Population genetic structure of *Scrobicularia plana* along the Atlantic coast and comparison with *Macoma balthica*

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Table of contents

1. Introduction 6
2.1 Sampling 6
2.2 Mitochondrial DNA extraction 7
2.3 Amplification and sequencing 7
2.4 Morphological data 8
2.5 Data analysis 8
2.5.1 Molecular data 8
2.5.2 Demographic and divergence inference 8
2.5.3 Morphological data 9
3. Results 9
3.1 Sequence variation 9
3.2 Population genetic analysis 16
3.3 Divergence rate 17
3.4 Morphological analyses 19
4. Discussion 20
References 22

Abstract

Scrobicularia plana (da Costa, 1778) is a burrowing bivalve species with a wide distributional range. It inhabits muddy sediments from the Norwegian Sea to Senegal as well as the Mediterranean Sea. It is commercially harvested in several countries.

The aim of this study was to assess *S. plana*'s population genetic structure. For that, a 507 bp fragment of the cytochrome-c-oxidase I gene from 283 individuals was analysed. These were retrieved from 17 locations and a total of 42 unique haplotypes were detected. In addition, variation in several morphological traits, assuming a genetic component, was studied.

Two hypotheses were formulated to better understand the results of observed patterns and they were compared with *Macoma balthica* results that were published earlier.

Comparing the distribution of the two species, *M. balthica* (continuous distribution) should have higher population connectivity than *S. plana* (patchy distribution). Therefore, *M. balthica* populations should be less differentiated (first hypothesis). Furthermore, *M. balthica* has larger populations, meaning that it is expected to harbour more variation at the intra-population level when compared to *S. plana* (second hypothesis).

Concerning the first hypothesis (population differentiation) the results were contrary to expectations. Both for molecular and morphological data, *M. balthica* was more differentiated than *S. plana*.

The second hypothesis (intra-population variability), in contrast, was confirmed. Within populations, nucleotide diversity was higher for *M. balthica* than for *S. plana*, consistent with the smaller population size of the latter. Morphological data showed the same trend, although these results were not statistically significant.

This study demonstrates that *S. plana* has limited population structure as well as low levels of intra-population variability. This is consistent with both a recent population expansion and with high population connectivity. Possibly, migration-mutation-drift balance has not yet been reached since *S. plana*'s post-glacial colonization of the European Atlantic.

Keywords: bivalve; *Scrobicularia plana*; *Macoma balthica*; COI; population divergence; distribution

1. Introduction

Invasions by non-indigenous species (NIS) are increasing in marine and estuarine habitats in the entire world (Ruiz et al., 1997; Occhipinti-Ambrogi & Savini, 2003). These invasions are related to global change or are directly human-mediated, mostly associated with mariculture (Reise et al., 1997).

This brings drastic changes in the community structure of coastal ecosystems, affecting the communities of organisms that live in the marine sediments (soft-bottom benthos) (Smith et al., 2000; Wehrmann et al., 2000). An example is the expected decrease in endemic bivalve populations due to food competition and effects of climate change. From an economic point of view, it is important to consider how commercial species will be affected.

Scrobicularia plana (Da Costa, 1778), commonly named the Peppery Furrow Shell, belongs to the Class *Bivalvia*, Superfamily *Tellinoidea*, Family *Semelidae* ([1], [2]) being sometimes included in the family *Scrobiculariadae* ([3], 2008; Casagranda & Boudouresque, 2005). This species is a member of the Order *Veneroida* which is characterized by thick, equal valves and adductor muscles of equal size. Some molecular information is available on this order, revealing taxonomy and systematic classification that are difficult to discern (Giribet & Wheeler 2002; Kappner & Bieler 2006).

Scrobicularia plana lives in intertidal estuarine habitats, with preference for soft bottoms of clay and mud, with abundant organic detritus. The animals are vertically burrowed and are relatively immobile being subjected to variations in the environment (Green, 1957; Akberali et al., 1983; Casagranda & Boudouresque, 2005). It can be easily identified by the star-shaped marks made on the surface of the sediments by its inhalant siphon (Pizolla, 2002).

It is widely distributed, from the Norwegian Sea to Senegal including the Mediterranean (Tebble, 1976). In some countries like Spain and Portugal it is commercially exploited (Langston et al., 2007). Knowledge on its genetic structure would facilitate stock management decisions (Avise, 1998).

A significant characteristic of this species is its patchy distribution (Hughes, 1970; Bocher et al., 2007), which theoretically means a lower connectivity between populations.

The aims of this study were to describe *S. plana's* population genetic structure and view it in light of its patchy distribution. We describe molecular variation for a portion of the mitochondrial cytochrome-c-oxidase I gene among 280 individuals, for 17 locations across its entire distribution range. In addition, we analyse several morphometric traits for spatial structure. Furthermore, we test the hypothesis that the more patchy distribution of *S. plana* leads to a more structured population and lower intrapopulational levels of genetic variability. To do so, we compare the obtained results with comparable data published earlier on the Baltic tellin *Macoma balthica*, which has a more continuous distribution.

2. Material and methods

2.1 Sampling

Scrobicularia plana adults were collected in intertidal mudflats at 17 locations between July 2006 and July 2008, from Baltic and North Sea to the Atlantic Ocean and Mediterranean Sea (Fig. 1). All individuals were preserved in 95% ethanol immediately after collection. Many of the samples were kindly sent to us by colleagues (see Acknowledgments).

