Quaternary geographical sibling speciation and population structuring in the Eastern Atlantic skates (suborder Rajoidea) *Raja clavata* and *R. straeleni*

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ABSTRACT

Aim Geographical (allopatric) speciation is a dynamic process whose footprints in the living world are a continuum of stages of increasing divergence. Geographical speciation can also contribute to the evolution of marine taxa. This study looked for two of these evolutionary stages (i.e. structured populations and sibling species) in the diversification patterns of two Atlantic skates (*Raja*, suborder Rajoidea) which exhibited high morphological and ecological conservativism.

Location E Atlantic, Mediterranean, W Indian

Methods Phylogeographical and population genetic analyses were performed by surveying DNA variation of 10 population samples assigned to the European *Raja clavata* and to the S African *R. straeleni*. Polymorphisms were detected by sequencing a mtDNA control region (CR) fragment and genotyping amplified fragment length polymorphism (AFLP) loci. Several statistical tests were used to explore genetic differentiation and population demography.

Results CR haplotypes clustered in two clades consistent with taxon zoogeography. Mean sequence divergence between allopatric taxa amounted to 2% and Bayesian estimate of the time of the most recent common ancestor dated their separation between 0.155 and 1.3 Myr. Isolation-by-distance between European and S African demes was inferred from a significant correlation between coastal and genetic distances at AFLP loci. Null or low gene flow estimates suggested reproductive isolation between allopatric taxa. After separation, taxa have expanded moderately since 30-45 Kyr. Geographical subgrouping of CR haplotypes and significant genetic heterogeneity of samples at both markers featured the Atlantic and Mediterranean thornback skates, revealing pronounced levels of population structuring in this widely distributed taxon.

Main conclusions In spite of the pronounced morpho-anatomical conservativism, *R. clavata* and *R. straeleni* are allopatric sibling species which diverged in the Pleistocene. A recent southward dispersal of European *R. clavata*-like elements along with the W African shelf with the budding of S African *R. straeleni* is ostensible. The tumultuous Quaternary palaeoclimatic history of equatorial and tropical Africa with the succession of glacial and interglacial palaeoenvironments could have promoted the repeated geographical isolation of local demes in relatively restricted refugial areas. Within the evolutionary trajectories experienced by Rajoidea, structured populations and allopatric sibling species could frequently represent intermediate frames of the microevolutionary animation proposed by Ernst Mayr to model allopatric speciation.

Keywords: AFLP, Atlantic, biscuit skate, cartilaginous fish, control region, elasmobranches, historical demography, Mediterranean, Quaternary, thornback skate,

INTRODUCTION

According to the Biological Species Concept, speciation is the acquisition of reproductive isolation by natural populations (Mayr, 1942; Mayr, 1963). Geographical speciation (including allopatric, parapatric and/or peripatric speciation) is viewed as a dynamic process whose temporal trajectory is a continuum of stages of increasing divergence (Mayr, 1954; Palumbi & Lessios, 2005). After many years of investigation and a huge amount of evidences (for some examples see (Mayr, 1954; Palumbi, 1994; Dawson & Hamner, 2005), it is also known that geographic speciation plays a significant role in the diversification of marine taxa. The footprints of these stages in the living world can be ordered in a hierarchical series of increasing complexity, from homogenous populations to a secondary sympatry of well-isolated species (Mayr, 1954; Palumbi & Lessios, 2005). However, some discrepancies among the methodological proxies used to describe the evolutionary stages (for example, morphological vs. genetic traits) might blur the reconstruction of the natural history of taxa. Among these discrepancies, the most striking are undoubtedly those set by genetically diagnosable units, described by phylogeographers, set against the morphologically diagnosable units, formally named and recognized as species by taxonomists (Rocha *et al.*, 2007). Although the DNA-based approach cannot be considered a panacea for defining species boundaries, PCR-based studies surveying mtDNA variation exponentially contribute to the recognition of cryptic species (two or more distinct species that are erroneously classified and hidden under one species name) in the animal kingdom (Bickford *et al.*, 2007). Because of its mode of inheritance in most animal taxa, mtDNA is not suitable however to resolve for situations of hybridization. Nuclear DNA analysis coupled with mtDNA might instead provide a more reliable dataset of the extent of the taxonomic divergence and of reproductive isolation between cryptic species (Bowen *et al.*, 2005; van Herwerden *et al.*, 2006; Rocha *et al.*, 2007). In marine fish, the paradigmatic ecological group encompassing an extraordinary number of cases of cryptic species are undoubtedly the coral reef fishes and most of these cases represent the by-products of extensive phylogeographic work

(reviewed by (Rocha *et al.*, 2007). However, two general themes are featured in the groups prolific in cryptic speciation: the use of non-visual mating signals to recognize conspecific mating partners and a morphological stasis (namely, the lack of change in characteristics of gross external anatomy) that can accompany speciation (Bickford *et al.*, 2007). These two features are coupled by skates and rays (order Rajiformes, suborder Rajoidea; (Ebert & Compagno, 2007) making this taxonomic group a potential incubator for interesting cases of cryptic speciation.

Skates and rays are marine chondrichthyans distributed world-wide that exhibit an extraordinary species diversity paradoxically paired with high levels of morphological and ecological stasis (McEachran & Miyake, 1990a, b; Last & Yearsley, 2002; Valsecchi *et al.*, 2005; Ebert & Compagno, 2007; Froese & Pauly, 2009). According to these trends, skate taxonomy and systematics have been revised several times in the last two decades. Skates are bottom-dwellers mostly inhabiting sandy habitats of continental plates and shelves, frequently up to 1500 m in depth, and instead of visual signals, they use a highly efficient electro-sensorial system for mating recognition (Tricas *et al.*, 1995) and prey detection (Camperi *et al.*, 2007). Species diversity and zoogeography of skates are well known in the regional shelf areas intensely surveyed by scientific trawling programs (i.e. the NE Atlantic-Mediterranean; (Stehmann & Burkel, 1984) as well as in the SE Atlantic and W Indian Oceans; (Compagno & Ebert, 2007). The NE Atlantic and Mediterranean skate fauna has a great zoogeographic similarity with the Central-E Atlantic but unexpectedly also shares several skate elements with the SE Atlantic and W Indian faunas (Valsecchi *et al.*, 2005; Froese & Pauly, 2009). Evolutionary data inferred from mtDNA and fossil internal calibration of substitution rate indicated that NE Atlantic and Mediterranean skates diversified recently with respect to the ancient origin and worldwide displacement of the family, since almost all mtDNA lineages showed approximated divergence times from 17 to 1.8 Ma (Valsecchi *et al.*, 2005). Among such recently evolved lineages, the species of the genus *Raja* began to diversify in the N Atlantic as of 10-12 Ma (Valsecchi *et al.*, 2005). Although most are endemic at the regional or local geographic scale (Valsecchi *et al.*, 2005; Froese & Pauly, 2009), the two

