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Phylogeography and environmental diversification of a highly adaptable marine amphipod, *Gammarus duebeni*

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Genetic diversity and phylogeographic population structure in the gammarid amphipod, *Gammarus duebeni*, were investigated across its broad latitudinal distribution in the NE and NW Atlantic by analysis of mitochondrial DNA sequence. *Gammarus duebeni* has exceptional tolerance of salinity change and inhabits environments ranging from marine to freshwater. The longstanding debate on whether there are distinct marine and freshwater subspecies was assessed by sampling populations from sites characterized by different salinities. Our sequence data demonstrates that there are two major lineages, with little internal geographic structuring. Evidence is provided to suggest a pre-glacial divergence of these two clades, involving segregation between a region historically associated with the freshwater form and the majority of the marine localities on both sides of the Atlantic. A modern contact zone between the marine and freshwater forms is proposed in western Britain.
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Introduction

Rocky shore environments in the North Atlantic are characterized by dramatic cyclical and stochastic fluctuations in a range of physical parameters. In addition to these short-term fluctuations, the intertidal organisms that inhabit this challenging environment have also been subject to significant environmental fluctuations over evolutionary time scales. In particular, Pleistocene climate cycles (ice ages) are predicted to have had dramatic impacts upon the geographical ranges of North Atlantic coastal species (Avise, 1992; Graham *et al*., 2003).

Previous case studies indicate that the effect of Pleistocene climate cycles upon the genetic diversity of North Atlantic coastal species was far from uniform (e.g. Wares and Cunningham, 2001; Young *et al*., 2002; Luttikhuizen *et al*., 2003; Riginos *et al*., 2004; Roman and Palumbi, 2004; Addison and Hart, 2005; Jolly *et al*., 2005; Provan *et al*., 2005). Some of these species show a phylogeographic structure characterized by shallow genetic divergence between haplotypes, with no geographic pattern to their distribution and no genetic subdivision between the NW and NE Atlantic. This pattern has been attributed (Ingólfsson, 1992) to the fact that, on the European side, rocky shore taxa displaced by temperature or ice, would encounter suitable refugia in rocky habitats further south. On the NW Atlantic coast, however, there is relatively little suitable rocky habitat in the south, and it has been suggested that this might lead to the extinction of NW Atlantic populations after displacement from northern latitudes. Several examples support this process with the pattern of genetic diversity in marine species indicating a recent NE Atlantic origin for NW Atlantic haplotypes (Wares, 2001a; Wares and Cunningham, 2001). A second, contrasting phylogeographic pattern is geographic structuring of distinct haplotypes (Riginos *et al*., 2004; Roman and Palumbi, 2004; Provan *et al*., 2005), frequently involving division into NE versus NW Atlantic populations, as well as additional geographically restricted lineages. Such an occurrence of genetically distinct and geographically isolated populations in the Northern Atlantic is often considered indicative of survival and divergence in isolated refugia during the Pleistocene. A phylogeographic approach then, even if revealing very limited structure, can reveal much about the Pleistocene history of both species and regions.

Gammarid amphipods are a large and diverse group of crustaceans that are exceptionally abundant in the marine coastal environment of both the eastern and western North Atlantic, where they form an important component of aquatic food webs (Bousfield, 1978). The *Gammarus* genus, in particular, is highly adaptable, with species occupying intertidal and subtidal habitats and extending into estuaries and freshwater. Much of gammarid systematics remains unresolved, despite a century of detailed morphological taxonomy (e.g. Kinne, 1954; Holmes, 1975) and analysis of allozymes (Bulnheim

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and Scholl, 1980; Kolding and Simonsen, 1983; Siegismund *et al*., 1985; Skadsheim and Siegismund, 1986; Kane *et al*., 1992; Müller *et al*., 2000). Modern molecular techniques for determining phylogenetic relationships have, to date, been used on only a few gammarids, and these are almost exclusively from freshwater habitats (Meyran *et al*., 1997, 1998; Meyran and Taberlet, 1998; Sherbakov *et al*., 1999; Müller, 2000; Englisch *et al*., 2003; Macdonald *et al*., 2005; Kelly *et al*., 2006).