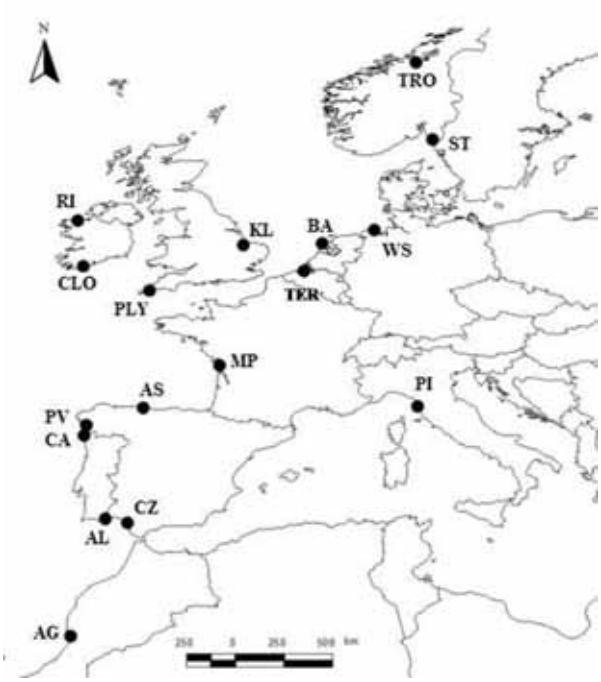

Figure 1: *Scrobicularia plana* sampling sites. The letter codes refer to locations as follows: TRO Trondheim Fjord; ST Tjärnö, Skagerak; WS German Wadden Sea; BA Balgzand, Dutch Wadden Sea; TER Terneuzen, Westerschelde; KL King's Lynn, southern North Sea; RI Ross Island, Atlantic; CLO Clonakilty, Atlantic; PLY Plymouth, Channel; MP Moeze Plaisance, Atlantic; AS Asturias, Atlantic; PV Ponte Vedra, Atlantic; CA Caminha, Atlantic; AL Algarve, Atlantic; CZ Cádiz, Atlantic; AG Agadir, Atlantic and PI Pisa, Mediterranean.

2.2 Mitochondrial DNA extraction

DNA was extracted from the mantle tissue using the GenElute™ Mammalian Genomic DNA kit from SIGMA®. Extracted DNA was visualized on 1% TBE agarose gels.

2.3 Amplification and sequencing

The universal primers HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA 3') and LCO1490 (5' GGTCAACAAATCATAAAGATATTGG 3') (Folmer et al., 1994) were used to amplify a fragment of roughly 710 base pairs (bp) from the mitochondrial cytochrome-c-oxidase subunit I region (COI). Each 50µl PCR reaction contained 5µl template DNA (1:10 dilution of DNA), 4.6 µl 10x reaction buffer (BiothermTM), 4.6 µl dNTPs (2.5 µmol), 0.3 of each primer (0.02µmol) and 0.25 µl Taq polymerase (5units/µl) (Biotherm PlusTM).

Amplifications were carried out in a Px2 thermo-cycler (Thermo Electron Corporation) with the following profile: one initial step of 5 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 45 s at 40ºC, 45s at 72ºC, then followed by a step of 7 min at 72ºC, and a final step of 5 min at 4ºC.

From the initial alignment of 23 sequences, specific internal primers were designed, that amplified a fragment of 507 bp, using the web-based program Primer3 (Rozen & Skaletsky, 2000): CO_SCROB_F (5' TTGGGAGTCTTTATTTTGTTTTAG 3') and CO_SCROB_R (5' AAGAAAGAAGTATTAAAATTACGATCA 3'). The newly designed primers were used for the remainder of the study.

For the specific primers, amplifications were carried out in the same thermo-cycler but with a different profile: one initial step of 5 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 45 s at 50ºC, 45s at 72ºC, then followed by a step of 7 min at 72ºC, and a final step of 5 min at 4ºC.

Amplification results were confirmed by 2% TBE agarose gel electrophoresis. Some PCR products had to be re-amplificatied from the TBE agarose gel, as follows: the ethidium bromide stained gel was viewed on a UV transilluminator, cut pipet tips (P100) were used to puncture a hole in the DNA band, this was transferred to a 1.5ml tube and to it was added 200µl sterile water. The tubes were then incubated for 2 minutes at 95ºC and 1µl of solution was used as a template for a second PCR reaction.

After confirmation, fragments were purified directly, using QuickClean 5M PCR Purification Kit (GenScript).

Sequencing was carried out by the company Macrogen, under BigDyeTM terminator cycling conditions, with the reacted products being run on an ABI3730XL automated sequencer. Only the forward primer was used (CO_SCROB_F).

In total, COI sequences were obtained from 280 individuals (Table 1).

Table 1. ***Scrobicularia plana*** **sampling information, including geographic region, GPS positions and date, population code and number (*n*)of individuals analyzed.**

