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Glacial-driven vicariance in the amphipod *Gammarus duebeni*

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ABSTRACT

We have examined the genetic diversity using mitochondrial COI and ND2 sequence data from 306 specimens of the amphi-Atlantic-distributed amphipod *Gammarus duebeni*. Marine populations from the Atlantic Ocean, the Baltic and North Sea, as well as freshwater populations from Ireland, Cornwall and Brittany were analysed.

G. duebeni is a complex of five allopatric lineages. Freshwater populations result from multiple invasions of marine ancestors, represented by distinct lineages. We interpret the recent distribution of lineages as the outcome of a series of spatio-temporal vicariant events caused by Pleistocene glaciations and sea level changes. The freshwater lineages are therefore regarded as 'glacial relicts'. Furthermore, inter-specific competition with, for example, *Gammarus pulex* (which is absent in Ireland and western Brittany) may be another important determinant in the distribution of freshwater *G. duebeni*. In Ireland and Brittany, three freshwater refugia are suggested. The significantly limited gene flow detected among marine populations is more likely due to inter-specific competition than to salinity. The *G. duebeni*-complex represents a model system for the study of allopatric speciation accompanied by major habitat shifts. The pattern of spatio-temporal origins of the freshwater entities we describe here provides an excellent system for investigating evolutionary adaptations to the freshwater environment. Our data did not confirm the presently used subspecies classification but are only preliminary in the absence of nuclear genetic analyses.

1. Introduction

It is accepted that several factors, including historical events or ecological skills, determine the geographic distribution of a species as well the spatial arrangements of genetic diversity within this species. The ice ages are one such potent historical factor. Habitat and population fragmentation, several consequences of the ice ages, are major factors in determining the present day distribution patterns of genetic variation within species (Hewitt, 1996, 2000, 2004; Schmitt, 2007). The study of the spatial adjustment of intraspecific diversity, also called phylogeography (Avise et al., 1987; Avise, 2009), is one key discipline for the identification of glacial refugia and for the detection of species subdivisions promoted by ice ages. Further such studies yielded insights into postglacial recolonisation patterns of animals and plants in Europe (Taberlet et al., 1998; Stewart and Lister, 2001; Provan and Bennett, 2008). The amphi-Atlantic-distributed amphipod *Gammarus duebeni* Lilljeborg, 1852 has a large natural distribution and its recent area was undoubtedly strongly influenced by the ice ages. Because of the resistance of *G. duebeni* to various stresses, such as aerial exposure, hypoxia, anoxia, hyperoxia, heavy metal contents and X-radiation (Gaston and Spicer, 2001), it is of interest for physiologists and ecologists. However, most striking is the exceptional tolerance to different salinity regimes, ranging from freshwater to full marine conditions. Like the ice ages as a historical factor, salinity as a limiting ecological determinant also defines the distribution of aquatic species. The interface between marine and freshwater habitats is a formidable barrier that few species are able to penetrate (reviewed by Lee and Bell, 1999). Biological transitions from marine to freshwater conditions are of particular interest because they are often accompanied by shifts of physiological, morphological and life-history traits. Such transitions have initiated the radiation and speciation of many taxa (Lee and Bell, 1999).

Marine or brackish water populations of *G. duebeni* are known from the Atlantic coasts of northern and mid-Europe as well as North America. Here it lives in the upper littoral zone of tidal areas, estuaries, shallow ponds, rock pools and other marginal habitats (Bulnheim, 1979). Aside from the marine or brackish water populations, freshwater populations of *G. duebeni* exist in Cape Breton Island, the Gulf of St. Lawrence, Canada (Shoemaker, 1930), Iceland (Poulsen, 1939), the Faroes (Poulsen, 1929), the Shetlands (Stephensen, 1928), the Outer Hebrides (Forrest et al., 1936), the Inner Hebrides (Beadle and Cragg, 1940), the Isle of Man (Jones, 1948), Ireland (Reid, 1939), Western Cornwall (Crawford, 1937) and Brittany, France (Pacaud, 1945; Hynes, 1954, 1959). Reid (1939) first described

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physiological differences between Irish freshwater *G. duebeni* and the brackish water form. Stock and Pinkster (1970) elevated these forms to the subspecies status. They designated the marine subspecies as *G. duebeni duebeni* and the freshwater subspecies as *G. duebeni celticus*. Sutcliffe (1971) found physiological evidence for two distinct *G. duebeni* forms by means of osmoregulatory differences, but it was suggested that the observed differences are "phenotypic in origin". Genetic investigations have been rare in *G. duebeni*. The first genetic study using allozymes (Bulnheim and Scholl, 1981) dealt with populations from the Baltic Sea, the North Sea (*G. d. duebeni*), and Lesneven (Brittany, France; *G. d. celticus*), and found no evidence of genetic differentiation within *G. d. duebeni* or between *G. d. duebeni* and *G. d. celticus*. Siegismund et al. (1985) studied three marine populations of *G. d. duebeni* from Limfjord (Denmark), Kattegat (Denmark) and Askö (Sweden). They investigated 19 enzyme-encoding loci and stated that *G. d. duebeni* shows a rather high but irregular differentiation among the populations without any clinal variation. Altogether, the biochemical and genetic studies did not reveal any phylogeographic patterns within *G. duebeni*.

Studies using mitochondrial DNA sequences seem to be better suited to detect genetic differentiation in *G. duebeni* (Ironside et al., 2003; Rock et al., 2007; this study). Rock et al. (2007) were the first to use cytochrome *c* oxidase subunit I (COI) gene sequences to gain insight into the population structure and phylogeography of *G. duebeni*. They analysed 88 specimens and found 11 haplotypes, which were divided into two distinct lineages. One lineage was found at marine and brackish sites and the other lineage in rivers and lakes of Ireland. Due to the low diversity of haplotypes spread across broad geographic areas, an ice age-shaped population structure with postglacial expansion for the marine lineage, *G. d. duebeni*, was suggested. The Irish freshwater lineage, *G. d. celticus*, showed little internal geographic structuring. Rock et al. (2007) assumed a preglacial divergence of the two *G. duebeni* lineages.

Two alternative hypotheses exist on the origin of the different freshwater populations. Hynes (1954, 1955) proposed that *G. duebeni* was formerly widespread in freshwaters in Western Europe and that it is now being eliminated from freshwaters through competition with *Gammarus pulex*. This suggests that *G. duebeni* was, and still is, pushed into brackish areas by *G. pulex*. In contrast, Sutcliffe (1967) suggested a very different hypothesis that marine *G. duebeni* is in the process of colonising freshwaters and that it may gradually extend its range into suitable streams. Pinkster et al. (1970) also followed this argument. They proposed that, in pre-glacial periods, *G. duebeni* was a marine, cold temperature species that occurred along the coasts of Northern Europe and Eastern North America. However, broad geographic areas inhabited by *G. duebeni*, including various freshwater populations, remain uninvestigated by modern molecular DNA methods. One of the principal aims of this paper was to evaluate the freshwater vs. marine origin of the ancestral *G. duebeni*. If we determined this ancestor to be of marine origin, the second aim was to investigate whether the different freshwater populations of *G. duebeni* result from a single invasion of marine *G. duebeni* into freshwater or if multiple transitions account for the recent distribution. Further, we assessed the influence of the ice age on the genetic diversity, the distribution of genetic variability and the demographic patterns in *G. duebeni*.

2. Materials and methods

2.1. Collection of samples

A total of 306 *Gammarus duebeni* specimens were collected in marine and brackish water habitats of the Baltic Sea, the North Sea, and the Atlantic Ocean, and in freshwater habitats of Ireland, Brittany (France), and Cornwall (England) between July 2003 and

