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Phylogeographical analysis of *Ligia oceanica* (Crustacea: Isopoda) reveals two deeply divergent mitochondrial lineages

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Isopods of the species *Ligia oceanica* are typical inhabitants of the rocky intertidal of the northern European coastline. The aim of this study was to assess the genetic structure of this species using mitochondrial and nuclear sequence data. We analysed partial mitochondrial cytochrome *c* oxidase subunit I (CO1) and 16S rRNA gene sequence data of 161 specimens collected from ten sites ranging from Spain to Norway. For selected specimens, we also sequenced the hypervariable V7 expansion segment of the nuclear 18S rRNA gene as a supplementary marker. Furthermore, we studied the infection rate of all analysed specimens by the alphaproteobacterium *Wolbachia*. Our analyses revealed two deeply divergent mitochondrial lineages for *Ligia oceanica* that probably diverged in the late Pliocene to mid Pleistocene. One lineage comprised specimens from northern populations ('lineage N') and one primarily those from France and Spain ('lineage S'). Distribution patterns of the haplotypes and the genetic distances between both lineages revealed two populations that diverged before the Last Glacial Maximum. Given that we found no homogenization of mitochondrial haplotypes, our present results also reject any influence of *Wolbachia* on the observed mtDNA variability. © 2014 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **112**, 16–30.

ADDITIONAL KEYWORDS: 16S rRNA gene – cytochrome *c* oxidase subunit I – glacial refugia – long-distance dispersal – Oniscidea – phylogeography – *Wolbachia*.

INTRODUCTION

The distribution and genetic structure of the current European biota has been shaped significantly by Pleistocene glacial oscillations (e.g. Hewitt, 2004). When temperatures were at their lowest, high latitudes of Europe were covered with ice and permafrost, while temperate regions were restricted to the south. Under such conditions, populations of temperate-adapted species often persisted in geographically isolated ice-free refugia (Taberlet *et al*., 1998; Hewitt, 1999, 2000, 2004). The application of molecular markers in phylogeographical studies has provided valuable new insights into the Pleistocene glacial history of many species, especially with respect to the recolonization of more northerly habitats after the Last Glacial Maximum (LGM) (Hewitt,

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1999, 2000; Avise, 2000). During the glacial periods, the southern, refugial populations remained relatively small for thousands or tens of thousands of years and differentiated genetically due to the lack of migration, resulting in independent lineage sorting (Hewitt, 2000, 2004). Levels and patterns of genetic variation in a broad range of temperate plant and animal taxa provide evidence for the existence of three major refugia in the Mediterranean peninsulas of Iberia, Italy and the Balkans (e.g. Hewitt, 2004; Provan & Bennett, 2008; Schmitt & Varga, 2012). As the climate warmed and glaciers retreated, northern Europe was recolonized from these refugia by leading-edge expansion of populations (Taberlet *et al*., 1998; Hewitt, 2000), with various fossil and pollen data providing an empirical basis for this 'expansion–contraction' model of Pleistocene biogeography (Hewitt, 2000; Provan & Bennett, 2008).

In the marine environment, the binding of water in multi-annual ice sheets and land-based glaciers caused drastic and rapid changes in sea level, generating new coastlines up to several hundred kilometres seaward as well as creating connections or barriers and influencing oceanic currents (Lambeck & Chappell, 2001; Edwards, 2006; Ehlers, Gibbard & Hughes, 2011). As a result, in contrast to terrestrial organisms, the available molecular data for marine and strictly coastal species suggest that their response to the LGM has been less uniform across a broad range of taxa (e.g. Wares & Cunningham, 2001; Hickerson & Cunningham, 2006; Hoarau *et al*., 2007; Maggs *et al*., 2008). In this context, rocky shores represent a highly dynamic and interesting habitat for phylogeographical studies: these coastal habitats are characterized by abrupt changes from marine to terrestrial conditions where salinity, light and temperature alter gradually whereas submersion and emersion alter periodically and induce the development of a characteristic littoral zonation (Kronberg, 1988). Furthermore, these dynamic habitats are not continuously distributed along the European coasts but occur as fragmented patches that are often separated by many kilometres of sandy shoreline. Thus, although it remains a particularly interesting topic to analyse the connectivity of populations that are restricted to such spots, molecular studies analysing the genetic variability of typical intertidal species inhabiting rocky Atlantic and North Sea shores in Europe are still restricted to a few taxa including crustaceans and gastropods (e.g. Wares & Cunningham, 2001; Campo *et al*., 2010; Doellman *et al*., 2011; Panova *et al*., 2011; Krakau *et al*., 2012) and seaweeds (e.g. Coyer *et al*., 2003; Provan, Wattier & Maggs, 2005; Olsen *et al*., 2010). In this context, the isopod *Ligia oceanica* (Linnaeus, 1767) represents an interesting taxon for in-depth analysis. It is a typical and abundant species living along the rocky shores of the European North Sea and Atlantic coastlines (e.g. Nicholls, 1931; Gruner, 1993), but one that inhabits an extremely narrow vertical range of the shoreline from the wave-splash region to just a few metres above it (Carefoot & Taylor, 1995). In addition, as with all remaining species of this genus, *L. oceanica* has numerous characteristics that are transitional between ancestral marine and fully terrestrial isopods, such as their high sensitivity to desiccation or their pleopod morphology (e.g. Carefoot & Taylor, 1995; Schmidt & Wägele, 2001).

In the current study, we analysed two mitochondrial gene fragments to characterize the genetic structure of *L. oceanica* sampled along the European coast from Norway to the Iberian Peninsula. Furthermore, we also analysed the nuclear hypervariable V7 expansion segment of the nuclear 18S rRNA gene of selected specimens and the infection rate of the analysed isopods by the alphaproteobacterium *Wolbachia*. This intracellular bacterium infects many terrestrial arthropods, and woodlice species in particular (e.g. Bouchon, Rigaud & Juchault, 1998; Cordaux, Michel-Salzat & Bouèchon, 2001; Cordaux *et al*., 2012), causing a linkage disequilibrium of mtDNA followed by a homogenization of mtDNA haplotypes (e.g. Hurst & Jiggins, 2005; Werren, Baldo & Clark, 2008). Using a chi-square test, we tested for any systematic deviations from a homogeneous *Wolbachia* infection between the major identified lineages. In so doing, we document the present-day population structure and genetic variability of this isopod as well as some aspects of its evolutionary past.

