Molecular Ecology (2006) 15, 3693–3705 doi: 10.1111/j.1365-294X.2006.03043.x

Phylogeography and population structure of thornback rays (*Raja clavata* L., Rajidae)

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Abstract

The phylogeography of thornback rays (*Raja clavata*) was assessed from European waters, using five nuclear microsatellite loci and mitochondrial cytochome *b* sequences. Strong regional differentiation was found between the Mediterranean basin, the Azores and the European continental shelf. Allelic and haplotype diversities were high in Portuguese populations, consistent with the existence of a refugium along the Iberian Peninsula. Unexpectedly, high diversity was also found in the English Channel/North Sea area. The lowest genetic diversity was found in the Black Sea. Populations sampled from the Mediterranean, Adriatic and Black Seas were characterized by a single mitochondrial haplotype. This haplotype was also the most ancestral and widespread outside of the Mediterranean basin except for the Azores. Populations from the Azores were dominated by a second ancestral haplotype which was shared with British populations. Results from multidimensional scaling, amova and nested clade analysis indicate that British waters are a secondary contact zone recolonized from at least two refugia — one around the Iberian Peninsula and one possibly in the Azores. Links to a potential refugium known as the Hurd Deep, between Cornwall and Brittany, are discussed. Finally, a historical demographic analysis indicates that thornback ray populations started to expand between 580 000 and 362 000 years ago, which suggests that the Last Glacial Maximum (20 000 years ago) had mainly affected the distribution of populations rather than population size.

Keywords: cytochrome *b*, elasmobranchs, historical demography, Hurd Deep, microsatellites, phylogeography, Rajidae, thornback rays

Received 7 March 2006; revision received 5 May 2006; accepted 2 June 2006

Introduction

The distribution of the North Atlantic biota has been drastically affected by the Pleistocene glacial/interglacial cycles over the past 2.4 million years (Myr). The Last Glacial Maximum (LGM), which occurred *ca.* 20 000 years ago, shaped contemporary distributions of both terrestrial (Hewitt 1999) and shallow-water, marine organisms (Cunningham & Collins 1998). Along European coastlines, ice sheets and perennial sea ice, extended approximately to the Bay of Biscay (Frenzel *et al.* 1992; Svendsen *et al.* 2004). Marine populations either became extinct or were forced to retreat southward into one or more refugial areas potentially including Atlantic islands, the Spanish–Portuguese–North African coast and the Mediterranean. As the ice retreated, populations recolonized northward into previously ice-covered areas.

Marine species present a double challenge in our attempts to generalize recolonization patterns in relation to the LGM. Sessile organisms (e.g. seaweeds, seagrasses, and numerous invertebrates) experienced a different set of conditions from those encountered by coastal mobile and pelagic organisms (e.g. crabs, shrimp, and fishes). Shallow subtidal/intertidal organisms with short distance dispersal would have experienced direct loss of habitat due to ice scouring, local extinction and, eventually, strong genetic differentiation between isolated refugia. In contrast, highly mobile

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organisms with long-lived, pelagic larval stages may have been able to escape local extinction because their habitats were only marginally modified and/or the possibility to move away from the front was much stronger. In this case, recolonization of previously ice-covered areas would have been rapid with little population differentiation between regions. Coastal demersal species like skates and rays — with low fecundity, no pelagic larval stage, and benthic eggs — present an intermediate case between benthic, sessile taxa and pelagic, migratory taxa.

Using molecular signatures present in contemporary population genetic and phylogeographic structure of species, historical processes can be inferred with respect to recolonization pathways, location of refugia and demographic bottlenecks or expansions (Avise 2000). Recent phylogeographic studies in the northeastern Atlantic consistently separate the Mediterranean from the Atlantic regardless of a benthic or pelagic life history, e.g. marine algae and seagrasses (Coyer *et al.* 2003; Olsen *et al.* 2004; Provan *et al.* 2005), invertebrates (Zane *et al.* 2000; Wilke & Pfenninger 2002; Duran *et al.* 2004; Baus *et al.* 2005), and bony fishes (Borsa *et al.* 1997; Gysels *et al.* 2004; Cimmaruta *et al.* 2005; Nakadate *et al.* 2005). Along the northeastern Atlantic coast alone, however, patterns of recolonization are complex and still poorly understood although the Iberian peninsular region appears to be a refugium for most taxa so far studied, e.g. marine algae (Coyer *et al.* 2003; Olsen *et al.* unpublished), invertebrates (Luttikhuizen *et al.* 2003) and some bony fishes (Cortey & Garcia-Marin 2002; Gysels *et al.* 2004).

Raja clavata, the thornback ray, ranges throughout the eastern Atlantic Ocean from the Faeroe Islands to Mauritania, including adjacent waters the Mediterranean and Black Seas (Stehmann & Bürkel 1994). It has also been reported from South Africa and in the western Indian Ocean, although its taxonomic status in these areas remains equivocal. Interest in the phylogeography of *R. clavata* stems from the fact that ray populations are in steep decline as a consequence of both their life history type and their extreme vulnerability to trawl fisheries (Walker & Hislop 1998; Dulvy *et al.* 2000; Heessen 2004). Thornback rays have a long generation time of 9–12 years (Walker 1998), a slow growth rate, produce only 48–150 eggs/female/year (Holden 1975; Ryland & Ajayi 1984; Ellis & Shackley 1995), and have an oviparous reproductive mode. Eggs are deposited in shallow coastal waters (< 10 m deep), where they attach to substrate and young hatch 4–5 months later (Ellis & Shackley 1995). Although tagging studies have suggested a low migration rate (Walker *et al.* 1997), a recent population genetic study around the Britain indicated a higher level of gene flow between the North Sea, English Channel and Irish Sea (Chevolot *et al.* in press) than initially predicted. Insights from the larger geographical genetic pool are expected to provide a deeper temporal and wider spatial framework for understanding the history and future of thornback rays.

In the present analysis, we survey regional population genetic and phylogeographic structure in the thornback ray (*Raja clavata*) in European waters. We focus on two general questions: (i) how has recent climatic history shaped the regional distribution of *R. clavata* along northeastern Atlantic shores including the Mediterranean basin; and (ii) to what extent are historical imprints of refugia, recolonization and demographic expansion detectable?

Materials and methods

Sampling and DNA extraction

A total of 385 rays were sampled from 20 locations during bottom trawl surveys conducted between 2002 and 2005 (Fig. 1, Table 1) and from local fish markets in a few cases (Lisbon, Faro and Varna on the Black Sea). Muscle tissue was collected from each individual and preserved in 70% ethanol. Total genomic DNA was extracted using either a CTAB cetyltrimethyl ammonium bromide-modified (Hoarau *et al.* 2002) or a silica-based extraction protocol (Elphinstone *et al.* 2003).

