Integrating microsatellite DNA markers and otolith geochemistry to assess population structure of European hake (*Merluccius merluccius*)

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Abstract

Population structure and natal origins of European hake were investigated using microsatellite DNA markers and otolith geochemistry data. Five microsatellites were sequenced and otolith core geochemical composition was determined from age-1 hake collected in the northeast Atlantic Ocean and the Mediterranean Sea. Microsatellites provided evidence of a major genetic split in the vicinity of the Strait of Gibraltar, separating the Atlantic and the Mediterranean populations, with the exception of the Gulf of Cádiz. Based on classification models using otolith core geochemical values individuals' natal origins were identified, although with an increased error rate. Coupling genotype and otolith data increased classification accuracy of individuals to their potential natal origins while providing evidence of movement between the northern and southern stock units in the Atlantic Ocean. Information obtained by the two natural markers on population structure of European hake was complementary as the two markers act at different spatio-temporal scales. Otolith geochemistry provides information over an ecological time frame and on a fine spatial scale, while microsatellite DNA markers report on gene flow over evolutionary time scales and therefore act on a broader spatio-temporal resolution. Thus, this study confirmed the usefulness of otolith geochemistry to complement the assessment of early life stage dispersal in populations with high gene flow and low genetic divergence.

Keywords: movement, population structure, otolith geochemistry, microsatellites, European hake

1. Introduction

Knowledge of stock structure is a necessary prerequisite for the sustainable management of marine capture fisheries (Begg and Waldman, 1999). Lack of such information can lead to scientists missing changes in biological characteristics and productivity rates of a species that would otherwise trigger new management actions (Begg et al., 1999). Numerous definitions of population can be found in the literature, presently the terms population and stock are often used as synonyms in fisheries science (Begg and Waldman, 1999). However, to define and to identify a population or stock unit continues to be a challenge.

A wide array of methodological approaches have been employed to assess stock structure in marine fish populations, including distribution and abundance data, morphometrics and meristics, life history patterns, genetics, and artificial and natural tags (Pawson and Jennings, 1996). Among these techniques, genetic markers and otolith geochemistry represent two powerful tools that have often been used to determine the spatial structure of fish stocks (Feyrer et al., 2007).

Genetic markers are well established in studies of population structure and connectivity in terrestrial and aquatic environments (e.g. Luikart et al., 2011; Pita et al., 2011). Highly variable codominant microsatellite DNA markers have proved particularly useful for identifying structure in populations with low genetic differentiation such as marine fish species (White et al., 2010). The marine environment presents few physical barriers to dispersal of the different life history stages of fish allowing exchange of individuals among local groups of fish. Even very low exchange rates (ca. 10 individuals per generation) can prevent the accumulation of large genetic differences among groups of fish (Slatkin, 1987; Palumbi, 2003). The ecological implications of low but significant genetic differentiation among marine fish populations are difficult to assess, especially because estimates of gene flow are generally made on evolutionary time scales rather than an ecological time frame over which most management decisions are made (Palumbi, 2003).

Alternatively, otolith geochemistry has been used to examine stock structure (Campana et al., 2000; Bergenius et al., 2005), natal or juvenile origins (Thorrold et al., 2001; Tanner et al., 2013) and migration patterns (Campana et al., 2007; Walther et al., 2011) over ecological time scales. These applications are feasible due to the inertness of otoliths, their continuous growth that acts to record environmental information of the individual life history in a chronological manner and the fact that their chemical composition is significantly influenced by the physical and chemical properties of the surrounding water (Campana, 1999).

Stock structure is influenced by physical, biological and ecological processes and interactions that impact over a range of temporal scales. Therefore the best inference on stock structure may be achieved by using multiple and potentially complementary techniques that integrate over different scales (Begg and Waldman, 1999; Thorrold et al., 2002). Recently, a holistic approach integrating four different techniques (genetic markers, morphometry, parasites and life history traits) has provided reliable information on stock structure of Atlantic horse mackerel (*Trachurus trachurus*) throughout the species distribution range (Abaunza et al., 2008). The use of two different techniques for stock structure assessment, such as parasite assemblage composition combined with genetic markers or otolith shape analysis has more commonly been employed (McClelland et al., 2005; Vignon et al., 2008). The combination of genetic markers and otolith geochemistry has been increasingly used as they may provide complementary information on population structure and connectivity patterns over evolutionary and ecological time scales, respectively (Miller et al., 2005; Bradbury et al., 2008; Woods et al., 2010). In some cases the two techniques produced conflicting estimates on population structure and connectivity (e.g. Thorrold et al., 2001), most likely due to different temporal scales at which genetic markers and otolith chemistry are informative. The chronological properties of otoliths provide information on the aquatic environments experienced by an individual over its

