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Evidence for genetic differentiation in the European conger eel *Conger conger* based on mitochondrial DNA analysis

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ABSTRACT: The European conger eel *Conger conger* is an important marine benthic fish in the North-East Atlantic and represents a valuable fishery resource. However, little is known about its reproductive biology. In an attempt to gain a better understanding of the conger eel population structure, mitochondrial DNA (mtDNA) sequences were examined. A region with 432 bp of the control region of the mtDNA was sequenced from 40 individuals from six different locations around the central and eastern North Atlantic Ocean. Thirty variable positions defined 28 distinct haplotypes. The average sequence difference within samples (1.3–4.2%) was comparable to those between samples (1.4–3.6%). MtDNA sequence-based statistical tests showed significant geographic differentiation between some local population samples, suggesting that the conger eel does not comprise a single panmictic population. However, given our sample sizes, these preliminary results should be interpreted with caution and more individuals from more sites, including the Mediterranean Sea, should be analyzed in detail. The genetic variability detected in this study is an initial step to elucidate the genetic background of the conger eel population structure.

KEY WORDS: ***Conger conger*, genetic variation, mitochondrial DNA, population structure.**

INTRODUCTION

The European conger eel *Conger conger* (Linnaeus, 1758) is a common fish of the North-East (NE) Atlantic, Mediterranean and western Black Sea marine ecosystems.[1] Despite being a geographically widespread species and an important fisheries resource, there is relatively little known about the reproductive biology, ecology and migratory behavior of *C. conger*. The information obtained so far is not enough to elucidate the entire life cycle of this fish, namely the location of the spawning ground(s), the duration of the leptocephalus stage and the coastal recruitment larval mechanism, which is essential for rational stock assessment and management.

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Congers are important commercial and recreational fishing species of the central and eastern Atlantic, being caught primarily with bottom trawl, hook and line gear.[2] World total catch reported for this species to FAO for 2001 was 14 238 t. The countries with the largest catches were France (5225 t) and Portugal (3311 t). From previous studies, several spawning locations have been suggested for the conger eel, although naturally spawning conger eels have not yet been observed and reports about the capture of maturing specimens are scarce.[3,4] The absence of males and of ripe or spent females in the coastal inshore waters of the Atlantic suggests that sexual maturation in conger eels occurs during their migration towards their deep-sea spawning areas.[5,6] The Mediterranean Sea is thought to be the spawning area for European conger eel, indicated by catches containing small, 9–20 mm, leptocephali.[7] Several authors also suggested that conger eels spawn once during the

summer, at great depths (3000–4000 m), in the NE Atlantic, between Gibraltar and the Azores archipelago.[8–11] However, they did not mention neither references nor data to support their assumptions. Until now, the only well-known spawning ground for this species is in the waters south of the Island of Sardinia, in the Mediterranean Sea.[3] This location is supported by the length and age of developing leptocephali collected in the North and Central Atlantic Ocean.[12] Although the spawning area of *C. conger* in the North Atlantic is still hypothetical, the young age of leptocephali recently collected in the Azores, and the knowledge of the water current systems of the NE Atlantic, suggest that this species might spawn in an area closer to the Azores.[13,14] However, further information on the geographic distribution, age, growth and genetic structure of *C. conger* leptocephali is necessary in order to determine the actual spawning ground(s) of this population and its migration pathways to the European and North African coasts.

The population structure and possibility of multiple spawning grounds has been studied using mitochondrial DNA (mtDNA) analysis on several oceanic fish species, particularly in *Anguilla*[15–17] and *Conger* species.[18] Until now, these reports did not indicate significant genetic differentiation between location samples, suggesting an intensive gene flow between geographically distant areas. However, genetic evidence for distinct geographic populations as been recently found in one tropical eel species.[19]

In the present study, genetic variation of the European conger eel leptocephali collected from different locations around the Central and Eastern Atlantic Ocean is investigated using mtDNA sequencing. Our goal was to provide additional information to better understand the spawning ground, ecology, genetic structure and migration of conger eel leptocephali in the ocean.

