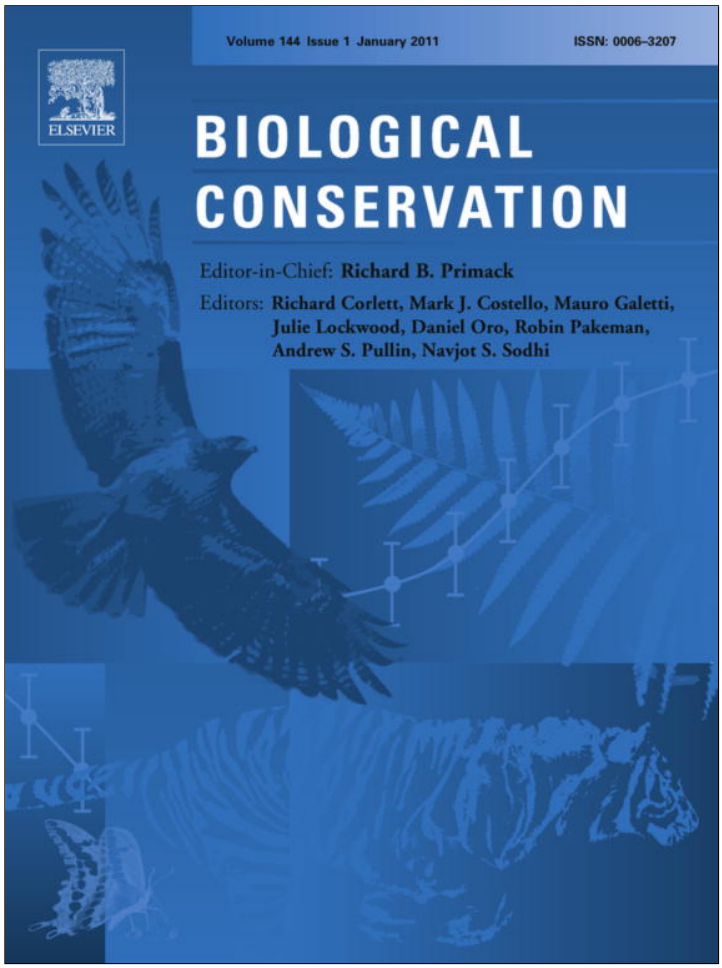

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Identifying unique populations in long-dispersal marine species: Gulfs as priority conservation areas

Gonzalo Machado-Schiaffino [a,*], Francis Juanes [b], Eva Garcia-Vazquez [a]

[a] Department of Functional Biology, University of Oviedo, C/Julian Claveria s/n 33006, Spain
[b] Department of Natural Resources Conservation, University of Massachusetts, Amherst, MA 01003, USA

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ABSTRACT

Identification of areas which should be a subject of protection is crucial for safeguarding the marine ecosystems. Amongst the reasons for protecting a region or location, the existence of unique populations or evolutionary significant units for one or more key species is a priority. The North American silver hake, *Merluccius bilinearis*, is currently managed as two stocks (northern and southern) without considering gulf areas separately. Employing microsatellite and mitochondrial markers we have detected significant F_{ST} values between hake individuals inhabiting gulfs and those distributed in the open sea, and asymmetric gene flow, higher from the gulf to the open sea than in the opposite direction. These differences can be interpreted as signals of separate populations in gulfs which may act as sources of variability for hake species. Occurrence of similar phenomena in Atlantic waters in both the northern and the southern Hemisphere, for these two pelagic–demersal hake species, suggests that gulfs may constitute a target for designing marine protected areas and confirms the adequacy of gulf-specific management already employed in Argentina.

1. Introduction

Identification of unique populations which represent biological units with particular genetic identity is a priority for conservation and management purposes (e.g. González-Suárez et al., 2009). Evolutionary significant units should be protected to preserve species biodiversity. At sea, although physical barriers are not as evident as in terrestrial ecosystems, some areas are at least partially isolated from the open ocean. The degree of isolation of a population depends on physical barriers and on the dispersal capacity of the organisms (e.g. Planes et al., 2009), as well as on historical processes like past climatic changes (e.g. Kenchington et al., 2009). Currents (Machado-Schiaffino et al., 2009), differences in salinity (Nielsen et al., 2004) and temperature (Banks et al., 2007), straits (Patarnello et al., 2007) and other geographical and physical–chemical factors can shape population structure of marine organisms and contribute to isolate stocks which may accumulate singular genetic diversity or variants absent in the rest of the species distribution. Marine protected areas and other zones subjected to special management measures should target identified valuable genetic resources for a species or a species assemblage (von der Heyden et al., 2008).

Gulfs seem to provide adequate conditions for the isolation of populations. They are usually shallower and exhibit higher salinity levels than the open sea. Substantial changes in physical conditions like salinity and temperature provide a mechanism for ecological isolation; individuals inhabiting gulfs can exhibit different patterns of growth, fertility and other biological characteristics (e.g. Houghton and Guptha, 1991; Rönkkönen et al., 2004). Gulf boundaries may therefore represent barriers to gene flow for some species. For example, fine-scale genetic structure associated with coastal geography has been described for various marine organisms in different localities worldwide, including Australian sea urchins (Banks et al., 2007), South African Clinid fish (von der Heyden et al., 2008), shrimp and fish from the Gulf of Mexico (Bernardi et al., 2003; Mathews, 2006), and others. Gulfs can also constitute shelters in disadvantageous climatic conditions and be the center of expansion after a species collapses (e.g. Kenchington et al., 2009). Populations inhabiting gulfs should thus be managed separately if they are identified as distinct biological units.