99 lifetime, whereas genetic markers resolve population structure over various time scales
100 depending on the rate that variation accumulates at a given locus (Woods et al., 2010).
101 Despite the progress in the application of different techniques used to identify stock
102 structure, the problem of defining the management units of many commercially exploited
103 species is far from resolved (e.g. Lleonart and Maynou, 2003; Abaunza et al., 2008).
104 European hake, *Merluccius merluccius* (Linnaeus, 1758), a commercially important
105 demersal, benthopelagic species is one such case. The species is distributed from Norway
106 to the Gulf of Guinea in the northeast Atlantic Ocean and throughout the Mediterranean
107 and Black Sea, with highest abundances from the British Isles to southern Spain (Murua,
108 2010). Although there is evidence of some gene flow between the Mediterranean and
109 Atlantic in the vicinity of the Strait of Gibraltar (Roldán et al., 1998), the Mediterranean
110 and Atlantic populations of hake are managed as different stocks due to differences in
111 biology, morphology and genetics (Abaunza et al., 2001; Lo Brutto et al., 2004; Mellon-
112 Duval et al., 2010). Moreover, in the northeast Atlantic Ocean, the International Council
113 for the Exploration of the Sea (ICES) divides the hake population into the northern and
114 southern stocks with Capbreton canyon (Bay of Biscay, SW France) delineating the
115 boundary between them. The establishment of these two stocks was based on management
116 considerations without a biological basis (ICES, 2011) and no stable genetic structure was
117 found either with mitochondrial DNA (Lundy et al., 1999) or with allozymes (Cimmaruta
118 et al., 2005) from Norway to the Mediterranean Sea, as well as among temporal samples
119 from Bay of Biscay using microsatellites (Lundy et al., 2000). Moreover, the large genetic
120 connectivity within the north-eastern Atlantic metapopulation of this species suggested by
121 recent spatio-temporal studies (Pita et al., 2011, 2013) is congruent with hake egg and
122 larvae dynamics in the Bay of Biscay (Alvarez et al., 2004) as well as with movements
123 detected between the two stocks in the northeast Atlantic Ocean based on an otolith
124 geochemistry approach (Tanner et al., 2012). In the Mediterranean Sea, European hake

populations are assessed and managed in geographical sub-areas (GSA) defined by GFCM (General Fisheries Commission for the Mediterranean) (Cardinale et al., 2011) which has led to numerous stock assessments of rather small stock units. Similarly to the populations in the Atlantic Ocean, European hake inhabiting the Mediterranean shelves and slopes are assumed to be connected over several GSA as has been shown by population genetic studies (e.g. Lo Brutto et al., 2004).

The aim of the present study was to use genetic markers and otolith geochemistry to investigate natal origins and population structure of European hake in the northeast Atlantic Ocean and the western Mediterranean Sea and to assess the complementarity of the information obtained by the two natural markers. Genetic structure of hake populations was assessed using microsatellite DNA markers. Geochemical composition of otolith cores, representing larval and early pelagic juvenile life stages, was used to investigate spatial separation at these early life stages.

2. Material and Methods

2.1 Fish sampling

Specimens of European hake were obtained from research surveys at seven locations in the northeast Atlantic Ocean and the western Mediterranean Sea (Fig. 1). Total length of the individuals was determined (Table 1), sagittal otoliths were extracted and fin tissue clips (ca. 1 cm^2) were obtained. Otoliths were rinsed with water, cleaned from adhering tissue and preserved dry. Fin tissue clips were stored in pure ethanol for genetic analysis. All individuals used in this study were classified into the age-class 1 given the age-length relationships provided by previous studies in the two areas based on increments in the otoliths (De Pontual et al., 2006; Mellon-Duval et al., 2010).

2.2 Microsatellite markers

151 2.2.1 DNA analysis

152 For DNA extraction and purification of European hake fin tissue samples a commercial kit
153 (MasterPure Complete DNA and RNA purification kit, EPICENTRE Biotechnologies) was
154 used. Five microsatellite markers, previously described for this species (Morán et al., 1999)
155 (*Mmer* UEAHk3b, *Mmer* UEAHhk9b, *Mmer* UEAHk20, *Mmer* UEAHk29 and *Mmer*
156 UEAHk34b) which were compatible with neutrality (Beaumont and Nichols, 1996) were
157 amplified following PCR reaction conditions outlined by Pita et al. (2011). The forward
158 primer of each marker was fluorescently labeled: *Mmer* UEAHk3b and *Mmer* UEAHk29
159 with 6FAM, *Mmer* UEAHhk9b and *Mmer* UEAHk34b with HEX and *Mmer* UEAHk20
160 with NED. Amplified fragments were analyzed by capillary electrophoresis (Applied
161 Biosystems) using an ABI 3130 automatic DNA sequencer and the internal sizer GeneScan
162 500 Rox Size Standard (Applied Biosystems). GeneMarker V1.97 software (SoftGenetics,
163 LLC) was used to determine the allele size and genotype of all individuals. Genotypes
164 were cross-checked among three independent readings to minimize genotyping errors.
165 Consistency of the allelic series was tested with MICRO-CHECKER 2.2.3 (van Oosterhout
166 et al., 2004).