The gammarid amphipod *Gammarus duebeni* (*Liljeborg*) has a particularly remarkable capacity to tolerate a wide range of salinity, from freshwater to hypertonic solutions of 70‰ (see Lockwood, 1992). *Gammarus duebeni* can be found in both marine and landlocked freshwater environments, and it has long been debated whether differentiation between these populations actually defines subspecies level divergence. The first proposal for a freshwater form (α, localized but abundant in rivers and lakes of Ireland and Brittany) and a brackish-marine form (β) (Reid, 1939) was later formalized by splitting *G. duebeni* into two subspecies (Stock and Pinkster, 1970): *G. d. celticus*, the freshwater form, and *G. d. duebeni*, the marine form. The original argument for subspecies-level differentiation in *G. duebeni* was based upon observations of higher tolerances for hypo-osmotic conditions in individuals from landlocked freshwater populations than in individuals from marine populations (Sutcliffe, 1971; but see Lockwood, 1992; Sutcliffe, 2000). However, subsequent laboratory experiments demonstrate that both marine and freshwater forms are capable of acclimating to a broad and overlapping range of salinities (Sutcliffe, 1978). Intermediate osmoregulatory capabilities also occur in individuals from freshwater sites in close proximity to a marine influence (i.e. streams on small islands or peninsulas including the Isle of Man, Cornwall and W Scotland). This suggests that marine and freshwater forms arise from a common gene pool by phenotypic plasticity (Sutcliffe, 2000). Recent physiological studies have, however, demonstrated that the ability of *G. d. duebeni* to adjust whole animal water permeability is far superior to the capabilities of *G. d. celticus* (C Lloyd Mills, personal communication), although it remains to be confirmed whether these osmoregulatory differences have any genetic basis.

Holmes (1975) examined 121 morphological features and failed to identify any that reliably distinguished between marine and freshwater forms of *G. duebeni*. However, other researchers have differentiated marine and freshwater forms on the basis of differences in leg morphology (Pinkster *et al*., 1970; Stock and Pinkster, 1970). Their results indicate that marine populations of *G. duebeni* have relatively high width to length ratios of the merus segment on pereopod 7 (mean log W/L $\geqslant$0.74) with the length being less than twice the width, whereas freshwater populations (Ireland and Brittany) have lower ratio values ($\leqslant$0.73), indicating a narrower overall leg segment (see Sutcliffe, 2000). However, intermediate values were again obtained for certain freshwater populations from small islands and peninsulas, suggesting that variation may demonstrate phenotypic plasticity in response to environmental variation (e.g. thermal regime and its effects on differential growth rate) rather than genetic divergence.

Individuals from marine and freshwater *G. duebeni* populations will successfully interbreed under laboratory conditions (Stock and Pinkster, 1970). A constraint on interbreeding between *G. d. duebeni* and *G. d. celticus* was proposed for some natural populations because of slight differences in breeding season (Hynes, 1954). However, freshwater populations in Ireland and Brittany occupy the warmest sites in the species' entire N Atlantic distribution and it has not been determined whether differences in reproductive cycles persist under common thermal regimes. Although a molecular study using allozyme electrophoresis also failed to distinguish between the putative subspecies (Bulnheim and Scholl, 1980), the degree of genetic divergence between the two forms of *G. duebeni* and its relationship to the morphological and physiological differences outlined above, remains to be resolved.

In addition to its ecological importance as a component of multiple aquatic energy cycles, *G. duebeni* has recently assumed importance as a model system for studies of environmental sex determination (ESD) (McCabe and Dunn, 1997), parasitic sex-ratio distortion (Ironside *et al*., 2003), parasite-mediated competition (MacNeil *et al*., 2004a) and intra-guild predation (MacNeil *et al*., 2004b). Recent evidence has indicated significant genetic variation within *G. duebeni* for ESD (Dunn *et al*., 2005) and for host-parasite coevolution (Hatcher *et al*., 2005) and future research within these areas will benefit from information relating to the genetic structure and evolutionary history of *G. duebeni* populations. This study uses mitochondrial DNA (mtDNA) sequence data to characterize the phylogeography of *G. duebeni* in the North Atlantic, and to evaluate levels of genetic divergence corresponding to freshwater and marine populations. The findings of this investigation resolve a long-running debate concerning *G. duebeni* taxonomy and further our understanding of how Pleistocene glacial cycles affected the genetic variation of intertidal and freshwater organisms.

