped, with a common widespread haplotype (H25). Lineage 2 was formed by all individuals from Iceland, Shetland, Orkney, Scotland, and Ardfry (W Ireland), one individual from Achill (W Ireland), and three Spanish individuals. This lineage had high haplotype diversity and contained several long branches. Two populations contained haplotypes from both main lineages: Spain and Achill (W Ireland). All Spanish 'typical' individuals had lineage 1 haplotypes, all but one of the Spanish '*reticulata*' individuals had lineage 2 haplotypes.

Average sequence divergence between lineages 1 and 2 is 2.24% (uncorrected *P*). Average divergence between lineages 1a and 1b is 1.51%. Populations showed a wide range of haplotype and nucleotide diversity (Table 1). Populations from lineage 2 (see below) showed higher haplotype diversities (average 0.80, range 0.67–0.90) than populations from lineage 1 (average 0.30, range 0–0.60). In addition, the nucleotide diversities of lineage 2 populations were much higher indicating that these populations comprise more divergent haplotypes. The two populations where both clades co-occurred had high haplotype diversity (0.68 for Spain and 0.81 for Achill Sound, W Ireland).

Global haplotype (gene) diversity (H_d) was 0.809 (variance: 0.00154). Global population differentiation (F_{ST}) *sensu* Hudson *et al.* (1992) in the 13 populations with sample sizes of four individuals or more was very high (0.64), and the estimated *Nm* using the formula for haploid data and assuming an island model is 0.28 migrants per generation.

Neutral expectations were not rejected by Tajima's *D* ($D = -1.72905$, $0.10 > P > 0.05$) or Fu and Li's *D** test statistic ($D^* = -2.24828$, $0.10 > P > 0.05$). However, Fu and Li's *F** test statistic was significantly different from neutrality expectations ($F^*: -2.43793$, $P < 0.05$) indicating an excess of low-frequency polymorphisms. These could indicate a recent selective sweep or nonequilibrium demographic effects such as range expansions or population subdivision (Tajima 1989).