167 2.2.2 Statistical analysis

168 Allele frequencies, observed and expected heterozygosities, and Hardy-Weinberg
169 equilibrium tests were performed using Genepop 4.0 (Raymond and Rousset, 1995). F_{STAT}
170 2.9.2.3 (Goudet, 1995) was used to calculate number of alleles, allelic richness, and
171 fixation indices within samples (F_{IS}) and between samples (F_{ST}). Hierarchical analysis of
172 molecular variance (AMOVA) implemented in Arlequin (ver. 3.11, Excoffier et al., 2005)
173 was used to examine differences among groups of collection locations (F_{CT}) and among
174 collection locations within groups (F_{SC}). In order to assess molecular variance at different
175 spatial scales, collection locations were pooled into the two hydrogeographic regions
176 (Atlantic Ocean and Mediterranean Sea) as well as to the currently implemented

177 management units in the Atlantic Ocean, i.e. northern and southern stocks and the western
178 Mediterranean populations pooled. A consensus star-like dendrogram describing the
179 relationships among collection locations was generated with the neighbor-joining
180 algorithm (Saitou and Nei, 1987) using Cavalli-Sforza and Edwards (1967) chord distances
181 implemented in PHYLIP package (Felsenstein, 2005). One thousand bootstrap replicates of
182 allele frequencies were used as nodal support of tree branches and the software
183 TREEVIEW (Page, 1996) was used for tree editing. A Bayesian clustering algorithm
184 implemented in STRUCTURE 2.3.1 (Pritchard et al., 2000) was used to determine the
185 most probable number of genetic clusters (*K*) within the dataset. *K* was selected a priori
186 ranging from 1 to 8 populations and a correlated allele frequency model was chosen. The
187 'no admixture' algorithm was used with information on collection location included to
188 assist the clustering, enabling a better performance for data with weak genetic structure
189 (Hubisz et al., 2009). In total, 10^4 burn-in and 10^4 MCMC (Markov Chain Monte Carlo)
190 repetitions were used and each independent run was iterated 5 times. The most appropriate
191 *K* was predicted from plots of *ad hoc* posterior probability models of both Pr(X|K) and ΔK
192 as recommended by Evanno et al. (2005). Assignment tests of hake individuals were
193 performed using the statistical package ONCOR (Kalinowski et al., 2008). Based on leave-
194 one-out cross validation, mixture genotypes are simulated and their probability of
195 occurring in the baseline samples is estimated. Individuals were assigned to their collection
196 locations, to their management unit in the Atlantic Ocean and the western Mediterranean
197 Sea, and finally to the hydrogeographic region (Atlantic Ocean and Mediterranean Sea).
198
199 2.3 Otolith geochemistry
200 2.3.1 Geochemical analysis
201 Preparation of otolith samples, analytical procedures and precisions of elemental analysis
202 and stable isotope analysis of otoliths are described in detail in Tanner et al. (2012).

Briefly, elemental ratios of Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca were quantified by measuring ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{88}Sr and ^{138}Ba in the otolith material ablated at the otolith core using a Thermo Finnigan Element2 single collector inductively coupled plasma mass spectrometer (ICP-MS) coupled to a New Wave Research 193 nm excimer laser ablation system. For stable isotope analysis ($\delta^{13}C$ and $\delta^{18}O$), a computer-controlled micromill was used to remove otolith material from the core which was then analysed on a Thermo Finnigan MAT253 equipped with a Kiel III carbonate device. The otolith core area sampled for both elemental and stable isotope analysis was defined as the region between the primordium and the accessory growth centres that correspond to the larval and early juvenile pelagic pre-settlement period (Morales-Nin and Moranta, 2004; Hidalgo et al., 2008).

2.3.2 Statistical analysis

Linear discriminant function analysis based on otolith core composition was used to investigate natal origins of European hake. As in the assignment tests based on genotype data, individuals of European hake were classified to their collection locations, to their management unit in the Atlantic Ocean and the western Mediterranean Sea, and finally to their hydrogeographic region (Atlantic Ocean and Mediterranean Sea). Classification accuracy of the discriminant functions was evaluated by calculating cross-validated classification success using a jacknife (leave-one-out) approach. The assumptions of LDFA, i.e. normality and homogeneity of variance–covariance matrices, were met after log10 transformation of the variables.

2.4 Combination of microsatellite markers and otolith geochemistry

Microsatellite marker data cannot be combined directly with non-genetic data (otolith geochemical data) due to their co-dominant nature. To deal with the incompatibility of the two datasets, genetic data were transformed to probabilities of belonging to a cluster (*K*) determined using STRUCTURE 2.3.1 (Stefánsson et al., 2009; Higgins et al., 2010) as

229 described previously in section 2.2.2. This approach converted qualitative data (genotypes)
230 into quantitative data and allowed the use of the two datasets in the same statistical
231 analysis. Otolith geochemical data and genotype-based probabilities were used in a LDFA
232 to assess the power of their combined application. As for the analyses using each dataset
233 individually, hakes were classified to their collection locations, to their management unit in
234 the Atlantic Ocean and the Mediterranean Sea, and finally to their hydrogeographic region
235 (Atlantic Ocean and Mediterranean Sea).
236
237 3. Results
238 3.1 Microsatellite markers
239 Genetic parameters including number of alleles (A), gene diversity (H_e) and allelic richness
240 (R_S) are presented in Appendix 1. Systematic deviations from Hardy-Weinberg
241 expectations were observed in most populations at two loci, *Mmer* UEAHk9b and *Mmer*
242 UEAHk29b. Such deviations consisted in high heterozygote deficits and were probably
243 caused by null alleles which are common to microsatellite markers (e.g. O'Connell and
244 Wright, 1997) including those of European hake (e.g. Pita et al., 2011).
245 Global differentiation test among collection locations (10^4 Markov chain iterations) was
246 not significant ($p = 1.0$). Pair-wise F_{ST} values revealed that European hake collected in the
247 Mediterranean Sea were genetically differentiated from those collected in the Atlantic
248 Ocean with the exception of the samples of the Gulf of Cádiz (Table 2). Within the
249 Atlantic Ocean, only the Gulf of Cádiz samples differed significantly from Celtic Sea and
250 Portugal samples. AMOVA analyses showed that most variation among collection
251 locations (2.25%) was due to differences between the Atlantic Ocean and the
252 Mediterranean Sea and to a lesser extent (1.60%) among the two management units
253 currently implemented in the northeast Atlantic and the Mediterranean Sea (Table 3). The
254 neighbour-joining dendrogram showed a major branching between Atlantic and

