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Incomplete cryptic speciation between intertidal and subtidal morphs of *Acrocnida brachiata* (Echinodermata: Ophiuroidea) in the Northeast Atlantic

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Abstract

The brittle-star *Acrocnida brachiata* (Montagu) lives in sandy-bottom habitat of both intertidal and subtidal zones along the coasts of the northwestern Europe. An allozyme frequency-based survey (five enzyme loci) was combined with a mitochondrial (mt) COI haplotype analysis (598-bp sequences) on 17 populations to trace back past colonization pathways from the actual population structure of the species. Both genetic markers display a sharp genetic break between intertidal (clade I) and subtidal populations (clade S). This break corresponds to an allele frequency inversion at three enzyme loci (*Hk*, *Pgm* and *Pgi*) and a deep divergence of about 20% in mtCOI sequences between most of the intertidal populations and other samples. The geographic distribution of clade I seems to be more restricted than clade S as it is absent from the intertidal of the eastern English Channel and North Sea and may be replaced by clade S in south Brittany. Applying previously published rates of mutation, divergence between the two clades is estimated to pre-date 5 million years ago and may be due to allopatric speciation processes at the Mio–Pliocene transition. The occurrence of putative hybrids in a few localities, however, suggests incomplete cryptic speciation with secondary contact zones. The relative importance of colonization history vs. habitat specialization are discussed in the light of neutral evolution as tested from mtCOI gene sequences. While differential selection seems to have contributed little to the separation of the lineages, it may have played a role in the emergence of adaptive polymorphisms in the hybrid zone. Furthermore, congruent spatial patterns of differentiation were observed in both clades suggesting a recent increase in population size. These findings are in agreement with a recent expansion of the populations during or after the formation of the English Channel, from a southern refuge for the subtidal clade whereas the intertidal clade may have persisted further north. As previously suspected for a species with a very short pelagic larval phase, contemporary gene flow between distant or adjacent populations appears to be extremely reduced or even absent.

Keywords: allopatric speciation, allozyme, ecotypic specialization, hybridization, mtCOI

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Introduction

Understanding by which processes and mechanisms speciation operates is of paramount importance and remains a major challenge facing evolutionists. Although there is still an open debate about the definition of a species (Wilson 1999) and on the relative contributions of allopatry (Mayr 1942; Turelli *et al*. 2001) and sympatry (Johannesson 2001; Via 2001) on speciation processes, only few studies have attempted to infer probability and time to speciate (Gavrilets 2003). It appears that the time to speciate, which is driven by mutation and drift, is typically very long while the speciation transition is relatively short. When changes affect genes underlying speciation, species' isolation occurs very rapidly. If selection contributes to speciation, time to

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speciate decreases significantly (Gavrilets 2003). An increasing number of authors appear to agree on the importance of fluctuating environmental conditions in triggering sympatric isolation (and maybe speciation) over very small spatial scales (Wiens 2004). Data sets on the Carribean lizard, *Anolis roquet* (Ogden & Thorpe 2002), or the rough periwinkle, *Littorina saxatilis* (Cruz *et al*. 2004), support local adaptation as the major evolutionary force acting on population divergence and fit with previously proposed ecological models of speciation (Orr & Smith 1998; Schluter 2001). In such cases, adaptation and ecological specialization may prevent gene flow (via differential mortality) between lineages that inhabit adjacent but contrasted microenvironments, thereby driving ecological speciation through the fixation of advantageous alleles (Bierne *et al*. 2003). Environmental selection is, however, not the only force facilitating speciation. Selective processes associated with local hybridization due to secondary contact zones are also known to prevent neutral gene flow between previously isolated demes. Reinforcement has thus often been proposed to be another major evolutionary mechanism in the maintenance of discrete breeding units during the time of speciation, leading to stable clines of allele frequencies at loci directly involved in reproductive isolation (Barton & Hewitt 1989). The way ecological factors may impact speciation mechanisms is thus a multifaceted question implying genes involved in either pre- or postzygotic processes of reproductive isolation (Palumbi 1992; Johannesson 2001).

The existence of sibling species provides means of understanding how speciation may work. Recent advances in development of the genetic markers help identify cryptic species and may lead to understanding the relative contributions of evolutionary forces promoting towards. Many sibling species have been discovered within all major marine taxa (Knowlton 2000). Genetic studies have investigated possible causes of speciation in the case of marine crypticism (Knowlton 1993). Recently, a study on the polychaete *Pectinaria koreni* revealed the existence of monophyletic lineages along the north coast of France (Jolly *et al*. 2005) involving allopatric processes. Likewise, Kruse & Reise (2003) revealed that the polychaete *Scoloplos armiger* in the Wadden Sea was actually composed of two sibling species living in distinct intertidal and subtidal areas. The genetic break between populations of *P. koreni* matches with the biogeographic boundary between Lusitanian and Boreal provinces whereas the distribution of sibling lineages in *S. armiger* coincides with a bathymetric preference. The occurrence of two opposing reproductive strategies (brooding vs. pelagic larvae) in this latter species has reinforced the lineages divergence, supporting the idea of an environmental role in speciation processes (Kruse *et al*. 2004).

In marine habitats, recently colonized areas can also be very informative in terms of mechanisms whereby a species expands and competes for space and thus to test whether crypticism is reflecting overlapping ranges or habitat specialization. Indeed colonization mainly depends on factors such as the dispersal ability of species (Goldson *et al*. 2001), local hydrodynamic regimes (Wares *et al*. 2001; Waters & Roy 2004) and ecological preferences (Wiens 2004). The English Channel is a relatively young geographic area, which was only completely open as a channel some 10 000–8000 years ago (Smith 1989; Lambeck 1997). Since the last glacial maximum (18 000–24 000 years), many ecological niches became available for native species from both the Atlantic Ocean and the North Sea. Moreover, the western entrance of the English Channel is a well-known biogeographic limit between the Lusitanian and the Boreal provinces (Cabioch *et al*. 1977; see Dinter 2001 and cited references) and thus an interesting area to explore when considering vicariance or ecotypic differentiation. The goal of this study is to examine how each of these processes may have influenced speciation in invertebrates associated with highly fragmented habitats such as the sandy-bottom habitat.

Sibling species have been massively reported within the echinoderm group (Matsuoka & Hatanaka 1991; Baric & Sturmbauer 1999) and even observed for a sea urchin species inhabiting sandy sediments along the coasts of northwestern Europe (Chenuil & Féral 2003). Sibling species have also been observed in polychaete species of the *Abra alba–Pectinaria koreni* community (Jolly *et al*. 2006). These latter findings allowed us to focus on the most commonly found echinoderm in these sandy-bottom habitat community, the brittle-star *Acrocnida brachiata* (Montagu) (Gentil *et al*. 1986), which can be suspected to also hide cryptic species. It is widely distributed at the subtidal level in the Northeast Atlantic, from the Mediterranean to the British Isles and to the North Sea, but is never encountered above 56°N (Koehler 1921). This brittle star is often observed as densely populated aggregates in sandy-bottom habitat in subtidal (i.e. 1000 individuals per m^2 in Ireland, Keegan & Konnecker 1979; 900 individuals per m^2 on the coasts of Normandy, Gentil & Zakardjian 1989). Its presence at the intertidal level seems much more restricted, to the Irish and Brittany areas where populations are sparsely distributed (less than 100 individuals per m^2 on the coasts of Brittany, Bourgoin 1987; D. Muths, personal observation). Unlike the polychaete species *Pectinaria koreni* and *Owenia fusiformis* studied by Jolly *et al*. (2005, 2006), with which it co-occurs, *A. brachiata* is suspected to have poor dispersal capacities. *A. brachiata* therefore constitutes an ideal candidate to investigate whether taxa exhibiting different life-history traits but the same habitat share a vicariant history and possibly, to evaluate whether local adaptation (intertidal vs. subtidal habitats) or local hybridization could interfere with these historical patterns.

This study was conducted on 17 populations of *A. brachiata* sampled among subtidal and intertidal areas along the

Table 1 Geographic position and habitat of *Acrocnida brachiata* samples and sample sizes used in the allozymes and mitochondrial surveys

Population	Label	Habitat	Latitude	Longitude	*N* allozymes	*N* COI
Pouldu	PO	Subtidal	47°44.06′N	4°31.91′W	26	18
Concarneau	CC	Subtidal	47°51.50′N	3°57.50′W	—	7
Bay Forest	BF†	Intertidal	47°53.42′N	3°57.48′W	48	25
Sub Forest	SF	Subtidal	47°52.30′N	3°58.12′W	36	12
Morgat	Mo†	Intertidal	48°13.54′N	4°30.30′W	35	26
Aber	PA†	Intertidal	48°13.661′N	4°25.891′W	48	20
Lannion-I	LI†	Intertidal	48°40.75′N	3°36.41′W	47	27
Lannion-S	LS	Subtidal	48°44.95′N	3°36.50′W	11	9
Saint Brieuc	SB†	Intertidal	48°33.08′N	02°41.24′W	48	15
Baie des Veys	BV	Subtidal	49°32.00′N	1°15.50′W	48	26
Utah Beach	UB†	Intertidal	49°25.04′N	1°10.32′W	48	23
Baie de Seine	BS	Subtidal	49°28.30′N	0°02.28′E	35	31
Cardigan Bay	CB	Subtidal	52°51.23′N	4°11.42′W	35	21
Redwharf Bay	RB	Subtidal	53°20.10′N	4°07.13′W	48	39
Colwyn Bay	Co	Subtidal	53°25.75′N	3°30.06′W	20	19
Rye Bay	RY	Subtidal	50°53.44′N	0°53.45′E	—	19
St Austell Bay	SA	Subtidal	50°18.97′N	4°44.94′W	—	21

†indicates that populations were collected in the intertidal zone.

coasts of Britanny, the English Channel and the Irish sea using five polymorphic enzyme loci and a 598-bp portion of the mitochondrial cytochrome oxydase I (mtCOI) gene.

Materials and methods

Larval development of Acrocnida brachiata

To have a rough idea of real dispersal capacities, observations of larval development in *Acrocnida brachiata* have been performed. They were made under a light microscope by F. Gentil in July 1993. Larvae were obtained in aquaria after a spontaneous spawning of adults collected at low tide on the shore of Morgat Bay (see Table 1 for site information). Larvae were regularly sampled from water collection during 4 days to follow larval development until subsequent metamorphosis.