Materials and methods

Sample collection

Gammarus duebeni were collected from populations on eastern ($n=3$) and western ($n=15$) sides of the N Atlantic, across a range of latitudes (Table 1, Figure 1). Individuals were collected with fine-meshed dip nets from under stones in the inter-tidal zone. *G. duebeni* is typically found in habitats where freshwater crosses the intertidal zone (e.g. streams and other low output natural runoffs, or storm drains and ditches). At low tide, populations in these habitats can encounter hypo-saline water owing to freshwater input, or hyper-saline water owing to evaporation, but are generally considered to be marine populations. In Wales, one collection (A Rhoscolyn) was also made from storm pools above the mean high water mark. In addition, several samples were obtained from freshwater sources of constant low salinity (0‰) in Ireland (A: River Lagan; B: Lough Neagh) and on the Isle of Man (from a stream 2.5 miles inland without brackish influence; J Ironside, personal observation). All specimens were stored in 95% ethanol following collection. At two locations (Isle of Skye and Wales, Beaumaris), reference specimens were collected for

confirmation of species identification by morphological comparison after Lincoln (1979) (M Lohe, Natural History Museum, London, personal communication); these individuals appear in subsequent analyses in this study as 'Wales mus' and 'Isle of Skye mus'.

Extraction/PCR/sequencing

MtDNA was extracted by either the Chelex method (Walsh *et al*., 1991) or by the alkaline lysis procedure (Hot Shot; Truett *et al*., 2000) on 1–3 legs of each specimen. A fragment of the cytochrome *c* oxidase subunit 1 (*CO1*) gene was amplified using universal primers (LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′, HCO02198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer *et al*., 1994). Each 20 μl reaction contained 1–3 μl DNA, 1X PCR buffer (NH2, Bioline), 2 mM $MgCl^2$, 80 mM deoxynucleoside triphosphate mix, 0.5 μM of each primer and 0.6 U DNA polymerase. Amplification consisted of an initial denature for 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min at 50°C, 2 min at 72°C; cycling ended with a final extension time of 10 min at 72°C. Amplicons were purified using ExoSAP (Fermentas) and sequenced bi-directionally on a Beckman Coulter CEQ 2000XL DNA Analysis System. Sequences were aligned and edited in CodonCode Aligner v. 1.3.4 (CodonCode, Dedham, MA, USA).

Table 1 Details of sampling locations, size (*n*) and population codes

Population	*Latitude and longitude*	n	*Code*
Eastern Atlantic			
England, Plymouth,	50 23 00 N 04 10 00 W	5	Ply
Wales: Bangor	53 13 51 N 04 08 14 W	8	Wsl
A Rhoscolyn	53 14 29 N 04 35 38 W	3	Wrcsp
B Rhoscolyn	53 14 35 N 04 35 29 W	2	Wrcst
Beaumaris	53 17 03 N 04 04 24 W	2	Wmus
Isle of Man	54 05 49 N 04 42 18 W	5	IoM
Ireland: A	*54 39 43 N 06 24 11 W*	*2*	*Ireland A*
B		*5*	*Ireland B*
Scotland: S. (Forth Clyde)	55 46 00 N 04 56 00 W	5	Ssc
Isle of Skye	57 24 00 N 06 12 00 W	8	IoS
Poland, Gdynia	54 31 04 N 18 32 11 E	2	Gdy
Sweden	57 43 00 N 11 58 00 E	1	Swe
Faroes	62 01 00 N 06 46 00 W	5	Far
Norway: Trondheim	63 25 00 N 10 25 00 E	5	Trn
Tromsø	69 40 00 N 18 58 00 E	11	Tro
Western Atlantic			
Rhode Island	41 29 00 N 71 19 00 W	11	RI
Maine	43 40 00 N 70 15 00 W	3	Me
Newfoundland	47 34 00 N 52 43 00 W	5	NF

The landlocked freshwater populations (Ireland) are shown in italics.

Phylogenetic analyses

Analyses of relationships among taxa were conducted using neighbour joining, maximum parsimony, maximum likelihood and Bayesian inference analyses in Paup 4.0b (Swofford, 2000), PHYML v2.4.4 (Guindon and Gascuel, 2003) and MrBayes v3.1.1 (Ronquist and Huelsenbeck, 2003). The model of nucleotide substitution was determined using Modelgenerator (Keane *et al*., 2004). Bayesian inference was conducted with data partitioned by codon positions for 2×10^6 generations (sample freq = 100, burnin = 2000). Substitution matrix was set at General Time Reversible (nst = 6) with other parameters optimized as part of the run. A Nested Clade Phylogeographical Analysis (NCPA) was conducted after Templeton's (2004) most current method to further discriminate between the contemporary processes and historical events. The haplotype cladogram was constructed using Templeton, Crandall, Sing (TCS); (Templeton *et al*., 1992; Clement *et al*., 2000) and Geodis (Posada *et al*., 2000) provided tests of relationships between relative clade distances.