A strong, significant positive relationship was found between genetic and geographical distance in *C. hyalina* (Fig. 4). Mantel tests for the geographical and genetic distance matrices showed significant Pearson correlation coefficients ($r = 0.373$, $P = 0.005$). The pattern produced by the regression of genetic distance on geographical distance was approximately wedgelike, with the variance in genetic distance increasing with geographical distance, therefore resembling Case I of Hutchison & Templeton (1999). However, there was a wide scatter of points, including distant
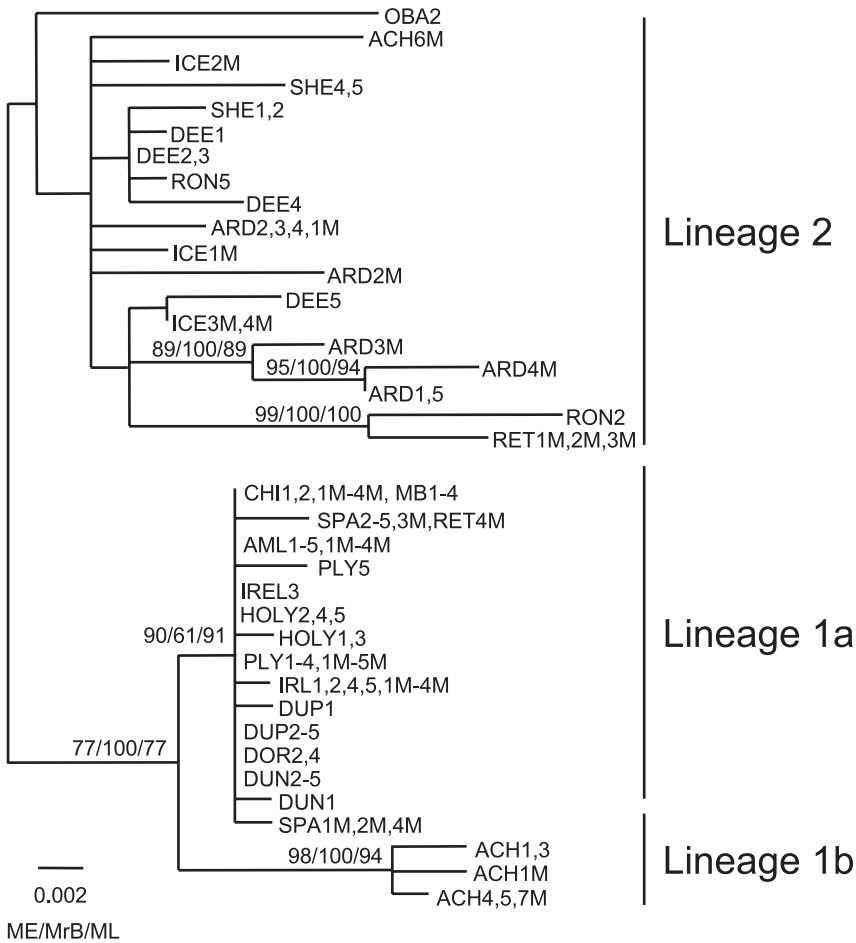
Fig. 3 Midpoint-rooted NJ phylogram representing the relationships of mtDNA sequences found in NE Atlantic *Celleporella hyalina*. Numbers represent percent bootstrap support/posterior probability in the minimum evolution, Bayesian inference and maximum-likelihood analyses. Only nodes with ME bootstrap values = 70 are labelled and those less than 50% are collapsed. The individuals sequenced are represented by the sample code and a number, followed by an M if the particular individual was used in the mating trials.
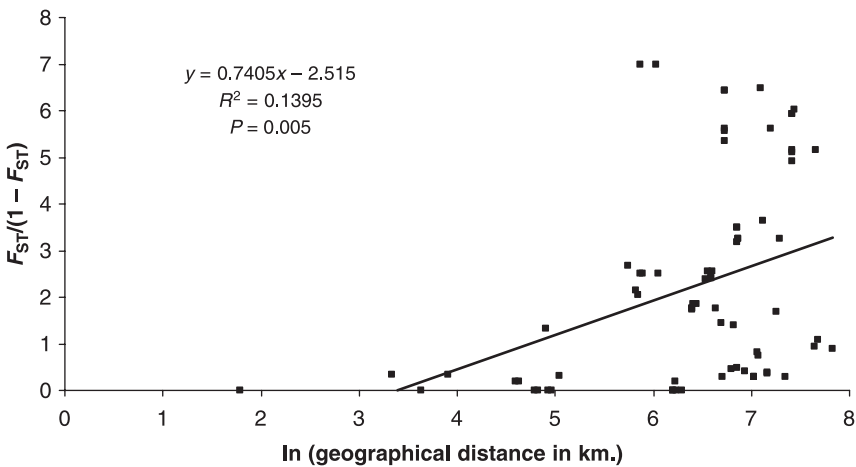

Fig. 4 Plot showing isolation by distance in *Celleporella hyalina* in the Northeast Atlantic. The analysis includes the 13 populations with sample size > 4.

points with very low F_{ST} (some of those possibly involved in the population expansion, see above). In addition, the presence of some population pairs with no or little haplotype diversity (fixed for distinct haplotypes no matter how related) yielded values of F_{ST} estimates close to 1. Consequently, the large F_{ST} found between some distant populations made residual analyses impossible because of boundary effects.