255 Mediterranean locations with 98.8% bootstrap support (Fig. 2). Within the branch of the
256 Atlantic locations, samples from the Gulf of Cádiz were positioned apart with 83.2%
257 bootstrap support.
258 Based on the clustering approach performed in STRUCTURE, individuals were assigned
259 to two hypothetical clusters ($K = 2$) (Fig. 3). The first cluster was composed by individuals
260 collected in the Atlantic Ocean however, only about one third of the individuals collected
261 in the Gulf of Cádiz were allocated to this cluster. The remaining individuals collected in
262 the Gulf of Cádiz were placed in the second cluster with the individuals collected in the
263 Mediterranean Sea (Fig. 4).
264 Overall accuracy of assignment tests of individuals to their collection locations based on
265 microsatellite DNA markers was very low (23.4%) and only increased slightly when
266 assigning to the management units in the Atlantic and to the western Mediterranean Sea
267 (48%) (Table 4). Acceptable values of accuracy were achieved when European hake were
268 assigned to their hydrogeographic regions of collection (77.8%) (Table 4).
269
270 3.2 Otolith geochemistry
271 Overall classification accuracy of European hake to the collection locations, Atlantic
272 management units and western Mediterranean Sea, as well as the hydrogeographic regions
273 was good, ranging from 77.1% to 82.9% (Table 4). Error rates were much higher when
274 classifying individuals to their collection locations, particularly for samples collected in the
275 Celtic Sea, Galician Shelf, Gulf of Cádiz and Sardinia where misclassification rates were
276 between 30% and 40%. While individuals collected in the Galician Shelf and Gulf of
277 Cádiz were generally misclassified to neighboring or proximate locations, those from the
278 Celtic Sea and Sardinia were classified as originating from the most distant locations. For
279 example, 30.0% of the misclassified individuals collected in Sardinia were classified as
280 originating from the Celtic Sea and the Armorican Shelf. A similar tendency was observed

281 when classifying individuals to their management units in the Atlantic Ocean and the
282 Mediterranean Sea. Individuals collected in the northern Atlantic stock were classified as
283 belonging to the Mediterranean Sea and vice versa (Table 4). Finally, error rates were 24%
284 when classifying individuals from the Atlantic Ocean to hydrogeographic region of
285 collection (Table 4).
286
287 3.3 Combination of microsatellite markers and otolith geochemistry
288 Combining microsatellite DNA markers and otolith geochemistry improved the overall
289 classification accuracy in all the analyses (Table 4). Yet, classification accuracy to some
290 collection locations did not improve or improved only slightly when compared to
291 classification accuracy based on otolith geochemistry alone (Celtic Sea, Armorican Shelf
292 and Galician Shelf). A high misclassification rate was observed between the Celtic Sea and
293 the Galician Shelf. Classification accuracy to the Atlantic management units and the
294 Mediterranean Sea generally improved through the combined use of the two markers and
295 misclassification only occurred within the Atlantic Ocean. Finally, classification accuracy
296 to the hydrogeographic regions was very good with only one individual collected in the
297 Atlantic Ocean (Gulf of Cádiz) misclassified to the Mediterranean Sea (Table 4).
298
299 4. Discussion
300 The application of microsatellite DNA markers and otolith geochemistry enhanced the
301 identification of potential natal origins and population structure of the European hake.
302 Microsatellite DNA markers report on gene flow over short evolutionary time scales and
303 therefore act at broad spatio-temporal resolutions. Alternatively, otolith geochemistry
304 generally provides information on finer spatial and temporal scales. In this instance,
305 Atlantic and Mediterranean hake populations were generally distinguished based on

306 microsatellite markers while otolith geochemistry differed significantly among locations
307 over the full geographical range of this study.
308 The parameters of the microsatellite DNA markers employed were within the range of
309 previous studies in European hake (e.g. Lundy et al., 1999; Pita et al., 2011; Pita et al.,
310 2013). The heterozygote deficit observed for two loci in most collection locations has been
311 previously reported for this set of markers (e.g. Lundy et al., 1999). Pita et al. (2011)
312 suggested that the existence of multiple null alleles co-segregating at low-frequency as the
313 most parsimonious explanation for the absence of null–null homozygotes in genotypes
314 given that technical artifacts (e.g. drop-out effects) had been minimized. Heterozygote
315 deficits caused by null alleles can introduce bias in estimates of divergence in highly
316 structured species (i.e. different null alleles segregating at different frequencies in different
317 populations). However, for closely related populations of highly homogeneous species
318 such as hake, it can be assumed that the impact of null alleles is evenly distributed across
319 samples and therefore the underestimation of gene diversity due to null alleles can be
320 ignored (Lado-Insua et al., 2011) as is usually done under homoplasy (Estoup et al., 1995).
321 Evidence of a major genetic split was found in the vicinity of the Strait of Gibraltar,
322 separating the Atlantic and the Mediterranean populations, with the exception of
323 individuals collected in the Gulf of Cádiz. These latter individuals showed an admixture of
324 genetic attributes from the Atlantic and Mediterranean populations suggesting gene flow
325 across this partial barrier. Similarly, Roldán et al. (1998) showed, based on allozyme data,
326 that European hake collected in Moroccan waters close to the Strait of Gibraltar were
327 genetically closer to the Mediterranean samples than to Atlantic samples. Lundy et al.
328 (1999) obtained comparable results using microsatellite DNA markers. These studies
329 supported the hypothesis that unidirectional passive larval drift from the Atlantic Ocean
330 into the Mediterranean Sea was likely responsible for the gene flow across the Strait of
331 Gibraltar, as suggested in other marine organisms such as mussels (Diz and Presa, 2008).