Comparison of limb morphology

In order to test whether genetic divergence corresponded to divergence in morphological characters used to differentiate the two putative *G. duebeni* subspecies, a comparison of limb morphology was performed between individuals collected from the Bangor and A Rhoscolyn populations. These populations are located in close proximity and experience similar environmental conditions but molecular analysis partitions the two populations between the two major genetic clades (see Results for sequence variation and divergence of haplotypes). A random sample of 50 individuals was collected from each population and width to length ratios of the merus segment on pereopod 7 were measured to the nearest 10th of a millimetre using a dissecting microscope and graticule eyepiece. Significance was tested using analysis of covariance (ANCOVA) with total body length as a covariate.

Figure 1 Map of sampling sites for marine (x) and freshwater (o) sites in the North Atlantic.

Table 2 Distribution of the 11 haplotypes among the 88 individuals collected from 18 different populations of *Gammarus duebeni* (see Table 1 for nomenclature of populations)

Population	*Haplotypes*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
England, Plymouth						5					
Wales: Bangor					8						
A Rhoscolyn		1			1				2		
B Rhoscolyn									2		
Beaumaris	1										
Isle of Man		3								2	
Ireland: Ireland A					*2*						
Ireland B							*5*				
Scotland: S. (Forth Clyde)	1	1							3		
Isle of Skye	5	2									1
Poland, Gdynia	1	1									
Sweden	1										
Faroes	5										
Norway: Trondheim	3	2									
Tromsø		1	10								
Rhode Island	2	5						4			
Maine				3							
Newfoundland				5							

The landlocked freshwater populations (Ireland) are shown in italics.
The nucleotide sequences of these haplotypes are available from international sequence databases under accession numbers EF468643–EF468653.

Table 3 HKY distances within haplotypes

	Hap1	*Hap2*	*Hap3*	*Hap4*	***Hap5***	*Hap6*	***Hap7***	*Hap8*	*Hap9*	*Hap10*
Hap1	—									
Hap2	0.0016	—								
Hap3	0.0046	0.0031	—							
Hap4	0.0015	0.0031	0.0061	—						
Hap5	*0.0388*	*0.0411*	*0.0372*	*0.0404*	—					
Hap6	0.0031	0.0016	0.0046	0.0046	*0.0411*	—				
Hap7	*0.0431*	*0.0447*	*0.0414*	*0.0447*	0.0031	*0.0454*	—			
Hap8	0.0031	0.0016	0.0046	0.0046	*0.0409*	0.0031	*0.0453*	—		
Hap9	0.0033	0.0016	0.0016	0.0049	*0.0382*	0.0032	*0.0417*	0.0033	—	
Hap10	0.0031	0.0015	0.0046	0.0046	*0.0427*	0.0031	*0.0464*	0.0031	0.0032	—
Hap11	*0.0404*	*0.0427*	*0.0388*	*0.0420*	0.0015	*0.0427*	0.0047	*0.0425*	*0.0399*	*0.0444*

Haplotypes for freshwater populations are in bold and comparisons between fresh and marine forms are shown in italics.

Results

Sequence variation and divergence of haplotypes

A 608 bp fragment of the *CO1* gene was obtained for a total of 88 specimens from the 18 populations studied. Average base composition for this sequence was 23% A, 36% T, 22% C and 20% G. Overall nucleotide diversity (Pi) was 0.0191, and 0.003 within each of the marine and freshwater populations (described below). Thirty variable nucleotide sites were identified (5%) of which 23 were parsimony informative mutations. Eleven haplotypes were distinguished (Table 2). Pairwise comparison of haplotype sequence divergence (measured by Hasegawa, Kishino, Yano (HKY) distances; Table 3) revealed a genetic distance ranging from 0.0015 (between haplotypes 1 and 4, 2 and 10, and 5 and 11) to 0.0464 (between haplotypes 7 and 10). For maximum likelihood and neighbour-joining analyses, HKY was determined to be the best-fit model of nucleotide substitution (using the Akaike information criterion in Modelgenerator (Keane *et al*., 2004).