However, the importance of gulfs as special habitats or shelters for unique populations does not seem to be extended to species with long dispersal capacity and vast migratory movements, which are able to pass through weak physical barriers as open gulf mouths often are. Hakes are an example of such species. Hakes of the genus *Merluccius* are demersal–pelagic species of huge economic and ecological importance (Alheit and Pitcher, 1995). They migrate long distances during their life cycle following the

* Corresponding author. Tel.: +34 985102726; fax: +34 985103534.
E-mail address: gonzamachado@yahoo.com (G. Machado-Schiaffino).

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seasonal movements of their prey. Mature hakes aggregate at spawning sites. They spawn near the shelf edge and their larvae sink and stay at a variable depth (depending on the species) until completing their early development about 1 year later (yearlings), then start seasonal migrations for feeding until maturity. There is little information on genetic population structure of hakes. In some

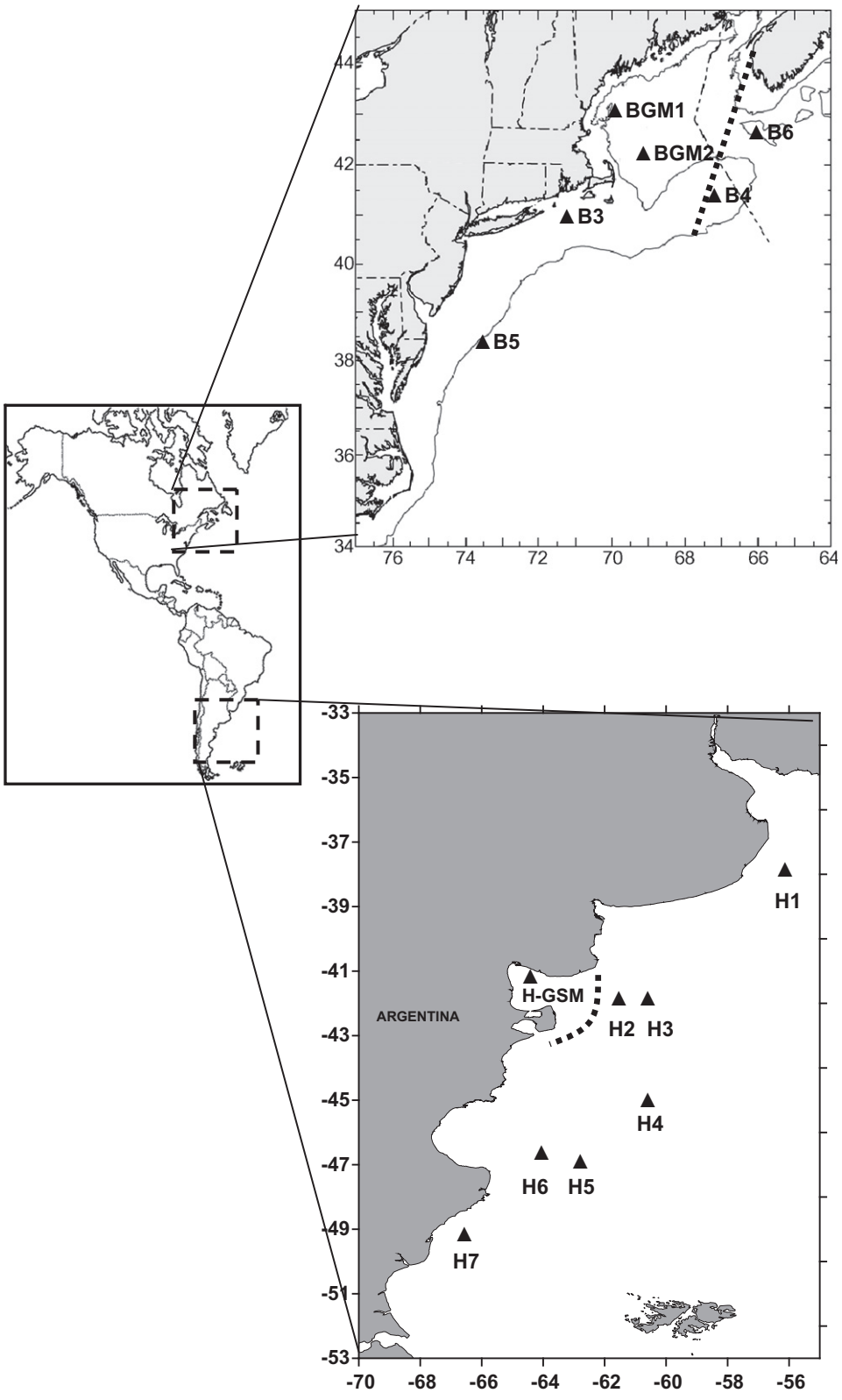

Fig. 1. Map with the sampling locations in the Atlantic Ocean. *Merluccius bilinearis* sampling locations: BGM1 and BGM2, Gulf of Maine populations; B3–B6, open sea. *M. hubbsi* sampling locations: H1–7, samples from the open sea; HGSM, samples from the San Matias Gulf. Dotted lines represent the main significant barrier found with the software Barrier V2.2 (Manni et al., 2004) for each species.

well-studied species like *Merluccius merluccius*, a type of homing has been described, resulting in isolation-by-distance population models in areas such as south European waters (Castillo et al., 2005) with a clear separation of the Mediterranean stock (Roldan et al., 1999; Lundy et al., 1999; Castillo et al., 2004; Cimmaruta et al., 2005). Other species like *Merluccius paradoxus* also exhibit a defined population structure in the South African region, whereas the sympatric species *Merluccius capensis* is almost panmictic (von der Heyden et al., 2007). Subtle genetic differentiation, probably associated with latitudinal distribution, has been recently proposed for the offshore hake *Merluccius albidus* (Machado-Schiaffino et al., 2010). Genetic structure was not detected in the Pacific *Merluccius gayi* (Galleguillos et al., 2000), although strong genetic differentiation has been recently described for the other hake species in its Pacific distribution area, *Merluccius australis* (Machado-Schiaffino et al., 2009). In all the cases above, genetic isolation, if it exists, occurs between stocks situated at relatively long distances, or with strong physical barriers like currents or straits. The high migratory capacity of the genus *Merluccius* makes these species good candidates for overcoming more subtle geographical barriers.