Genotyping, SSCP and sequencing

All individuals were genotyped for five microsatellite loci as described in Chevolot *et al.* (2005). Polymerase chain reaction (PCR) products were separated on a 6% polyacrylamide gel and visualized with an ABI PRISM-377 automatic sequencer (Applied Biosystems). Allele size was determined using an internal lane standard and GENESCAN software.

A 290-bp fragment of the mitochondrial cytochrome b gene was amplified by PCR using the primers Cb-F (5′-CACAGATAAAATCCCATTCC3′), fluorescently 5′ labelled with 6-FAM and Cb-R (5′CCGCCCAATCACTCAAACC-3′), fluorescently 5′ labelled with HEX. PCR reactions were performed in a 10-μL total vol containing 1–3 μL of extracted DNA (< 1 ng), 1× reaction buffer (Promega), 0.2 mM of each dNTP, 0.25 U *Taq* DNA polymerase (Promega), 2 mM $MgCl_2$ and 0.5 μM of each primer. PCR amplifications were performed with either a PTC-100 Thermocycler (MJ Research) or Mastercycler gradient cycler (Eppendorf). PCR conditions were: initial denaturation for 1 min at 94 °C, followed by 30 cycles of denaturation for 30 s at 94 °C, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min 30, and followed by a final extension step at 72 °C for 10 min.

Single-strand conformation polymorphism (SSCP) (Orita *et al.* 1989; Sunnucks *et al.* 2000) was used to detect sequence variation in mitochondrial DNA (mtDNA) fragments. Point mutations affect conformation of the single DNA strand which can be visualized on nondenaturing polyacrylamide gels. Because mutations can affect the mobility of one or both strands differently, different fluorescent labelling of

Table 1 Sampling locations for *Raja clavata*

Locations	Code	GPS coordinates		Period	Sample size	Collector*
North Sea/English Channel/Irish Sea						
Outer Wash	Ow	53.50	0.67	Jan 2002	23	IBTS
North Thames Estuary 2	Nt2	52.06	2.14	Jan 2002	54	IBTS
		52.09	2.01			
		52.02	2.00			
		52.08	2.01			
North Thames Estuary 1	Nt1	52.06	2.14	July 2003	25	CORY
		52.09	2.01			
		52.02	2.00			
		52.08	2.01			
English Channel	Ec	50.75	−0.67	April 2002	19	RIVO
		50.77	−0.73			
East English Channel	Ee	49.99	1.13	Nov 2003	43	CGFS
		49.99	1.18			
Tremadog Bay	Tb	52.33	−4.35	Sept 2003	18	CORY
		52.63	−4.29			
		52.72	−4.51			
Liverpool Bay	Lb	53.58	−3.36	Sept 2003	25	CORY
		53.62	−3.62			
		53.64	−3.30			
Gulf of Biscay						
Ajo	Aj	43.71	−5.20	April 2004	17	DEMERSALES
		43.75	−5.38			
Penas	Pe	43.46	−3.37	April 2004	13	DEMERSALES
		43.48	−3.34			
Estaca	Es	43.70	−7.05	April 2004	21	DEMERSALES
		43.62	−7.04			
		43.77	−6.91			
		43.80	−6.57			
Portugal						
Algarve	Al	—	—	June 2005	6	Fish market Faro
Lisboa	Li	—	—	June 2005	9	Fish market
Mediterranean Basin						
Corsica	Co	41.58	9.54	July 2003	9	MEDITS
		41.82	9.44			
		42.18	9.60			
		41.95	9.57			
Adriatic Sea	Ad	44.77	12.69	Dec 2002	25	GRUND
		43.36	14.30			
		42.16	16.69			
Black Sea						
Varna	Bs	—	—	Nov 2004	35	Fish market
Azores Islands						
Faial	Fa	38.64	−25.66	May 2001	7	ARQDAÇO
Graciosa	Gr	39.10	−27.97	Spring 2002	10	ARQDAÇO
		39.11	−28.05			
		39.10	−28.00			
Sao Miguel	Sa	37.67	−25.45	Spring 2001	8	ARQDAÇO
		37.77	−25.77			
		37.76	−25.77			
		37.79	−25.80			
Terceira	Te	38.75	−27.40	Spring 2002	7	ARQDAÇO
		38.66	−27.30			
		38.66	−27.31			
		38.63	−27.24			
Flores	Fl	39.39	−31.14	Spring 2002	8	ARQDAÇO
		39.42	−31.28			
		44.42	−31.28			
		39.72	−31.08			

*IBTS, International Bottom Trawl Survey; CORY, Center for Environmental Fisheries and Aquaculture Science (CEFAS) ground fish survey; MEDITS, Mediterranean International Trawl Survey; GRUND, Italian National Survey program; RIVO, The Netherlands Institute for Fisheries special survey; CGFS, Channel Ground Fish Survey; ARQDAÇO, Azores Archipelago Survey; DEMERSALES, demersal fish survey.