Sampling sites

Subtidal samples of *A. brachiata* were obtained by sampling the top 10 cm of sediment with a 0.25 m² Hamon grab. Populations from the English Channel, the Irish Sea and South Brittany were sampled during the PECTGENE 2000, the PECTIRL 2004, and the OPHIROIS 2005 cruises, respectively. All six intertidal populations were collected along Brittany and Normandy coasts at low tide in 2004 or 2005. Geographic coordinates, sample labels and sizes are given in Table 1 and reported in Fig. 1. Individuals were frozen in liquid nitrogen, except samples from southern England and Concarneau, which were stored at –32 °C.

Enzyme electrophoresis

Each individual was divided into two parts: the disk-shaped body for enzyme electrophoresis and arms for DNA extraction. Electrophoresis procedures were performed according to the methods described by Pasteur *et al*. (1987). Frozen samples of the body were homogenized using a potter with 250 µL of grinding buffer (10 mM Tris-HCl, 2 mM EDTA, 0.05% β-mercaptoethanol, 0.1 mM PMSF, 0.25 mM sucrose; pH 6.8), prior to centrifugation at 6500 *g* for 12 min. The supernatant was stored at –80 °C until electrophoresis. Horizontal enzyme electrophoresis was conducted on 12% starch gels for the five enzyme systems that were found polymorphic using the following buffers: (1) Tris-Borate-EDTA, pH 8.6 for the glucose phosphate isomerase (*PGI*, E.C. 5.3.1.9), mannose phosphate isomerase (*MPI*, E.C. 5.3.1.8) and malic enzyme (*ME*, E.C. 1.1.1.40), and (2) Tris-HCl, pH 8.5 for the phosphoglucomutase (*PGM*, E.C. 5.4.2.2) and hexokinase (*HK*, E.C. 2.7.1.1). Buffer systems were run at constant amperage, during 5 h [160 V, 35 mA for system (1) and at 150 V, 40 mA, for system (2)]. The most frequent allele was called '100' and other alleles were labelled according to their relative mobility from allele 100.

Mitochondrial COI sequencing

Total genomic DNA was extracted according to the cetyltrimethyl ammonium bromide (CTAB) extraction protocol as described in Jolly *et al*. (2003). Specific polymerase chain reaction (PCR) primers (Ab-COIf: 5′-ATTTGGAAACTG-ACTYGTCC-3′ and Ab-COIr: 5′-GTYGCTGCTGTRAAR-TAGG-3′) were designed from conserved regions of

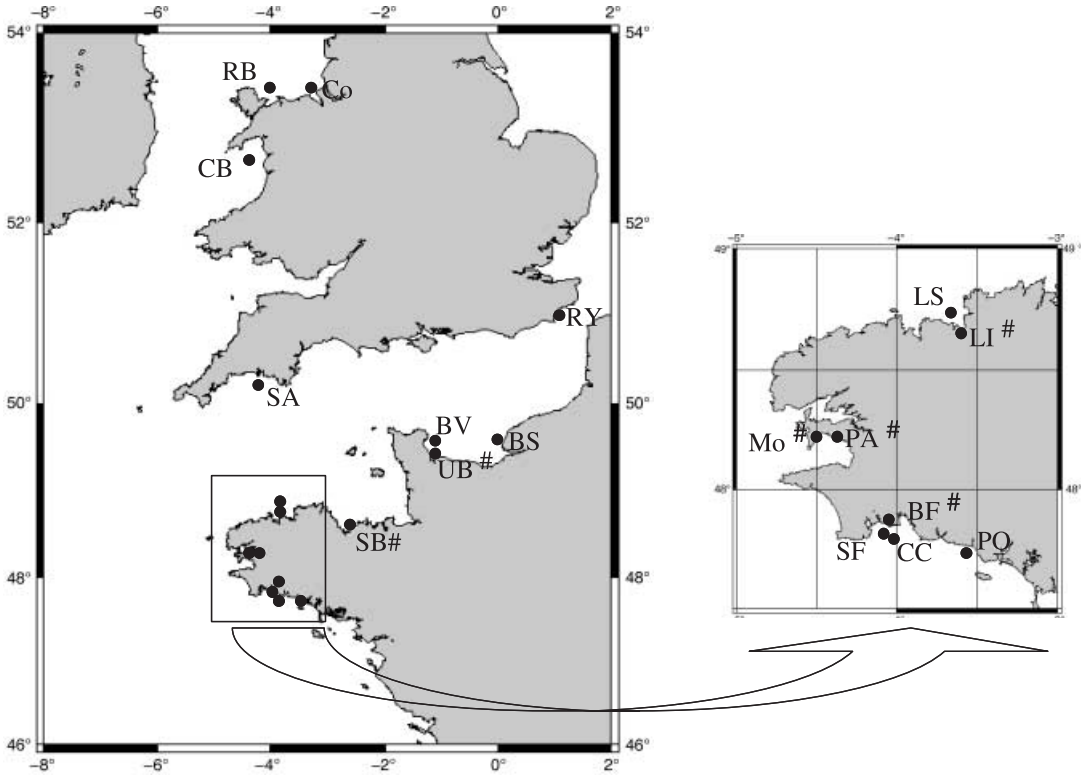

Fig. 1 Geographic location of *Acrocnida brachiata* populations used in the study. Population labels are presented in Table 1. # indicates that populations were collected in the intertidal.

sequences of the whole cytochrome oxydase I (COI) gene previously obtained from 10 specimens of ophiuroids using primers based on COI regions conserved in invertebrates (McMullin *et al*. 2003). Ab-COIf and Ab-COIr amplify a 598-bp fragment of the COI gene. Reactions were performed in 27 µL containing 1× PCR buffer, 2 mM $MgCl_2$, 25 µM of each dNTP, 0.2 µM of each primer, 0.5 U of Thermoprime Plus *Taq* polymerase (Abgene), 25 ng CTAB-extracted genomic DNA. Cycling parameters were 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 54 °C for 60 s, and 72 °C for 70 s and a final elongation at 72 °C for 7 min. PCR products were purified before sequencing using BigDye terminator chemistry (PerkinElmer) on an ABI 3100 sequencer, following the manufacturer's protocol. Sequences were run in both directions. They were checked and edited using CHROMAS version 1.6 (McCarthy 1997) and aligned using CLUSTAL W (Thompson *et al*. 1994) in BIOEDIT Sequence Alignment Editor (Hall 1999). Sequences were submitted to GenBank (Accession nos DQ507450–507799).

Data analyses

Allozymes. For each population, allele frequencies, the average number of alleles per population (N_{all}), the observed (H_O) and expected (H_{nb}) heterozygosities were estimated using GENEPOP 3.3 software (Raymond & Rousset 1995) whereas allelic richness (R_s) was estimated with FSTAT 2.9.3.2 (Goudet 1995). The null hypothesis of independence of loci was tested within each sample based on a statistical genotypic disequilibrium analysis across loci using GENEPOP 3.3 (Raymond & Rousset 1995). An UPGMA-based tree was constructed with POPULATION 1.2.28 software (Langella 2002) using Reynolds's distance (Reynolds *et al*. 1983). Homogeneity of allele distributions across samples was examined using exact tests using GENEPOP 3.3 (Raymond & Rousset 1995). Deviations from Hardy–Weinberg equilibrium were tested in each population, at each locus, by calculating Wright's fixation index F_{IS} as estimated by Weir & Cockerham (1984) and a Fisher's exact test was also calculated with this software. The pairwise level of genetic differentiation was analysed by calculating the estimator θ of Weir & Cockerham (1984) for each locus. An F_{ST}-based hierarchical analysis was used to determine the hierarchical relative part of the genetic variance using ARLEQUIN 2.0 (Schneider *et al*. 2001). Partition was made between 'among groups' (Φ_{SC} fixation index), 'among populations within groups' (Φ_{CT}) and 'within populations' (Φ_{ST}). Groups were defined according to geography: South Brittany (Po, BF, SF, PA), English Channel (LS, BV, BS) and Irish Sea (CB, RB, Co). Isolation by

distance was tested using a Mantel test with GENEPOP 3.3 (Raymond & Rousset 1995). Geographic distances between localities were estimated by calculating linear distances along the coastline using Great Circle Calculator (available at www.gb3piorg.uk/great.html). Geographic distances were plotted against $\theta/(1-\theta)$ estimates to test for a linear relationship following the recommendations of Rousset (1997).

Mitochondrial COI sequences. For each population, haplotype ($H_{e\text{-}hap}$) and nucleotide (π) diversities were estimated with DNASP 4.0 (Rozas *et al*. 2003). A neighbour-joining tree, based on Kimura 2-parameter distance was constructed using MEGA 2.1 (Kumar *et al*. 2001), and *Amphiura filiformis*, its nearest related species, as the outgroup. Relative constancy rate of a molecular clock was tested and average pairwise distances between populations were estimated using the same software. Pairwise values of genetic differentiation (Φ_{ST}) were calculated with ARLEQUIN 2.0 (Schneider *et al*. 2001). This latter software was also used to perform an analysis of molecular variance (AMOVA) on populations. Partition of the data set was similar to that performed for allozymes with the St Austell Bay population added to the Irish Sea group and the Rye Bay one added to the English Channel group. Median-joining networks (Bandelt *et al*. 1999) were constructed using NETWORK 4.1.0.7 (available at www.fluxus-technology.com/).

Asymmetric gene flow and migration patterns among populations were estimated using the coalescent approach as implemented in the software package MIGRATE-N (Beerli & Felsenstein 2001). Past gene flow was obtained from a *n*-island migration model using 10 short chains with 500 steps and 3 long chains with 5000 steps, with 10 000 non-retained genealogies sampled at the beginning of each chain. A first run was made with our theta estimates (i.e. π values). A 10-replicate run was then conducted using results of the first run with random tree as data type option. Runs were compared until results became convergent.

Tajima's (1989) *D* statistic was calculated as an index of departure from population equilibrium using DNASP 4.0 (Rozas *et al*. 2003). Mismatch curves were created with the same software package. A sliding-window analysis was conducted along sequences to further examine the distribution of nucleotide diversity (π) and Tajima's *D* statistic over the sequences in order to localize local departures from neutrality. Departure from neutral evolution was examined using a McDonald & Kreitman (1991) test, using DNASP 4.0 (Rozas *et al*. 2003). This method compares the distribution of synonymous and nonsynonymous differences that have been fixed between two taxa (K_N/K_S ratio) and the distribution of synonymous and nonsynonymous polymorphic sites accumulated within each species (θ_N/θ_S ratio). If K_N/K_S is significantly greater than θ_N/θ_S then at least one lineage may have evolved under positive Darwinian selection whereas, in the opposite case, it may indicate either the selective maintenance of ancestral polymorphism between species or the emergence of an adaptive polymorphism in one species.