Geographic region	Location	Latitude	Longitude	Sampling date	Code	n
Northeastern Atlantic	Trondheim	63º18'60.88''N	10º11'1.74''E	April 2008	TRO	21
Northeastern Atlantic	Tjärnö	58º53'32.94''N	11º10'04.14''E	October 2007	TJ	27
Northeastern Atlantic	Ross Island	54º13' 34.62''N	9º12'43.14''W	March 2008	RI	23
Northeastern Atlantic	Wadden Sea	53°42'49''N	7°48'04''E	May 2007	WS	6
Northeastern Atlantic	Balgzand	52º 56'9''N	4º48'7.2''E	March 2008	BA	8
Northeastern Atlantic	King's Lynn	52º49'14.30''N	0º17'16.60''E	February 2008	KL	11
Northeastern Atlantic	Clonakilty Estuary	51º37'17.50''N	8º52'40.39''W	April 2008	CLO	18
Northeastern Atlantic	Terneuzen	51º20'47.76''N	3º47'44.52''E	May 2008	TER	24
Northeastern Atlantic	Plymouth	50º12'35''N	5º05'27''W	January 2008	PLY	20
Northeastern Atlantic	Moeze plaisance	45º55'15.29''N	1º4'31.11''W	March 2008	MP	19
Northeastern Atlantic	Asturias	43º28´00''N	5º 26´00''W	April 2007	AS	19
Mediterranean Sea	Pisa	43°34'59.79"N	10°18'0.43"E	Jully 2008	PI	28
Northeastern Atlantic	Ponte Vedra	42º28'00.57''N	8º42'10.50''W	April 2007	PV	9
Northeastern Atlantic	Caminha	41º53'8.00''N	8º50'50.40''W	February 2008	CA	26
Northeastern Atlantic	Algarve	37º07´37.6"N	7º36´36.4"W	April 2008	AL	11
Northeastern Atlantic	Cádiz	36°52'31.50''N	6°20'44''W	May 2007	CZ	11
Northeastern Atlantic	Agadir	30°21'50.03"N	9°35'41.80"W	Jully 2006	AG	2

2.4 Morphological data

Individuals were measured according to their shell length, height, width and shell mass, except for the Plymouth sample since no shells were available. A total of 479 individuals were analysed.

2.5 Data analysis

2.5.1 Molecular data

DNA sequences were aligned using the software BioEdit Sequence Alignment Editor (Hall, 1999). Ambiguous sites were scored conservatively using IUPAC ambiguity codes.

Haplotypes were detected with the help of sequence analysis program MEGA4 (Tamura et al., 2007). A minimum spanning network among the haplotypes was constructed using Arlequin 3.1 (Excoffier & Schneider, 2005).

Analysis of molecular variance (AMOVA) was carried out in Arlequin. Nucleotides diversities (π; Tajima, 1983) were calculated for each sample, and exact tests of population differentiation based on haplotypes frequencies were carried out, also using Arlequin 3.1.

2.5.2 Demographic and divergence inference

Mismatch distributions were produced in Arlequin to estimate demographic parameters and coalescence times for all sites as well as the two major phylogeographic groups. This was calculated using a model of pure demographic expansion. A least squares approach is used in this model to test for deviation from exponential population size expansion and it produces a maximum likelihood estimate for coalescence time ($\tau=2\mu t$, where μ is the mutation rate for whole haplotype per generation and t equals the number of generations) of the haplotypes. Generation time, defined as the age at first reproduction (note that the model is based on non-overlapping generations), was assumed to be two years (Paes-da-Franca, 1956; Bachelet, 1981; Rodríguez-Rua et al., 2003). The mutation rate used was the one calibrated for bivalve COI by Luttikhuizen et al. (2003).

Average uncorrected p-distances and nucleotide diversities within each Atlantic Ocean sample were calculated and compared with the *Macoma balthica* data supplied by Luttikhuizen et al., 2003 (see Table 4 and 5). Due to the fact that *M. balthica* does not exist in the Mediterranean (Beukema & Meehan, 1985; the sample PI (Pisa) from *S. plana* was not included in this comparison.

2.5.3 Morphological data

Morphological data were analysed in SYSTAT 12 (Wilkinson, 1997). Shell measures were log10-transformed (henceforth called loglength, logheight, logwidth and logmass) and corrected for loglength to enable comparisons between samples and between species. This was done by taking residuals from linear regression models on the log transformed data against loglength. Comparisons between *S. plana* and *M. balthica* were done using a General Linear Model (GLM), with the goal to test for differences between species in levels of intrapopulation and interpopulation variability.

3. Results

3.1 Sequence variation

From a total of 283 COI fragments sequenced for *S. plana*, forty-two haplotypes were obtained across all the different regions. Ninety one polymorphic sites were detected, 12 of them representing amino acid substitutions and the other 79 silent substitutions (Table 2a, b, c and d). Two replacement mutations in a single haplotype was observed in h25 (both Val replaced by Ala), in h35 (Val replaced by Ala and Ala by Thr) and in h41 (Ala replaced by Ser and Thr by Ser). The other replacement mutations were observed in h08 (Met by Thr), h22 (Ile by Met), h36 (Val by GYA), h37 (Val by Ala), h40 (Val by Glu) and h42 (Val by Glu). All these amino acid substitutions occurred only once, except in h37. Four of the replacement substitutions were observed in Norway (h25, h35, h36, h37), two in England (h22 and h40) and one in Ireland (h08), Italy (h41) and Sweden (h42).

In the case of h36 the substitution was not so clear because a double peak existed in that position in electropherograms. This can be due to (1) one of the templates representing a nuclear copy of a mitochondrial sequence (NUMT), (2) contamination, (3) DNA from two individuals, (4) gene duplication within the mtDNA, (5) heteroplasmy, or the presence of two mitochondrial lines in a single individual (for an elaborate discussion see Luttikhuizen et al., 2008).

Haplotype h38 was highly deviant (66 in 507 or 13% compared to h01); all differences were silent substitutions (Table 2c and d).

Most of the polymorphisms occurred at the third codon position (83.3%) being only 8.73% at the second codon and 7.97% at the first one. 86 transitions and 5 transversions were observed (T_i/T_v=17.2).

Table 2a. Variable sites in the COI haplotypes identified for *Scrobicularia plana*. The polymorphic sites that represent amino acid substitutions are in bold.