Nested clade analyses

The median haplotype network and nested design used for the nested clade analysis is shown in Fig. 5, with the geographical distribution of the haplotypes, and the haplotype network is shown in Fig. 2 to aid in the interpretation of NCA. The maximum number of parsimonious connections was 10. The nested design had five levels
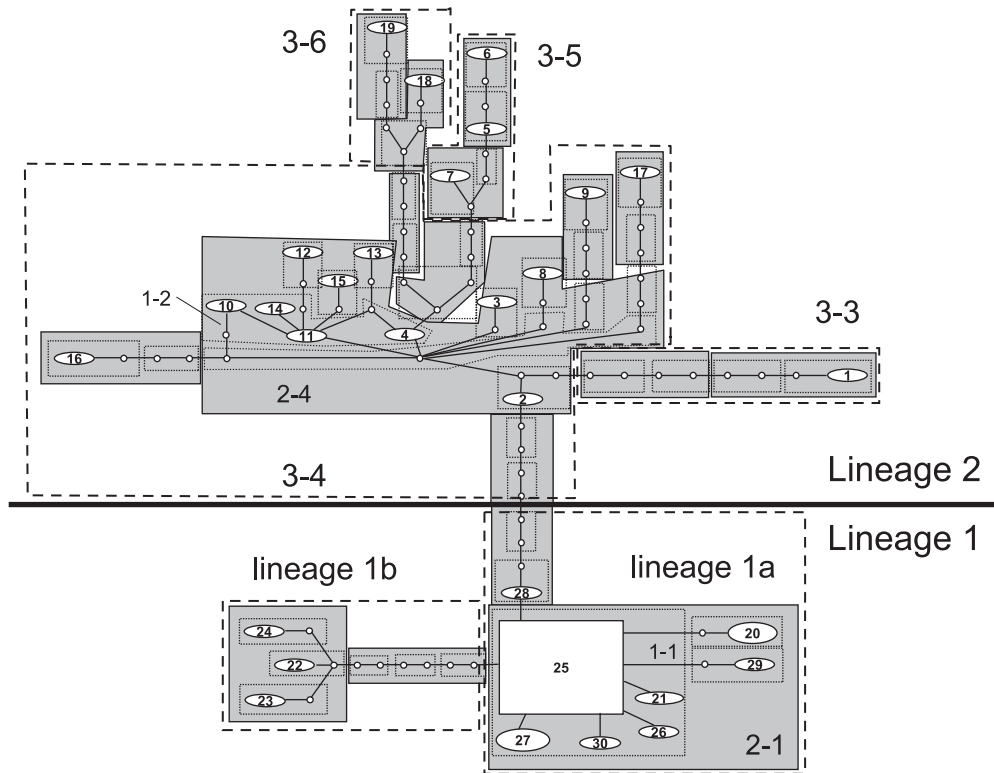
Fig. 5 Nested design for the 30 mtDNA haplotypes found in NE Atlantic *Celleporella hyalina* overlayed on the minimum-spanning network. Haplotypes are indicated by their numbers. See Table 1 for haplotype distribution. Unfilled circles represent missing intermediate haplotypes. For clarity, only informative clades (for which there is both geographical and genetic variation) have been designated by the *x.y* system, where *x* represents the nesting level (from the haplotype level to the 4th level) and *y* represents the number assigned to that particular clade. Each line represents one mutation. All connections indicated are supported to be parsimonious at the 95% level.

including the total cladogram (Fig. 5). The highest-level clades correspond to lineages 1 and 2. Lineage 2 contained two loops involving haplotypes from Iceland, Shetland and Orkney, but the resolution of these loops did not affect the nested design. The nine nested haplotype groups for which we had geographical and genetic information were used in the GEODIS input. Seven nested haplotype groups yielded significant associations of haplotype and geography. NCA inferences for these groups are given in Table 3. We found evidence for two past fragmentation events, one of them involving the total cladogram, which supports the allopatric fragmentation of lineages 1 and 2, which are now parapatric. The other past fragmentation event involves lineage 1a (found in England, Wales, E and S Ireland and Spain) and lineage 1b (found in Achill, and W Ireland). In the two other nested lower level clades within lineage 1a (1-1 and 2-1), a highly significant association between haplotype groups was found, but the factors involved in creating these patterns could not be conclusively determined because of the reduced number of clades involved. The deepest, oldest event inferred by NCA is a contiguous range expansion in lineage 2 as a whole, affecting nested clade 3-4, found in Iceland, Ireland, Shetland and Orkney

(Fig. 2). This event could explain the secondary contact between this clade and lineage 1 (Fig. 2). In addition, restricted gene flow with isolation by distance was inferred in clade 3-4, distributed in Iceland, W Ireland, Shetland and Orkney. Finally, significant geographical associations with inference of past fragmentation or long-distance colonization was inferred for clade 2-4 (found in Iceland, Ardfry, Shetland and Orkney), the (marginally) significant event seems related to the divergent haplotype (H8) found in Ardfry, Ireland, which was either isolated allopatrically from the rest of its nested clade or migrated into Ireland from a more northern latitude.

