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Genetic monitoring of the declining European stony sea urchin *Paracentrotus lividus* from the central Bay of Biscay (Asturias, northwest Spain) and attempts to restore its wild populations

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Abstract

1. *Paracentrotus lividus* is a sea urchin with an important ecological role in the Cantabrian Sea ecosystem, where its populations are in severe decline and the regional government has implemented a population restoration strategy with the aim of preserving this valuable marine resource.
2. In this study, genetic monitoring was conducted for the first time in the central area of the southern Bay of Biscay to describe the genetic diversity patterns of *P. lividus* and to assess the potential impacts of conservation and mitigation actions on the wild gene pool. Genetic analyses were performed using the mitochondrial cytochrome b gene and microsatellite loci.
3. Asturian localities showed significant genetic heterogeneity, possibly due to genetic drift, and seemed to constitute a differentiated management unit with regard to other areas of the species' distribution. The genetic diversity analyses comparing wild samples with those subjected to restoration experiments did not show significant negative effects on restored localities.
4. Sea urchins from hatcheries represented 3.5% of the total recaptured individuals (95% accuracy). Even when low hatchery contributions were detected in this work, the results pointed to the necessity of improving the initial autochthonous breeder genetic pool and the aquaculture strategies applied when restoring wild populations to avoid future unwanted negative effects on wild gene pools.

KEYWORDS

conservation aquaculture, management unit, microsatellites, mitigation aquaculture, mitochondrial DNA, Ryman–Laikre, stony sea urchin

Marina Parrondo and Samuel López have contributed equally.

1 | INTRODUCTION

Sea urchins play a critical role in the general functioning of marine ecosystems (Pearse, 2006) by controlling the abundance and distribution of algae and seagrasses in shallow-water marine environments (Boudouresque & Verlaque, 2020). Their role can be especially important on reefs, where they graze on algae and provide settlement surfaces for corals (Knowlton, 2001); moreover, sea urchins help reef recovery after acute disturbance (such as storms or bleaching events) and from overfishing-induced reductions in the number and size of herbivorous fishes (Lessios, 1988). In temperate and subpolar waters, which have high potential for production but are subject to high disturbance from water turbulence (Lawrence, 2020), there is a balance between sea urchin grazing and kelp forest productivity, which leads to stable states that alternate between kelp forests and sea urchin 'barrens' (Pearse, 2006). Moreover, sea urchins are preyed upon by many predators (Pearse, 2006), such as starfish, crabs, fish, and lobsters (Tourón-Besada, 2012), although the main predator of sea urchins is humans (Boudouresque & Verlaque, 2020).

The stony sea urchin, *Paracentrotus lividus*, is widely distributed along the coast of the Mediterranean Sea and the north-eastern coast of the Atlantic Ocean and extends from Ireland and Scotland to southern Morocco, as well as to the Azores, Canary Islands, Madeira, and Cape Verde (Boudouresque & Verlaque, 2020; Lawrence, 2020). It mainly inhabits hard substrates in shallow waters (from the intertidal to 10–20 m depth), where individuals group together in high-density clusters (Tuya et al., 2007).

Stony sea urchins are of commercial interest throughout their distribution range, especially in France and Spain; however, a general decline in *P. lividus* catches has been observed in most producing countries due to overexploitation of their populations as a result of increased market demand and non-selective harvesting practices (Allain, 1975; Byrne, 1990; Ouréns, Naya & Freire, 2015). In Spain, the tradition of *P. lividus* consumption is limited to coastal areas and sea urchin fishery development varies among regions (Ballesteros & García-Rubies, 1987; Sánchez-España, Martínez-Pita & García, 2004; Fernández-Boán, Fernández & Freire, 2012; Ouréns, Naya & Freire, 2015). Currently, Galicia (north-western Iberian Peninsula) leads in *P. lividus* catches, with approximately 700 tons annually (historical data average and standard deviation: 643 ± 121 t; min–max: 301–765 t) (Ouréns, Naya & Freire, 2015; Instituto Galego de Estatistica, 2021); however, Asturias (north-western Iberian Peninsula) is the region where stony sea urchins are most appreciated (Haya de la Sierra, 1989; Ouréns, 2013; de la Uz, Carrasco & Rodríguez, 2018a).

An initial evaluation and mapping of the *P. lividus* populations on the Asturian coast was carried out in 1990 and 1991 to determine the extent and location of the sea urchin banks within the area (de la Hoz et al., 1991). In 2006 and 2007, the Regional Ministry of Rural Development and Natural Resources (RMRDNR) reviewed previous evaluations in which stony sea urchin densities remained stable on the eastern coast of Asturias but suffered a significant decline of 44% on the western coast (Álvarez-Raboso, 2006; Álvarez-Raboso, 2007). Despite the prestige that *P. lividus* have in Asturias, their harvesting was not professionalized, and a large proportion of the stony sea urchins exploited in this region came from recreational harvesting (Ouréns, 2013). Although harvesting was only allowed in the intertidal zone, there had always been very little control of *P. lividus* exploitation. As early as 1981, it was reported that over 90% of the people harvesting stony sea urchins were unauthorized (Alcázar et al., 1981), which reinforces that most of this fishery was unmanaged and a hidden economy. Frequently, harvesters sold their catch directly to local consumers without auctioning it in a fish market, which is where the official data of landed sea products that are used for management by the administration are gathered. Nonetheless, the reported official captures have declined dramatically in recent years, from more than 72 tons in 2011 to less than 5 tons in 2016 (Dirección General de Pesca Marítima del Gobierno del Principado de Asturias, 2019).