	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	1	1	2	3	4	5	5	5	6	7	8	9	9	0	0	1	1	1	2	2	2	2	3	3	4	5	5	6	7	7	7	7	8	9	9	0	0	0	1	1	1	1	2	3	3	4	4	5	6	8	9	9	9
haplotype	1	7	6	8	7	0	6	8	2	6	6	3	5	4	4	0	3	8	1	5	7	8	4	7	0	2	5	4	0	4	6	9	5	1	4	0	3	7	0	3	5	8	1	5	9	5	8	1	6	7	0	3	6
h 01	A	C	T	T	A	T	T	T	T	T.	T	T	A	G	G	T	G	G	C	T	T	T	G	A	T	A	A	G	T	C	T	C	A	A	A	A	T	T	C	C	T	A	T	A	T	T	G	T	A	T	C	T	A
h 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Table 2b. Variable sites in the COI haplotypes identified for *Scrobicularia plana*. The polymorphic sites that represent amino acid substitutions are in bold.

	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5
	0	1	2	2	3	4	4	4	5	5	6	7	9	9	0	0	1	1	2	3	3	3	4	5	5	5	6	6	7	7	7	8	8	9	9	9	9	0	0
haplotype	2	8	0	3	8	1	4	7	3	9	2	7	2	8	1	7	0	6	8	0	1	4	6	5	6	9	6	8	0	3	9	0	8	1	2	5	9	4	6
h 01	T	C	T	A	T	A	T	A	T	G	T	A	G	G	T	G	T	G	T	T	A	T	T	T	T	T	T	G	T	G	A	T	A	G	T	A	T	C	T
h 02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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h 21	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
h 22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 2c. Variable sites in the COI haplotypes identified for *Scrobicularia plana*. The polymorphic sites that represent amino acid substitutions are in bold.

haplotype	0 1 1 7	0 2 6	0 3 8	0 4 7	0 5 0	0 5 6	0 5 8	0 6 2	0 7 6	0 8 6	0 9 3	0 9 5	0 0 4	0 0 4	1 1 0	1 1 3	1 1 8	1 2 1	1 2 5	1 2 7	1 2 8	1 3 4	1 3 7	1 4 0	1 5 2	1 5 5	1 6 4	1 7 0	1 7 4	1 7 6	1 7 9	1 8 5	1 9 1	1 9 4	2 0 0	2 0 3	2 0 7	2 1 0	2 1 3	2 1 5	2 2 8	2 3 1	2 3 5	2 3 9	2 4 5	2 4 8	2 5 1	2 6 6	2 8 7	2 9 0	2 9 3	2 9 6
h 23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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h 38	.	.	.	.	G	C	.	C	A	.	A	C	T	T	T	C	A	A	A	.	A	.	A	G	A	G	G	T	.	T	A	T	.	G	T	T	A	.	T	T	A	.	A	G	C	G	C	A	T	.	.	A	C
h 39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Table 2d. Variable sites in the COI haplotypes identified for *Scrobicularia plana*. The polymorphic sites that represent amino acid substitutions are in bold.

	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5
	0	1	2	2	3	4	4	4	5	5	6	7	9	9	0	0	1	1	2	3	3	3	4	5	5	5	6	6	7	7	7	8	8	9	9	9	9	0	0
haplotype	2	8	0	3	8	1	4	7	3	9	2	7	2	8	1	7	0	6	8	0	1	4	6	5	6	9	6	8	0	3	9	0	8	1	2	5	9	4	6
h 23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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h 35	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**C**	**A**	.	.	.	.	.	.	.	.	.	.	.
h 36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	?	.	.	.	.	.	.	.	C	.	.	.	.
h 37	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**C**	.	.	.	.	.	.	.	C	.	.	.	.
h 38	.	T	A	T	.	G	G	T	C	T	.	G	A	A	A	T	.	T	.	.	T	G	.	C	C	C	.	.	A	A	T	C	.	T	C	.	.	T	G
h 39	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
h40	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.
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h42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.

Table 3a. Geographic distribution of haplotypes h1 through h24for *Scrobicularia plana*.

Code	Site	h 01	h 02	h 03	h 04	h 05	h 06	h 07	h 08	h 09	h 10	h 11	h 12	h 13	h 14	h 15	h 16	h 17	h 18	h 19	h 20	h 21	h 22	h 23	h 24
TRO	Trondheim, Norway	12	0	0	0	0	1	0	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0
TJ	Tjärnö, Sweden	21	0	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
WS	German Wadden Sea	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BA	Balgzand, Netherlands	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TER	Terneuzen, Netherlands	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
RI	Ross Island, Ireland	17	0	0	1	0	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CLO	Clonakilty Estuary, Ireland	15	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
PLY	Plymouth, England	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1
KL	King's Lynn, England	8	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0
MP	Moeze Plaisance, France	13	2	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
AS	Astúrias, Spain	15	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PV	Ponte Vedra, Galicia, Spain	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1	0	0	0	0
CA	Caminha, Portugal	18	0	0	0	0	1	1	0	1	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0
AL	Tavira, Algarve, Portugal	3	1	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	2	0	0	0	0	0
CZ	Cádiz, Spain	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0
PI	Pisa, Italy	8	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AG	Agadir, Morocco	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	sum	195	4	1	3	11	7	2	1	4	4	1	2	1	1	4	3	1	1	4	1	1	1	1	1

Table 3b. Geographic distribution of haplotypes for *S. plana*, nucleotide diversity (π) and gene diversity (h) for the Cytochrome Oxidase subunit I (COI) fragment. Standard deviations (SD) are in parentheses.