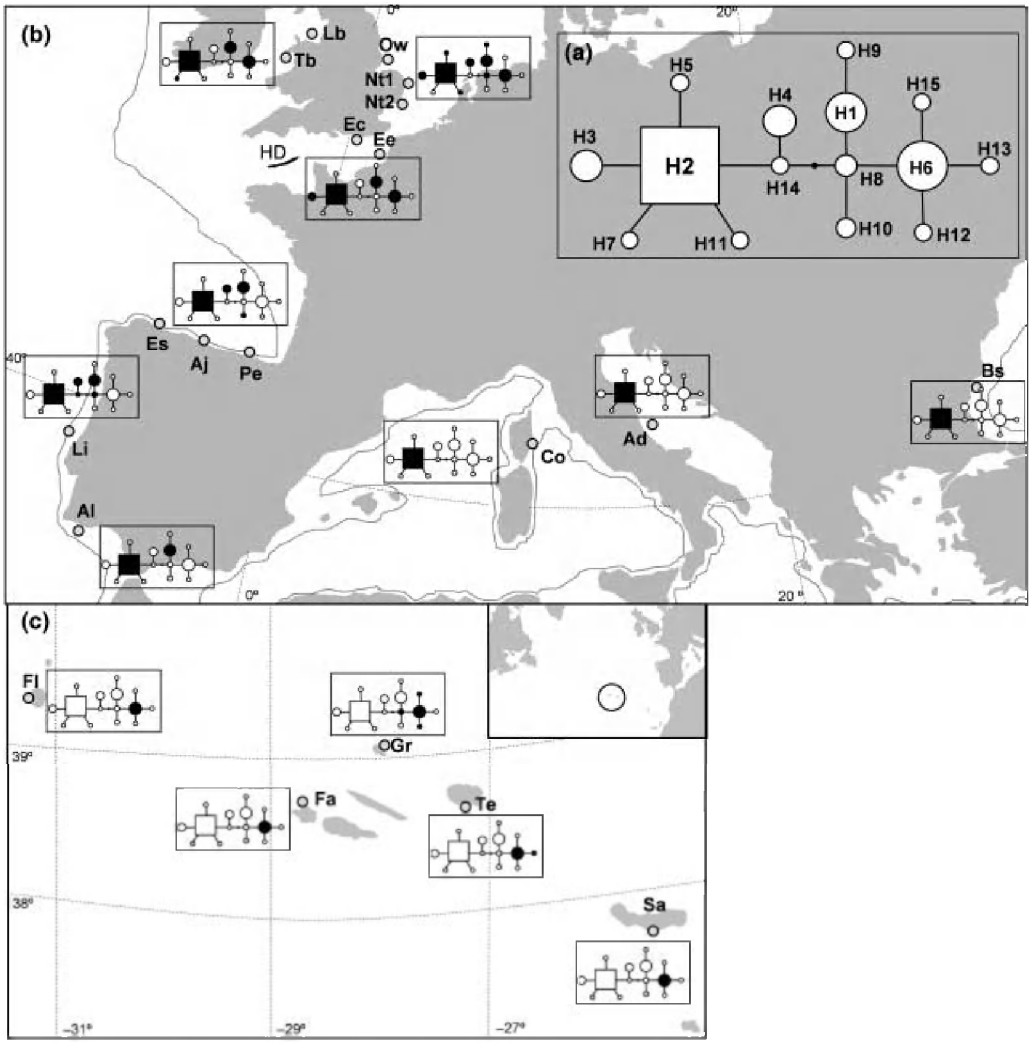

Fig. 1 Sampling locations (see Table 1) and distribution of mtDNA haplotypes. The heavy grey line delimits the European continental shelf, and the black line delimits the Hurd-Deep (HD). (a) Statistical parsimony network of haplotypes (95% confidence level) in which the square indicates the most likely ancestral haplotype; other haplotypes are indicated by circles; and one hypothesized haplotype is shown by the black dot; square and circle sizes are proportional to haplotype frequencies. (b) Sampling locations and haplotype distribution along the continental shelf, Mediterranean and Black Seas in which the filled circles or square indicate the presence of the haplotype. (c) Sampling locations and haplotype distribution in the Azores (1300 km west of Portugal).

each DNA strand was used to increase the sensitivity of detection (Lescasse 1999). SSCP gels were run on an ABI PRISM-377 automatic sequencer (Applied Biosystems) as described in Coyer *et al.* (2002), except that we used a 0.3× MDE (mutation detection enhancement gel solution; BMA Bioproducts) concentration and added 5% glycerol to the gel. SSCP gels were analysed independently and all differing haplotypes were subsequently sequenced. When > 5 individuals had the same SSCP haplotype, at least two individuals were sequenced. PCR products were cleaned with ExoSapIt (USB Corporation) enzyme following the provider's instructions. Sequencing reactions were performed using the Big Dye Terminator Kit (Applied Biosystems) and run on an ABI PRISM-377 automatic sequencer (Applied Biosystems). Both strands were sequenced. Sequences were edited using BIOEDIT version 7.0.1 (Hall 1999), aligned with CLUSTALW and then checked by eye.

Data analysis

Microsatellite loci were assessed for null alleles, stuttering and large allele dropout using MICRO-CHECKER 2.2.1 (Van Oosterhout *et al.* 2004). GENETIX 4.05 (Belkhir *et al.* 2004) was used for the following calculations: linkage disequilibrium for all loci and for all locations using the LinkDis procedure (Black & Krafsur 1985); observed (H_O) and nonbiased expected (H_E) heterozygosities (Nei 1978); allelic richness for each locus individually and as a multilocus

estimate per location; and single and multilocus F_{IS} estimates using Weir & Cockerham *f* (1984). Significance was tested against 3000 permutations. GENECLONE β version (Arnaud-Haond & Belkhir, available on request) was used to correct for unequal sample sizes in determining mean allele number. The method utilizes a jackknife resampling procedure of individuals at each location. The corrected allelic diversity (N_c) was based on 1000 resamplings and corrected for the smallest sample size (See Table 1). Haplotype (*h*) and nucleotide (π) (Nei 1987) diversities of mtDNA were estimated using DNASP version 3.53 (Rozas & Rozas 1999).

Population differentiation was estimated using Wright's F_{ST} (Wright 1969) for both the microsatellite and mtDNA data. Global and pairwise F_{ST} were estimated using the Weir & Cockerham (1984) θ estimator with GENETIX 4.05 (Belkhir *et al.* 2004) and significance was tested against 3000 permutations.

For the mtDNA sequence data, a model for molecular evolution was selected using MODELTEST version 3.06 (Posada & Crandall 1998). A hierarchical test of likelihood was performed under 56 models. The HKY model, which takes account for unequal nucleotide frequencies and two substitution types (transition and transversion) was selected as it was supported by the likelihood ratio test (LRT) ($P < 0.0001$). The parameters of the model were f(A) = 0.2581; f(T) = 0.2676 f(C) = 0.3566; f(G) = 0.1177, and transition/transversion ratio = 6.46. For the mtDNA data, pairwise genetic distances between populations were estimated in ARLEQUIN 2.0 (Schneider *et al.* 2000) with Tamura and Nei genetic distance, as the HKY model is not implemented in ARLEQUIN 2.0 and the Tamura and Nei model was the next best model in the LRT. For the microsatellite data, pairwise genetic distances were estimated using the GenDist procedure implemented in the PHYLIP version 3.5c package (Felsenstein 1989). Cavalli-Sforza & Edwards (1967) genetic distances were used. To assess whether any group structure could be detected in the data set, a multidimensional scaling analysis (MDSA) was computed in STATISTICA 7.0 (StatSoft Inc. 2004) using the estimated pairwise genetic distances as described above. Next, a hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN 2.0 (Schneider *et al.* 2000) to test for the significance of the groups observed in the MDSA.

Isolation by distance (IBD) was tested among the 20 locations using a Mantel test (Mantel 1967) as implemented in GENETIX 4.05 with the log of geographical distance and $\theta/1-\theta$ for the microsatellite data and the Tamura and Nei's genetic distance for the mtDNA (Rousset 1997). In the same way, IBD was tested among the European/Azores locations and among the European continental shelf locations only. Significance was tested using 10 000 permutations.

Intraspecific relationships among the mtDNA haplotypes were inferred using statistical parsimony in the software package TCS version 1.13 (Clement *et al.* 2000) followed by a nested clade analysis (NCA) following the nesting rules of Templeton *et al.* (1995). This analysis provides a way to separate contemporary and historical events in the observed population structure by testing for geographical associations between haplotypes and nested clades. Significance of clade distances (D_c), which measures the geographical spread of a clade; and nested clade distances (D_n), which measures how a clade is distributed in comparison to other clades with same nested clade-level was calculated in GEODIS 2.4 (Posada *et al.* 2000) with 1000 permutation tests. The output was interpreted using the latest Templeton Inference key (Templeton 2004).