MATERIALS AND METHODS

Fish sampling

A total of 40 *C. conger* leptocephali, already used in oceanic larval aging and migration studies,[13,14,20,21] were collected from six locations in the Central and Eastern Atlantic Ocean: Azores ($n = 13$; two sample points), North Portuguese Continental Slope ($n = 8$), Bay of Biscay ($n = 4$; two sample points) and Minho River ($n = 15$) (Fig. 1 and Table 1).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from ethanol-preserved muscle tissue of each individual of *C. conger* using proteinase k digestion, ammonium acetate separation of proteins and cold-ethanol DNA precipitation.[22] A 5′- end fragment of the mtDNA control region was first amplified by the polymerase chain reaction (PCR) with a pair of primers designed for this study, L15013 (or CR1) (5′-CGGTTTTGTAATCCGAAG-3′) and H15590 (5′-ATAGGAACCAGATGAAAG-3′). These primers were designed based on the transfer RNA (tRNA)-Thirosine and control region nucleotide sequences from the mitochondrial genome of *Conger myri-*

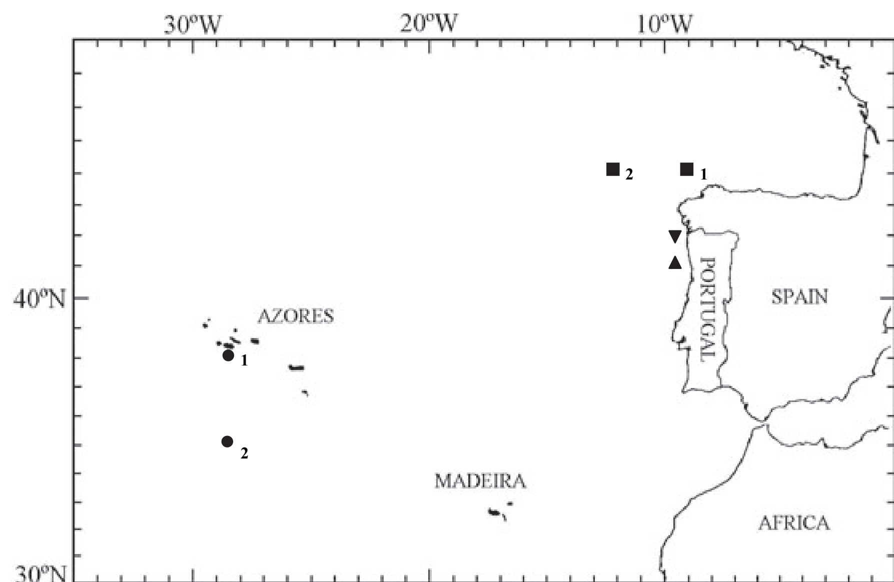

Fig. 1 Sampling locations of the 40 leptocephali collected in the central and eastern Atlantic Ocean. Numbered (●), Azores 1 and 2; numbered (■), Bay of Biscay 1 and 2; (▼), Minho River; (▲), North Portuguese Continental Slope. See also Table 1.

Table 1 Location (area and geographic position), sampling date, number (*n*), total length (TL), developmental stage (PL premetamorphic and ML metamorphic leptocephali), and age of the leptocephali used in this study (data extracted from Correia *et al.*;[13,14,20] Antunes and Correia[21])

Area	Position	Capture date	*n*	TL range (mm)	Stage	Age (days)
A1	38°00′N/29°00′W	October 1999	10	51.5–125.5	PL	76–275
A2	35°00′N/29°00′W	August 2000	3	49.0–76.0	PL	56–93
MR	42°00′N/9°00′W	February 1999	15	108.0–136.0	ML	Unknown
NPCS	41°00′N/9°00′W	May/June 1989	8	89.0–123.0	PL	250–385
BB1	44°00′N/9°00′W	June 1989	2	102.0–125.0	PL	253–301
BB2	44°00′N/12°00′W	August 2000	2	92.9–96.0	PL	240–260

A1, Azores 1; A2, Azores 2; MR, Minho River; NPCS, North Portuguese Continental Slope; BB1, Bay of Biscay 1; BB2, Bay of Biscay 2. See also Figure 1.