Additionally, over the last decades, there has been a significant change in the composition of algae in the intertidal region of Asturias, where a westward retreat of dense populations of various macroalgae, such as *Saccorhiza polyschides*, *Laminaria ochroleuca*, *Laminaria hyperborea* and *Fucus serratus*, has been reported (Anadón et al., 2009; Álvarez-Losada et al., 2020). The disappearance of stony sea urchins along the Asturias coast took place at the same time as the retreat of macroalgae. It has been argued that the disappearance of these macroalgae was due to an increase in water temperature in the area (Fernández, 2011). Other environmental phenomena, such as the frequency and intensity of storms, factors related to emersion, or a limited availability of nutrients due to the temporal variation and intensity of upwelling, have also been suggested as causes of this decline (Álvarez-Losada et al., 2020). The reduction in these algae, which represented available biomass for the stony sea urchin populations, caused a rapid biotic homogenization with functional and ecological impoverishments of the whole region (Álvarez-Losada et al., 2020) and may be a key factor in the reduction of *P. lividus* populations (Fernández, 2011). In addition, increased ocean temperatures are known to promote disease emergence (Harvell et al., 1999; Lafferty, Porter & Ford, 2004) and reduce pathogen resistance to equinoids (Scheibling & Stephenson, 1984; Miller, 1985). Thus, the decline in *P. lividus* may be related to overfishing and climate change and promoted by potential diseases such as bald sea urchin disease (Maes & Jangoux, 1984), which has strongly affected the southernmost populations of *P. lividus* (Girard et al., 2012).

Overexploitation and a changing environment have led to a critical situation for stony sea urchins in Asturias; therefore, a ban was imposed on both professional and recreational fishing from 16 April to 14 December 2013 (Gobierno del Principado de Asturias, 2013). In 2016, technicians from the RMRDNR observed that the abundance had barely increased, especially regarding individuals of commercial size (i.e. horizontal test diameter (htd) >55 mm; which were very rare), and the decision to reinstate a year-round ban was made (Gobierno del Principado de Asturias, 2016). Since then, the RMRDNR has conducted annual surveys to assess the status of the resource, and they have recommended that the government of Asturias extend the

reviewable ban each year because the populations have not yet recovered (Gobierno del Principado de Asturias, 2016; Gobierno del Principado de Asturias, 2020).

The alarming shortage of sea urchins in Asturias led to a reaction by the government of Asturias, which decided to undertake a population restoration strategy through the RMRDNR (Figure 1) with the aim of preserving this marine resource, which has such an important role in the ecology, economy, and culture of the region. The regional government efforts to safeguard *P. lividus* fall within classical conservation and mitigation aquaculture processes. This strategy was planned to preserve and restore the self-sustainability of the species and compensate for reduced natural production associated with lost habitats or functional elements of the ecosystem (Utter & Epifanio, 2002). With the goal of safeguarding stony sea urchins in mind, juvenile *P. lividus* began to be reared at the facilities of the Centre for Fisheries Experimentation in Castropol (Figure 1) (de la Uz et al., 2013; de la Uz, Carrasco & Rodríguez, 2018a). Briefly, adults were collected from the natural environment and spawning was induced individually in beakers. Then, the oocytes were pooled and 2–3 ml of sperm from each selected male were added. After 48 hours, the larvae were distributed in common tanks (200 L) for onward larval culture (de la Uz et al., 2013). Competent larvae were obtained by 18 days and they were transferred into tanks containing benthic diatoms where they completed metamorphosis to the juvenile stage. When the juveniles had grown to > 5 mm they were fed with macroalgae until release.

Juveniles were released at two shallow subtidal sites along the west coast of Asturias: Punta Focicón (Lluarca) and Ensenada de La Arquina (Cuideiru) (Figure 1). Site selection included areas that had previously showed significant aggregations of *P. lividus* (de la Hoz et al., 1991; Álvarez-Raboso, 2006; Álvarez-Raboso, 2007). Predation is the greatest obstacle to the survival of released juveniles, and it is known that the complexity of the substrate and the abundance of adults are important factors determining the success of sea urchin recruitment (Hereu, 2005; Clemente et al., 2013; Oliva et al., 2016). To improve the settlement of juveniles and the recovery of sea urchin aggregations, the RMRDNR decided to carry out a complementary strategy that included a prior translocation of adults to the selected sites (de la Uz et al., 2018b). In June 2015, 800 kg (∼13,000 specimens) of adult sea urchins (htd: >55 mm) brought from Cape San Lorenzo (Xixón) were released at Punta Focicón (Lluarca). Two days later, 8,664 juveniles (htd: 10–15 mm, 5,060 (58.4%); htd: 15–20 mm, 2,891 (33.4%); htd: 20–25 mm, 713 (8.2%)) were released at that location (Figure 1a). In June 2016, a further 13,233 juveniles (htd: 10–15 mm, 6,456 (48.8%); htd: 15–20 mm, 5,918 (44.7%); htd: 20–25 mm, 802 (6.1%); htd: 25–30 mm, 57 (0.4%)) were released at the same location in Lluarca (Figure 1b). In September 2016, 700 kg of adult stony sea urchins (∼12,000 specimens) from Cape San Lorenzo (Xixón) were released in Ensenada de La Arquina (Cuideiru), and in October 2016, after checking that the adults had become established, another 8,730 juveniles were released at that location (htd: 10–15 mm, 6,722 (77%); htd: 15–20 mm, 1,509 (17.3%); htd: 20–25 mm,