species *R. clavata* and *R. miraletus* appeared to be distributed more widely than expected given their ecological traits (i.e. limited migration of adults and juveniles and lack of egg dispersal) and as compared to most of the close relatives. The thornback skate *Raja clavata* L. 1758, whose origin dated approximately to the Late Miocene - Early Pliocene (Valsecchi *et al.*, 2005), spreads from Iceland southward to Morocco, Namibia and S Africa, including the Mediterranean and the Black Sea, from 20 to 300 m depth (Froese & Pauly, 2009). Because of the very high levels of morphological stasis across the geographic range (Fig. S1 in Appendix 1), in the past individuals and populations from S Africa were specifically assigned to *R. clavata*. More recent zoogeographic and taxonomic studies assigned the S African *clavata*-like individuals to the biscuit skate *Raja straeleni* Poll 1951 (Compagno *et al.*, 1991; Compagno, 1999; Compagno & Ebert, 2007) but without resolving the evolutionary relationships between the two taxa. Phylogeographic analysis of European *R. clavata* based on variation of cytochrome-*b* (cyt-*b*) sequences and neutral microsatellite loci detected strong genetic differences among three geographical groups corresponding to populations from the NE Atlantic shelf, Mediterranean and Azores (Chevolot *et al.*, 2006). The genetic divergence of the Azores samples accounts for a pronounced population structuring of this species along the W African coastal shelf. On the other hand, no significant genetic subdivision was detected in the Mediterranean even if only three geographical samples (Corsica, Adriatic Sea and Black Sea) were analyzed. Based on the lowest levels of genetic diversity at both mtDNA and nuclear markers, a relictual and isolated condition was assumed for the Mediterranean *R. clavata* populations (Chevolot *et al.*, 2006). This condition could be strongly affected by the Last Glacial Maximum (LGM) that likely allowed extinction and recent recolonization events in several areas of the Mediterranean Sea (Cunningham & Collins, 1998; Patarnello *et al.*, 2007).

This paper deals with the phylogeographical and population genetic analyses of the European *R. clavata* and S African *R. straeleni* using two DNA markers: the nucleotide sequence of the mtDNA control region (CR) and the genomic amplified fragment length polymorphisms (AFLP). The

Quaternary sibling speciation of *R. straeleni* and the recent population structuring in the European and Mediterranean *R. clavata* might represent the two main stages of allopatric speciation. Geographical structuring of populations allopatric and sibling speciation may play a considerable role in the diversification process of skates and rays.

METHODS

Sampling of *Raja clavata* and *R. straeleni*

A total of 288 individual skates were collected from 10 areas of NE Atlantic (3), Mediterranean (5), SE Atlantic (1) and W Indian (1) (Table 1, Fig. 1). Although all individuals shared the gross external morphology (Fig. S1 in Appendix S2), we putatively assigned 230 European individuals to *R. clavata* (Stehmann & Burkel, 1984) and 58 S African individuals to *R. straeleni* according to zoogeographic data (Compagno *et al.*, 1991; Compagno & Ebert, 2007). Eight population samples were analysed for variation at each marker and six of them were analysed at both markers.

Control region sequencing

Total genomic DNA was prepared from ethanol-stored muscle tissue according to a standard cetyl trimethyl ammonium bromide (CTAB) extraction procedure (Winnepenninckx *et al.*, 1993). The 5'-end of the mtDNA CR (~460 pb) was amplified and cycle-sequenced using the newly designed primers *Rcla*intF 5'-CACCATTTTGACGTGTTAG-3' and *Rcla*intR 5'-AATGAGATGGGGTAATTGAG-3'. The polymerase chain reaction (PCR) and DNA sequencing conditions are detailed in the Appendix S1 of the Supporting Information. The nucleotide polymorphism of variant haplotypes was confirmed by the sequencing of both strands. Control region haplotypes were aligned using the CLUSTALX v1.83 (Thompson *et al.*, 1997). Gaps resulting from the alignment were excluded from data analysis.

Amplified Fragment Length Polymorphism profiling

The AFLP profiles were generated according to (Congiu *et al.*, 2001). We used four *EcoR*I/*Mse*I primer combinations in the final selective amplification (selective nucleotides ACG/CTA; ACG/CAG; ACG/CTT and ACG/CAA) to generate markers from 80 to 500 bp. In each case, the *Eco*RI selective primer was labelled with a fluorescent dye PET, HEX, TAMRA (Sigma) and FAM (MWG). Selective PCR products were resolved on an ABI310 automated sequencer (Applied Biosystems) with GS-500LIZ internal size standard. Electropherograms were then analyzed using Peak Scanner software v.1.0 (Applied Biosystems). The intensity of each individual peak was normalized on the basis of the total signal intensity and a peak was included in the analysis only if its intensity exceeded a fixed threshold (100 Fluorescent Units) and its size above 100 bp. To test the reproducibility of the AFLP procedure, from 5 to 10 individuals from each sample were completely replicated starting from the same DNA extraction and no differences between the two temporal analyses were observed.

Data analysis

For CR, the number of polymorphic sites (*S*), haplotype diversity (*H*), and the nucleotide diversity (π) (Nei, 1987) with their standard deviations were computed using DNASP 4.0 (Rozas *et al.*, 2003). The phylogenetic relationships between unique CR haplotypes found in *R. clavata* and *R. straeleni* were inferred using parsimony network analysis implemented in the software package TCS 1.13 (Clement *et al.*, 2000). Mean haplotype genetic distances were computed with the MEGA4.0 software (Tamura *et al.*, 2007) applying the TN93 (Tamura & Nei, 1993) model. Standard error (SE) of genetic distance were calculated using bootstrapping with 1,000 replicates. The CR haplotype of the outgroup *Raja polystigma* (GenBank: EU515624) was used to estimate D between related but morphologically well-separated *Raja* species.

Divergence time of the siblings *Raja clavata* and *R. straeleni* was estimated using a Bayesian-coalescent approach, as implemented in BEAST v1.5.3 (Drummond & Rambaut, 2007). To obtain consistency in the results and due to the large values for the confidence intervals obtained with the CR analyses, we decided to perform two independent analyses using both CR and 16S rDNA sequence data sets. In the CR analyses, we used a matrix from a restricted 32-taxon data set and 453 bp representing the main mitochondrial lineages within Rajini (GenBank accession numbers: AY167923, AY218358, EU515624, AY218372, AY218364, AY218362, AY218363, AY218365, AY218354, AY218355). We specified the rate prior to having a normal distribution with a mean of 0.009 and standard deviation of 0.007, and started the search with an unweighted pair group method with arithmetic mean (UPGMA) tree. In the 16S rDNA analyses, we used a matrix from a restricted 19-taxon set of 1429 bp (GenBank accession numbers: GU597951-GU597968). Normal distribution priors were used for the times to the most recent common ancestor (MRCA) of *R. clavata*, *R. straeleni*, *R. radula*, *R. asterias* (mean 5.5 Ma; SD 0.2) and for the MRCA of *R. clavata*, *R. straeleni*, *R. radula*, *R. asterias*, *R. undulata*, *R. polystigma* and *R. montagui* (mean 8.2 Ma, SD 0.5), all based on the divergence times obtained for the NE Atlantic and Mediterranean Rajidae reported by (Valsecchi *et al.*, 2005). Analyses were performed for both data sets using the Hasegawa, Kishino and Yano (HKY) model of evolution (Hasegawa *et al.*, 1985) and an uncorrelated relaxed clock (Drummond *et al.*, 2006), as implemented in BEAST v1.5.3. A Yule tree prior was used, following the recommendation of (Drummond & Rambaut, 2007) for species-level phylogenies. To ensure convergence of the posterior distributions, three independent Markov chain Monte Carlo (MCMC) runs of 50,000,000 generations, with 500,000 generations of burn-in followed by sampling every 5,000 generations were performed and subsequently combined in the module LOGCOMBINER (distributed as part of the BEAST software package). All effective sample size (ESS) values exceeded 2,000, suggesting a sufficient run length.