Differentiation between freshwater and marine populations

Phylogenies produced by maximum parsimony, maximum likelihood, neighbour-joining and Bayesian Inference had very similar topologies, revealing two distinct and well-supported clades (Figure 2). One clade included the two Irish freshwater populations, as well as one population from Wales (Bangor) from a typical marine habitat. The second major clade included all of the other marine populations, as well as one population

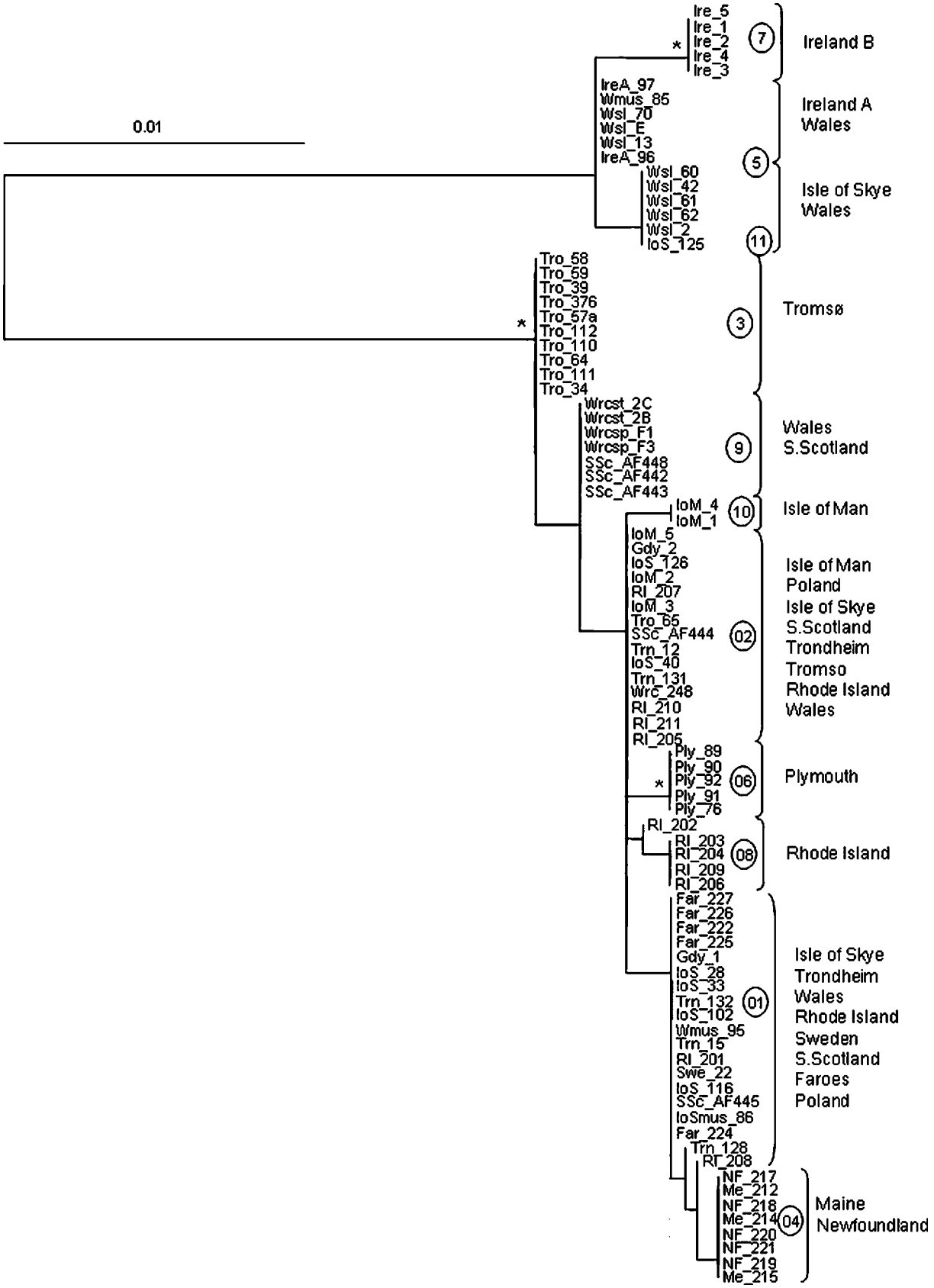

Figure 2 Tree constructed by Maximum Likelihood Analysis for *G. duebeni*. Bootstrap analysis consisted of 1000 pseudoreplicates; nodes with >70% support are marked with an asterisk. Haplotype assignment number (after Table 2) is given within a circle to the left of each bracket.

from the previously described Isle of Man site (see Materials and methods). Within this clade, one marine population in Wales (Beaumaris) and one marine population in Scotland (Isle of Skye) contained single individuals with haplotypes belonging to first (freshwater) clade.