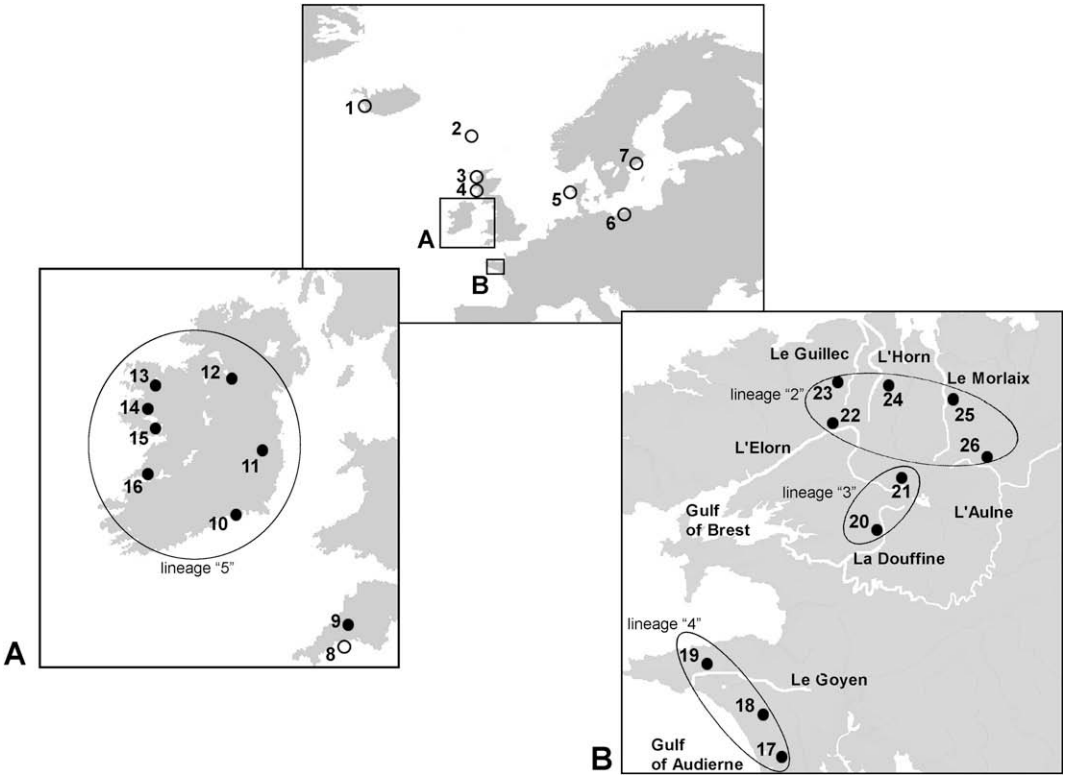

Fig. 1. Sampling sites (1–26) for *Gammarus duebeni*. Open circles are marine or brackish sites and filled dots are freshwater sites. Areas of the freshwater lineages "2", "3", "4" and "5" are indicated. Lineage "1" was found at sites 1–9. Table 1 includes information on the number of specimens analysed per site and which haplotypes were found at the particular sites.

Table 1
Distribution of *Gammarus duebeni* haplotypes (K1–K69) of the combined dataset (COI + ND2, 896 bp). Numbers (1–26) of column "site" refers to sampling sites in Fig. 1. Haplotypes in bold are those that were shared between populations; "*n*" refers to the number of specimens.

Site	Population	*n*	Haplotypes (*n*)
1	Iceland, Atlantic Ocean	7	K67 (7)
2	Faroe, Atlantic Ocean	9	**K64** (9)
3	Isle of Sky, Atlantic Ocean	7	K38 (2), **K41** (4), K69 (1)
4	Dunstaffnage, Atlantic Ocean	15	K9 (8), K31 (3), K35 (1), **K41** (1), **K47** (2)
5	Ringkøbing, North Sea	8	K61 (7), K63 (1)
6	Greifswald, Baltic Sea	10	K39 (1), **K44** (8), K46 (1)
7	Island of Askö, Baltic Sea	11	**K44** (5), **K47** (3), K62 (1), **K64** (2)
8	England, Cornwall, marine	7	**K57** (7)
9	England, Cornwall, freshwater	30	K37 (1), K42 (1), K45 (11), K56 (4), **K57** (8), K58 (5)
10	Ireland, Annestown river	5	K3 (2), K4 (3)
11	Ireland, Brittas river	11	K18 (11)
12	Ireland, Lough Erne	9	K10 (7), K19 (2)
13	Ireland, Lough Conn	10	K7 (3), K8 (5), K11 (1), K13 (1)
14	Ireland, Lough Mask	7	**K15** (1), K16 (6)
15	Ireland, Lough Corrib	11	K14 (2), **K15** (1), K17 (8)
16	Ireland, Shannon river estuary	7	K6 (1), K12 (5), K43 (1)
17	Brittany, Tréogat, small rivulet	6	K5 (6)
18	Brittany, Kervinon, small rivulet	1	K30 (1)
19	Brittany, Le Goyen	6	K28 (4), K29 (2)
20	Brittany, La Douffine	13	K33 (1), K34 (12)
21	Brittany, L'Elorn 1	10	K65 (2), K68 (8)
22	Brittany, L'Elorn 2	9	K49 (3), K55 (6)
23	Brittany, Le Guillec	15	K22 (2), K27 (6), K32 (7)
24	Brittany, L'Horn	31	K1 (1), K2 (3), K23 (13), K25 (1), K26 (1), K52 (1), K53 (1), K54 (4), K59 (1), K60 (5)
25	Brittany, Le Morlaix	30	K20 (2), K21 (1), K24 (5), K36 (5), K40 (1), K48 (8), **K50** (1), K66 (7)
26	Brittany, L'Aulne	21	**K50** (10), K51 (11)
	Overall	306	

March 2007 (Fig. 1 and Table 1). In Cornwall, we found *G. duebeni* in a rivulet (Cornwall freshwater) as well as in a rock pool (brackish habitat) about 500 m apart. All specimens were fixed in 95% ethanol immediately after sampling. The specimens were examined morphologically and a sample from the middle of the body was taken for DNA extraction. The remaining parts of the specimens were preserved in ethanol and deposited in the Zoological Collection of Rostock University.

A total of 26 populations of *G. duebeni* were analysed. In some cases, such as some Breton populations, samples were taken from different parts of a particular river and regarded as one population. The exact sampling coordinates for every specimen is given in Table S1 (Supplemental material).

2.2. Outgroup choice

Recently, Hou et al. (2007) published a molecular phylogeny of the genus *Gammarus*, including *G. duebeni*. In their study the phylogenetic position of *G. duebeni* varied dependent on the marker and tree building method used, and no final conclusion could be made. In some analyses, *Gammarus locusta* and *Gammarus oceanicus* were relatively closely related. However, *G. locusta* was polyphyletic in some of their calculations. For selection of an appropriate outgroup taxon for rooting our mitochondrial gene trees, we sequenced a part of the 18S rRNA gene for *G. duebeni* and 11 other *Gammarus* species, including *G. locusta* and *G. oceanicus*. This tree was rooted with *Corophium multisetosum* Stock, 1952 (Amphipoda, Corophiidae) and *Leptocheirus pilosus* Zaddach, 1844 (Amphipoda, Aoridae). The analysis of 1002–1036 base pairs (bp) of the nuclear gene for the 18S rRNA showed that *Gammarus finmarchicus* Dahl, 1938 was very closely related to *G. duebeni*, whereas *G. locusta* was not related (Fig. S3). Therefore *G. finmarchicus* was chosen as the outgroup and two specimens were used. *G. finmarchicus* is widely distributed along the coasts of North America and Europe. It is often found in tide pools and has a limited tolerance for reduced salinity (Lincoln, 1979). *G. finmarchicus* was sampled from the Atlantic Ocean of Rockland, Maine, USA (44°06′N 69°06′W). This species was not included in the work of Hou et al. (2007).

2.3. DNA extraction, primer design, PCR, and sequencing

Total DNA was extracted using a silica spin column procedure with the NucleoSpin® Tissue Kit (Macherey–Nagel) following the protocol provided by the manufacturer. Partial sequences of the COI gene were amplified with the universal primers LCO 1490 and HCO 2198 (Folmer et al., 1994), resulting in a fragment of 708 bp. However, for many specimens, this primer combination did not give positive PCR results. Therefore, we designed the new primers LCO3 and COI-MZ1-rev, which gave a fragment of 933 bp.

PCR amplifications for both primer combinations were performed with a denaturation step for 60 s at 94 °C, followed by 38 cycles of: 30 s at 94 °C, 30 s at 50 °C and 60 s at 72 °C, and completed with 5 min at 72 °C as a final extension step. PCR was performed in a 40 µl reaction volume consisting of 4 µl DNA template, 1.7 U of Moltaq (Molzym GmbH & Co.KG), 10× PCR buffer, 2.5 mM $MgCl_2$ (final concentration), 62.5 µM dNTP each and 1 pmol of each primer.

No suitable primers were available for amplification of a fragment of the mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit II (ND2) gene. We developed the primers ND2-171fw-33 and ND2-722-rev based on various previously published crustacean ND2 gene sequences. This primer combination yields a fragment of 569 bp from the mitochondrial ND2 gene of *G. duebeni*. The PCR profile and concentrations of PCR reagents were the same as for the COI PCR, with the exception of an annealing temperature of 52.5 °C and a final $MgCl_2$ concentration of 4.0 mM.

The PCR products were extracted from agarose gels according to the protocol of the NucleoSpin® Extract Kit (Macherey–Nagel) or the innuPREP Gel Extraction Kit (Analytik Jena). All PCR products were sequenced using the DTCS Quick Start Kit (Beckman Coulter) according to the manufacturer's protocol and electrophoresed on an automated DNA sequencer (CEQ™ 8000; Beckman Coulter).

The COI fragment was sequenced uni-directionally with the LCO or the LCO3 primer, depending on the primer used in the PCR. The ND2 fragments were sequenced bi-directionally using the PCR primers or with the internal sequencing primer ND2-193fw instead of ND2-171fw-33. All primers used in this study are summarised in Table S7.

2.4. Alignments, datasets, and phylogenetic analyses

The sequences were automatically analysed using the software CEQ™ 8000 (Beckman Coulter) and aligned using the BioEdit software (Hall, 1999). All variable positions in a sequence were checked on the basis of the corresponding electropherogram.