In this study, we have identified two hake species with seasonal migratory movements and high dispersal capacity which inhabit two almost symmetrical oceanographic areas: the Atlantic North and South American waters. The species are *Merluccius bilinearis* and *Merluccius hubbsi* respectively. Argentine hake (*M. hubbsi*) is a demersal–pelagic species distributed from southern Brazil (23°S) to the Patagonian shelf bordering Argentina (54°S) at depths between 50 and 500 m (Bezzi et al., 1995). Silver hakes (*M. bilinearis*) are distributed on the continental shelf of the north-west Atlantic ranging from Newfoundland to South Carolina (around 55–24°N; Helser et al., 1995; Morse et al., 1999). In the distributional areas of each species there are various conspicuous gulfs, like the Gulf of Maine in North America and the Gulf of San Matias (Argentina) in South America, which are located approximately at the same latitude in each hemisphere (42°N and S respectively; Fig. 1). In the present study, we employ microsatellite loci and mitochondrial variation at the control region to test the null expectation of no genetic structure at microscales due to high mobility, analyzing individuals of the two species sampled inside and outside gulfs.

2. Materials and methods

2.1. Sample collections

We tried to collect adult samples during the pre-spawning season (the month/s just before spawning) because that is the moment when individuals aggregate in reproduction areas and population structure may be better defined for these highly migratory species. Adults of *M. bilinearis* were collected from six locations in North American waters as part of the NMFS-NEFSC

Table 1
Summary of genetic variation at the studied microsatellite loci. Coordinates: latitude and longitude of the central point of each marine area sampled for each species. *N*: sample size. Na: number of alleles per locus. HWE: deviation from Hardy–Weinberg equilibrium. He and Ho, heterozygosities expected and observed, respectively. SD, standard deviation. NS, no significant; $^{**}P < 0.01$. For *M. bilinearis*: BGM1 and BGM2, Gulf of Maine populations; B3–B6, open sea. For *M. hubbsi*: H1–7, samples from the open sea; HGSM, samples from the San Matias Gulf. Samples obtained in 2006 are identified with −06.

Samples	Coordinates	*N*		Microsatellite loci							Over all loci		
				Maus4	Maus7	Maus30	Maus32	W01	Hk20	Hk03	Mean Na (SD)	He (SD)	Ho (SD)
M. bilinearis													
BGM1	43°0′ N 69°45′ W	54	Na	22	27	22	23	13	23	–	21.67 (4.63)	0.90 (0.30)	0.85 (0.09)
			HWE	**	**	**	NS	**	*				
BGM2	42°15′ N 69°0′ W	28	Na	18	26	17	23	14	21	–	19.83 (4.36)	0.89 (0.05)	0.89 (0.03)
			HWE	NS	**	NS	NS	NS	**				
B3	41°0′ N 71°30′ W	34	Na	18	29	16	24	13	23	–	20.50 (5.89)	0.90 (0.05)	0.87 (0.10)
			HWE	NS	NS	NS	NS	NS	NS				
B4	41°20′ N 67°0′ W	69	Na	22	34	21	30	17	32	–	26.00 (6.90)	0.91 (0.03)	0.87 (0.07)
			HWE	NS	NS	NS	NS	NS	NS				
B5	38°30′ N 73°30′ W	26	Na	21	29	15	22	12	24	–	20.50 (6.16)	0.91 (0.04)	0.87 (0.09)
			HWE	NS	NS	NS	NS	NS	NS				
B6	42°40′ N 66°0′ W	24	Na	12	24	12	19	13	22	–	17.00 (5.37)	0.88 (0.07)	0.88 (0.12)
			HWE	NS	NS	NS	NS	NS	NS				
M. hubbsi													
H1	37°45′ S 56°30′ W	50	Na	–	19	14	7	16	21	17	15.67 (4.88)	0.85 (0.10)	0.75 (0.18)
			HWE		NS	**	NS	*	NS	NS			
H1–06	37°45′ S 56°30′ W	20	Na	–	14	12	6	15	15	14	12.67 (3.44)	0.85 (0.10)	0.77 (0.25)
			HWE		NS	**	NS	NS	NS	NS			
H2	42°0′ S 61°30′ W	52	Na	–	15	21	10	14	20	17	16.17 (4.07)	0.87 (0.08)	0.74 (0.23)
			HWE		NS	**	NS	**	NS	NS			
H3	42°0′ S 60°20′ W	37	Na	–	12	14	8	15	17	15	13.50 (3.15)	0.84 (0.01)	0.77 (0.20)
			HWE		NS	**	NS	NS	NS	NS			
H4	45°0′ S 60°30′ W	50	Na	–	14	19	8	14	21	18	15.67 (4.68)	0.85 (0.11)	0.73 (0.12)
			HWE		NS	**	NS	**	NS	NS			
H4–06	45°0′ S 60°30′ W	34	Na	–	12	15	8	17	18	14	14.00 (3.63)	0.86 (0.06)	0.81 (0.15)
			HWE		NS	**	NS	NS	NS	NS			
H5	47°0′ S 62°45′ W	50	Na	–	13	19	9	15	18	16	15.00 (3.66)	0.85 (0.09)	0.76 (0.17)
			HWE		NS	**	NS	NS	NS	NS			
H6	46°45′ S 64°0′ W	50	Na	–	15	16	10	16	22	14	15.50 (3.89)	0.87 (0.07)	0.77 (0.21)
			HWE		NS	**	NS	*	NS	NS			
H6–06	46°45′ S 64°0′ W	35	Na	–	15	16	6	16	20	15	14.67 (4.63)	0.86 (0.10)	0.79 (0.19)
			HWE		NS	**	NS	NS	NS	NS			
H7	49°15′ S 66°40′ W	31	Na	–	12	17	8	15	20	14	14.33 (4.13)	0.87 (0.08)	0.79 (0.12)
			HWE		NS	**	NS	NS	NS	NS			
H-GSM	41°40′ S 64°20′ W	54	Na	–	17	18	9	15	22	14	15.83 (4.35)	0.87 (0.08)	0.84 (0.11)
			HWE		NS	**	NS	NS	NS	NS			