Mating compatibility and paternity analyses

We performed 24 between-population mating trials in which we estimated resulting fecundity, viability of the F_1 and fecundity of the F_1 in both backcrosses and $F_1 \times F_1$ crosses. We used four genotype replicates for each pair of populations tested. Eight of the trials performed were unsuccessful, with no production of viable larvae, and all of them involved the Icelandic population (belonging to lineage 2) both as sperm donor and as sperm receptor, crossed with Spanish '*reticulata*'

Table 3 Results of nested clade analysis for *Celleporella hyalina* NE Atlantic using 'as the larvae swims' distances. Only clades with genetic and geographical information are shown. The probability refers to the frequency with which the 1000 randomly generated chi-square statistics were equal to or greater than the observed chi-square. GEODIS key version November 2005 was used to infer the cause of geographical structure

Clade	Subclades	Distribution	Probability	Inference chain	Inferred pattern
1-1	H25	SE Ireland, Wales, PLY	0.0000	1-2-3-5-6-7-8-NO	insufficient resolution to discriminate between isolation by distance vs. long-distance dispersal
	H21	SPA			
	H26	DUP			
	H30	HOLY			
	H27	IRL			
2-1	H29	PLY	0.0010	1-2-3-5-6 TOO	insufficient resolution to discriminate between range expansion and restricted gene flow
	H20	SPA, RET		FEW CLADES	
	1-1	SE Ireland, Wales, PLY, SPA			
Lineage 1a (3-1)	H28	DUP	0.2770	—	—
	2-1	SE Ireland, Wales, PLY, SPA			
Lineage 1 (4-1)	3-2	ACH	0.0000	1-19-20-2-3-5-15-NO	past fragmentation
	3-1	SE Ireland, Wales, PLY, SPA			
2-4	H2	ICE	0.0120	1-2-3-5-15-NO-21 YES (partial)	past fragmentation or long-distance colonization into W Ireland (ARD sample)
	H3	ICE			
	H8	ARD			
	H12	DEE			
	H13	DEE			
	H15	SHE			
	1-2	ICE, Orkney			
3-4	H9	ARD	0.0370	1-2-3-4-NO	restricted gene flow with isolation by distance
	H16	SHE			
	H17	ACH			
	2-4	ICE, Orkney, SHE, ARD			
3-6	H18	RET	0.2330	—	—
	H19	RON			
Lineage 2 (4-2)	H1	OBA	0.0000	1-2-11-12-NO	contiguous range expansion
	3-4	ACH, ARD, ICE, Orkney, SHE			
	3-5	ARD			
	3-6	RET, RON			
Total	4-1	ACH, SE Ireland, Wales, PLY, SPA, RET	0.0000	1-2-3-4-9-NO	allopatric fragmentation
	4-2	ICE, Orkney, SHE, OBA, W Ireland, RET			

form (mostly from lineage 2), and SW Ireland, Spain typical form and Amlwch (Wales) populations (lineage 1).

