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Genetic differentiation among *Parastichopus regalis* populations in the Western Mediterranean Sea: potential effects from its fishery and current connectivity

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Abstract

Parastichopus regalis (Cuvier, 1817) is the most expensive seafood product on the Catalonian market (NE Spain), with prices at approximately 130 €/Kg (fresh weight). Despite its ecological and economic importance, biological and genetic information on this sea cucumber species is scarce. Here, we provide both the first insight on the genetic structure of *P. regalis* using sequences of cytochrome oxidase I (COI) and 16S genes and a morphological description of its population. Individual sea cucumbers were collected in six locations along the Spanish Mediterranean coast, including an area under fishery pressure (Catalonia). We found high haplotype diversity and low nucleotide diversity for both genes, with higher levels of genetic diversity observed in the COI gene. The population pairwise fixation index (F_{ST}), AMOVA and correspondence analysis (CA) based on the COI gene revealed significant genetic differentiation among some locations. However, further analysis using nuclear markers (e.g., microsatellites) is necessary to corroborate these results. Moreover, the genetic and morphological data may indicate fishery effects on the Catalonian population with a decrease in the size and weight averages and lower genetic diversity compared with locations that lack fishery pressure. For the appropriate management of this species, we suggest the following: 1) accurately assessing the stock status along the Spanish coasts; 2) studying the reproductive cycle of this target species and the establishment of a closed fishery season according to the reproductive cycle; and 3) establishing protected areas (i.e., not take zones) to conserve healthy populations and favour recruitment in the nearby areas.

Keywords: Holothurian, genetic structure, mitochondrial DNA, NW Mediterranean, resource management, fisheries.

Introduction

Sea cucumbers play a very important role in marine ecosystems and are considered target fishery species with high economic value (González-Wangüemert *et al*., 2014a; 2015; Purcell, 2014). Holothurians, as primarily deposit feeders, ingest large amounts of sediment (Uthicke & Karez, 1999) and provide important ecosystem services that enhance nutrient cycling and local productivity (Ramón *et al*., 2010). Indeed, by converting organic detritus into animal tissue and nitrogenous wastes, they increase the local productivity and produce more available food for herbivores (Purcell *et al*., 2013).

At least 66 species of holothurians are globally exploited, especially in the Indo-Pacific Ocean (Toral-Granda, 2008; Purcell, 2010; 2014), and they are primarily exported to Asian markets, where they are considered important and a traditional food resource (Bruckner, 2006; Purcell *et al*., 2013). Several species have also been investigated by pharmaceutical companies because of the biological activities of some components from the wall, guts and Cuvier tubules (Kerr, 2000; Bordbar *et al*., 2011). The high commercial value of sea cucumbers and its growing international demand have increased fishing pressure over the last few years, reaching an annual total global catch in the form of 80,000 tonnes of live animals (Purcell, 2010; Purcell *et al*., 2013). Holothurians are prone to overfishing due to their life history, including their limited mobility as adults, late sexual maturity, reproduction depending on density and low rates of recruitment (Uthicke *et al*., 2004).

Parastichopus regalis (Cuvier, 1817) is the only holothurian species that belongs to the family Stichopodidae, which is present in the Mediterranean Sea. It is a benthic species and can be found from intertidal and shallow areas to abyssal depths; its range has been described from 10 to 800 meters, and it inhabits preferably sandy and rubble sea bottoms (Clark, 1922; Cutress & Miller, 1982; Pawson *et al.*, 2009). There is a general information gap regarding this species. One of the few exceptions is a study describing its population structure on the Balearic islands (Spain) (Ramón *et al.,* 2010). In this geographic area, *P. regalis* has shown a heterogeneous spatial distribution, high levels of aggregation, a length ranging from 6.5 cm to 29.5 cm and a multimodal size-frequency distribution. A recent study also revealed the

occurrence of pearlfish into several North Atlantic and Mediterranean sea cucumber species, including *P. regalis* (González-Wangüemert *et al*., 2014b). This species was the only one that had a *Carapus acus* as a commensalist, but no relationship between fish length and host weight/length was found. In some individuals of *P. regalis,* two specimens of *C. acus* were registered, which suggested that coupling could occur inside sea cucumbers.

P. regalis is one of the sea cucumber species that is caught and sold for human consumption in the Mediterranean Sea. For most of the twentieth century, the *P. regalis* species was cooked mainly by fishermen as a rice accompaniment, particularly in Catalonia and the Balearic Islands. It is commercialised in Spain, especially in Catalonia, where it currently represents the most expensive seafood product, reaching 130 €/kg fresh weight (Ramón *et al*., 2010). The parts of the animal that are collected and consumed are the internal muscle bands ("espardenya" or "llongo"), which are considered a delicacy in luxury restaurants. This species is captured as by-catch in trawl fisheries (González-Wangüemert *et al*., 2014a; b) and sold in Catalonia when a minimum number of 20 individuals are caught. This corresponds to a weight of approximately 0.5 kg of internal muscle bands, whereas the mean catch per unit effort is approximately 1.78 kg per boat and day (Ramón *et al*., 2010). In the last years, its fishery has been extended to other Mediterranean countries such as Turkey (Aydin, 2008: González-Wangüemert *et al*., 2014a; 2015) and Greece (Dr. Chatzinikolaou personal communication), where they are caught using trawls at depths of 80–100 meters.

Therefore, considering the increasing fishery pressure on *P. regalis* and the lack of basic information on its biology and population genetics, the main objective of this work was to assess the genetic diversity and population structure of *P. regalis* along the Mediterranean Spanish coast, using two mitochondrial DNA markers (i.e., cytochrome oxidase 1 [COI] and 16S genes) and to provide a morphological description of its populations. Specifically, this study had the following five aims: (i) to assess the pattern of genetic diversity of *P. regalis* along the Mediterranean Spanish coast; (ii) to establish the existence of different populations *versus* stocks under a genetic perspective; (iii) to determine the connectivity patterns among populations; (iv) to discuss the possible fishery effects; and (v) to suggest first recommendations towards the sustainable management of this sea cucumber fishery.