Code	Site	h 25	h 26	h 27	h 28	h 29	h 30	h 31	h 32	h 33	h 34	h 35	h 36	h 37	h 38	h39	h40	h41	h42	sum	π (SD)	h (SD)
TRO	Trondheim, Norway	1	0	0	0	0	0	0	0	0	0	1	1	2	0	0	0	0	0	21	0.002254 (0.001697)	0.6762 (0,1110)
TJ	Tjärnö, Sweden	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	27	0.000865 (0.000890)	0.3989 (0.1172)
WS	German Wadden Sea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0
BA	Balgzand, Netherlands	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0
TER	Terneuzen, Netherlands	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0.000479 (0.000631)	0.2355 (0.1093)
RI	Ross Island, Ireland	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	23	0.001029 (0.000998)	0.4625 (0.1283)
CLO	Clonakilty Est., Ireland	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0.000657 (0.000769)	0.3137 (0.1376)
PLY	Plymouth, England	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	20	0.001381 (0.001214)	0.4474 (0.1367)
KL	King's lynn, England	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0.001434 (0.001301)	0.4909 (0.1754)
MP	Moeze plaisance, France	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19	0.001638 (0.001365)	0.5380 (0.1330)
AS	Astúrias, Spain	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	19	0.015646 (0.008529)	0.3860 (0.1389)
PV	Ponte Vedra, Spain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0.001644 (0.001458)	0.5556 (0.1653)
CA	Caminha, Portugal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	26	0.001924 (0.001503)	0.5231 (0.1160)
AL	Tavira, Algarve, Portugal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0.003514 (0.002478)	0.9273 (0.0665)
CZ	Cádiz, Spain	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0.001371 (0.001265)	0.6000 (0.1539)
PI	Pisa, Italy	0	0	1	0	5	1	1	1	1	1	0	0	0	0	0	0	1	0	28	0.002703 (0.001913)	0.8252 (0.0441)
AG	Agadir, Morocco	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
	sum	1	1	2	4	5	1	1	1	1	1	1	1	2	1	2	1	1	1	283		

3.2 Population genetic analysis

The sequence variation among the haplotypes is shown as a minimum spanning network (Fig. 3). Most of the networks are connected to a main haplotype, i.e., a haplotype with higher frequency that is common to all or most sampling sites (Posada & Crandall, 2001). This kind of network is known as a star-like phylogeny and is typical of a recent population expansion following a population bottleneck (Magoulas et al., 2006). To confirm this supposition, the test statistic R2 of Ramos-Onsins and Roza, implemented in the DnaSP program (Rozas et al., 2003), was estimated. It equaled 0.16 and the null hypothesis of stable population size was significantly rejected ($p<0.01$), validating the star-shaped network as indicative of population expansion.

The occurrence of haplotypes among samples as well as nucleotide and gene diversity and their standard deviations are shown in Tables 3a and b. In the last table it is possible to observe that the highest nucleotide diversity is from Astúrias and the highest gene diversity from Algarve. However, the high nucleotide diversity in Astúrias mainly depends on one deviating sequence (h38). If this sequence is omitted, the highest nucleotide diversity is from Algarve (0.003514±0.002478).

Nucleotide diversities were also calculated for *Macoma balthica* and compared between the two species (Fig. 2). Nucleotide diversity was significantly higher in *M. balthica* than in *S. plana* (Kruskal-Wallis, $P<0.05$).

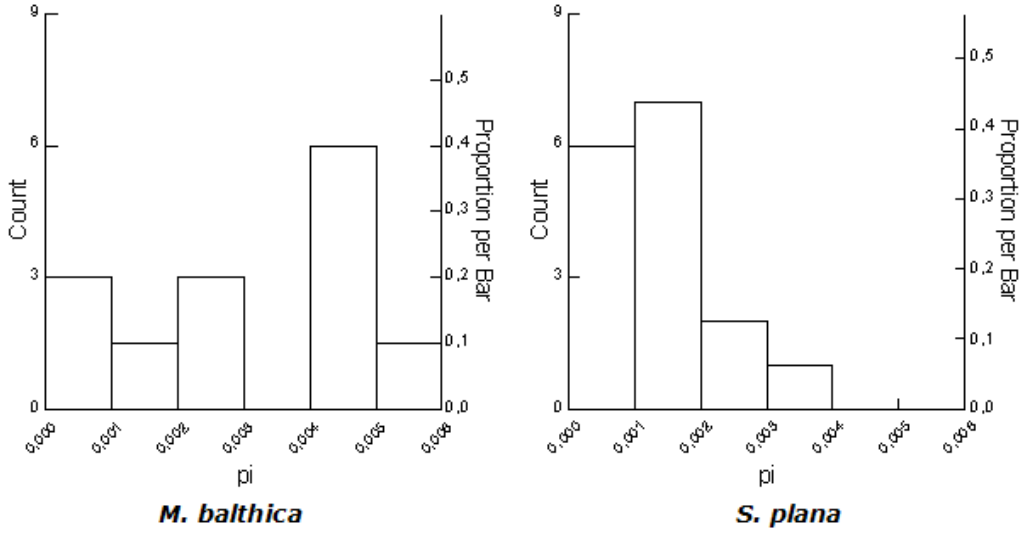

Figure 2. Distribution of nucleotide diversities (π) for cytochrome oxidase I in population samples of Macoma balthica and *Scrobicularia plana*.

On the basis of a single-level AMOVA, population differentiation in *S. plana* was not significant; the percentage of total molecular variation within samples was 97.64% and among samples 2.36%.

