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Population structure and connectivity of the European conger eel (*Conger conger*) across the north-eastern Atlantic and western Mediterranean: integrating molecular and otolith elemental approaches

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Abstract Genetic variation (mtDNA) of the European conger eel, *Conger conger*, was compared across five locations in the north-eastern Atlantic (Madeira, Azores, South Portugal, North Portugal and Ireland) and one location in the western Mediterranean (Mallorca). Genetic diversity of conger eel was high, and differentiation among regions was not significant. Additionally, comparisons of element:Ca ratios (Sr:Ca, Ba:Ca, Mn:Ca and Mg:Ca) in otolith cores (larval phase) and edges (3 months prior to capture) among the Azores, North Portugal, Madeira and Mallorca regions for 2 years indicated that variation among regions were greater for edges than cores. Therefore, while benthic conger may display residency at regional scales, recruitment may not necessarily be derived from local spawning and larval retention. Furthermore, data from otoliths suggest a separated replenishment source for western Mediterranean and NE Atlantic stocks. The combination of genetics and otolith chemistry suggests a population model for conger eel involving a broad-scale dispersal of larvae, with limited connectivity for benthic juvenile life stages at large spatial scales, although the existence of one or multiple spawning grounds for the species remains uncertain.

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Introduction

Marine species with a prolonged larval phase are a priori expected to display high levels of dispersal resulting in panmixia (i.e. interbreeding leading to little or no structuring) at oceanic, regional and subregional scales. In benthic marine species in which adults do not move extensively, planktonic larvae are the only vehicles for dispersal and gene flow between populations. Notwithstanding, even if both larvae and adults tend to move extensively, oceans are not a continuum and soft barriers, such as oceanographic currents and gyres or salinity and temperature gradients, may restrict their movements, and consequently, gene flow among populations (Floeter et al. 2008). Several population types have been postulated to exist between two extremes, including populations extending over large expanses of ocean (i.e. open populations receive and pass on individuals to other populations) to closed populations (i.e. populations that do not exchange individuals to an appreciable extent) (Hellberg et al. 2002) and numerous examples of marine species can

be found along this gradient in gene flow (Ayre et al. 2009).

The European conger eel (*Conger conger* Linnaeus, 1758) is a deep-water marine fish widely distributed along sandy and rocky shores of the NE Atlantic, Mediterranean and western Black Sea (Bauchot and Saldanha 1986). The species constitutes an important commercial and recreational fishery resource that is caught by bottom trawl, hook and line (Figueiredo et al. 1996). Despite being a geographically widespread species and an important fisheries resource, little is presently known about the life history traits of this species, namely its reproductive biology, spawning area(s) and migratory patterns. Indeed, data on the *C. conger* spawning area(s), duration of the leptocephalus phase and larval migratory routes are scarce (Correia et al. 2002, 2003, 2006a). After spawning, the leptocephalus larvae can persist up to 2 years in pelagic environments (Correia et al. 2006a), presumably sustained by consuming dissolved organic carbon, faecal pellets, waste products of zooplankton and other larvae (Mochioka and Iwamizu 1996; Bishop and Torres 1999). As the spawning season approaches, congers cease to feed (Cau and Manconi 1984), which prevents capture by longlines, and only trawler fishing is effective in capturing conger mature specimens (Fannon et al. 1990). As a consequence, most conger eels captured in the coastal inshore waters of the Atlantic are immature (Sbaihi et al. 2001; Sullivan et al. 2003; Correia et al. 2009). An earlier extensive survey in the eastern Mediterranean did capture sexually mature specimens, suggesting the existence of a breeding area for the European conger eel in deep waters southeast of Sardinia (Cau and Manconi 1983). This was also supported by the record of small larvae (>30 mm) near the Gibraltar strait (Strehlow et al. 1998). The capture of small (ca. 50 mm) and young larvae (ca. 2½ months) in the Mid-Atlantic region, near the Azores Islands, also indicated that spawning occurred in the vicinity of the Azorean archipelago (Correia et al. 2002, 2003) during the summer season. These various observations of larval stages lead to the hypothesis that congers can spawn across a larger area between Gibraltar and the Azores, as suggested in some textbooks (e.g. Hayward and Ryland 1995) and that one single and highly localized spawning aggregation site for this species is unlikely. Otherwise, the presence of conger eel early life stages in these remote regions can be explained only if the larvae are capable of active and oriented swimming against the North Atlantic oceanographic currents of opposite direction (Correia et al. 2002). However, some other studies have suggested that leptocephalus larvae largely depend on currents for their migration (Tsukamoto 2006; Bonhommeau et al. 2008) and lend support to an alternative hypothesis of a single spawning area and passive larval drift. A genetic survey of six central and eastern Atlantic locations including Azores, North Portuguese Continental Slope, Bay of Biscay and Minho River (North of Portugal) on a limited number of samples (40 individuals in total) did not reveal significant differences among sampling sites (Correia et al. 2006b). Recently, the oxygen and carbon isotopic signatures in the otoliths of *C. conger* clearly discriminated between the NE Atlantic and the Mediterranean fishery areas, suggesting low levels of connectivity between these fishing grounds and a relatively sedentary lifestyle after larval settlement (Correia et al. 2011).

Resolving population structure of meta-population complexes is a general problem in fisheries management due to potential for larval dispersal and migration and the difficulty of quantifying connectivity rates or interdependency of replenishment among populations in different areas. This question has to be addressed in the light of global depletion of fish stocks and is essential for sustainable management. Otolith chemistry analysis is an innovative approach that can complement other stock discrimination techniques, such as the traditional tag/recapture and genotypic approaches, as an effective method to assess stock structure, migration patterns and connectivity between adult populations and spawning/nursery sources (Campana et al. 2000; Elsdon et al. 2008). The integration of molecular and otolith-based techniques have proven powerful tools in resolving population structure in high gene flow systems where environmental heterogeneity exists (Bradbury et al. 2008; Selkoe et al. 2008; Smith and Campana 2010).

The major goal of this work was to apply both molecular and otolith-based analyses to provide new information on the population structure and connectivity of the European conger eel across the north-eastern Atlantic and western Mediterranean. Firstly, we analyzed the genetic structure of the conger eel to test for clinal variation, to infer the demographic history of the species and to provide evidence for the existence of at least two spawning sites. Secondly, we examined the variation in otolith trace elements at large spatial scales for both the otolith core (larval pelagic stage) and edge (adult benthic stage) to complement the molecular approach in inferring population structure and also evaluated the annual temporal stability of elemental signatures for different fishery regions.

Materials and methods

Fish collection

The congers used in this study were collected from the local artisanal fisheries mostly captured by hook and pots in shallow coastal waters of the NE Atlantic and western

Mediterranean between December 2005 and September 2008.

In the laboratory, total length (TL, cm) and whole weight (M, g) were measured for all fish sampled. Sagittal otoliths were removed with plastic forceps, to avoid metallic contamination, cleaned with Milli-Q water, air-dried in a laminar-flow cabinet, weighed (0.0001 g) and stored dry in acid-washed vials. Muscle tissue samples collected near the dorsal tip were preserved in 96 % ethanol and stored at −20 °C. For Irish and South Portugal locations, pectoral or caudal fin clips were taken and immediately placed in 96 % ethanol. No otoliths were available for these locations. For molecular studies, a total of 347 specimens of *C. conger* were sampled covering the geographic range of the species. For otolith analyses, we used 160 specimens (TL: 95 ± 3 cm; size range: 75–115 cm) sampled in two consecutive years (2006 and 2007) from four widely separated regions: Azores, North Portugal, Madeira and Mallorca (Fig. 1). Further details on sampling localities and geographic range of specimens are shown in Table 1.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from white muscle tissue, using a saline extraction method (Sambrook and Russell 2001). Primers pairs published for the mitochondrial control region of *C. conger* (Correia et al. 2006b) did not amplify as expected. Combinations of a number of other teleostean mitochondrial control region primers also yielded negative PCRs. The conger eel mtDNA has an arrangement of genes that prevents the use of universal primers to amplify the target region. New modified primers based on L15774 (Kocher et al. 1989) (CCTGAAGTAGGAACCAGATGT CAGTA) and H16498 (Kocher et al. 1989) (ACATGAA TTGGAGGAATACCCGTTG) were used to amplify a fragment of *c*. 700 base pairs (bp). We intended to use nuclear intron primers to further assess population structure. However, the widely used "universal" intron S7 primers (Chow and Hazama 1998) did not yield readable sequences under a wide range of PCR profiles. The cloning of some of the amplicons also did not produce useful sequences.