Comparison of limb morphology

Mean merus ratios were 0.72 ± 0.11 s.d. in the Bangor population sample and 0.67 ± 0.08 s.d. in the A Rhoscolyn population sample. No significant difference in merus ratio values was found ($F_{1,96} = 1.22$, $P = 0.47$; with body size used as a covariate to control for the significant relationship between body length and merus width:-length; $F_{2,96} = 15.99$, $P = 0.0001$) and no significant interspecific difference was found for the interaction between body size and merus dimensions ($F_{1,96} = 3.55$, $P = 0.063$; ANCOVA).

Geographic distribution of haplotypes

No sequence variation (homogeneity) was observed within nine populations: Maine, Newfoundland, Faroes, Wales Bangor, Sweden, Ireland B, Ireland A, Wales B and Plymouth (Table 2; although sample sizes for the latter three were all small and may compromise analyses of diversity). In addition, Plymouth and Ireland B were fixed for a unique haplotype, whereas all others possessed a haplotype shared with at least one other population. Four haplotypes were restricted to single populations: hap 3 in Tromsø, hap 11 in Isle of Skye, hap 10 in the Isle of Man and hap 8 in Rhode Island. Two haplotypes were shared across the Atlantic (amphi-Atlantic), whereas seven were exclusive to the NE Atlantic and two were exclusive to the NW Atlantic.

Two separate haplotype networks were generated by TCS, as this programme is only able to resolve haplotype connections with at least 95% parsimony (<11 mutational steps) (Figure 3). The two separate networks correspond to those haplotypes mainly associated with the freshwater Ireland samples (hereafter referred to as the freshwater clade) and those haplotypes associated with the predominant marine populations (hereafter, the marine clade). Biological NCPA interpretation of the structuring of three internal clades for the larger marine clade invoked both recent and historical processes. Past fragmentation and subsequent range expansion, with the potential of contributions from long distance colonization, characterize the divergence of both the Tromsø population (hap 3) from the hap 9 assemblage (S Scotland, Wales A and B) and the Newfoundland/Maine populations (hap 4) from the amphi-Atlantic grouping (hap 1, Table 2, Figure 2). A process involving restricted gene flow with some long distance dispersal was invoked for divergence between the basal amphi-Atlantic grouping (hap 2; see Table 2, Figure 2) and populations in Rhode Island (hap 8), Plymouth (hap 6) and Isle of Man (hap 10). Divergence within the overall marine clade was characterized by restricted gene flow with isolation by distance from the ancestral haplotype (hap 2). Ancestral status of this haplotype as the source of genetic diversity is supported under coalescence theory (Crandall and Templeton, 1993) by its high frequency of occurrence in different populations and wide geographic distribution.

Discussion

Phylogenetic significance of mtDNA variation: *G. d. duebeni* and *G. d. celticus*

The results of this study reveal significant intraspecific differentiation (4% sequence divergence) for *G. duebeni*. Two major clades were resolved by a range of phyloge-

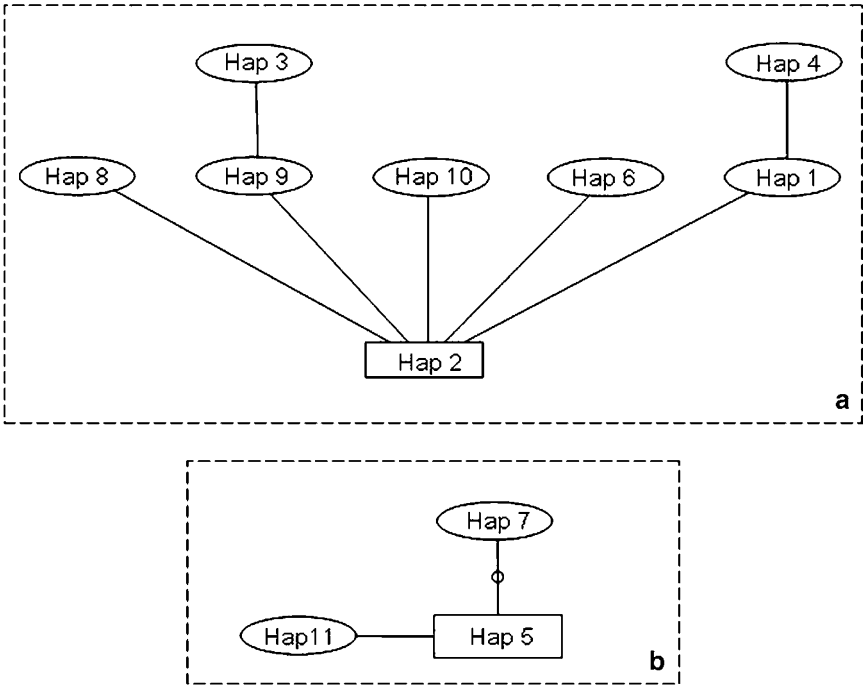

Figure 3 Network of the 11 *G. duebeni* haplotypes. (**a**) Represents the marine form, and (**b**) represents the freshwater form.