Genetic markers were also used to perform assignment tests in order to detect potential hybridization in *A. brachiata*. Assignment tests were done with the software GENECLASS version 2 (Piry *et al*. 2004) using Rannala & Mountain's criterion (1997) and Paetkau *et al*.'s simulation algorithm (2004) with 10 000 simulated individuals. First, partition of the data set was made on the basis of mtCOI signature (two groups defined; see Results) and then assignment tests were performed on multilocus allozyme data. At the end of the first simulation, misassigned individuals (i.e. individuals displaying a mitochondrial signature characteristic from one group and a nuclear genotype typical of the other group) were removed from the data set and additional simulations were carried out until all individuals were successfully assigned.

Results

Larval development of Acrocnida brachiata

Observations of larval development of *Acrocnida brachiata* are presented in Fig. 2. The two-arm pluteus stage was reached after only 6 h. The length of the arms rapidly increased then and was followed by the growth of the body until metamorphose. Because of abbreviated development, changes in the pluteus shape are very limited, e.g. post-erodorsal arms are absent, in comparison with other echinoderms. Complete development occurred in 4 days

Table 2 Allozyme diversities in populations of *Acrocnida brachiata*. N_{all}, number of alleles per locus; H_O, observed heterozygosity; H_{nb}, unbiased heterozygosity (Nei 1987); R_s, allelic richness estimated from a minimal sample size of 10 individuals

	PO	SF	LS	BV	BS	CB	RB	Co	BF†	Mo†	PA†	LI†	SB†	UB†
N	26	38	11	48	35	35	48	20	47	35	48	47	48	48
N_{all}	3.2	3.6	2.6	3.8	3	4	4	3.2	3.8	3.8	3.2	3.6	3.6	3.4
H_{nb}	0.460	0.457	0.425	0.393	0.437	0.502	0.536	0.494	0.470	0.366	0.328	0.471	0.442	0.407
H_O	0.423	0.4847	0.382	0.383	0.406	0.406	0.421	0.320	0.327	0.383	0.300	0.447	0.367	0.400
R_s	2.7	3.0	2.6	2.9	2.6	3.3	3.1	3.1	3.1	2.9	2.6	2.9	3.0	2.8

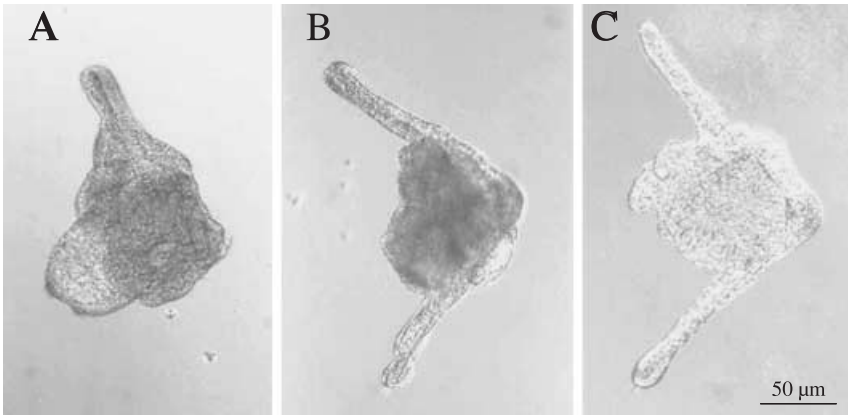

Fig. 2 Photographs depicting the larval development of intertidal specimens of *A. brachiata* made under light microscope. Less than three days separate the 1st from the 3rd picture: (A) 6-h-old larva; (B) 36-h endotrophic pluteus with two arm; and (C) 60-h-old pluteus, metamorphosis started and the juvenile skeletal architecture is appearing.

in aquaria under *in situ* conditions (i.e. aquaria were directly supplemented with nonfiltered open sea water) and is thus thought to be very reduced in natural conditions. This coincides with the type II of ophiuroid developmental mode with a short-lived lecitotrophic larva (Mortensen 1921).

Allozymes

Five polymorphic enzyme loci were screened during this study. The number of alleles varied from 3 to 7 (see Table 2), with an average of 5.4 (± 1.8). Allelic richness was quite similar between populations. Allele frequencies (*f*) highlight the occurrence of a marked genetic structure for at least three out of five loci (*Hk*, *Pgm*, *Pgi*) and for two to four alleles per locus (Fig. 2). While five of the six intertidal populations (all except Bay Forest) were characterized by high frequencies of alleles *Hk-90* and *Hk-95* ($f > 0.95$), the most frequent alleles in the subtidal populations were *Hk-100* and *Hk-105* ($f > 0.95$). Although alleles *Hk-95*, *Hk-100* and *Hk-105* appeared in nearly all populations, *Hk-80* and *Hk-90* were exclusive of intertidal populations whereas *Hk-110* was only found in subtidal ones. The *Pgm* and *Pgi* loci show similar, but less pronounced, patterns of frequency inversions (see Fig. 3). Moreover, allele frequencies of Lannion-intertidal vs. Lannion-subtidal samples or Utah Beach vs. Baie des Veys samples clearly showed an allozyme profile that was less similar between two close populations inhabiting different bathymetric levels than between two populations of similar depth but distant by thousands of kilometres. This important allele-frequency break between intertidal and subtidal populations suggests the existence of two distinct genetic groups that does not correspond to the geographic position of samples but to their bathymetry over the shore. However, unlike the other intertidal populations, population from Bay Forest showed an allozyme frequency profile similar to that of the subtidal populations, which contrasted with the other intertidal populations.

The UPGMA tree obtained using Reynolds distances highlights a major genetic split between intertidal and subtidal populations (Fig. 4), with the exception of the intertidal population from Bay Forest grouping within the clade S with subtidal populations. However most bootstrap values were weak. The position of Bay Forest inside the subtidal clade may be indicative of a range limit of the intertidal clade in the Atlantic (one clade being replaced by the other in this context). A hierarchical analysis of variance was used to test the significance of the clustering by partitioning the genetic variance between and within these two clades. Although most of the genetic variation was explained by population heterogeneity (76.02% of the total variation: $\Phi_{ST} = 0.24$; $P < 0.01$), variations between clades (18.90%) or between populations within clades (5.08%) were also significant ($\Phi_{CT} = 0.19$ and $\Phi_{SC} = 0.06$; $P < 0.01$).

Genotypic disequilibrium was not detected across loci when calculated within each clade ($P > 0.05$), indicating that loci give independent information. Association among loci was, however, detected at some subtidal populations located in the Irish Sea and some intertidal populations along the Brittany coastline: *Me-Mpi* in Colwyn Bay, Cardigan Bay and Redwharf Bay populations ($P < 0.05$), for *Hk-Pgi* at Redwharf Bay ($P < 0.05$), for *Pgm-Pgi* at Morgat ($P < 0.01$) and *Pgi-Me* at Lannion-I ($P < 0.01$). Monolocus and multilocus F_{IS} values are presented separately for each clade in Table 3 (A, B). Heterozygote deficiencies were observed in all populations. These departures from Hardy–Weinberg proportions were highly significant for six out of the nine populations of clade S and four out of five populations of clade I. Values in clade S ranged from –0.048 for Sub Forest to 0.416 for Colwyn Bay. The three Irish Sea populations together with Bay Forest displayed the highest F_{IS} values compared to the five other populations. Values in clade I ranged from –0.003 for Utah Beach to 0.241 for Saint Brieuc and were highest along the Brittany coast.

Using Weir & Cockerham's (1984) estimator θ, pairwise genetic differentiation was calculated separately for each clade; values are presented in Table 4 (A, B). All pairwise θ values were significantly different from zero in clade I

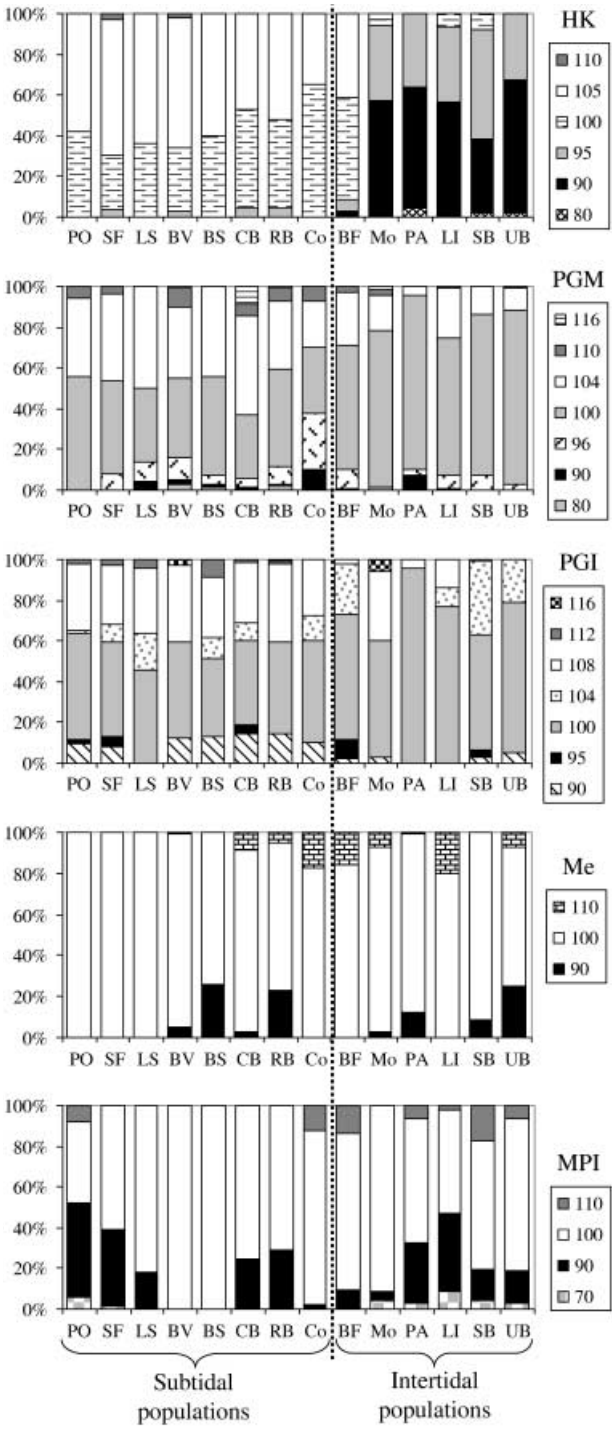

Fig. 3 Allele frequencies at the five enzyme loci in populations of *Acrocnida brachiata* sampled in subtidal and in intertidal habitats.