aster, respectively.[23] A new internal reverse primer CR2 (5′-TTGTCCCTGATTATCAATAAAC-3′) was designed based on a mtDNA sequence of *C. conger*, and used in all subsequent amplification reactions. The PCR amplifications were carried out in a T3 Thermocycler (Biometra, Goettingen, Germany) under the following conditions: initial 3 min denaturation at 94°C, 35 alternating cycles of 30 s at 94°C for denaturation, 30 s at 59°C for annealing and 30 s at 72°C for extension. A final extension was made during 2 min at 72°C. A total of 2 μL of each PCR product was used for 2% agarose gel electrophoresis for verifying the amplified fragment length with a standard size marker (Marker 5, Eurogentec, Southampton, UK). Band visualization was made using ultraviolet lights after a bath in ethidium bromide. The rest of the PCR products were purified by enzyme cleaning (Exosap-IT, Amersham, Piscataway, NJ, USA). Purified double-stranded DNA was used as template for automated sequencing reactions performed using a T3 Thermocycler and run on an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The primers used for sequencing were the same as those for PCR amplification. The nucleotide sequences reported in this paper are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB183719 to AB183746.

Data analysis

All sequences were aligned using the sequence alignment editor software Bio Edit 5.0.9,[24] with subsequent refinement by eye by means of the Chromas 2.23 program (Technelysium, Tewantin, Australia). Average sequence differences within and among samples were calculated from pairwise sequence differences obtained by MEGA 2.1.[25] Insertions and deletions were treated as the fifth character and no mutations were weighted for sequence difference estimation. Two distinct classes of test statistics, Hudson's haplotype (*Hst*) and sequence-based statistical tests (*Kst**, *Z** and *Snn*), were applied to detect genetic differentiation of subpopulations.[26,27] The haplotype statistic (*Hst*) is based on haplotype frequencies in the samples and does not matter whether two haplotypes differ by one or hundreds of nucleotides. The sequence-based statistics (*Kst**, *Z** and *Snn*) use the information on the number of nucleotide differences between haplotype sequences. This class of statistics is a more appropriate method when the haplotype diversity is very high and the sample sizes are small.[26] The *Snn* (nearest-neighbor statistic), for instance, appears to represent the most powerful test for detecting genetic differentiation in this case.[27] Analyses were performed between locations with a sample size ≥8 (Azores 1, Minho River and North Portuguese Continental Slope) using DnaSP 4.0 with 1000 permutations.[28] Genetic variation within each population was quantified by mtDNA haplotype diversity (*h*) and nucleotide diversity (π)[29] using MEGA 2.1.[25] A maximum parsimony network was constructed by using TCS 1.6.[30] This program implements Templeton's algorithm,[31] and provides a 95% plausible set for all haplotype linkages in an unrooted tree. Demographic population inferences were assessed by analysis of mismatch distributions of pairwise differences among all haplotypes[32] using DnaSP 4.0.[28]

RESULTS

MtDNA variation

The aligned mtDNA sequence data consisted of part of the control region containing 432 base pairs (bp) (Fig. 2). A total of 30 variable positions and 28 haplotypes were found in the 40 individuals. Of those variable positions, one is an insertion (haplotype 21, position 270) and two are deletions (haplotypes 14 and 17, positions 38 and 64,

ACACGACA**T**ATGGACATATAACACA 25
AATAACTGTATA**A**TTACATAAATGA 50
TATACAACCTACC**T**ATGTGTATGTT 75
GTATTTCAC**T**TATGTAATATATACA 100
TAA**T**ATGTAAATACAACATACACCT 125
ATGTAATCA**A**TACATAACATGTATT 150
GATTACAAAGGTGTATGTAAA**CT**A**T** 175
CTG**T**AAATGTAAACGTTACATACCC 200
AT**A**CCAGATTTTAAAT**C**AATGAAGT 225
ATAACATA**C**ATATTAATG**G**ACC**TC**T 250
AATA**AC**ATTAATAATGA**C**T**T**AAAGA 275
A**C**TGCAGCAAATAGTATTAAA**T**CTA 300
TAAATATTGGACA**G**T**G**AT**T**CATGAT 325
TTGAATGA**T**AAATGCA**C**AAACCAAG 350
TTTCCATGAAGAAT**G**ACATTAACTG 375
GACCTAAACCAGCAT**G**CGCAGTAAG 400
AAACCACCAACCAGCACAA**T**TCAGG 425
AAAATAT

Fig. 2 Nucleotide sequence of a 432 bp fragment of the mtDNA control region from one individual collected in the Minho River. Bold letters indicate the variable sites in 28 haplotypes. DDBJ/EMBL/GenBank accession number AB183739.

