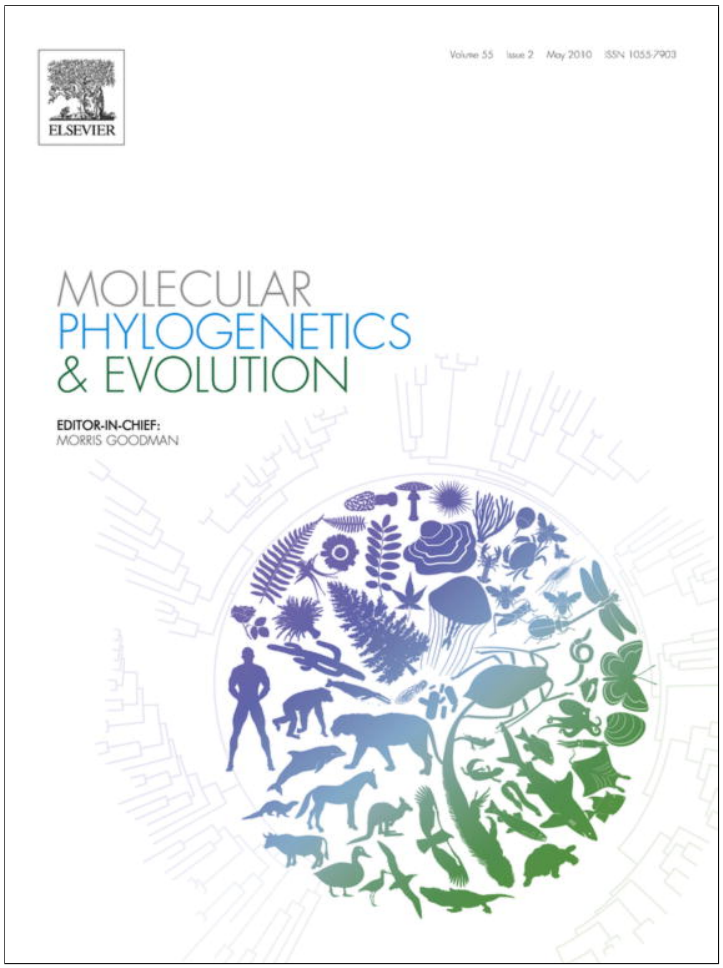

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Introgressive hybridization in North American hakes after secondary contact

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ABSTRACT

High levels of bidirectional introgressive hybridization were found between the two Atlantic North American hakes *Merluccius albidus* and *M. bilinearis* in their overlapping distribution area between the 34 and the 42°N parallels, employing mitochondrial and nuclear DNA markers. Absence of F_1 hybrids, and varied levels of bidirectional introgression, indicate long-time hybridization and backcrossing. Based on the evolutionary history of the genus *Merluccius*, originated in this area from the ancestor of the present *M. bilinearis* by the rise of the Panama Isthmus, secondary contact between the two species has probably been promoted by northwards displacement of *M. albidus*. Higher introgression rates in southern areas of *M. albidus* could be explained by restricted gene flow in that area which may allow long-term accumulation of introgressed genes.

1. Introduction

Hybridization followed by interspecific introgression of nuclear genes is an evolutionary path which remains poorly understood in the context of the biological definition of species. Classically, species are reproductively isolated taxonomic units, two separate genetic pools (Dobzhansky, 1935; Mayr, 1942; Coyne, 1992; Mallet, 1995). With the advent of molecular genetics there is a growing body of evidences of at least some interchange of genes between related species, which in some cases can be beneficial (Mallet, 2005), although it usually constitutes a population load due to lower fitness of F_1 and post-F_1 hybrids (Mayr, 1963; Coyne and Orr, 2004). Two species may maintain their separate integrity with a low-level interchange of genes (e.g. Wiley et al., 2009; Kappas et al., 2009), but hybridization may also interfere in the process of speciation. For example, when introgressive hybridization is intense, speciation may revert and formerly separate species may fuse together (e.g. Wolfe, 2003; Seehausen, 2006; Seehausen et al., 2008).

The spatial distribution of species determines opportunities for hybridization. Some species are entirely sympatric and exhibit a certain level of hybridization, although maintain their integrity (Mallet, 1995; Coyne and Orr, 2004). Other species are partially allopatric, and hybridize in the overlapping distribution areas forming hybrid zones which are maintained more or less stable without compromising the rest of the species (e.g. Pastorini et al., 2009). Hybridization can be promoted when two species enter in secondary contact, especially if those species are relatively young and reproductive isolation is still not complete. In a changing world where geographical barriers are often broken by human activity, hybridization becomes increasingly frequent when allopatric species establish new contacts (Vellend et al., 2007). But anthropogenic activities are not the only cause of secondary contacts between species. Geological and climate changes largely drive variations in the geographical distribution of species and may allow secondary contacts and opportunities for hybridization; many examples have been demonstrated employing a vast variety of genetic tools in many taxa (e.g. Saetre et al., 2001; Lu et al., 2001; Gava and Freitas, 2003; Good et al., 2003).

In the marine realm warmer temperatures are causing rapid northwards expansion of species adapted to temperate climate (e.g. McFarlane et al., 2000; De Young et al., 2004). Exploring the overlapping areas of partially allopatric marine species can contribute to understanding the extent and evolutionary implications of secondary contacts and hybrid zones. During the expansion of a species, genetic drift occurs at the wavefront, where alleles of the colonizing populations can reach high frequencies even if they have no selective advantage (Klopfstein et al., 2006). Petit and Excoffier (2009) suggested that high rates of intraspecific gene flow can efficiently mitigate interspecific introgression, because introgressed genes will compete with genes migrating from the interior of the wavefront, making it less likely that introgressed genes reach high frequencies.