MATERIALS AND METHODS

Sampling and DNA extraction

In total, 161 specimens were collected by hand from ten intertidal sites from its distribution range along the European North Atlantic/North Sea coast of Norway to the Iberian Peninsula between 1999 and 2008 (see supporting information Table S1). Representatives of two other ligiid isopod species, *Ligidium hypnorum* (Cuvier, 1792) and *Ligia italica* Fabricius 1798, were used as outgroup taxa. All specimens were preserved in 96% ethanol directly after collection. Total genomic DNA was extracted from legs using a commercial extraction kit (QIAmp Blood and Tissue Kit; Qiagen) following the tissue extraction protocol. Specimens and DNA extracts were listed and stored in the North Sea Fauna collection of the German Centre for Marine Biodiversity Research (DZMB) in Wilhelmshaven, Germany.

PCR amplification and sequencing

Amplification reactions for all gene fragments were carried out on a Thermal Cycler GeneAmp PCR System 2700/2720 (Applied Biosystems) or Mastercycler pro S (Eppendorf). Detailed summaries of amplification reactions and temperature profiles for both mitochondrial fragments can be found in a previous study (Raupach *et al*., 2010a). The hypervariable V7 expansion segment of the nuclear 18S rRNA gene, which has been shown to vary between even closely related species (e.g. Raupach *et al*., 2007, 2010b), was amplified for 13 selected specimens (see Table S1). For amplification, the newly designed primer pair LoV7F (forward; 5′-GGGACCACCAGGAGTG-3′) and LoV7R (reverse; 5′-GGCCCAGAACATCTAAGG-3′) was used. Amplification reactions were carried out on a Thermal Cycler GeneAmp PCR System 2700/2720 (Applied Biosystems) in a 20-μL volume, containing 4 μL Q-Solution, 2 μL 10× Qiagen PCR buffer, 2 μL dNTPs (2 mmol μL^{-1}), 0.1 μL of each primer (both 50 pmol μL^{-1}), 1 μL of DNA template with an amount between 2 and 150 ng μL, 0.2 μL^{-1} Qiagen Taq polymerase (5 U μL^{-1}), and filled up to 20 μL with sterile H_2O. The PCR protocol consisted of 94 °C for 5 min (initial denaturation), 38 cycles of 94 °C denaturation for 45 s, 56 °C annealing for 45 s and 72 °C extension for 80 s, followed by a final 72 °C extension for 7 min. Details of PCR product purifications can be found in Raupach *et al*. (2007). All purified PCR products were outsourced for sequencing to a contract sequencing facility (Macrogen) using the same primer sets as for PCR. Both sequencing reads were assembled with Seqman II (DNASTAR) and the identity of all new sequences was confirmed with BLAST searches (Zhang *et al*., 2000; Morgulis *et al*., 2008). All new sequences were deposited in GenBank [cytochrome *c* oxidase subunit I (CO1) haplotypes: accession numbers JQ814406–JQ814468; 16S rRNA haplotypes: JQ814385–JQ814404; 18S rRNA V7: JQ814405: see Table S1]. The presence of *Wolbachia* bacteria was checked using published primers and amplification protocols for the *wsp* gene region of the *Wolbachia* genome (Braig *et al*., 1998; Cordaux *et al*., 2001).

Phylogenetic and population structure analyses

All sequences for each marker were aligned separately using MUSCLE version 3.6 (Edgar, 2004) using the default settings. Because of some sequence ambiguities at the 5′ and 3′ ends of some CO1 sequences we removed 74 bp (5′: 34 bp; 3′: 40 bp), generating a final alignment with a length of 584 bp. Aligned CO1 sequences were translated to amino-acid sequences to check for possible nuclear mitochondrial pseudogenes (numts) using Geneious (Drummond *et al*., 2010). Fragment lengths of the 16S rRNA gene varied between 492 and 494 bp with no significant differences in base composition. The 16S rRNA alignment consisted of 495 bp, whereas the V7 alignment was 595 bp in length. Both mitochondrial gene fragments were tested for nucleotide bias using a chi-square test of base composition homogeneity across taxa as implemented in PAUP* 4.0b10 (Swofford, 2002).

Phylogenetic relationships of the CO1 alignment were inferred using Bayesian analysis in MrBayes 3.2.1 (Ronquist *et al*., 2012). The most appropriate model was determined beforehand using the Bayesian information criterion (BIC) as implemented in jModeltest 2.1.1 (Darriba *et al*., 2012), indicating TPM1uf + I + G to be the optimal nucleotide substitution model with the following parameters: nucleotide frequencies A: 0.27, C: 0.19, G: 0.17, T: 0.37; substitution rates R_{AC}: 1, R_{AG}: 13.33, R_{AT}: 4.63, R_{CG}: 4.63, R_{CT}: 13.33, R_{GT}: 1; gamma distribution shape = 0.48; and proportion of invariable sites = 0.38. The Bayesian analysis employed 10 000 000 Markov chain Monte Carlo (MCMC) generations in two parallel runs, each with three cold chains and one hot chain. Trees were sampled every 500th generation, with the number of burn-in generations being determined using the likelihood scores over all generations in Tracer 1.5 (Rambaut *et al*., 2013). Determination of the split frequencies in both independent runs (< 0.01) with 2 × 20 001 tree samples showed good convergence after 5000 sampled trees (split frequency < 0.01), which was therefore set as the burn-in. Phylogenetic analyses were also conducted in a maximum-likelihood framework using a combined rapid bootstrap and likelihood search in RAxML v7.2.8 (Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008) using a GTR + CAT model. Support for individual clades was estimated using 1000 replicates of a non-parametric bootstrap (Felsenstein, 1985). Relationships between haplotypes and their geographical distribution were explored with haplotype networks. All alignments and topologies were deposited in DRYAD (Raupach *et al*., 2014).

We used TCS 1.21 (Clement, Posada & Crandall, 2000) to create maximum-parsimony networks for both CO1 and 16S rRNA fragments and overlaid these networks with the geographical distribution of the haplotypes. The analysis using the default setting of a 95% connection limit resolved the two lineages as unconnected but with highly complex sub-networks. Lowering the connection limit to 90% revealed a putative connection between these sub-networks via 18 mutational steps. For CO1, additional population structure and diversity analyses, including haplotype diversity (h), nucleotide diversity (π) and the calculation of fixation indices (F), were performed using Arlequin 3.5.1.2 (Excoffier & Lischer, 2010). We also

employed an interpolation-based graphic method to visualize genetic variation across the landscape using Alleles in Space (Miller, 2005). A three-dimensional surface plot of the set of interpolated genetic distances was produced where x and y values are latitude and longitude within the Delaunay network, respectively, and surface plot heights (z) correspond to the genetic distance. Using Monmonier's algorithm as implemented in the software, we tested for prominent geographical barriers present in the data set. To provide a first, rough estimate of the timing of the split between the two distinct mitochondrial lineages, we calibrated the amount of molecular divergence using both a 'standard' molecular clock rate of 1.15% divergence per million years per lineage (as reported for butterflies of the genus *Heliconius*; Brower, 1994) and a fast rate of 6.6% per million years per lineage (as reported for the stomatopod crustacean *Haptosquilla pulchella*; Crandall *et al*., 2012). For both analyses, we applied strict molecular clocks and a standard coalescent model in BEAUTI v.1.7.5 (Drummond & Rambaut, 2007) and analysed the CO1 data in BEAST v.1.7.5 (Drummond & Rambaut, 2007) with a GTR + I + G model with four gamma categories for 100 000 000 generations sampling every 1000th tree to estimate the time to the most recent common ancestor (tMRCA) with 95% highest posterior density (HPD) intervals. Effective sampling sizes (ESSs, > 200 for all parameters) and convergence of the parameter estimates were assessed in Tracer 1.5 (Rambaut *et al*., 2013). A consensus tree was calculated using TreeAnnotator 1.7.5 (Rambaut *et al*., 2013).