The COI gene is widely used for inter- and intraspecific diversification questions concerning amphipod taxa. In contrast, the ND2 gene has not been the subject of diversity studies within the Amphipoda and only rarely has it been used in crustaceans to date. Therefore, some basic measurements of diversity were calculated to obtain an initial overview of the phylogenetic performance of the two genes. Three datasets, COI, ND2, and combined COI + ND2 were built. The MEGA4 programme (Tamura et al., 2007) was used to calculate minimum and maximum genetic distances (uncorrected *p*-distances) for each dataset. DnaSP 4.50.3 (Rozas et al., 2003) was used to determine the nucleotide and haplotype diversity (Nei, 1987) as well as the standard deviation for both measurements for all three datasets.

To uncover phylogenetic relationships among *G. duebeni* haplotypes, Neighbour-joining (NJ) and Maximum Parsimony (MP) trees were constructed (both using MEGA4) on the basis of the combined data of COI and ND2 (896 bp). The NJ tree was constructed using uncorrected *p*-distances. Branch support for the nodes was calculated from 1000 bootstrap replicates. Settings for the MP tree are available in the Supplementary material.

Intraspecific data can often produce a variety of possible trees using conventional tree building methods due to the small genetic distances among taxa. In these cases, the relationship among taxa is best expressed by a network that is able to show alternative potential phylogenetic relationships within a single figure (Bandelt et al., 1999). Further networks allow the identification and illustration of ancestral alleles whereas phylogenetic trees treat all sequences as terminal taxa (Posada and Crandall, 2001). For that reason a Median-Joining (MJ) network analysis was performed with the combined COI and ND2 data. The MJ networks were calculated using the NETWORK software (Bandelt et al., 1999; www.fluxus-engineering.com) using default parameters. The first MJ network analysis was conducted with the complete combined dataset (69 haplotypes), resulting in a network with the same lineages as found with NJ. However, lineages were often multiple connected through derived haplotypes and few involved median vectors (not shown). Derived haplotypes were haplotypes that are only one mutational step away from the ancestral (the putative founder) haplotype (Templeton, 2005), while median vectors represent unsampled or extinct haplotypes. The linking of major lineages through derived haplotypes is possible but very unlikely. Such putative "incorrect linking" of lineages is rather the consequence of accumulated homoplasy in derived haplotypes of different lineages (Templeton, 2005; Bandelt, Hamburg, personal communication). The larger the genetic and evolutionary distances among taxa the higher the chance of homoplasy and "incorrect linking" of lineages within a phylogenetic network. The presented combined dataset depicted genetic distances of up to 7.48%, which are high considering this is a putative intraspecific study.

It is possible to avoid the influence of lineage linking through homoplasy by removing most of the derived haplotypes of a given lineage (Templeton, 2005; Bandelt, Hamburg, personal communication). Proceeding in this way resulted in a network with a more reasonable connection of the five lineages (Fig. 3). Subsequently, we re-analysed the intra-lineage relationships of haplotypes from those lineages that were reduced in the first network analyses into two separate networks (Figs. 4 and 5). For completeness, we also calculated a MJ network containing the COI sequence data of Rock et al. (2007) and our COI sequence data (Fig. S1).

2.5. Population structure and differentiation

Population genetic structure was explored through the spatial analysis of molecular variance (SAMOVA) approach, which defines groups of populations that are geographically homogeneous and maximally differentiated from one another (Dupanloup et al., 2002). This clustering method is based on a simulated annealing procedure that aims to maximise the proportion of the total genetic variation that is due to differences between *k* groups of populations, without any a priori definition of the *k* groups of populations. The grouping that maximised the F_{CT}-value (among group variance) is assumed to be the most probable geographic subdivision. This optimal grouping was identified by running SAMOVA repeatedly with 2–20 groups. Statistical variance was evaluated with 1000 random permutations.

The genetic population structure was further examined using an analysis of molecular variance (AMOVA; Excoffier et al., 1992) implemented in ARLEQUIN version 3.1 (Excoffier et al., 2005). The AMOVA was performed at different hierarchical levels. Populations were first pooled into a marine and a freshwater group (structure 1). A second AMOVA was run with populations grouped into lineages according to results of the phylogenetic analyses (structure 2). Finally populations were grouped according to the suggested grouping of the SAMOVA. All AMOVA tests were performed with corrected Φ-estimates (Tamura–Nei+G, with shape parameter $\alpha = 2$). Statistical significance was assessed by 1000 permutations. Since only one specimen was available for site 18, this site was excluded from SAMOVA and AMOVA analysis.

Pairwise Φ_{ST}-values as a measure of population differentiation among the marine populations of *G. duebeni* (sites 1–8), were calculated with Arlequin version 3.1, taking into account not only haplotype frequencies but also the differences among them. Therefore a model of base substitution among haplotypes was selected with Modeltest 3.7 (Posada and Crandall, 1998) according to the Akaike Information Criterion using likelihood scores estimated in PAUP 4.0b10 (Swofford, 2003). The chosen model was GTR+I+G. However, this model is not implemented in Arlequin and so the next simpler model available, Tamura–Nei+G (with shape parameter $\alpha = 2$), was used to correct genetic distances. Statistical significance was assessed by 1000 permutations.

2.6. Inference of demographic history

To determine the historical demographic patterns of *G. duebeni,* we applied two approaches. First, we evaluated whether the sequenced section (COI + ND2) of the mtDNA evolved under neutrality by applying Tajima's *D* test (Tajima, 1989a,b) and Fu's F_S test (Fu, 1997) in ARLEQUIN version 3.1. Both tests tend to be negative if an excess of the number of rare haplotypes is present in a sample. Such an excess of rare haplotypes might be the consequence of low frequency maintenance of deleterious mutations in the population (selection). However, another explanation is that the population is not in mutation-drift equilibrium, which is common after a population expansion following a bottleneck. During an expansion, the population deviates from mutation-drift equilibrium because new mutations are created faster than can be removed by genetic drift. Significantly negative *D* or F_S values are therefore suitable to detect demographic population expansion.

The significance of these statistics was evaluated using 1000 permutations.

The distribution of pairwise nucleotide differences (mismatch distribution) under the expectations of a sudden-expansion model was calculated as second test for demographic expansion (Rogers and Harpending, 1992), also in ARLEQUIN version 3.1. The validity of the model was analysed by the sum of squared deviations (SSD) between observed and expected mismatches. A significant SSD value (p-value < 0.05) is taken as evidence for departure from the model of demographic population expansion. Also, the degree of approximation between the observed mismatch distribution and that expected under population growth was tested using Harpending's raggedness statistic (Hri; Harpending, 1994). This index has greater values for distributions that are multimodal, as expected for stationary, or non-expanding, populations. The pairwise mismatch distribution analysis produces an age expansion parameter (τ), which is a relative measure of the time in generations since the population expansion, and is a useful measure of the starting point of rapid population growth (Rogers and Harpending, 1992). The effective population size at the start (θ_0) and at the end (θ_1) of expansion was also calculated with the pairwise mismatch distribution analysis.

The analysis of demographic history was applied for lineages within *Gammarus duebeni*. This is justified due to the absence of lineage admixture (allopatric lineages, see Section 3).

3. Results

3.1. mtDNA sequence diversity

For 306 *G. duebeni* and two *G. finmarchicus* specimens, the nucleotide sequences of the amplified COI and ND2 gene fragments were obtained. For *G. finmarchicus* no variation was found in either of the genes.

The COI dataset comprised 423 unambiguous aligned positions and 41 *G. duebeni* haplotypes (C1–C41). The GenBank accession numbers are listed in Table S5. Uncorrected sequence differences among *G. duebeni* haplotypes ranged from 0.24% to 5.67%. The haplotype diversity for the ingroup was 0.914 (±0.011) and the nucleotide diversity was 2.1% (±0.2).

The ND2 dataset comprised 473 unambiguous aligned positions and 55 *G. duebeni* haplotypes (N1–N55). The GenBank accession numbers are listed in Table S6. Uncorrected sequence differences among *G. duebeni* haplotypes ranged from 0.21% to 9.94%. The haplotype diversity for the ingroup was 0.963 (±0.004) and the nucleotide diversity was 4.1% (±0.4). All measurements of diversity were higher for ND2 than for COI.

The combined dataset of COI and ND2 had a sequence length of 896 bp and comprised 69 *G. duebeni* haplotypes (K1–K69). The COI and ND2 haplotypes contributing to the particular combined haplotypes are listed in Table S2 (Supplemental material). Uncorrected sequence differences among *G. duebeni* haplotypes ranged from 0.1% to 7.48%. The haplotype diversity for the ingroup was 0.979 (±0.002) and the nucleotide diversity was 2.9% (±0.3).

3.2. Geographic distribution of haplotypes

The distribution of haplotypes for the combined dataset is described below (Table 1). The distribution of the haplotypes for the individual COI and ND2 genes is given in Tables S3 and S4.

In general, we found that very few haplotypes are shared among populations. Only one haplotype occurred at a freshwater site as well as a marine site: haplotype K57 was found at the Cornwall marine site 8 and at the Cornwall freshwater site 9 (Table 1). However, these sites are very close to each other (Fig. 1). With the exception of two populations (Cornwall marine, site 8 and Faroe, site 2) all populations exhibit at least one private haplotype. Within the combined dataset, only seven out of the 69 haplotypes (K15, K41, K44, K47 K50, K57 and K64) were shared between two populations, and none of these seven haplotypes were found in more than two populations. At marine sites, haplotypes K47 and K64 are of particular interest because both were found in the Baltic Sea as well as in the Atlantic Ocean (K47 in sites 4 and 7, and K64 in sites 2 and 7).