Material and Methods

Sampling

Most of the samples used in this study were collected during the Mediterranean International Trawl Survey (MEDITS) campaign (*http://www.ieo.es*), which was conducted on the trawl fishing grounds along the continental shelf and slope off the Spanish coast. The surveys (May–July 2013) were carried out at several sampling sites (Supplementary Table 1) along the Mediterranean Spanish coast, stretching from the Gibraltar Strait (South Spain) to Catalonia (Northeastern Spain), and were clustered into six localities, according to geographical and hydro-physical criteria. A total of 241 individuals of *P. regalis* were collected: 39 individuals from Catalonia (CT), 74 from Valencia (VL), 37 from Alicante (AL), 9 from Cabo de Gata (CG), 9 from Castell de Ferro (CF) and 73 from Alborán Sea (AB) (Fig. 1; Supplementary Table 1). All individuals were preserved in labelled plastic bags containing absolute ethanol (99%) and stored in sealed containers.

Additionally, 6 individuals from Sicily (Italy) and 4 individuals from Quarteira (South Portugal) were sampled and analysed for inclusion in the haplotype network. In this way, we could assess the presence or absence of the most common haplotypes and the detection of exclusive haplotypes in these localities outside the Western

Table 1. Molecular diversity measures of *Parastichopus regalis* for each location, using 561-bp of COI and 471-bp of 16S rRNA. (N: sample size; Hap: number of haplotypes; Ex.Hap: among brackets the number of exclusive haplotypes; *h*: haplotype diversity; π: nucleotide diversity). (VL: Valencia; CT: Catalonia; AL: Alicante; CF+CG: Castell de Ferro and Cabo de Gata; AB: Alborán Sea).

Locations	N	Hap (Ex.Hap)	Polymorphic Sites	*h*	π
COI					
AB	19	10 (4)	10	0.8830 ± 0.0563	0.0034 ± 0.0023
CG + CF	16	13 (6)	18	0.9750 ± 0.0295	0.0060 ± 0.0037
AL	17	9 (3)	8	0.7868 ± 0.1011	0.0024 ± 0.0017
VL	22	12 (6)	12	0.8528 ± 0.0648	0.0027 ± 0.0019
CT	17	9 (4)	8	0.8603 ± 0.0684	0.0028 ± 0.0019
Total	91	35	36	0.8845 ± 0.0230	0.0035 ± 0.0022
16S					
AB	23	8 (2)	7	0.7470 ± 0.0655	0.0023 ± 0.0017
CG + CF	18	6 (2)	4	0.6524 ± 0.1020	0.0017 ± 0.0014
AL	19	7 (3)	7	0.6608 ± 0.1143	0.0023 ± 0.0018
VL	22	10 (5)	9	0.8587 ± 0.0481	0.0029 ± 0.0021
CT	24	7 (1)	6	0.7489 ± 0.0666	0.0022 ± 0.0017
Total	106	24	18	0.7557 ± 0.0289	0.0024 ± 0.0017

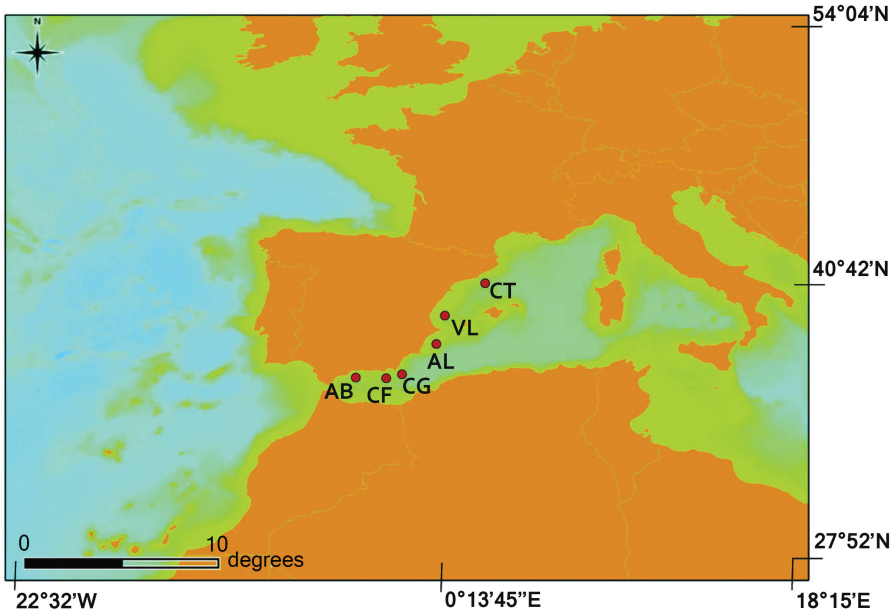

Fig. 1: Map showing the sampling locations in the Mediterranean Spanish coast for *Parastichopus regalis*. (AB: Alborán Sea; CF: Castell de Ferro; CG: Cabo de Gata; AL: Alicante; VL: Valencia; CT: Catalonia).

Mediterranean Sea, thereby evaluating the evolutionary history of this species on a wider spatial scale.

The total length was registered dorsally from the mouth to the anal orifice with an accuracy of ± 0.1 cm. Most of the individuals did not have internal organs due to the evisceration processes of sea cucumbers under stress conditions (e.g., fishery). From each individual, a small piece of internal muscle bands (2x1 cm) was taken for genetic analysis, and the eviscerated weight without water (accuracy ± 0.01 g) was recorded.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from a fragment of muscle following the protocol based on Sambrook *et al.* (1989) with minor modifications (i.e., increasing the time of proteinase K digestion to 24 h and adding 8 µl of proteinase K per sample).

Fragments of the mitochondrial gene COI and the large ribosomal subunit 16S rRNA were amplified by polymerase chain reaction (PCR) using the following primers: COIceF and COIceR, which are specific for echinoderms (Hoareau & Boissin, 2010), and 16Sar-L and 16SB, which are universal primers for invertebrates (Palumbi, 1996). For both genes, double-stranded DNA was PCR amplified in 25 µl volume reaction containing 14.8 µl H_2O, 2.5 µl amplification buffer (10X), 2.5 µl of each primer (forward and reverse; 10 mM), 1.0 µl of $MgCl_2$ (50 mM), 0.2 µl of dNTP mix (25 mM), 0.5 µl of 1 U Taq polymerase (Ecogen) and 1 µl of DNA diluted 1:400. Amplifications were performed using an Applied Biosystems® 2720 Thermal Cycler. For the COI gene, amplification proceeded with an initial denaturation temperature at 95°C for 3 min, then 40 cycles of denaturation at 94°C for 45 s, annealing to 45°C for 1 min and 10 s and extension at 72°C for 1 min and 10 s, followed by a final extension at 72°C for 5 min. For 16S, amplification proceeded with an initial denaturation temperature at 95°C for 3 min, then 40 cycles of denaturation at 94°C for 40 s, annealing to 43°C for 40 s and extension at 72°C for 40 s, followed by a final extension at 72°C for 10 min. A 5-µl sample of each PCR product was mixed with 3 µl of gel red and run on a 2% agarose gel. The products of successful amplifications were sent to the Molecular Biology Services of the Centre of Marine Sciences (CCMAR, Faro, Portugal) and sequenced using an ABI Prism 3130 automated genetic analyser (Applied Biosystems).