bottom trawl survey (Fig. 1). A minimum of five different stations were sampled per location, with a latitudinal range of 37–44°N. The samples were collected in spring and winter surveys between 2002 and 2005. Winter and spring samples are considered pre-spawning seasons (Helser et al., 1995).

Adults of *M. hubbsi* were collected from eight locations in Argentinean waters as part of the EU Project MARINEGGS in May 2004 (Table 1). They could be considered, with the exception of temporal samples, pre-spawning samples (Bezzi et al., 1995). Temporal samples (juveniles) were taken from three of the eight locations in 2006 (H1, H4 and H6). Sampling stations covered the distributional area of the species between 37 and 49°S (Fig. 1).

Sample sizes for each location (ranging from 20 to 67) are shown in Table 1. A small piece of muscle or fin was taken from each individual and preserved in pure ethanol until analysis.

2.2. DNA extraction and genetic analysis

DNA was extracted from tissue samples following a Chelex-based protocol (Estoup et al., 1996). Genetic variation at six dinucleotide microsatellite loci was analyzed: Mmer UEAW01 (Rico et al., 1997); Mmer-Hk20 and Mmer-Hk03 (Morán et al., 1999); Maus7, Maus30 and Maus32 (Machado-Schiaffino and Garcia-Vazquez, 2009). PCR amplifications were performed following Machado-Schiaffino and Garcia-Vazquez (2009).

Sizes of the labeled PCR products were determined using an ABI 3100 Genetic Analyzer (Applied Biosystems). The results were visualized employing the GENEMAPPER v3.5 software (Applied Biosystems).

The primers MmerHK01 and MmerHK02 (Lundy et al., 2000) were employed to amplify the control region. PCR reactions were carried out as described by Lundy et al., (2000). PCR was performed using the GeneAmp PCR system 2720 by Perkin Elmer Cetus with the following conditions: an initial denaturing step at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 20 s, annealing (for 20 s) at 53 °C and an extension at 72 °C for 30 s, ending with a final extension at 72 °C for 10 min. PCR products were visualized in 50 ml 1.5% agarose gels with 3 μl of 10 mg/ml ethidium bromide. Stained bands were excised from the gel and DNA was purified with an Eppendorf PerfectPrep Gel CleanUp Kit prior to sequencing. Automated fluorescence sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 Terminator system. Both strands (direct and reverse) of each DNA fragment were sequenced.

2.3. Sequence editing

Mitochondrial control region sequences were edited using the BioEdit Sequence Alignment Editor software (Hall, 1999). Sequences were aligned with the ClustalW application (Thompson et al., 1994) included in BioEdit. The different haplotypes found for each species were obtained with the program Collapse 1.2 (Posada, 2004).

2.4. Statistical analysis

2.4.1. Microsatellite data analysis

Scoring errors, large allele dropout and null alleles were checked with MICROCHECKER (Van Oosterhout et al., 2004). Exact *P*-values for testing conformity of genotypes with Hardy–Weinberg proportions and linkage disequilibrium were estimated by the Markov chain method (1000 dememorization steps, 1000 batches, 10,000 iterations per batch) employing the GENEPOP program (Raymond and Rousset, 1995). Genetic differentiation between populations is generally measured with the F_{ST} statistics, which is based on differences in allele frequencies. The program ARLEQUIN (Schneider et al., 2000) was employed for estimates of F_{ST} values and their statistical significance between samples pairs, i.e. the significance of population differentiation, with the following settings: 1000 permutations for significance, 10,000 steps in Markov chain. Levels of significance for multiple tests were determined using sequential Bonferroni adjustments for simultaneous tests (Rice, 1989) whenever relevant. The program ARLEQUIN (Schneider et al., 2000) was also employed to carry out the hierarchical analysis of molecular variance (AMOVA).

The program migrate version 2.4.3 (Beerli and Felsenstein, 1999) was used to estimate dispersal rates and long-term effective population size (N_e) from the microsatellite data, using a maximum-likelihood (ML) coalescent approach and averaging five runs. The program estimates a parameter called Θ, which is the product of the effective population size and mutation rate: $4N_e\mu$, where μ is the mutation rate per generation (10^{-4}; Estoup and Angers, 1998); and N_em, which is the effective number of migrants per generation. As program settings we employed a stepwise-mutation model (Brownian motion approximation) and default settings for other parameters. For each run, starting estimates for Θ were based on F_{ST} values, with burn-in = 10,000 trees, 14 short chains with a total of 100,000 genealogies sampled, and three long chains with 1000,000 genealogies sampled, for each locus. Adaptive Chain heating, with four different temperatures, was employed to get an efficient exploration of the data.