However, the hypothesis of unidirectional dispersal of hake larvae from the Atlantic Ocean into the Mediterranean Sea has yet to be confirmed. In the Atlantic Ocean, individuals collected in the Celtic Sea, the Armorican Shelf and the Galician Shelf showed very little genetic divergence. This result is in agreement with recent studies suggesting consistent gene flow between hake grounds in Porcupine Bank and Great Sole Bank (Celtic Sea), and northern grounds of the Iberian Peninsula over a two year period (Pita et al., 2011; Pita et al., 2013). The congruence with these studies that have a temporally more intensive sampling design supports the results obtained in this study.

While otolith geochemistry proved to be a useful natural tag to track collection locations in European hake (Swan et al., 2006; Tanner et al., 2012), disentangling individuals' natal origins based on otolith core values over the full geographical range of our study was more challenging. Elemental and isotope ratios in otolith cores of individuals collected in the most distant locations (i.e. Celtic Sea and Sardinia) resembled each other leading to high misclassification rates of individuals among these distant locations. Migration between these locations within one year of life of European hake seems improbable, rather environmental conditions in the two locations were likely similar during the early stages of life thus producing congruent geochemical signatures in their otoliths. On the other hand, hake spawning areas in the Atlantic Ocean are thought to be continuous from the French coast to the Celtic Sea and along the northwestern coast of the Iberian Peninsula (Domínguez-Petit et al., 2008), so misclassification of individuals to neighboring or proximate locations was expected as spawning and early life development is not restricted to collection locations.

The combined application of otolith geochemistry and microsatellite DNA markers has provided information on population structure (Miller et al., 2005), early life stage dispersal (Bradbury et al., 2008) and natal origins of a number of species, particularly salmonids (Barnett-Johnson et al., 2010; Miller et al., 2010; Perrier et al., 2011). The results obtained

358 in the present study using the two techniques yielded complementary information on natal
359 origins and population structure of European hake. The classification model based on
360 genetic and geochemical data substantially decreased classification errors between distant
361 locations or areas, such as Celtic Sea and Sardinia. However, misclassification rates
362 continued to be high among individuals collected in the Celtic Sea, Armorican Shelf and
363 Galician Shelf as well as between the northern and southern stock units in the Atlantic
364 Ocean providing further evidence of movement of individuals of European hake between
365 these locations and areas. Pita et al. (2011) proposed directional gene flow from northern
366 to southern stocks based on the genetic similarity of Porcupine and Galician samples, large
367 recruitment in the southern stock relative to its depleted spawning stock biomass and
368 predominant current directions in the Bay of Biscay. However, integrated results of
369 microsatellite DNA markers and otolith geochemistry suggested that movement of
370 European hake might occur in both directions. Further, one individual collected in the
371 Atlantic Ocean (Gulf of Cádiz) was misclassified to the Mediterranean Sea which might
372 indicate migration through the Strait of Gibraltar. However, we can say little about the
373 extent or direction of probable movements through the Strait of Gibraltar due to the low
374 number of samples used in the otolith chemistry analysis. Analyzing a higher number of
375 samples would increase the probability of identifying migrants given that only a few
376 migrants per generation are necessary to prevent genetic divergence among populations
377 (Palumbi, 2003). Nevertheless, our results show that the northern and southern hake stocks
378 in the northeast Atlantic are connected and disclose the high complexity of population
379 structure of European hake in the Atlantic Ocean.
380 The integration of genetic markers and otolith geochemistry clearly added to the study of
381 natal origins and population structure of European hake providing information at different
382 spatial resolutions. Furthermore, we confirmed that otolith geochemistry is a useful
383 technique to complement the assessment of early life stage dispersal in populations with

high gene flow and low genetic divergence (Campana, 1999; Thorrold et al., 2001). Nevertheless, the sampling design should cover several years to assess the stability of the results obtained. For the genetic markers, studies have determined temporal consistency in this species (Lundy et al., 2000; Pita et al., 2011; Pita et al., 2013) however, to our knowledge, no otolith geochemistry study has assessed otolith composition in European hake over more than a year. Temporal stability in otolith geochemical composition is rare, even in rather homogenous environment such as the ocean (reviewed in Elsdon et al., 2008). Furthermore, to obtain a reliable estimate of dispersal, hake larvae need to be systematically sampled to constrain a baseline dataset for retrospective determination of natal origin of adults. The extent and direction of larval dispersion and migration of juvenile and adult hake might be further unraveled by combining biophysical models, additional otolith chemistry studies and using artificial tags given that successful tag-recapture experiments have been conducted with European hake with the objective of validating growth rate and age estimation based on otolith interpretation (e.g. De Pontual et al., 2003; Piñeiro et al., 2007). The integrated use of genetic and other natural markers (e.g. otolith chemistry, parasites), combined where possible with biophysical models and artificial tagging, shows great promise to resolve connectivity patterns over the entire life history of European hake.