3.3. Phylogenetic analyses

Both the NJ and the MP analyses resulted in a topology containing five partially well supported lineages (lineages "1–5"; Figs. 2 and S2). The five lineages are allopatric (Fig. 1). Lineage "1" was found at marine sites (1–8) and at the freshwater site in Cornwall (9). Haplotype K43, found in a specimen from the Irish Shannon river estuary (site 16), also phylogenetically belongs to this lineage. Within lineage "1" the haplotypes from the Cornish freshwater site are very closely related. Lineage "2" is restricted to the L'Elorn, Le Guillec, L'Horn, Le Morlaix and L'Aulne rivers in Brittany (sites 22–26). In many cases, haplotypes from the same population are more closely related to each other than to haplotypes found in other populations of this lineage. Lineage "1" is most closely related to lineage "2". Lineage "3" is well supported and contains haplotypes from specimens of the Breton rivers La Douffine (site 20) and L'Elorn (site 21). Lineage "4" is also found exclusively in Breton rivers (sites 17–19). Lineage "5" is the sister taxon to lineages "1" through "4" and was found in Irish lakes and rivers (sites 10–16), as well as in one marine site (site 4) close to Ireland. The lineages "1" and "5" were also found by Rock et al. (2007).

For the first MJ network analyses, the number of haplotypes belonging to lineages "1", "2" and "5" were reduced to overcome the effects of homoplasy. This network revealed that very long branches lead to lineages "3", "4" and "5", indicating that they are not closely related to each other, or to the other two lineages (Fig. 3). Lineages "1" and "2" are separated by only two mutations. However, their allopatry and the salinity differences of their habitats (freshwater vs. marine or brackish waters) indicate that they are two distinct entities.

The subsequent analyses of the full lineages "1" and "2" (Fig. 4) and lineage "5" (Fig. 5) yielded a star-like shape for lineage "2", thought to be a sign of a population expansion. For all three lineages we found evidence for monophyly for haplotypes within the same water system, for example the river Horn, river Morlaix, L'Elorn and Cornwall freshwater (Fig. 4) as well as Lough Erne, Lough Corrib, Lough Conn, Brittas river and Annestown river (Fig. 5), indicating an independent evolution event within these catchments.

Haplotype diversity was comparable for lineages "1", "2" and "5", ranging from 0.92 to 0.94. The remaining lineages "3" and "4" exhibited considerably lower haplotype diversities of 0.625 and 0.718, respectively. The nucleotide diversities of lineages ranged from 0.4% to 0.7%, with lineages "1" and "4" showing the lowest values and lineage "5" the highest (Table 3).

3.4. Genetic differentiation of populations

The SAMOVA failed to find a geographical structure. The optimal configuration (number of groups) that maximised the F_{CT}-value was 20, close to the number of tested populations (25). Only the following populations were forming geographically homogeneous groups in these SAMOVA: 1 + 2; 8 + 9; 3 + 6 + 7 and 25 + 26. All 16 other populations were separate groups. A total of 85.21% (p = 0.00) of the variation was due to differences among these 20 groups. A total of 1.12% (p = 0.00) was responsible for differences among populations within groups and 13.66% (p = 0.00)

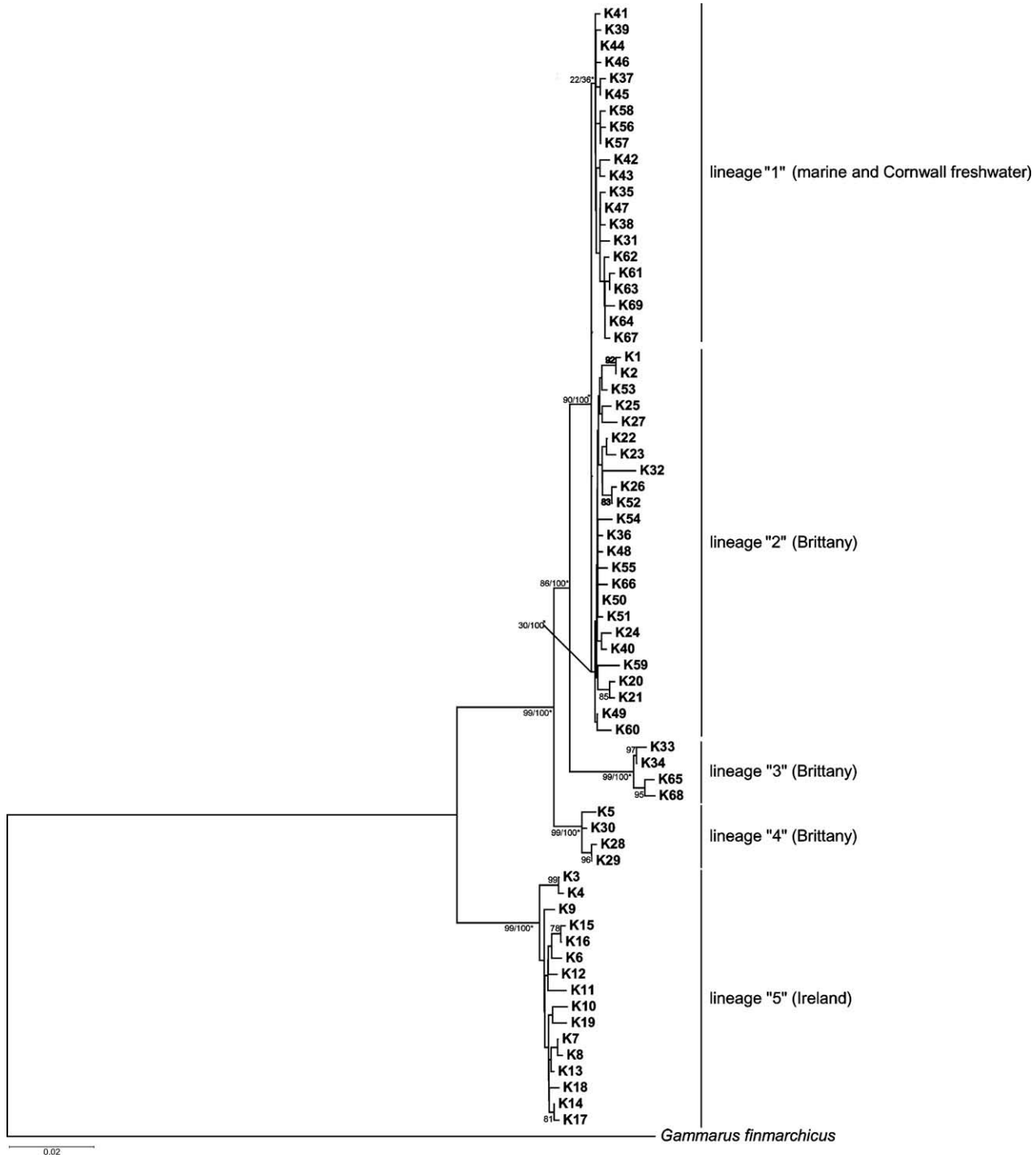

Fig. 2. Neighbor-joining tree showing the relationship among *Gammarus duebeni* haplotypes (K1–K69) of the combined dataset (COI + ND2, 896 bp) using uncorrected *p*-distances. Nodal support is indicated by bootstrap values (estimated from 1000 replications). NJ-bootstrap values over 75% are displayed (except for lineages "1" and "2"). For deeper branches the consensus values from the MP analyses are also given (marked with an asterisk). The tree was rooted with *Gammarus finmarchicus*.

for within population variation. Average fixation indices were 0.852 for Φ_{CT}, 0.076 for Φ_S, and 0.863 for Φ_{ST}. The performance of the SAMOVA algorithm is relatively low when gene flow among populations is low (Dupanloup et al., 2002). In the present case gene flow among populations is indeed almost absent as indicated by the high total number of private haplotypes and the low number of shared haplotypes among populations (Table 1). We interpret the failure of the SAMOVA as additional evidence of reduced gene flow between *G. duebeni* populations in general.