2.5. mtDNA data analysis

Population variation of mitochondrial sequences was analyzed employing the program ARLEQUIN (Schneider et al., 2000). Different weight was given to transitions and transversions (1:2 respectively; Graur and Li, 2000). Variation within samples was estimated by nucleotide diversity (π) and haplotype diversity (h) (Nei, 1987). Genetic differentiation between populations was based on haplotype frequencies; population differentiation was estimated by Markov chain methodology (10,000 steps, 1000 dememorization steps).

Deviation from equilibrium expectations were tested with Tajima's D (Tajima, 1989) and Fu's *Fs* (Fu, 1997) neutrality tests based on an infinite-site model without recombination. To investigate the possibility of demographic change, mismatch distributions (Harpending, 1994) were analyzed. A median-joining network (Bandelt et al., 1999) was constructed to represent the intra-specific genealogy of the large data set of haplotypes and their relative frequencies in the sampled population using the software NETWORK 4.5 (available at http://www.fluxus-technology.com).

Long-term effective population size (N_e) was estimated as described above for microsatellite loci, using a constant mutation rate per generation of 4×10^{-2} (Machado-Schiaffino et al., 2009) to transform estimates of Θ into N_e.

2.6. Detection of barriers to gene flow

We employed Monmonier's maximum difference algorithm (Manni et al., 2004) to highlight geographical features that are associated with pronounced genetic discontinuities, using the program BARRIER (version 2.2). Geographical coordinates of each sample were connected by Delauney triangulation using a pairwise F_{ST} genetic matrix obtained from microsatellite loci results. Putative genetic boundaries were therefore identified across the oceanographic areas considered (Manni et al., 2004).

3. Results

3.1. Merluccius bilinearis

3.1.1. Microsatellite loci variation

Scoring errors or allele dropouts were not detected in the data set obtained for microsatellite loci except for the locus Mmer-Hk03 which showed signs of null alleles (Chakraborty estimation = 0.33) and was excluded from population structure analysis. High genetic variability at all microsatellite loci was found (Table 1). The number of alleles per locus ranged from 12 (loci Maus4 and W01 in B6 and B5 respectively) to 34 (locus Maus7 for B4). The mean number of alleles per locus ranged from 17 (B6) to 26 (B4). Seven tests out of 36 were significant indicating deviation from HWE, due to heterozygote deficit in all cases (Table 1).

Temporal differentiation between years was not found from the analysis of molecular variance (AMOVA) for *M. bilinearis* ($P > 0.05$ in the AMOVA) and the samples from the same location were pooled.

With respect to geographical differentiation, significant pairwise F_{ST} values (=significant genetic differentiation between population pairs) ranging from 0.006 to 0.009 were obtained only between the samples caught within the Gulf of Maine (BGM1 and BGM2) and two samples taken outside the gulf (B4 and B5) (Table 2A). The main spatial barrier to gene flow (Manni et al., 2004) was significant ($P < 0.05$), and was detected between the offshore samples B4 and B6 and the rest of the samples obtained nearer the coast (Fig. 1).

3.2. Mitochondrial DNA variation

The length of the analyzed control region sequence was 437 nucleotides. Eighteen variable sites were found which correspond to 33 haplotypes (GenBank accession numbers GQ204141 to GQ204166; EU410453 to EU410455 and GQ275350 to GQ275354). Both haplotypic and nucleotidic diversities were slightly higher for Gulf of Maine samples than for the rest (Table 3). Nine (64% of the haplotypes found in that area) and four (44%) haplotypes were exclusive to the gulf and open sea respectively. Fu's test of selective neutrality was significantly negative ($P < 0.01$ and $P < 0.05$ for gulf and open-sea samples respectively). Based on mismatch analyses, the sudden expansion model of population growth could not be rejected for the gulf or the open-sea populations (non-significant SSD and raggedness values).

The haplotype network (Fig. 2) provided two equally frequent haplotypes which could be considered two lineages, H1 and H3, which were present inside and outside the Gulf of Maine. The rest of the haplotypes were derived from them yielding a two star-shaped network. Most Gulf of Maine private haplotypes derived from the haplotype 1, indicating that the population established there has derived principally from one of the two main lineages, H1. Most interesting is the succession of the four gulf private haplotypes H29–32, derived by consecutive single mutations from the haplotype 25. In contrast, the private haplotypes present only in the open sea were derived from the two central haplotypes (five and nine from Haplotype 1 and 3 respectively).

Table 2
Tests for genetic differentiation between populations. Above diagonal: pairwise genetic distances (F_{ST}) based on five microsatellites. Below diagonal, F_{ST} significance. $P < 0.05$ and $P < 0.01$ levels of significance are marked with one and two asterisks respectively. NS, not significant. (A) *M. bilinearis.* BGM1 and BGM2, Gulf of Maine populations; B3–B6, open sea. (B) *M. hubbsi.* H1–7, samples from the open sea; HGSM, samples from the San Matias Gulf.

	BGM1	BGM2	B3	B4	B5	B6
(A)						
BGM1	–	0.004	0.003	0.009	0.007	0.005
BGM2	NS	–	0.001	0.006	0.007	0.007
B3	NS		–	0.004	0.003	0.003
B4	**	*	NS	–	0.004	0.004
B5	*	*	NS	NS	–	0.007
B6	NS	NS	NS	NS	NS	–

	H1	H2	H3	H4	H5	H6	H7	H-GSM
(B)								
H1	–	0.001	0.003	0.002	0.002	0.001	0.003	0.011
H2	NS	–	0.001	0.001	0.001	0.002	0.001	0.011
H3	NS	NS	–	0.003	0.002	0.002	0.004	0.010
H4	NS	NS	NS	–	0.004	0.001	0.007	0.012
H5	NS	NS	NS	NS	–	0.002	0.001	0.015
H6	NS	NS	NS	NS	NS	–	0.001	0.009
H7	NS	NS	NS	NS	NS	NS	–	0.017
H-GSM	**	**	*	**	**	*	**	–

Table 3
mtDNA haplotypes found in *M. bilinearis and M. hubbsi* at the sampling locations considered. n = sample size, h = gene diversity, SE = standard error, π = nucleotide diversity, D = Tajima's test of selective neutrality, Fs = Fu's test of selective neutrality. SSD tests the validity of a stepwise expansion model based on the sum of squares deviations between the observed and expected mismatch, non-significant mismatch values suggest population expansion. Raggedness Index is calculated similarly, non-significant raggedness values suggest population expansion. Significance level: NS, non-significant.