For AFLP, the percentage of polymorphic loci (at the 5%-level) and unbiased estimates of genetic diversity (H_j, analogous to H_e) were computed using the AFLP-SURV v. 1.0 software

(Vekemans *et al.*, 2002). Allelic frequencies at AFLP loci were calculated from the observed frequencies of fragments using the Bayesian approach proposed by (Zhivotovsky, 1999) for diploid species. A non-uniform prior distribution of allelic frequencies was assumed with its parameters derived from the observed distribution of fragment frequencies among loci (Zhivotovsky, 1999). The allelic frequencies were used as the input for the analysis of genetic diversity within and between samples following the method described in (Lynch & Milligan, 1994). Average fragment size and Pearson correlation coefficients between fragment sizes and fragment frequencies (together with the *P*-value associated with the correlation) were inferred on the overall sample.

Patterns of genetic differentiation among population samples were disentangled by an array of statistical tests and software. The overall genetic heterogeneity of population samples was assessed by an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) using the program ARLEQUIN v. 3.01 (Excoffier *et al.*, 2005) on both markers. Groupings of population samples for AMOVA were based on *a priori* geographical subdivision of sampling locations in regional and sub-regional areas (Table 4). Total genetic variation of samples was computed based on a frequency distribution analysis [equivalent to an *F* analysis, (Cockerham, 1973)] of CR haplotypes and of AFLP alleles. The haplotype frequency distribution analysis was corrected for inter-haplotype sequence divergence. The overall and pair-wise genetic differentiation were assessed by fixation indices. For CR, we calculated Φ, an analogue to the Cockerham *F* estimates of genetic variation, using ARLEQUIN v. 3.01 (Excoffier *et al.*, 2005). The statistical significance of the total and pair-wise Φ_{ST}s was estimated by comparing the observed distribution with a null distribution generated by 20,000 permutations, in which individuals were randomly re-distributed into samples. The significance threshold of pairwise comparisons ($P<0{\cdot}05$) was adjusted with the sequential Bonferroni's correction for multiple simultaneous comparisons (Rice, 1989). For AFLP, F_{ST} was calculated using the AFLP-SURV software v. 1.0 (Vekemans *et al.*, 2002). This program uses the approach of (Lynch & Milligan, 1994) to calculate population genetic parameters on the basis of the expected heterozygosity of dominant marker loci.

Further analyses to identify outlier AFLP loci (i.e. loci potentially under selection) and to explore the genetic structuring of population samples using a Bayesian clustering approach were carried out. Methods and software used in these analyses are detailed in the Appendix S1 (see Supporting Information).

On both markers, we tested the isolation by distance of samples using a Mantel test (Mantel, 1967) as implemented in GENEPOP 3.1 software (Raymond & Rousset, 1995). The natural logarithm of coastal distance was plotted against the $\Phi_{ST}/(1-\Phi_{ST})$ for the CR data and $F_{ST}/(1-F_{ST})$ for the AFLP data. Correlations and associated *P* values were calculated using 10,000 permutations.

The NE Atlantic demes of thornback ray likely expanded in the Middle Pleistocene (580-362 kya; (Chevolot *et al.*, 2006). Thus, we thoroughly investigated the historical demography of the Mediterranean populations of *R. clavata* and of the S African *R. straeleni* using two different approaches. Firstly, we carried out the Tajima's D (Tajima, 1989) and the Fu's (Fu, 1997) F_s tests of neutrality, as implemented in DNASP 4.0 (Rozas *et al.*, 2003). For neutral markers and under a population expansion model, significant negative values would be expected. Secondly, past demographic patterns for the two taxa were also inferred using the coalescent Bayesian skyline plot (BSP) model (Drummond *et al.*, 2005) as implemented in BEAST v 1.5.3 (Drummond & Rambaut, 2007) and visualized in TRACER v1.4.1 (Rambaut & Drummond, 2007). We assumed a relaxed molecular clock and used the HKY model (Hasegawa *et al.*, 1985). Substitution rate and run conditions were identical to those used in the TMRCA analysis.

The coalescence-based Markov Chain Monte Carlo (MCMC) methods implemented in MIGRATE v1.7.3 (Beerli & Felsenstein, 1999; Beerli & Felsenstein, 2001) were used to estimate $\Theta = 2N_{ef}\mu$ (where N_{ef} is the effective female population size and μ is the mutation rate) and the gene flow among samples (as the product of N_{ef} and migration rate *m*) based on CR sequence data. A slowed-down CR mutation rate of 0.97×10^{-8} substitution/site/generation was estimated from (Valsecchi *et al.*, 2005) and present data, assuming a generation time approximately at 9 years for

R. clavata (Froese & Pauly, 2009). The Φ_{ST} estimates of effective population sizes and migration rates were used as initial values. A MCMC run consisted of ten short and three long chains with 10,000 and 100,000 genealogies, respectively, with a burn-in of 10,000 genealogies and a sampling rate of constructed genealogies of 1%. One of every 100 reconstructed genealogies was sampled. The Θ and gene flow estimates were the average of the five MCMC runs which were performed to verify consistency of results.

RESULTS

Genetic diversity

Control region sequence data gave an initial alignment of 421 bp that was further reduced to 419 bp after exclusion of two positions with gaps. The nucleotide polymorphisms defined totally 50 CR haplotypes (GenBank: EU515625-EU515683) whose variation was due to 48 (11%) polymorphic sites and 17 (4%) were parsimony-informative. Mean genetic polymorphism was higher in the S African *R. straeleni* samples than in the European *R. clavata* samples though the highest *h* and π values were shown by the EM *R. clavata* sample (Table 1).

With respect to primer-enzyme combination we obtained different numbers of AFLP bands (ranging from 76 to 102 bands in the *EcoRI-ACG/MseI-CTA* and *EcoRI-ACG/MseI-CTT* combinations, respectively) for a total of 351 fragments with a related mean polymorphism of 44.5%. We detected evidence of fragment size homoplasy in the data set since there was a correlation between fragment size and banding frequency (the Pearson's product-moment correlation coefficient r = - 0.4559; P = 0.000). In contrast to CR results, the AFLP genetic polymorphism was higher in the European *R. clavata* than in the S African *R. straeleni* (Table 1).