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In this study we have chosen as model species two hake species (genus *Merluccius*) distributed along the northwest Atlantic Ocean, the offshore hake *Merluccius albidus* and the silver hake *M. bilinearis*. *M. bilinearis* is distributed on the continental shelf of the northwest Atlantic ranging from Newfoundland to South Carolina (Helser et al., 1995; Morse et al., 1999). This is the area where the genus arose (Roldan et al., 1999; Quinteiro et al., 2000; Campo et al., 2007), during the middle Oligocene, around 30 million years ago (MYA). *M. albidus* likely appeared in the southern Hemisphere because in phylogenetic trees it clusters with South American species (Campo et al., 2007, 2009). Its overlapping distribution with *M. bilinearis* could be attributable to a northerly expansion of *M. albidus* along North American waters to reach its present distribution, from the Caribbean, the Gulf of Mexico, Suriname and French Guiana to the continental shelf and slope of the northwest Atlantic up to 42°N (Chang et al., 1999). The two species overlap in the southern and northern distribution area of *M. bilinearis* and *M. albidus*, respectively, approximately between the 34 and the 42°N parallels.

Employing five microsatellite loci, the nuclear locus 5S rDNA and the mitochondrial control region (CR) and cytochrome oxidase I (COI) gene as molecular markers we examined the level of intraspecific gene flow in *Merluccius albidus* and *M. bilinearis*, and the extent and direction of introgressive hybridization of these two species, to determine if there is an association between intraspecific and interspecific gene flow as suggested by Petit and Excoffier (2009), in an area of extensive secondary contact.

2. Materials and methods

2.1. Samples

A total of 380 samples were collected from the NMSF-NEFSC (National Marine Sanctuary Foundation-Northeast Fisheries Science Center) bottom trawl survey. Fish were identified *de visu* as 137 *M. albidus* and 243 *M. bilinearis*. The species was determined based on morphological characteristics, principally by the number of gill rakers (Chang et al., 1999). They were weighed and measured, and a small sample of tissue (1 g of gill or muscle) was preserved in 95% ethanol and stored at room temperature until DNA extraction. A total of 58 sampling stations were considered to provide extensive latitudinal coverage of the overlapping areas of the two species (Fig. 1). For the analysis we initially joined the *M. bilinearis* and *M. albidus* samples by latitudinal region in four and three groups, respectively (Table 1). The samples were collected in spring, fall and winter surveys in 2001, 2002, 2004 and 2005.

2.2. DNA analysis

DNA was extracted from the tissue (1 mm^3) with a Chelex-based protocol following Estoup et al. (1996). The species-specific marker 5S rDNA (partial region) was amplified using the primers 5S A 5′-TACGCCCGATCTCGTCCGATC-3′ and 5S B 5′-CAGGCTGG TATGGCCGTAAGC-3′ (Pendas et al., 1995). PCR reactions and conditions were as described by Garcia-Vazquez et al. (2009).

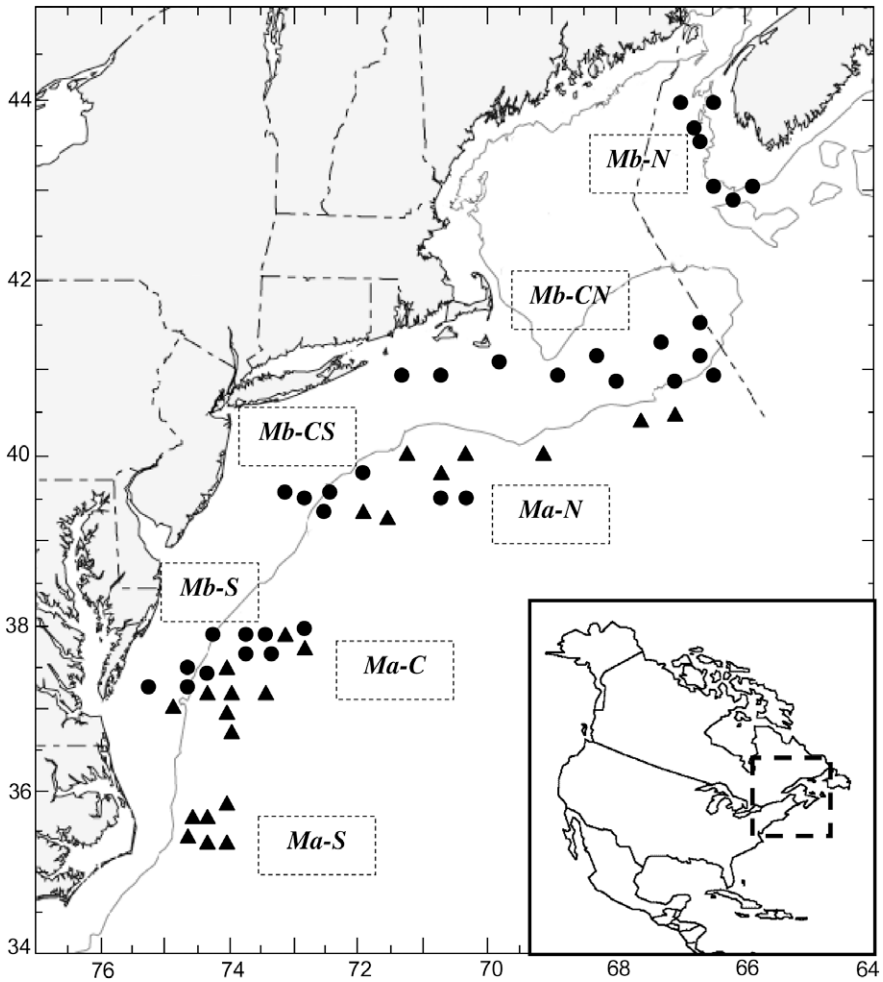

Fig. 1. Map with the sampling sites for *Merluccius albidus* (triangles) and *M. bilinearis* (circles). N: North; CN: Central North; C: Central; CS: Central South; S: South.