Of the 16 successful trials, 13 showed high reproductive compatibility, with high fecundity scores, F_1 viability, and with fertile F_1 in both backcross and $F_1 \times F_1$ trials in most replicates (see Table 2). However, some crosses showed consistent evidence of outbreeding depression, often asymmetric, notably those trials involving the Spain 'reticulata' population. This population showed limited F_1 fecundity indicating partial reproductive isolation when mated with lineage 1 populations (Spain and Amlwch). In trials between Spanish forms, a high fecundity and F_1 viability in all replicates when 'reticulata' genotypes act as female was accompanied by variable F_1 fertility. When Spanish 'reticulata' genotypes acted as male, only one cross

out of three produced F_1 larvae. These reached sexual maturity, but viability was reduced. In this cross, F_1 fecundity could not be estimated because of poor growth and survival of the colonies. In the other crosses involving 'reticulata' form genotypes (RET × aml and AML × ret), similar results were found, with the AML genotypes failing to release larvae when paired with 'reticulata' genotypes acting as males. When 'reticulata' genotypes acted as female, however, high viability and fecundity was observed. Some evidence of outbreeding depression was found in the cross Spain 'typical' form (maternal) × Ireland (paternal), where only one out of four genotype combinations showed high fecundity and viability. The other three combinations released no or very few larvae and none of these reached sexual maturity. Limited outbreeding depression was also found

in the cross between Amlwch (maternal) and Plymouth (paternal) with only one out of four crosses yielding over 100 viable larvae. However, the F_1 fertility and backcrosses showed little or no indication of reduced fitness.

In all crosses, the simultaneous controls showed that the involved genotypes were reproductively active at the time the experiment took place. Self-fertilization was rare and often resulted in embryo mortality before larvae were released, and little or no fecundity. Most parental genotypes failed to brood any embryos when kept in isolation. Only two genotypes, one Irish (IRL2) and the other one from Amlwch (AML4), brooded embryos regularly when kept in isolation, but they released very few larvae (AML4, 1 larvae released; IRL2, 3 larvae released).

Twenty CHY1 alleles were found, ranging in size from 144 to 286 bp, and broadly coincident with Hoare *et al.*'s (1999) study. A total of 532 individuals were screened, 434 of which could be directly confirmed as the product of outcrossing, with one allele from each of the maternal and paternal acting genotypes. Out of the remaining amplifying individuals, only six had genotypes incompatible with being the offspring of a single outcrossed colony, five having a single amplified maternal allele and therefore feasibly the product of self-fertilization (but see below), the remaining one probably being an artefact of extracting DNA from two offspring growing next to each other (we found three alleles in the DNA extraction). A large proportion of the colonies whose paternity could not be ascertained (47) belonged to mating trials where both parental genotypes shared one allele. In these trials, those F_1 colonies containing the paternal allele not found in the mother genotype were confirmed as outcrossed, as the maternal genotype was known with certainty, while the rest (*c.* 50% of the scored offspring) were considered 'unassigned'. Two genotype combinations could not be scored as the parental genotypes shared both alleles (SPA4 × ret4 and RET4 × spa4). Overall, over 98% of the F_1 colonies whose paternity could be ascertained were confirmed as being the result of outcrossing.

Microsatellite scoring of F_1 offspring from the Irish population was problematic as their alleles showed little or no amplification when combined with non-Irish alleles, suggesting differences in the primer sites. All parental genotypes, including the Irish, could be amplified reliably with the standard PCR conditions described above; however, when Irish F_1 offspring was amplified, a lower annealing temperature (58 °C) had to be used to obtain amplification of the Irish alleles. In a particular combination (AML4 × irl4), Irish alleles failed to be amplified at all, even in those more relaxed conditions.

Discussion

Celleporella hyalina shows strong phylogeographical structuring in the NE Atlantic congruent with its relatively low dispersal abilities. Populations displayed high genetic diversity and a large proportion of private haplotypes. The data do not suggest that human-mediated dispersal has impacted significantly on *C. hyalina* populations. Two main parapatric lineages, with contact zones in Spain and W Ireland were found. NCA found significant evidence of past allopatric fragmentation between them.

Lineage 2 is strongly subdivided, with four third-level clades. Of these, one was found in Scotland with a single individual sequenced, another in W Ireland (Ardfry), another formed by the Spain '*reticulata*' (H18) and an Orkney haplotype, and a widely distributed and diverse one (clade 3-4) in W Ireland, Orkney, Shetland and Iceland. Two of these clades co-occur in W Ireland (Ardfry). The depth of the divergence between these clades is similar to that found for the subclades in Lineage 1, and therefore they seem to suggest (i) that the structuring of this clade took place before the LGM, and (ii) that haplotypes belonging to this clade survived the LGM in several refugia.