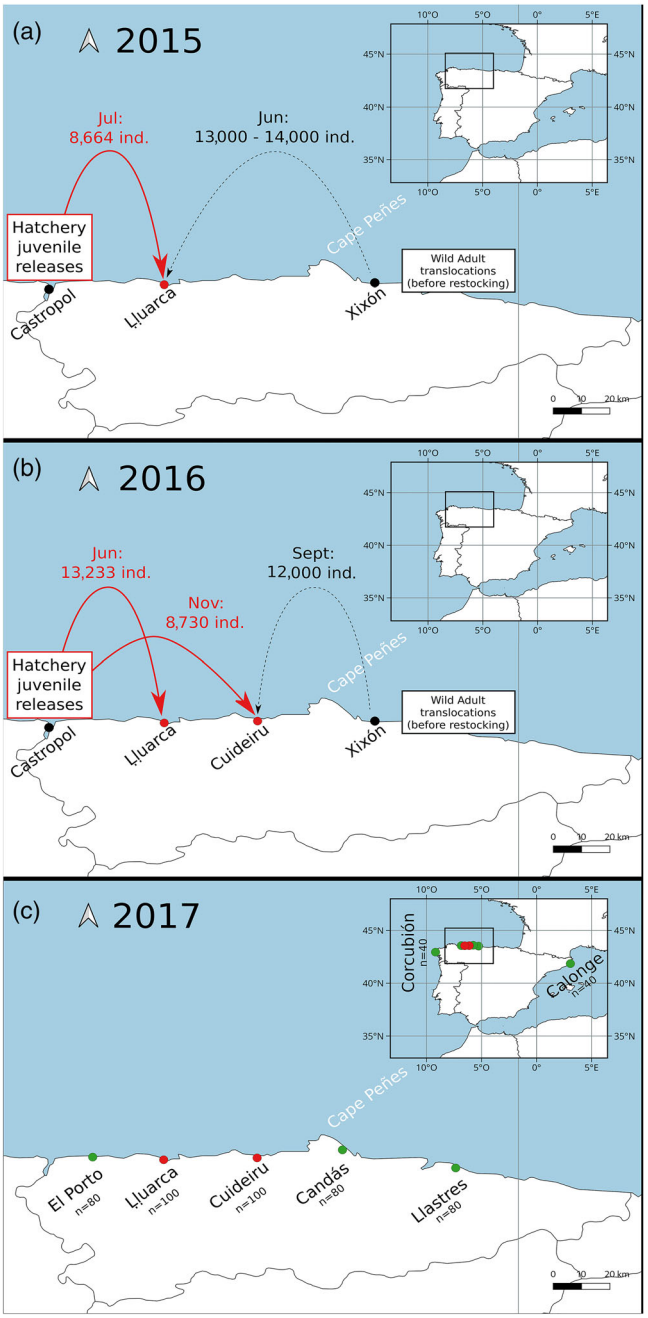

FIGURE 1 Description of the population restoration actions conducted in the southern central area of the Bay of Biscay (Asturias) for the species *Paracentrotus lividus* and sampling sites in this study from the Atlantic and Mediterranean areas. (a) Population restoration action conducted on Lluarca in 2015 with hatchery juveniles obtained from wild adults from Castropol and Lluarca within the hatchery (in red). The dashed black line represents the preceding adult's translocation from Xixón to the locality. (b) Population restoration action conducted on Lluarca and Cuideiru in 2016 with hatchery juveniles obtained from wild adults from Castropol within the hatchery (in red). The dashed black line represents prior adult's translocation from Xixón. (c) Localization and sample sizes for the non-restored (in green) and restored (in red) localities sampled in this study. The localization for Atlantic (Corcubión) and Mediterranean (Calonge) samples are also indicated

395 (4.5%); htd: 25–30 mm, 90 (1%); htd: 35–40 mm, 14 (0.2%)) (Figure 1b).

The major risks of conservation/mitigation aquaculture actions are potential negative interactions with wild individuals from the native populations. These practices may entail a number of genetic risks that are widely recognized and documented in the literature and are summarized as (i) loss of genetic diversity, (ii) loss of fitness, (iii) change in population composition, and (iv) change in population structure (Laikre et al., 2010). In addition, in combined captive–wild systems, what is known as the Ryman–Laikre effect can occur, which is an increase in inbreeding and a reduction in total effective population size that arises when a few captive parents produce a large number of offspring (Ryman & Laikre, 1991; Christie et al., 2012; Waples et al., 2016). Previous studies on *P. lividus* have shown that hatchery-reared populations were less diverse and diverged significantly from wild populations, and they showed a very small effective population size and high degree of relatedness between individuals (Segovia-Viadero et al., 2016), which can severely damage the natural populations to which they are introduced. However, despite the recognition and documentation of adverse genetic impacts on wild populations, little effort has been devoted to their actual monitoring (Laikre et al., 2010) and the studies into the impacts on wild conspecifics and other competitive species are missing from the literature (Kitada, 2018).

Unfortunately, the threatened *P. lividus* gene pool in the Cantabrian Sea has not been deeply studied. This knowledge gap hinders a better understanding of the biogeography of the species, effects of past and current demographic events within the area, the size of the population, and effects of evolutionary processes (selection, genetic drift, mutation and gene flow) on that gene pool. All these data (when available) can help to preserve and manage marine resources in terms of conservation and sustainable management. There are no data about the effects of the recent pilot restoration programme conducted by RMRDNR across the Asturias coast. In this work, genetic monitoring of the *P. lividus* populations in the central area of the Bay of Biscay (Asturias) was undertaken using the cytochrome b gene (CytB) from mitochondrial DNA and microsatellite markers previously described by Calderón, Turón & Pascual (2009). The main aims were (i) to obtain a deeper understanding of the population history and current dynamics of *P. lividus* within the area, and (ii) to gather data on the effects of the population restoration strategy for *P. lividus* conducted in Asturias that could aid in making future conservation and management decisions by the regional government.

2 | MATERIALS AND METHODS

All experiments performed in the present study were approved by the Research Ethics Committee of the Principality of Asturias (ref. no. 166/19).

2.1 | Study area and sampling methods for genetic diversity analyses within/among wild and restored localities

A total of 520 individuals were collected from Atlantic and Mediterranean wild sea urchin populations in 2017 (Figure 1c). This consisted of 80 individuals from each of the non-restored localities in Asturias (Bay of Biscay) of El Porto (also known as Viavélez; htd min–max: 55.070–81.610 mm), Candás (htd min–max: 55.900–72.800 mm), and Llastres (htd min–max: 50.500–71.400 mm), and 40 individuals each from Corcubión (Galicia, Atlantic Ocean, htd min–max: 56.640–73.710 mm) and Calonge (Catalonia, Mediterranean Sea, htd min–max: 56.600–70.080 mm). *Paracentrotus lividus* maximum size is normally described as approximately 75 mm htd (González-Irusta, 2009), and it is important to note that there is large size variation in individuals of the same age (Crapp & Willis, 1975; Allain, 1978; Haya de la Sierra, 1989; Turon et al., 1995). In addition, a sample of 200 juvenile individuals was collected from restored localities in Asturias; namely, Punta de Focicón, Lluarca (100 individuals) and Ensenada de La Arquina, Cuideiru (100 individuals) in 2017 (1 year and 2 years after the restoration actions in 2016 and 2015 respectively). For calculating the size of the *P. lividus* juvenile individuals to be sampled in the restored localities, the annual growth rate in the natural environment was taken into account. In Asturias, Haya de la Sierra (1989) reported a maximum growth rate of 9 mm htd per year (at 3 years of age); and in Galicia, Ouréns et al. (2013) reported a maximum growth rate of 15 mm htd per year (also at 3 years of age). For sample collection, an approximate growth rate of 10 mm per year was estimated. Divers were instructed to collect samples of approximately 30 mm htd in Lluarca, and approximately 20 mm htd in Cuideiru. Thus, in Lluarca, those stony sea urchins released in 2015 would have grown to 30–35 mm htd and the ones released in 2016 would have grown until 20–25 mm htd. The size range of individuals sampled from Lluarca was 18.40–38.11 mm htd, so would include the urchins from the two restocking actions. In the case of Cuideiru, with a single restocking in 2016, individuals in the range 10.14–26.68 mm htd were sampled (Figure 1c, Tables 1 and 2).