The first AMOVA dividing populations into a marine and a freshwater group (structure 1) showed that only 0.50% ($p = 0.257$) of the variation was due to differences between habitat types (Table 2). However, this value was not significantly different from zero and no variance on this level can be assumed. Differences between populations accounts for 85.98% ($p = 0.00$) of the total variability, whereas 13.52% ($p = 0.00$) was due to intra-population differences. This AMOVA supports the hypothesis that the division of *G. duebeni* into a single freshwater and a single marine form is an un-natural

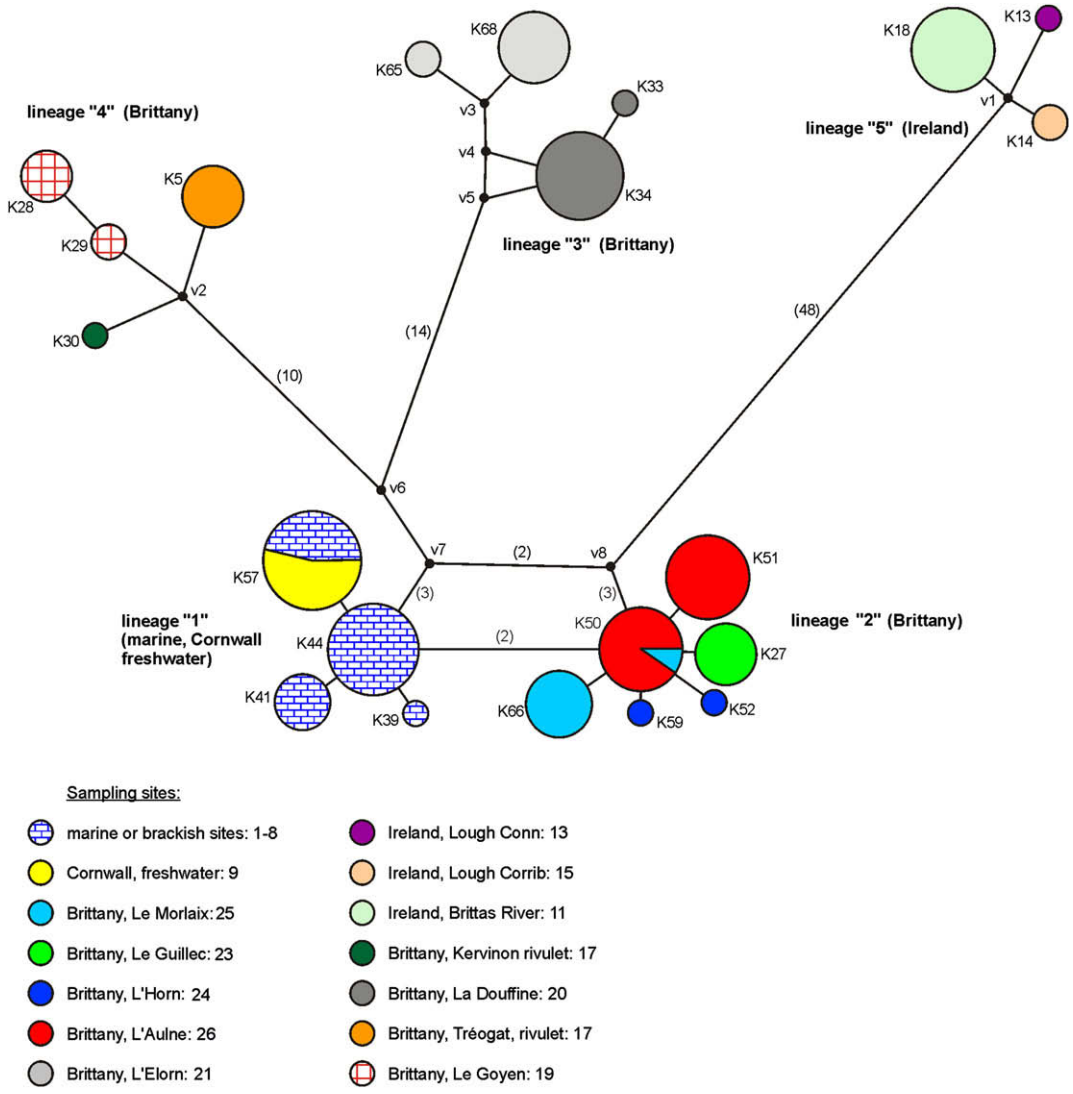

Fig. 3. Median-joining network of *Gammarus duebeni* haplotypes of the combined dataset (COI + ND2, 896 bp) for all lineages. Circles represent haplotypes and the size is proportional to the relative frequencies. v1–v8 are median vectors and represent possible extant unsampled sequences or extinct ancestral sequences. Numbers in parentheses at connecting branches between haplotypes are selected counts of mutational steps. Lineage nomenclature is the same as in Fig. 2. Numbers behind sampling sites are the same as used in Table 1 and Fig. 1. Lineages "1", "2" and "5" are reduced to overcome the effect of homoplasy among derived haplotypes.

one. Average fixation indices were 0.005 for Φ_{CT}, 0.864 for Φ_{SC}, and 0.865 for Φ_{ST}.

Grouping the samples according to the results of the phylogenetic analyses (five allopatric lineages; structure 2) showed that 77.03% ($p = 0.00$) of the total variation occurred among these allopatric lineages. Intra-population differences account for 11.93% ($p = 0.00$) of the total variability, whereas 11.05% ($p = 0.00$) was due to intra-population differences. Average fixation indices were 0.770 for Φ_{CT}, 0.519 for Φ_{SC}, and 0.890 for Φ_{ST}.

Pooling the data into the configuration suggested by SAMOVA (20 groups, structure 3) yielded the highest between group variance of all AMOVA analyses (85.74%, $p = 0.00$). However, this configuration of 20 groups is close to the total number of tested populations (25) and limited gene flow among *G. duebeni* populations may have reduced the performance of the SAMOVA (Dupanloup et al., 2002). The maximum number of possible groups in a SAMOVA of population genetic structure is 20 groups. It is very likely, were SAMOVA able to run with 25 groups, the best structure found would be greater than 20 or even up to 25. Therefore, we favour structure 2 as the most likely one. Since no lineage admixture (with two exceptions of secondary contact) was evident in our samples, the division in the allopatric lineages, and hence in the geographic areas of the lineage, seems to be the most natural one.

The marine populations (sites 1–8) were generally highly differentiated with pairwise Φ_{ST} values ranging from 0.150 to 1, all values being significant (Table 4).

3.5. Demographic inference

For lineage "1" Fu's F_S was significantly negative (−8.636, $p = 0.004$) and Tajima's D nearly significantly negative (−1.235, $p = 0.092$). For lineage "2" Fu's F_S was not significantly negative (−4.371, $p = 0.123$) in contrast to the negative value of Tajima's D (−1.454, $p = 0.049$). Further the plots of pairwise nucleotide differences (mismatch distribution) were unimodal or bell-shaped for lineages "1" and "2". In addition, Harpending's raggedness index was small and associated with a lack of significance for both lineages (Hri = 0.027, $p = 0.700$ for lineage "1" and Hri = 0.015, $p = 0.600$ for lineage "2") supporting the unimodal or bell-shaped interpretation of the mismatch distribution for these lineages. This is in agreement with a demographic population expansion. Further, the observed distribution did not deviate significantly from the null hypothesis

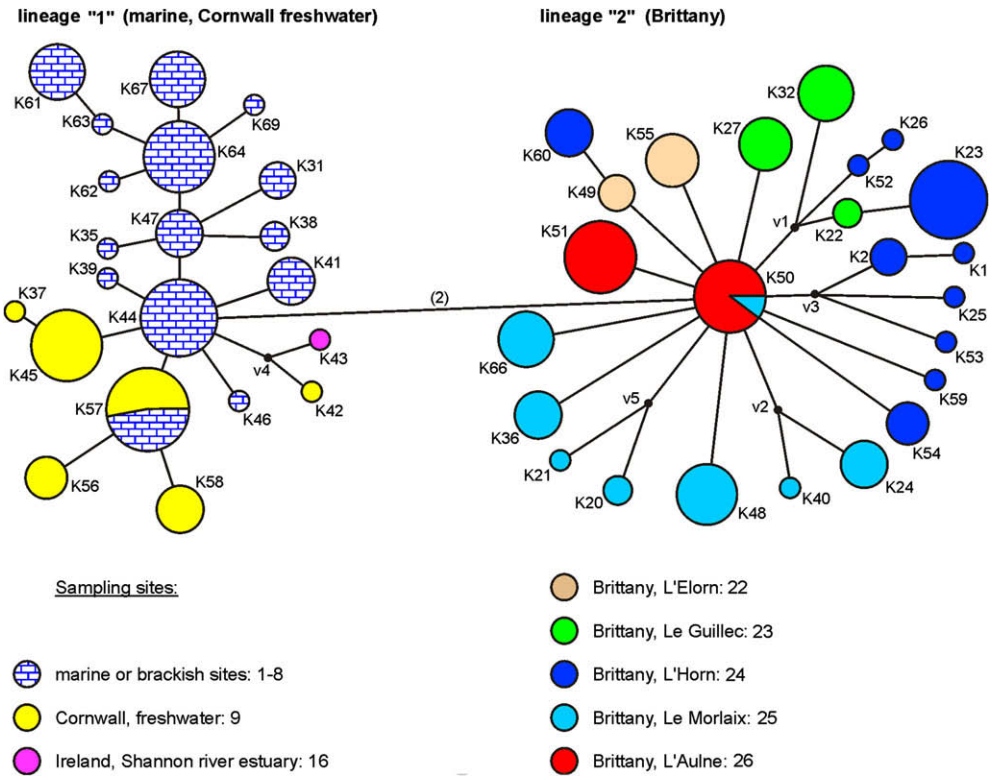

Fig. 4. Median-joining network of *Gammarus duebeni* haplotypes of the combined dataset (COI + ND2, 896 bp) for all taxa belonging to lineages "1" and "2". Circles represent haplotypes and the size is proportional to the relative frequencies. v1–v5 are median vectors and represent possible extant unsampled or extinct ancestral sequences. Numbers in parentheses at connecting branches between haplotypes are selected counts of mutational steps. Lineage nomenclature is the same as in Fig. 2. Numbers behind sampling sites are the same as used in Table 1 and Fig. 1. One reticulation between haplotypes K41 and K49 was resolved arbitrarily. This connection between derived haplotypes from two distinct lineages is thought to be the result of homoplasy.

of population expansion under the expansion model in both lineages (SSD = 0.001, $p = 0.450$ for lineage "1" and SSD = 0.004, $p = 0.450$ for lineage "2"). In summary, we found evidence for a demographic population expansion for lineages "1" and "2".