Data analysis

mtDNA

The chromatograms were analysed using the FinchTV software v. 1.3.1 (Geospiza Inc), and sequences were aligned using Seaview software v. 4.5.0 (Gouy *et al*., 2010). Genetic diversity was estimated from the haplotype (*h*) and nucleotide (π) diversities (Nei, 1987) using the DnaSP software v. 5.10 (Rozas *et al*., 2003). The ARLEQUIN software v. 3.11 (Excoffier *et al*., 2005) was used to test the genetic differentiation between pairs of localities evaluating the rate of fixation (F_{ST}) using 10,000 random permutations. An exact test of population differentiation based on haplotype frequencies (Raymond & Rousset, 1995) was also performed to test the null hypothesis that the observed haplotype distribution was random with respect to sampling location (10,000 random permutations). Analysis of molecular variance (AMOVA) using haplotype frequencies and implemented

in ARLEQUIN was carried out to examine the hierarchical population structure by pooling the samples into the following geographical groups: i) according to the geographic distribution of the samples, we considered CG+CF & AB (first group) and VL & CT & AL (second group); and ii) according to F_{ST} values and CA results, we defined AB & AL (first group) and CG+CF & VL & CT (second group).

Moreover, populations were spatially clustered using correspondence analysis (CA) conducted with BiodiversityR package in the R software (R Development Core Team, 2010), which employed the haplotype frequencies of populations as variables to visualise the similarities among locations. The correspondence analysis summarised all of the variation in the study area and accommodated each population as a study unit. A statistical parsimony network of the haplotypes was estimated using the TCS software v. 1.21 (Clement *et al*., 2000). This method uses coalescence theory (Hudson, 1990) to determine the limits of parsimony by defining a set of plausible connections among haplotypes with an accumulative probability (>95%) of being true (Templeton *et al*., 1992). Neutrality tests and mismatch distribution analyses were carried out using ARLEQUIN to detect population expansion or bottleneck events and to test for deviations from a strictly neutral model of evolution (Tajima, 1989; Fu, 1997; Roger & Harpending, 1992). To estimate the approximate time of expansion for *P. regalis*, the formula $\tau = 2\mu t$ was used (Rogers & Harpending, 1992). We considered a mutation rate of 0.84% per nucleotide per million years, which was calculated previously for the Holothuriidae family, for both the 16S and COI genes (Borrero-Pèrez *et al.,* 2010).

Morphometric data

The relationship among total length (TL) and eviscerated weight (EW) was established by adopting the linear regression analysis, EW= $a + b$ (TL), where a is the intercept of the regression line (coefficient related to body form) and b is the regression coefficient (exponent indicating isometric growth when equal to 3) (González-Wangüemert *et al*., 2014a; 2015). Differences in the length and weight parameters among locations were analysed by an ANOVA test considering "location" to be a factor. These analyses were performed using the "ade4" (Chessel, 1992) and "mgcv" (Wood, 2006) packages in the R statistical software (R Development Core Team, 2010).

Results

Genetic diversity

A total of 91 individuals of *P. regalis* were analysed for COI and 106 individuals for 16S with a final fragment length of 561 bp and 471 bp, respectively. To have similar sample sizes from each location, samples from Cabo de Gata and Castell de Ferro (CG + CF) were combined for further genetic analysis after verifying that there were no significant genetic differences between the localities (estimating F_{ST} value and genetic distance).

The COI sequences were characterised by low nucleotide (π) and high haplotype (h) diversity; these values were higher than those observed in the 16S dataset. Overall, 35 different haplotypes and 36 polymorphic sites were found using the COI gene data (GENBANK accession numbers: KM048347-KM048381). Two haplotypes (COI-1 and COI-5) were found in all localities, 6 haplotypes were shared between 2 to 4 locations, and 27 haplotypes were singletons. CG + CF showed the highest number of haplotypes (13) and haplotype diversity (0.9750). AL and CT revealed the lowest number of haplotypes (9) and CT exhibited also the lowest haplotype diversity (0.7868). Using the 16S data, a total of 24 haplotypes and 18 polymorphic sites were found (GENBANK accession numbers: KM048323-KM04346). Two haplotypes (16S-1 and 16S-4) were shared among all locations, 6 haplotypes were found among 2 to 4 sites, and 16 were singletons. The highest number of haplotypes (10) and haplotype diversity (0.8587) were found in VL; CG + CF showed the lowest number of haplotypes (6) and haplotype diversity (0.6524; Table 1).

Population genetic structure

The exact test of population differentiation based on haplotype frequencies for the COI gene suggested no population differentiation among the five locations (P = 0.11465). However, the fixation index (F_{ST}) between pairs of locations for the same data set ranged from -0.0079 to 0.0626, finding significant differentiation between AL and CG + CF (F_{ST} = 0.0646, $P < 0.05$) and between AB and CG + CF (F_{ST} = 0.0466, $P < 0.05$) (Table 2). A similar pattern was found using the 16S gene data. No significant differences were revealed using the exact test (P = 0.2390). The F_{ST} values showed significant differentiation between AL and GC + CF (F_{ST} =0.1386, $P < 0.05$), which corroborated the results obtained from the COI gene, and between AL and VL (0.1005, $P < 0.05$) (Table 2).

The analysis of molecular variance (AMOVA) showed the highest differentiation for the COI data when considering AB with AL as the first group, and VL, CT and CG + CF as the second group, according to the F_{ST} results. The test noted no significant differences between the groups (F_{CT} = 0.0363; percentage of variance = 3.63; P = 0.09) but significant differences both between populations within the groups (F_{SC} = 0.0040; percentage of variance = 0.39; $P < 0.05$) and within the populations (F_{ST} = 0.0402; percentage of variance = 95.98; $P < 0.05$). For 16S, neither of the groupings tested showed significant differences either among or within the groups (Table 3).