All individuals were labelled and stored in absolute ethanol at room temperature. The sexes of all adult individuals were determined by examining the fresh gonads following Gago, Range & Luis (2003) and Rocha et al. (2019).

2.2 | Sampling methods for genetic diversity and parentage analyses conducted on available hatchery samples

During the course of the restoration activities in 2015 and 2016, the RMRDNR froze and stored a partial representation of the breeders used to obtain progenies in the hatchery (91% of the 101 parents involved) and a representative number of the siblings of the stony sea

TABLE 1 Genetic diversity in wild and restored localities of for *Paracentrotus lividus* populations in the Atlantic and Mediterranean areas using mitochondrial DNA

Region	Origin	Location	Code	N	n_h	n_{hs}	D_h	π	S	TD	F^*	AY*	Fs	R_2	r_g	Pi(a)/Pi(s)
Asturias (Bay of Biscay)	W	El Porto	Ep	22	18	12	0.978	0.008	28	−1.443 (0.061)	−2.101* (0.028)	−0.250 (0.440)	−11.234* (0.000)	0.039* (0.000)	0.006* (0.001)	0.152
	R	**Lluarca**	**Lu**	**24**	**21**	**16**	**0.986**	**0.009**	**36**	**−1.621* (0.038)**	**−1.903* (0.044)**	**−1.450 (0.066)**	**−14.762* (0.000)**	**0.039* (0.000)**	**0.006* (0.000)**	**0.184**
	R	**Cuideiru**	**Cu**	**31**	**25**	**15**	**0.985**	**0.012**	**48**	**−1.553* (0.045)**	**−1.274 (0.121)**	**−1.664* (0.041)**	**−14.467* (0.000)**	**0.039* (0.000)**	**0.006* (0.000)**	**0.326**
	W	Candás	Ca	21	21	18	1.000	0.019	56	−1.136 (0.131)	−1.417 (0.100)	−0.526 (0.329)	−12.513* (0.000)	0.039* (0.000)	0.006* (0.001)	0.691
	W	Llastres	La	20	18	15	0.984	0.009	27	−1.238 (0.114)	−1.744 (0.071)	−0.564 (0.322)	−12.496* (0.000)	0.039* (0.000)	0.006* (0.000)	0.263
Galicia (Atlantic)	W	Corcubión	At	23	21	17	0.988	0.007	24	−1.194 (0.127)	−1.328 (0.118)	−0.841 (0.230)	−18.719* (0.000)	0.039* (0.000)	0.006* (0.000)	0.157
Catalonia (Mediterranean)	W	Calonge	Me	28	27	24	0.997	0.017	50	−0.840 (0.227)	−0.642 (0.254)	−0.920 (0.196)	−18.416* (0.0000)	0.039* (0.000)	0.006* (0.001)	0.353

Note: Data in bold for restored localities.

Abbreviations: AY*, Achaz Y^*; D_h, haplotype diversity; F^*, Fu & Li's F^*; Fs, Fu's Fs; N, sample size; n_h, number of haplotypes; n_{hs}, number of site-specific haplotypes; π, nucleotide diversity; Pi(a)/Pi(s), proportion of non-synonymous by synonymous mutations using Jukes and Cantor corrections; R_2, Ramos-Onsins & Roza's's R_2; r_g, raggedness; R, restored; S, number of segregating sites; TD, Tajima's D; W, wild.

*$P < 0.05$ for neutrality tests after 1,000 coalescent simulations and using theta estimates from raw data (DnaSP 6.11.1 (Librado & Rozas, 2009)).

urchins released at the different locations. The following were available:

- Ninety-two wild breeders (B; htd min–max: 38.200–73.300 mm), which were used to obtain juveniles in the restoration campaigns of 2015 and 2016 (Table 3).
- A total of 250 offspring (O) with known broodstock origins were also available. These juveniles were raised in hatchery facilities (Castropol) and comprised 50 individuals from each broodstock and year (htd min–max: 12.10–27.20 mm) (Table 3). In this case, "known origin" indicates that they were siblings of the stony sea urchins released in the restored areas under study and that the broodstock from which these offspring were obtained was known.

It was not possible to obtain samples from the specimens used to carry out prior translocations of adult stony sea urchins from Cape San Lorenzo (Xixón) to the restored areas.

2.3 | DNA extractions

A portion of muscle was taken from each urchin's Aristotle's lantern, and genomic DNA was extracted using the Mini QIAamp DNA Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. Once the DNA was extracted, it was stored at −20 °C until use.

2.4 | Mitochondrial and microsatellite polymerase chain reaction amplifications

Mitochondrial DNA analyses were conducted on 200 samples using approximately 30 individuals that were randomly sampled from each wild and restored population. The primer pairs described by Maltagliati et al. (2010) for partial amplifications of the gene cytochrome b (CytB) were used. The optimal reagent concentrations for polymerase chain reaction (PCR) were 0.5 μM primers, 250 μM deoxynucleoside triphosphates, 2 mM magnesium chloride, Green GoTaq® Flexi Buffer (Promega Corporation, Fitchburg, WI, USA) (1×) and 0.03 U μl^{-1} GoTaq G2 Flexi Polymerase (Promega Corporation). Reactions were carried out in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using an amplification profile of 35 cycles of 30 s denaturing phase at 94 °C, 30 s annealing phase at 42 °C, and 2 min extension phase at 72 °C, followed by 7 min at 72 °C for the final extension. The PCR products were sent to Macrogen Spain to be sequenced in the forward and reverse orientations using the classic Sanger sequencing method.