All PCRs had the following profile: 2 min at 95 °C, 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C (repeated for 40 cycles) and 10 min at 72 °C. Amplification reactions were carried out in 25 µL total volume, including 0.5 µL of each 10 µM primer, 0.5 µL of 10 mM dNTP mix, 2.5 µL of 10 × reaction buffer, 18.8 µL of purified water and 0.2 µL of 5 U μL^{-1} Go*Taq* DNA polymerase (Promega, USA). Negative controls (no template) were included in each set of reactions. PCR amplicons were purified by ethanol and sodium acetate precipitation and directly sequenced. Forward and reverse DNA strands were sequenced by Macrogen, Inc. Chromatograms were obtained from an automated sequencer (ABI 3130XL-Genetic Analyzer-Applied Biosystems).

Molecular data analyses

Sequences were aligned in Geneious software version PRO 4.5.6, and the alignment was further optimized by eye.

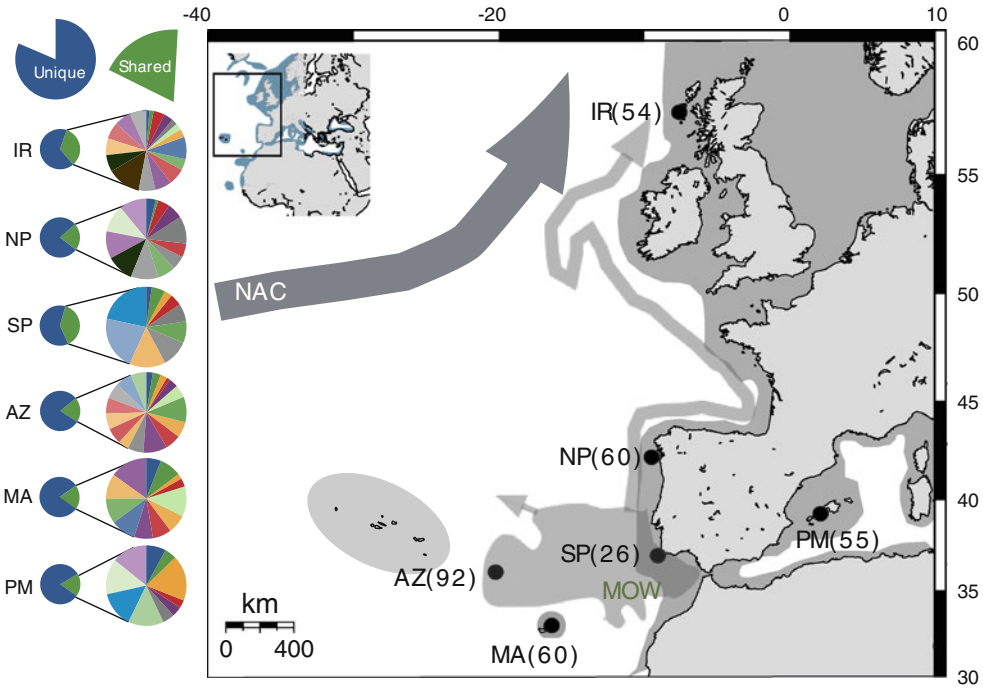


Fig. 1 Sampling locations (sampling codes) and number of individuals in parenthesis. *Inset*, *darker shaded area*: putative natural range in the north-eastern Atlantic Ocean of conger eel (*C. conger*). *IR* Ireland, *NP* North Portugal, *SP* South Portugal, *AZ* Azores, *MA* Madeira, *PM* Mallorca. *Currents depicted*: *NAC* North Atlantic surface current, *MOW* Mediterranean outflow centred near 1,000 m water depth, adapted from Reid 1979 (in Khélifi et al. 2009). *Left pies* represent the proportion of shared and private haplotypes in each sampling location

Table 1 Geographic locations, date of sampling, code, sample sizes (N), total length (TL), number of haplotypes (NH); private haplotypes (PH), haplotype diversity (h) and percentage nucleotide diversity (π) and standard deviations (SD) and mean number of nucleotide differences (k) for the studied populations of *C. conger*

Locations	Code	Location	Date	*N*	TL (cm)	NH	PH	*h* (±SD)	% π (±SD)	*k*
Ireland	IR	56°26′N/09°34'W	September 2008	54	53–141	39	36	0.97 ± 0.02	0.50 ± 0.30	2.99
Azores*	AZ	36°00′N/20°00′W	April 2006 April/May 2007	92	55–173	56	73	0.95 ± 0.02	0.45 ± 0.27	2.69
Madeira*	MA	32°47′N/16°44′W	May 2006 April/May 2007	60	39–146	35	49	0.89 ± 0.04	0.38 ± 0.23	2.29
Mallorca*	PM	39°15′N/02°20′W	April 2006 April 2007	55	55–122	18	45	0.74 ± 0.06	0.21 ± 0.15	1.24
North Portugal[a]	NP	41°11′N/08°58′W	April 2006 April 2007	60	83–117	40	47	0.96 ± 0.03	0.43 ± 0.26	2.40
South Portugal	SP	36°55′N/09°15′W	December 2005 April 2006	26	Not available	20	16	0.92 ± 0.03	0.40 ± 0.25	2.38
All		56°26′N/09°34′W	December 2005–September 2008	347	39–173	156	129	0.90 ± 0.02	0.40 ± 0.24	2.38

[a] Also used for otolith elemental signatures (see section "Materials and methods" for further details and for the meaning of sampling codes see Fig. 1)

Genetic parameters were estimated with DnaSP version 5 (Librado and Rozas 2009) and Arlequin version 3.5 (Excoffier and Lischer 2010). Collapse v.1.2 (Posada 2008a) was used to reduce the sequence data file into the haplotype file.

Different methods were used to assess whether group structure could be detected in the data set. First, a Kruskal's non-metric multidimensional scaling analysis (NMDS) (Kruskal 1964) was performed with the SMACOF (Scaling by MAjorizing a COnvex Function) that minimizes the "Normalized Stress" (De Leeuw 1977) included in XLSTAT 2010 (Addinsoft, TM). The NMDS was based on the interindividual Euclidean distances calculated from the matrix of corrected genetic distances estimated under the general time reversible (GTR) model (Tavaré 1986) with proportion of invariable sites ($I = 0.5011$) and gamma distribution ($\gamma = 0.7355$) as selected by JModelTest v. 0.1.1 (Posada 2008b). Second, pairwise genetic distances (Φ_{ST}) were estimated between samples using Arlequin 3.5 (Excoffier and Lischer 2010). Because we tested multiple hypotheses about population structure, we adjusted the a priori significance value ($\alpha = 0.05$) for Type I error using the modified false discovery rate (FDR) correction (Benjamini and Yekutieli 2001). Third, an AMOVA was performed on every possible combination of locations with SAMOVA (Dupanloup et al. 2002). This procedure aimed at identifying groups of populations that are geographically homogeneous and maximally differentiated in terms of among-group component (Φ_{CT}) of the overall genetic variance, without the prior assumption of group composition that is necessary for AMOVA. Runs consisted of 10,000 iterations for each of 100 random initial conditions to test all the grouping options (pre-defined number of groups (K) ranging from 2 to 5).