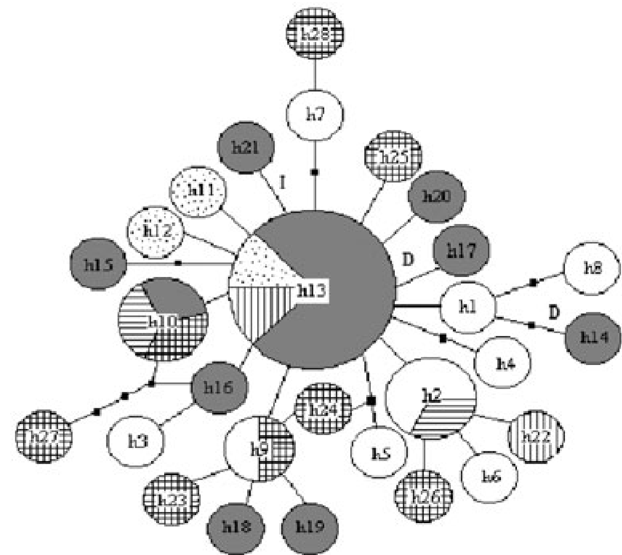

Fig. 3 MtDNA haplotype network estimated with the 95% statistical limits of parsimony using the algorithm in Templeton *et al.*[31] Haplotypes are represented in circles with their size proportional to their frequencies. Each (■) on the lines connecting circles represents an additional mutational event; letters D and I represent deletions and insertions, respectively; Azores 1, white area; Azores 2, dotted; Minho River, gray; Bay of Biscay 1, horizontal lines; Bay of Biscay 2, vertical lines; North Portuguese Continental Slope, square-hatching.

respectively). The frequency of variable sites in this part of the mtDNA control region was 6.9% (30/432 bp). Haplotype diversity (h) and nucleotide diversity (π) were highest in the Bay of Biscay (1.000 ± 0.500) and North Portuguese Continental Shelf (0.010 ± 0.006) samples, respectively (Table 2). The average sequence divergence and its standard error in the overall data was 2.464 ± 0.516%. Average sequence divergences in overall data within samples varied from 1.333 ± 0.906% (Azores 2) to 4.214 ± 1.060% (North Portuguese Continental Slope), and those between samples ranged from 1.400 ± 0.508% (between Minho River and Azores 2) to 3.650 ± 0.835% (between North Portuguese Continental Shelf and Azores 1) (Table 3).

Haplotype network

The geographic distribution of haplotypes was non-random and some samples showed private haplotypes (Fig. 3). Haplotypes h1, h3–h8, h11, h12, h14–h21, h22, and h23–h28 were only present at Azores 1, Azores 2, Minho River, Bay of Biscay 1, and North Portuguese Continental Slope, respectively. The most frequently observed haplotype (h13, $n = 8$) was not detected in Azores 1, Bay of Biscay 2 and North Portuguese Continental slope (Table 2). The network shows a star-like pattern, typical of a recent expansion, with a more common ancestral haplotype (h13) and multiple rare alleles. The mismatch distribution analysis showed that the observed and expected (under population growth scenario) pairwise nucleotide differences produce a unimodal distribution, suggesting population expansion.

Genetic differentiation analysis

The results of the haplotype and sequence-based statistics (Table 4) show the existence of significant genetic differentiation between local population samples. Azores (A1) and North Portuguese Continental Slope (NPCS) subpopulations are not genetically differentiated for all statistics ($P \geq 0.05$), but there is a significant genetic differentiation between the Minho subpopulation and the other two subpopulations ($P < 0.05$). All sequence-based statistics gave similar results, except for the Snn test that only showed a significant genetic differentiation between A1 and NPCS.