Nine microsatellite markers previously described by Calderón, Turón & Pascual (2009) were also used in this work. All microsatellite markers were individually amplified in seven individuals to test the markers. PCR was conducted in a 20 μl total volume with Green GoTaq® Flexi Buffer (Promega Corporation) (1×), magnesium chloride (2.5 mM), deoxynucleoside triphosphates

TABLE 2 Genetic diversity in wild and restored localities of for *Paracentrotus lividus* populations in the Atlantic and Mediterranean areas using microsatellites markers

Region	Origin	Location	Code	*N*	N_A	A_P	A_R	H_O	H_E	F_{IS}	R_{xy}	TPM
Asturias (Bay of Biscay)	W	El Porto	Ep	80	22.375	2	11.300	0.572	0.898	0.365*	0.008	0.679
	R	**Lluarca**	**Lu**	**100**	**24.375**	**5**	**11.400**	**0.560**	**0.899**	**0.379***	**0.007**	**0.962**
	R	**Cuideiru**	**Cu**	**100**	**25.125**	**3**	**11.500**	**0.577**	**0.901**	**0.361***	**0.004**	**0.986**
	W	Candás	Ca	80	23.375	7	11.400	0.550	0.888	0.382*	0.018	0.902
	W	Llastres	La	80	24.875	5	11.500	0.565	0.906	0.377*	0.000	0.972
Galicia (Atlantic)	W	Corcubión	At	40	19.500	2	11.200	0.619	0.899	0.315*	0.008	0.769
Catalonia (Mediterranean)	W	Calonge	Me	40	17.750	4	10.900	0.575	0.895	0.360*	0.010	0.726

Note: Data in bold for restored localities.

Abbreviations: A_P, number of private alleles; A_R, allelic richness for the minor possible number of diploid individuals by sample, $n = 11$; F_{IS}, degree of departure from expected Hardy–Weinberg proportions within samples ($P = 0.0004$); H_E, expected heterozygosity; H_O, observed heterozygosity; *N*, sample size; N_A, number of alleles per locus; R, restored; R_{xy}, relatedness values following Queller & Goodnight (1989); TPM, bottleneck TPM one-tailed Wilcoxon tests *P*-values; W, wild.

*$P < 0.05$.

(0.5 mM), 0.2 μM of each primer, 0.1 U of GoTaq G2 Flexi Polymerase (Promega Corporation), 50 ng of DNA and water. The PCR programme included an initial 5 min denaturation at 95 °C, 30 cycles of denaturation at 95 °C for 30 s, followed by annealing at an optimal temperature for 30 s (Calderón, Turón & Pascual, 2009) and elongation at 72 °C for 30 s. The PCR products were visualized using electrophoresis on a 2% agarose gel stained with SimplySafe™ (EURx, Gdańsk, Poland). The PCR products were sent to Scientific and Technical Services from the University of Oviedo for fragment analysis using capillary electrophoresis with an ABI PRISM 3130xl DNA analyser (Applied Biosystems) and the GeneScan 500 LIZ standard (Applied Biosystems). One of the previously reported markers (Pl-Hist) was not reliable due to the difficulty of achieving correct genotyping; thus, it was discarded from further analyses.

To reduce costs and time, two new multiple PCRs were designed with Multiplex Manager software (Holleley & Geerts, 2009). The multiple PCR designs were first tested on seven individuals, and according to the needs of each process, the concentration of each marker was varied separately until reaching an optimal fluorochrome signal intensity for the genotyping of all individuals. The amplification cycles were performed in a 2720 Thermal Cycler (Applied Biosystems) following the manufacturer's specific instructions for multiple PCR on a final volume of 13 μl containing 50 ng of DNA template, Qiagen Multiplex PCR Kit (1×), and water. Forward primers were fluorescently labelled. The primer combinations and initial concentrations C_I were as follows. PLM5: Pl-32 (PET™, $C_I = 0.5$ μM), Pl-F (NED™, $C_I = 0.4$ μM), Pl-L (VIC™, $C_I = 0.2$ μM), and Pl-C (6-FAM™, $C_I = 0.3$ μM); and PLM4: Pl-B (PET, $C_I = 0.3$ μM), Pl-T (NED, $C_I = 0.2$ μM), Pl-28 (VIC, $C_I = 0.6$ μM), and Pl-15 (6-FAM, $C_I = 0.2$ μM). The PCRs were carried out with initial denaturation for 15 min at 95 °C, followed by 40 cycles of denaturation for 30 s at 94 °C, annealing for 90 s at 60 °C, and elongation for 60 s at 72 °C, and a final elongation step for 30 min at 60 °C. The PCR products were sent for analysis to Scientific Technical Services of the University of Oviedo. Individual genotypes were scored after analysing the amplification products using Genemapper 4.0 (Applied Biosystems).

2.5 | Mitochondrial genetic analysis

BioEdit 7.0.5.3 software (Hall, 1999) was used to manually edit and check every mitochondrial sample sequence. Afterwards, the BLAST (Basic Local Alignment Search Tool from NCBI) web service was used to confirm that the samples' genetic identity was indeed *P. lividus*, with 98% certainty of identity used as the cut-off limit (Madden, 2013). The MUSCLE algorithm (Edgar, 2004) was used to align the sequences, and DnaSP 6.11.1 (Librado & Rozas, 2009) was used to obtain the diversity data. This software also provided information about the samples' past demographics and dynamics through neutrality tests, such as Tajima's *D* (Tajima, 1989), *F** (Fu & Li, 1993), *Y** (Achaz, 2008), Fu's *F* (Fu, 1997), Ramos-Onsins and Rozas's R_2 (Ramos-Onsins & Rozas, 2002), and raggedness statistic r_g (Harpending et al., 1993).