RESULTS

The CO1 data set of 161 specimens of *Ligia oceanica* from ten different populations comprised 61 haplotypes with no significant differences in base composition (Table 1). Most haplotypes (48, or 79%) were obtained from single specimens only. Of the 65 polymorphic sites in total, 12, four and 49 were located at the first, second and third codon positions, respectively. In total, 16 amino-acid replacement substitutions (first position: 7, second position: 4, third position: 5) were detected. A majority-rule consensus tree of the remaining 2 × 15 001 trees showed strong evidence for the existence of two distinct mitochondrial lineages, which are named as 'lineage N' and 'lineage S' from here on (Fig. 1). Interestingly, we did not find two monophyletic lineages. Instead of this, the monophyletic lineage S is nested within lineage N, representing a para- or polyphylum. Lineage N comprised 39 haplotypes, pri-

Table 1. Sampled regions, number of analysed specimens (N), and number of identified CO1 and 16S rRNA gene haplotypes for the sampled populations of *Ligia oceanica*; statistical parameters indicate mean haplotype h (± SD) and nucleotide diversity π (± SD) observed for the CO1 fragment of the studied populations

Marker	Sample locality	N	No. of haplotypes	Haplotype diversity h	Nucleotide diversity π
COI	Kvinnherad	9	4	0.6944 ± 0.147	0.0016 ± 0.0014
	Spiekeroog	13	2	0.1538 ± 0.1261	0.0003 ± 0.0004
	Juist	8	1	0	0
	Wilhelmshaven	34	15	0.7772 ± 0.0741	0.0081 ± 0.0045
	Galway	15	8	0.8476 ± 0.0712	0.0161 ± 0.0088
	West Dale	29	13	0.7365 ± 0.0892	0.0084 ± 0.0047
	Battenoord	8	3	0.4643 ± 0.2	0.0009 ± 0.0009
	Plouguerneau	30	17	0.8161 ± 0.0725	0.015 ± 0.0079
	Concarneau	5	5	1 ± 0.1265	0.0176 ± 0.0114
	Ferrol	10	5	0.6667 ± 0.1633	0.0051 ± 0.0033
	Total	161	61	0.9031 ± 0.0156	0.0175 ± 0.0089
16S rRNA	Kvinnherad	9	1	0	0
	Spiekeroog	13	1	0	0
	Juist	8	1	0	0
	Wilhelmshaven	34	7	0.4528 ± 0.1008	0.0023 ± 0.0017
	Galway	15	4	0.7429 ± 0.0636	0.0069 ± 0.0042
	West Dale	29	5	0.6478 ± 0.0663	0.0031 ± 0.0021
	Battenoord	7	3	0.4643 ± 0.2	0.002 ± 0.0017
	Plouguerneau	30	4	0.6368 ± 0.0639	0.005 ± 0.0031
	Concarneau	5	3	0.7 ± 0.2184	0.0065 ± 0.0047
	Ferrol	10	2	0.2 ± 0.1541	0.0004 ± 0.0006
	Total	161	18	0.7172 ± 0.0347	0.0057 ± 0.0033

marily from northern sampling sites, whereas lineage S lineage consisted of 22 haplotypes from mostly southern sampling sites (Fig. 2). Uncorrected pairwise genetic distances (*p*-distances) among the CO1 haplotypes within lineage N ranged from 0.0017 to 0.0274, and from 0.0017 to 0.0257 for those within lineage S; distances between the lineages ranged from 0.0325 to 0.0514. Three haplotypes (numbers 1 and 5 in lineage N and 47 in lineage S) were present at high frequencies, representing 59% of all haplotypes in lineage N and 30% in lineage S, respectively. The results of the AMOVA (Table 2) showed that most of the variation (60.5%) occurred between the two major regions (South: France and Spain; North: all seven other populations; $F_{CT} = 0.61$, $P < 0.01$). Significant proportions of genetic variance were also found between populations within regions (8.6%, $F_{SC} = 0.22$, $P < 0.01$) and between all populations (30.9%, $F_{ST} = 0.69$, $P < 0.01$), indicating strong population structuring together with the high and significant F_{ST} values (Table 3). Both haplotype ($h = 0.9031 \pm 0.0156$) and nucleotide diversity ($\pi = 0.0175 \pm 0.0089$) were high, with the highest values occurring for populations around Ireland and the French coast (Table 1). The genetic landscape shape analysis of the CO1 data supports a geographical barrier between the southern populations of Ferrol/Plouguerneau and all other populations examined (Fig. 3). Genetic diversity within populations was highest for those around Ireland and the French coast (see Fig. 3 and Table 1) and decreased in a north-easterly direction. In particular, populations from Battenoord, Juist, Spiekeroog and Kvinnherad presented low genetic diversity and consisted of haplotypes from the northern lineage only. Genetic diversity was lower for the southernmost population from the Iberian coast (Ferrol).

With its 18 haplotypes, the 16S rRNA data supported the splitting of the CO1 network, albeit with a somewhat lower degree of genetic variability and resolution (Fig. 4). Eight haplotypes (44%) were obtained from single specimens. We found 22 substitutions, including 16 transitions and five transversions as well as two indels. Due to the lower mutation rate compared with CO1, values for both haplotype ($h = 0.7172 \pm 0.0347$) and nucleotide diversity ($\pi = 0.0057 \pm 0.0033$) were somewhat lower for this marker. Similarly, pairwise distances were 3.3-fold lower than those of CO1.