The correspondence analysis (CA) using the COI haplotype frequencies showed that 61.59% of the total variance was explained by the two first ordination axes and revealed geographic structuring with the three major groups: i) CG + CF on the positive side of Axis I, representing the

Table 2. Population pairwise fixation indices (F_{ST}) for *Parastichopus regalis* based on mtDNA COI (below diagonal) and 16S rRNA (above diagonal). Significant F_{ST} values (*: $P < 0.05$); in bold significant F_{ST} values after Bonferroni correction. (VL: Valencia; CT: Catalonia; AL: Alicante; CF+CG: Castell de Ferro and Cabo de Gata; AB: Alborán Sea).

Locations	AB	CG + CF	AL	VL	CT
AB	-	0.02983	0.00175	0.00986	-0.02466
CG + CF	**0.04661***	-	**0.13861***	-0.01065	-0.00328
AL	-0.01651	**0.06264***	-	**0.10046***	0.04650
VL	0.02451	0.01857	0.02315	-	-0.01258
CT	0.04502	0.01676	0.03719	-0.00787	-

Table 3. Analysis of molecular variance (AMOVA) performed for *Parastichopus regalis* considering two different groups: i) VL & CT & AL / CF+CG & AB; ii) AB & AL/CF+CG & VL & CT. (VL: Valencia; CT: Catalonia; AL: Alicante; CF+CG: Castell de Ferro and Cabo de Gata; AB: Alborán Sea).

gen	Source of variation	Total variance (%)	Fixation indices	*P* value
16S	Among groups (VL&CT&AL/ CF+CG&AB)	-2.08	F_{CT} = -0.01161	0.8241
	Among populations within groups	3.29	F_{SC} = 0.01830	0.0578
	Within populations	98.80	F_{ST} = 0.55036	0.0000*
16S	Among groups (AB&AL/ CG+CF&VL&CT)	5.51	F_{CT} = 0.03167	0.0635
	Among populations within groups	-1.20	F_{SC} = -0.00691	0.7576
	Within populations	95.69	F_{ST} =0.55036	0.0000*
COI	Among groups (VL&CT&AL/ CF+CG&AB)	-0.21	F_{CT} = 0.00203	0.5357
	Among populations within groups	2.73	F_{SC} = 0.02674	0.0362*
	Within populations	97.48	F_{ST} = 0.95524	0.0361*
COI	Among groups (AB&AL/ CG+CF&VL &CT)	3.63	F_{CT} =0.03629	0.0997
	Among populations within groups	0.39	F_{SC} =0.00403	0.0000*
	Within populations	95.98	F_{ST} =0.04018	0.0000*

* significant values (P<0.05)

most different population, ii) VL and CT on the negative side of Axis II, and iii) AB and AL on the positive side of Axis II (Fig. 2a). Furthermore, the CA using the 16S gene data, with the two first ordination axes explaining the 69.29% of the total variance, exhibited a different pattern: CG + CF were located together with CT on the positive side of both axes, and Axis I discriminated VL and AL from the other sites, showing VL on the positive side and AL on the negative side. AB was located on the positive side of Axis II, close to the origin (Fig. 2b).

Haplotype network analysis

The COI statistical parsimony network consisted of two main central haplotypes (COI-1 and COI-5) that were shared among all locations, including QU (South Portugal), and several descendant haplotypes that were closely connected to them in a typical star-like phylogeny (Fig. 3a). All haplotypes were separated by a few mutational steps. Two more haplotypes (COI-3 and COI-23) were shared among all of the Spanish locations except CT. Neither of these haplotypes was found in the QU samples. Exclusive haplotypes were found in all locations: AB (5), CG + CF (7), AL (3), VL (6), CT (5) and QU (1).

The haplotype network obtained for the 16S dataset had the same structure as for the COI data set. One of the two main central haplotypes (16S-4) was shared among all of the locations, including SI and QU. The other main one (16S-1) was present in all of the Mediterranean locations, although it was not found in QU (Fig. 3b). Two other haplotypes (16S-14 and 16S-13) were also shared. All locations exhibited exclusive haplotypes: AB (2), CG + CF (2), AL (3), VL (5), CT (1), QU (1) and SI (2). Most of haplotypes displayed only one or two mutational changes.

Mismatch distribution

The mismatch analysis of both genes in the different samples showed, in general, a pattern of unimodal distribution that was associated with a typical sudden population expansion, although the skewness was different depending on the time since expansion (Fig. 4a-b). Both the COI and 16S datasets displayed negative Tajima's D (only significant in Valencia considering COI gene and Alicante for 16S gene) and Fu's F_S values (all highly significant with $p<0.01$), which suggested population expansion (Table 4).

Morphometry

Two hundred forty-one (241) individuals that were obtained along the Mediterranean Spanish coast were measured. The entire sample had an average length of 13.35 cm (± 3.49) and an average weight of 90.18 g (±