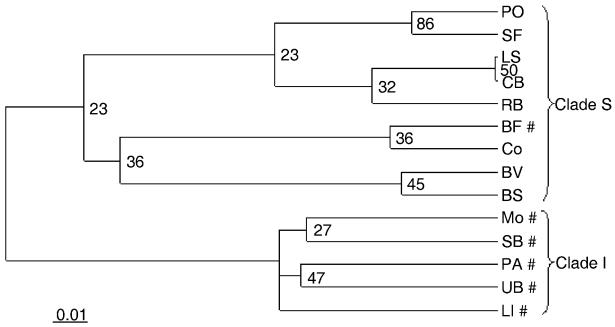

Fig. 4 UPGMA tree showing the genetic relationship between populations of *Acrocnida brachiata* using allozyme data and Reynold *et al.*'s (1983) distance. The number above branches indicates bootstrap confidence values, with 1000 resampling of the data set.

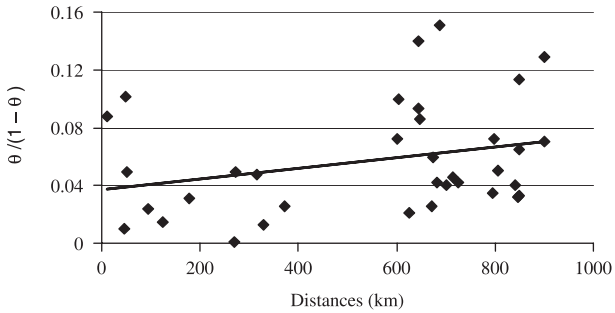

Fig. 5 Isolation-by-distance graph showing pairwise genetic distance $\theta/(1-\theta)$ plotted against geographical distances for populations of clade S of *Acrocnida brachiata* (test Mantel, $P = 0.06$)

($0.04 < \theta < 0.11$; all $P < 0.01$) whereas several were not in clade S ($0 < \theta < 0.13$; see P values in Table 4A). The overall multilocus θ estimate was lower for clade S than for clade I (0.051 and 0.071, respectively). An AMOVA based on geography was performed for clade S but was not significant across the three main geographic groups ($\Phi_{CT} = 0.02$; $P > 0.05$). More variance was found 'within populations' than 'among populations' (93.8% and 4.3%, respectively). Values of fixation indices were weak ($\Phi_{SC} = 0.04$ and $\Phi_{ST} = 0.06$). Although marginally significant, a pattern of isolation by distance was observed in clade S ($P = 0.06$, Mantel test; see Fig. 5), but not in clade I ($P = 0.94$).

Mitochondrial COI

The sequencing of the 598-bp mtCOI gene revealed a high level of nucleotide and haplotype polymorphism. Sequence analysis indicated the occurrence of 168 polymorphic sites, from which 151 different haplotypes could be detected. From this data set, 77.9% of the sites were parsimoniously informative and 22.1% were singletons. A neighbour-joining tree based on average pairwise distances between mtCOI sequences across populations is presented in Fig. 6 and shows a deep nucleotide divergence (19.6%) between intertidal populations (with the exception of Bay Forest) and subtidal populations. The Aber population was split into two subpopulations, each within one clade (PA-a in

Table 3 Mono- and multilocus estimates of the fixation index F_{IS} within each population of (A) the subtidal clade of *Acrocnida brachiata* and (B) the intertidal clade. Test of significance were performed with GENEPOP 3.3 (Raymond & Rousset 1995), *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

A	PO	SF	LS	BV	BS	CB	RB	Co	BF†
Hk	−0.084	−0.143	−0.538	−0.121	0.299	0.273	0.193	−0.073	0.269***
Pgm	0.232	0.053	−0.000	0.182*	0.004	0.269***	0.270*	0.358	0.188
Pgi	0.016	−0.138	0.484	−0.093	0.032*	−0.074*	0.150	0.329*	0.290***
Me	—	—	—	0.305	−0.033	0.460*	0.377**	0.835**	0.299
Mpi	0.143**	−0.012	0.429			0.314	0.103	0.633*	0.567***
Multilocus average	0.061*	−0.048	0.075	0.055*	0.060	0.248***	0.219**	0.416***	0.323***

B	Mo†	PA†	LI†	SB†	UB†
Hk	−0.002	0.035	−0.032*	0.252*	−0.021
Pgm	0.022	−0.101	0.267*	0.053	−0.118
Pgi	−0.386**	−0.033	−0.205	−0.386***	−0.167
Me	0.238	0.567***	0.284	0.732***	0.350**
Mpi	0.477**	0.032	−0.008*	0.554***	−0.061
Multilocus average	0.070**	0.100*	0.061***	0.241***	−0.003

Table 4 Pairwise values of genetic differentiation of *Acrocnida brachiata* clade S (A) and of clade I (B). Multilocus Weir & Cockerham's (1984) θ values obtained from the enzyme data set are in bold, below the diagonal with test of significance performed with GENEPOP 3.3 (Raymond & Rousset 1995) Pairwise φ_{ST} values obtained from the mtCOI sequences data set are above the diagonal with test of significance performed with ARLEQUIN 2.0 (Schneider *et al*. 2001). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (— indicates unavailable values)

A	PO	CC	BF,4	SF	LS	BV	BS	CB	RB	Co	SA	RY
PO		0.000	0.000	0.017	0.000	0.007	0.011	0.000	0.009	0.000	0.029	0.003
CC			0.005	0.048	0.003	0.000	0.009	0.000	0.012	0.000	0.000	0.000
BF #	**0.092****	—		0.002	0.000	0.021	0.019*	0.006	0.016	0004	0.046*	0.009
SF	**0.010**	—	**0.081****		0.101**	0.140**	0.113**	0.100	0.082*	0.132	0.203*	0.106*
LS	**0.045***	—	**0.047****	**0.001**		0.013	0.000	0.000	0.001	0.002	0.045	0.001
BV	**0.123****	—	**0.091****	**0.067****	**0.013**		0.018	0.000	0.011	0.000	0.000	0.004
BS	**0.131****	—	**0.079****	**0.085****	**0.025***	**0.023****		0.000	0.000	0.002	0.042**	0.014
CB	**0.044****	—	**0.056****	**0.025****	**−0.010**	**0.040****	**0.040****		0.000	0.000	0.016	0.003
RB	**0.039****	—	**0.067****	**0.034****	**0.021**	**0.048****	**0.031****	**0.014***		0.002	0.043	0.006
Co	**0.114****	—	**0.032***	**0.102****	**0.039**	**0.061****	**0.066****	**0.030****	**0.047****		0.000	0.000
SA	—	—	—	—	—	—	—	—	—	—	—	0.027

B	Mo†	PA-b†	Ll†	SB†	UB†
Mo #		0.100*	0.019	0.078*	0.045*
PA-b #	**0.105****		0.036	0.066	0.005
Ll #	**0.080****	**0.043****		0.029	0.005
SB 9	**0.090****	**0.092****	**0.065****		0.011
UB 9	**0.074****	**0.043****	**0.057****	**0.060****	

clade S and PA-b in clade I). This deep divergence contrasted greatly with the average pairwise distances: within each clade, populations showed less than 1% of divergence. Relative constancy rate of a molecular clock was not rejected ($P > 0.05$) when considering the neighbour-joining tree, allowing us to estimate the time elapsed since the divergence of the two clades. Applying the substitution rate previously published by Bermingham & Lessios (1993) for sea urchins (i.e. 2% substitutions per million years), a value commonly used for animals (Rand 1994), divergence of 19.6% corresponds to a divergence time of about 5 million years. As a comparison, divergence between *A. brachiata* and *Amphiura filiformis* (the outgroup), its nearest related species, was about 29.8%.

Since divergence between groups was very deep, each clade was analysed separately. Levels of genetic diversities

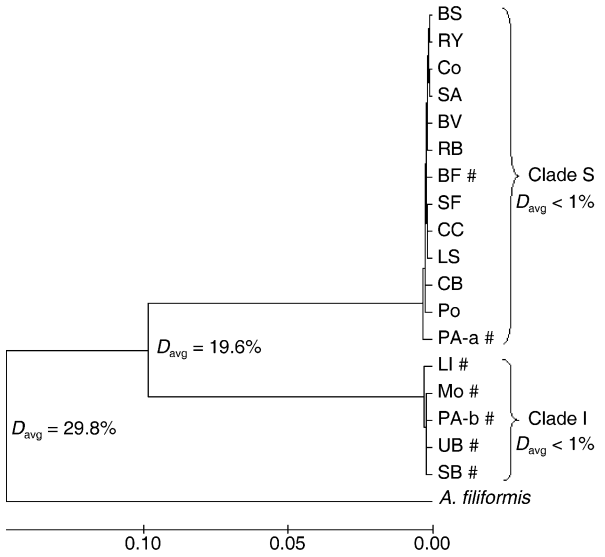

Fig. 6 Neighbour-joining tree of *Acrocnida brachiata* populations based on average pairwise distances between mtCOI sequences of *A. brachiata* individuals across populations using Kimura 2-parameter distance method (Kimura 1980). D_{avg} is the average divergence between the two clades using the same distance method.

within populations of each clade are provided in Table 5A (clade S) and Table 5B (clade I). For both clades, the mean haplotype diversity ($H_{e\text{-}hap}$) and mean nucleotide diversity (π) were of the same order of magnitude, approximately 0.92 and 0.005, respectively.