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612

Table 1. Collection location, date of collection, median and standard deviation (SD) of fish total length (Lt) in cm and sample sizes for geochemical (n_a) and genetic analysis (n_b) of *Merluccius merluccius*.

Collection location	Abbreviation	Date of collection	Lt (cm)		n_a	n_b
			Median	SD		
Celtic Sea	CS	November 2010	22.3	1.4	10	50
Armorican Shelf	AS	May 2010	22.7	1.1	10	42
Galician Shelf	GS	October 2010	22.2	1.5	10	50
Portugal	PT	June 2010	20.7	0.8	10	50
Gulf of Cádiz	GC	November 2010	22.3	0.7	10	50
Balearic Islands	BI	May 2010	21.2	1.3	10	50
Sardinia	SA	October 2010	23.5	1.3	10	47

Table 2. Pair-wise F_{ST}-distance between collection locations of *Merluccius merluccius* in the northeast Atlantic Ocean and the western Mediterranean Sea. Asterisks indicate *p*-values smaller than the adjusted nominal level for multiple comparisons α=0.0023 obtained after 420 permutations.

Collection location	CS	AS	GS	PT	GC	BI	SA
Celtic Sea (CS)	-	0.0016	0.0016	0.0066	0.0191*	0.0485*	0.0402*
Armorican Shelf (AS)		-	-0.0013	0.0048	0.0145	0.0372*	0.0292*
Galician Shelf (GS)			-	0.0041	0.0150	0.0384*	0.0301*
Portugal (PT)				-	0.0061*	0.0214*	0.0167*
Gulf of Cádiz (GC)					-	0.0084	0.0070
Balearic Islands (BI)						-	-0.0003

Table 3. Hierarchical analysis of molecular variance (AMOVA) among the whole dataset, the hydrographic regions (northeast Atlantic Ocean and western Mediterranean Sea) and the management units in the Atlantic Ocean (northern and southern stock) and western Mediterranean Sea. Asterisk indicates $p<0.01$.

Hierarchical level	Source of variation	df	Sum of Squares	Variance components	% of variation	Fixation indices	
Whole dataset	Among locations	6	37.13	0.0412	1.85	F_{ST}=	0.018*
	Within locations	671	1472.50	2.1945	98.15		
Hydrogeographic regions	Among groups	1	17.97	0.5103	2.25	F_{CT}=	0.0225*
	Among locations	5	19.16	0.0169	0.75	F_{SC}=	0.0075*
	Within locations	671	1472.50	2.1994	97.00	F_{ST}=	0.0300*
Management units and Mediterranean Sea	Among groups	2	22.91	0.0359	1.60	F_{CT}=	0.0160*
	Among locations	4	14.22	0.0140	0.63	F_{SC}=	0.0063*
	Within locations	671	1472.50	2.1944	97.77	F_{ST}=	0.0223*

Table 4. Correct classification (%) of individuals of *Merluccius merluccius* based on microsatellite DNA markers using the ONCOR software, otolith geochemical values using linear discriminant function analysis (LDFA) and the two datasets combined using LDFA.

	Correct classification (%)		
	Microsatellite markers	Otolith geochemistry	Combination
Sampling locations			
Celtic Sea	16.0	60.0	60.0
Armorican Shelf	21.4	80.0	80.0
Galician Shelf	10.9	70.0	80.0
Portugal	30.0	100.0	100.0
Gulf of Cádiz	22.4	70.0	90.0
Balearic Islands	34.7	100.0	100.0
Sardinia	27.7	60.0	90.0
Overall	23.4	77.1	85.7
Management units and Mediterranean Sea			
Northern stock	42.4	70.0	90.0
Southern stock	40.0	96.7	93.3
Mediterranean Sea	65.6	75.0	100.0
Overall	48.0	82.9	94.3
Hydrogeographic regions			
Atlantic Ocean	79.3	76.0	98.0
Mediterranean Sea	74.0	90.0	100.0
Overall	77.8	80.0	98.6