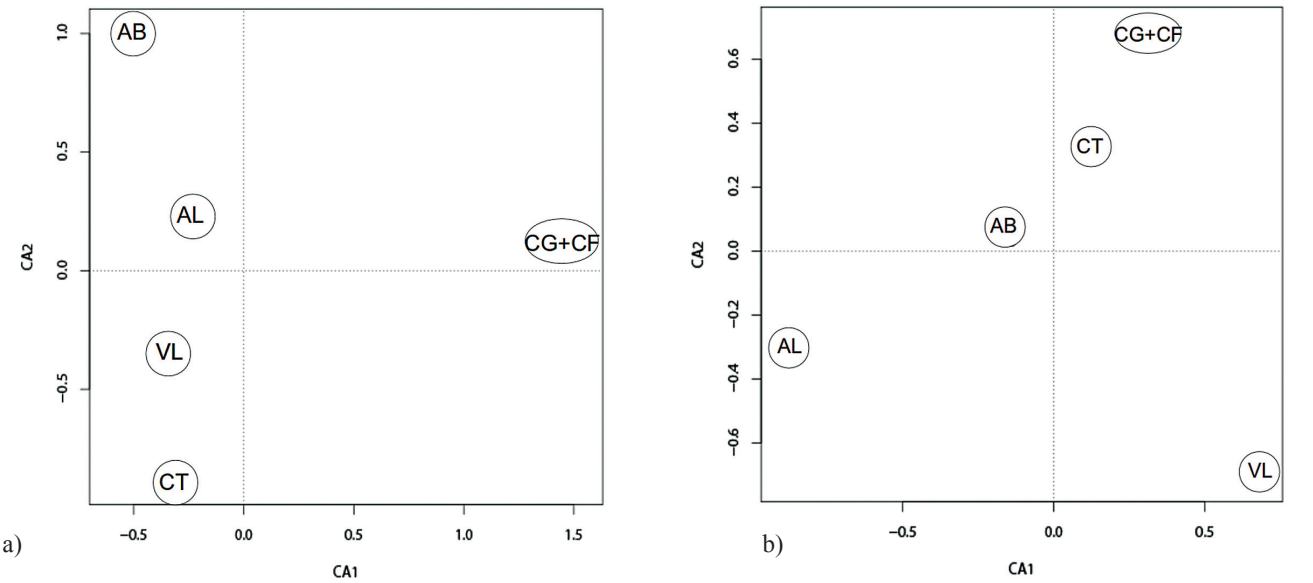

Fig. 2: Correspondence analysis using COI (a) and 16S (b) haplotype frequencies of *Parastichopus regalis.* (AB: Alborán Sea; CG + CF: Cabo de Gata and Castell de Ferro; AL: Alicante; VL: Valencia; CT: Catalonia).

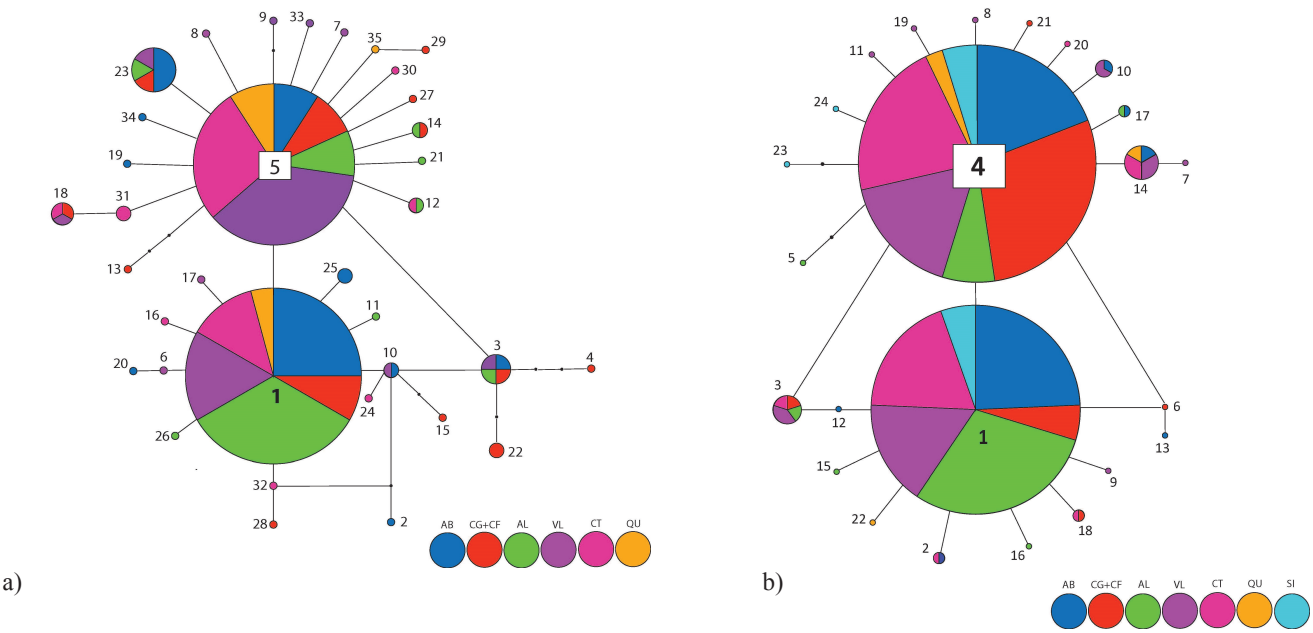

Fig. 3: Statistical parsimony network based on COI (a) and 16S (b) sequences from *Parastichopus regalis*. Each haplotype is defined by a corresponding number. The size of the circle is proportional to the number of individuals. The partitions inside the circle represent the frequency of the haplotype in each locality. White circles surrounding the corresponding number of the haplotype indicate the ancestral haplotype. The black dots correspond to mutational changes.

56.39). The total length of specimens ranged from 6 to 26 cm, and the weight ranged from 3.80 to 273.58 g. Table 5 displays a summary of the main morphometric features from each location. Considering the size-frequency distribution, CT exhibited the most frequent class in 7.5–10 cm, with the highest occurrence of small individuals. AB, AL and VL exhibited a normal distribution, where the middle size organisms were prevalent. The largest sea cucumbers were found in CG + CF. The pattern exhibited by the eviscerated weight frequency distributions corroborated the higher abundance of lighter (smaller) individuals in CT and heavier (bigger) individuals in CG + CF. The eviscerated weight data could be more accurate considering the capture methodology of sea cucumbers and their behaviour after collection.

The total length-eviscerated weight relationship exhibited a significant fitting through linear regression ($y=13.054x-83.831$, $R^2 = 0.6495$; $p<0.05$). Values of *b* equal to 3 indicated that holothurian grew isometrically, whereas those different from 3 indicated allometric