Arlequin 3.5 software (Excoffier, Laval & Schneider, 2005) was used to study the mitochondrial population parameters based on the genetic data. A comparison between localities was performed using Φ_{ST} index. Analysis of molecular variance tests were performed to determine the degree of molecular differences within and between the localities and between the groups of defined populations. Finally, the 'adegenet' and 'vegan' packages in R were again used to calculate the genetic differentiation Φ_{ST} and clustering with a discriminant analysis of principal components (DAPC; Jombart, 2008; Jombart, Devillard & Balloux, 2010; Oksanen et al., 2018) and to test the stress of the DAPC. Tolerable stress levels were considered below 0.2 (Oksanen et al., 2018). Moreover, the Network 10.2 program using the median-joining model (Bandelt, Forster & Röhl, 1999; Fluxus Technology Ltd, 2021) was applied to obtain a haplotype network in

TABLE 3 Genetic diversity in breeders and offspring of *Paracentrotus lividus* after genetic analysis using eight microsatellites in the hatchery from Asturias, Bay of Biscay

Region	Origin	Location	Spawning	Code	*N*	*M*	*F*	N_A	A_R	H_O	H_E	F_{IS}	R_{xy}	TPM	N_e	CI
Breeders																
Asturias	Wild	La Cruz, Castropol	April 28, 2014	C_2014_B	30	15	15	16.625	11.000	0.577	0.896	0.360*	0.009	0.962	∞	107.7/∞
Asturias	Wild	Cape Bustu, Lluarca	February 11, 2015	L_2015_B	12	6	6	9.750	9.500	0.615	0.892	0.321*	0.009	0.003*	9.3	1.6/∞
Asturias	Wild	La Cruz, Castropol	May 20, 2015	C_2015_B	14	6	8	12.625	11.300	0.571	0.911	0.382*	−0.006	0.230	214.7	32.4/∞
Asturias	Wild	La Cruz, Castropol	March 11, 2016	C_2016_B	20	10	10	13.875	10.600	0.506	0.879	0.431*	0.013	0.808	∞	107.5/∞
Asturias	Wild	El Porto	May 17, 2016	P_2016_B	16	5	11	13.000	11.100	0.586	0.915	0.367*	−0.012	0.097	326.4	57.3/∞
				Average	18.4			13.175	10.700	0.571	0.899	0.372*				
Offspring																
Asturias	Culture	—	—	C_2014_O	50	—		17.000	9.700	0.650	0.857	0.244*	0.073	1.000	30.0	16.8/63.5
Asturias	Culture	—	—	L_2015_O	50	—		10.750	8.100	0.574	0.838	0.318*	0.098	0.191	9.3	5.3/14.3
Asturias	Culture	—	—	C_2015_O	50	—		12.875	8.200	0.575	0.801	0.283*	0.176	0.808	24.9	16.7/39.1
Asturias	Culture	—	—	C_2016_O	50	—		13.375	8.800	0.600	0.848	0.294*	0.077	0.808	18.1	12.6/26.4
Asturias	Culture	—	—	P_2016_O	50	—		11.625	8.100	0.639	0.838	0.240*	0.103	0.472	15.4	10.1/23.6
				Average	50			13.125	8.580	0.608	0.836	0.276*				

Abbreviations: A_R, allelic richness for the minor possible number of diploid individuals by sample, $n = 11$; CI, N_e 95% confidence interval; F_{IS}, degree of departure from expected Hardy–Weinberg proportions within samples ($P = 0.0004$); H_E, expected heterozygosity; H_O, observed heterozygosity; *N*, sample size; N_A, number of alleles per locus; N_e, effective population sizes; R_{xy}, relatedness values following Queller & Goodnight (1989); TPM, bottleneck TPM one-tailed Wilcoxon tests *P*-values.

*$P < 0.05$.

P. lividus. Microsoft Office Excel 2016 and R Commander 2.4 (Fox, 2005) were used for the average comparisons. Bonferroni corrections were applied to multiple comparisons.

2.6 | Microsatellite genetic analysis

Genetic diversity analyses were also conducted in wild and restored areas using microsatellites. The allele frequencies, number of alleles per population N_A, observed heterozygosity H_O, and unbiased expected heterozygosity H_E were calculated using Genetix 4.05 (Belkhir et al., 2004). The polymorphic information content of each of the microsatellite markers was estimated using Cervus 3.0 (Kalinowski, Taper & Marshall, 2007). Possible deviations from the expected proportions in Hardy–Weinberg equilibrium and linkage disequilibrium for each locus and population were assessed using FSTAT 2.94 (Goudet, 1995). In addition, possible genotyping errors and null allele frequency estimates were determined using Micro-checker 2.2.3 (Van Oosterhout et al., 2004) and FreeNA (Chapuis & Estoup, 2007), with the number of replicates fixed to 10,000.

FSTAT 2.94 software was used to determine the fixation indexes (*F*-statistics). The allelic richness A_R and total variation in gene frequency F_{IT} separated into components of variation occurring within the F_{IS} and among the F_{ST} for the samples for each locus were determined following the method described in Weir & Cockerham (1984). The significance levels of F_{IS} were estimated by randomizing the alleles within samples 10,000 times, after which the Bonferroni correction was applied (Rice, 1989). In addition, F_{ST} values were estimated using FreeNA, which estimated the unbiased F_{ST} following the ENA method (Chapuis & Estoup, 2007). The F_{ST} values among the samples and *P*-values were also calculated using FSTAT 2.94 (Goudet, 1995). To assess the significance levels of F_{ST}, multilocus genotypes were randomized between pairs of samples (10,000 permutations), and the significance was then calculated by applying the Bonferroni correction (Rice, 1989). The differences among wild and restored localities for several statistics (A_R, H_O, H_E, F_{IS}, F_{ST}, relatedness *R*, and corrected relatedness) were determined using the two-sided statistical analysis included in the FSTAT software. Moreover, for each population, the number of private alleles was calculated with GenAlEx 6.503 (Peakall & Smouse, 2012). The 'adegenet' package in R was used to estimate the genetic differentiation and visualize individual clustering with discriminant analysis of principal components (DAPC; Jombart, 2008; Jombart, Devillard & Balloux, 2010) among study localities and including breeders and siblings of the offspring used for population restoration strategies in Asturias. Structure 2.3.4 (Pritchard, Stephens & Donnelly, 2000) was used to assess possible clustering in all samples from the three regions (Galicia, Asturias, and Catalonia) using microsatellite data. The settings used were an admixture model from $K = 1$ to $K = 7$, with a burn-in period of 50,000 iterations in 500,000 Markov chain Monte Carlo repetitions. The most likely value of *K* was chosen following Evanno, Regnaut & Goudet (2005) and Structure Harvester (Earl & vonHoldt, 2012).