When haplotype diversity is high and most haplotypes are unique, it is not feasible to assess the genetic structure solely from the haplotype frequency data. The nearest-neighbours statistic (*Snn*) (Hudson 2000) has been proposed as a more powerful method for analyzing loci with high haplotype diversity. Values of *Snn* are expected to be close to 0.5 if individuals from different locations are panmictic, and closer to 1 if they are highly differentiated (Hudson 2000). Genetic differentiation between populations was calculated using the *Snn* statistic implemented in the program DnaSP version 5.0 (Librado and Rozas 2009).

A haplotype network was constructed using the median-joining approach on Network, version 4.0, available at http://www.fluxus-engineering.com/sharenet.htm (Bandelt et al. 1999). Mantel test performed by XLSTAT (Addinsoft^{TM}) was used to test the null hypothesis of no correlation between corrected $\phi_{st}/(1 - \phi_{st})$ genetic distances and the natural logarithm of the waterway geographic distance between pairs of sampling locations.

Inferences on demographic history were made from the results of neutrality tests and the mismatch distribution. Tajima's D (Tajima 1989), Fu's F_s (Fu 1997) and Ramos-Onsins and Rozas' R_2 (Ramos-Onsins and Rozas 2002) statistics test for departures from equilibrium between mutation and drift. Changes in population size were estimated from the mismatch distribution (the analysis of differences between pairs of mitochondrial haplotypes) using DnaSP version 5.0 (Librado and Rozas 2009). The mismatch distribution is usually multimodal for sequences

sampled from populations at demographic equilibrium, but typically unimodal for populations having passed through a recent demographic expansion (Slatkin and Hudson 1991; Rogers and Harpending 1992).

Otolith preparation

After mounting in epoxy resin (ATM, KEM 91), transverse sections of approximately 300 μm width were taken through the core region of the sagittal otoliths with a diamond saw (Buehler, Isomet low-speed saw) lubricated with Milli-Q water. The otoliths were ground to expose the core with a series of graded silicon carbide papers (Hermes 1,200 and 2,500 grits) and regular viewing through a metallographic microscope (Meiji ML7100), further polished with diamond pastes (Magnum-top-plus, DP-paste: 6, 3, 1 and 0.25 μm) and then mounted on acid-cleaned microscope slides with epoxy resin. Once cured, the otolith slides were sonicated for 3 min in Milli-Q water, further rinsed with copious amounts of Milli-Q water, and then dried in a laminar-flow cabinet before storage in sealed containers.

Otolith chemistry

Otoliths were analysed with a New Wave 213 nm Nd:YAG ultraviolet laser microprobe in conjunction with a Finnigan ELEMENT 2 high-resolution inductively coupled plasma mass spectrometer (HR-ICP-MS). After a preliminary investigation we decided to focus on the isotopes ^{88}Sr, ^{138}Ba, ^{55}Mn and ^{25}Mg, along with ^{43}Ca as the internal standard. The concentration of Ca in otoliths was taken as 38.8 % by weight or 388.000 ppm (Yoshinaga et al. 2000). We also collected data for ^{7}Li, ^{63}Cu, ^{85}Rb, ^{139}La, ^{208}Pb and ^{66}Zn; however, these elements were below the detection limits for more than 50 % of the otoliths analysed and so were not included in further statistical analyses.

Ablation was conducted in helium that was mixed with argon for transport to the plasma. Laser samples were taken in the core region (two ablations per otolith, pelagic larval phase) and adjacent to, but not overlapping, the proximal margin of otoliths (one ablation at dorsal and ventral tip regions, benthic adult phase). The ablations were circular and of approximately 100 μm diameter, with a repetition rate of 6 Hz and an output energy of 10 J cm^{-2}. Ablation sample positions were standardized across otoliths to reduce any influences of within otolith variation on comparisons. The data from the edge and core samples were treated separately. Preliminary analyses of the data indicated that variation among the geographic regions where fish were collected was similar irrespective of which ablation location at the core or edge was used in comparisons (i.e. based on ANOVA interaction term being non-significant). Therefore, we chose to average the data from the two ablations in the core and edge to provide the elemental concentrations used in statistical analyses.

To eliminate the influence of any biases due to instrument drift, we randomized and blocked the daily analysis sequences with respect to geographic sampling region. At the time of this study, no certified, otolith-based standard that was suitable for laser analyses was available. The National Institute of Standards (NIST) 612 glass standard reference material (SRM) was used for the quantification of elemental concentrations (Pearce et al. 1997). This calibration standard was analysed every 16 otolith ablations (i.e. every 4 otoliths) to further eliminate possible short-term drift effects. Samples were pre-ablated prior to data acquisition to remove any residual surface contamination. The average counts of a 20-scan blank acquired prior to each ablation were subtracted from the average sample counts from 30 scans during laser ablation of the sample. The ablation cell was flushed with He for 1 min prior to each blank/sample acquisition to remove any residue from the previous samples and pre-ablation. Data reduction was conducted offline. Raw counts of data (counts s^{-1}) were converted to elemental concentrations against calcium (μg element g^{-1} Ca) (Ludden et al. 1995).

A blank of 70 scans was acquired at the start and end of each analysis day. The concentration equivalent to three times the standard deviation of the blank was used as the detection limit. Detection limits also depend on the amount of material ablated and were adjusted for each ablation based on ablation yield estimates (Lahaye et al. 1997). Average detection limits (μg g^{-1}) were: ^{7}Li: 0.526, ^{25}Mg: 0.756, ^{55}Mn: 0.098, ^{63}Cu: 0.353, ^{66}Zn: 0.164, ^{85}Rb: 0.103, ^{88}Sr: 0.821, ^{138}Ba: 0.025, ^{139}La: 0.013 and ^{208}Pb: 0.011. Accuracy and precision were estimated on a daily basis for the NIST SRM 612 (analysed as an unknown). Precision estimates for individual elements from repeated sampling of the NIST SRM 612 were, mean relative standard deviation (RSD); Li: 7 %, Mg: 4 %, Mn: 4 %, Cu: 7 %, Zn: 5 %, Rb: 4 %, Sr 3 %, Ba: 4 %, La: 3 % and Pb: 4 %. Accuracy estimates based on recovery for individual elements in the NIST SRM 612 were 99–100 % recovery for all elements.

Otolith chemistry: statistical analyses

After testing for normality (Shapiro–Wilk's test) and homogeneity of variances (Levene's test), the data (μg element g^{-1} of calcium) were analysed through univariate and multivariate analysis. To meet model assumptions, some analyses were performed on log10 transformed data. A few outliers detected using Grubbs' test were eliminated or transformed (truncated).

Fish length overlapped between the sampling regions for each year allowing further comparisons (TL of 55–118 cm and 66–130 cm for 2006 and 2007 years, respectively). To ensure that any size differences among samples did not influence differences in edge otolith chemistry among regions, thereby potentially confounding the spatial variation in elemental fingerprints (Fowler et al. 1995; Bath et al. 2000; Reis-Santos et al. 2008), we performed linear regressions between trace element concentration and otolith weight (Campana et al. 2000). However, ANCOVAs for the elements presenting a significant relationship between both variables showed that otolith weight was not a significant influence on the differences among regions. Therefore, we elected not to adjust otolith concentrations for otolith weight prior to further statistical analyses. For core analysis, otolith weight is not a relevant covariate for natal retrospective regional comparisons (Hamer et al. 2003).