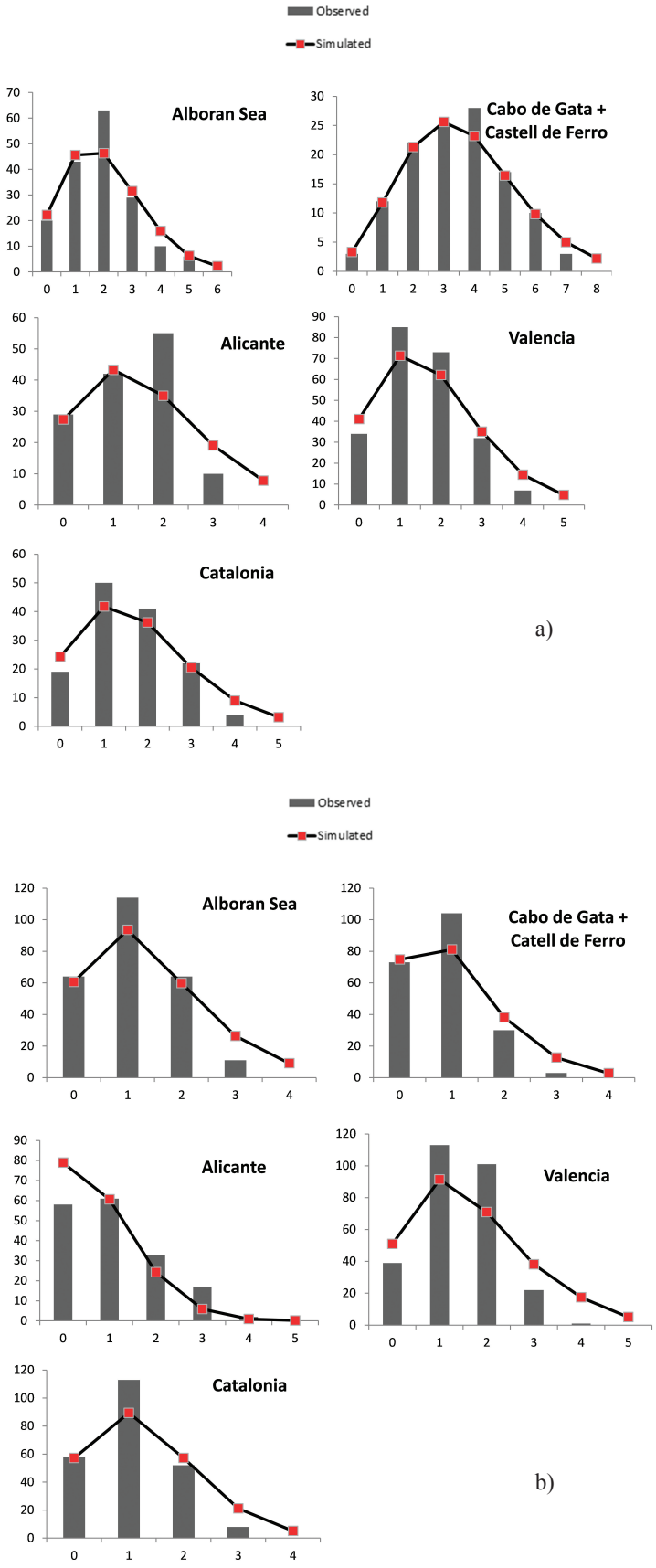

Fig. 4: Pairwise mismatch distribution of *Parastichopus regalis* (COI (a) and 16S (b) genes). Bars represent the observed frequencies of nucleotide differences between pairs of individuals and continuous lines with red dots correspond to the distribution fitted to the data under a model of population expansion.

Table 4. Neutrality tests statistics and demographic expansion parameters for *Parastichopus regalis* (SSD: Sum of square deviations; * significant values (P<0.05)). (VL: Valencia; CT: Catalonia; AL: Alicante; CF+CG: Castell de Ferro and Cabo de Gata; AB: Alborán Sea).

		AB	CG+CF	AL	VL	CT
COI						
	SSD	0.0113	0.0021	0.0257	0.0084	0.0078
	P	0.4000	0.8500	0.0000*	0.2500	0.5000
	Tajima's	-1.1833	-1.4817	-1.5366	-1.8680	-1.1849
	P	0.1060	0.0560	0.0550	0.0190*	0.1160
	Fu's	-5.0829	-8.1511	-5.8432	-9.0040	-5.0312
	P	0.0020*	0.0000*	0.0000*	0.0000*	0.0010*
16S						
	SSD	0.0130	0.0203	0.0107	0.0286	0.0215
	P	0.3000	0.1000	0.3500	0.0500*	0.1000
	Tajima's	-1.3495	-0.7381	-1.5251	-1.3805	-1.1346
	P	0.0760	0.2380	0.0470*	0.0710	0.1600
Fu's		-4.2465	-2.7891	-3.3511	-5.7708	-3.1777
	P	0.0020*	0.0110*	0.0060*	0.0010*	0.0090

Table 5. Length and weight of *P. regalis* from the sampled locations (AB, Alborán Sea; CF, Castell de Ferro; CG, Cabo de Gata; AL, Alicante; VL, Valencia; CT, Catalonia). N: sample size; Min: minimum value; Max: maximum value; Mean: mean values; Std: standard deviance.

Total length (cm)		**N**	**Min**	**Max**	**Mean**	**Std**
	Total	241	6	26	13.35	3.49
	AB	73	8.5	22	13.87	2.70
	CF	9	16	23	19.33	2.19
	CG	9	8	26	18.39	5.48
	AL	537	7.5	17.3	13.89	3.00
	VL	74	7.5	19	13.28	2.65
	CT	39	6	13	9.44	1.60
Eviscerated weight (g)						
	Total	241	3.8	273.58	90.18	56.39
	AB	73	19.05	273.58	116.70	48.24
	CF	9	91.38	257.09	153.39	54.81
	CG	9	11.19	223.9	124.61	81.70
	AL	37	17.33	178.7	104.03	45.16
	VL	74	15.41	200.36	80.74	44.66
	CT	39	3.80	80.00	22.78	16.52

growth. The exponent *b* found in this study suggested the allometric growth of the species.

Furthermore, the differences in the total length and the eviscerated weight were analysed between localities using ANOVAs (Fig. 5 a-b). Significant differences for both parameters (length: F-ratio=31.85, p = 2.2e-16; weight: F-ratio=28.62, p = 2.2e-16) were found. Individuals from CT and VL were smaller and lighter than were animals from the other sites, whereas individuals from CG + CF displayed the largest and the heaviest specimens.

Discussion

The genetic study of *P. regalis* populations along the Spanish Mediterranean coast revealed significant genetic differentiation, primarily between the AB/AL and CG + CF samples (based on the COI marker), whereas the absence of population differentiation between AB and AL and between VL and CT indicated high gene flow among these localities. However, the correspondence analysis based on COI haplotype frequencies suggested that the AB and AL samples may also differ from VL and CT (Fig. 2a). These results were not conclusive and should be confirmed using microsatellite markers to identify the actual stocks of *P. regalis* along the Spanish Mediterranean coast.