The haplotype distribution for each clade over the sampling area is shown in Fig. 7(A, B). Populations of clade S only shared 20 haplotypes out of 104 although at very different frequencies. The proportion of 'private' haplotypes varied from 35% to 63% with smaller values in the Irish Sea populations and higher values in South Brittany populations. Populations from clade I shared 6 haplotypes out of 47. Pairwise Φ_{ST} estimates obtained from mtCOI sequences are presented in Table 4 (A, B). Most Φ_{ST} values were weak and nonsignificant in both clades. Sub Forest appeared very different from other subtidal populations, mainly due to a haplotype (HS1) for which the frequency was twice as low as in the other populations. An AMOVA was also performed according to geography within clade S. Even if most of the variance (69%) was still associated with the 'within population' level ($\Phi_{ST} = 0.31$, $P < 0.01$), differentiation was also significant across geographic groups ($\Phi_{CT} = 0.15$, $P < 0.05$).

Estimated gene flows between the three geographic regions were very asymmetric (Table 6). Most migrants originated from South Brittany with more individuals colonizing the Irish Sea than the English Channel. Although slight, migrations appeared also asymmetric between the Irish Sea and the English Channel with gene flow orientated towards the English Channel by one order of magnitude.

Haplotype networks are presented in Fig. 8. The network drawn for the clade S revealed a 'star-like distribution' with one common haplotype (HS1), found in all populations (Fig. 8A). Most divergent haplotypes directly originated from this haplotype with a maximum divergent branch length of nine mutational steps. Nonsynonymous substitutions were homogeneously distributed over the network. The network drawn for clade I revealed a more complex structure (Fig. 8B). The five intertidal populations shared four main haplotypes plus a highly divergent branch made of eight haplotypes presenting a maximum length of 14 mutational steps from haplotype HI1 that only occurred in Lannion-I specimens. As opposed to clade S, nonsynonymous mutations were mostly found around haplotype HI2.

Mismatch distributions created for each clade are presented in Fig. 9(A, B). Distributions were unimodal with negative and significant Tajima's D values ($D = -2.455$, $P < 0.001$ for clade S, and $D = -1.956$, $P < 0.05$ for clade I). These negative values indicated an excess of low frequency variants that could be due to either a selective sweep or a population size expansion.

Table 5 Mitochondrial COI gene diversities for the 13 populations of *Acrocnida brachiata* of clade S (A) and of clade I (B) (PA-a and PA-b corresponds to individuals of the population from Aber that display, respectively, a subtidal or an intertidal mtCOI signature). *N*, number of sequences; *h*, number of haplotypes by populations; $H_{e\text{-}hap}$, haplotype diversity; π, nucleotide diversity

A	PO	CC	BF†	SF	PA-a†	LS	BV	BS	CB	RB	Co	RY	SA	clade S
N	18	7	25	12	4	9	26	31	21	39	19	19	21	243
h	15	S	20	11	3	7	14	18	14	21	13	9	13	104
$H_{e\text{-}hap}$	0.961	0.857	0.973	0.985	0.833	0.944	0.874	0.916	0.938	0.938	0.906	0.778	0.929	0.927
π	0.0064	0.0038	0.0060	0.0064	0.0047	0.0046	0.0054	0.0047	0.0068	0.0060	0.0041	0.0033	0.0015	0.0054

B	Mo†	PA-b†	LI†	SB†	UB†	clade I
N	26	16	27	15	23	107
h	15	11	18	9	10	47
$H_{e\text{-}hap}$	0.898	0.933	0.960	0.914	0.885	0.923
π	0.0052	0.0047	0.0072	0.0051	0.0048	0.0058

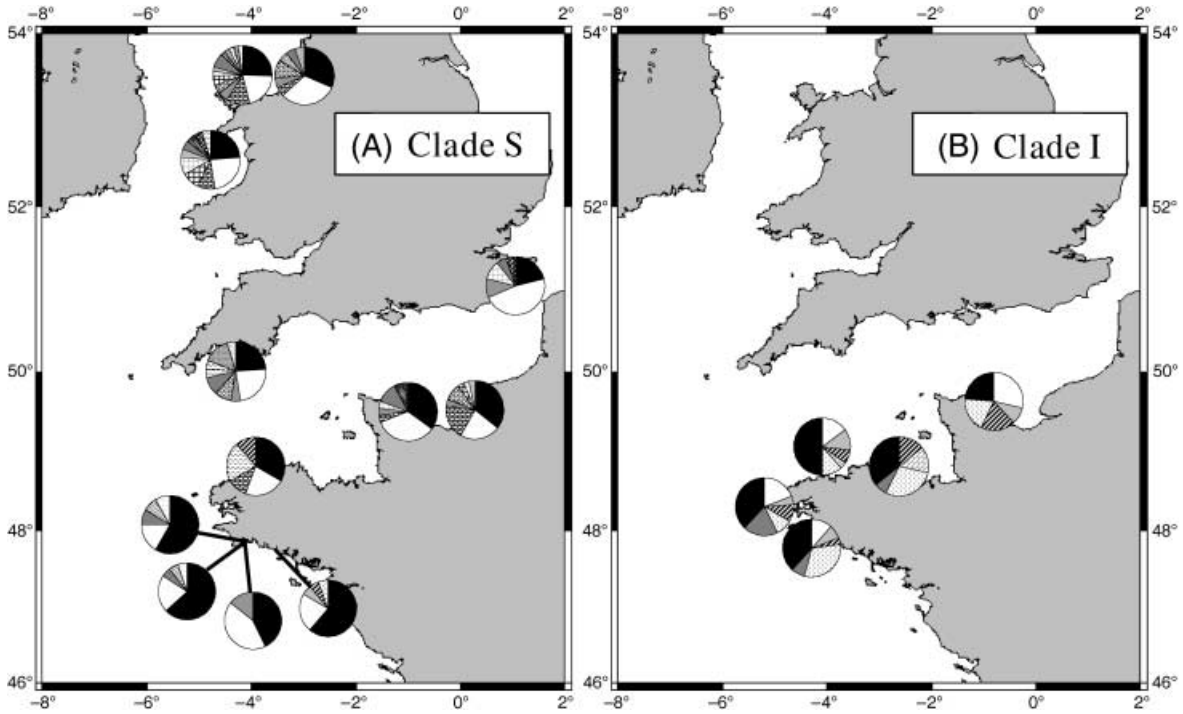

Fig. 7 Map showing the distribution of mtCOI haplotypes among populations of *Acrocnida brachiata* that belong to clade S (A) and clade I (B). Black slice symbolizes private haplotype and other slices represent shared haplotypes between populations within each clade.

Table 6 Gene flow estimates (*Nm*) between main geographic regions obtained by MIGRATE-N simulations for clade S of *Acrocnida brachiata*. Donating populations are in columns (with estimates of Θ, the parameter representing the effective population size) and receiving populations are in rows

	South Brittany (0.0858)	English Channel (0.0014)	Irish Sea (0.0062)
South Brittany	*	0.03	0.58
English Channel	525.82	*	207.94
Irish Sea	1432.83	19.13	*

Nearly all substitutions (89.4%) occurred on the third codon position but 22 were found to be nonsynonymous. Because only two nonsynonymous mutations were fixed between clades, a search for a positive selective effect within clades was performed along sequences. No significant variation was observed either in the distribution of the nucleotide diversity (π) or in the Tajima's *D* statistic along sequences. However, nonsynonymous/synonymous substitution ratios deviated significantly from neutral expectations (McDonald's *G*-statistic, $G = 7.017$; $P < 0.01$) with $\theta_N/\theta_S >> K_N/K_S$ suggesting that positive selection may have occurred at the 'within-clade' level. The mean value of θ_N/θ_S ratio at the intraclade level was of 0.02 for both clades indicating the emergence of adaptive polymorphisms in both lineages.

Search for hybrids between the clades

Sixteen individuals (*c*. 3% of the all sample) appeared to be misassigned across the two clades. These individuals were all sampled in the intertidal. Out of these 16 brittle-stars, eight individuals [four from Aber (PA-a) and four from Bay Forest (BF)] displayed a subtidal haplotype but presented an intertidal-like nuclear background. On the contrary, eight individuals from Morgat (2), Lannion-I (3) and Saint Brieuc (3) possessed an intertidal haplotype but were assigned to the subtidal clade based on their allozyme genotype.

Discussion

Emergence of ecotypes in Acrocnida brachiata

Both allozymes and mtCOI sequences suggest the existence of a marked habitat-dependent genetic break in the brittle-star *Acrocnida brachiata* along the coasts of northwestern Europe. Inversion of allele frequencies occurred at three enzyme loci, segregating most of the intertidal populations (except the Bay Forest population) from subtidal ones. Mitochondrial COI sequences were clustered into two deeply divergent lineages (*c*. 20% of divergence), which differentiates the same populations, intertidal vs. subtidal. Although morphologically *A. brachiata* is an easily identifiable