Table 2 Absolute and relative frequencies of the haplotypes, number of haplotypes per site (n_h), number of individuals sequenced (n_t), haplotype (h) and nucleotide (π) diversity (± standard deviation). See also Figure 3

Site/haplotype	A1	A2	BB1	BB2	MR	NPCS	Total (Abs.)	Frequency (%)
h1	1	0	0	0	0	0	1	2.5
h2	2	0	0	1	0	0	3	7.5
h3	1	0	0	0	0	0	1	2.5
h4	1	0	0	0	0	0	1	2.5
h5	1	0	0	0	0	0	1	2.5
h6	1	0	0	0	0	0	1	2.5
h7	1	0	0	0	0	0	1	2.5
h8	1	0	0	0	0	0	1	2.5
h9	1	0	0	0	0	1	2	5.0
h10	0	0	0	1	1	1	3	7.5
h11	0	1	0	0	0	0	1	2.5
h12	0	1	0	0	0	0	1	2.5
h13	0	1	1	0	6	0	8	20.0
h14	0	0	0	0	1	0	1	2.5
h15	0	0	0	0	1	0	1	2.5
h16	0	0	0	0	1	0	1	2.5
h17	0	0	0	0	1	0	1	2.5
h18	0	0	0	0	1	0	1	2.5
h19	0	0	0	0	1	0	1	2.5
h20	0	0	0	0	1	0	1	2.5
h21	0	0	0	0	1	0	1	2.5
h22	0	0	1	0	0	0	1	2.5
h23	0	0	0	0	0	1	1	2.5
h24	0	0	0	0	0	1	1	2.5
h25	0	0	0	0	0	1	1	2.5
h26	0	0	0	0	0	1	1	2.5
h27	0	0	0	0	0	1	1	2.5
h28	0	0	0	0	0	1	1	2.5
n_h	9	3	2	2	10	8		
n_t	10	3	2	2	15	8		
h	0.978 ± 0.054	1.000 ± 0.272	1.000 ± 0.500	1.000 ± 0.500	0.857 ± 0.090	1.000 ± 0.062		
π	0.007 ± 0.005	0.003 ± 0.003	0.005 ± 0.006	0.005 ± 0.006	0.004 ± 0.003	0.010 ± 0.006		

Table 3 Average number (± standard error) of pairwise differences in mtDNA sequences within and between six sample locations of the European conger eel.

	MR	NPCS	A1	A2	BB1	BB2
MR	1.448 ± 0.448	2.817 ± 0.698	2.327 ± 0.621	1.400 ± 0.508	1.733 ± 0.707	1.667 ± 0.698
NPCS		4.214 ± 1.060	3.650 ± 0.835	2.917 ± 0.741	3.125 ± 0.878	2.875 ± 0.846
A1			3.133 ± 0.906	2.367 ± 0.663	2.400 ± 0.803	2.400 ± 0.804
A2				1.333 ± 0.906	1.667 ± 0.780	1.667 ± 0.788
BB1					2.000 ± 1.329	1.500 ± 0.794
BB2						2.000 ± 1.359

A1, Azores 1; A2, Azores 2; MR, Minho River; NPCS, North Portuguese Continental Slope; BB1, Bay of Biscay 1; BB2, Bay of Biscay 2. See also Figure 1.

Table 4 Analysis of the genetic differentiation between Azores 1 (A1), Minho River (MR) and North Portuguese Continental Slope (NPCS) samples

	Hd	Hst	Kst*	Z*	Snn
A1 *vs* MR	0.903	0.085	0.049	4.641	0.640
($n_1 = 10$; $n_2 = 15$)		(0.004**)	(0.005**)	(0.005**)	(0.025*)
MR *vs* NPCS	0.885	0.077	0.060	4.455	0.630
($n_1 = 15$; $n_2 = 8$)		(0.013*)	(0.007**)	(0.005**)	(0.061 *ns*)
NPCS *vs* A1	0.987	0.000	0.002	4.066	0.513
($n_1 = 8$; $n_2 = 10$)		(0.742 *ns*)	(0.387 *ns*)	(0.360 *ns*)	(0.418 *ns*)

Values for mean haplotype diversity (*Hd*) and for haplotype (*Hst*) and sequence-based statistics (*Kst**, *Z**, *Snn*) calculated according to Hudson *et al.*[26] and Hudson;[27] *ns* not significant; *$0.01 < P < 0.05$; **$0.001 < P < 0.01$.