Our results suggested a higher level of gene flow among CG + CF and VL/CT populations than among CG+CF and AB/AL populations, despite the closer geographical location of the latter. This preferential gene flow is shaping the population genetic structure of this species in the Spanish Mediterranean coast. In marine benthic invertebrates, gene flow is driven primarily by dispersal of pelagic larvae; in sea cucumbers, the larvae remain in the water column for 13–26 days (Navarro *et al*., 2012; Domínguez-Godino *et al*., 2015). Therefore, sea cucumbers could have high genetic connectivity and show low population differentiation over large geographical scales (Vergara-Chen *et al*., 2010; Valente *et al.,* 2015). Nev-

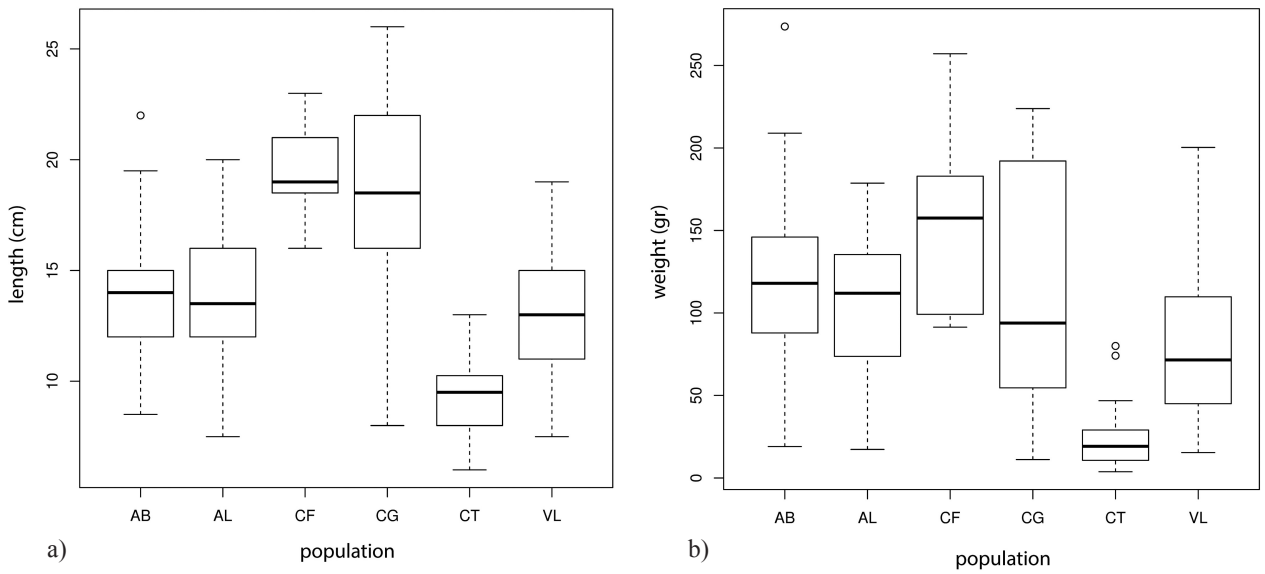

Fig. 5: Mean length (a) and weight (b) in the different sampled locations of *Parastichopus regalis.*

ertheless, genetic differentiation among marine populations is also possible even in species with pelagic larvae, when geographical barriers or biological factors cause some restrictions in gene flow (Riginos & Nachman, 2001; González-Wangüemert *et al.,* 2004; De Croos & Pálsson, 2010; González-Wangüemert *et al.,* 2010; 2011; Borrero-Pérez *et al.*, 2011; González-Wangüemert & Pérez-Ruzafa, 2012; González-Wangüemert & Vergara-Chen, 2014). Thus, our data demonstrated the existence of gene flow barriers among the studied locations, which could be represented by hydrological (eddies and currents), bathymetrical (different depths and slopes influencing the continuity of the habitat for our target species) or environmental factors (temperature, salinity, and chlorophyll). In particular, the current and temperature patterns in the studied area seemed to be in accordance with the genetic differentiation pattern detected among our *P. regalis* populations, especially the differentiation among AB and CG + CF populations and the connectivity between AB and AL (Renault *et al*., 2012; Sayol *et al*., 2013; http://www.socib.eu/?seccion=modelling). The West Alboran gyre close to the AB locality is separated from (or shows only few connections to) the East Alboran gyre close to the CG + CF locality. In fact, the surface temperature pattern showed similar temperatures among the Western gyre (AB locality) and AL area and thus isolated CG + CF with a different temperature register, which could affect the recruitment of the larvae, survival rate, and availability of food resources.

The *P. regalis* populations exhibited high levels of haplotype diversity and low levels of nucleotide diversity. This pattern of genetic diversity can be attributed to a recent population expansion after a period of low population size, thereby enhancing the retention of new mutations (Grant & Bowen, 1998). Previous studies carried out on other Atlantic-Mediterranean holothurians, such as *Holothuria mammata* and *H. arguinensis*, or Mediterranean species, such as *H. polii*, showed a similar genetic pattern (Borrero-Pérez *et al*., 2010; Vergara-Chen *et al*., 2010; Borrero-Pérez *et al*., 2011; Valente *et al*., 2015; Rodrigues *et al*., 2015). Both markers, i.e., COI and 16S genes, have also been used to clarify taxonomic uncertainties, species relationships, biogeography and evolution of the family Stichopodidae (Byrne et al., 2010) and have showed high diversity, especially for the COI gene. This trend was also observed in *P. regalis*; i.e., higher levels of genetic diversity were observed using the COI gene rather than the 16S gene, which can be explained by the higher mutational rate of the former marker and their functional constraints (Calderón *et al*., 2008; Borrero-Pérez *et al.*, 2010; Vergara-Chen *et al*., 2010; Valente *et al*., 2015).

The obtained parsimony networks (based on COI and 16S genes) with the star-like shape also indicated a pattern of demographic expansion, which is in accordance with the mismatch distribution analyses and the Tajima's D and Fu's F_S tests. Two hypotheses could be considered to explain the network pattern: 1) a single expansion event that could have affected both central haplotypes (present in one population) in a similar manner or 2) two expansion events that could have produced the observed star-like pattern if the two main haplotypes were each present in different populations, which were later connected by gene flow. These two expansion events could be linked with colonisation/expansion processes between Mediterranean and Atlantic populations. According to our data, the historical population expansion of *P. regalis* predates the Last Glacial Maximum (LGM), dating to 454 or 203 kya (16S) and 381 or 170 kya (COI), depending on the populations. However, it is not possible to establish the geographic origin of the ancestral populations and the direction of the colonisation events, considering the available data, and more samples from Atlantic localities are

needed to test this hypothesis. These processes (colonisation and recolonisation) have already been described for other sea cucumber species, such as *Holothuria mammata* and *H. arguinensis*, which inhabit this same geographic area (Borrero-Perez *et al.,* 2011, Rodrigues *et al*., 2015), although more complicated networks were found in that species that were perhaps influenced by its evolutionary history and/or by their ecology and biology.