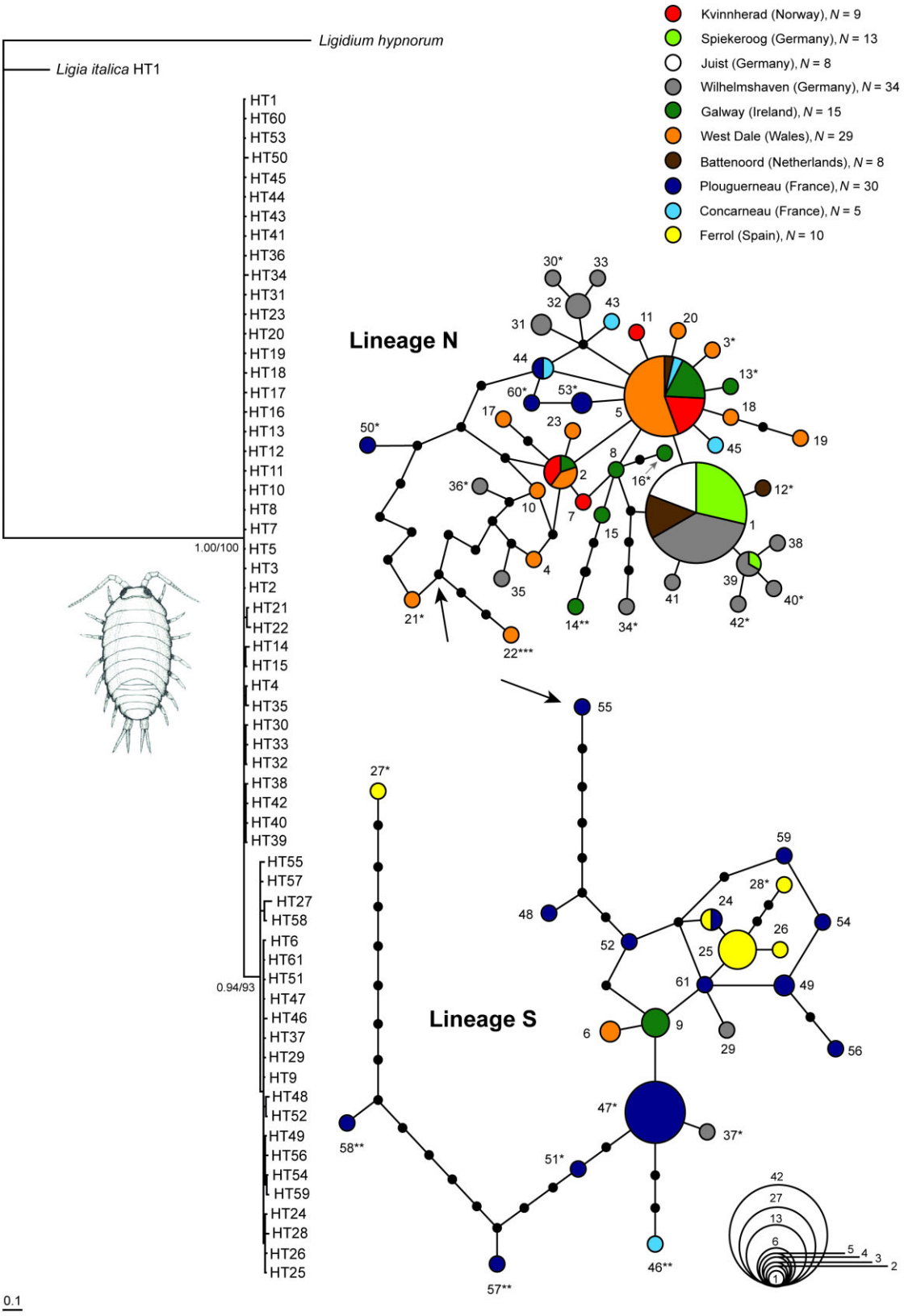
The average tMRCA for the two lineages was estimated to be 3.524 Myr (95% HPD: 1.981–5.353 Myr) using the standard clock of 1.15% per million years per lineage, and 0.597 Myr (95% HPD: 0.339–0.919 Myr) using the fast clock rate of 6.6%. There was no variation among the examined V7 expansion segment sequences.

Examining for putative *Wolbachia* infection showed a total infection rate of 21% (Table 4), being present in 14 different CO1 haplotypes. Here, specimens bearing haplotypes 9 and 47 had the highest infection rates of all haplotypes (three of four: 75%, and nine of 13: 69%) (Fig. 5). We also found infections in seven specimens bearing singleton haplotypes (Table 3). Based on sampling sites, individuals from Juist had the highest infection rate (63%), whereas specimens from Battenoord and Kvinnherad showed no *Wolbachia* infection (see supporting information Fig. S1). The infection rate for lineage S (41%) was significantly higher than that for lineage N (13%; chi-square test, $P < 0.001$), but did not correlate with the sex of the isopods or with a specific haplotype. In total, 14% of all examined males ($N = 58$) and 23% of all examined females ($N = 91$) were infected.

DISCUSSION

Our analyses revealed two deeply divergent and distinct mitochondrial clades within the analysed populations of *Ligia oceanica* along the European coastline (Fig. 1). Whereas haplotypes of lineage S were primarily found in Spain and France, haplotypes of lineage N dominated on the British Isles, the Netherlands, Germany and Norway (Fig. 2). This distribution is also supported by the genetic landscape shape analysis: for the CO1 data this revealed high genetic distances between Ferrol/Plouguerneau and all other populations examined (Fig. 3). Genetic diversity within populations was highest for those around Ireland and the French coast (see Fig. 3 and Table 1) and decreased in a north-easterly direction. In particular, populations from Battenoord, Juist, Spiekeroog and Kvinnherad presented low genetic

Figure 1. Bayesian tree and statistical parsimony networks for the 61 CO1 haplotypes of *Ligia oceanica*. Numbers on the major nodes of the phylogram represent posterior probabilities (left) and bootstrap values (right). For the networks, each circle represents a separate haplotype. The size of each circle is proportional to the number of sampled individuals, with numbers for each haplotype corresponding to the given haplotype classification. Each line between haplotypes represents one mutational step; putative intermediate haplotypes not recovered in our sampling are indicated by small black dots. Black arrows indicate putative connection points between the two networks separated by 18 mutational steps. Sampled regions are colour-coded (see legend) and the number of analysed specimens (*N*) is listed. Asterisks indicate the number of amino-acid replacement substitutions in comparison with the ancestral haplotype (no. 1).