netic analyses, one of which contained the two landlocked Irish freshwater populations, and very little internal structure or divergence (0.1–0.4%) was detected within each clade. Varying degrees of intraspecific diversity have been previously reported for other, mainly freshwater, gammarids. The degree of intraspecific diversity observed for *G. duebeni* compares favourably with that of *G. lacustris* (*Sars*) (0.2–1.6%; Meyran and Taberlet, 1998) and for populations of *G. fossarum* (*Koch*) distributed within a narrow geographic area varying in environmental calcium ($\approx$0.3–4.7%; Meyran *et al*., 1998). On a broader scale, populations of *G. fossarum* distributed across Europe are highly divergent (8–11%; Meyran *et al*., 1997; Müller, 2000) and a similar level of divergence (5–9%) was used to differentiate subspecies in *G. pulex* (*Linnaeus*) (Hadfield, 2002). Molecular variance in *G. duebeni* does in fact approach the minimum interspecific distances reported for other amphipod taxa (4–18%; France and Kocher, 1996), although minimum distances often appear to be higher for gammarids (9–12%; Meyran *et al*., 1997; Kelly *et al*., 2006). Thus, values of 4% divergence within *G. duebeni* might arguably be substantial enough to indicate divergence into two biological species. However, although the level of divergence between congenerics in other crustaceans may even be $<$4% (see Müller, 2000) (with typical among-genus level of divergence of at least 20%; Cunningham *et al*., 1992), preliminary phylogenetic data from a broad sampling of marine *Gammarus* indicates that 24% sequence divergence is common between species in the N Atlantic (J Rock and DH Lunt; unpublished observation). Understanding whether the lineages identified in this work represent full species or intraspecific units will require additional work that considers mating compatibility, gene flow at nuclear loci and ecological and physiological divergence. Such approaches are currently ongoing (Rock *et al*., unpublished). The depth of differentiation between the two central clades in *G. duebeni* suggests that it is a fairly old divergence. Although there is no molecular clock specifically calibrated for amphipods, Quek *et al*. (2004) surveyed the literature for arthropod COI rates calibrated by fossils or biogeography and reported rates ranging from 1.3 to 1.9% per million years. Thus, although precise dating will probably require fossil calibrations and more sophisticated analyses of additional data, it seems likely that divergence between the *G. duebeni* clades is much older than the last glacial maximum (LGM) and may be early Pleistocene or Pliocene with the lineages having experienced separate phylogeographic histories during the Pleistocene climate cycles. Based on both historical precedence (Sutcliffe, 2000) and physical barriers to a marine influence, it is reasonable to assume that the Irish populations sampled in this study represent the putative freshwater subspecies, *G. d. celticus*. Our results indicate that populations from this region are indeed associated with a haplotype highly divergent from the majority of marine populations sampled in the N Atlantic. The observed values of sequence divergence (4% between versus 0.3% within clades) provide the first molecular support for *G. duebeni duebeni* and *G. duebeni celticus*.

Preliminary morphological data on merus width:-length ratios for *G. d. duebeni* and *G. d. celticus* collected from nearby, environmentally similar populations in Wales (Bangor and A Rhoscolyn) does not support the hypothesis that *G. d. celticus* has lower merus ratios. Indeed, considering the combined effects of allometric growth patterns (Sutcliffe, 1972, 2000) and interpopulation variation in thermal environment in *G. duebeni*, it is doubtful that any morphological features can be considered diagnostic for differentiating the two subspecies.