The network based on the 16S gene, which included individuals from QU (South Portugal, Atlantic) and SI (Middle Mediterranean), allowed us to theoretically suggest several scenarios of connectivity at wider spatial scales, although the samples sizes are small for drawing reliable conclusions. Most of the samples from SI showed the two main haplotypes, which were present in all of the localities from the Western Mediterranean Sea, highlighting the potential lack of phylogeographic structure between the Western and Middle basins of the Mediterranean Sea for this species. In the Atlantic locality (QU), only one of the two central haplotypes (16S-4) was found (in 3 of the 4 specimens). The absence of the other central haplotype (16S-1) from the Atlantic, if it is confirmed from broader sampling, would suggest that the second expansion could have occurred only in the Mediterranean Sea. Nevertheless, the singleton found in QU (16S-22) was linked to the second main haplotype (16S-1), which was present in the Mediterranean, suggesting that this central haplotype may also be present in the Atlantic.

Another interesting finding was that the CT sample showed few recent and exclusive haplotypes compared with the other locations studied. This trend could be found in species or populations under fishing pressure (Pérez-Ruzafa *et al*., 2006; González-Wangüemert *et al*., 2012; González-Wangüemert *et al.,* 2015). The absence in CT of shared haplotypes (as COI-23 and COI-3) that were present among all Western Mediterranean populations (AB, CG + CF, AL and VL) may be due to the loss of haplotypes that occurs because of the fishing pressure or may suggest restricted gene flow and isolation with respect to the other populations. Catalonia is an exploited area for this species with an annual mean value of approximately 3 648 Kg landing, and 237 fishing days per year (Ramón *et al*., 2010). Sea cucumber species are especially vulnerable to overfishing because of their low and infrequent recruitment, late age at maturity, high longevity, density-dependent reproductive success and slow growth rate (Uthicke *et al*., 2004; González-Wangüemert *et al*., 2014a).

The potential genetic effects of the fishery pressure on CT were also supported by the morphometric results, which showed that smaller and lighter individuals inhabited this location, as previously noted in another sea cucumber species under heavy fisheries in the Eastern Mediterranean Sea (González-Wangüemert *et al.,* 2014a; 2015). According to Anderson *et al*. (2011), one of the most important fishery effects is the decrease in the size and weight of individuals. Our results in CT, compared to the records from the other sampled locations without fishery pressure, showed exactly this trend, i.e., the loss of individuals belonging to larger size classes and a high frequency of small individuals. The fishery effects on *P. regalis* from CT seem to be the same as those registered by González-Wangüemert *et al*. (2014a) on *H. polii* from Turkey. Those authors assessed the status of three target species (i.e., *H. polii, H. tubulosa* and *H. mammata*) through the distribution of size classes and weight and found the lighter and smaller individuals in heavy fishery areas, mainly for *H. polii,* because this species represents the 80% of the sea cucumber catches in Turkey. Similar results were found when protected and non-protected populations of sea cucumbers were compared in Turkey (González-Wangüemert *et al*., 2015). Despite the scarce information about length, weight and size frequencies of *P. regalis*, our results could be compared with those obtained by Ramón *et al*. (2010). Indeed, they collected the same species along the continent shelf and slope off Mallorca and Menorca islands (non-fishery areas for this species) between 50 m and 800 m in depth and used the same methodology (bottom trawling during MEDITS and BALAR surveys). The lengths of sea cucumbers ranging from 6.5 to 29.5 cm and a mean length of 18.7 cm. The differences with our results (mean length of 13.35 ranging from 6 to 26 cm) could be due to the presence of smaller individuals from the Iberian Peninsula or to holothurian contraction after collection because the estimation of body length is subjected to larger error than weight (González-Wangüemert *et al*., 2014a; 2015).

Focusing on weight, it is notable that there was an occurrence of bigger individuals in the Balearic islands (mean weight of 165.8±77.7 g; Ramón *et al.,* 2010) than along the Spanish continental coast (mean weight of 90.18±56.39 g), although we stress the high standard deviation shown in our data was affected by the low weights registered in individuals from CT (Table 5). The differences in weight among continental and island locations could also have been influenced by the depth of the samplings. Ramón *et al.* (2010) found the heaviest individuals at 200–299 meters, but only the *P. regalis* individuals from CF + CG and AB were sampled close to this depth (202 meters), and these locations had the highest means of weight (153.39 g, 124.61 g and 116.70 g, respectively).

On the other hand, the recent models of chlorophyll concentrations in Alborán Sea and the neighbouring areas showed the highest values in AB and CF + CG (Ruíz *et al.,* 2013), which favoured the diatom-dominated communities and perhaps providing higher availability of food resources to the sea cucumbers inhabiting this area. This hypothesis could help explain the presence of bigger individuals in AB and CF + CG. However, further studies linking environmental variables and descriptors of the *P. regalis* populations are necessary to validate this hypothesis.

Our length-weight relationship corroborated the allometric growth of *P. regalis* detected by Ramón and colleagues, a pattern previously described for most holothurians (Bulteel *et al.,* 1992; Herrero-Pérezrul & Reyes-Bonilla, 2008; González-Wangüemert *et al*., 2014a; 2015). The relationship between length and weight differs in each species and depends primarily on their body shape, thickness of the wall and, within a species, according to the conditions of individuals (González-Wangüemert *et al*., 2014a; 2015).

In conclusion, our results suggested a restricted gene flow between some populations for our target species along the Mediterranean Spanish coast. Nevertheless, several clues lead to considering the existence of gene flow between the Western and Middle basins of Mediterranean Sea and among Atlantic and Mediterranean populations. Therefore, to assess the evolutionary history of this species on a wider spatial scale and to increase the knowledge of its connectivity pattern, it is necessary to undertake an analysis of more samples from Atlantic sites and the Eastern Mediterranean distribution.

This study has supplied initial knowledge on the genetic diversity and structure of this high-priced sea cucumber species and on the potential fishery effects (genetic and morphological) demonstrated in the CT location. Therefore, in our opinion, the first proposals of management for *P. regalis* could be as follows: 1) assessing populations based on their geographical distributions; it is mandatory to determine their general degree of exploitation; 2) establishing a closed fishery season that should be based on the reproductive cycle of the species; and 3) founding protected areas (i.e., not take zones) to conserve healthy populations and favour recruitment in the nearby areas.

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