The high haplotype diversity and structure of W Irish populations for both lineages (Fig. 2) suggests W Ireland as a refugial area for *C. hyalina*. It is possible that this refugium was large enough to include already subdivided populations that maintained their differentiation throughout the LGM (note the large differentiation between the three W Irish populations, with F_{ST} ranging from 0.57 to 0.73), or the refugial populations maintained a large effective population size. Moreover, W Ireland has been proposed as a northern glacial refugium for trees, *Pinus sylvestris* (Stewart & Lister 2001).

Two populations (Spain and Achill) contained individuals from both lineages and secondary contact was probably created by past episodes of range expansion. An old episode of range expansion (which could have included some cases of long-distance colonization) affected the entire lineage 2, most likely predating the LGM. The Spanish population is relatively isolated by the warmer waters of the Bay of Biscay (Álvarez 1991) where it seems to be replaced by *Celleporella angusta*. The '*reticulata*' form has only been found in the Ría de Ferrol (Fernández-Pulpeiro *et al.* 1992). The strong differentiation, the disjunct distribution when compared to the rest of lineage 2 and its limited range are consistent with the '*reticulata*' form being either a relict from a past interglacial or a recent new arrival from northern waters. The Spanish lineage 1, given the absence of the common H25 haplotype involved in the expansion into the Irish Sea and English Channel, may have survived the LGM in the area. Since reproductive isolation between both Spanish forms is partial, mtDNA introgression could explain the presence of one of the Spanish 'typical' haplotypes from lineage 1 in one of the '*reticulata*' isolates, and it would be interesting in future studies to characterize the extent and nature of gene flow (if any) between these forms using much greater sample sizes and both nuclear and mtDNA markers.

Because the Irish Sea formed a glaciated land bridge between Ireland and Britain (Dawson 1992), *C. hyalina* must have recolonized this part of its range after the LGM, as supported by our data. In particular, lineage 1a which is distributed from Spain to N Wales and S and E Irish coasts shows a 'star-phylogeny' with closely related haplotypes (Fig. 2), and a common widespread genotype (H25). The geographical distribution of haplotypes in lineage 1a supports a recent population expansion in the area. NCA, however, did not find significant evidence of a range expansion in this clade. Previous work has indicated that NCA might be too conservative for inferring recent range expansions when the ancestral haplotypes are still present in the expanded populations, which is likely to happen when there is insufficient time for drift to 'purge' the ancestral haplotypes (Alexandrino *et al.* 2002; Printzen *et al.* 2003). Given the presence of some unsampled areas or areas without known populations of *C. hyalina*, we cannot determine which refugial population initiated the range expansion into the Irish Sea and the English Channel. The closest relatives to the haplotypes involved in the expansion (lineage 1a) are present in W Ireland (Achill), a population with high haplotype diversity (including lineage 1b and lineage 2 individuals). The only population containing lineage 1a that did not contain the ancestral haplotype H25 is the Spanish 'typical' form, suggesting that a yet unsampled area between NW Spain and Plymouth might have been the source of the range expansion (see Discussion below).

The main structuring for both lineages 1 and 2 seems to be much older than the LGM. In the case of lineage 1, this suggests that survival occurred in several glacial refugia, one of them most likely in W Ireland (which gave rise to lineage 1b), a second one further south (which gave rise to lineage 1a), and possibly another one on the Spanish coast. The ancestral population of lineage 1a probably exploited the newly available habitat after the LGM, and was involved in a large recent range expansion as supported by haplotype diversity patterns. The position of the refugium/refugia giving rise to lineage 2 cannot be inferred from our data.