The relatedness between individuals R_{xy} was estimated with the 'related' package in R (Pew et al., 2015). The relative performances of the Wang (Wang, 2002), LynchLi (Li, Weeks & Chakravarti, 1993), LynchRd (Lynch & Ritland, 1999), and QuellerGT (Queller & Goodnight, 1989) relatedness estimators were tested through comparison of the observed values with expected values generated from a simulated sample set of 400 individuals of known relatedness (100 each of parent–offspring ($R_{xy} = 0.500$), full-sib (0.500), half-sib (0.250), and unrelated pairs (0.000)). The results (data not shown) showed that QuellerGT (Queller & Goodnight, 1989) gave the most consistent estimates through all possible levels of kinship; therefore, QuellerGT was chosen to estimate the relatedness within the empirical data set, and 500 iterations were performed.

Possible bottlenecks were tested using Bottleneck 1.2.02 (Piry, Luikart & Cornuet, 1999) under a two-phased model of mutation. This method tests for the departure from mutation–drift equilibrium based on heterozygosity excess or deficiency. A model with 90% single-step mutations was used for the two-phased model of mutation, and the remaining 10% were multistep mutations with a variance of 12.

2.7 | Parentage studies on hatchery samples and on restored localities

Cervus 3.0 software (Kalinowski, Taper & Marshall, 2007) was used to infer the kinship relationships between the different broodstocks and offspring. First, whether the microsatellite markers would have sufficient ability to assign paternity in the two restored locations was tested using simulated and real offspring with known origins. The proportion of possible incorrectly genotyped loci was fixed at 0.125 (one out of eight total loci), which reduces the impact of two other possible causes of mismatches in parent–offspring relationships, such as mutations and null alleles (Wang, 2018). The Cervus simulation module was used to obtain reference critical log of the likelihood ratio (LOD) and Delta values. The LOD score is obtained by taking the natural log (log to base e) of the overall likelihood ratio, and a positive LOD score means that the candidate parent is more likely to be the true parent (Kalinowski, Taper & Marshall, 2007). Delta is defined as the difference in LOD scores between the most likely candidate parent and the second most likely candidate parent, and it is especially useful when multiple candidate parents have positive LOD scores (Kalinowski, Taper & Marshall, 2007). Ten thousand 'virtual' descendants were simulated and assigned. The same procedure was conducted with the 250 available offspring with known origins and for the parentage analysis of the 200 juveniles sampled in restored localities following previous parentage studies (Read et al., 2012; Borrell et al., 2014; Couvray et al., 2015).

NeEstimator V.2.0.1 software (Do et al., 2014) was also used to obtain estimates of the effective population sizes N_e from breeders and the offspring produced in the Centre for Fisheries Experimentation hatchery. The method based on linkage disequilibrium was used as recommended by Gilbert &

Whitlock (2015), and the allelic frequencies were restricted to those below 5% ($P_{crit} = 0.05$). The confidence intervals were obtained with the Waples & Do (2008) jack-knife method.

3 | RESULTS

3.1 | Mitochondrial DNA analyses on wild and restored localities

A total of 169 high-quality CytB sequences were obtained from the 200 samples under analysis and tested for genetic identity (>98%) with the species *P. lividus*. The amplification of the other samples failed or the electropherogram had unacceptable levels of noise, and they were not considered for the subsequent analyses. Aligning and manual editing produced an alignment with a consensus sequence length of 598 bp. A total of 101 different haplotypes resulting from 92 variable sites were found in this study (GenBank ID: MN600716–MN600846). The haplotype D_h and nucleotide π diversities were very high (Asturias' mean value $D_h = 0.987$, and $\pi = 0.0116$; Table 1). The different localities contained a considerable number of site-specific haplotypes, with Candás showing the highest D_h and π values (Table 1). However, neither the haplotype nor nucleotide diversities were significantly different ($P > 0.05$) between the samples from restored localities (Lluarca and Cuideiru) and the rest of the Asturian populations. The same result was evident when analysing the proportions of site-specific haplotypes (Table 1).

There was a significant global index of $\Phi_{ST} = 0.0541$ ($P < 0.05$) (Figure 2). The pairwise Φ_{ST} values showed that Calonge (Mediterranean) was a different entity, although this was also found for the Asturian locality of Candás (Figure 2). A suprapopulation structure within three groups (Calonge, Candás; and the remaining localities) was tested using analysis of molecular variance. The results indicated that the differences among the localities within groups were not significant ($P = 0.094$), wheress the differences among groups were significant ($P = 0.045$), which validated the proposed structure. This finding was corroborated by the DAPC, which also showed the same three different clusters (Calonge, Candás, and the remaining localities) (Figure 3a; stress value: 0.1369).

The inclusion of previously published genetic data by Maltagliati et al. (2010) for the western and eastern Mediterranean, Atlantic, and Adriatic populations in the structuring analyses again suggested three major genetic clusters. The clusters produced by these analyses were: the Mediterranean cluster (including the samples from Calonge), the Atlantic cluster (including Corcubión samples), and the Bay of Biscay cluster (pool of all Asturian samples) (Figure 3b; stress value: 0.0758).

The haplotype analyses using the network also suggested population expansion for *P. lividus* (Figure 4). A reticulated pattern was found with three widespread Atlantic haplotypes (H_27, MN600742; H_29, MN600744; H_30, MN600745) connected to numerous less frequent haplotypes (Figure 4). The Calonge (Mediterranean) and Candás (Bay of Biscay) samples revealed haplotypes that were clearly distinct from the most frequent Atlantic haplotypes, whereas the restored localities (Lluarca, Cuideiru) shared distinctive haplotypes among them (e.g. H_26, MN600741) (Figure 4).