For lineages "3" and "4" both Fu's F_S and Tajima's D were positive (Table 3) and the mismatch distribution was ragged for both (Fig. 6) suggesting a stable historical effective population size. Harpending's raggedness index was high and significant for lineage "3" (Hri = 0.547, $p = 0.000$) and also high but non-significant in lineage "4" (Hri = 0.173, $p = 0.200$). For both lineages we found significant deviations from the model of demographic expansion (SSD = 0.235, $p = 0.000$ for lineage "3" and SSD = 0.089, $p = 0.000$ for lineage "4") giving further support for the rejection of an expanding population.

For lineage "5" both Fu's F_S and Tajima's D were negative but neither was significant (Table 3). Therefore a population growth is not probable, consistent with a significant Harpending's raggedness index (Hri = 0.045, $p = 0.000$) and a significant deviation of the observed distribution from that expected under the sudden-expansion model (SSD = 0.026, $p = 0.000$) in the mismatch analyses. The observed mismatch distribution, however, was unimodal.

4. Discussion

4.1. The ancestral G. duebeni, phylogenetic relationships and glacial refugia

On the basis of our data, we cannot rule out either of the two alternatives concerning the ancestral *G. duebeni* (freshwater vs. marine). However, taking into account the following two arguments, we favour a marine origin for the ancestral *G. duebeni*. First, if the ancestral *G. duebeni* was a freshwater species and widespread in European freshwaters, then it is hard to explain why we found only one lineage in the marine region that is distinct from the freshwater lineages. In the case of a freshwater ancestry of *G. duebeni*, we would expect a variety of lineages in the sea, reflecting the complete freshwater diversity of the species. Second, the Cornish freshwater population (site 9) reflects exactly the picture of a recent immigration by marine invaders into a freshwater habitat and the subsequent appearance of a new freshwater lineage. This Cornish freshwater population is characterised by five private haplotypes that are phylogenetically very closely related to each other and to haplotypes from marine source populations (Table 1; Figs. 2 and 4). Although this interpretation is far from parsimonious in the simple terms of the number of habitat shifts, it is more likely than a freshwater ancestral *G. duebeni*.

The phylogeographic structure seen within *G. duebeni* can be interpreted as the result of a series of spatial vicariant events during the Pleistocene, as originally suggested by Pinkster et al. (1970), who suggested that the ancestral *G. duebeni* was a euryhaline species in pre-glacial times. In the glacial periods, no inland water was present, but glacier lakes were occasionally dammed up against the ice-walls. These water bodies turned brackish by contact with the sea water in coastal areas, and *Gammarus pulex*, which was shown to eliminate *G. duebeni*, was not present in such brackish lakes. During the inter-glacial periods, the melting ice sweetened the lakes and the populations of *G. duebeni* living in these waters gradually adapted to the decreasing salinity. These adapted *G. duebeni* populations could easily invade fresh inland waters. Therefore the diverse and geographically vicarious freshwater *G. duebeni* lineages can be considered 'glacial relicts', created through repeated allopatry in time and space. Based on the 4.64%

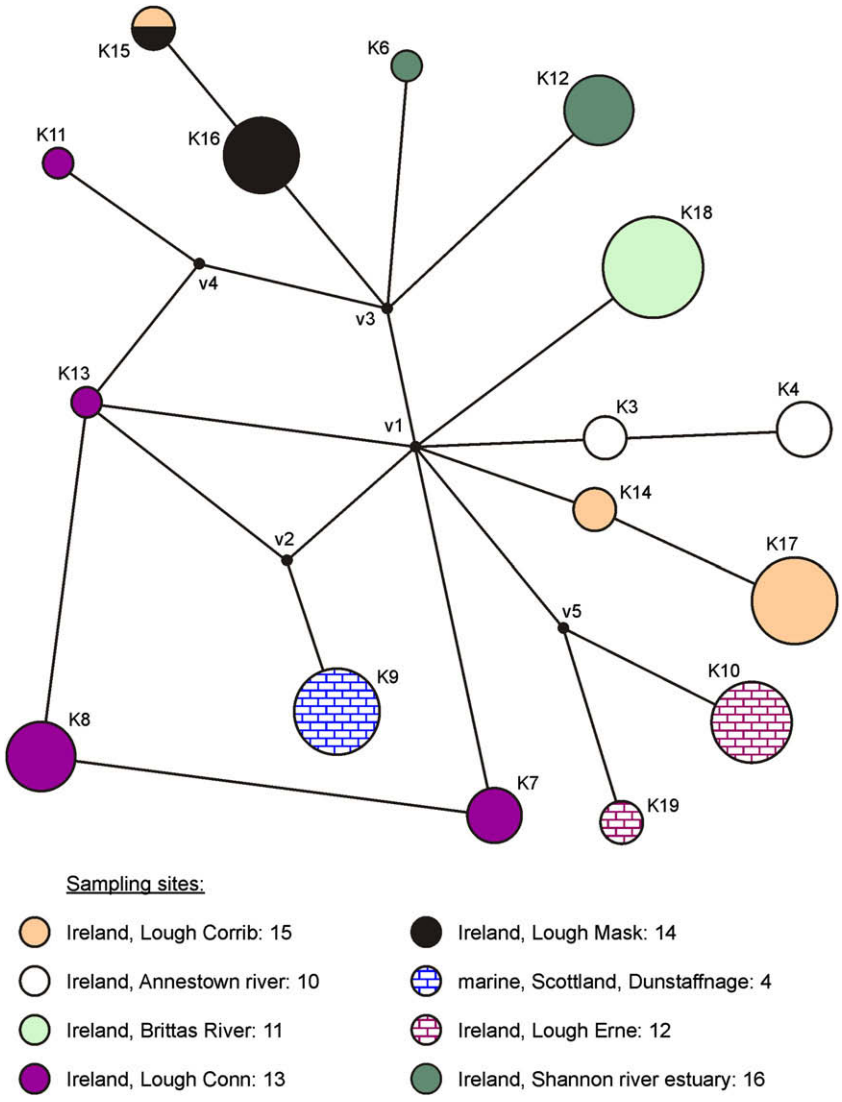

Fig. 5. Median-joining network of *Gammarus duebeni* haplotypes of the combined dataset for all taxa belonging to lineage "5" (COI + ND2, 896 bp). Circles represent haplotypes and size is proportional to the relative frequencies. v1–v5 are median vectors and represent possible extant unsampled sequences or extinct ancestral sequences. Numbers behind sampling sites are the same as used in Table 1 and Fig. 1.

COI sequence divergence between lineages "5" and "1", Rock et al. (2007) suggested a pre-Pleistocene origin for lineage "5". We found maximum uncorrected *p*-distances of up to 5.67% for the COI gene and this value is in agreement with Rock et al. (2007). Dividing the data into two groups, the Irish lineage "5" vs. all other lineages, yielded a mean uncorrected COI distance of 4.1%. Gently applying several mitochondrial DNA molecular clocks (1.4% divergence per million years (Myr), Knowlton and Weigt, 1998; 2% per Myr, Brown et al., 1979; 2.3% per Myr, Schubart et al., 1998) to that 4.1% sequence divergence, results in a predicted date of the oldest lineage split (lineage "5") of approximately 1.8–2.9 Myr. This would imply a pre-Pleistocene, or early Pleistocene origin for lineage "5".

Table 2
Analysis of molecular variance (AMOVA) of *Gammarus duebeni* (combined dataset, COI + ND2, 896 bp). For structure 1 the populations were grouped on the basis of habitat type (marine vs. freshwater, two groups), for structure 2 the samples were grouped according to phylogeny (five allopatric lineages, five groups) and for structure 3 the samples were grouped according to the suggestion of the SAMOVA (20 groups).

	Source of variation	d.f.	% Of total variation	Fixations index (*p*-value)
Structure 1	Among groups	1	0.50	Φ_{CT} = 0.005 (*p* = 0.257)
	Among populations within groups	23	85.98	Φ_{SC} = 0.864 (*p* = 0.000)
	Within populations	280	13.52	Φ_{ST} = 0.865 (*p* = 0.000)
Structure 2	Among groups	4	77.03	Φ_{CT} = 0.770 (*p* = 0.000)
	Among populations within groups	20	11.93	Φ_{SC} = 0.519 (*p* = 0.000)
	Within populations	280	11.05	Φ_{ST} = 0.890 (*p* = 0.000)
Structure 3	Among groups	19	85.74	Φ_{CT} = 0.857 (*p* = 0.000)
	Among populations within groups	5	1.00	Φ_{SC} = 0.070 (*p* = 0.000)
	Within populations	280	13.26	Φ_{ST} = 0.867 (*p* = 0.000)

Table 3
Number of segregating sites (S), haplotype diversity (h), nucleotide diversity (π) and the mean number of pairwise nucleotide differences (K), standard deviations (SD) in parentheses. Neutrality tests (Fu's F_S and Tajima's D) and their p-values in parentheses. Results of mismatch distribution under the under the sudden-expansion model: coalescence time in mutational units (τ) and the effective population size at the start (θ_0) and at the end (θ_1) of the expansion, Harpending's raggedness statistic (Hri) and the sum of squared deviations (SSD) and their p-values in parentheses. All statistics are based on the combined dataset (COI + ND2, 896 bp) for the five allopatric *Gammarus duebeni* lineages.