Table 1
Samples available from *M. bilinearis* (Mb) and *M. albidus* (Ma). Coordinates, latitude and longitude of the central point each marine area sampled for each species. N: North; CN: Central North; C: Central; CS: Central South; S: South. Sampled stations, number of sites where each species was caught within a marine area; *n*, sample size.

Sample	Coordinates	Sampled stations	*n*
M. bilinearis			
Mb-N	43°N 66.5°W	7	29
Mb-CN	41°N 69°W	11	143
Mb-CS	39.5°N 72°W	7	36
Mb-S	37.5°N 74°W	10	35
M. albidus			
Ma-N	40°N 70°W	8	47
Ma-C	37.5°N 74°W	9	55
Ma-S	35.5°N 74.5°W	6	35

The samples were genotyped for five microsatellite loci: Maus7, Maus30 and Maus32 (Machado-Schiaffino and Garcia-Vazquez, 2009); Mmer-UEAW01 (Rico et al., 1997) and Mmer-Hk20 (Morán et al., 1999). PCR protocols and genotyping followed Machado-Schiaffino and Garcia-Vazquez (2009).

The mitochondrial CR was amplified employing the primers MmerHK01 and MmerHK02 (Lundy et al., 2000) with the PCR reactions and conditions described therein. In addition, the COI gene was amplified in ten individuals of each species which yielded the typical species-specific CR sequences, to confirm species identification. The primers CO1Fish-F1 and CO1Fish-R1 were employed for PCR amplification as described in Ward et al. (2005). PCR products were visualized, purified and sequenced (both forward and reverse DNA strands) as described in Machado-Schiaffino et al. (2009).

2.3. Statistical methodology

Mitochondrial CR and COI sequences were edited using the BioEdit Sequence Alignment Editor software (Hall, 1999). Sequences were aligned with the ClustalW application included in BioEdit. The different haplotypes found for each species were obtained with the program DnaSP (Rozas et al., 2003). To examine possible spatial differentiation within species, population variation was analyzed with the program ARLEQUIN version 3.01 (Excoffier et al., 2005), weighting 1:2 transitions and transversions, respectively. Within-population variation was estimated by nucleotide diversity (π) and haplotype diversity (h) (Nei, 1987). Differentiation between populations within species based on haplotype frequencies was estimated by Markov chain methodology (10,000 steps, 1000 dememorization steps). Deviations from equilibrium expectations were tested with Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) neutrality tests based on infinite-site model without recombination. Under a model of sudden population expansion, both Fu's F and Tajima's D are expected to be significantly negative.

For microsatellites, scoring errors, large allele dropout and null alleles were checked employing the program MICROCHECKER (Van Oosterhout et al., 2004). Allelic richness was estimated with the program FSTAT (Goudet, 1995) with 10,000 permutations. Conformity of genotypes with Hardy–Weinberg (HWE) and linkage disequilibrium were estimated by the Markov chain method (1000 dememorization steps, 1000 batches, 10,000 iterations per batch) employing the program ARLEQUIN version 3.01 (Excoffier et al., 2005). Sequential Bonferroni adjustments for simultaneous tests (Rice, 1989) were applied whenever relevant.

The software STRUCTURE (v2.2, Pritchard et al., 2000) was employed for determining K, the number of clusters in the dataset (an indicator of the number of genetic units, independent of population and locality information), using Bayesian algorithm. Ten independent runs were performed between $K = 1$ and $K = 7$ using admixture model (each individual draws some fraction of its genome from each of the K populations) and correlated allele frequencies model. A burn-in period of 30,000 steps followed by 300,000 Markov chain Monte Carlo (MCMC) iterations were enough to ensure convergence. The classical model choice criterion (Ln $P(D)$), which is the mean of the log likelihood of the data at each step of the MCMC from which half of the variance is subtracted, was used to estimate K. Maximum value of Ln $P(D)$ gives an estimation of the K which best fits the data. STRUCTURE software provides the estimation of the membership fraction in each of the K inferred clusters (Q), for each individual. ΔK, a measure of the second order rate of change in the likelihood between successive K values, was found to accurately detect the most pronounced genetic subdivisions (the uppermost hierarchical levels of population structure) (Evanno et al., 2005). The height of the modal value of ΔK can be used as an indicator of the strength of the detected signal. Consequently, we calculated and plotted ΔK to validate K estimates and to detect eventual hierarchical structures in the dataset analyzed.

Spatial differentiation within species based on microsatellite loci, once discarded hybrids, was detected based on the statistical significance of F_{ST} values (10,000 permutations for significance, 10,000 steps in Markov chain) employing the program ARLEQUIN version 3.01 (Excoffier et al., 2005). Sequential Bonferroni adjustments for simultaneous tests (Rice, 1989) were applied whenever relevant.

3. Results

The mitochondrial DNA yielded clearly different control region sequences for *Merluccius bilinearis* (GenBank Accession Nos. GQ204141 to GC204166 and EU410453–EU410455) and *M. albidus* (GQ204121–GQ204140). They differed in 33 positions which can be considered species-specific polymorphisms, as they always exhibited the same nucleotides for each species. In addition there were also within-species polymorphisms distributed all along the CR sequences. In general *M. bilinearis* was more variable than *M. albidus*, exhibiting higher nucleotide diversity in the region considered (Table 2). No spatial differentiation was found within each species based on this mitochondrial sequence ($F_{ST} = 0.0162$ with $P = 0.337$ and $F_{ST} = 0.0135$ with $P = 0.32$ for *M. bilinearis* and *M. albidus*, respectively). Although Fu's tests were significant for three areas, *M. bilinearis* did not exhibit significant values of D (Tajima's test) in any sample (Table 2), indicating that the species was not subjected to a recent expansion. In contrast *M. albidus* provided highly significant negative values in both Tajima's and Fu's tests, strong signals of recent population expansion.