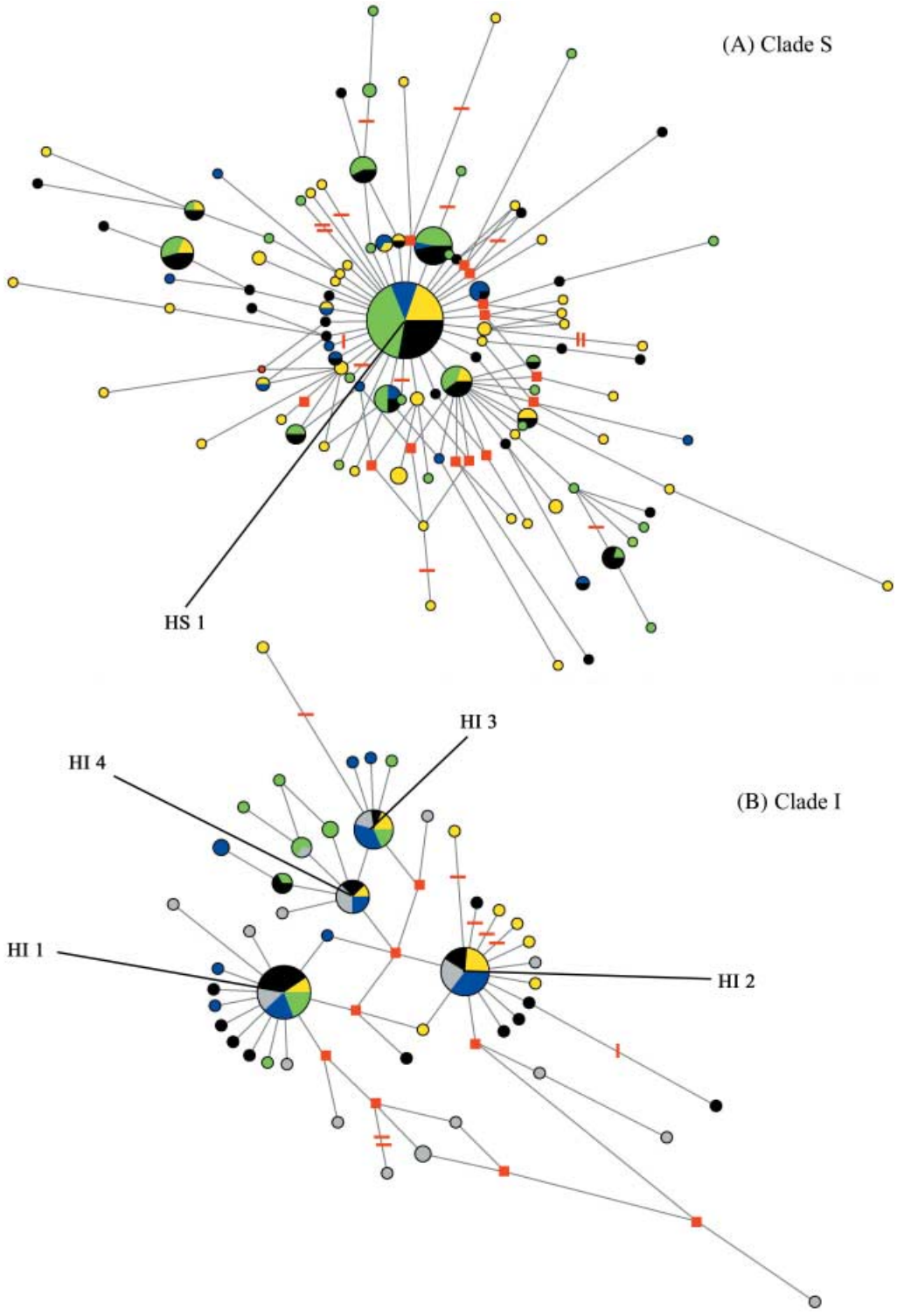

Fig. 8 Haplotype networks representing the evolutionary relationships between mitochondrial haplotypes within clade S (A) and clade I (B). Connecting lines correspond to the number of mutational steps between haplotypes. Red square represents hypothetical haplotype not detected in the study. Red bar indicates a nonsynonymous mutation. Names of the most common haplotypes are indicated. The size of a circle is proportional to the number of individuals observed for a given haplotype and the colour of pie charts represents the regional origin of the haplotype. For clade S, black corresponds to Irish populations (CB, Co, RB), yellow to South Brittany populations (Po, CC, SF, BF), blue to Eastern English Channel populations (BV, BS, RY), green to Western English Channel (SA, LS). For clade I, black corresponds to Morgat, yellow to Aber, blue to Utah Beach, grey to Lannion and green to Saint Brieuc.

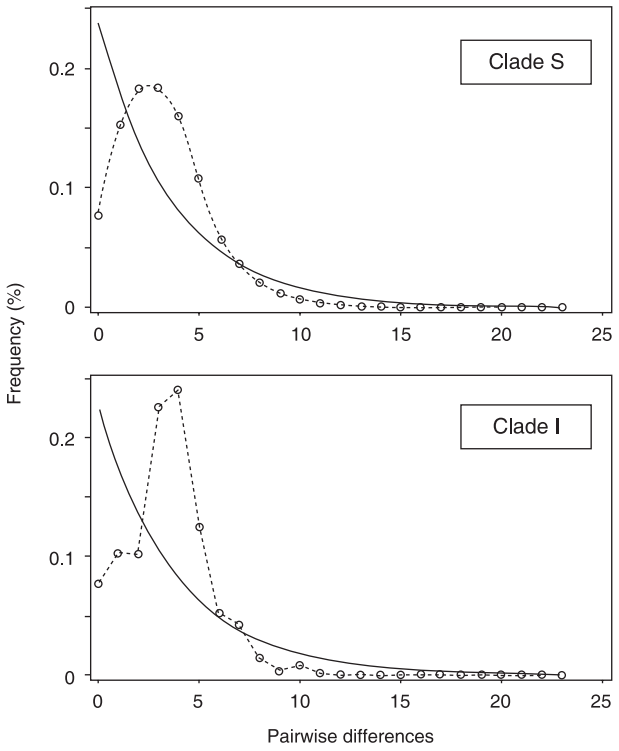

Fig. 9 Mismatch curves obtained for the two clades of *Acrocnida brachiata*. Expected curve under a constant population size is represented by a continuous line and the observed one by a dotted line.

species (Koehler 1921), with no reported plasticity, it is now clear that this echinoderm species should not be regarded as a unique species but as a complex of two old 'sibling' lineages. The average level of genetic differentiation between two populations from the same clade but from different localities is three to five times lower than that found between two populations of the same locality but inhabiting different bathymetric levels (e.g. $\theta = 0.22$ between Lannion-S and Lannion-I whereas $\theta = 0.02$ and 0.06 between Lannion and other populations of clade S and I, respectively). The same level of divergence was encountered across the whole sampling area. Hence, the divergence between these two lineages is probably due to an ancient unique speciation event followed by expansion rather than the result of local selective pressures acting independently within each locality. This poses the intriguing question about speciation mechanisms leading to the emergence of these two lineages. Did populations separate geographically (i.e. allopatric speciation followed by secondary contacts), or did they progressively diverge from a single ancestral area (i.e. sympatric speciation) in which contrasted habitats have favoured reduced gene flow and local adaptation?

Based on previous published substitution rates in echinoderms (Bermingham & Lessios 1993), the level of divergence found between the two clades was estimated to date back to *c*. 5 million years ago (divergence of 20% with a mutation rate of 2% per million years), approximately at the time of the Mio–Pliocene transition. This period was characterized by a continentalization of Western Europe (Meulenkamp & Sissingh 2003) as well as major climatic and tectonic changes (Brault *et al*. 2004). During this period, no marine corridors existed between the Atlantic Ocean and the North Sea with the exception of a passage towards the North of the British Isles. Haq *et al*. (1987) showed numerous sea level changes during this period with dramatic increases possibly reaching 100 m in amplitude, 6–5 and 4–3 million years ago, with a period of low levels in between. As a consequence, exchanges were temporarily reduced between the North Sea and the Atlantic Ocean and may have greatly affected migration patterns of marine invertebrates. Continental uplift and decreases in sea level may have influenced processes of differentiation between the two *A. brachiata* clades. One possible scenario is that genetic isolation started during a period of decreasing sea level: one group may have been isolated under colder conditions whereas the other group took refuge in warmer waters (i.e. in different latitudes or different depths). Secondary contacts between the two groups may have then been possible owing to an increase in sea level, the opening of the North Sea Basin and/or the opening of the English Channel (10 000–8000 years). In this case, the introduction of each group to the other group's habitat would have led to local maladaptation, thus reinforcing each clade at a given depth.

Polychaete genera associated with sandy-bottom habitat of Northeastern Atlantic exhibit a similar deep genetic break of about 20% either between closely related species of *Pectinaria* or between the subtidal and intertidal clades of *Owenia fusiformis* (25.9% and 19.2%, respectively; Jolly *et al*. 2006). At present, *A. brachiata* clade I and *O. fusiformis* clade 3 seem to share the same geographic distribution, restricted in the intertidal (Jolly *et al*. 2006). Moreover, divergence of 20.6% was found between two well-identified tubeworm polychaete species from the genus *Sabellaria* that live separately in the subtidal or the intertidal area (F. Rigal, unpublished). The similar level of divergence encountered within several species complexes of the sandy-bottom habitat suggests the occurrence of vicariant events. These species seem to share a similar history at the Mio–Pliocene transition, suggesting allopatric processes through an ancestral separation of the sandy-bottom habitat communities into two distinct refuges, one being probably shallower than the other.

While the Mio–Pliocene transition may have played an important role in allopatric speciation, sympatric differential selective pressures could have contributed to the splitting of *A. brachiata* lineages. Long-term adaptation of brittle stars to different environments may be associated with contrasting levels of physiological tolerance to desiccation,

temperature, or hypoxia, as it is the case for the polychaete *S. armiger* (Kruse *et al*. 2004) and thus may reinforce habitat segregation. However, the sympatric hypotheses could be considered if regarding only the allozyme data but not in the light of our results on mitochondrial sequences. First, the McDonald and Kreitman test was significant but nonsynonymous substitutions accumulated within each lineage, not between them. Second, no variation in the distribution of the nucleotide diversity and of the Tajima's *D*-statistic was detected along sequences. While both findings invalidate (1) the possibilities of ecotypic specialization hypotheses and (2) the maintenance of ancestral polymorphism, they strengthen the supposition of the emergence of adaptive polymorphisms within each lineage after the splitting of the ancestral species. As a consequence, speciation in *A. brachiata* does not seem to have been driven by diversifying selective processes on ecological interactions but is likely the reflection of allopatric events at the Mio–Pliocene transition.

Nonetheless, ecological interactions were probably of crucial importance in the reinforcement and in the bathymetric positioning of the two lineages after they separated. Even if not directly implicated in the emergence of the two lineages, ecological interactions may explain their co-occurrence and relative differentiation within a given locality. A few studies have already shown the influence of bathymetry on the spatial distribution of enzyme alleles (Kirby *et al*. 1994; Schmidt & Rand 2001; Veliz *et al*. 2004). Moreover, postzygotic selection by the way of local retention of pre-adapted larvae or young settlers and selective elimination of nonadapted ones may reinforce the maintenance of the two clades (Bierne *et al*. 2003). A previous fine-scale survey of population dynamics in the Bay of Morgat revealed subtle differences in recruitment periods and gamete maturation between intertidal and subtidal populations (Bourgoin *et al*. 1991). Thus, mismatch in reproductive periods may also contribute to preferential reproduction within lineages and to the continuing isolation of the divergent lineages within a locality. However, ecophysiological experiments are necessary to determine whether the maintenance of *A. brachiata* ecotypes is favoured by differing environmental conditions (e.g. Gardner & Thompson 2001; Edmands & Deimler 2004).