Genetic differentiation and population structuring

The parsimony network subdivided CR haplotypes into two main haplogroups according to putative species boundaries (Fig. 2). Both haplogroups showed a slightly deep star-like topology, indicating somewhat older relationships among haplotypes. The first group included 35 haplotypes found in the European *R. clavata* samples whereas the second was formed by the 15 haplotypes shown by the S African *R. straeleni* individuals. The *R. clavata* and *R. straeleni* haplogroups differed by four missing haplotypes. However, it should be noted that they were interconnected via a subclade only formed by NE Atlantic haplotypes (Rcla_31, 32, 34 and 35; Fig. 2). In the *R. clavata* group, the most abundant Rcla_01 was shared by all NE Atlantic and Mediterranean samples other than EM. In contrast, the sample EM possessed the Rcla_15 as the most frequent. The Rcla_15 was also found in all other *R. clavata* samples except the AS. All the remaining haplotypes showed very low frequencies and most are unique. Other than a NE Atlantic subclade, three weakly differentiated Mediterranean *R. clavata* subgroups of haplotypes were identified. The most relevant was formed by the Rcla_04 (found in all Mediterranean samples but not in EM) and the Adriatic haplotypes Rcla_02, 03 and 10. The other two subclades were detected in the Eastern Mediterranean (formed by Rcla_26 and 29) and in the Tyrrhenian Sea (formed by Rcla_13 and 14). In the *R. straeleni* cluster, the haplotypes Rstr_36 (the most common) and Rstr_40 were shared by both SE Atlantic (CT) and W Indian (SC) samples. The remaining 13 *R. straeleni* haplotypes were sharply sorted in the two sampling locations (Fig. 2). The mean CR sequence divergence between *R. clavata* and *R. straeleni* (2% of TN93 genetic distance; Table 3) was three to four fold greater than the within-species value (0.5-0.7%), while it was six fold lower than that obtained from the comparison with the outgroup species *R. polystigma* (12.3-13.1%).

The MRCA of *R. clavata* and *R. straeleni* was estimated at 1.3 Ma (95% confidence interval = 1.1×10^{-3} – 2.3 Ma) and at 155 kya (95% confidence interval = 31 – 330 kya) from the CR and 16S rDNA data set, respectively. Although slightly different, both estimates strongly support a very recent genetic differentiation of the siblings *R.clavata* and *R. straeleni*.

A great level of overall genetic differentiation among samples was detected with both CR and AFLP markers by AMOVA (Table 4; analysis 1). Such structuring is fully consistent with species boundaries and zoogeography (analysis 2: CR, Φ_{ST} = 0.8427, P = 0.033; AFLP, F_{ST} = 0.1396, P < 0.0001). The European *R. clavata* population samples were differentiated by both markers (analysis 3; CR: Φ_{ST} = 0.0521, P < 0.0001; AFLP: F_{ST} = 0.0528, P < 0.0001), though a significant geographical structuring into NE Atlantic and Mediterranean was detected only by AFLPs (analysis 4). Within the Mediterranean, *R. clavata* samples are differentiated (analysis 5) but regardless of any possible geographical grouping of samples (e.g. W and E Mediterranean, analysis 6).

The great genetic divergence of *R. clavata* with respect to *R. straeleni* was also shown by the high fixation indexes (P < 0.01) obtained in the between-species pair-wise comparisons using both markers (Table 5). On the contrary, the detection of significant within-species population differences were marker-dependent. In *R. clavata*, most pair-wise Φ_{ST}s were not significant different from zero after applying the Bonferroni sequential correction, with the exception of those involving the EM sample. In contrast, an opposite pattern was shown by AFLP-based *F* statistics where significant genetic differences were detected either between NE Atlantic and Mediterranean samples or among most of the Mediterranean samples (Table 5). Significant pairwise fixation indexes were never observed within NE Atlantic *R. clavata* and *R. straeleni* (Table 5). Seven AFLP loci gave F_{ST} values different from the expected values (i.e. loci potentially under selection; Figure S2 in the Appendix S2). After the removal of these loci the overall F_{ST} value decreased to 0.0503; however, the significance of the F_{ST} values did not change. The Bayesian clustering analysis of AFLP data found two distinct genetic clusters (K=2) corresponding to the European and S African samples (Fig. S3 in Appendix S2). Evidence of significant isolation-by-distance among samples was detected only using AFLP markers (Figure 3). Significant correlation between genetic differentiation estimated by AFLP and coastal distance was obtained including in the test all

samples (Mantel test, $P < 0.001$) and the European *R. clavata* samples (Mantel test, $P = 0.012$). On the contrary, the Mediterranean samples did not show significant isolation by distance..

Demographic patterns

Since neutrality tests gave negative and significant results (Table 3), a sudden demographic expansion model can not be rejected for both Mediterranean *R. clavata* and S African *R. straeleni*. However, BSP analyses showed pattern that suggest moderate demographic expansions for both *R. clavata* and *R. straeleni*, more indicative of a stationary population size than a sudden expansion. While the Mediterranean thornback rays have expanded slowly since 30 kya, the S African *R. straeleni* populations have grown since 45 kya (Figure 4).

The effective female population sizes estimated from CR-based Θ values using the coalescent methods implemented in Migrate v1.7.3 were highly homogeneous and ranged between 4.38×10^5 and 1.23×10^6 individuals (Table 6). Migration (estimated as number of migrants per generation) was low or absent between the European *R. clavata* and the S African *R. straeleni*. In contrast, it was higher among populations within each group (Table 6), even if values were greatly variable depending on the population sample pair.

DISCUSSION

Geographical sibling speciation in Eastern Atlantic skates

Among cartilaginous fish, skates and rays showed the highest species diversity (Ebert & Compagno, 2007). In spite of a pronounced conservativism of morpho-anatomical, biological and ecological traits, members of such ancient cartilaginous evolutionary lineage radiated in all marine habitats, apparently regardless of water temperature, salinity and latitude (McEachran & Miyake, 1990b; Ebert & Compagno, 2007). Macroevolution of skates occurred predominantly during the Tertiary (Long, 1994; Dolganov, 2002; Kriwet *et al.*, 2009), displacing greatly differentiated regional faunas characterized by a high proportion of endemic taxa (Last & Yearsley, 2002;

Valsecchi *et al.*, 2005; Froese & Pauly, 2009) and has continued in the Quaternary, with diversification at the within-species level (Chevolot *et al.*, 2006; Chevolot *et al.*, 2007).

Here, we obtained evidence that two closely-related skates *R. clavata* and *R. straeleni*, showing a high level of morphological and ecological stasis, are recently-diverged allopatric sibling species. The 2% of mean sequence divergence observed between the European and S African CR haplotype lineages appears to be sufficient to delineate them as species within the genus *Raja*. The mean genetic distance estimated between the thornback ray and biscuit skate based on the CR variation fully agreed with those estimated with the same marker in other recently diverged *Raja* species of the NE Atlantic and Mediterranean (Valsecchi *et al.*, 2005). The geographical clustering of CR haplotypes is consistent with species boundaries defined by morphological taxonomy and zoogeography (Compagno & Ebert, 2007). The absence of gene flow between European and S African populations as well as the high and significant AFLP-based *F* values strongly suggest that these taxa are reproductively isolated. In our study, the lack of thornback samples from the Central African shelves is a limitation for reconstructing a complete phylogeographical and micro-evolutionary track of these taxa. The genetic divergence and the distinct natural history of Azores samples with respect to those of the NE Atlantic shelves and Mediterranean (Chevolot *et al.*, 2006) reasonably allow us to suspect that more than one evolutionary lineage of *Raja clavata* has spread in the southernmost part of its distribution area (Central W African shelves).