A generalized skyline plot was used to infer historical demographic changes in thornback rays. This method is particularly useful with partially resolved phylogenies and low sequence variation (Strimmer & Pybus 2001). The first step in the analysis is to generate genealogical trees with branch lengths proportional to time. Maximum likelihood (ML) trees were estimated under the HKY model (see above) and a molecular clock assumption in PAUP version 4.0b10 (Swofford 1998). Next, the generalized skyline plot was generated in GENIE 3.0 (Pybus *et al.* 2000) in which the ML trees were subjected to a smoothing algorithm. The ε parameter governing this algorithm was chosen using the 'maximize optimization' option which must be large enough to reduce the noise in the data, but small enough to preserve the demographic signal in the data. The third step involved dating the estimated demographic expansion. To do this, we first estimated the substitution rate for the 290-bp fragment of the cytochrome *b* gene between sequences of Rajini (*R. clavata*, *R. brachyura*, *R. montagui*, *R. undulata* and *R. asterias*) and Amblyrajini (sister group: *Amblyraja radiata* and *Leucoraja naevus*). Nucleotide substitution rates ranged from 0.15 and 0.24 per site. The divergence time between the Rajini and Amblyrajini has been previously estimated at 31 million years (Myr) based on mt 16S sequence divergence scaled with fossil records (Valsecchi *et al.* 2005). Therefore, the nucleotide substitution rate could be estimated at between 0.008 and 0.005 per site/Myr.

Results

Genetic diversity

None of the microsatellite loci showed evidence for null alleles, stuttering or large allele dropout. Pairwise comparisons between loci revealed no linkage disequilibrium after sequential Bonferroni corrections. The mean number of alleles per locus ranged from 4.2 (locus Rc-B3) to 15.4 (locus Rc-B6) (Table 2). The mean allelic richness per location was corrected for sample size to allow comparison between sampling sites. The lowest genetic diversity was found in the Azores samples (3.7–4.4) and in the Black Sea samples (4.5) (Table 2). The highest allelic diversities were observed in southern Portugal at Al (6.4) and in the English Channel

Table 2 Summary statistics of the genetic variability for five microsatellite loci and cytochrome *b* data at 20 sampling sites for *Raja clavata*

Loci	Locations	Ow	Nt2	Nt1	Ec	Ee	Tb	Lb	Aj	Pe	Es	Li	Al	Co	Ad	Bs	Fa	Gr	Sa	Te	Fl	Mean N_A/locus
Rc-B3	N_a	4	5	4	4	6	5	6	4	3	5	4	4	4	5	4	3	3	3	4	4	4.2
	H_E	0.602	0.496	0.552	0.627	0.617	0.595	0.558	0.562	0.541	0.624	0.675	0.636	0.699	0.666	0.492	0.582	0.631	0.341	0.714	0.691	
	H_O	0.636	0.428	0.458	0.556	0.561	0.529	0.400	0.562	0.308	0.500	0.625	0.667	0.778	0.560	0.412	0.428	0.615	0.975	0.714	0.750	
	f	–0.060	0.138	0.170	0.120	0.090	0.113	**0.288**	0.000	**0.440**	0.200	0.078	–0.050	–0.120	0.170	0.170	0.280	0.020	–0.100	0.000	–0.090	
Rc-B4	N_a	14	30	19	19	24	17	18	17	14	16	7	10	11	16	9	9	10	10	12	10	14.6
	H_E	0.886	0.941	0.938	0.95	0.938	0.951	0.942	0.952	0.942	0.941	0.867	0.955	0.935	0.913	0.837	0.934	0.890	0.925	0.978	0.925	
	H_O	0.739	0.870	0.880	1.000	0.954	0.833	0.826	0.941	0.923	0.952	0.500	1.000	1.000	0.880	0.827	0.857	0.846	0.625	1.000	0.875	
	f	**0.167**	**0.070**	0.060	–0.050	–0.010	**0.120**	**0.125**	0.010	0.020	–0.013	**0.440**	–0.050	–0.070	0.030	0.010	0.090	0.050	**0.340**	–0.020	0.006	
Rc-B6	N_a	19	25	19	22	27	20	24	21	17	18	13	10	12	22	16	3	6	6	4	4	15.4
	H_E	0.935	0.913	0.921	0.954	0.911	0.950	0.935	0.961	0.939	0.948	0.954	0.970	0.954	0.951	0.855	0.582	0.705	0.775	0.692	0.641	
	H_O	0.957	0.865	0.920	0.947	0.977	1.000	0.875	1	1	0.905	0.667	0.833	0.778	0.920	0.743	0.714	0.842	0.750	0.714	0.375	
	f	–0.020	0.050	0.001	0.010	–0.070	–0.050	0.060	–0.040	–0.060	0.040	**0.31**	**0.150**	**0.190**	0.030	**0.130**	–0.250	–0.210	0.030	–0.030	**0.430**	
Rc-E9	N_a	6	8	6	5	7	6	4	4	5	4	5	4	4	5	5	2	3	2	1	1	4.35
	H_E	0.534	0.467	0.384	0.543	0.363	0.544	0.367	0.362	0.455	0.398	0.533	0.636	0.529	0.399	0.700	0.143	0.1508	0.125	0	0	
	H_O	0.609	0.389	0.320	0.631	0.325	0.625	0.333	0.235	0.385	0.381	0.500	0.667	0.444	0.417	0.645	0.143	0.1538	0.125	0	0	
	f	–0.140	**0.160**	0.170	–0.160	0.110	–0.160	0.090	**0.360**	0.160	0.040	0.06	–0.05	0.170	–0.05	0.030	0.000	–0.020	0	—	—	
Rc-G2	N_a	5	9	5	5	7	6	7	5	3	7	4	4	4	5	3	4	3	3	3	4	4.8
	H_E	0.691	0.677	0.598	0.665	0.641	0.638	0.665	0.449	0.514	0.638	0.542	0.560	0.601	0.595	0.510	0.651	0.428	0.700	0.385	0.600	
	H_O	0.696	0.630	0.760	0.790	0.651	0.833	0.640	0.353	0.615	0.571	0.444	0.500	0.667	0.520	0.457	0.500	0.539	0.625	0.429	0.500	
	f	–0.007	0.070	–0.280	–0.190	–0.160	–0.320	0.040	**0.220**	–0.200	0.100	0.19	0.120	–0.120	0.128	0.110	0.250	–0.270	0.110	–0.130	0.200	
Mean N_A		9.6	15.4	10.6	11	14.2	10.8	11.8	10.2	8.4	10	6.6	6.4	7	10.6	7.4	4.2	5	4.8	4.8	4.6	8.7
Mean N_c		5.3	5.4	5.1	5.9	5.3	5.6	5.2	5.6	5.3	5.5	5.1	6.4	5.6	5.5	4.5	3.7	3.7	4	4.4	3.8	
Multilocus H_E	0.730	0.701	0.679	0.748	0.697	0.731	0.693	0.657	0.678	0.710	0.714	0.752	0.744	0.707	0.678	0.578	0.561	0.573	0.554	0.575		
Multilocus f	0.003	**0.090**	0.017	–0.050	0.000	–0.040	**0.120**	0.060	0.050	0.070	**0.250**	0.020	0.015	0.070	0.080	0.090	–0.070	0.140	–0.030	0.140		
Cytb	N_h	5	6	4	3	4	4	3	2	3	3	5	2	1	1	1	1	3	1	2	1	2.65
	N_C	3	2.6	2.3	2	2.3	1.9	1.7	1.6	2.3	2.6	2.7	2	1	1	1	1	2	1	1.8	1	
	h	0.640	0.510	0.420	0.368	0.460	0.310	0.240	0.220	0.410	0.600	0.720	0.530	0.000	0.000	0.000	0.000	0.420	0.000	0.290	0.000	
	π	0.006	0.006	0.004	0.005	0.005	0.003	0.003	0.002	0.006	0.006	0.007	0.007	0.000	0.000	0.000	0.000	0.002	0.000	0.001	0.000	