Lineage (N)	S	h (SD)	π (SD)	K (SD)	Fu's F_S	Tajima's D	τ	θ_0	θ_1	Hri	SSD
lineage "1" (97)	23	0.920 (0.010)	0.004 (0.000)	2.605 (1.407)	−8.636 (0.004)	−1.235 (0.092)	2.801	0.02637	56.563	0.027 (0.700)	0.001 (0.450)
lineage "2" (106)	48	0.940 (0.007)	0.006 (0.001)	4.935 (2.42)	−4.371 (0.123)	−1.454 (0.049)	4.739	0.768	21.006	0.015 (0.600)	0.004 (0.450)
lineage "3" (23)	9	0.625 (0.069)	0.006 (0.001)	2.996 (1.623)	3.514 (0.935)	0.758 (0.781)	6.023	0.0000	4.795	0.547 (0.000)	0.235 (0.000)
lineage "4" (13)	7	0.718 (0.089)	0.004 (0.001)	3.308 (1.817)	2.472 (0.885)	1.769 (0.975)	6.502	0.0018	7.900	0.173 (0.200)	0.089 (0.000)
lineage "5" (67)	31	0.920 (0.013)	0.007 (0.001)	5.410 (2.640)	−0.569 (0.496)	−0.535 (0.330)	6.109	0.0000	99999	0.045 (0.000)	0.026 (0.000)

The most recent freshwater lineage is therefore lineage "2", separated by only two mutations from the founder of the marine lineage "1" (Figs. 2–4). Its origin seems to be associated with the Last Glacial Maximum (LGM). It is likely that lineage "2" is even younger than the LGM. The palaeo-shorelines in Brittany and Cornwall were approaching their present position approximately 8000 years before present (BP) (Lambeck, 1997). Therefore, the present day northern Breton rivers, now colonised by lineage "2", were stable in the present form for no more than 8000 years. Lineage "2" is an offshoot of lineage "1" as indicated by the phylogenetic relationship of haplotypes (Figs. 2–4). Because lineage sorting through drift and mutation is complete as evidenced by the reciprocal monophyly of the two lineages (Fig. 2), and because of the assumed short time since the origin of lineage "2", it can be assumed that only very few individuals from a marine population invaded the northern Breton region (founder effect). This view is also supported by the signs of a demographic population expansion in this lineage probably as a consequence of a range expansion by means of the colonisation of northern Breton rivers after the LGM (Table 3, Fig. 6). The star-like haplotype network of lineage "2" supports this model as this network organisation is typical for expanding populations after a founder event or bottleneck (Beebee and Rowe, 2008).

A pre-Pleistocene origin for lineage "5" seems to conflict with the 'glacial relict' theory. However, for a few other taxa considered to be 'glacial relicts', a pre-Pleistocene separation of inland populations from their marine counterparts has also been demonstrated. For example, Audzijonyte et al. (2005) found that the inland invasion of members from the *Mysis relicta* species group may have occurred in the Miocene or Pliocene, rather than the Pleistocene, as previously supposed. Similar results were found by Dooh et al.

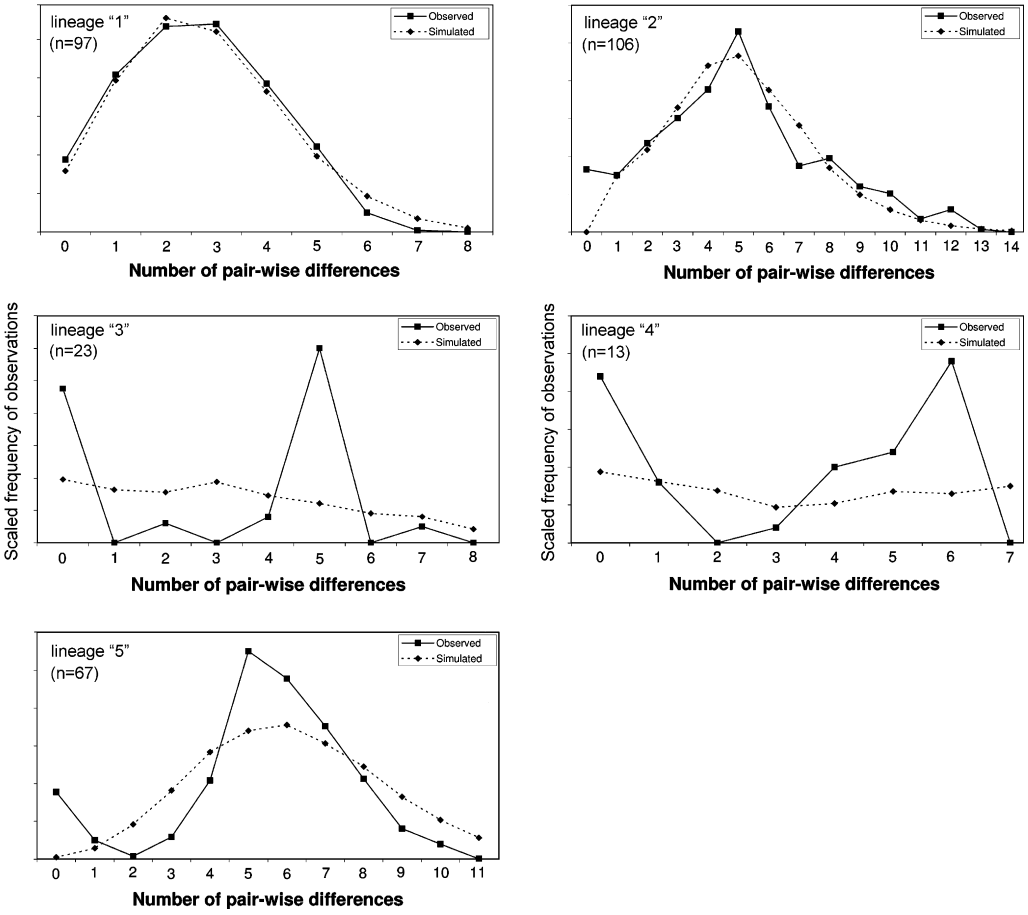

Fig. 6. Distribution of pairwise nucleotide differences (mismatch distribution) in the five allopatric lineages within *Gammarus duebeni*. The expected values were calculated under the assumption of a recent demographic expansion. The statistic was calculated on the basis of the combined dataset (COI + ND2, 896 bp).

(2006). The authors found the north-American inland lacustrine *Mysis diluviana* was separated by an average divergence of 8.2% in COI from its circumarctic relatives. Dooh et al. (2006) suggested that many cladogenetic events could have occurred in the sea prior to the transition to inland waters, whereas Audzijonyte et al. (2005) assumed that Miocene–Pliocene cooling and Pliocene glaciation processes may be responsible for the observed deep branches within *Mysis*. In the case of *G. duebeni*, either of these two explanations could explain the observed patterns.

Several 'glacial relicts' studies have dealt with a variety of taxa: the crustaceans *Limnocalanus macrurus* (Dooh et al., 2006), *Mysis* spp. (Audzijonyte et al., 2005; Audzijonyte and Väinölä, 2005; Dooh et al., 2006), *Gammaracanthus* spp. (Väinölä et al., 2001) and fishes like the sculpin *Myoxocephalus quadricornis* (Kontula and Väinölä, 2003). These 'glacial relicts' are mostly confined to lakes of the arctic, boreal zones, or temperate zones of Europe and America, all of which are also potential habitats for *G. duebeni*. However, when Hynes (1954) listed records of freshwater populations known for *G. duebeni*, no continental lakes in either Europe or America were mentioned by the author. Therefore, it is possible that a second factor besides the glacial history may influence the recent distribution of *G. duebeni* in freshwater. Indeed, past studies were able to show that *G. duebeni* is out-competed by other gammarids, especially by *Gammarus pulex* in freshwaters (Pinkster et al., 1970; Dick et al., 1990; Kelly et al., 2003). In Ireland and western Brittany, where *G. duebeni* is present (see Fig. 2 in Pinkster et al., 1970), *G. pulex* is not or was only recently introduced by man in Ireland (Strange and Glass, 1979). Most likely, the scenario of vicariant *G. duebeni* freshwater populations was common during the Pleistocene in northern Europe and North America, but most of these freshwater populations became extinct through subsequent glaciations or through competition with other, later arriving, freshwater gammarids.