The separation of *R. straeleni* from *R. clavata* dated approximately from Late (ca. 155 kya) to Early Pleistocene (1.1 Ma). Such TMRCA estimates support a recent budding of the S African *R. straeleni* from the European *R. clavata*. The BSP analysis suggests that S African populations have expanded in size moderately in size since 45 kya and that divergence of both taxa were not followed by significant population growth, but a more moderate expansion. A southward dispersal of *R. clavata*-like European elements along with the W African shelf and, across the region of the Cape, into the W Indian Ocean is ostensible. The tumultuous Quaternary palaeoclimatic history of equatorial and tropical Africa with the succession of glacial and interglacial palaeoenvironments

(Krueger *et al.*, 2008), could have promoted the formation of relatively restricted refugial areas promoting repeated geographical isolation of local demes for this close bottom-dweller (Chevolot *et al.*, 2006). The Late Quaternary and present oceanographic discontinuities occurring along the W African continental shelf (e.g. Cape Blanc and the Angola–Benguela Front(Gasse *et al.*, 2008) may contribute to the maintenance of low or null levels of gene flow between such closely related siblings, even if the genetic and zoogeographic breakups remain unidentified. The African Atlantic marine faunas can be subdivided into three zoogeographic provinces by two main oceanographic discontinuities corresponding to steep thermal gradients in the upwelling areas of Cape Blanc at 21°N and of Cape Frio at 18°S (Briggs, 1974). While Cape Blanc (Mauritania) acts as a physical barrier against the southward dispersal of endemic skate taxa from Mediterranean and NE Atlantic shelves, Cape Frio's upwelling and the Angola-Benguela front are considered the southern limit of the tropical skate faunas (Hulley, 1972). The accumulated genetic differences between the thornback ray and biscuit skate and their parallel morphological stasis may be the micro-evolutionary outputs of stabilizing selection which tend to conserve a well-fitting phenotype across the wide ranging distribution of the clade (Williamson, 1987) in relation to the stasis of marine communities on evolutionary time scales (Jackson & Sheldon, 1994; Colborn *et al.*, 2001).

The tendency toward allopatric cryptic speciation as an evolutionary stage of the natural history could be rather diffused among *Raja* species and, at higher taxonomic levels, among the suborder Rajoidea (i.e. skates and rays). Experimental evidence supporting this issue includes some significant cases that have been documented by phylogeographical analyses. The Mediterranean *R. polystigma* and the prevalently E Atlantic *R. montagui* exhibited high levels of morphological stasis (Stehmann & Burkel, 1984; Serena, 2005) and in parallel showed 2-3% of divergence at the mitochondrial 16S rDNA and CR gene fragments (Valsecchi *et al.*, 2005). Similarly, the wide distributed *R. miraletus* (spreading from the Mediterranean to S Africa; (Stehmann & Burkel, 1984; Froese & Pauly, 2009) likely corresponds to a complex of species exhibiting very similar rough external morphology but also discrete differences in the meristic-morphometric characters

(McEachran *et al.*, 1989) and in genetic traits (F. Tinti personal communication). In addition, several new and cryptic rajoid taxa have been discovered using DNA-based investigative approaches (Ward *et al.*, 2005; Spies *et al.*, 2006; Smith *et al.*, 2008; Iglésias *et al.*, 2009). The low levels of sequence divergence that characterizes the mtDNA-based species delineation in skates are likely related to the slowing of elasmobranch mtDNA substitution rate (Martin & Palumbi, 1993).

Within the evolutionary trajectories experienced by skates and rays, allopatric sibling species could frequently represent intermediate frames in the micro-evolutionary animation proposed by Ernst Mayr to model allopatric (geographical) speciation (Mayr, 1954). Such a continuum in the axis of the allopatric speciation process begins with polytypic species inhabiting a continuous range and ends with super-species, by means of a group of allopatric species with a complete separation in rough morphological traits. The abundance of cryptic species in a marine fish group naturally exhibiting life-history traits enhancing vulnerability and extinction risks (Dulvy *et al.*, 2003) also have relevant downstream consequences for the conservation of genetic and species diversity (Dulvy *et al.*, 2000; Bickford *et al.*, 2007; Dulvy & Reynolds, 2009; Iglésias *et al.*, 2009).

Population structuring and history of Mediterranean *Raja clavata*

In the (Mayr, 1954)'s temporal trajectory of geographical speciation, the frame of "allopatric populations" (that may be considered either species or subspecies and that likely include also the allopatric sibling species) follows the frame of polytypic species whose populations are geographically isolated. Efficient geographical/hydrogeographical barriers should limit gene flow between populations, leading to significant genetic structuring.

In the older sibling *R. clavata*, populations separated by physical barriers such as the Gibraltar Strait, large deep oceanic areas or long coastal distances have accumulated significant levels of neutral genetic divergence, as shown by the overall patterns of genetic structuring and isolation-by-distance inferred from both mitochondrial (cyt-*b* and CR sequences, [(Chevolot *et al.*, 2006), this study]) and nuclear markers (microsatellites (Chevolot *et al.*, 2006); AFLPs, this study). The pattern

of genetic differences exhibited by European *R. clavata* largely corresponds to the prominent phylogeographical breaks detected at the regional scale level between Atlantic and Mediterranean populations of several marine species (Patarnello *et al.*, 2007).

At a smaller spatial scale (e.g. the Mediterranean regional level), where there was an apparent lack of effective barriers against dispersal, the extent of genetic divergence among thornback skate populations may be null or reduced as within the Mediterranean and NE Atlantic shelf samples, respectively (Chevolot *et al.*, 2006). In the Mediterranean and Black Sea *R. clavata* samples, the presence of a unique ancestral cyt-*b* haplotype and the lack of significant variation at the microsatellite loci allowed (Chevolot *et al.*, 2006) to hypothesize a relictual distribution of this species in these basins. This was likely related to strong bottleneck events and maintained by restricted gene flow with populations inhabiting the European Atlantic shelves.

In the Mediterranean *R. clavata*, our study has newly identified *i*) noticeable levels of genetic diversity, *ii*) slight but detectable genetic structuring and *iii*) signals of moderate demographic growth.

Mediterranean population samples of *R. clavata* possessed levels of genetic diversity at the CR and AFLP loci that are comparable to those shown by the Atlantic samples and, exceptionally, the Eastern Mediterranean sample showed the highest CR polymorphism. Such relatively high levels of genetic diversity seem to contradict the relictual distribution and bottlenecked condition of Mediterranean *R. clavata* populations predicted by (Chevolot *et al.*, 2006), based on the presence of only a single, widespread, ancestral cyt-*b* haplotype and a microsatellite allelic diversity that probably predates the LGM. Genetic responses in bottlenecked populations are variable, context-dependent and balanced by several counteracting factors (e.g. the generation time and life history of the species, the severity of the bottleneck event, the current levels of gene flow, and the nature of demographic rebound (Nei *et al.*, 1975; Garza & Williamson, 2001). Despite this, some ecological and environmental traits may help fish populations to retain genetic diversity, such as the overlap of

generations (Waples, 1990), reduced variance in reproductive success (Ardren & Kapuscinski, 2003), the abundance of high-quality spawning habitats, the genetic contribution of precocial males and high levels of gene flow (Neville et al. 2007). Based on CR variation, we obtained homogeneous and large estimates of effective female population size (approximately 438,000-613,000 individuals) in the Mediterranean samples, which might suggest population census size of several millions of individuals (Avise, 1994).