N_A, number of alleles; N_c, number of alleles corrected for sample size (N = 6 individuals); H_E, nonbiased expected heterozygosity (Nei 1978), H_O, observed heterozygosity; f, inbreeding coefficient (Weir & Cockerham 1984). N_h, number of haplotypes; h, haplotype diversity (Nei 1987); π, nucleotide diversity (Nei 1984). Nt2, Lb and En showed a significant heterozygote deficiency (corrected $P < 0.05$) after sequential Bonferroni corrections.

at Ec (5.9) (Table 2). The mean expected heterozygosity was lowest in the Azores samples (0.554–0.578) but relatively uniform among the other sampling sites, ranging from 0.68 to 0.75 (Table 2). Locations Nt2, Lb and Li showed a significant departure from Hardy–Weinberg equilibrium (Table 2) (multilocus $f = 0.09$, 0.12 and 0.25, respectively).

Fifteen mtDNA haplotypes were detected among the 385 individuals sampled from 20 locations (Fig. 1, GenBank Accession nos DQ408224–DQ408238). The difference among the haplotypes was due to 13 polymorphic sites of which nine were informative. All differences were due to substitutions; no indels were found. The global haplotype and nucleotide diversities were 0.503 and 0.006, respectively. The highest haplotype diversities were found in central Portugal at Li ($h = 0.72$) and in the North Sea at Ow ($h = 0.64$). The lowest diversities were found in three locations in the Azores (Fa, Sa, Fl), Adriatic and Black Seas (Table 2). The geographical distribution of haplotype diversities and microsatellite allelic diversities were generally congruent.

Population differentiation

Thornback ray populations are significantly differentiated throughout European waters. The global θ across all samples was 0.042 for the microsatellite (θ_{msat}) and 0.348 for the mtDNA (θ_{mtDNA}): both were highly significant ($P < 0.0001$). Pairwise comparisons further revealed three regional groupings separating the Azores, the Atlantic continental shelf and the Mediterranean/Black Seas. Pairwise θ_{msat} and θ_{mtDNA} between the Azores and European continental shelf populations were 0.086 ($P < 0.0001$) and 0.6 ($P < 0.0001$), respectively, between the Mediterranean and European continental shelf, $\theta_{msat} = 0.04$ ($P = 0.006$) and $\theta_{mtDNA} = 0.15$ ($P < 0.0001$). A hierarchical analysis molecular of variance (AMOVA) was performed with the following groups: (i) a British water group with Ow, Nt1, Nt2, Ec, Ee, Tb and Lb; a southern Bay of Biscay group with Aj, Es, Pe; (ii) a Portuguese group with Li and Al; (iii) an Azores group with Gr, Fl, Sa, Te, Fa; and (iv) a Mediterranean/Black Sea group with Co, Ad, Bs. The amount of variation explained by the differences within locations was high and highly significant for both molecular markers (94.1% for the microsatellite data and 53.63% for mtDNA data) as well as the amount of variation explained by the differences among groups (4.7%, $P < 0.0001$; 46.32%, $P < 0.0001$, respectively).

The MDSA was performed only on European continental shelf locations as the Azores and the Mediterranean/Black Sea sampling sites were highly genetically distant. The microsatellite data set recovered three groups: (i) the British water (Ow, Nt1, Nt2, Ec, Ee, Tb and Lb) and southern Bay of Biscay locations (Aj, Es, Pe); (ii) central Portugal (Li); and (iii) southern Portugal (Al) (Fig. 2a). The mtDNA data set further subdivided the southern Bay of Biscay and British waters locations (Fig. 2b).

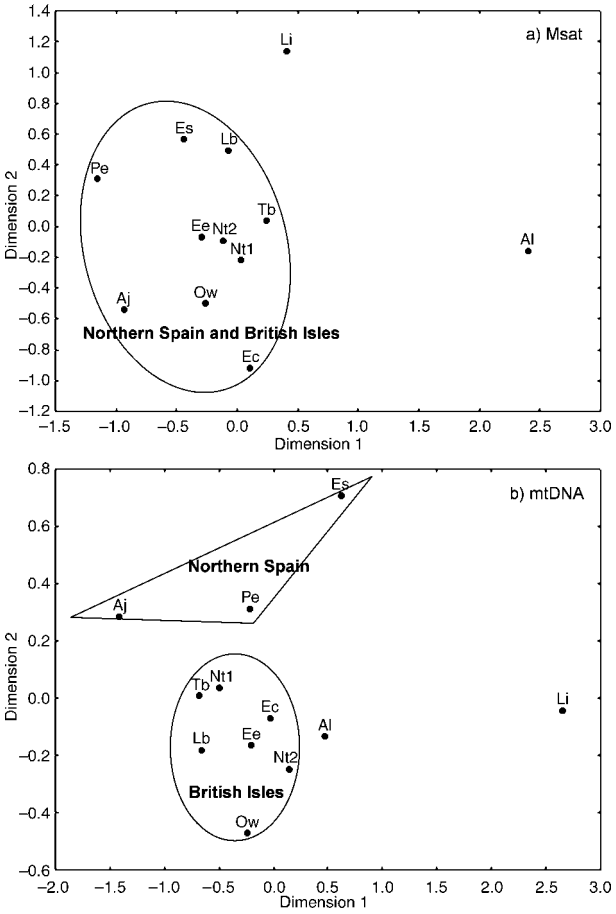

Fig. 2 Multidimensional scaling plot for continental shelf locations based on (a) microsatellite (Cavalli-Sforza & Edwards) genetic distances (Stress value = 0.002); and (b) mtDNA (Tamura & Nei) genetic distances (Stress value = 0.05). Sample abbreviations are given in Table 1. Mediterranean and Azores samples were extremely distant and therefore not included.