For microsatellites, MICROCHECKER did not detect dropouts or null alleles. Linkage disequilibrium was not found for any of the 40 and 30 possible pairwise comparisons for *M. bilinearis* and *M. albidus*, respectively, thus the loci can be considered independent. However many of the loci were significantly in discordance with HWE in the three *M. albidus* and four *M. bilinearis* populations considered (data not shown). The program STRUCTURE allowed us to identify two main genetic units (K) in the dataset (Fig. 2), as the highest Ln $P(D)$ and ΔK were obtained for $K = 2$. These two genetic units roughly corresponded to the two different species (as classed by morphological characteristics and the mitochondrial CR) with a proportion of individuals of clearly mixed origin (Fig. 3). Ten of the individuals of each species which seemed to be of hybrid origin (exhibiting mitochondrial CR of one species and more than 90% membership of the other species based on microsatellites) were analyzed for the COI gene which yielded sequences corresponding to the same species assigned by mitochondrial CR (GenBank Accession Nos. of COI sequences: GQ204167–GC204169 and

Table 2
Mitochondrial control region haplotypes found for silver (*M. bilinearis*) and offshore (*M. albidus*) hake 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. Significance level: NS, non-significant. Sample locations are identified in Table 1.

	M. bilinearis				*M. albidus*		
	Mb-N	Mb-CN	Mb-CS	Mb-S	Ma-N	Ma-C	Ma-S
n	29	117	32	30	27	28	35
Haplotypes	13	23	7	5	11	10	10
Exclusive haplotypes	4	11	1	1	8	5	3
h	0.855	0.827	0.699	0.637	0.655	0.548	0.694
SE	0.051	0.022	0.055	0.054	0.105	0.114	0.081
π	0.0047	0.0044	0.0029	0.0024	0.0798	0.0669	0.0793
D	-0.617^{NS}	-0.730^{NS}	-0.399^{NS}	-0.455^{NS}	-1.444^{**}	-2.029^{**}	-1.463^{**}
Fs	-6.887^{**}	-14.320^{**}	-1.782^{*}	-0.523^{NS}	-7.417^{**}	-6.740^{**}	-5.017^{**}

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

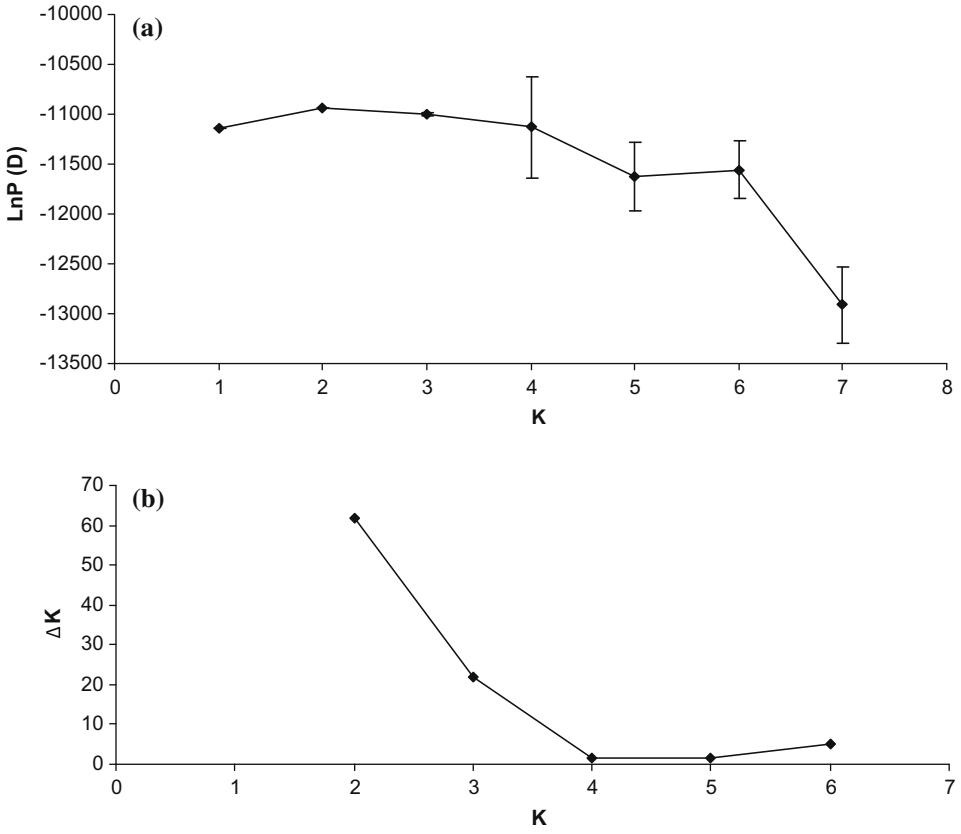

Fig. 2. (a) Values of Ln $P(D)$ for each number of putative genetic units (K) in the North American *Merluccius* samples analyzed for microsatellite loci. Error bars correspond to the different runs. (b) Values of ΔK (rate of change of the likelihood function estimated as in Evanno et al. (2005)) plotted against K for the same samples.

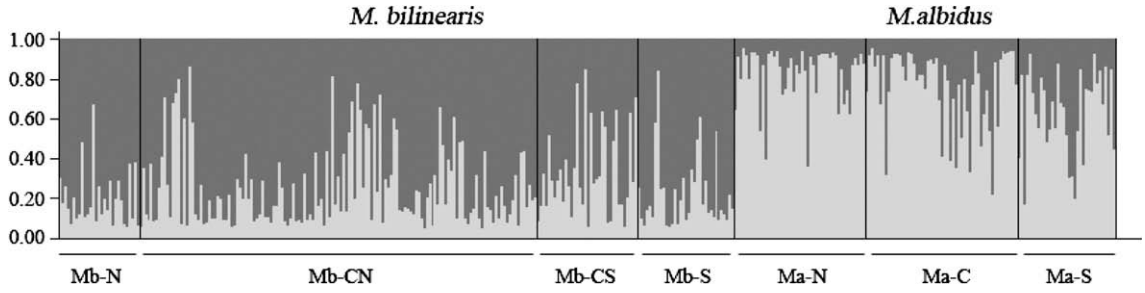

Fig. 3. Estimation of the membership fraction to each of the two inferred clusters for North American hakes, based on microsatellite loci. Each vertical bar represents one individual and horizontal lines mark species limits as deduced from mitochondrial DNA. Each cluster is represented by one gray color: cluster 1 is dark gray, cluster 2 is light gray. N: North; CN: Central North; C: Central; CS: Central South; S: South.