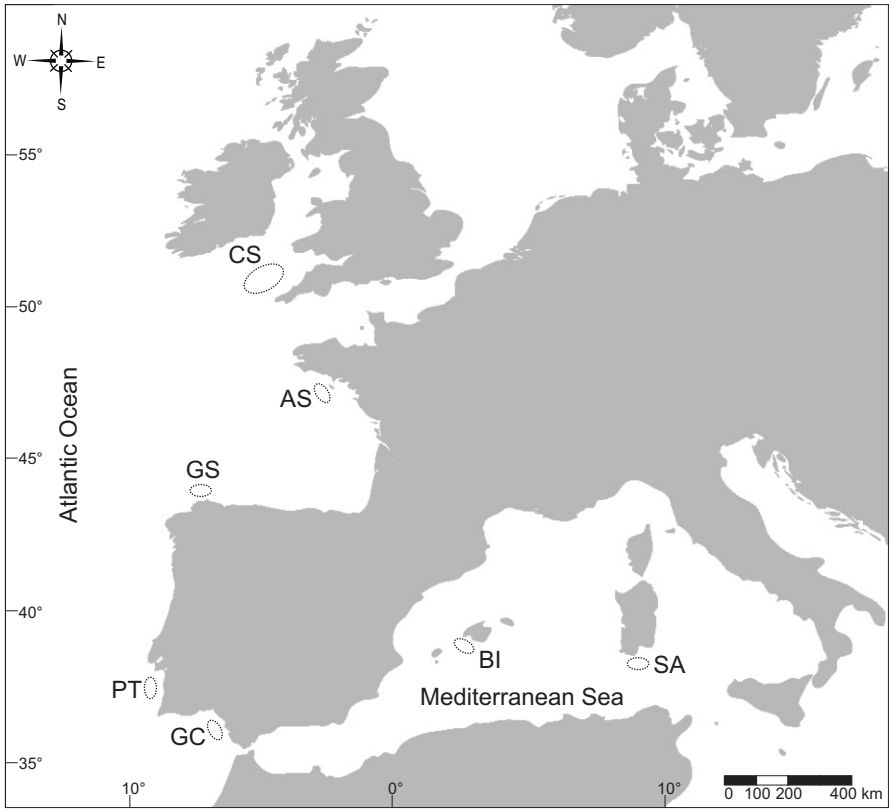
Figure legends

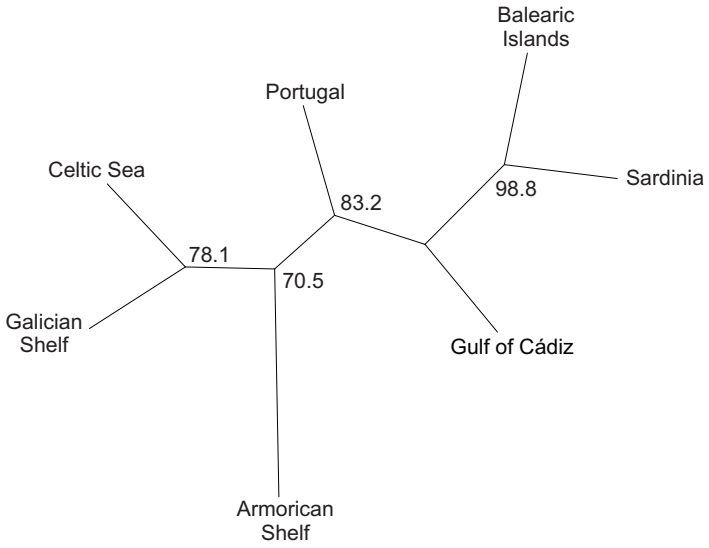
Figure 1. Collection locations of *Merluccius merluccius* in the northeast Atlantic Ocean and the western Mediterranean Sea: CS – Celtic Sea, AS – Armorican Shelf, GS – Galician Shelf, PT – Portugal, GC – Gulf of Cádiz, BI – Balearic Islands, SA – Sardinia.

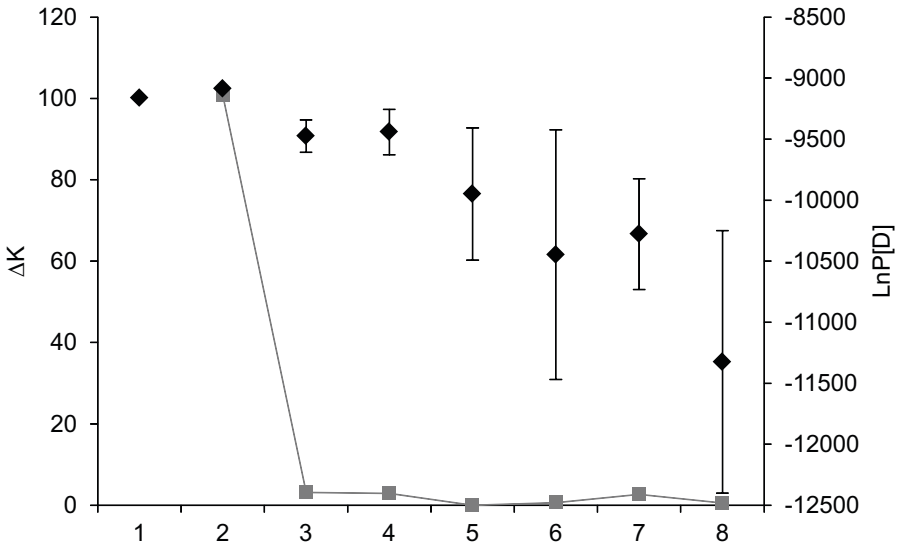
Figure 2. Relative relationships between *Merluccius merluccius* collected in the northeast Atlantic Ocean and the western Mediterranean Sea according to unrooted neighbour-joining tree based on Cavalli-Sforza and Edwards chord distance. Bootstrap support was generated from 1000 replicates.