In the present case, hybridization followed by selection against hybrids could also explain the maintenance of both clades at different bathymetric levels after secondary contact. Differences in allele frequencies between intertidal and subtidal ecotypes were quite modest for four allozyme loci (compared to locus *Hk*), possibly reflecting hybridization and different levels of introgression between lineages depending on localities. Moreover, the occurrence of 16 individuals displaying a haplotype from one clade but a nuclear background from the other clade strongly suggests that the splitting of the two lineages is incomplete. Varying degrees of introgression may explain the observed genotypic disequilibria and high heterozygote deficiencies that were found locally, especially in the Irish Sea (subtidal clade) and in South Brittany (intertidal clade). Furthermore, the level of gene introgression may also alter the patterns of nuclear vs. cytoplasmic differentiation, possibly explaining the greater pairwise population differentiation for allozymes compared to the mitochondrial gene and the lack of clear evidence for an isolation-by-distance pattern in either clade. In any case, the occurrence of hybridization strengthens our scenario of ancestral separation of two ecotypic lineages due to allopatry and reinforcement after secondary contact — as it is the case for other hybrid zones (Kirby *et al*. 1997) — rather than to sympatric differential selection.

Additional studies based on larger sample sizes at a microspatial scale (e.g. in the Bay of Douarnenez) as well as reproductive compatibility trials between the two lineages are now needed to evaluate the level of both hybridization and environmental selection in the positioning of the clades.

Substantial geographic isolation of populations of both ecotypes since their recent range expansion

Analyses of allozyme and mtCOI polymorphisms provided interesting information about the genetic structure and geographic expansion within each clade. High percentage of singletons, low level of intraclade divergence among and within populations (< 1%) and, as previously described, negative significant Tajima's *D* values characterized each clade. Under neutral hypotheses, these characteristics suggest a recent increase in population sizes. The star-like distribution of subtidal haplotypes with a few mutation steps suggests that DNA sequences have diverged little over the Quaternary ice ages, and that the observed mutations are mostly due to postglacial diversification (Hewitt 2000). Demographic explosion could be due to the recent formation of the English Channel and to the colonization of new territories. The catastrophic opening of the Dover Strait with a great volume of water spilled out of the North Sea (Smith 1989) could have promoted the spread of both *A. brachiata* lineages and their admixture over the whole or part of the study area. Water originating from melting ice did not enter directly into the ocean but was accumulated over continental masses and released precipitously into marine habitat (Pirazzoli 1998), driving important sediment movements and the reorganization of communities. However, the two lineages show different colonization patterns, with a more restricted expansion for the clade I. Tajima's *D* value is lower for clade S than for clade I (–2.46 and –1.96, respectively). Accordingly, clade I appears to have a more limited range and may be restricted, in fact, to the western approach of the English Channel and possibly the Irish Sea. Indeed, *A. brachiata* has never been observed in the intertidal area in the Eastern English Channel

and the North Sea (Ursin 1960). Furthermore, the intertidal population from Bay Forest displayed a subtidal genetic signature, which may suggest the replacement of clade I by clade S further south. In addition, because of its limited range, it might be possible that the actual distribution of the lineage also includes its initial location. The occurrence of a large brackish lake (i.e. the Solent lake) prior to the opening of the English Channel could have played the role of such a glacial refuge, as it has been proposed for other intertidal species in the English Channel (Gysels *et al*. 2004; Provan *et al*. 2005). Conversely, the high proportions of 'private' haplotypes in the subtidal populations from South Brittany are concordant with the patterns of asymmetric gene flow estimated using MIGRATE-N towards the English Channel (Table 6): both indicate the occurrence of a massive colonization event of both the Irish Sea and the English Channel from the south, suggesting that clade S may come from a more southern glacial refuge. This scenario fits the one revealed by the work of Jolly *et al*. (2006) where *P. koreni* clade 2 and *O. fusiformis* clade 1 may have persisted in a southern glacial refuge during the last glacial maximum.

Most pairwise enzyme θ values between populations within clade S or within clade I were significantly different from zero, with a high overall level of genetic differentiation. Even in light of some level of hybridization, genetic structure of both clades strongly supports the fact that *A. brachiata* populations are geographically isolated from each other. Despite the lack of isolation-by-distance pattern in clade I, there is a trend towards isolation by distance in clade S. Moreover, hierarchical analyses of differentiation clearly indicate that populations from the Irish Sea, the Atlantic Ocean and the English Channel are significantly isolated from each other ($\Phi_{CT} = 0.15$, $P < 0.05$). The high proportions of 'private' haplotypes in all populations of both clades are congruent with a high degree of isolation between populations. Isolation may be promoted by weak dispersal capacities. Indeed, larval observations in aquaria indicate the occurrence of a very short pelagic developmental stage in *A. brachiata*, at least for clade I. Moreover, *A. brachiata* larvae are absent from plankton and previous studies document low fecundities and large yolky eggs in the species adults for both habitats (Webb & Tyler 1985; Bourgoin 1987; Gentil & Zakardjian 1990). All these observations highlight the very restricted dispersal abilities of this brittle star, for both clade. The influence of the larval planktonic phase on population structuring is increasingly documented (e.g. Siegel *et al*. 2003). Many studies have emphasized the relationship between larval pelagic phase, effective dispersal and population structure (McMillan *et al*. 1992; Hoskin 1997; Arndt & Smith 1998; Goldson *et al*. 2001), even if some short-developing species present considerable levels of gene flow that contradicts such a dogma (Colson & Hughes 2004). As a short pelagic larval phase is commonly known in gregarious species to represent an advantage for not dispersing away from favourable habitats (Pechenik 1999), it could minimize the risk that numerous *A. brachiata* populations become rapidly extinct as sandy-bottom habitat are highly fragmented and scattered along the European coastlines.

In *A. brachiata*, past glacial history, ecological selection and dispersal capacities have together produced a very contrasted and complex population structure. This study poses intriguing questions about the origin of speciation for the shallow-water endobenthic fauna in Europe during the last glacial episodes. As stated by Bermingham & Moritz (1998), comparative approaches using species sharing the same kind of habitat but presenting contrasted life history traits should be a valuable tool to better understand the relative importance of vicariance and local selection in shaping populations.

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References

Arndt A, Smith MJ (1998) Genetic diversity and population structure in two species of sea cucumber: differing patterns according to mode of development. *Molecular Ecology*, **7**, 1053–1064.

Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biological Evolution*, **16**, 37–48.

Baric S, Sturmbauer C (1999) Ecological parallelism and cryptic species in the genus *Ophiothrix* derived from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, **11**, 157–162.

Barton NH, Hewitt GM (1989) Adaptation, speciation and hybrid zones. *Nature*, **341**, 497–503.

Beerli P, Felsenstein J (2001) Maximum likelihood estimation of a migration matrix and effective population sizes in subpopulations using a coalescent approach. *Proceedings of the National Academy of Sciences, USA*, **98**, 4563–4568.

Bermingham E, Lessios HA (1993) Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama. *Proceedings of the National Academy of Sciences, USA*, **90**, 2734–2738.

Bermingham E, Moritz C (1998) Comparative phylogeography: concepts and applications. *Molecular Ecology*, **7**, 367–369.

Bierne N, Bonhomme F, David P (2003) Habitat preference and the marine-speciation paradox. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **270**, 1399–1406.

Bourgoin A (1987) Ecologie et démographie d'*Acrocnida brachiata* (Montagu) (Echinodermata: Ophiuroidea) en baie de Douarnenez (Bretagne) Thesis, University of Occidental Brittany, Brest (France).

Bourgoin A, Guillou M, Glémarec M (1991) Environmental instability and demographic variability in *Acrocnida brachiata* (Echinodermata: Ophiuropidea) in Douarnenez Bay (Britanny: France). *Marine Ecology*, **12**, 89–104.

Brault N, Bourquin S, Guillocheau F *et al.* (2004) Mio–Pliocene to Pleistocene paleotopographic evolution of Britanny (France) from a sequence stratigraphic analysis: relative influence of tectonics and climate. *Sedimentary Geology*, **163**, 175–210.

Cabioch L, Gentil F, Glaçon R, Retière C (1977) Le Macrobenthos des fonds meubles de la Manche: distribution générale et écologie. In: *Biology of Benthic Organisms* (eds Keegan BF, O'Cedigh P, Boaden PJS), pp. 115–128. Pergamon Press, Oxford.

Chenuil A, Féral JP (2003) Sequences of mitochondrial DNA suggest that *Echinocardium cordatum* is a complex of several sympatric or hybridizing species: a pilot study. In: *Echinoderms Research 2001* (eds Féral JP, David P), pp. 15–21. Swets & Zeitlinger, Lisse, The Netherlands.

Colson I, Hughes RN (2004) Rapid recovery of genetic diversity of dogwhelk (*Nucella lapillus* L.) populations after local extinction and recolonization contradicts predictions from life-history characteristics. *Molecular Ecology*, **13**, 2223–2233.

Cruz R, Carballo M, Conde-Padin P, Rolan-Alvarez E (2004) Testing alternative models for sexual isolation in natural populations of *Littorina saxatilis*: indirect support for by-product ecological speciation? *Journal of Evolutionary Biology*, **17**, 288–293.

Dinter WP (2001) *Biogeography of the OSPAR Maritime Area*. Federal Agency for Nature Conservation, Bonn, Germany.

Edmands S, Deimler JK (2004) Local adaptation, intrinsic co-adaptation and the effects of environmental stress on inter-population hybrids in the copepod *Tigriopus californicus*. *Journal of Experimental Marine Biology and Ecology*, **303**, 183–196.

Gardner JPA, Thompson RJ (2001) The effects of coastal and estuarine conditions on the physiology and survivorship of the mussels *Mytilus edulis*, *M. trossulus* and their hybrids. *Journal of Experimental Marine Biology and Ecology*, **265**, 119–140.

Gavrilets S (2003) Models of speciation: what have we learned in 40 years? *Evolution*, **57**, 2197–2215.

Gentil F, Zakardjian B (1989) Premières données sur la dynamique de population *d'Acrocnida brachiata* (Montagu) en Baie de Seine orientale. *Vie marine HS*, **10**, 148.

Gentil F, Zakardjian B (1990) Reproductive cycle of the ophiuroid *Acrocnida brachiata* (Montagu) in the Bay of Seine (English Channel). In: *Echinoderms Research* (eds De Ridder C, Dubois P, Lahaye MC, Jangoux M), pp. 83–89. Balkema, Rotterdam, The Netherlands.