GQ204170–GQ204171 for *M. bilinearis* and *M. albidus*, respectively). This confirmed discrepancy between mitochondrial and nuclear markers. When using nuclear markers, some individuals of both species were completely assigned to the species different from that identified based on morphological characteristics and mtDNA. Other individuals exhibited intermediate values of membership of the two species.

Based on microsatellite loci, the proportion of individuals with significant (more than 50%) membership in the other species ranged from 4.3% to 28.6% for the northernmost and the southernmost sampling areas of *M. albidus*, respectively (Table 3). These proportions were similar to the three southern samples of *M. bilinearis*, but we detected stronger differences among the three *M. albidus* samples. Considering the entire overlapping area of these two species as one region, the proportion of individuals with genetic components from the other species was similar for both species at around 15%.

When examining the population structure of these two hake species as revealed by microsatellite variation, we found some differences between the species after removing from the analysis the individuals with hybrid components. For the five microsatellite loci analyzed *M. bilinearis* exhibited slightly more variation than *M. albidus* (Table 4), with more alleles per locus and generally higher heterozygosity. *M. bilinearis* had significantly higher allelic richness than *M. albidus* (average allelic richness of 18.40 and 13.59, respectively; $P = 0.028$). With respect to population structuring, *M. bilinearis* did not exhibit between-population spatial variation of allele frequencies (non-significant F_{ST} values between pairs of samples; Table 5) whereas for *M. albidus* the southern sample provided significant F_{ST} values with the two other samples, indicating spatial within-species differentiation. As expected the microsatellites analyzed provided highly significant differences between *M. bilinearis* and *M. albidus* samples caught from the same and from distinct areas, confirming their value for differentiating the two species.

5S rDNA, a nuclear marker with functional significance, detects first-generation hybrids. *M. bilinearis* yields two amplification fragments 703 and 586 bp long whereas *M. albidus* provides 593 and 383 bp long fragments (Garcia-Vazquez et al., 2009). In the samples analyzed, hybrid amplification patterns exhibiting 5S rDNA fragments of the two species simultaneously were not found, which suggests that we have found high degree of interspecific introgression at nuclear loci but no direct evidence of first-generation (F_1) hybrids.

4. Discussion

The results obtained in this study depict a situation of extensive introgression between the two Atlantic North American hake species *Merluccius bilinearis* and *M. albidus*. Introgression has been detected with five microsatellite markers. Four microsatellites, including the markers employed here, have provided unambiguous species-specific identification in the genus *Merluccius* (Castillo et al., 2003), thus the tool can be considered adequate for our purpose. The levels of interspecific introgression found here were unexpectedly high. Although introgressive hybridization is common in contact zones of partially overlapping species (e.g. Alves et al., 2008; Trigo et al., 2008), such cases are very rare in oceanic species (e.g. Roques et al., 2001), and in the present case the introgression rate, as high as 29% in some areas, is likely the highest ever described for two sympatric but well differentiated species. The pattern of population differentiation and introgressive hybridization observed at microsatellites was in contrast with that revealed by mtDNA variation, as occurs for other fish lineages in secondary contact (Lu et al., 2001). Hybridization and recurrent backcrossing with parental species likely occurred for many generations, as deduced from individuals with diverse degrees of bidirectional introgression, from total mixture to greater proportion of one of the species. Coincidence of mitochondrial DNA with morphological identification suggests a phenotypic dominance of the maternal species, although this conclusion should be taken with caution since F_1 hybrids were not found.

For *M. bilinearis*, the level of introgression was higher in the three areas where the two species overlap and lower in the northern area where overlapping is not expected. Movements of hybrid adults around all distributional areas are expected, as this species is highly migratory (Helser et al., 1995), therefore occurrence of some hybrids in areas where the other species is not present can be considered normal.

For *M. albidus*, however, interspecific introgression rate was higher in the southern area (29%; Table 3). This species exhibited weak but significant spatial differentiation, and it should be remarked that *M. albidus* inhabiting the southern area were significantly different from those distributed in other regions (Table 5), indicating at least partial isolation and restricted gene flow. As suggested by Petit and Excoffier (2009), limited intraspecific gene flow would be accompanied by higher interspecific introgression rates. In the present case, the effect would not be directly related with gene drift favoring introgression of some alien genes (marine fish species have usually large breeding stocks and the effects of gene drift are likely weak, e.g. Ely et al., 2005; Machado-Schiaffino et al., 2009), but rather to the possibility of accumulating hybrids in areas with older contact. If *M. albidus* is advancing to the north (it exhibits signals of population expansion, Table 2), it has been in contact with *M. bilinearis* for more generations in the south, thus hybrids and backcrossed could have accumulated in that area for a longer time. Restricted gene flow (at least for males, as no differentiation has been found for mitochondrial DNA) would maintain the hybridization cline along the species distribution.

Are the observed hybridization events ancient or is hybridization still occurring? It is not easy to have complete certainty given that the analysis was carried out on adults (F_1 hybrids may exhibit high mortality at embryonic or larval stage as documented in other species, Rogers and Bernatchez, 2006). Absence of F_1 hybrid genotypes at the 5S rDNA can not be confirmed, as first-generation hybridization rates lower than 1% (described for other plant and animal taxa, e.g. Kay, 2006; Castillo et al., 2008) are not expected to be detected with sample sizes lower than 400 individuals as was the case in this study. In any case, lack of detection of F_1 hybrids supports the idea of ancient hybridization. In long-term hybridizing sympatric species, first-generation hybrids are generally the least frequent, due to reproductive barriers between closely related sympatric species (Coyne and Orr, 1997; Price and Bouvier, 2002). Recurrent hybridization between sympatric species has been described for taxa as diverse as for example for the bird skua (Ritz et al., 2008) and the plant *Malva* (Escobar García et al., 2009).