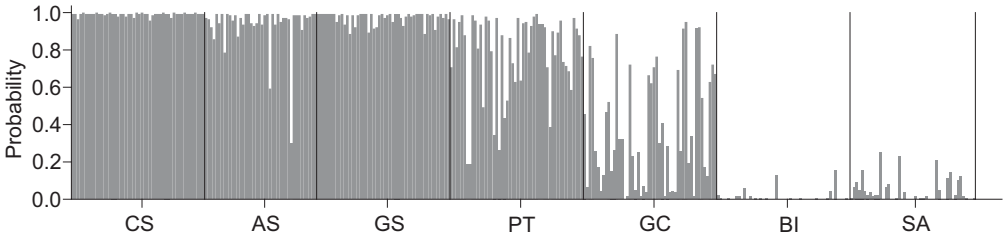
Figure 3. *Ad hoc* models (ΔK and LnP(D)) from STRUCTURE used to determine the number of hypothetical clusters (*K*) identified over the geographical range of the study. Predetermined *K* ranged from 1 to 8. ΔK values (grey) are shown on the primary y-axis and the log probability of LnP(D) (black) was averaged over 5 independent runs, the average and associated standard deviations are shown on the secondary y-axis.

Figure 4. Individual assignment based on Bayesian clustering method from STRUCTURE. Each bar represents an individual of *Merluccius merluccius* with its probability of membership to one of the hypothetical clusters ($K = 2$). Labels at the bottom indicate collection locations. See table 1 for abbreviations.


N
W
E
S
55°
50°
45°
40°
35°
Atlantic Ocean
CS
AS
GS
BI
SA
PT
GC
Mediterranean Sea
10°
0°
10°
0 100 200 400 km


Balearic
Islands
Portugal
Celtic Sea
Sardinia
98.8
83.2
78.1
70.5
Galician
Shelf
Gulf of Cádiz
Armorican
Shelf


120
100
80
60
40
20
0
ΔK
-8500
-9000
-9500
-10000
-10500
-11000
-11500
-12000
-12500
LnP[D]
1
2
3
4
5
6
7
8


Probability
1.0
0.8
0.6
0.4
0.2
0.0
CS
AS
GS
PT
GC
BI
SA

Appendix 1. Genetic parameters (number of alleles (A), mean allele size (Ā), allele size range (Range A), modal allele size (Modal A), allelic richness (R_S), expected heterozygosity (H_e), fixation index (F_{IS}) (Weir & Cockerham, 1984)) of five microsatellite loci analysed in *Merluccius merluccius* collected at seven locations in the Atlantic Ocean and the Mediterranean Sea. Bonferroni correction for significant departures from the Hardy-Weinberg expectations (P<0.001).

		Northern stock		Southern stock			Mediterranean stock	
Locus		Celtic Sea	Armorican Shelf	Galician Shelf	Portugal	Gulf of Cádiz	Balearic Islands	Sardinia
Mmer-hk3b	A	10	10	14	11	11	10	10
	Ā	334.9	335.5	336.2	334.8	334.1	332.4	332.6
	Range A	324-346	328-346	322-348	324-348	324-344	324-344	322-344
	Modal A	330-336	332-336	332-336	332-336	332	332	332
	R_s	9.68	10.00	13.18	10.66	10.79	9.29	9.76
	H_e	0.80	0.85	0.83	0.81	0.76	0.53	0.60
	F_{IS}	0.038	0.139	0.001	0.002	0.004	-0.005	-0.013
Mmer-hk9b	A	32	29	34	29	35	36	34
	Ā	150.5	144.1	147.5	146.3	150.3	155.4	147.5
	Range A	119-211	109-185	111-203	111-181	109-195	109-211	111-193
	Modal A	133-163	123-151	133-161	133-153	153	155	115-123
	R_s	30.55	29.00	32.67	27.98	33.23	34.06	33.04
	H_e	0.96	0.95	0.96	0.95	0.96	0.96	0.96
	F_{IS}	0.028	0.059	0.245*	0.229*	0.219*	0.156*	0.169*
Mmer-hk20b	A	18	17	19	17	18	20	18
	Ā	225.78	224.9	225.8	225.9	228.36	222.66	225.6
	Range A	213-249	213-247	213-251	213-249	213-247	211-253	211-253
	Modal A	221	221	221	221-237	221-237	223-239	221
	R_s	17.28	17.00	18.40	16.62	17.46	19.47	17.74
	H_e	0.89	0.90	0.88	0.89	0.92	0.91	0.90
	F_{IS}	0.177	-0.016	-0.057	0.070	0.310*	0.005	-0.026
Mmer-hk29b	A	12	13	15	14	13	15	15
	Ā	162.98	164.9	157.8	164.1	158.62	162.84	162.6
	Range A	146-172	152-178	146-180	146-180	148-176	140-174	146-184
	Modal A	162	168	168	166	160-166	166	164
	R_s	11.79	13.00	14.57	13.60	12.98	14.58	14.55
	H_e	0.89	0.88	0.88	0.87	0.88	0.89	0.86
	F_{IS}	0.400*	0.331*	0.534*	0.525*	0.407*	0.315*	0.467*
Mmer-hk34b	A	17	21	17	19	20	17	18
	Ā	128.9	131.8333	127.7	130.88	131.5	131.08	133.6596
	Range A	128	128-136	128-138	130	130-140	130-136	130-140
	Modal A	110-152	108-160	112-156	110-158	106-160	112-152	114-154
	R_s	16.58	21.00	16.65	18.15	19.04	16.63	17.35
	H_e	0.90	0.92	0.90	0.90	0.92	0.90	0.89
	F_{IS}	0.051	0.133	0.039	0.123	0.034	0.150	0.102