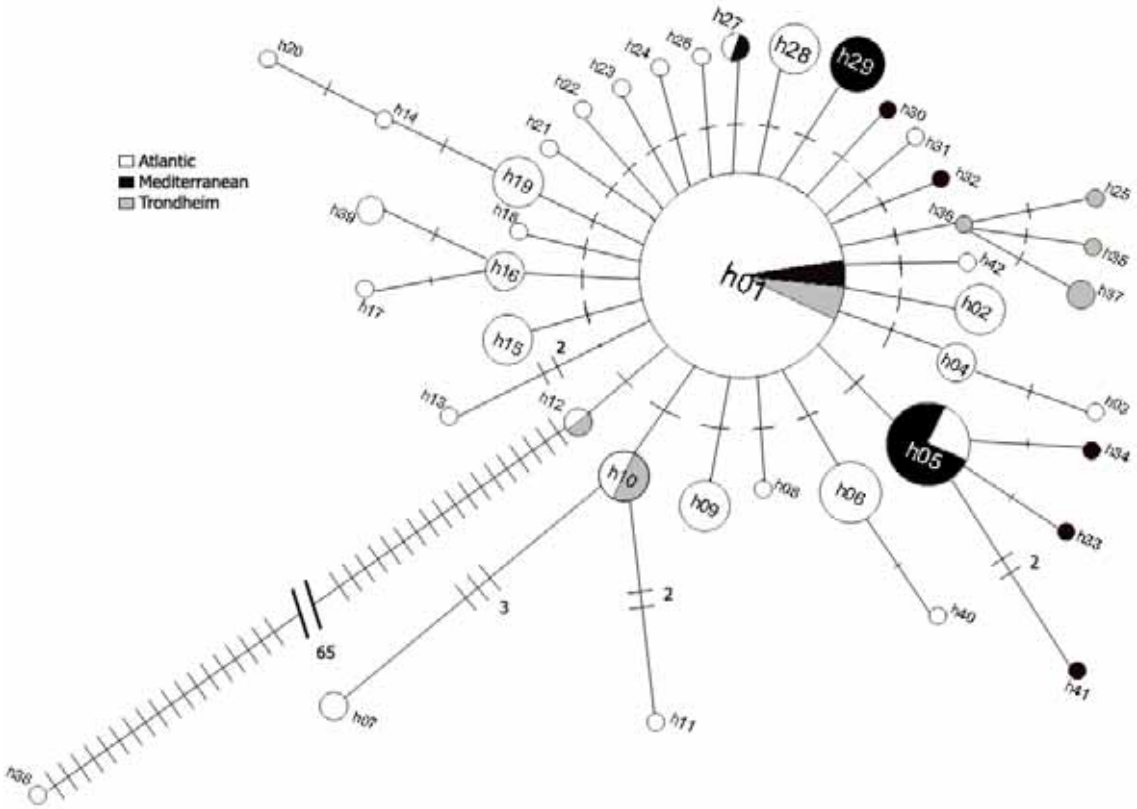

Figure 3: Minimum spanning network of all haplotypes detected, where each circle signifies a haplotype and its size denotes the frequency. The branch length is one substitution (one dash) unless otherwise indicated.

3.3 Divergence rate

The time since population expansion was calculated using a molecular clock of 0.14-0.52% sequence divergence per million years and a per-sequence mutation rate μ between $0.55x10^{-6}$ and $2.04x10^{-6}$ (Luttikhuizen et al., 2003), assuming a generation time of two years. This mutation rate was created for *Macoma balthica* for a fragment of 393 bp (Table 5). For this reason, these mutation rates were adapted for a length of 507 bp being $0.71x10^{-6}$ and $2.63x10^{-6}$.

Applying these mutation rates we obtained an estimated time since population expansion of 0.215-0.798 Mya for *S. plana* populations and for *M. balthica* 0.672-2.49 Mya (Tables 4 and 5).

Table 4. Estimation of population origin using a coalescent-based joint maximum likelihood estimation for time since expansion (Tau) of *Scrobicularia plana*. The population divergence times (PDT's) are calculated based on the molecular clock by Luttikhuizen et al., 2003.

	Time since expansion*		
		Actual time (million yrs)	
	Tau	lower	upper
TRO	1.9960	1.41	0.379
ST	0.5210	0.37	0.099
WS	0	0	0
BA	0	0	0
TER	3.0000	2.11	0.57
RI	0.6250	0.44	0.12
CLO	0.3690	0.26	0.070
PLY	1.8260	1.29	0.35
KL	1.1880	0.84	0.23
MP	1.2010	0.85	0.23
AS	3.0000	2.11	0.57
PV	0.9300	0.66	0.18
CA	0.4080	0.29	0.078
AL	1.8050	1.27	0.34
CZ	0.8750	0.62	0.17
PI	1.5080	1.06	0.29
AG	0	0	0
Mean*	1.18	0.83	0.23

***PI sample is not included in these analyses.**

Table 5. Estimation of population origin using a coalescent-based joint maximum likelihood estimation for time since expansion (Tau) of *Macoma balthica*. The population divergence times (PDT's) are calculated based on the molecular clock by Luttikhuizen et al., 2003.

	Time since expansion		
		Actual time (yrs)	
	Tau	lower	upper
White sea	0	0	0
Norway	3	2.73	0.74
Scotland	3	2.73	0.74
Terschelling	5.291	4.81	1.30
Vlieland	5.281	4.80	1.29
Kimstergat	0	0	0
Balgzand	5.363	4.88	1.31
Gironde	0	0	0
Mean	2.74	2.49	0.67

3.4 Morphological analyses

Figure 4 shows morphological variation for *Scrobicularia plana* organized according to latitude (Fig. 4).