Mediterranean thornback ray samples have drifted to a weak but detectable level of genetic differentiation. Genetic structuring was detected by means of significant overall F-statistic values, though a significant geographical grouping of Mediterranean samples was not observed from the AMOVAs. The occurrence of weakly differentiated geographical CR haplotype lineages suggests that Mediterranean *R. clavata* would have experienced different micro-evolutionary histories. This appears to be true for the Eastern Mediterranean, whose population sample showed a CR haplotype composition with the haplotype Rcla15 more frequent than the ancestral Rcla01 and two not rare endemic haplotypes (Rcla26 and 28). Similar patterns could be inferred for the Adriatic Sea, Algerian and Tyrrhenian Sea samples. Such genetic structuring in the Mediterranean thornback rays would be maintained by the reduced dispersal related to the limited movements of adult individuals (Walker *et al.*, 1997) and to the pronounced benthic ecology of eggs and hatchings (Ellis & Shackley, 1995). The AFLP-based estimates of genetic differentiation strengthened the evidence of population structuring given by CR variation, with all pairwise F_{ST} values high and significant with the exception of the comparison between two W Mediterranean samples (BI and TS). The AFLP markers performed better than CR in differentiating thornback ray samples between NE Atlantic and Mediterranean and in revealing a significant overall isolation-by-distance pattern. However, within Mediterranean thornback rays, neither of the two markers revealed significant structuring of samples in geographical groups (e.g. W and E Mediterranean) or significant correlations between coastal distance and genetic divergence. This pattern is opposite to that exhibited by thornback ray

populations inhabiting the extended NE Atlantic shelves, which have shown a stronger and significant correlation of genetic divergence with geographical distance at the mtDNA locus cyt *b* (Chevolot *et al.*, 2006). Comparatively, this issue suggests that in the Mediterranean, in addition to the reduced dispersal ability of adults, discontinuities in the shelf edges and pronounced variation of ocean depth might also contribute to the structuring of *R. clavata* populations (Hoarau *et al.*, 2002; Chevolot *et al.*, 2006; Chevolot *et al.*, 2007).

Finally, the Mediterranean thornback ray populations display historical demography characterized by moderate growth (closer to stationary population size). Although the pattern reconstructed by BSP analysis might also be explained by the lack of information in the data set, a slow and continuous population expansion in the last 30 kyr strengthened the prediction that *R. clavata* has been demographically resilient to Pleistocene paleoclimatic changes (Chevolot *et al.*, 2006), which in turn have shaped demographic features of populations of several bony fish species (see for example (Larmuseau *et al.*, 2009).

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BIOSKETCH

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Figure 1 - Geographical distribution of the sampling locations for the European *Raja clavata* (NE Atlantic and Mediterranean) and S African *R. straeleni* (SE Atlantic and E Indian Ocean). Acronyms are given as in Table 1.

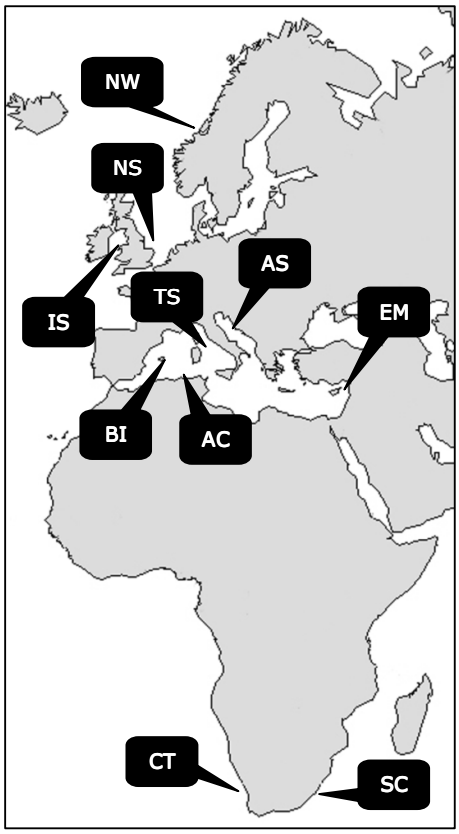


Figure 2 - Statistical parsimony network of the CR haplotypes of the European *Raja clavata* and S African *R. straeleni*. The confidence interval was at 95%. The size of the circles is proportional to the number of individuals sharing that haplotype. The haplotypes are indicated by numbers as given in Table 2.

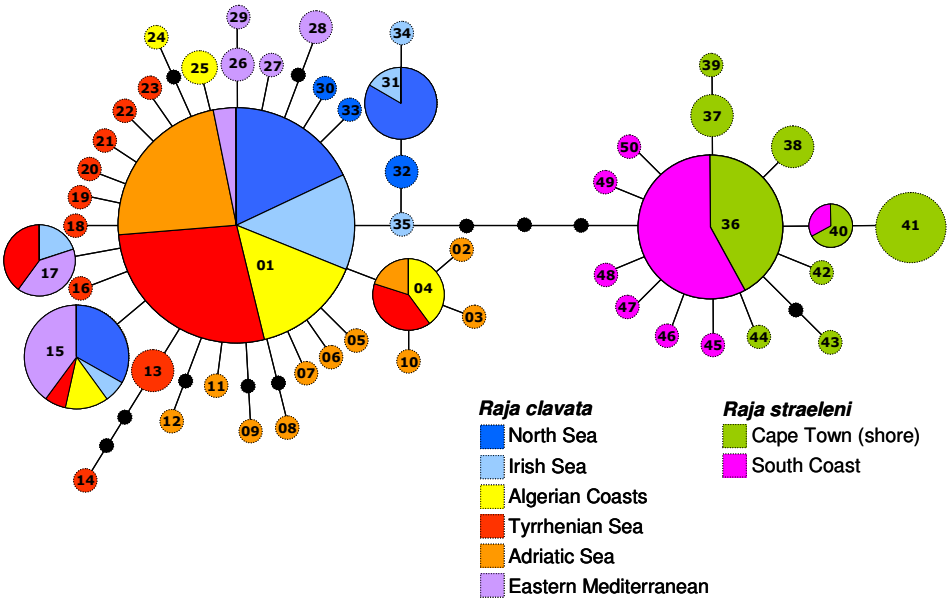


Figure 3 - Isolation by distance based on both European *Raja clavata* and S African *R. straeleni* (asterisks and solid line) and only on the European *Raja clavata* (dots and dashed line). Genetic distance expressed as $F_{ST}/(1- F_{ST})$ were based on AFLP data.