Kvinnherad (Norway), N = 9
Spiekeroog (Germany), N = 13
Juist (Germany), N = 8
Wilhelmshaven (Germany), N = 34
Galway (Ireland), N = 15
West Dale (Wales), N = 29
Battenoord (Netherlands), N = 8
Plouguerneau (France), N = 30
Concarneau (France), N = 5
Ferrol (Spain), N = 10
Ligidium hypnorum
Ligia italica HT1
HT1
HT60
HT53
HT50
HT45
HT44
HT43
HT41
HT36
HT34
HT31
HT23
HT20
HT19
HT18
HT17
HT16
HT13
HT12
HT11
HT10
HT8
HT7
1.00/100
HT5
HT3
HT2
HT21
HT22
HT14
HT15
HT4
HT35
HT30
HT33
HT32
HT38
HT42
HT40
HT39
HT55
HT57
HT27
HT58
HT6
HT61
HT51
0.94/93
HT47
HT46
HT37
HT29
HT9
HT48
HT52
HT49
HT56
HT54
HT59
HT24
HT28
HT26
HT25
0.1
Lineage N
Lineage S

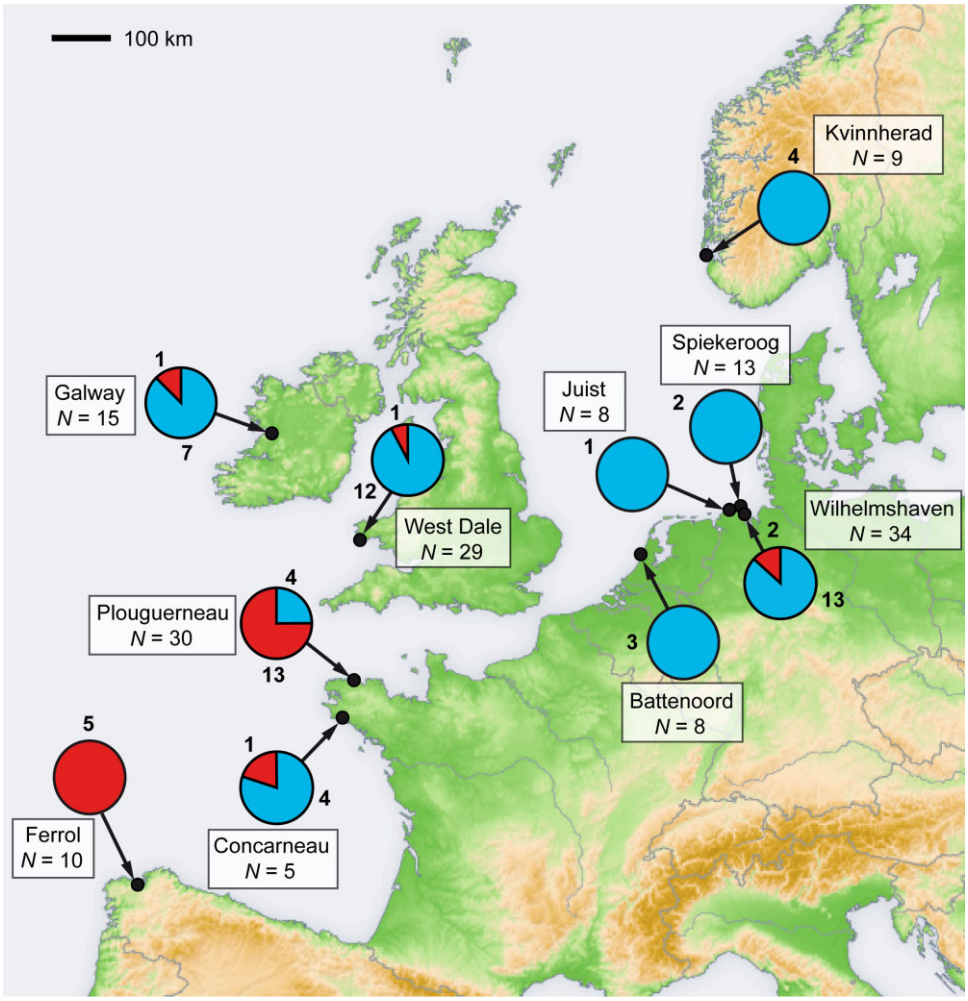


Figure 2. CO1 haplotype distributions of the sampled *Ligia oceanica* populations along the European coastline. The number of specimens per population is given in the white boxes, with numbers on the circles representing the number of haplotypes belonging to lineage N (blue) and S (red).

Table 2. Results of AMOVA testing for the partitioning of genetic variation in *Ligia oceanica* based on the CO1 data set (Spain and France vs. all others), with fixation indices (F) among groups of populations (F_{CT}), among populations within groups (F_{SC}), or among populations (F_{ST}); levels of statistical significance (P) based on randomization tests is also provided

Source of variation	d.f.	Sum of squares	Variance component	Per cent of total	F_{CT}	F_{SC}	F_{ST}	P
Between regions	1	338.1	4.9301	60.5	0.61			0.01
Among populations within regions	8	101.3	0.7008	8.6		0.22		0
Within populations	151	380.3	2.5185	30.9			0.69	0
Total	160	819.7	8.1494	100				

diversity and consisted of haplotypes from the northern lineage only. Genetic diversity was lower for the southernmost population from the Iberian coast (Ferrol).

Molecular studies of other *Ligia* species from North America and East Asia also revealed similar deep mitochondrial divergences (Jung *et al.*, 2008; Hurtado, Mateos & Santamaria, 2010; Markow & Pfeiler, 2010; Eberl, 2013; Eberl *et al.*, 2013; Yin *et al.*, 2013). In the case of *Ligia occidentalis* from the Pacific coast of North America, recent molecular studies also imply the existence of cryptic species

Table 3. Pairwise differentiations (F_{ST}) between all sampled populations

Population	Kvinnherad	Spiekeroog	Juist	Wilhelmshaven	Galway	West Dale	Battenoord	Plouguerneau	Concarneau	Ferrol
Kvinnherad	0									
Spiekeroog	**0.6912**	0								
Juist	**0.6812**	0.0417	0							
Wilhelmshaven	0.1129	0.0214	–0.0043	0						
Galway	0.1343	**0.2974**	**0.2363**	**0.1766**	0					
West Dale	–0.021	**0.2365**	0.1993	**0.1091**	0.0906	0				
Battenoord	**0.5297**	0.0302	0	–0.0148	**0.2095**	0.1571	0			
Plouguerneau	**0.6467**	**0.6943**	**0.6644**	**0.6412**	**0.3996**	**0.614**	**0.6576**	0		
Concarneau	0.1172	**0.3891**	**0.282**	0.099	–0.0866	0.0226	0.2281	0.4363	0	
Ferrol	**0.9093**	**0.9401**	**0.9273**	**0.8058**	**0.6193**	**0.7972**	**0.9177**	**0.1954**	**0.7121**	0

Significant values after Bonferroni correction for multiple comparisons are given in bold type ($P < 0.05$). F_{ST} values were calculated using haplotype frequencies and significance was assessed using 10 000 random permutations.