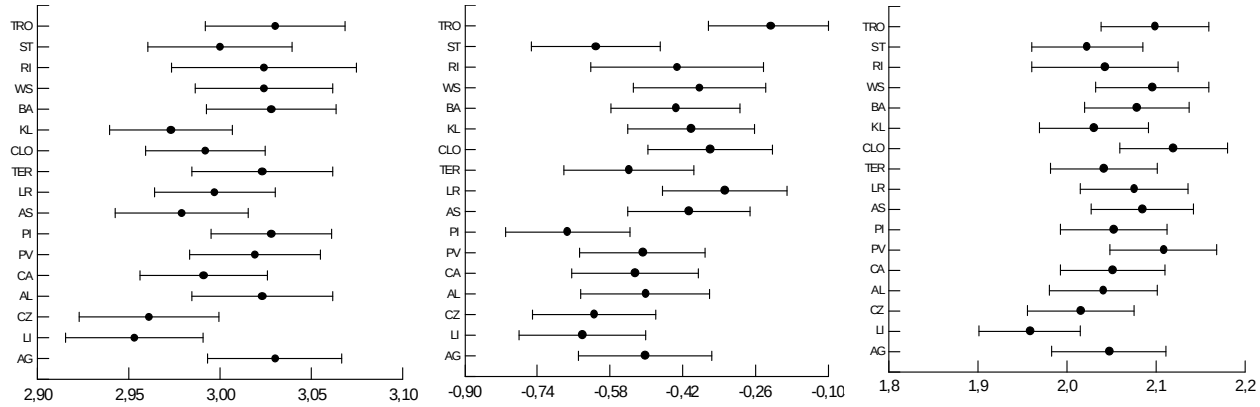

Figure 4. *Scrobicularia plana*. Morphological, loglength-corrected data (from left to right: logheight, dry mass and logwidth) organised from top to bottom according to decreasing latitude. Bars represent standard deviations.

While strong differences exist between samples/locations ($p<0.05$), no obvious relationship between morphological data and latitude is present.

A Generalized Linear Model (GLM) approach was used to enable comparing variance components of the morphological data between *M. balthica* (Luttikhuizen unpubl. data) and *S. plana*. Results are shown in Table 6.

Table 6. Variance components for morphological, loglength-corrected data compared between *M. balthica* and *S. plana*. 'Within' indicates levels of variation within populations; 'between' indicates amounts of variation between populations. 'NS' = not significant.

		M. balthica	S. plana	F ratio	Significance
shell mass	within	0.00504	0.00326	1.55	NS
	between	0.00573	0.00240	2.39	p<0.001
shell height	within	0.00032	0.00020	1.6	NS
	between	0.00039	0.00013	3	p<0.001
shell width	within	0.00066	0.00064	2.13	NS
	between	0.00045	0.00031	1.45	p<0.001

Comparing the two species it can be seen that all variance components (within and between populations) are higher for *M. balthica*. However, the differences between species are only significant when compared between populations.

4. Discussion

The first geographical survey of molecular variation in *Scrobicularia plana*, presented here, shows that its Atlantic populations show little differentiation in the mitochondrial gene COI, while a sample from the Mediterranean was significantly different.

For a better interpretation of the results of *S. plana*, a species with similar bio-geographical conditions was analyzed for comparison *(Macoma balthica)* (Beukema, 1993; Riera et al., 1999; Bocher et al., 2007). A marked difference between the species is their distribution types: while *S. plana* has a patchy distribution, *M. balthica* is more continuously distributed (Hughes, 1970; Bocher et al., 2007). This means that population size and population connectivity should be larger in *M. balthica*.

Theoretically this means that, between populations, *M. balthica* populations should be less differentiated than those of *S. plana* (first hypothesis).

Furthermore, higher levels of intrapopulational variation are expected for *M. balthica*. Given random mating and assuming that a population is in mutation-genetic drift equilibrium, a large population produces a large sample of successful gametes, which means a greater probability that the allelic frequencies of the offspring will represent the allelic frequencies of the parents. Thus, a large population traduces in a higher level of intra-population variation, (Tamarin, 1982). It was therefore expected that *M. balthica* populations should harbour more variation at the intrapopulation level than *S. plana* populations (second hypothesis).

However, evaluating the first hypothesis concerning molecular variation, the opposite of what was expected was observed, i.e., higher inferred connectivity for *S. plana* than for *M. balthica* (% of variation of *S. plana*: N.S.). Similarly, for the morphological data, a lower variation between populations was expected for *M. balthica* but the contrary was observed ($p<0.001$).

The second hypothesis, in contrast, was confimed. Nucleotide diversities (π) (Fig. 2) were higher for *M. balthica* than *S. plana*, and intrapopulational variance components for morphological data were higher for *M. balthica*, although only a non-significant trend was observed.

An overview of all results is shown in Table 3.

Table 3. Overview of results of comparing molecular and morphological data between *Scrobicularia.plana* (*S*) and *Macoma balthica* (*M*).