Table 3
Number (proportion) of individuals of each species assigned to the same species as identified by mitochondrial DNA (mt-nuclear coincidence) or with significant ($\geqslant 50\%$) membership of the other species (hybrid origin), based on microsatellite loci genotypes analyzed with the STRUCTURE software assuming two genetic units ($K = 2$). The percentage of individuals from each sample assigned to each species is shown in brackets. *n*, sample size.

	n	mt-nuclear coincidence	Hybrid origin
M. bilinearis	243	206 (0.848)	37 (0.152)
Mb-N	29	27 (0.931)	2 (0.069)
Mb-CN	143	121 (0.846)	22 (0.154)
Mb-CS	36	27 (0.750)	9 (0.250)
Mb-S	35	31 (0.886)	4 (0.114)
M. albidus	137	116 (0.847)	21 (0.153)
Ma-N	47	45 (0.957)	2 (0.043)
Ma-C	55	46 (0.836)	9 (0.164)
Ma-S	35	25 (0.714)	10 (0.286)

Table 4
Summary of genetic variation at the studied microsatellite loci. *n*, sample size; SR, size range of alleles in base positions; 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. Sample locations are identified in Table 1.

Samples	*n*	Microsatellite loci					Overall loci		
		Maus7	Maus30	Maus32	Hk20	UEAW01	Allelic richness (SD)	He (SD)	Ho (SD)
Mb-N	27								
	SR	125–225	205–233	177–223	223–295	232–262			
	Na	26	13	20	22	15	18.05 (4.76)	0.911 (0.031)	0.918 (0.071)
	HWE	NS	NS	NS	NS	NS			
Mb-CN	121								
	SR	115–265	203–281	175–245	217–307	220–260			
	Na	46	26	32	37	17	18.15 (4.33)	0.927 (0.026)	0.889 (0.060)
	HWE	**	NS	NS	NS	NS			
Mb-CS	27								
	SR	131–211	203–275	175–225	217–305	228–272			
	Na	21	16	20	21	16	17.8 2 (2.37)	0.915 (0.027)	0.893 (0.066)
	HWE	NS	NS	NS	NS	**			
Mb-S	30								
	SR	115–251	203–263	177–237	219–305	226–258			
	Na	31	15	24	26	13	19.58 (6.58)	0.918 (0.040)	0.893 (0.089)
	HWE	NS	NS	NS	NS	NS			
Ma-N	44								
	SR	127–143	205–241	177–245	223–287	226–274			
	Na	7	14	14	25	16	12.67 (5.39)	0.829 (0.138)	0.716 (0.169)
	HWE	NS	**	NS	NS	**			
Ma-C	46								
	SR	127–139	205–235	177–233	217–281	226–264			
	Na	5	15	20	26	16	13.66 (5.75)	0.841 (0.148)	0.736 (0.153)
	HWE	NS	**	**	NS	**			
Ma-S	24								
	SR	127–139	205–235	177–233	221–303	230–254			
	Na	4	14	22	21	12	14.45 (7.26)	0.812 (0.201)	0.775 (0.185)
	HWE	NS	NS	NS	NS	NS			

** $P < 0.01$.

Table 5
Tests for genetic differentiation between populations for *M. bilinearis* and *M. albidus*. Above diagonal: Pairwise genetic distances (F_{ST}) based on five microsatellites. NS, not significant. Sample locations are identified in Table 1.

	Mb-N	Mb-CN	Mb-CS	Mb-S	Ma-N	Ma-C	Ma-S
Mb-N	–	0.001	0.003	0.004	0.071	0.064	0.074
Mb-CN	NS	–	0.003	0.001	0.063	0.059	0.063
Mb-CS	NS	NS	–	0.007	0.061	0.056	0.070
Mb-S	NS	NS	NS	–	0.062	0.061	0.062
Ma-N	**	**	**	**	–	0.005	0.028
Ma-C	**	**	**	**	NS	–	0.019
Ma-S	**	**	**	**	**	*	–

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

Selection has been reported to be implicated in promoting and maintaining introgression in other marine species (Roques et al., 2001). However in the case studied here we have not found an asymmetrical pattern of introgressive hybridization, differential patterns of linkage disequilibrium among samples, restricted introgression areas or any other sign which could indicate selective introgression. It seems that introgression levels simply depend on the time elapsed since the secondary contact.

Hybridization in secondary contact areas has been suggested for other *Merluccius* species. For example, *M. australis* seems to be of hybrid origin based on discrepancy between mitochondrial and nuclear phylogenetic trees (Campo et al., 2009). The common ancestor of all *Merluccius* species likely inhabited the area now occupied by *M. bilinearis*. *M. albidus* and the rest of American species distributed in the South Hemisphere and in the Pacific Ocean would have originated in the south after the closure of the Panama Isthmus (Roldan et al., 1999; Campo et al., 2007). The contact of *M. albidus* with *M. bilinearis* would have occurred after migration of the former species from the south. The final outcome of recurrent introgressive hybridization may be a new species, or the fusion of two formerly separated genetic units through reversal speciation (Wolfe, 2003; Seehausen, 2006), or sympatric persistence of two reproductively isolated introgressed groups (Roques et al., 2001).

The current warming of sea temperatures would allow *M. albidus* to expand northwards as are many other marine species (McFarlane et al., 2000; De Young et al., 2004), progressively enlarging the area of overlap with *M. bilinearis*. The introgressive process observed in our dataset was likely initiated long time ago, but for other species could start in a next future, increasing opportunities for new hybridizations and introgression. Exploration of such processes will help to understand the role of interspecific gene flow in shaping species boundaries.