Based on the results of the MDSA clusters of sampling locations, a hierarchical AMOVA was performed to test the significance of these clusters. Because an AMOVA cannot be conducted with groups of less than two, in a first analysis we excluded Al, Li. In a second analysis, we subsequently grouped them together to form a Portuguese cluster, despite the fact that the MDSA shows that they were genetically distant. In the first analysis, the largest amount of variation was explained by the differences within location (98.52% for the microsatellite data and 96.85% for mtDNA data). Among clusters, the variance was not significant for the microsatellite data (0.55%, $P = 0.08$), but highly significant for the mtDNA data (3.15%, $P < 0.0001$). Results from the second analysis were similar with the largest amount of variation due to within-population variation (98.7% and 88.97%, for the microsatellite and mitochondrial data, respectively) and variation among groups was significant only for the mitochondrial data (10.54%, $P < 0.0001$) but

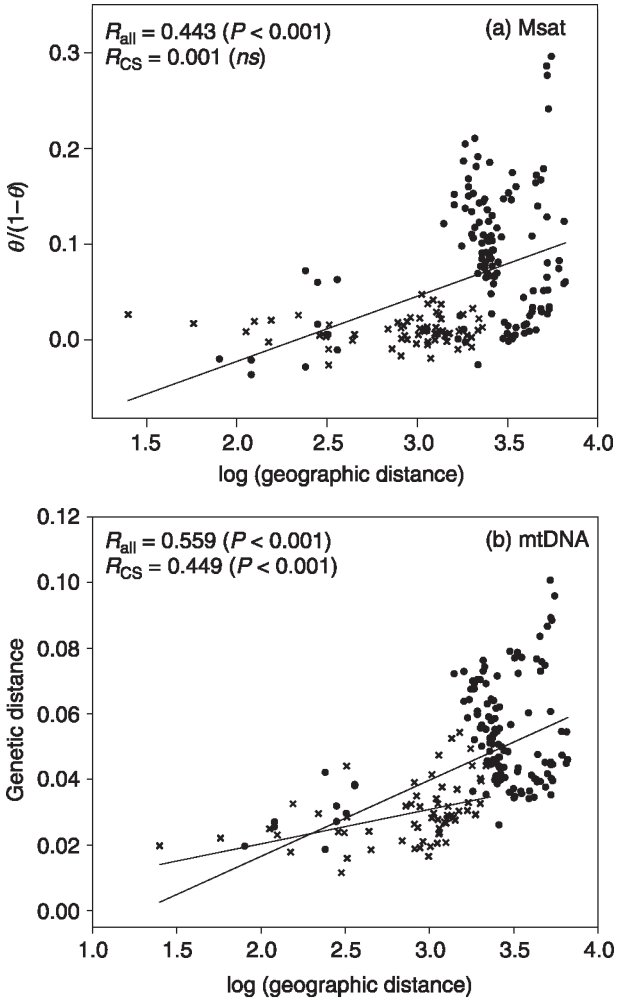

Fig. 3 Isolation by distance based on all locations (solid line) and within continental shelf locations only (dotted line). Crosses represent pairwise comparisons within continental shelf locations, dots represent all other pairwise comparisons. (a) Microsatellite distances and (b) mtDNA distances.

not for the microsatellites (0.46%, $P = 0.06$). This suggests that British waters, Southern Bay of Biscay and Portuguese locations are genetically differentiated for the mitochondrial marker only.

Isolation by distance

When all sampling locations were compared, there was significant isolation by distance (IBD) with both markers ($Z = 78.58$, $P < 0.001$ for microsatellites; $Z = 56.56$, $P < 0.001$ for mtDNA) (Fig. 3a, b, solid lines). Excluding the Mediterranean/Black Sea, a significant IBD with both sets of markers (not shown) was also found ($Z = 48.49$, $P = 0.02$ for microsatellites; $Z = 35.72$ $P < 0.001$ for mtDNA). When the analysis was restricted to the continental shelf locations alone, only the mtDNA showed a significant correlation

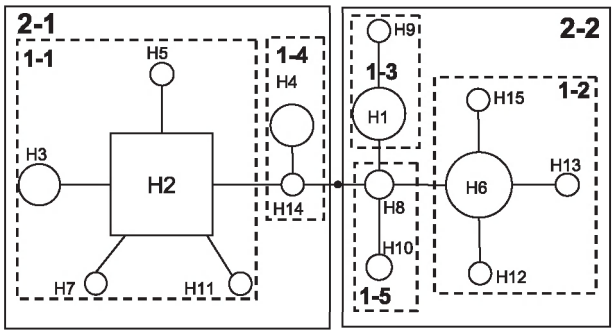

Fig. 4 Nested clade design for *Raja clavata* following the nesting rules of Templeton *et al.* (1995). Each branch represents one mutational step and the black dot denotes a missing haplotype. Lowest level clades are enclosed within dashed boxes. See Table 3 for phylogeographic inference.

($Z = 11.77$, $P < 0.001$) (Fig. 3a, b, dotted lines). These results are consistent with patterns of diversity and population differentiation described above.

Geographical distributions of the mtDNA haplotypes

The statistical parsimony networks (Figs 1 and 4) revealed a starlike genealogy with two main clades. Clade 2-1 contains the most common haplotype H2 (64%), which is found everywhere except in the Azores. This clade is connected to Clade 2-2 by a single-step mutation. Clade 2-2 contains the second most common haplotype H6 (12%), which is found only in the Azores and in British waters. The remaining haplotypes had more restricted geographical distributions, e.g. H3 was only detected in the North Sea and the English Channel and H4 in the southern Atlantic samples (i.e. Aj, Pe, Es, Li) and in the North Sea (Nt1). The Mediterranean, Adriatic and Black Sea samples (Bs, Co and Ad) were fixed for H2, and most of the Azores samples were fixed for H6, except Te and Gr (Fig. 1). The other haplotypes found in the Azores samples are different from H6 by one substitution (Fig. 1).

The NCA showed that Clades 1-1, 2-1 and 2-2 have a significant association with their geographical distribution. At the lowest clade-level (one-step), isolation by distance or fragmentation was suggested for Clade 1-1 (involving the Mediterranean/Black Sea, Iberian Peninsula and British waters locations). At the two-step level, allopatry and/or fragmentation was suggested for Clade 2-2 (involving the Azores and British waters locations) and a contiguous range expansion for Clade 2-1 (involving Mediterranean/Black Sea, Iberian Peninsula and British waters locations) (Fig. 2, Table 3).