	Expectation	Observation	
		Molecular data	**Morphological data**
between populations	higher in *S*	lower in *S* (N.S.)	lower in *S* (p<0.05)
within populations	lower in *S*	lower in *S* (p<0.05)	lower in *S* (trend)

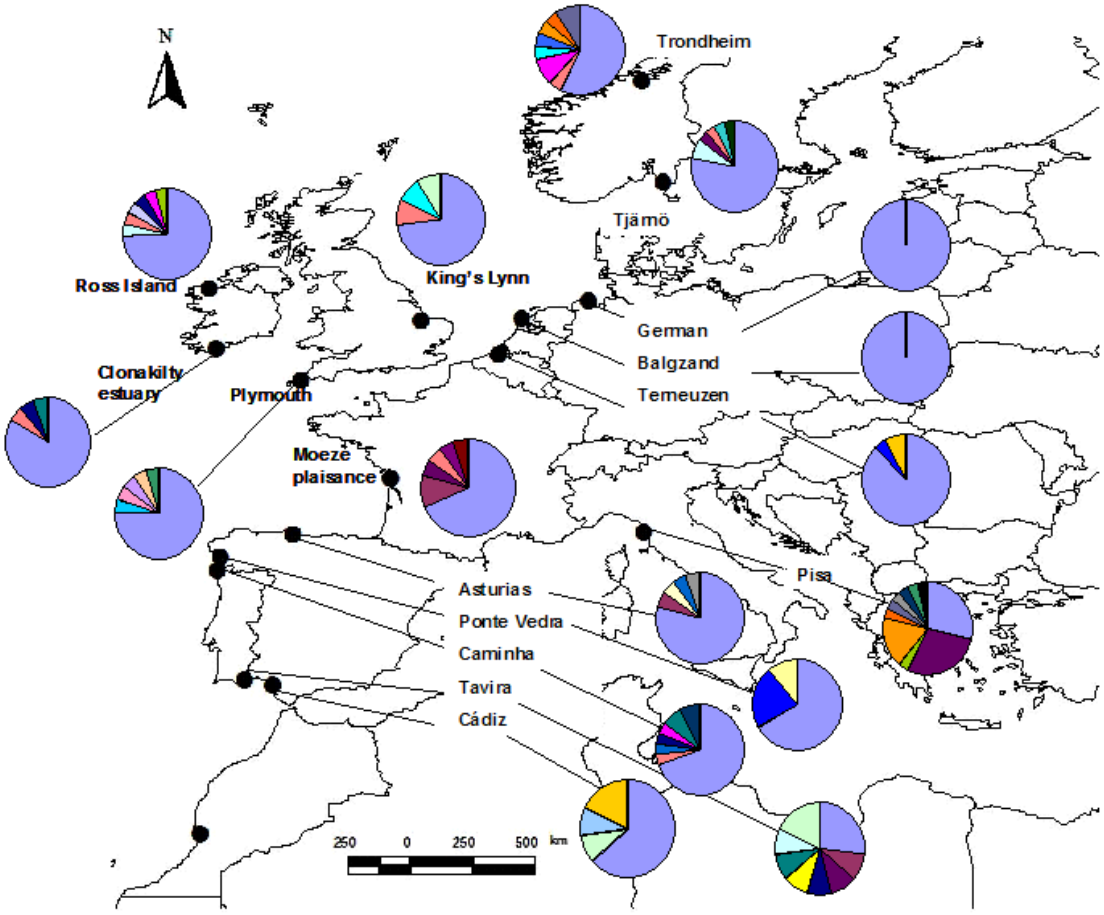

Figure 5. Distribution of COI haplotypes for *Scrobicularia plana* across Europe. Agadir (Morocco) is not included because of its small sample size.

The widespread occurrence of haplotype h01 for *Scrobicularia plana* COI can be seen in Figure 5 (in blue). This shows the apparent connectivity between locations and therefore an opposite result of the first hypothesis. It also demonstrates that the populations of these species have recently been expanding and possibly still are (Palumbi, 2003). Also, the sharing of the most common haplotype between the Atlantic Ocean and the Mediterranean Sea suggests ongoing gene flow. However, the possibility that populations may not yet be in mutation-genetic drift balance, after their post-glacial colonization of the European Atlantic, must be kept in mind, meaning that connectivity may actually be low but genetic differentiation has not yet had enough time to build up and show it (see also Patarnello et al., 2007).

Looking in detail at the data, southern samples (from Ponte Vedra to Cádiz and Pisa) appear to harbour lower frequencies of the blue haplotype than elsewhere. This implies a higher variability and frequency of other haplotypes in those places. Another aspect is the higher values for estimation of population origin (Table 4) at these places, suggesting that southern popualtions may be somewhat older.

This may indicate that southern Atlantic populations have been less severely bottlenecked, which is in accordance with southern confinement (Portuguese coast and southward) during the Pleistocene ice ages (18,000-10,000 years ago) (Nikula & Väinölä, 2003; Papadopoulos et al. 2005).

The fact that the Iberian Peninsula (Caminha, Tavira and Cadiz) and the Mediterranean Sea (Pisa) have higher haplotype diversities may also indicate that a connection existed through the Strait of Gibraltar. However for better supported conclusions, more samples from that area must be analysed.

Another important fact that must be considered is the h38 discordant mitochondrial lineage presented on Fig. 3. This is the only individual that belongs to the Astúrias sample, which morphologically had the same characteristics as h01 (most common) but not genetically, since 13% of the analysed mitochondrial DNA fraction are variable sites but silent substitutions. According to some authors (Lee, 2000; Goetze, 2003; Lee & Foighill, 2004), divergences of ~5-15% of the mitochondrial protein-coding genes of some marine species represent cryptic species. This individual can be an example of an unidentified sympatric sibling species or from a previously isolated population that started to interbreed (Peijnenburg et al., 2006). Several other individuals from Astúrias did not amplify well and should be analysed again but using universal primers.

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