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References

Alves, P.C., Melo-Ferreira, J., Freitas, H., Boursot, P., 2008. The ubiquitous mountain hare mitochondria: multiple introgressive hybridization in hares, genus *Lepus*. Philosophical Transactions of the Royal Society London B Biological Sciences 363 (1505), 2831–2839.

Campo, D., Machado-Schiaffino, G., Perez, J., Garcia-Vazquez, E., 2007. Phylogeny of the genus *Merluccius* based on mitochondrial and nuclear genes. Gene 406, 171–179.

Campo, D., Machado-Schiaffino, G., Horreo, J.L., Garcia-Vazquez, E., 2009. Molecular organization and evolution of 5S rDNA in the genus *Merluccius* and their phylogenetic implications. Journal of Molecular Evolution 68 (3), 208–216.

Castillo, A.G.F., Martínez, J.L., Garcia-Vazquez, E., 2003. Identification of Atlantic hake species by a simple PCR-based methodology employing microsatellite loci. Journal of Food Protection 66, 2130–2134.

Castillo, A.G.F., Ayllon, F., Moran, P., Izquierdo, J.I., Martinez, J.L., Beall, E., Garcia-Vazquez, E., 2008. Interspecific hybridization and introgression are associated with stock transfers in salmonids. Aquaculture 278, 31–36.

Chang, S., Berrien, P.L., Johnson, D.L., Zetlin, C.A., 1999. Offshore hake, *Merluccius albidus*, life history and habitat characteristics. NOAA Technical Memorandum NMFS-NE, vol. 130, pp. 1–24.

Coyne, J.A., 1992. Genetics and speciation. Nature 355, 511–515.

Coyne, J.A., Orr, H.A., 1997. Patterns of speciation in *Drosophila* revisited. Evolution 51, 295–303.

Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Sunderland, MA.

De Young, B., Heath, M., Werner, F., Chai, F., Megrey, B., Monfray, P., 2004. Challenges of modeling ocean basin ecosystems. Science 304, 1463–1466.

Dobzhansky, T., 1935. A critique of the species concept in biology. Philosophy of Science 2, 344–355.

Ely, B., Viñas, J., Alvarado Bremer, J.R., Black, D., Lucas, L., Covello, K., Labrie, A.V., Thelen, E., 2005. Consequences of the historical demography on the global population structure of two highly migratory cosmopolitan marine fishes: the yellowfin tuna (*Thunnus albacares*) and the skipjack tuna (*Katsuwonus pelamis*). BMC Evolutionary Biology 5 (1), 19.

Escobar García, P., Schönswetter, P., Fuertes Aguilar, J., Nieto Feliner, G., Schneeweiss, G.M., 2009. Five molecular markers reveal extensive morphological homoplasy and reticulate evolution in the *Malva* alliance (Malvaceae). Molecular Phylogenetics and Evolution 50 (2), 226–239.

Estoup, A., Largiader, C.R., Perrot, E., Chourrout, D., 1996. Rapid one-tube DNA extraction protocol for reliable PCR detection of fish polymorphic markers and transgenes. Molecular Marine Biology and Biotechnology 5 (4), 295–298.

Evanno, G., Regnault, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14, 2611–2620.

Excoffier, L., Laval, G., Schneider, S., 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1, 47–50.

Fu, Y.X., 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147, 915–925.

Garcia-Vazquez, E., Horreo, J.L., Campo, D., Machado-Schiaffino, G., Bista, I., Triantafyllidis, A., Juanes, F., 2009. Mislabelling of commercial North American hakes suggests underreported exploitation of *Merluccius albidus*. Transactions of the American Fisheries Society 138 (4), 790–796.

Gava, A., Freitas, T.R., 2003. Inter and intra-specific hybridization in tuco-tucos (*Ctenomys*) from Brazilian coastal plains (Rodentia: Ctenomyidae). Genetica 119 (1), 11–17.

Good, J.M., Demboski, J.R., Nagorsen, D.W., Sullivan, J., 2003. Phylogeography and introgressive hybridization: chipmunks (genus *Tamias*) in the northern Rocky Mountains. Evolution 57 (8), 1900–1916.

Goudet, J., 1995. A computer program to calculate F-statistics. Journal of Heredity 86, 485–486.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95–98.

Helser, T.E., Almeida, F.P., Waldron, D.E., 1995. Biology and fisheries of north-west Atlantic hake (silver hake: *M. bilinearis*). In: Alheit, J., Pitcher, T.J. (Eds.), Hakes: Biology, Fisheries and Markets. Chapman & Hall, London, pp. 203–237.

Kappas, I., Baxevanis, A.D., Maniatsi, S., Abatzopoulos, T.J., 2009. Porous genomes and species integrity in the branchiopod *Artemia*. Molecular Phylogenetics and Evolution 52, 192–204.

Kay, K.M., 2006. Reproductive isolation between two closely related hummingbird-pollinated Neotropical gingers. Evolution 60 (3), 538–552.

Klopfstein, S., Currat, M., Excoffier, L., 2006. The fate of mutations surfing on the wave of a range expansion. Molecular Biology and Evolution 23, 482–490.

Lu, G., Basley, D.J., Bernatchez, L., 2001. Contrasting patterns of mitochondrial DNA and microsatellite introgressive hybridization between lineages of lake whitefish (*Coregonus clupeaformis*); relevance for speciation. Molecular Ecology 10 (4), 965–985.

Lundy, C.J., Rico, C., Hewitt, G., 2000. Temporal and spatial genetic variation in spawning grounds of European hake (*Merluccius merluccius*) in the Bay of Biscay. Molecular Ecology 9, 2067–2079.

Machado-Schiaffino, G., Garcia-Vazquez, E., 2009. Isolation and characterization of microsatellite loci in *Merluccius australis* and cross-species amplification. Molecular Ecology Resources 9, 585–587.