Historical demography

The generalized skyline plot (Fig. 5) indicates that the most likely model was piecewise expansion (Pybus *et al.* 2000)

Table 3 Phylogeographic inference from the nested clade analysis (Templeton 2004) of *Raja clavata*

Clade	Steps followed in inference key	Inference
1-1	1,2,3,4,9,10 No	Unable to distinguish between fragmentation and isolation by distance
2-1	1,2,11,12 No	Contiguous range expansion
2-2	1,2,3,5,9 No	Fragmentation/allopatry

Only clades with significant association between haplotype/clades and geographical distribution are shown. See Fig. 4 for the nested design.

with the following estimated parameters: $\theta = 0.73$ (population size at present); $\rho = 1990.01$ (exponential growth rate); and $\alpha = 0.003$ (population size prior to change). From these parameters, we could estimate the time of expansion, which gave $t = 0.0029$ substitutions per site. Using a substitution rate ranging between 0.005 and 0.008/Myr (see Materials and methods), the estimated time of expansion for *Raja clavata* was estimated to begin between 580 000 and 362 000 BP with relatively stable population size since then.

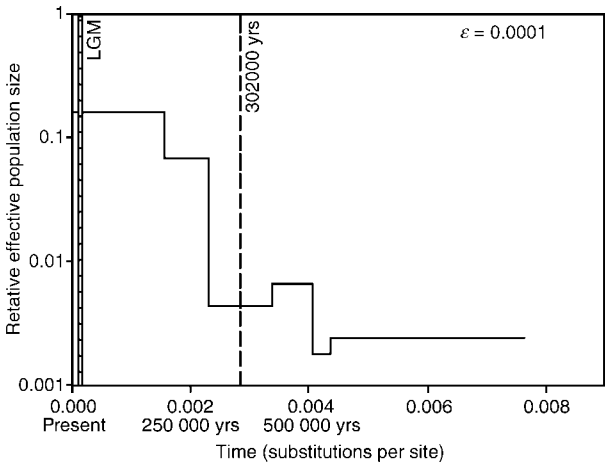

Fig. 5 Generalized skyline plot for *Raja clavata*. The *x*-axis represents the time since the present in substitutions per site and the *y*-axis the estimated effective population size scaled to substitution rate. The ε-parameter governing the smoothing algorithm was selected from the Akaike information criterion (AIC) (see Materials and methods). The dashed vertical line represents the estimated time of expansion for *R. clavata* and hatched bar the Last Glacial Maximum (LGM).

Discussion

Population structure is typically weak in most commercial marine fishes (Waples 1998). For baseline characterization, it is generally recommended that at a 50 adults/haul be sampled on the spawning grounds to avoid large sampling variance (Waples 1998). This is simply not possible for *Raja clavata* where even with conservative pooling of hauls (or no pooling), the number of individuals/location averaged around 20 (range 6–54). This means that our ability to detect significant population structure is relatively weak and appropriate caution should be exercised in interpreting the results. With these caveats in mind, however, population differentiation, MDSA and the isolation by distance revealed strong regional groupings corresponding to the Mediterranean basin, the Azores Islands and the European continental shelf. We define the Iberian Peninsula populations to include the two Portuguese samples and the three Spanish samples, the British water populations to include the samples collected in the North Sea, English Channel and the Irish Sea. Together, these represent the European continental shelf populations.

The Mediterranean has certainly served as a refugium for *R. clavata* based on microsatellite allelic diversity that probably predates the LGM. The presence of only a single, widespread, ancestral mitochondrial haplotype further supports this relictual distribution. It also suggests a strong bottleneck, subsequent isolation from the Atlantic and continually restricted gene flow between the Atlantic and the Mediterranean ($\theta_{msat} = 0.04$, $P = 0.006$ and $\theta_{mtDNA} = 0.15$, $P < 0.0001$). The low diversity observed in the Black Sea population for the microsatellite and mitochondrial data is also the probable result of a strong bottleneck due to past and current isolation. The Black Sea was a freshwater lake during the LGM (Ryan *et al.* 1997) and inhospitable for marine species. Therefore, recolonization of the Black Sea could only have occurred within the past 10 000 years.

The Azores were little affected by the LGM as compared with continental Europe (Rogerson *et al.* 2004) (Morton & Britton 2000) and almost certainly served as an offshore refugium for *R. clavata* populations. Indeed, the finding of the H6 haplotype in the Azores (but not in the Iberian or Mediterranean populations) is consistent with a divergent refugium. The low nuclear allelic diversity and the high genetic differentiation of the Azores are consistent with a strong bottleneck and physical isolation of the Azores with other locations, which are further supported by the NCA and the significant isolation by distance. The Azores lie some 1300 km from Portugal and 580 km from Madeira. Ocean depth apparently acts as a physical barrier to dispersal for thornback rays, as has been shown between continental shelf and Icelandic populations in flatfish (Hoarau *et al.* 2002).

The continental shelf populations form the third major regional group. Further differentiation within the continental shelf was only detectable with mtDNA including significant isolation by distance between the Iberian Peninsula and the more northerly British water populations. This suggests restricted gene flow between northern and southern

European populations. The smaller effective population size of the mtDNA and the stronger effects of genetic drift may explain the higher level of differentiation. Similar results have been found in plaice (Hoarau *et al.* 2004), the blacktip shark (Keeney *et al.* 2005) and the common goby (Gysels *et al.* 2004). The stronger level of genetic differentiation found with mtDNA marker in comparison to the nuclear one may also suggest a male-biased dispersal. Indeed, whereas microsatellites are biparentally inherited, mtDNA is generally only maternally inherited, and thus population structure with mtDNA represents limited female dispersal (Avise 2000). If a species show a high male dispersal and a low female dispersal, then higher population differentiation can be expected with mtDNA locus. Male-biased dispersal has been strongly suggested in several shark species from molecular markers, such as in the great white shark (Pardini *et al.* 2001), the shortfin mako (Schrey & Heist 2003), and the blacktip shark (Keeney *et al.* 2005). Recent tagging studies based on DST (data storage tags) showed that thornback rays were philopatric, although they could not make any distinction between males and females (Hunter *et al.* 2005).