	M. bilinearis		*M. hubbsi*	
	Gulf	Open sea	Gulf	Open sea
n	62	42	37	283
Haplotypes	14	9	13	27
Exclusive haplotypes	9	4	2	16
h	0.7996	0.7282	0.7643	0.8259
SE	0.0374	0.0466	0.0509	0.0123
π	0.0032	0.0024	0.1736	0.1640
D	-0.5724^{NS}	-0.6488^{NS}	1.0964^{NS}	0.2084^{NS}
Fs	-5.5967^{**}	2.8289^{*}	-0.2898^{NS}	-9.0456^{**}
SSD	0.0016^{NS}	0.0033^{NS}	0.0384^{NS}	0.0066^{NS}
Raggedness	0.0238^{NS}	0.0344^{NS}	0.0940^{NS}	0.0229^{NS}

* $P < 0.05$.
** $P < 0.01$.

3.3. Relationship between gulf and open-sea samples

For *M. bilinearis* (Table 4), maximum-likelihood estimates of dispersal between the Gulf of Maine and the open sea were clearly asymmetric: higher dispersal values were obtained from the gulf to open sea than in the opposite direction (less than 1 versus 1.776 immigrants/generation respectively).

An estimator of the relative weight of hake populations inhabiting gulfs with respect to the rest of populations distributed in the open sea could be the relative effective sizes, N_e, assuming that the N_e/N ratios are homogeneous across areas. For *M. bilinearis*, N_e estimates for the population inhabiting the Gulf of Maine were slightly higher of those obtained for open sea locations, either based on microsatellite or mitochondrial loci (Table 5), in spite of the fact that sampling in the open sea area was much broader. The estimations obtained were lower than 10,000 in all cases.

3.4. Merluccius hubbsi

3.4.1. Microsatellite loci variation

Significant scoring errors or allele dropouts were detected at the locus Maus30 for this species. It exhibited signs of null alleles (Chakraborty estimation = 0.33) and was excluded from population structure analysis. The number of alleles per locus ranged from 6 (locus Maus32 in H1–06) to 22 (locus HK20 in H6 and H-GSM), and the mean number of alleles per locus from 12.7 (H1–06) to

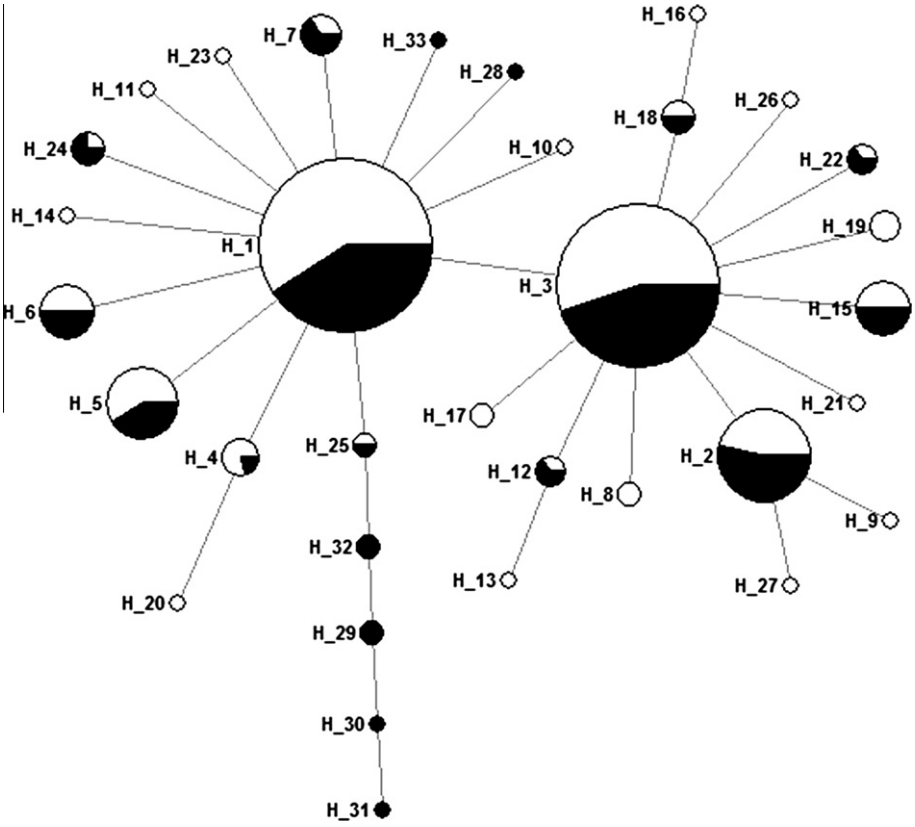

Fig. 2. Median-joining network showing the relationships among the 33 *M. bilinearis* haplotypes defined by mitochondrial control region sequence variation. H_1, H_2, etc. are the haplotype names. Areas in the circles are proportional to the frequency of each haplotype. In black and white background, haplotypes from Gulf of Maine and open sea respectively.

Table 4
Maximum likelihood estimates of effective dispersal rate (N_em) among regions based on microsatellite data using Migrate program. Confidence intervals (95%), CI.