(Eberl *et al*., 2013). Nevertheless, we would argue that *L. oceanica* represents a single species despite similarly deep mitochondrial lineages given that the hypervariable V7 expansion segment, which is known to vary between even closely related species (e.g. Raupach *et al*., 2007, 2010b), was identical for all analysed specimens. Nevertheless, the analysis of other faster evolving nuclear markers and detailed morphological and/or morphometric studies is prudent and will shed additional light on the species status of *L. oceanica*.

The high mitochondrial variability and deep divergence of most *Ligia* species has been interpreted as a consequence of repeated population contractions and expansions due to recurring glacial cycles. No calibrated molecular clocks or robust calibration points exist for *L. oceanica* and therefore dating of ancestral divergence events must be regarded with caution. To counteract this to some degree, we used two very different reported substitution rates for arthropods (1.15 and 6.6% per lineage per million years) for the CO1 gene as a form of sensitivity analysis. Even with the faster rate, the upper 95% HPD interval estimate of 338 500 years pre-dates the LGM and most estimates suggest that the splitting actually occurred in the early or mid Pleistocene or even in the late Pliocene.

In terms of other European coastal species, various studies have inferred past expansion events that are definitely older than the LGM (e.g. Hoarau *et al*., 2007; Campo *et al*., 2010; Francisco *et al*., 2011), something that has been also demonstrated for some species colonizing the North Sea after the LGM (Debes, Zachos & Hanel, 2008; Francisco *et al*., 2009; Coscia & Mariani, 2011). For many populations of marine and coastal organisms of the European coasts, it has commonly been suggested that these species moved up and down the western shore with the glacial cycles (Francisco *et al*., 2011). However, recent phylogeographical studies also support the possible existence of several, previously unknown and therefore 'cryptic' northern refugia (Stewart & Lister, 2001; Hoarau *et al*., 2007; Maggs *et al*., 2008; Provan & Bennett, 2008; Stewart *et al*., 2010), with these periglacial refugia being located in areas of sheltered topography that provided suitable, stable microclimates. For *L. oceanica*, which today inhabits a dynamic habitat that is strongly influenced by sea-level changes or other factors (e.g. storm surges, human harbour constructions), populations may have survived in such isolated rocky supralittoral localities and functioned later as source populations for the recolonization of formerly ice-covered or inaccessible habitats. Although *L. oceanica* is principally terrestrial and does not usually spend time in seawater, there are occasions when they enter this environment (e.g. when foraging for intertidal seaweeds or being washed by waves into the water during storms; Carefoot & Taylor, 1995). Indeed, experiments showed that specimens can survive immersed in sea water for up to 80 days (Nicholls, 1931) and, given that these isopods feed on seaweeds of the genus *Fucus* (Gruner, 1993), rafting on floating seaweeds can also represent a feasible dispersal mechanism in some cases (e.g. Highsmith, 1985; Thiel & Gutow, 2005a, b; Thiel & Haye, 2006). In addition, like other members of the Peracarida, female isopods possess a brood pouch (marsupium) in which the eggs (*c*. 50 for *L. oceanica*; Gruner, 1993) are incubated until hatching such that even single pregnant females can easily contribute significantly to already established populations or even found new ones. The lack of significant differentiation between populations in close proximity (e.g. West Dale and Galway; Wilhelmshaven and Spiekeroog) adds further support for regular dispersal events. However, due to the limited sampling of some

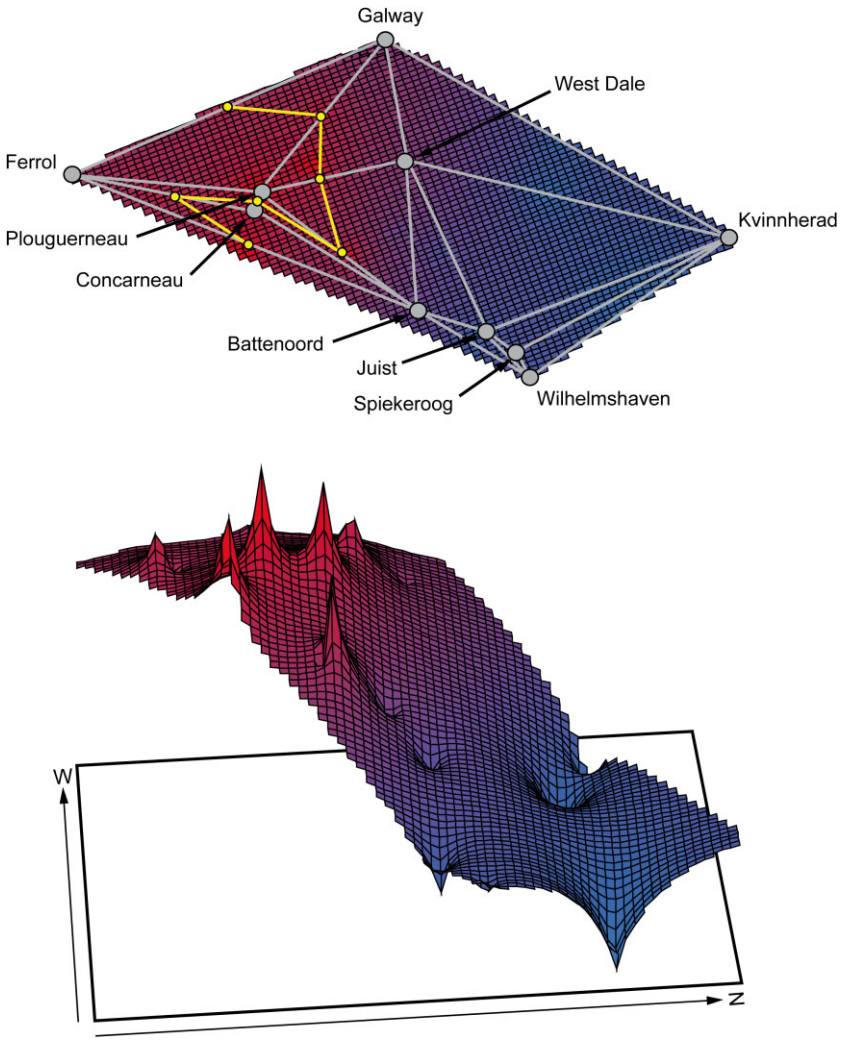


Figure 3. Results of the genetic landscape shape interpolation analyses using a 75 × 75 grid and a distance weight of 1 (default setting). The *x*- and *y*-axes show the geographical coordinates of the Delaunay triangulation network constructed for the sampled populations, with a top view that includes the connectivity network (grey lines) and genetic boundaries (yellow lines) obtained with Monmonier's maximum difference algorithm (above) and a side view (below). Peaks (red) and valleys (blue) on the *z*-axis indicate areas with high or low pairwise genetic distances between the analysed individuals.

populations, the lack of significant differentiation in some cases should be regarded with caution (e.g. comparisons with the population from Concarneau, for which only five specimens were analysed).