To detect regional differences in the concentrations of particular elements and multielemental fingerprints, we performed analyses of variance (ANOVA) and multivariate analyses of variance (MANOVA), respectively. Tukey's HSD test was used to detect a posteriori differences among single elemental means. MANOVA was used to test for spatial and temporal differences in otolith elemental signatures. Region and year were treated as fixed factors. For the MANOVA, we reported the approximate F-ratio statistic for the most robust test of multivariate statistics (Pillai's trace). Post hoc multivariate pairwise comparisons between locations were performed using the Hotelling T-square test. Significant interannual differences in single elemental signatures were tested using Student's *t* tests performed for each element comparing 2006 and 2007 years for edge analysis. Quadratic discriminant function analyses (QDFA) were used to visualize spatial differences and to examine the re-classification accuracy success of fishes to this original location. QDFA was chosen as opposed to linear DFA as it does not require homogeneity of within-group covariance matrices (Quinn and Keough 2002). Cross-validations were performed by using jackknife ("leave one out") procedures.

All these statistical analyses were performed using Systat (version 12.0). Results are presented as means ± standard errors (SE). The statistical level of significance (α) for all tests was 0.05.

Results

Genetic analyses

The partial nucleotide sequences of the mitochondrial control region of 347 specimens of *C. conger* produced an alignment of 607 bp. The alignment revealed 103 (17 %) polymorphic sites, 38 (6 %) of which were parsimony informative. We identified a total of 156 haplotypes, 127 (81 %) of which occurred as singletons. Four of the twenty-nine shared haplotypes showed a wide geographic distribution, with the most common haplotype shared by 97 (28 %) individuals from all six locations. Overall haplotype diversity was 0.9, ranging from 0.74 in Mallorca to 0.96 in both Ireland and South Portugal (Table 1). Overall nucleotide diversity (π) was 0.2 % for all samples combined and ranged from 0.2 % in Mallorca to 0.5 % in Ireland (Table 1).

The distribution of mitochondrial control region sequences among collections of the conger eel from all locations refuted the null hypothesis of no genetic differences. The NMDS plots showed no geographically meaningful associations among samples (Fig. 2). Sample homogeneity measured by Snn could not be rejected (Snn = 0.1995, $P = 0.073$). All pairwise values of *Snn* were close to 0.5 (Table 2), indicating a panmictic population structure (Hudson 2000). Moreover, all pairwise Φ_{ST} comparisons were not significant, except those involving the sample from Mallorca (Table 2). After correction for multiple tests, all comparisons were non-significant except for the Mallorca South of Portugal pair. Results from the single genepool AMOVA returned a non-significant minute proportion of the overall genetic variability partitioned among locations ($\Phi_{ST} = 0.00352$; $P = 0.064$). Independently of the group scheme tested with SAMOVA, all hierarchical tests yielded non-significant results indicating that the bulk of the mitochondrial variation occurred within sampling sites (>98 %), with only up to 1.9 % among sites (Table 3).

The median-joining network (Fig. 3) depicted a "star-like" phylogeny with a large number of singletons closely related to the common central haplotype and showing no obvious phylogeographic structure.

There was no statistically significant correlation between genetic distance and geographic distance, as assessed by the Mantel test (slope 0.1, $R2 = 0.239$, $P = 0.183$). Genetic differentiation was clearly not linear with log-transformed geographic distances or untransformed geographic distances (data not shown). The unimodal distribution of sequence mismatches indicated no deviations from the expectations under a population growth-decline model (Fig. 4). The neutrality tests produced significant values (Tajima's $D = -2.55, P < 0.001$; and $Fs = -26.33, P < 0.0001$, and $R^2 = 0.0102, P < 0.0001$) indicating that the C. conger may have experienced a population expansion.

Otolith chemistry

Cores

Sr and Mg were the most abundant elements in otolith cores, followed by Mn and Ba. There were no significant

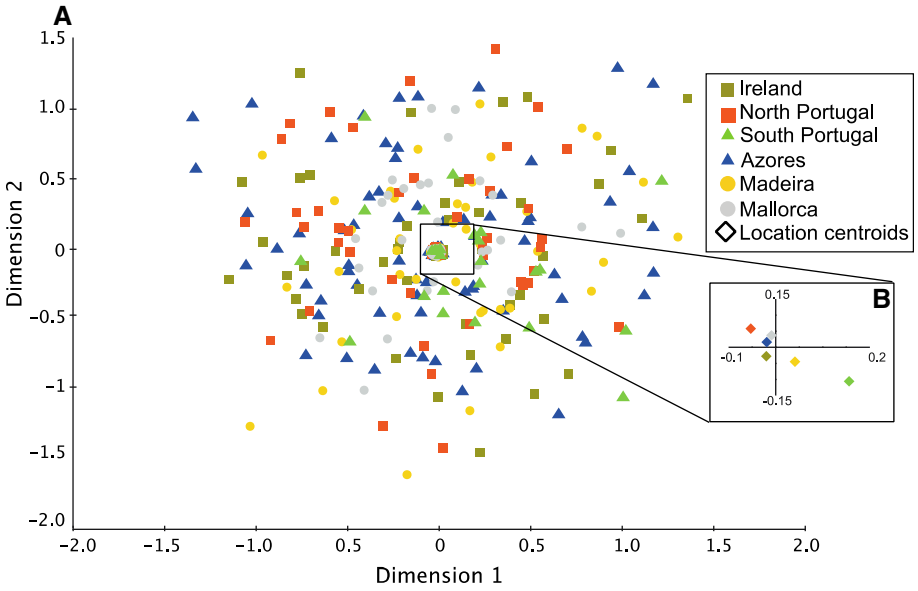


Fig. 2 Multidimensional scaling plots of pairwise Euclidean distances calculated from the matrix of corrected genetic distances of *Conger conger* mtDNA samples (**a**). Inset displays centroids only (**b**)

Table 2 Divergence among locations: Φ_{ST} values above diagonal, bold values after correction for multiple tests; *Snn* values below diagonal

	Ireland	Azores	Madeira	Mallorca	North Portugal	South Portugal
Ireland		−0.0014	−0.0060	0.0243	−0.0061	−0.0112
Azores	0.55		0.0002	0.0184	−0.0022	−0.0092
Madeira	0.49	0.52		0.0275	−0.0056	−0.0044
Mallorca	0.56	0.58	0.53		0.0261	0.0254
North Portugal	0.42	0.55	0.47	0.52		−0.0097
South Portugal	0.54	0.64	0.54	0.59	0.58	

differences among regions for Sr for both years (2006: ANOVA, $F_{3,76} = 1.797$, $P = 0.155$; 2007: ANOVA, $F_{3,76} = 2.401$, $P = 0.074$), although the Mallorca region showed the highest values for this element (Fig. 5). Mg showed some regional differences between Madeira and Azores in 2006 (Tukey's test, $P = 0.009$), but in 2007, there were no statistically significant regional differences (ANOVA, $F_{3,76} = 0.927$, $P = 0.432$). Although there were no statistically significant differences for *Mn* in 2006 (ANOVA, $F_{3,76} = 1.182$, $P = 0.322$), this element showed some regional differences between Mallorca and Azores in 2007 (Tukey's test, $P = 0.023$). Ba showed some significant regional variation in both years, with consistently higher values in Mallorca samples compared with N-Portugal (2006: Tukey's test, $P = 0.013$; 2007: Tukey's test, $P = 0.014$).

Multielemental composition (Sr, Ba, Mn and Mg) of otolith cores differed significantly among regions for both years (2006: MANOVA, Pillai's trace, $F_{12,225} = 0.507$, $P < 0.001$; 2007: MANOVA, Pillai's trace, $F_{12,225} = 0.338$, $P = 0.005$). Pairwise comparisons indicated no significant differences between samples from Azores and Portugal (Hotelling's T-square, $P = 0.629$) and also between Mallorca and Madeira (Hotelling's T-square, $P = 0.175$) for 2006 data; but in 2007, Mallorca samples were significantly different from the other regions (Hotelling's T-square, $P < 0.05$).

The canonical plot resulting from the QDFA procedure indicated a high overlap of sample data from all regions for both years (Fig. 6). Jackknifed classification accuracies were also generally low; mean values were 49 and 46 % for 2006 and 2007 years, respectively. Classification accuracies were relatively similar for all regions for both years, although Madeira and N-Portugal presented a slight low discrimination in 2006 (45 %) and 2007 (30 %), respectively (Table 4).