Dispersal per generation	N_em	95% CI
M. bilinearis		
From gulf to open sea	1.776	1.544–2.044
From open sea to gulf	0.966	0.841–1.104
M. hubbsi		
From gulf to open sea	1.356	1.134–1.619
From open sea to gulf	0.616	0.532–0.712

Table 5
Effective population size (N_e) estimated using likelihood method (migrate program) based on microsatellite loci (left) and on mitochondrial sequences (right). Confidence intervals (95%) are shown between brackets.

	Effective population size estimates			
	Microsatellite loci		mtDNA	
	Open sea	Gulf	Open sea	Gulf
M. bilinearis				
Long-term N_e	3771	5143	3150	3600
	[3593–3958]	[4833–5481]	[2575–3900]	[1900–7925]
M. hubbsi				
Long-term N_e	11,536	13,957	3000	4725
	[11,016–12,225]	[12,444–15,634]	[2450–3700]	[1475–34,375]

16.2 (H2) (Table 1). Significant deviations from Hardy–Weinberg equilibrium (HWE) were found at the locus Maus30 (possible due to null alleles, as were detected with the MICROCHECKER program) due to heterozygosity deficit (Table 1).

Significant temporal differences were not detected for *M. hubbsi* from the analysis of molecular variance (AMOVA) comparing 2004 and 2006 samples ($P > 0.05$), allowing us to pool samples from the same location for the general analysis. Pairwise F_{ST} tests for genetic differentiation between samples provided highly significant *P*-values ranging from 0.009 to 0.015 among samples from the Gulf of San Matias (H-GSM) and the rest of the samples after Bonferroni correction (Table 2B). Overall, these data indicate spatial differentiation due to inside–outside gulf genetic differences. This differentiation was confirmed with the program Barrier (Manni et al., 2004). The main barrier to gene flow ($P < 0.05$) was found between the Gulf of San Matias sample and the rest of the hake caught in the open sea.

3.5. Mitochondrial DNA variation

In the 438 nucleotides analyzed within the control region for *M. hubbsi* 17 variable sites were found, corresponding to 29 different haplotypes (GenBank Accession numbers EF362866 to EF362870 and EU860404 to EU860427). The samples obtained inside and outside the Gulf of San Matias exhibited similar within-population diversity indices (Table 3), for both π and h values, despite much smaller gulf sample size. Two private haplotypes were found within the Gulf of San Matias (15.4% of haplotypes present in the gulf were exclusive of that area) and 15 in the rest of the locations considered, in low frequency in all cases: around 58% of haplotypes

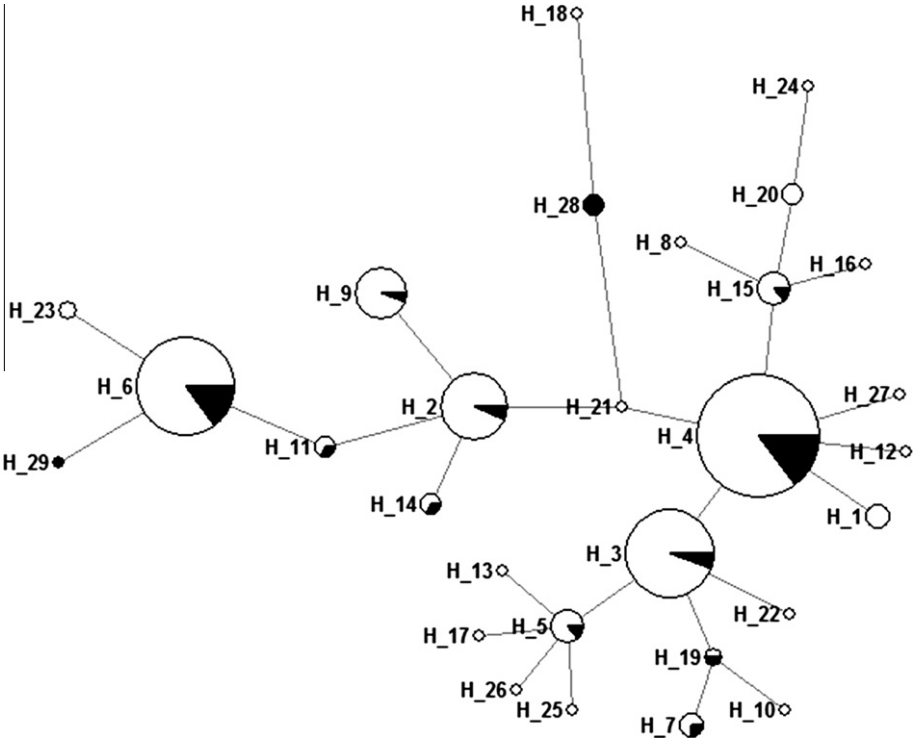

Fig. 3. Median-joining network showing the relationships among the 29 *M. hubbsi* haplotypes defined by mitochondrial control region sequence variation. H_1, H_2, etc. are the haplotype names. The size of the circles is proportional to the frequency of each haplotype. In black and white background, haplotypes from San Matías Gulf (H-GSM) and open sea respectively.

present in open seas were relatively rare and exclusive. Deviations from equilibrium were not statistically significant for gulf samples ($P > 0.05$ for both Tajima's and Fu's tests). However, Fu's test was significant for open-sea Argentinean hake ($F = -9.05$, $P < 0.01$). Mismatch analyses did not allow us to reject a sudden expansion model of population growth. The haplotype network had a complicated shape with four main haplotypes, from which all the rest derived by consecutive mutations (Fig. 3). Exclusive alleles found in the gulf and in open seas were interspersed in the network without a clear assignment to a cluster.