Geographic distribution and molecular variation in the *G. duebeni* complex

Although divergence of *G. duebeni* subspecies is likely to fall well before the LGM ($\sim$18–20 kya) for both eastern and western sides of the Atlantic, intra-lineage mtDNA variation in this taxon has undoubtedly been shaped by the ice ages. For *G. d. duebeni*, molecular diversity is low with haplotypes shared across broad geographic areas, including the coasts of both N America and Europe. Such a pattern is indicative of taxa experiencing postglacial expansion (Ibrahim *et al*., 1996; Hewitt, 2000; Fedorov *et al*., 2003; Lessa *et al*., 2003). A degree of genetic differentiation does exist among some populations, most noticeably at the species' latitudinal extremes (e.g. N: Tromsø, Newfoundland, Maine; S: Plymouth), suggesting lack of gene flow owing to geographic isolation. Phylogeographic analyses (NCPA) indicated that the divergence of these populations was attributable to past fragmentation and subsequent range expansion, with some likelihood of contributions from long distance colonization. However, the source and directionality of these observed divergences remains unclear with the present data set. In general, however, we observe low genetic diversity even between Eastern and Western Atlantic populations, some sharing COI haplotypes. This is likely to indicate a recent shared history associated with a post-glacial expansion, such as has been observed in other N Atlantic coastal species (Wares, 2001b; Wares and Cunningham, 2001; Riginos *et al*., 2004). An intensive sampling effort will be required on both sides of the Atlantic to further characterize the subtleties of the historic processes of divergence in *G. duebeni*. Its broad distribution, ease of sampling and amenability to phylogeographic study makes *G. duebeni* a powerful system for study of patterns of N Atlantic recolonization. Further, this taxon exhibits marked tolerance for low temperatures, living in Arctic locations such as Tromsø (Lat 69° 40′ N) where they are active in brackish waters of -2°C, and is *a priori* a good candidate to survive the glacial maximum at northern latitudes. However, because our data indicates a very recent common ancestor for amphi-Atlantic populations, it seems unlikely that it has in fact persisted in northern areas throughout the Pleistocene. Study of such northern and cold-tolerant taxa is potentially a productive direction for phylogeographic studies aimed at better understanding the response of communities to climate cycles (Fedorov *et al*., 2003; Hewitt, 2004).

We have detected two genetic lineages of *G. duebeni* that appear to match closely the previously proposed subspecies (Reid, 1939; Stock and Pinkster, 1970). The pre-LGM divergence levels of *G. d. celticus* and *G. d. duebeni* however contradicts the suggestion that *G. d. celticus* represents a postglacial invasion of freshwater habitats by a marine form (Sutcliffe, 2000), suggesting instead that they possess a much more extensive independent history. Furthermore, expansive sampling

of marine habitats around the N Atlantic revealed only one population of *G. d. celticus* in a marine intertidal site (Wales, Bangor), a site novel in its association with freshwater outflow from a decommissioned pumping station. We also found only one site in which a population of *G. d. duebeni* occupied a freshwater stream (Isle of Man), but this site's proximity to a marine influence suggests a case of upstream migration of a marine population. Taken together with the depth of the divergence between *G. duebeni* subspecies (~4%), and the apparent lack of biological barriers to gene flow (i.e. similarity in morphology and osmoregulatory ability), it seems that a physical barrier to gene flow must have remained in place for a considerable time. One possible scenario is that populations of *G. duebeni* were isolated during sea level fluctuations in the late Pliocene/early Pleistocene, when periods of extremely low sea levels caused coastal estuaries to drain. Some populations may then have persisted in freshwater refugia in the ice-free regions of Ireland, Brittany, and possibly in the extreme western isles and peninsulas of Wales, England and Scotland. (See Hänfling *et al*. (2002) for discussion of a glacial refugium for temperate/cold-tolerant species in the extreme southwest of England.) Concomitantly, other populations of *G. duebeni* may have persisted in a coastal refugium, presumably at more southerly latitudes (e.g. rocky shore habitat of the Iberian Peninsula).

The current data also provide preliminary evidence for several contemporary contact points, creating a potential for modern introgression between *G. d. duebeni* and *G. d. celticus*. In two instances, a freshwater haplotype was found for an individual sampled from populations that were otherwise marine in habitat type and haplotype (Isle of Skye; Wales, Beaumaris). It may be that sporadic storm events will occasionally flush *G. d. celticus* individuals downstream into the marine environment, where key traits including osmotic adaptability and longevity may provide a potential opportunity for interbreeding, albeit at a low frequency. A more likely explanation for the mixed Beaumaris population is its proximity to the previously described Bangor population, which is situated on the same strait and connected by high current interchange; few barriers to gene flow are obvious between the subspecies in these environments. However, the divergence levels between freshwater and marine forms of *G. duebeni* indicate that they may have independent histories spanning more than one glacial cycle. This lack of exchange of mitochondrial haplotypes, despite broad tolerance of osmotic conditions, indicates that there may well be a practical barrier to genetic exchange (whether behavioural, ecological or genetic). Further ecological, reproductive compatibility tests and genetic study with nuclear markers may help to clarify the mechanisms behind such a barrier and elucidate important processes of divergence and interchange between marine and freshwater communities.

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