Edges

Significant regional differences in otolith edge chemistry were detected for all elements (Fig. 5), with the exception of Mg for the 2006 year (ANOVA, $F_{3,76} = 0.188$, $P = 0.904$). In 2007, Mg was, however, significantly lower in samples from the Azores compared with Mallorca (Tukey's test $P = 0.011$). Sr showed the highest values for Mallorca and Azores samples in 2006 (ANOVA,

Table 3 Results from SAMOVA analysis with different pre-defined numbers of groups. Group composition, per cent of variation, fixation indices and associated *P* values are reported (for the meaning of sampling codes see Fig. 1)

Number of groups (K)	Group composition	% Variation	Fixation	Index	*P*
1	(IR, MA, AZ, SP, NP, PM)				
	Among populations	0.35			
	Within populations	99.65	FST	0.00351	0.064
2	(IR, MA, AZ, SP, NP) (PM)				
	Among groups	1.9	FCT	0.01786	0.167
	Among populations within groups	−0.28	FSC	−0.00242	0.966
	Within populations	98.38	FST	0.01548	0.770
3	(IR, MA, SP, NP)(AZ)(PM)				
	Among groups	1.28	FCT	0.01278	0.066
	Among populations within groups	−0.55	FSC	−0.00561	0.991
	Within populations	99.28	FST	0.00724	0.075
4	(IR, SP, NP)(MA)(AZ)(PM)				
	Among groups	1.12	FCT	0.01120	0.051
	Among populations within groups	−0.63	FSC	−0.00641	0.988
	Within populations	99.51	FST	0.00486	0.074
5	(SP, NP)(IR)(MA)(AZ)(PM)				
	Among groups	1.29	FCT	0.01285	0.067
	Among populations within groups	−0.90	FSC	−0.00912	0.921
	Within populations	99.61	FST	0.00386	0.073

$F_{3,76} = 5.619$, $P = 0.002$) and 2007 (ANOVA, $F_{3,76} = 2.753$, $P = 0.048$), respectively. Ba showed significant regional variation in both years (2006: ANOVA, $F_{3,76} = 23.031$, $P < 0.001$; 2007: ANOVA, $F_{3,76} = 10.871$, $P < 0.001$), with consistently higher values in Mallorca. Portugal recorded the highest values in Mn for both years (2006: ANOVA, $F_{3,76} = 4.356$, $P = 0.007$; 2007: ANOVA, $F_{3,76} = 6.996$, $P < 0.001$).

Multielemental composition of otolith edges differed significantly among regions for both years (2006: MANOVA, Pillai's trace, $F_{12,225} = 0.756$, $P < 0.001$; 2007: MANOVA, Pillai's trace, $F_{12,225} = 0.887$, $P < 0.001$). Pairwise comparisons indicated significant differences between all region pairs for both years, with the only exception of Portugal and Madeira for 2006 data that showed no significant differences (Hotelling's T-square, $P = 0.023$).

The canonical variate plot resulting from the QDFA procedure depicted four separated groups, but with a significant overlap of Madeira and Portugal regions for both years (Fig. 6). Overall jackknifed classification accuracies were moderate, with means of 64 and 68 % for 2006 and 2007, respectively (Table 4). Individual classification accuracies were relatively similar for all regions in both years, although Madeira presented a relatively low per cent in 2006 (50 %).

All the elements displayed a general trend of lower concentrations for the edges compared to cores (Fig. 5). This relationship was statistically significant in the case of Mn and Mg for both years and for all regions; in the case of Ba also for both years, but only for Azores and Mallorca regions; and in the case of the Sr for Portugal and Mallorca, but only in the year of 2007 (Student's *t* tests, $P < 0.05$).

Temporal Stability

Interannual differences in multielemental signatures detected for the Azores (MANOVA: $F_{4,35} = 0.325$, $P = 0.007$), Portugal (MANOVA: $F_{4,35} = 0.264$, $P = 0.027$) and Madeira (MANOVA: $F_{4,35} = 0.501$, $P < 0.001$) regions were due to variation of Ba, Mg and Mn, respectively. However, for Mallorca samples, the multielemental differences detected between years (MANCOVA: $F_{4,35} = 0.540$, $P < 0.001$) were due to all elements with the exception of the Mn (Student's *t* test, $t_{38} = 0.227$, $P = 0.822$) (Fig. 5). Univariate tests shows that Sr content in the edge of otolith was lower in the year 2007 comparatively to 2006, although this difference was only statistically significant in Mallorca (Student's *t* test, $t_{38} = 3.238$, $P = 0.002$). Ba showed an opposite tendency, that is, higher values in 2007, but only statistically significant for Mallorca (Student's *t* test, $t_{38} = -2.507$, $P = 0.017$) and Azores (Student's *t* test, $t_{38} = -3.645$, $P < 0.001$). Mg was also statistically significant higher in Mallorca (Student's *t* test, $t_{38} = -3.857$, $P < 0.001$) and Portugal (Student's *t* test, $t_{38} = -2.444$, $P < 0.031$) samples from 2007. Madeira samples were statistically significantly lower in Mn in 2007 (Student's *t* test, $t_{38} = 4.098$, $P < 0.001$).

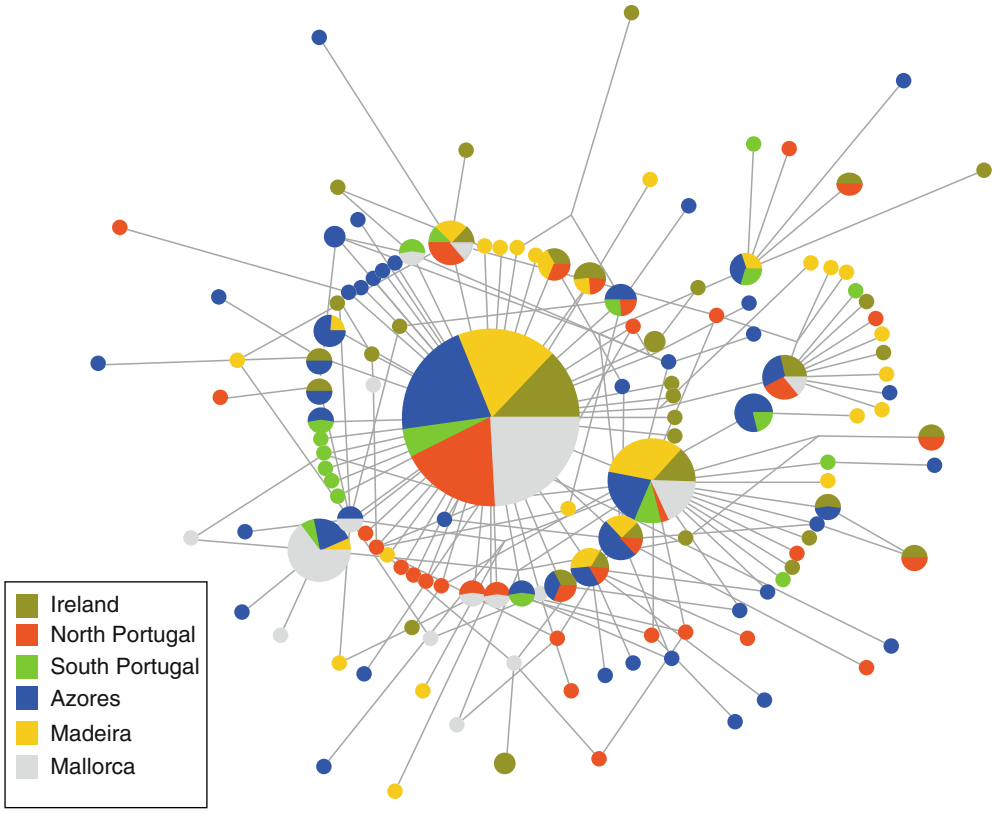


Fig. 3 Median-joining network representing the genealogical relationships of the 156 mtDNA haplotypes found in the 347 individuals of *Conger conger* sequenced for the study. *Circle* size is proportional to haplotype frequency. Differences between haplotypes are proportional to branch length. Median-joining vectors (hypothesized sequences, extinct or not sampled haplotypes) are represented by *black lozenges*