3.6. Relationship between gulf and open-sea samples

For *M. hubbsi* (Table 4), the situation was similar to that found for *M. bilinearis*, with higher migration rate from the Gulf of San Matias to the open sea (1.356 immigrants/generation) than vice versa (0.6 immigrants/generation). Also similar to *M. bilinearis*, long-term N_e estimates based on microsatellite loci suggested that the number of *M. hubbsi* breeding adults inside the Gulf of San Matias (about 14,000 or 4700 estimated based on microsatellite loci and mitochondrial DNA respectively) was higher than outside (Table 5).

4. Discussion

Our results suggest a role of gulfs as shelters for hake populations. On the one hand, we have found subtle but very clear signals of differentiation between hake populations inhabiting the inside and outside of gulfs, like significant F_{ST} values for microsatellite loci. A significant barrier to gene flow (Manni et al., 2004) was detected between gulf and open-sea samples for *M. hubbsi* (Fig. 1b), reinforcing this idea and representing one more example of barriers to migration in the marine environment (Palumbi, 1994) that contribute to population structuring in species with high dispersal capacity. For *M. hubbsi* there is no consensus about stock composition (Roldan, 1991), but the presence of a separate stock in the Gulf of San Matias has been postulated (Di Giácomo et al., 1993; Ehrlich, 1998) based on differentiation in parasite composition (Sardella and Timi, 2004). The results obtained in this study confirm such differentiation.

For *M. bilinearis* however, the main barrier to gene flow seems to separate offshore samples (B4 and B6) from those obtained closer to the coast (Fig. 1a), including the samples caught inside the Gulf of Maine. There is little information about the population genetic structure of *M. bilinearis*. The species is managed as two different stocks (north and south stocks), defined based on morphological and demographic characters; the boundary dividing the stocks is located between Nantucket Shoals and the northern edge of Georges Bank (Lock and Packer, 2004). The results obtained in this study suggest a separation between offshore and inshore hake instead, as reported for the long-tailed South American hake *Macruronus magellanicus* (D'Amato, 2006).

In the two hake species considered, relatively large effective population sizes and asymmetric gene flow suggest large contributions of gulf stocks to the whole population. Population size estimates were smaller when based on mitochondrial DNA, as expected given that mitochondrial DNA effective population size is ¼ of that for nuclear loci (Birky et al., 1989), but they were consistently larger inside than outside gulfs for both *M. bilinearis* and *M. hubbsi* (Table 5). Both *M. bilinearis* and *M. hubbsi* exhibit a consistent pattern of significant migration from the gulf to the open sea but not significant migration in the opposite direction, as populations with less than one immigrant per generation can be considered separated from the rest (Mills and Allendorf, 1996). This asymmetric gene flow strongly suggests that gulfs have historically acted as a source of breeders for the rest of the population. Higher gene flow outwards than inwards recalls a source-sink model,

where the gulf is the source for the open sea. Populations naturally structured following source-sink models should be managed protecting the sources (i.e. Crowder et al., 2000). The fact that the model was similar in hakes inhabiting completely separated seas in different hemispheres reinforces the idea of considering gulfs as targets for conservation measures given their condition of shelters for genetic variation (Game et al., 2009).

Another question is if the genetic differentiation between gulf and open-sea populations is a consequence of reproductive barriers associated with environmental factors or rather a sort of homing. Some species of marine fishes have been found to exhibit some population subdivision if the species also show a degree of spawning fidelity to natal areas. For example, Ruzzante et al., (1998) found genetic structure in north-west Atlantic cod (*Gadus morhua*) spawning banks, which was interpreted as evidence for homing behaviour. There are spawning areas inside and outside the gulfs which are relatively near to each other, for the two hake species considered here (Bezzi et al., 1995; Helser et al., 1995). High gene flow between them could be expected, but here we have evidenced that such expected gene flow is restricted, suggesting homing. Other authors have reported the influence of selection in population structuring of marine species (Nielsen et al., 2004; Banks et al., 2007), and selective factors may also account for spatial population differentiation in the two species studied here. However, as we have employed supposedly neutral markers, such possibility can not be tested with the present dataset.

The high frequency of rare exclusive mtDNA haplotypes is an indirect indicator of a process of evolutionary expansion after a bottleneck (Fu, 1997). For both *M. bilinearis* and *M. hubbsi*, such rare haplotypes were more abundant in open sea than in gulf samples (Figs. 2 and 3). Populations inhabiting the open sea may have been exposed to more environmental hazards, including climate-related events, than the more protected gulf stocks, which can even constitute expansion centers after bottlenecks (Kenchington et al., 2009). The footprints of bottlenecks can be diluted or erased for nuclear DNA, subjected to less gene drift by higher N_e and also transported by highly migratory males, but may be better conserved for mtDNA, with only one quarter N_e of nuclear DNA (Birky et al., 1989).

In conclusion, we have identified genetic signals of at least partial isolation of hake populations inhabiting gulfs for two species, *M. hubbsi* and *M. bilinearis*, distributed in the South and the North Atlantic Ocean respectively. Asymmetric gene flow between the gulf and the open-sea populations indicates that gulf hakes significantly contribute to effective population size and consequently to maintain genetic variation of these species. Therefore this mechanism may well be important for the evolutionary legacy of the species. In the long-term nature of the process, total isolation of gulf sub-populations is not expected as long as gene flow occurs. However, if gene flow decreases or is interrupted due to more abrupt differentiation of gulfs by environmental changes (warming, oscillations of sea level, El Niño phenomena, etc.) creating stronger barriers between gulfs and open sea, gulfs could become hotspots of incipient speciation, as reported for example for the Gulf of California (Bernardi et al., 2003).

As a final remark, due to their conditions of shelters of genetic variation and nursery areas, we suggest considering gulf areas as management units subjected to special measures given their value for conservation.

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