DISCUSSION

The use of leptocephalus larvae for genetic studies can be advantageous, as they may better reflect the genetic substructure of the spawning adults. Conversely, the use of juveniles may underestimate the genetic substructure, because of the high rate of migration during this stage of development.[18] The mtDNA segment sequenced in the present study comprised a portion of the control region assuming that there is a similarity between the mtDNA of the European conger eel, *Conger conger* and the Japanese conger eel, *Conger myriaster.*[23] A total of 30 variable sites were observed in this conserved region, in 40 animals, defining 28 different haplotypes. The frequency of variable sites in the control region (6.9%, 30/432 bp) was lower than that of *C. myriaster* (19.8%, 89/450 bp).[33] Haplotype diversity is very high, but nucleotide divergence is very low, which is a common feature of marine species.[34,35] Although h13 haplotype was the most frequent in our samples, it was absent in A1 and NPCS samples. This effect could be simply the result of the low and unequal sample sizes analyzed or the effect of genetic drift operating during ocean migration of larvae. Alternatively, its high frequency in Minho contributes to the smaller haplotype diversity observed in this population. The haplotype network has the star shape typical of many marine species, suggesting a high effective population size, a low divergence between haplotypes, and a possible recent expansion. The bell-shaped mismatch distribution is consistent with a demographic population expansion scenario.

The geographic genetic variation of the Japanese conger eel *C. myriaster* has been investigated using mtDNA sequencing and nuclear DNA fingerprinting by amplified fragment length polymorphism analysis.[36] This study reported that there were apparently no genetically isolated populations in this species. However, since this study has been inconclusive, five polymorphic microsatellite markers are being developed in an attempt to better understand the recruitment mechanisms and population structure of this species, whose spawning sites and migration routes are unknown.[37] Recently, phylogenetic analysis based on mtDNA sequence data of leptocephali indicated no significant genetic differentiation between samples collected from different locations along the east coast of central Japan, implying intensive gene flow between geographically distant areas.[18] Although some molecular biology studies have shown no genetic difference among distantly separated geographic samples in some anguillid eels,[15–17] a recent study showed genetic evidence for multiple geographic population of the temperate eel *Anguilla*

marmorata.[19] Concerning the European conger eel *C. conger* the only well-known spawning ground is in the Mediterranean Sea.[3,7,12] However, some evidence (length and age of leptocephali) exists on the early life history of *C. conger* suggesting the possibility of another spawning area near the Azores.[13,14] Therefore, a first step should be to clarify the population structure of the European conger eel to provide further knowledge about its reproductive biology, which is essential for rational stock assessment and management.

It is unknown whether *C. conger* eel includes multiple populations or a single panmictic population. If the population isolation occurred recently, or if gene flow among local populations continues to occur, little or no genetic difference is expected, even when using more powerful molecular approaches. Although the duration of the leptocephali larval stage of the European conger eel remains unknown and speculative,[14] the conger eel should take about 2 years to drift inshore and to reach the juvenile form.[20] This long larval life enables the leptocephali to be transported by the ocean currents and to be dispersed over a wide area. The large-scale dispersion of the leptocephali and the North Atlantic currents could explain a gene flow among geographically distant areas, as for example between the Azores and northern Portugal. Some individuals with the same mtDNA haplotype were found in widely separated locations. However, the present mtDNA sequencing analyses provided some evidence for the existence of a significant genetic differentiation among some sample populations, suggesting that the conger eel does not comprise a single panmictic population. Nevertheless, since the number of individuals examined in the present study was small, and therefore the power for detecting significant genetic difference was low, these results should be interpreted with caution. Further, knowledge about the reproductive biology of conger eel, although limited, suggests that males and females could have different premigratory behavior.[38] In that case, nuclear DNA in addition to mtDNA data could provide a superior tool for testing for genetic differentiation of subpopulations, since mitochondrial genomes are mostly transmitted maternally.

The main purpose of the present work was not to examine in detail the genetic population structure of the Atlantic population of the European conger eel, but to detect molecular genetic variations in *C. conger* as an initial step for more extensive molecular genetic research. Although the oceanographic sampling of leptocephali was difficult and expensive, the genetic composition and the age of leptocephali may raise some questions about the location and time of spawning. In conjunction with leptocephali, the analysis of adult specimens collected more intensively and from a wide area of the NE Atlantic (including Madeira and the Canary Islands) and Mediterranean Sea, is essential to understanding the population structure and the genetic variation of this species.

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