The identification of the spatial structuring of distinct genetic lineages can deliver evidence for the existence of glacial refugia (Hewitt, 1996). In the present case our data imply that Brittany and Ireland must have acted as glacial refugia for lineages "3", "4" and "5", at least for the LGM. A small part of southwest Ireland remained un-glaciated during the LGM (Frenzel et al., 1992; Bowen et al., 2002) and might have served as a refugium for the Irish lineage "5". A terrestrial (and hence freshwater) glacial refugium in south-western Ireland was also suggested by recent phylogeographical studies of the Scots pine (Sinclair et al., 1998) and natterjack toads (Rowe et al., 2006). Since lineages "3" and "4" are again allopatrically distributed, two distinct freshwater refugia in western Brittany likely explain the differential distribution. The demographic patterns of these lineages are congruent among one another and consistent with those of populations in long-term demographic stability, as predicted for a Pleistocene refugium without a subsequent range extension (Table 3, Fig. 6). Therefore a recent freshwater invasion of marine *G. duebeni* seems unlikely. Brittany was not glaciated at the LGM but permafrost extended south to 47°N (Hewitt, 2004) or even further southwards in France (van Vliet-Lanoë et al., 2004). There is a growing body of literature on cryptic northern terrestrial and hence freshwater refugia. Besides Ireland, evidence for cryptic northern or central European terrestrial refugia exists for the Norwegian coast between 12000 and 10000 years BP (Larsen et al., 1987), for the Belgian Ardennes between 13000 and 11000 years BP (Cordy, 1991), for the Devon, UK between 60000 and 25000 years BP (Lister, 1984; Bocherens et al., 1995), for north-east Hungary 27000 years BP (Willis et al., 2000), for western Slovakia 18100 years BP (Litynska-Zajac, 1995), and for the Dordogne in south-western France and the Carpathian region at the LGM (Sommer and Nadachowski, 2006). Our results may add the most western parts of Brittany to that list (Fig. 1).

The marine and brackish regions of Europe and America must have been re-colonised from a single, marine and to date unknown refugium (Rock et al., 2007). It is possible that lineage "1" outlived the LGM in the English Channel palaeoriver system, which has been shown to be a LGM refugium for other marine taxa like the seaweeds *Palmaria palmata* (Provan et al., 2005) and *Fucus serratus* (Hoarau et al., 2007).

Transitions from marine to freshwater habitats constitute dramatic shifts between adaptive zones that initiated the radiation and speciation of many taxa (Lee and Bell, 1999). Therefore, the data described here seems to make *G. duebeni* an ideal model organism for the study of allopatric speciation. The allochronic origin of the freshwater lineages offers an excellent system for observing evolutionary adaptations to the freshwater environment at different stages. Such a pattern of multiple invasions of freshwater allows us to ask whether independent invasions are governed by similar evolutionary processes, and whether the same course of trait gain and loss occurs during independent colonisations (Lee and Bell, 1999).

4.2. Post glacial population differentiation and reduced gene flow in the sea

The marine or brackish populations (sites 1–8, Fig. 1) are characterised by many private haplotypes (Table 1) also reflected in the high and significant pairwise Φ_{ST}-values among the marine populations (Table 4). This pattern of population structure is somewhat surprising, considering the European climate history of the last 2 million years (see Hewitt, 2000, 2004; Lambeck et al., 2002). All sampled marine populations were within the area influenced by ice ages, during the LGM, approximately 25000 to 18000 years BP. During this time, the European ice sheet extended south to 52°N (Hewitt, 2004), which means that most sampled marine populations must have been established after the LGM. It is very unlikely that each marine population derived from a separate refugium. Therefore it is suggested that the private haplotypes found in sea-populations evolved after the LGM. The lack of deep branches within the lineage "1" suggests a single refugium origin. For the North American amphipod *Gammarus tigrinus,* similar patterns of population structuring were observed among 19 estuarine populations along the coast of the Atlantic Ocean (Kelly et al., 2006). Limited dispersal in *G. tigrinus* was attributed to an oligo-mesohaline salinity distribution and a direct development without vagile or planktonic larvae. For *G. duebeni,* salinity does not seem to be responsible for the observed genetic differences of marine populations, as this species can tolerate oligo- to polyhaline conditions (Segerstråle, 1946; Kinne, 1959). There are several other possible explanations for the highly differentiated marine populations. Like *G. tigrinus*, *G. duebeni* has a direct development without vagile or planktonic larvae (Ward, 1985). This should reduce gene flow

Table 4
Pairwise Φ_{ST} values among the eight marine or brackish populations of *Gammarus duebeni*, based on Tamura–Nei genetic distance. Statistical significance was assessed by 1000 permutations. Φ_{ST} values were calculated on the basis of the combined dataset (COI + ND2, 896 bp). Numbers 1–8 are site numbers and the same as used in Table 1 and Fig. 1. All values were significant ($p < 0.05$).

Site	1	2	3	4	5	6	7	8
1 ($N = 7$)	–							
2 ($N = 9$)	1.000	–						
3 ($N = 7$)	0.653	0.564	–					
4 ($N = 15$)	0.414	0.427	0.378	–				
5 ($N = 8$)	0.953	0.938	0.709	0.423	–			
6 ($N = 10$)	0.954	0.905	0.298	0.458	0.919	–		
7 ($N = 1$)	0.700	0.513	0.150	0.449	0.755	0.288	–	
8 ($N = 7$)	1.000	1.000	0.544	0.544	0.973	0.805	0.641	–

and promote population differentiation in comparison with species showing other reproductive strategies (Bilton et al., 2002). Interspecific competition is another factor that greatly influences the local distribution of *G. duebeni*. In the Baltic Sea, Kolding and Fenchel (1981) showed that in the presence of other *Gammarus* species, *G. duebeni* is almost exclusively confined to high shore pools. Generally, *G. duebeni* does rather well on its own in "extreme" environments but is out-competed in locations where it could co-exist with other *Gammarus* species (Gaston and Spicer, 2001). This may also causes reduced gene flow among marine populations leading to high Φ_{ST}-values.

4.3. Implications for taxonomy

Based on the presented phylogenetic analyses (Figs. 2 and 3) and AMOVA (Table 2, structure 1) the assumption of two subspecies according to a habitat division into freshwater (*G. duebeni celticus*) and marine (*G. duebeni duebeni*) is quite simple and artificial. The situation is more complex and there are at least five distinct units within *G. duebeni* (Figs. 2 and 3; Table 2, structure 2). If at all, the name *G. duebeni celticus* should only be used for the Irish freshwater populations (lineage "5") and *G. duebeni duebeni* for the Breton and marine *G. duebeni* (lineages "1" to "4").

It is an open question whether the given mtDNA differences among the five lineages are even signatures of one or more speciation events. Comparing our genetic distances observed for the COI gene (5.67%, uncorrected *p*-distance) with those that are published for other amphipod taxa suggest that *G. duebeni* is a single species: Meyran et al. (1997) found 36.4% genetic distance between *Gammarus pulex* and *Gammarus marinus*; Witt et al. (2006) found 35.2% sequence divergence (K2P distance) between *Hyalella* species; Hou et al. (2007) described maximum uncorrected genetic distances of 33.6% for *Gammarus* spp. from North America, Europe and Asia; Cristescu and Hebert (2005) found 28% COI sequence divergence (Tamura–Nei distance) among Ponto-Caspian amphipod species and Müller et al. (2002) found 16,7% between *Dikerogammarus villosus* and *Dikerogammarus haemobaphes*. On the other hand, some intraspecific studies dealing with amphipod taxa found COI sequence divergences that are well below the 5.67% we found within *G. duebeni*: Müller et al. (2002) found 0.6% COI sequence divergence within *D. haemobaphes* and 0.4% within *D. villosus*; Meyran and Taberlet (1998) detected that *Gammarus lacustris* haplotypes from various alpine lakes varied by a maximum of 1.62% and finally Meyran et al. (1997) observed a 2.25% K2P distance within *Gammarus fossarum*. Therefore, additional studies involving genetic (nuclear markers), physiological, biochemical and ecological aspects are necessary to gain deeper insights into the evolutionary history and the taxonomic status of the lineages within the *G. duebeni* complex.

4.4. Conservation issue

The *G. duebeni* complex is an excellent example of cryptic biodiversity and evolutionary transitions to different habitat types at different time scales. Furthermore, it is a model object for speciation and adaptive radiation. The term "evolutionarily significant unit" is a population unit that merits separate management and has a high priority for conservation (Ryder, 1986). However, there are some definitions what "evolutionary significant units" are (Ryder, 1986; Waples, 1991; Moritz, 1994). We found five allopatric lineages. Four of them are distributed in freshwaters and one in marine habitats indicating various and repeated independent adaptations according to habitat salinity. The lineages are reciprocally monophyletic according to mtDNA. But to demonstrate that they are real cryptic and reproductively isolated species and not just mitochondrial phylogroups, nuclear evidence is essential and must be examined.

The diversity of the *G. duebeni* complex is threatened by natural and human-mediated intraguild competition between endemic *G. duebeni* and other freshwater gammarids. In Brittany and Normandy, it was shown that *Gammarus pulex*, which is invading from the east, gradually replaces *G. duebeni* (Pinkster et al., 1970; Piscart et al., 2007). In Ireland, where man introduced *G. pulex*, similar processes take place (McLoughlin et al., 2000; MacNeil et al., 2004). The conservation of *G. duebeni* as a complex of different units is not only desirable but absolutely essential.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.07.034.

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