Given the overall marked population subdivision we can assume that these populations are strongly affected by genetic drift but show little influence of gene flow (Slatkin 1993). This contrasts with the strong correlation between genetic and geographical distance found. Given the reduced ongoing gene flow, and the evidence for strong historical effects (population range expansions, past allopatric fragmentation, etc.) supported by NCA and additional analyses (see below), we find it highly unlikely that *C. hyalina* populations are at migration–drift equilibrium. The usual interpretation for the association between genetic and geographical distance (isolation by distance) is that equilibrium has been reached where gene flow is dependent on geographical distance (Rousset 1997; Hutchison *et al.* 1999). We suggest instead that such an association might have been created by serial founder events (Ramachandran *et al.* 2005), as populations expanded from Pleistocene refugia. It remains to be determined whether the common isolation-by-distance patterns found in low dispersal marine species (Hellberg 1996; Wilke & Davis 2000) might be due to such cases of nonequilibrium conditions. Our results suggest that phylogeographical analysis, including NCA, can help to determine if equilibrium conditions are warranted and therefore, to elucidate the underlying causes for the association between genetic and geographical distances in other marine organisms.

The divergence between mtDNA lineages, together with the allopatric fragmentation inferred by NCA, suggests that several *C. hyalina* populations survived the LGM. Even the northernmost populations (Iceland, Orkney and Shetland), in areas thought to be heavily affected by the Pleistocene glaciations, have high haplotype diversity, despite the relatively small sample sizes obtained. As *C. hyalina* can be considered a cold-tolerant taxon, its ecological requirements and hence the geographical areas suitable for its population growth during glacial maxima would obviously be different from those expected for less cold-tolerant species, indicating that *C. hyalina* was impacted differently when compared with other temperate taxa.

Several studies of marine phylogeography in the NE Atlantic seem to challenge the expectations of postglacial colonization from southern refugia as found in terrestrial taxa (Luttikhuizen *et al.* 2003; Marko 2004). Instead, it has been found necessary to postulate northern refugia, located from Scotland, W and S Ireland to the English Channel (Coyer *et al.* 2003; Roman & Palumbi 2004; Provan *et al.* 2005) (Fig. 1). Several reasons might explain this discrepancy between marine and terrestrial phylogeography (but see Stewart & Lister 2001 for a review of the evidence for terrestrial refugia). Studies on the Pleistocene dynamics of Californian kelp forests suggest that temperate coasts were much more productive during glacial periods than currently. The response of temperate marine and terrestrial ecosystems to glacial–interglacial cycles therefore could be out of phase, with glacial maxima affording more abundant resources and more opportunities for the growth of marine populations than interglacial periods (Graham *et al.* 2003). If such patterns applied also to the NE Atlantic, we should expect many coastal species to have occupied northern localities during glaciations. In addition, the vertical structure of littoral communities might favour differential survival of species depending on their favoured position on the shore. Thus, species living in the subtidal would be more likely to survive in northern localities as they are normally exposed to less extreme temperatures than species living higher on the shore (Coyer *et al.* 2003; Marko 2004). Moreover, kelp — and presumably its epiphytes including *C. hyalina* — can live below sea ice for most of the year (Dunton *et al.* 1982), and so glacial survival

of such organisms in northern refugia is expected. The concept of marine 'refugia' must itself be taken with caution, since populations need not suffer range contraction in response to climatic cooling so acutely as do terrestrial organisms. Furthermore, marine species may be able to track suitable habitat during cold periods by moving into deeper water (isothermal submergence) analogous to altitudinal shifts of terrestrial species (Hewitt 1996). In addition, given the continuous distribution of many marine taxa and the discontinuous nature of sampling, along with the often uncertain location and nature of the Pleistocene coastline it is difficult to pinpoint exactly where refugia might have occurred (Roman & Palumbi 2004).