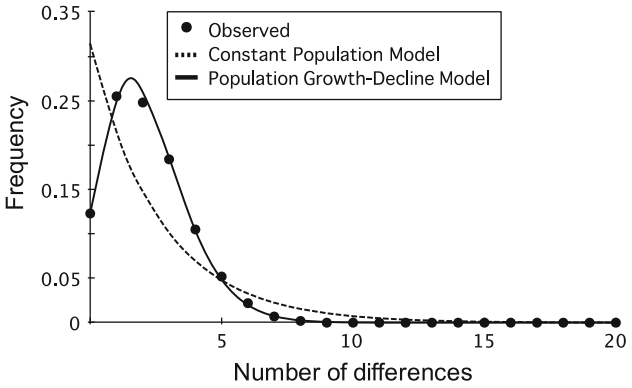


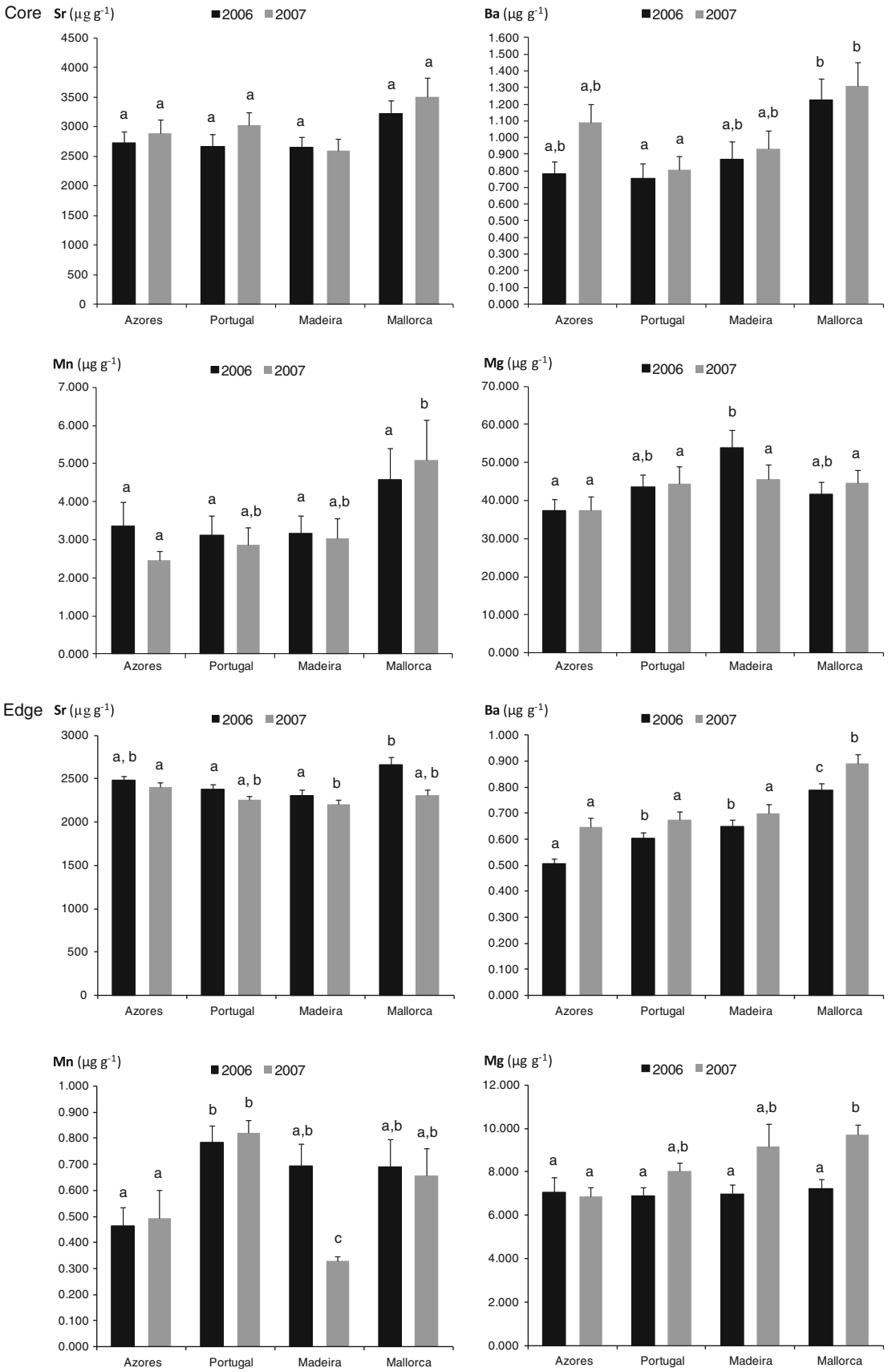
Fig. 4 Mismatch distribution. *Conger conger* control region. Observed distribution of pairwise differences (*dots*) plotted against the expected *curve* predicted in the case of past population growth-decline model (*dark line*) and the constant population model (*dashed line*)

Discussion and conclusions

Genetics

Prior to dissecting results, we must acknowledge the implicit caveat in the interpretation of data derived from a single mitochondrial marker. This implies that only the maternal lineage history is being under scrutiny and also that parameter estimates are very much dependent on the extent that this locus is a true reflection of the population history. Having these limitations in mind, conclusions should be addressed with caution.

The mtDNA control region genealogy indicates that *C. conger* populations consist of a highly abundant haplotype shared among all locations, and a geographically widespread assemblage of mostly unique haplotypes separated by a small number of mutations with no obvious geographic structure. Similar results have already been reported in preliminary and sample size-limited MtDNA study using conger eel larvae (Correia et al. 2006b). A number of different analyses return the same conclusion of no differentiation among locations: (1) one genepool AMOVA, resulted in a small and non-significant Φ_{ST} value; (2) hierarchical AMOVA with different group arrangements did not yield significant results and the bulk of the mtDNA variation occurred within sampling sites; (3) *Snn* statistic revealed a non-significant association between sequence similarity and geographic location, suggesting that migration between neighbouring populations is not


Core
Sr (μg g-1)
2006 2007
Azores Portugal Madeira Mallorca
Ba (μg g-1)
Mn (μg g-1)
Mg (μg g-1)
Edge
Sr (μg g-1)
Ba (μg g-1)
Mn (μg g-1)
Mg (μg g-1)

◀ **Fig. 5** Elemental concentrations (mean ± SE) recorded in the core and edge of the otoliths in *Conger conger* from juvenile fishes collected in four sampling locations (Azores, N-Portugal, Madeira and Mallorca) during 2006 and 2007. Concentrations are given in μg element μg^{-1} calcium. The locations marked with the *same letter* above the *error bars* are not significantly different from each other ($P > 0.05$)

restricted. Mallorca seems to display some degree of differentiation, only detected by pairwise Φ_{ST} values. This last result may be explained by particular oceanographic conditions of the Mediterranean and its Atlantic most adjacent site rendering asymmetrical exchange of individuals. It is known that the permeability of the Atlantic–Mediterranean barrier acts differently on different species (e.g. Patarnello et al. 2007; Galarza et al. 2009).