Our results indicate that specimens of *L. oceanica* survived at least the LGM in different refugia. Although we are presently unable to determine the exact locations of these refugia because of the limited number of specimens and localities analysed, other studies provide some hints. Comprehensive analyses of mtDNA and microsatellite data sets designed to identify glacial refugia and recolonization pathways in the brown seaweed *Fucus serratus* (Coyer *et al*., 2003; Hoarau *et al*., 2007), which acts as food for *L. oceanica* (Gruner, 1993; Carefoot & Taylor, 1995), identified three different, cryptic glacial refugia using haplotype networks and the pattern of genetic diversity: (1) the south-west coast of Ireland, (2) the Brittany/Hurd Deep region in the English Channel and (3) the northern Iberian Peninsula (Hoarau *et al*., 2007). Similar results were also shown for other seaweed species (Provan *et al*., 2005) as well as other marine taxa (e.g. Gysels *et al*., 2004; Casu *et al*., 2011). In our study, CO1 haplotype diversity for *L. oceanica* was highest in Galway/Ireland (0.8476 ± 0.0712) with 15 analysed specimens, supporting the hypothesis of an Irish refugium. By contrast, haplotype diversity was only moderate for Ferrol/Spain (0.6667 ± 0.1633), although the limited number of analysed specimens ($n = 10$) makes a

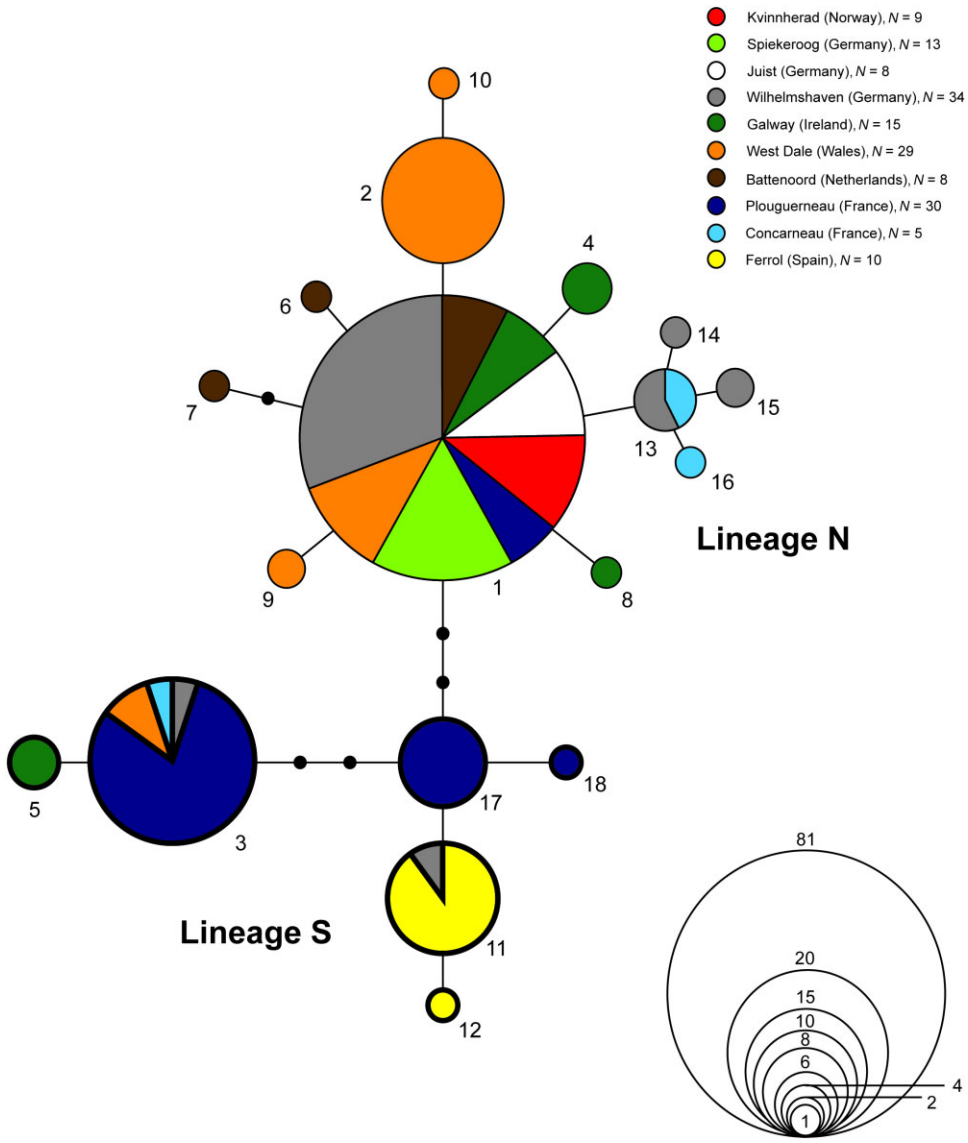


Figure 4. Statistical parsimony network of the sampled mitochondrial 16S rRNA gene haplotypes of *Ligia oceanica*. Each line in the network represents a single mutational change; small black dots indicate missing haplotypes. Numbers for each haplotype correspond to the given haplotype classification. The diameter of the circles is proportional to the number of haplotypes sampled and the haplotypes are colour-coded according to the region (see legend; *N* = number of sampled specimens). Haplotypes that correspond with the CO1 haplotype lineages N and S are bounded with lines of normal width and bold lines, respectively.

precise estimate difficult. Therefore, additional specimens from different localities have to be analysed to test this hypothesis in more detail.

Beside phylogeographical processes, mitochondrial genetic variability can be also influenced by other factors, including introgressive hybridization (e.g. Nunes *et al*., 2010; Barber *et al*., 2012), incomplete lineage sorting (e.g. Carstens & Knowles, 2007; McGuire *et al*., 2007), heteroplasmy and maternally inherited endosymbionts such as *Wolbachia* (Dobson, 2004; Duron *et al*., 2008). Although heteroplasmy has been reported in various terrestrial isopod species (Doublet *et al*., 2008), there is no evidence for heteroplasmy within *L. oceanica* (Doublet *et al*., 2012). With respect to *Wolbachia*, our analyses revealed some interesting aspects of infection frequencies within isopods or even arthropods in general, where most *Wolbachia* studies analyse high numbers of species but only low numbers of specimens per species (e.g. Jeyaprakash & Hoy, 2000; Gerth, Geißler & Bleidorn, 2011). In our study, no lineage was completely infected by or free of