Gentil F, Irlinger JP, Elkaim B, Proniewski F (1986) Premières données sur la dynamique du peuplement macrobenthique des sables fins envasés à *Abra alba* de la baie de Seine orientale. *Actes de Colloques IFREMER*, **4**, 409–420.

Goldson AJ, Hughes RN, Gliddon CJ (2001) Population genetic consequences of larval dispersal mode and hydrography: a case study with bryozoans. *Marine Biology*, **138**, 1037–1042.

Goudet J (1995) FSTAT (version 1.2): a computer program to calculate *F*-statistics. *Journal of Heredity*, **86**, 485–486.

Gysels ES, Hellemans B, Pampoulie C, Volckaert FAM (2004) Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea. *Molecular Ecology*, **13**, 403–417.

Hall TA (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Haq BU, Hardenbol J, Vail PR (1987) Chronology of fluctuating sea levels since the Triassic. *Science*, **235**, 1156–1166.

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature*, **405**, 907–913.

Hoskin MG (1997) Effects of contrasting modes of larval development on the genetic structures of populations of three species of prosobranch gastropods. *Marine Biology*, **127**, 647–656.

Johannesson K (2001) Parallel speciation: a key to sympatric divergence. *Trends in Ecology & Evolution*, **16**, 148–153.

Jolly MT, Viard F, Weinmayr G *et al.* (2003) Does the genetic structure of *Pectinaria koreni* (Polychaeta: Pectinariidae) conform to a source-sink metapopulation model at the scale of the Baie de Seine? *Helgoland Marine Research*, **56**, 238–246.

Jolly MT, Jollivet D, Gentil F, Thiébaut E, Viard F (2005) Sharp genetic break between Atlantic and English Channel populations of the polychaete *Pectinaria koreni*, along the north coast of Britanny, France. *Heredity*, **94**, 23–32.

Jolly MT, Viard F, Gentil F, Thiébaut E, Jollivet D (2006) Comparative phylogeography of two coastal polychaete tubeworms in the North East Atlantic supports shared history and vicariant events. *Molecular Ecology*, **15**, 1841–1855.

Keegan BF, Konnecker G (1979) Aggregation in echinoderms on the West coast of Ireland. An ecological perspective. *Proceedings of the Royal Irish Academy*, **86B**, 199.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Kirby RR, Bayne BL, Berry RJ (1994) Phenotypic variation along a cline in allozyme and karyotype frequencies, and its relationship with habitat, in the dog-whelk *Nucella lapillus* L. *Biological Journal of the Linnean Society*, **53**, 255–275.

Kirby RR, Berry RJ, Powers DA (1997) Variation in mitochondrial DNA in a cline of allele frequencies and shell phenotype in the dog-whelk *Nucella lapillus* (L.). *Biological Journal of the Linnean Society*, **62**, 299–312.

Knowlton N (1993) Sibling species in the sea. *Annual Review of Ecological System*, **24**, 186–216.

Knowlton N (2000) Molecular genetic analyses of species boundaries in the sea. *Hydrobiologia*, **420**, 73–90.

Koehler R (1921) Echinodermes. *Faune de France*, **1**, 1–210.

Kruse I, Reise K (2003) Reproductive isolation between intertidal and subtidal *Scoloplos armiger* (Polychaeta, Orbiniidae) indicates sibling species in the North Sea. *Marine Biology*, **143**, 511–517.

Kruse I, Strasser M, Thiermann F (2004) The role of ecological divergence in speciation between intertidal and subtidal *Scoloplos armiger* (Polychaeta, Orbiniidae). *Journal of Sea Research*, **51**, 53–62.

Kumar S, Tamura K, Jakobsen I, Nei M (2001) MEGA 2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics*, **17**, 1244–1245.

Lambeck K (1997) Sea-level change along the French Atlantic and Channel coasts since the time of the Last Glacial Maximum. *Palaeogeology, Palaeoclimatology, Palaeoecology*, **129**, 1–22.

Langella O (2002) POPULATION *1.2.28. Logiciel de génétique des populations*. Laboratoire Populations, génétique et évolution, CNRS UPR 9034, Gif sur Yvette, http://www.cnrs-gif.fr/pge/.

Matsuoka N, Hatanaka T (1991) Molecular evidence for the existence of four sibling species within the sea-urchin, *Echinometra mathaei* in Japanese waters and their evolutionary relationships. *Zoological Science*, **8**, 121–133.

Mayr E (1942) *Systematics and the Origin of Species*. Columbia University Press, New York.

McCarthy C (1997) *Chromas*, Version 1.41. Griffith University, Brisbane, Queensland.
McDonald JH, Kreitman M (1991) Adaptive protein evolution at the Adh locus in *Drosophila*. *Nature*, **351**, 652–654.
McMillan WO, Raff RA, Palumbi SR (1992) Population genetic consequences of developmental evolution in sea urchins (genus *Heliocidaris*). *Evolution*, **46**, 1299–1312.
McMullin ER, Hourdez S, Schaeffer SW, Fisher CR (2003) Phylogenetics and biogeography of deep-sea vestimentiferan tuberworms and their bacterial symbionts. *Symbiosis*, **34**, 1–41.
Meulenkamp JE, Sissingh W (2003) Tertiary palaeogeography and tectonostratigraphic Evolution of the Northern and Southern Peri-Tethys platforms and the intermediate domains of the African–Eurasian convergent plate boundary zone. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **196**, 209–228.
Mortensen T (1921) *Studies of the Development and Larval Forms of Echinoderms*. Gotlieb Ernst Clausen Gad Publishers, Copenhagen.
Nei M (1987) *Molecular Evolutionnary Genetics*. Columbia University Press, New York.
Ogden R, Thorpe RS (2002) Molecular evidence for ecological speciation in tropical habitats. *Proceedings of the National Academy of Sciences, USA*, **99**, 13612–13615.
Orr MR, Smith TB (1998) Ecology and speciation. *Trends in Ecology & Evolution*, **13**, 502–506.
Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology*, **13**, 55–65.
Palumbi SR (1992) Marine speciation on a small planet. *Trends in Ecology & Evolution*, **7**, 114–118.
Pasteur N, Pasteur G, Bonhomme F, Catalan J, Britton-Davidan J (1987) *Manuel Technique de Génétique par Électrophorèse des Protéines*. Lavoisier, Paris.
Pechenik JA (1999) On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. *Marine Ecology Progress Series*, **177**, 269–297.
Pirazzoli P (1998) Sur la vitesse des variations du niveau de la mer. *Annales de Géographie*, **600**, 220–232.
Piry S, Alapetite A, Cornuet J-M *et al.* (2004) GENECLASS 2: a software for genetic assignment and first-generation migrant detection. *Journal of Heredity*, **95**, 536–539.
Provan J, Wattier RA, Maggs CA (2005) Phylogeographic analysis of the red seaweed *Palmaria palmata* reveals a Pleistocene marine glacial refugium in the English Channel. *Molecular Ecology*, **14**, 793–803.
Rand DM (1994) Thermal habit, metabolic rate and the evolution of mitochondrial DNA. *Trends in Ecology & Evolution*, **9**, 125–131.
Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences, USA*, **94**, 9197–9201.
Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.
Reynolds J, Weir BS, Cockerham CC (1983) Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics*, **105**, 767–779.
Rousset F (1997) Genetic differentiation and estimation of gene flow from *F*-statistics under isolation by distance. *Genetics*, **145**, 1219–1228.
Rozas J, Sanchez-DeI, Barrio JC, Messeguer X, Rozas R (2003) DNASP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.
Schluter D (2001) Ecology and the origin of species. *Trends in Ecology & Evolution*, **16**, 372–380.
Schmidt PS, Rand DM (2001) Adaptive maintenance of genetic polymorphism in an intertidal barnacle: habitat- and life-stage-specific survivorship of Mpi genotypes. *Evolution*, **55**, 1336–1344.
Schneider S, Roessli D, Excoffier L (2001) *ARLEQUIN (version 2.0): A software for population genetics data analysis*. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva, Switzerland.
Siegel D, Kinlan B, Gaylord B, Gaines S (2003) Lagrangian descriptions of marine larval dispersion. *Marine Ecology Progress Series*, **260**, 83–96.
Smith AJ (1989) The English Channel — by geological design or catastrophic accident? *Proceedings of the Geological Association*, **100**, 325–337.
Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.
Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.
Turelli M, Barton NH, Coyne JA (2001) Theory and speciation. *Trends in Ecology & Evolution*, **16**, 330–343.
Ursin E (1960) A quantitative investigation of the Echinoderm fauna of the central North Sea. *Meddelser fra Danmarks Fiskeri-og Havundersogelser*, **2**, 1–204.
Veliz D, Bourget E, Bernatchez L (2004) Regional variation in the spatial scale of selection at MPI* and GPI* in the acorn barnacle *Semibalanus balanoides* (Crustacea). *Journal of Evolutionary Biology*, **17**, 953–966.
Via S (2001) Sympatric speciation in animals: the ugly duckling grows up. *Trends in Ecology & Evolution*, **16**, 381–390.
Wares JP, Gaines SD, Cunningham CW (2001) A comparative study of asymmetric migration events across a marine biogeographic boundary. *Evolution*, **55**, 295–306.
Waters JM, Roy MS (2004) Phylogeography of a high-dispersal New Zealand sea-star: does upwelling block gene-flow? *Molecular Ecology*, **13**, 2797–2806.
Webb CM, Tyler PM (1985) Post-larval development of the common north-west European brittle-star *Ophiura ophiura, O. Albida* and *A. Brachiata* (Echinodermata: Ophiuroidea). *Marine Biology*, **89**, 281–292.
Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.
Wiens JJ (2004) Speciation and ecology revisited: phylogenetic niche conservatism and the origin of species. *Evolution*, **58**, 193–197.
Wilson RA (1999) *Species. New Interdisciplinary Essays*. MIT Press, Cambridge, Massachusetts.

This work is part of D. Muths's doctoral research on population structure of two brittle-star species, *A. brachiata* and *O. fragilis*. Her research involves the use of various genetic markers to study processes acting at different scales, from evolutionary history to fine scale spatio-temporal genetic structure, as well as demographic survey of several populations. This work was co-directed by Dr D. Jollivet and Pr D. Davoult, from the 'EGPM' and the 'Benthic Ecology' teams respectively at the Station Biologique de Roscoff (www.sb-roscoff.fr). This teams carry out wide ranging and interconnected projects on coastal marine algae and invertebrates as well as hydrothermal vent fauna.