The conger eel may have a large effective population size not sensitive to genetic drift, or it may have suffered a recent population expansion, as shown by the haplotype network. Both scenarios are supported by several lines of evidence: high levels of genetic diversity in the conger eels, a lack of differentiation over a large spatial scale (i.e. about 3,500 km from the most northern location to the Mediterranean); star-shaped haplotype network, unimodal mismatch distribution and significant neutrality statistics (D, F_s and R_2). However, both possibilities are difficult to disentangle on the basis of genetic parameters alone. Paleoclimatic data show that during the last glacial maximum (LGM) seawater at the latitude of Ireland was covered with ice, and the polar front reached the western shores of the Iberian Peninsula (Dias et al. 1997). Therefore, it is plausible that the Ireland population results from a recent colonization, if seawater temperature was affected at the depth ranges that the conger eels live or if affected the abundance of its prey. However, the genetic make-up of the sample from Ireland does not show particular signs of recent population expansion, such as the decrease of genetic diversity, compared with the more southerly locations. One can postulate that most individuals have moved further south as a consequence of the marked temperature decrease, and when conditions were re-established, all locations were re-colonized. This scenario is more compatible with the results, as when all locations are taken as a whole, the population expansion signal becomes evident. The lack of differentiation may be due to an insufficient lack of time since the LGM for lineage sorting.

The lack of geographic structure in marine organisms over a wide range has been reported in several species with different life history traits, including fish (e.g. Ely et al.

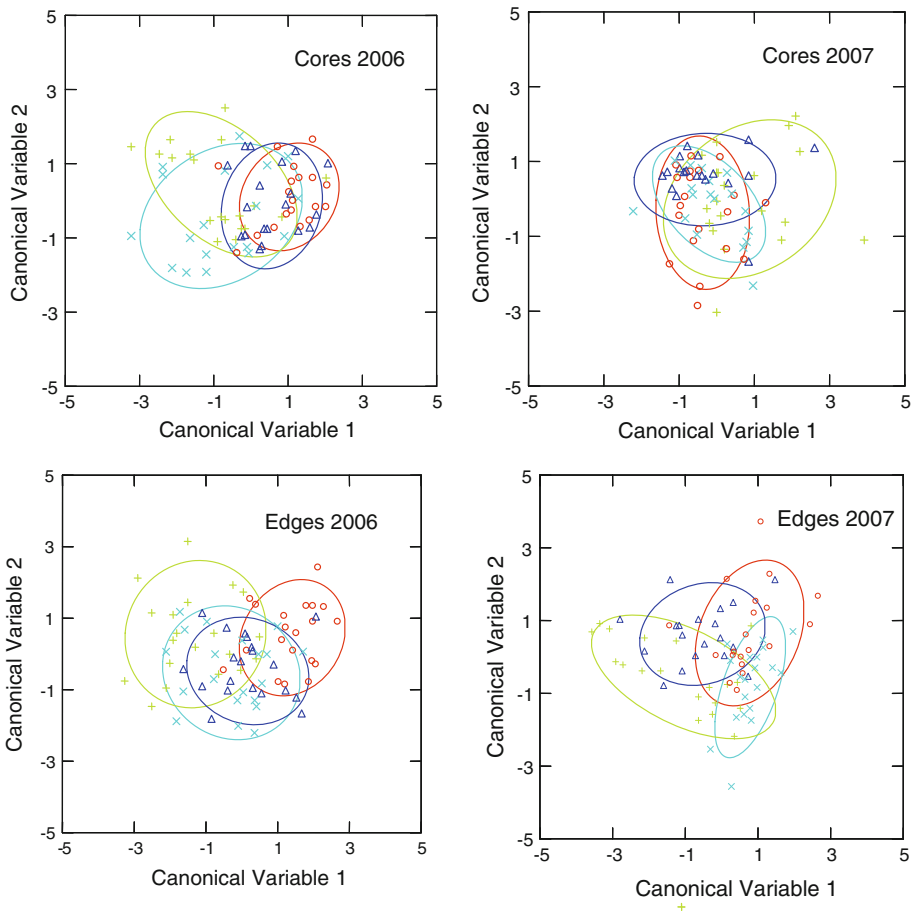


Fig. 6 Canonical variate plots displaying the differences of chemical elements concentrations imprinted in the otolith's core and edge of *Conger conger* juveniles collected in the Azores (*circle*), Madeira (*times*), Mallorca (*plus sign*) and N-Portugal (*triangle*). *Ellipses* represent 95 % confidence intervals around each locations data, and *data points* represent individual fish. The two canonical variables explain at least 90 % of the existing variation

Table 4 Jackknife classification matrix of *C. conger* specimens based on otolith's core and edge fingerprints used in quadratic discriminant function analyses for years 2006 and 2007, respectively

	Predicted location				
	Azores	N-Portugal	Madeira	Mallorca	% Correct
Cores					
2006					
Location of collection					
Azores	10	6	2	2	50
N-Portugal	7	10	2	1	50
Madeira	3	3	9	5	45
Mallorca	2	4	4	10	50
Total	22	23	17	18	49
2007					
Location of collection					
Azores	10	3	5	2	50
N-Portugal	6	6	5	3	30
Madeira	4	4	10	2	50
Mallorca	2	2	5	11	55
Total	22	15	25	18	46
Edges					
2006					
Location of collection					
Azores	15	2	2	1	75
N-Portugal	2	13	4	1	65
Madeira	2	4	10	4	50
Mallorca	0	2	5	13	65
Total	19	21	21	19	64
2007					
Location of collection					
Azores	14	2	2	2	70
N-Portugal	2	13	1	4	65
Madeira	5	1	14	0	70
Mallorca	2	1	4	13	65
Total	23	17	21	19	68

2005; Martinez et al. 2006; Klanten et al. 2007). Moreover, previous information revealed that American and European eels although having both broad geographic distributions, they mature and spawn in a single, or perhaps several areas, in deeper oceanic waters yet displaying intraspecific near-panmixia (Als et al. 2011). This peculiar life history pattern suggests a mechanism for maintaining large-scale genetic homogeneity even when adult populations in inshore waters are segregated, which is not the case of the conger eels.

Connectivity among populations of relatively sedentary benthic marine organisms depends mainly on the successful transport of larvae and/or pelagic juveniles to appropriate habitats by ocean currents.

North-eastern Atlantic currents can act as a vehicle for the transport of conger eel larvae at regional scales (Correia et al. 2002), even if some movement restriction exists between Mediterranean and NE Atlantic populations. The trend of the major currents in the North-eastern Atlantic Ocean with a general direction towards the North could imply that with a passive drift larval mode, as Tsukamoto (2006) and Bonhommeau et al. (2008) suggested, the populations could be prone to a source (south)–sink (north) metapopulation effect, where northern locations would recruit from the southern locations. However, predictions for the source–sink model including (1) the existence of significant differences in genetic diversity between sub-populations (Tero et al. 2003); (2) absence of unique haplotypes in sink populations because individuals originate in source populations and (3) sink populations will have the representatives of the most abundant and older haplotypes of the source populations (Rex et al. 2005) do not hold. The absence of genetic differentiation throughout sample locations may imply that the conger eel larvae can

eventually exert some degree of swimming control against dominant currents moving vertically (Leis 2006) and escaping currents transport regardless their pelagic larval duration (PLD). Alternatively, the lack of observed differentiation between locations may result from migratory displacements of adults following prey movements. This last statement seems, however, unlike since otolith data indicate that site fidelity of adults is high.

Our results are inconclusive regarding the number and location of the spawning areas. In fact, the genetic identification of a spawning area is only possible if the species displays spawning-site fidelity (i.e. philopatry). The tendency of individuals to breed in their natal range can reduce gene flow, if there is more than a single spawning area. In some instances, isolation might promote local adaptations and drive populations to differentiate from one another (Dittman and Quinn 1996). This feature, which is a trademark of species in the order Anguilliformes, is not supported in the conger eel. If this philopatric behaviour is not actually present in the conger eel then the genetic identification of a spawning area is not realistic, and the previous claims of two spawning areas cannot be genetically substantiated.