Machado-Schiaffino, G., Campo, D., Garcia-Vazquez, E., 2009. Strong genetic differentiation of the Austral hake (*Merluccius australis*) across the species range. Molecular Phylogenetics and Evolution 53, 351–356.

Mallet, J., 1995. A species definition for the modern synthesis. Trends in Ecology and Evolution 10, 294–299.

Mallet, J., 2005. Hybridization as an invasion of the genome. Trends in Ecology and Evolution 20, 229–237.

Mayr, E., 1942. Systematics and the Origin of Species from the Viewpoint of a Zoologist. Columbia University Press, New York.

Mayr, E., 1963. Animal Species and Evolution. The Belknap Press of Harvard University Press, Cambridge, MA.

McFarlane, G.A., King, J.R., Beamish, R.J., 2000. Have there been recent changes in climate? Ask the fish. Progress in Oceanography 47, 147–169.

Morán, P., Lundy, C.J., Rico, C., Hewitt, G.M., 1999. Isolation and characterization of microsatellite loci in European hake, *Merluccius merluccius* (Merlucidae, Teleostei). Molecular Ecology 8, 1357–1358.

Morse, W.W., Johnson, D.L., Berrien, P.L., Wilk, S.J., 1999. Silver hake, *Merluccius bilinearis*, life history and habitat characteristics. NOAA Technical Memorandum NMFS-NE, vol. 135, pp. 1–42.

Nei, M., 1987. Molecular Evolutionary Genetics. Columbia University Press, New York.

Pastorini, J., Zaramody, A., Curtis, D.J., Nievergelt, C.M., Mundy, N.I., 2009. Genetic analysis of hybridization and introgression between wild mongoose and brown lemurs. BMC Evolutionary Biology 9, 32.

Pendas, A.M., Moran, P., Martinez, J.L., Garcia-Vazquez, E., 1995. Applications of 5S rDNA in Atlantic salmon, brown trout, and in Atlantic salmon × brown trout hybrid identification. Molecular Ecology 4, 275–276.

Petit, R.J., Excoffier, L., 2009. Gene flow and species delimitation. Trends in Ecology and Evolution 24 (7), 386–393.

Price, T.D., Bouvier, M.M., 2002. The evolution of F1 postzygotic incompatibilities in birds. Evolution 56, 2083–2089.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inferences of population structure using multilocus genotype data. Genetics 155, 945–959.

Quinteiro, J., Vidal, R., Rey-Mendez, M., 2000. Phylogeny and biogeographic history of hake (genus *Merluccius*), inferred from mitochondrial DNA control region sequences. Marine Biology 136, 163–174.

Rice, W.R., 1989. Analyzing tables of statistical tests. Evolution 43, 223–225.

Rico, C., Ibrahim, K.M., Rico, I., Hewitt, G.M., 1997. Stock composition in North Atlantic populations of whiting using microsatellite markers. Journal of Fish Biology 51, 462–475.

Ritz, M.S., Millar, C., Miller, G.D., Phillips, R.A., Ryan, P., Sternkopf, V., Liebers-Helbig, D., Peter, H.U., 2008. Phylogeography of the southern skua complex-rapid colonization of the southern hemisphere during a glacial period and reticulate evolution. Molecular Phylogenetics and Evolution 49 (1), 292–303.

Rogers, S.M., Bernatchez, L., 2006. The genetic basis of intrinsic and extrinsic post-zygotic reproductive isolation jointly promoting speciation in the lake whitefish species complex (*Coregonus clupeaformis*). Journal of Evolutionary Biology 19 (6), 1979–1994.

Roldan, M.I., Garcia-Marin, J.L., Utter, H.M., Pla, C., 1999. Genetic relationships among *Merluccius* species. Heredity 83, 79–86.

Roques, S., Sévigny, J.M., Bernatchez, L., 2001. Evidence for broadscale introgressive hybridization between two redfish (genus *Sebastes*) in the North-west Atlantic: a rare marine example. Molecular Ecology 10 (1), 149–165.

Rozas, J., Sánchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–2497.

Saetre, G.P., Borge, T., Lindell, J., Moum, T., Primmer, C.R., Sheldon, B.C., Haavie, J., Johnsen, A., Ellegren, H., 2001. Speciation, introgressive hybridization and nonlinear rate of molecular evolution in flycatchers. Molecular Ecology 10 (3), 737–749.

Seehausen, O., 2006. Conservation: losing biodiversity by reverse speciation. Current Biology 16 (9), R334–R337.

Seehausen, O., Takimoto, G., Roy, D., Jokela, J., 2008. Speciation reversal and biodiversity dynamics with hybridization in changing environments. Molecular Ecology 17 (1), 30–44.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Trigo, T.C., Freitas, T.R., Kunzler, G., Cardoso, L., Silva, J.C., Johnson, V.E., O'Brien, S.J., Bonatto, S.L., Eizirik, E., 2008. Inter-species hybridization among Neotropical cats of the genus *Leopardus*, and evidence for an introgressive hybrid zone between *L. geoffroyi* and *L. tigrinus* in southern Brazil. Molecular Ecology 17 (19), 4317–4333.

Van Oosterhout, C., William, F., Hutchinson, D.P., Wills, M., Shipley, P., 2004. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4, 535–538.

Vellend, M., Harmon, L.J., Lockwood, J.L., Mayfield, M.M., Hughes, A.R., Wares, J.P., Sax, D.F., 2007. Effects of exotic species on evolutionary diversification. Trends in Ecology and Evolution 22, 481–488.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., Hebert, P.D.N., 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society London B Biological Sciences 360, 1847–1857.

Wiley, C., Qvarnström, A., Andersson, G., Borge, T., Sætre, G.P., 2009. Postzygotic isolation over multiple generations of hybrid descendents in a natural hybrid zone: how well do single-generation estimates reflect reproductive isolation? Evolution 63, 1731–1739.

Wolfe, K., 2003. Evolutionary biology: speciation reversal. Nature 422, 25–26.