Table 4. *Wolbachia* infection rate based on the presence of the amplified *wsp* gene fragment of the investigated *Ligia oceanica* populations; results are also presented separately for each mtDNA lineage (left: number of specimens, right: number of infected specimens; dashes indicate no infection)

Sample locality	Lineage N	Lineage S	Total
Kvinnherad	9/–	–	9/–
Spiekeroog	13/1 (7%)	–	13/1 (7%)
Juist	8/5 (63%)	–	8/5 (63%)
Wilhelmshaven	32/6 (19%)	2/–	34/6 (18%)
Galway	11/–	4/3 (75%)	15/3 (20%)
West Dale	27/2 (7%)	2/1 (50%)	29/3 (10%)
Battenoord	8/–	–	8/–
Plouguerneau	5/–	25/11 (44%)	30/11 (37%)
Concarneau	4/1 (25%)	1/–	5/1 (20%)
Ferrol	–	10/3 (30%)	10/3 (30%)
Total	**117/15 (13%)**	**44/18 (41%)**	**161/33 (21%)**

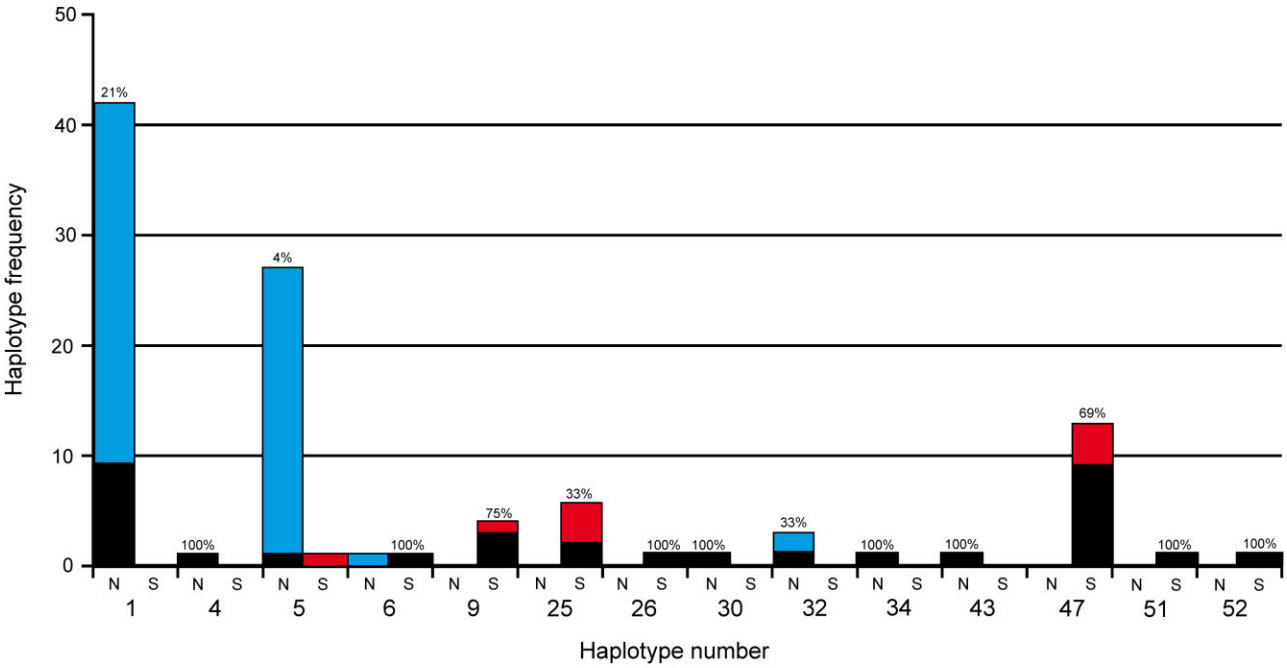


Figure 5. *Wolbachia* infection rates in correlation with CO1 haplotype frequency of both mitochondrial lineages (N: blue, S: red).

Wolbachia, with a significantly higher infection rate of the southern lineage compared with the northern one (41 vs. 13%), which might explain the lower genetic diversity of the former clade. However, because of the lack of *Wolbachia* sequence data and limited number of analysed specimens of the southern lineage, this inference is subject to discussion. It also remains unclear if the analysed isopods were infected only once or multiple times by the same or different *Wolbachia* strains. Furthermore, we cannot exclude with certainty that temporal sampling effects (sampling period 1999–2008) may have biased the observed rates of *Wolbachia* infections of the studied populations, limiting a direct comparison of the infection rates of the different studied populations (e.g. while Kvinnherad was sampled in 2000, specimens from Ferrol were collected in 2006). In this context, the expected life of a female of *L. oceanica* is suggested to be about 3 years, with the first being one of active growth, maturity being reached early in the second with the production of perhaps two broods, and three broods during the third and last year (Nicholls, 1931; Gruner, 1993). The average number of young produced in each brood is about 80 (Nicholls,

1931). Nevertheless, there was no evidence for a homogenization of mtDNA haplotypes. While the number of given CO1 haplotypes was low for some localities (e.g. Juist, Spiekeroog or Battenoord), there was with one exception (Juist) no correlation of high *Wolbachia* infection rates and low haplotype diversity. For example, 12 of 13 (93%) specimens collected at Spiekeroog bore haplotype 1, but only one out of these 12 isopods was infected (see Table S1). In the case of Juist, all eight specimens bore haplotype 1, and five of them showed positive *wsp* amplifications. However, the total number of analysed specimens was not very high, and the given results may represent a sampling artifact. Based on the available data, we have to reject the hypothesis that the observed genetic variability of mtDNA within *L. oceanica* was influenced by the presence of *Wolbachia* even if we cannot explain the observed infection patterns at present.

CONCLUSIONS

Our data provide novel and important insights into the genetic variability and population structure of the European coastal isopod *Ligia oceanica*. Phylogenetic analysis revealed two distinct and 'old' mitochondrial lineages that have been clearly separated since before the LGM. We also found some evidence for a putative cryptic northern refugium during the LGM located around Ireland. Based on our results, we also exclude an influence of *Wolbachia* on the observed mtDNA variability.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Geographical distributions of *Wolbachia*-infected specimens of *Ligia oceanica* (black, in %). The total number of specimens per population is given in the white boxes.

Table S1. Table of all specimens used in this study, including individual codes, GenBank accession numbers (left) and haplotype numbers (right) of both mitochondrial gene fragments, collection sites with coordinates and dates, gender and status of *Wolbachia* infection of the analysed isopod specimens. Specimens are marked with asterisks when nuclear V7 regions were additionally analysed (accession number JQ814405).

ARCHIVED DATA

Data deposited in the Dryad digital repository (Raupach *et al*., 2014).