Otoliths

In marine species, molecular assignment-based methods may be limited by high connectivity, high gene flow and resultant low genetic structure of fishes (Manel et al. 2005). Otolith chemistry does not require genetic diversity among populations and have proven successful in resolving dispersal and mixture separation in high gene flow systems where environmental heterogeneity exists (Campana et al. 1999). These techniques are based on the fact that otoliths are metabolically inert structures that grow continuously throughout a fish's life time and that some non-physiologically regulated elements are integrated into the calcium carbonate matrix based on ambient water concentrations or physical environmental conditions (Campana 1999). Elements such as Sr, Ba, Mn and Mg are commonly used in the studies of stock differentiation in fishes (Thresher 1999; Gillanders 2002; Bergenius et al. 2005), while elements under strong physiological regulation (e.g. Na, K, S, P and Cl) appear to be of limited value (Thresher et al. 1994; Proctor et al. 1995). The incorporation of trace elements is a complex process that remains poorly understood, and to date, only a few studies have been able to clearly link incorporation of some elements (e.g. Sr and Ba) into otoliths with specific exogenous environmental factors (e.g. Bath et al. 2000; Campana and Thorrold 2001). However, knowledge of the underlying causes of variation in otolith elemental chemistry is not necessary for the use of measured differences as an aid in delineating fish stocks and population structure (Thresher 1999). The success of these methodologies is dependent on the observation of distinct differences in otolith chemistry that support the hypotheses of population structure and are of limited use when no variation is detected, which can often occur where spatial environmental variance is low irrespective of population separation (Bradbury et al. 2008).

Five elements (Ca, Sr, Ba, Mn and Mg) were present at levels above the detection limits both in the core and edge portions of the conger eel otoliths. In general, the conger eel otoliths showed some natural variability of the elements examined across sampling years and regions, and in general, mean otolith concentrations of elements such as Ba, known to be primarily influenced by ambient concentrations, were low consistent with expectations for an oceanic species.

The aim of the otolith core analyses was to investigate the potential for discrete spawning and replenishment pathways for different regional benthic conger populations. An ablation diameter of 100 μm in the otolith core would represent the leptocephalus larval stage of less than 4 months of age (i.e. pre-metamorphic stage), which is typically found on the continental slope (Correia et al. 2002). The observation that otolith core compositions are relatively homogenous between regional samples, with the exception of Mallorca, suggests that the benthic conger in each region were derived from a single spawning area or alternatively they may have been derived from multiple spawning areas, but experienced similar environmental conditions during the leptocephalus stage. The higher mean Ba and Mn in the otolith cores of Mallorca, however, lends some support to the hypothesis that these fish were derived from a separated replenishment source within the Mediterranean that the Atlantic fish (i.e. separate stock idea: Mediterranean vs NE Atlantic). However, this issue should be interpreted with caution because of the limitations of having Mallorca as only site within the Mediterranean.

By ablating the outer surface (otolith edges) of the otolith, the most recently produced material incorporating a period prior to capture is analysed. For a 100 μm ablation, we estimate that this period of time represents, at least, the 3 months of life prior to capture r [i.e. otolith growth of 1.20 μm day^{-1}, Correia et al. (2003)]. MANOVA and DFA analyses show the differences among regions for otolith edge analysis. All fish samples were accurately discriminated, and reclassification success was reasonably high in Azores for both years. The most notable differences observed in otolith edge chemistry among regions, similar to cores, was for Mallorca samples that had slightly higher mean Ba levels, a pattern that was also similar across years. This could be related to higher ambient Ba levels in the enclosed Mediterranean waters compared with the oceanic Atlantic regions. For many elements, coastal, estuaries and

inland waters have generally much higher concentrations, sometimes by orders of magnitude, than that of the open ocean (Balls et al. 1993; Davis 1993). Inshore areas, for instance, have been found to be markedly enriched with Mn and Ba relative to adjacent offshore areas that are thought to reflect the influence of freshwater inflow from surrounding rivers (Balls 1986). In our study, discrimination was largely driven by differences in Ba (low at Azores and high for Mallorca samples for both years) and was important for both the edge and otolith core comparisons. Otolith Ba is a known reliable indicator of water chemistry, with variation often linked to terrestrial sources and upwelling (Patterson et al. 2004; Elsdon and Gillanders 2006; Hamer et al. 2006). It would be expected higher Ba concentrations in Mediterranean than in offshore Atlantic locations (Dehairs et al. 1987). The comparisons of the otolith edge chemistry suggest that congers are likely to show resident behaviour, at least at the scale of months, in juvenile habitats. The data support a previous work that suggests that benthic juveniles show high fidelity to regional growing areas (Correia et al. 2011). However, in the former work, whole otolith isotopic (oxygen and carbon) composition proved to be a better location-specific environmental proxy for conger (mean classification accuracy rate of 88 %).

Otolith elemental compositions may not be temporally stable between year-classes, since interannual variation in water chemistry can affect the chemical composition of otoliths even if fish remain in the same locations (Milton et al. 1997). Some studies that have examined the temporal stability in otolith chemistry indicate that stock-specific signatures vary among years (Campana et al. 2000; Hamer et al. 2003; Swearer et al. 2003). Temporal variation in otolith multielemental tags can have significant implications for their application to retrospective determination of adult origins and can confound inferences of population structure (Gillanders 2002). Temporal stability of the otoliths signatures was examined by investigating the elemental properties of otolith's edge sections from all geographic regions across 2 years of sampling (2006 and 2007). Interannual variation across the two consecutive years for the otolith analysis edges suggested limited variation among single elemental ratios at most regions, with the exception of Mallorca. However, in the case Mallorca, the most informative element for discriminating this region, Ba, was consistently higher than the other regions across years. Furthermore, gross differences between the otolith core and edge elemental concentrations are at least partly related to endogenous/ontogentic differences (Patterson et al. 2004). Similar to our observations for conger, and for other species, it has been shown that the core can be highly enriched in manganese (Mn) and magnesium (Mg) relative to adjacent regions of the otoliths (Brophy et al. 2004; Ruttenberg et al. 2005; Warner et al. 2005). However, changes in water temperature, experienced for instance during the different conger eel life stages, or habitats colonization, might also lead to characteristic changes in otolith element composition (Marohn et al. 2011).

Summary

Both the molecular and otolith approaches support the hypothesis of a broad-scale dispersal of larvae, with limited connectivity between juvenile fish populations at large spatial scales, although the existence of one or multiple spawning areas for the species remains uncertain. Based on the results of this study, we propose a model of population structure for conger eel in the NE Atlantic/Mediterranean that involves significant mixing of larval stages derived from an unknown number of spawning areas, with local site fidelity of benthic life stages. As such, replenishment of localized fisheries could be linked to reproduction by multiple distant populations, although the population in the Mediterranean may be more dependent on local reproductive sources than those in the Atlantic. Determining the nature and consistency of such spatial connections is essential to understanding resilience of each local fishery to fishing pressure, but may require alternative approaches such as larval dispersal modelling and the scoring of highly variable nuclear markers. Future genetic studies could also focus on the analyses of leptocephali recruiting to particular areas in relation to genetic features of adults from the same areas, and comparisons of the level of the genetic variation among samples of leptocephali and benthic juveniles in different regions of the NE Atlantic/Mediterranean. This may provide further detail on whether local benthic populations are replenished solely by settlement of leptocephali or a combination of leptocephali and active dispersal of older benthic stages. Furthermore, if there are multiple spawning stocks with different spawning grounds, spawning seasons and/or migration routes, recruitment of leptocephali collected from different regions may show different dynamics, and the leptocephali may display different life history traits, such as age and growth rate, or stage durations within their otolith microstructure (Kimura et al. 2004; Ma et al. 2008). Additional strategic studies of juvenile and larval otolith microstructure are also likely to play an important role in further resolving meta-population structure of conger eel in the NE Atlantic/Mediterranean.

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