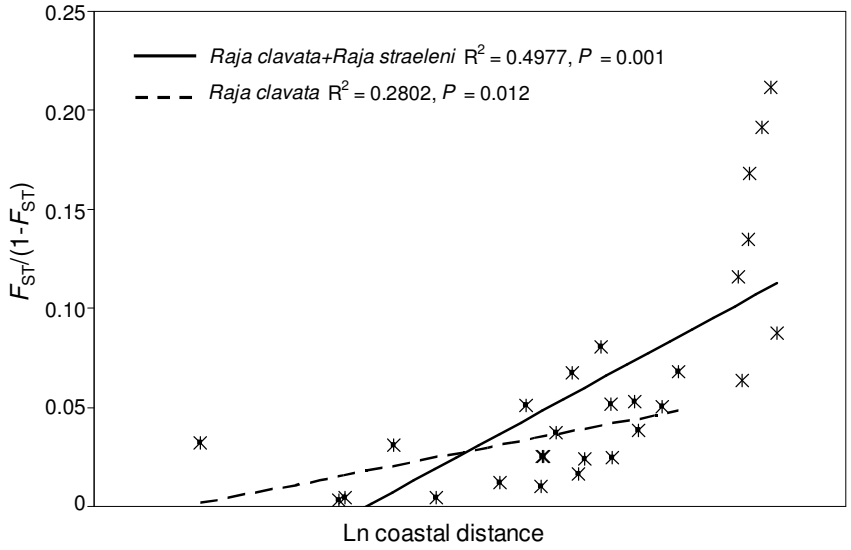
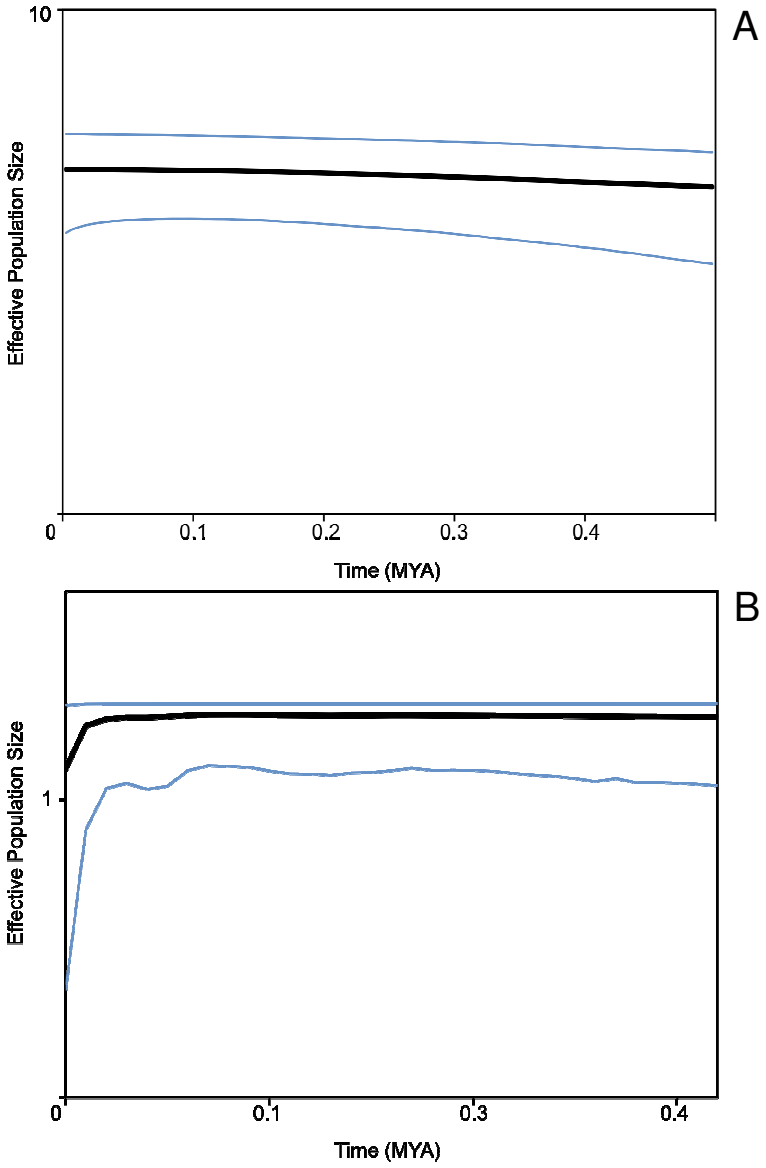


Figure 4 - Bayesian skyline plots of *R. straeleni* (A) and Mediterranean *R. clavata* (B). The curves show posterior median (solid black lines) and 95% credible interval (blue lines) of the effective population size. We used the log transformation of the effective population size axis to plot Ne.



TABLES

Table 1 Sampling data and genetic polymorphisms of *Raja clavata* and *R. straeleni*

Sampling data					CR polymorphism					AFLP polymorphism			
Species/Sampling area	Code	Year	Source	Coordinates	N	n	S	*h* ± SD	π ± SD	N	S	PLP	*Hj* ± SE
Raja clavata					*199*	*35*	*36*	*0.554 ± 0.043*	*0.0023 ± 0.0003*	*146*	*151*	*43.0*	*0.164 ± 0.010*
Norwegian Sea	NW	2005	ST	64° 13' N 9° 40' E	-	-	-	-	-	11	180	51.3	0.183 ± 0.011
North Sea	NS	2002	ST	52° 06' N 2° 14' E	38	6	6	0.578 ± 0.084	0.0027 ± 0.0006	31	171	48.7	0.181 ± 0.010
Irish Sea	IS	2005	ST	53° 58' N 3° 36' O	22	6	5	0.411 ± 0.131	0.0021 ± 0.0008	24	154	43.9	0.178 ± 0.010
Algerian Coasts	AC	2003	LF	not available	27	5	5	0.450 ± 0.114	0.0014 ± 0.0004	18	153	43.6	0.158 ± 0.009
Balearic Islands	BI	2006	ST	39° 55' N 3° 32' E	-	-	-	-	-	16	149	42.5	0.163 ± 0.010
Tyrrhenian Sea	TS	2003	ST	43° 39' N 10° 03' E	52	13	14	0.521 ± 0.085	0.0018 ± 0.0004	26	154	43.9	0.156 ± 0.010
Adriatic Sea	AS	2004	ST	42° 09' N 16° 41' E	42	12	13	0.460 ± 0.097	0.0020 ± 0.0005	-	-	-	-
E Mediterranean	EM	2004	LF	not available	18	7	7	0.843 ± 0.058	0.0039 ± 0.0006	20	156	44.4	0.150 ± 0.009

- continued

Table 1 - continued

Sampling data					CR polymorphism					AFLP polymorphism			
Species/Sampling area	Code	Year	Source	Coordinates	N	n	S	*h* ± SD	π ± SD	N	S	PLP	*Hj* ± SE
Raja straeleni					*55*	*15*	*14*	*0.674 ± 0.070*	*0.0025 ± 0.0003*	*33*	-	-	-
Cape Town (shore)	CT	2006	ST	33° 27' S 31° 30' E	30	9	9	0.782 ± 0.064	0.0034 ± 0.0005	33	121	34.5	0.118 ± 0.008
South Coast	SC	2006	ST	29° 24' S 17° 38' E	25	8	7	0.490 ± 0.123	0.0013 ± 0.0004	-	-	-	-

ST, sample collected by scientific trawl; LF, sample collected by landing fishery; N, number of individuals analyzed per area; n, number of haplotypes; S, number of polymorphic sites; *h*, haplotype diversity; π, nucleotide diversity; PLP, Percentage of polymorphic loci; *Hj*, genetic diversity; SD, standard deviation; SE, standard error

Table 2 Frequency distribution of CR haplotypes in the *Raja clavata* and *R. straeleni* samples.