Taking the above points into account, only the occurrence of northern refugia can seemingly explain cases such as *Carcinus maenas*, where a distinctive clade inhabits the Faroe Islands and Iceland (Roman & Palumbi 2004) (Fig. 1). The English Channel area has been identified as a potential refugium in a several taxa including the algae *Fucus serratus* (Coyer *et al*. 2003), *Ascophyllum nodosum*, and *Palmaria palmata* (Provan *et al*. 2005). A refugium in the S. North Sea has been postulated for the gobies *Pomatoschistus microps* and *Pomatoschistus minutus*, although given the absence of samples in the southern Channel or Brittany the authors could not rule out an English-Channel refugium (Gysels *et al*. 2004a; Gysels *et al*. 2004b). In addition, SW Ireland has been proposed to be a refugium in *P. palmata* (Provan *et al*. 2005) and *F. serratus* (Coyer *et al*. 2003). The distribution of suitable marine habitats in the English Channel and North Sea during the LGM is, however, unclear since most of the area may have been dry or of very low salinity due to ice-runoff. An alternative explanation to refugia for areas of high genetic diversity is the presence of a contact zone between two discrete lineages. Unfortunately, not all of the studies above use markers with genealogical information to distinguish between these two alternatives. Surprisingly, current Iberian populations of some of the investigated taxa seem to be relict, but with little genetic diversity despite inferred Pleistocene survival (Coyer *et al*. 2003). On the other hand, high-diversity refugial populations of *P. microps* have been recognized in the Iberian Peninsula (Gysels *et al*. 2004a).

All *C. hyalina* populations were reproductively competent under the experimental conditions, albeit with lower fecundity in the case of Iceland (Table 2). Our results indicate that complete reproductive isolation has developed between Icelandic (lineage 2) and all other populations tested from lineage 1. In addition, reproductive isolation has also developed between two of subclades within lineage 2 (Spain '*reticulata*' vs. Iceland). However, postzygotic reproductive isolation is not complete between '*reticulata*' and lineage 1 populations. Strongly asymmetrical outbreeding depression exists between these populations: when Spain '*reticulata*' acts as a male, the crosses are unsuccessful, but

crosses are successful when it acts as a female. Therefore, in the NW Spanish site where these two forms are sympatric, introgression through backcrossing is to be expected. Our data allow us to infer that introgression and gene flow are likely to be occurring between both clades, with morphological '*reticulata*' individuals bearing 'typical' form haplotypes.

From the genetic and mating compatibility data we can consider that *C. hyalina* is formed by several lineages at different stages of speciation, with some populations having achieved full reproductive isolation (i.e. species status) (Gómez *et al*. 2006), with others representing incipient species. Phylogeographical analysis combined with mating tests allows us to conclude that speciation is taking place in allopatry, but may not be developing at the same pace in different lineages.

In her review of cryptic species in the sea, Knowlton (1993) stated that tests of reproductive isolation in the sea are rare, and studies of F_2 offspring are even rarer (but see Edmands 2002). Although this statement still holds true today, reproductive isolation and speciation is well known in some case studies. Recent studies have used mating trials, including to a limited extent F_2 crosses, to support reproductive incompatibility between putative cryptic copepod species (Lee 2000), and in the *Tigriopus* species complex (Edmands 1999). Several hybrid zones of the blue mussel complex, *Mytilus* are now very well known (Bierne *et al*. 2002; Bierne *et al*. 2003; Riginos & Cunningham 2005). The relationship between reproductive isolation and genetic divergence is often positive, but exceptions abound, and *C. hyalina* illustrates that this pattern can be complex within a single clade.

Here we present among the first studies combining phylogeography and mating compatibility in a marine species. *Celleporella hyalina* is a genetically subdivided taxon that occupies a NE Atlantic habitat greatly affected by the Pleistocene glaciations. Our assessment of mating compatibility between these phylogeographical lineages demonstrates some complete and some partial reproductive isolation, indicating that genetic subdivision in this complex may represent incipient speciation. The distribution of the lineages suggests they did not simply retreat to southern refugia during glaciations, and the survival of cold-tolerant marine species such as *C. hyalina* does not solely depend on surface water temperature. Our results emphasize the complexity of marine phylogeography and that major trends cannot always be transferred from the terrestrial to the marine realms.

Acknowledgements

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