Under the leading edge hypothesis (Ibrahim *et al.* 1996), genetic diversity is expected to decrease northward following the recolonization front, thus producing a latitudinal gradient of genetic diversity. Simultaneous with the northward expansion, the southern refugial areas may have experienced a bottleneck due to relatively sudden local warming (Hewitt 1999). In such a case, a unimodal distribution would be produced, i.e. low diversity at the southern (refugial) and northern (leading edge) boundaries with high diversity in the middle. Neither a latitudinal or unimodal distribution was observed for thornback rays. Instead, the highest diversity occurs at the putative leading edge, being the British waters and at the trailing edge being the Iberian Peninsula; and lowest diversity was found in the Azores and the Mediterranean Basin. Looking more carefully at the distribution of the two mtDNA haplotypes H2 and H6, they are both ancestral and both link to two different regions: the Mediterranean and Azores (Fig. 1). The high haplotype and allelic diversities and the absence of H6 haplotype in Iberian Peninsula populations strongly suggest the Iberian Peninsula to be a third refugium for *R. clavata* populations. Finally, the haplotype distribution shows that H2 and H6 haplotypes are only found together in British waters, consistent with British waters being a recent contact zone between Iberian Peninsula (H2) and Azores (H6) populations. This is supported by the starlike haplotype network involving H2 and H6. It is also supported by the contiguous range expansion and significant isolation by distance (mtDNA) found at the European continental shelf scale (Table 3, Fig. 3). Migrations of rays from the Azores to the British waters may be linked to today's winter-time circulation patterns, in which the northern branch of the Azorean current joins the North Atlantic current, forming the southwestern European current which reaches British waters (Morton & Britton 2000; Rogerson *et al.* 2004). Thus, it is likely that at least a few individuals carrying the H6 haplotype had contributed to the recolonization of the British waters at the same time that the H2 haplotype arrived from the continental coast.

Much of the outer continental shelf was exposed as a result of the sea-level drop during the last LGM (Frenzel *et al.* 1992) including a region of the nascent English Channel known as the Hurd Deep (Lericolais *et al.* 1995, 2003). The Hurd Deep is a depression 150 km long and 100 m deeper than the adjacent seafloor. It extends approximately between the southwestern tips of England (Cornwall) and northwest France (Brittany; see Fig. 1). During the LGM (and possibly earlier glaciations), this depression, including the surrounding exposed shelf, may have served as a huge marine inlet in which marine organisms were able to persist (Provan *et al.* 2005). A number of recent studies have detected high genetic diversity in the Hurd Deep area (i.e. both sides of the English Channel), i.e. the red alga *Palmaria plamata* (Provan *et al.* 2005), the fucoid algae *Fucus serratus* and *Ascophyllum nodosum* (Coyer *et al.* 2003; Olsen *et al.* unpublished), or in the North Sea the seagrasses *Zostera marina* and *Zostera noltii* (Coyer *et al.* 2004; Olsen *et al.* 2004) and the common goby *Pomastoschitus microps* (Gysels *et al.* 2004). *R. clavata*, too, follows this pattern with the highest haplotype diversity now found in this region. However, we did not find a third ancestral haplotype that would strengthen the arguments for another independent refugium, although we cannot fully reject the hypothesis that haplotype H6 has by chance been maintained in a British water refugium. Nevertheless, as the ice retreated and the North Sea began to form, the entire area, which is shallow and sandy, developed into excellent habitat for demersal species. With or without the invocation of a Hurd Deep refugium, it is clear that the area became a secondary contact zone involving newcomers from the Azores and the Iberian coast.

Historical population expansion of *R. clavata* predates the LGM reaching back to between 580 000 and 362 000 years ago, which corresponds to the beginning of the Holstenian, one of the longest warm periods of the Quaternary; and the Cromerian complex, a succession of long glacial periods followed by small warmer periods (Zagwijn 1992; Svendsen *et al.* 2004). Unlike most terrestrial species in northwestern Europe, most marine species (so far investigated) follow the pre-LGM expansion model, e.g. the common goby (Gysels *et al.* 2004), the Atlantic swordfish (Bremer *et al.* 2005), the Atlantic bluefin tuna (Bremer *et al.* 2005), the red alga *Palmeria palmata* (Provan *et al.* 2005), the bivalve *Macoma balthica* (Luttikhuizen *et al.* 2003) and the estuarine fish, *Ethmalosa fimbriata* (Durand *et al.* 2005), where the date of expansion was estimated between 536 000 (for the common goby) to 128 000 BP years (for the red alga, *Palmeria palmata*). Exceptions include Northern Atlantic mollusks,

like *Nucella lapillus*, *Semibablanus balanoides*, *Littorina obtusata*, *Idoltea balthica* where the date of expansion for the North American clade was always estimated after the LGM (Wares & Cunningham 2001). Reasons for this difference are thought to be related to the direct effects of ice sheets and scouring effects. Mobile species and/or those able to shift in the subtidal clearly fared better in the many glacial–interglacial periods. The major effects of the LGM were therefore to reshape *R. clavata*'s distribution with large populations persisting in at least three refugial areas (Azores, Iberian Peninsula and Mediterranean Basin) and subsequently recolonizing new areas as the North Sea basin formed.

In conclusion, the phylogeographic distribution of *R. clavata* falls into three well-defined regional groups: the Mediterranean basin, the Azores and the Atlantic continental shelf. Some evidence from the data suggests a secondary contact zone between at least two divergent refugia in the British waters region. The LGM had little demographic effect on population size of *R. clavata* but great effects on its phylogeographic distribution. Finally, in the timeframe of tens of thousands of years, thornback rays have proven themselves resilient to Pleistocene glacial and interglacial periods. Over the past century, however, thornbacks have fallen victim to over-fishing, especially in the North Sea (Dulvy & Reynolds 2002; Heessen 2004). Despite this threat, their high genetic diversity (in the most heavily fished areas) suggests that recovery is possible if demersal fisheries are heavily reduced.

Acknowledgements

We thank Jim Ellis from the CEFAS (CORY survey, UK), Jean-Paul Delpech from IFREMER- Boulogne sur Mer (CGFS survey, France), Henk Heessen from the RIVO (IBTS survey, the Netherlands), Francisco Sanchez and Cristina Rodriguez-Cabello from the Spanish Institute of Oceanography in Santander (DEMERSALES survey, Spain), Alexandra Rosa and Mario Rui Pinho from the University of the Azores (ARQDAÇO survey, Portugal), Pedro Machado and Bárbara Serra-Pereira from the Portuguese Research Institute for Sea and Fisheries (IPIMAR, Lisbon, Portugal), Arnauld Souplet from IFREMER-Sète (MEDITS survey, France), Chiara Raggiani from the University of Bologna (samples from the Adriatic Sea) and Petya Ivanova from the Institute of Fisheries and Aquaculture of Varna (Bulgaria) for their help in the sampling and to J.A. Coyer for his useful comments on previous versions of this manuscript, as well as two anonymous reviewers and E.B. Taylor. This research was supported by NWO-PRIORITEIT programma SUSUSE, Project Nr. 885-10-311.

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This study is part of Malia Chevolot's PhD thesis on population genetic structure of *Raja clavata*. Galice Hoarau is a post-doc interested in population genetics and evolutionary biology of marine organisms. Adriaan D. Rijnsdorp has a long-standing interest in population dynamics and management of exploited marine fishes. Wytze T. Stam is interested in molecular phylogenetics and ecology of marine benthic organisms. Jeanine L. Olsen is interested in dispersal, phylogeography and life-history evolution of marine organisms.