Haplotype	NS	IS	AC	TS	AS	EM	CT	SC	frequency (%)
Rcla_01	24	17	20	36	31	4			52.0
Rcla_02					1				0.4
Rcla_03					1				0.4
Rcla_04			2	2	1				2.0
Rcla_05					1				0.4
Rcla_06					1				0.4
Rcla_07					1				0.4
Rcla_08					1				0.4
Rcla_09					1				0.4
Rcla_10					1				0.4
Rcla_11					1				0.4
Rcla_12					1				0.4
Rcla_13				3					1.2
Rcla_14				1					0.4
Rcla_15	5	1	2	1		6			5.9
Rcla_16				1					0.4
Rcla_17		1		2		2			2.0
Rcla_18				1					0.4
Rcla_19				1					0.4
Rcla_20				1					0.4
Rcla_21				1					0.4
Rcla_22				1					0.4
Rcla_23				1					0.4
Rcla_24			1						0.4
Rcla_25			2						0.8
Rcla_26						2			0.8
Rcla_27						1			0.4
Rcla_28						2			0.8
Rcla_29						1			0.4
Rcla_30	1								0.4
Rcla_31	5	1							2.4
Rcla_32	2								0.8

- continued

Table 2 - continued

Haplotype	NS	IS	AC	TS	AS	EM	CT	SC	frequency (%)
Rcla_33	1								0.4
Rcla_34		1							0.4
Rcla_35		1							0.4
Rstr_36							13	18	12.2
Rstr_37							3		1.2
Rstr_38							3		1.2
Rstr_39							1		0.4
Rstr_40							2	1	1.2
Rstr_41							5		2.0
Rstr_42							1		0.4
Rstr_43							1		0.4
Rstr_44							1		0.4
Rstr_45								1	0.4
Rstr_46								1	0.4
Rstr_47								1	0.4
Rstr_48								1	0.4
Rstr_49								1	0.4
Rstr_50								1	0.4

Sample codes are given as in Table 1. Haplotypes are identified according to putative species (Rcla: *R. clavata*; Rstr: *R. straeleni*) and to geographical distribution within species

Table 3 CR sequence divergence and results of neutrality tests of *Raja clavata* and *R. straeleni* samples

	Raja clavata	*Raja straeleni*
TN93, within-species	0.007 ± 0.001	0.005 ± 0.001
TN93, between-species	0.020 ± 0.006	
TN93, versus *R. polystigma*	0.123 ± 0.018	0.131 ± 0.019
D	**-2.48**[a]	**-2.04**
Fs	**-50.02**[a]	**-12.60**

TN93; mean Tamura-Nei's genetic distance; D, Tajima's D-test; Fs, Fu's Fs-test. Values given in bold showed $P < 0.01$. [a] Estimate referring only to the Mediterranean *Raja clavata*

Table 4 Genetic structure of *Raja clavata* and *R. straeleni* samples

AMOVA - Groupings	CR			AFLP		
	Total variation (%)	Φ statistics	P	Total variation (%)	F statistics	P
AMOVA1 – All samples, no groups						
Among samples	69.59	ST = 0.6959	0.000	10.63	ST = 0.1063	0.000
Within samples	30.41			89.37		
AMOVA2 – All samples, two groups: *R. clavata* , *R. straeleni*						
Among groups	84.27	CT = 0.8427	0.033	13.96	CT = 0.1396	0.000
Among samples within groups	0.84	SC = 0.0537	0.000	4.82	SC = 0.0560	0.000
Within samples	14.88	ST = 0.8512	0.000	81.23	ST = 0.1878	0.000
AMOVA3 – *R. clavata* samples, no groups						
Among samples	5.20	ST = 0.0521	0.000	5.28	ST = 0.0528	0.000
Within samples	94.80			94.72		
AMOVA4 - *R. clavata* samples, two groups: NE Atlantic, Mediterranean						
Among groups	3.66	CT = 0.0366	0.134	4.42	CT = 0.0442	0.003
Among samples within groups	3.21	SC = 0.0336	0.005	2.59	SC = 0.0271	0.001
Within samples	93.12	ST = 0.0688	0.000	92.98	ST = 0.0702	0.000
AMOVA5 - Mediterranean *R. clavata* samples, no groups						
Among samples	4.60	ST = 0.0460	0.000	3.50	ST = 0.0350	0.000
Within samples	95.40			96.50		
AMOVA6 - Mediterranean *R. clavata* samples, two groups: W and E Mediterranean						
Among groups	-3.40	CT = - 0.0340	1.000	0.90	CT = 0.0090	0.501
Among samples within groups	6.99	SC = 0.0676	0.001	3.04	SC = 0.0306	0.152
Within samples	96.41	ST = 0.0359	0.000	96.06	ST = 0.0394	0.002

Table 5 Pair-wise fixation indices (CR, above diagonal; AFLP below diagonal) between *Raja clavata* and *R. straeleni* samples.

	NW	NS	IS	AC	BI	TS	AS	EM	CT	SC
NW		ne	ne	ne	ne	ne	ne	ne	ne	nc
NS	0.0032		-0.0177	*0.0618*	ne	**0.0637**	**0.0783**	*0.0730*	**0.8284**	0.8688**
IS	0.0120	0.0041		*0.0402*	ne	0.0230	*0.0407*	*0.0818*	**0.8355**	0.8943**
AC	**0.0748**	**0.0632**	**0.0483**		ne	0.0105	0.0027	**0.0970**	**0.8581**	0.9145**
BI	**0.0492**	**0.0234**	**0.0247**	**0.0310**		ne	ne	ne	ne	nc
TS	**0.0500**	**0.0238**	**0.0159**	**0.0302**	0.0041		***0.0172***	**0.1214**	**0.8599**	0.8963**
AS	ne	ne	ne	ne	ne	ne		**0.1298**	**0.8507**	0.8912**
EM	**0.0637**	**0.0480**	**0.0367**	**0.0101**	**0.0358**	**0.0244**	ne		**0.8026**	0.8586**
CT	**0.1745**	**0.1606**	**0.1441**	**0.0600**	**0.1037**	**0.1186**	ne	**0.0803**		**0.0630**
SC	ne	ne	ne	ne	ne	ne	ne	ne	ne	nc

Sample codes are given as in Table 1; ne, not estimated. Bold, P-value < 0.01; italics, values not significant after the Bonferroni's sequential correction ($\alpha = 0.0$

Table 6 Effective female population size and gene flow estimate (based on CR variation) in *Raja clavata* and *R. straeleni* samples

	$\Theta = [2N_fm]$	N_f	$2N_fm$							
			NS	IS	AC	TS	AS	EM	CT	SC
NS	0.0089	4.59×10^5	-	206	420	153	67	835	0	0
IS	0.0124	6.39×10^5	4873	-	382	1101	362	282	0	0
AC	0.0111	5.72×10^5	80	587	-	353	989	456	0	0
TS	0.0113	5.82×10^5	11	299	542	-	365	432	1	0
AS	0.0119	6.13×10^5	0	0	915	970	-	153	0	0
EM	0.0085	4.38×10^5	348	320	5	87	77	-	0	0
CT	0.0094	4.85×10^5	6	0	0	0	6	3	-	529
SC	0.0239	1.23×10^6	5	0	0	2	3	0	1133	-

Sample codes are given as in Table 1; N_f, effective female population size; m, migration rate.

SUPPORTING INFORMATION

Appendix S1 - Additional information on the control region fragment amplification and sequencing, the AFLP outlier loci analysis and the Bayesian clustering analysis.

Appendix S2 - Supplementary figures.