Almost all neutrality statistics calculated from the data showed negative values, indicating past expansion or bottleneck recovery (Table 1). Tajima's *D* was negative but only barely significant in the wild populations, whereas it was negative and significant in the restored localities (Lluarca and Cuideiru) (Table 1). The high number of singletons could bias statistics due to sequencing errors and lead to incorrect inferences of evolutionary scenarios; however, Achaz's statistics, which was proposed to strengthen conclusions in suspicious data sets (Achaz, 2008), were consistent with Tajima's *D* (Table 1). The *F* and R_2 statistics, which are the most powerful tests for detecting population growth (Fu, 1997; Ramos-Onsins & Rozas, 2002), were both significant. Finally, the proportion of non-synonymous (Pi(a)) and synonymous mutations (Pi(s)) were in the

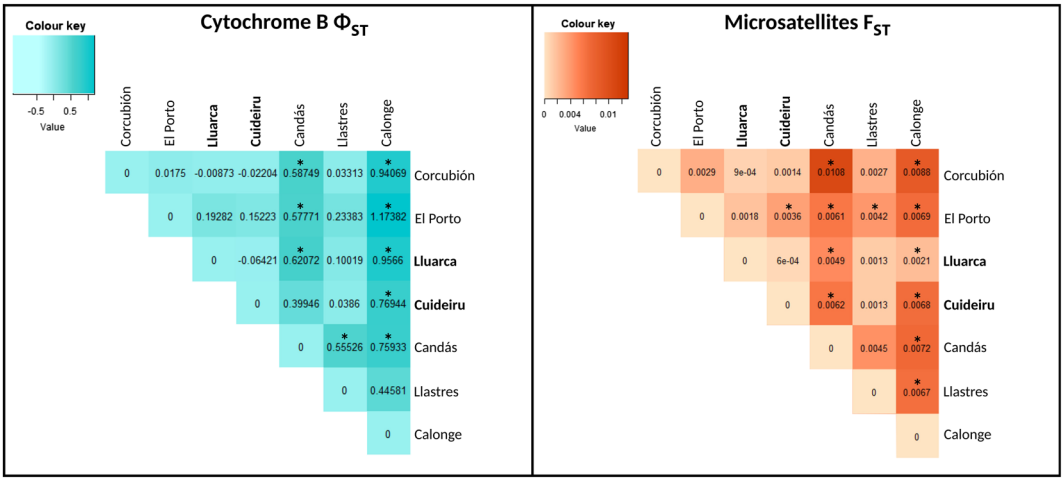

FIGURE 2 Heatmap representing the pairwise cytochrome B Φ_{ST} and microsatellite F_{ST} values among localities for the species *Paracentrotus lividus*. The darker the colour, the higher the value. Asterisks indicate those differences that had significant *P*-values after a Bonferroni correction. Restored localities are in bold

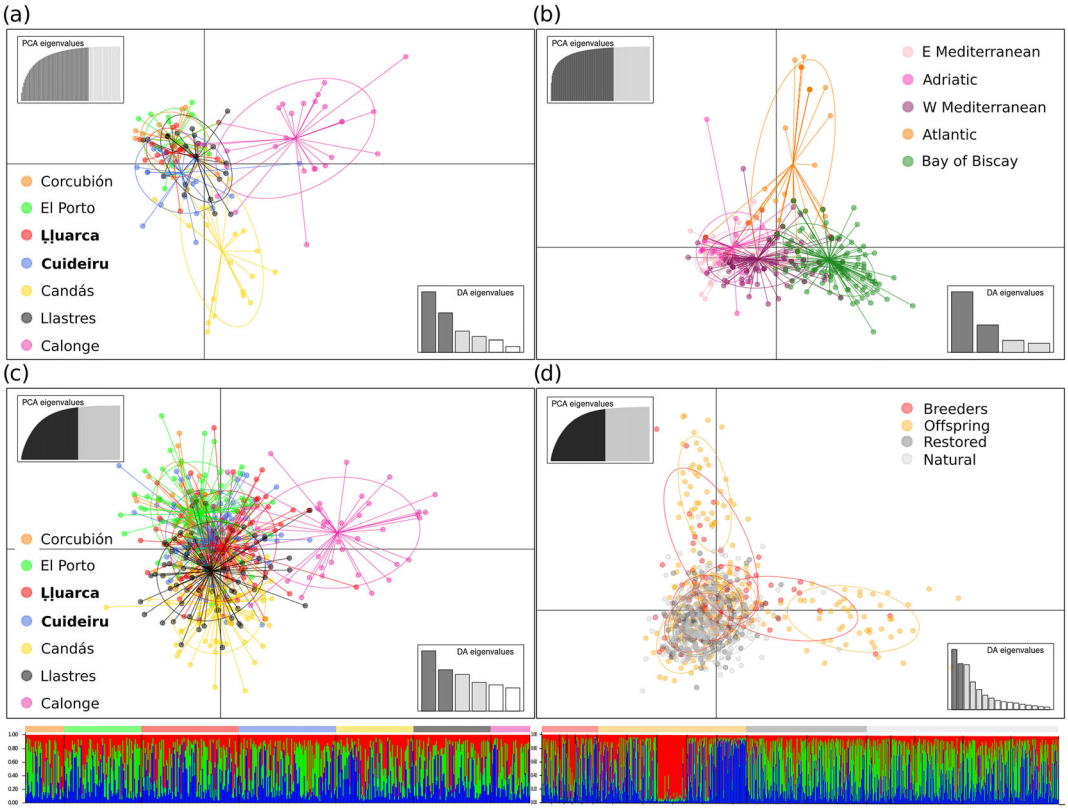

FIGURE 3 Genetic clustering using discriminant analysis of principal components and using Bayesian analyses (microsatellites) in *Paracentrotus lividus* samples in this study. (a) Clustering among study localities in the Atlantic and Mediterranean area (Galicia, Asturias, Catalonia) using CytB. (b) Clustering including Maltagliati et al. (2010)'s samples, using CytB. (c) Clustering among wild and restored localities in the Atlantic and Mediterranean area using microsatellites markers. (d) Clustering among study localities in the Atlantic and Mediterranean area and including breeders and siblings of the offspring used for population restoration strategies in Asturias using microsatellites markers. Restored localities are in bold

range 0.152–0.691 (Table 1), indicating that most selection eliminates deleterious mutations and retains the protein as is (purifying selection).

3.2 | Microsatellites analyses on wild and restored localities

Eight microsatellite loci were successfully arranged into two multiplex PCRs in this study (named PLM5 and PLM4). The eight microsatellite loci showed medium and high levels of genetic variation in the assayed samples, ranging from 19 to 54 alleles per locus (Table S1). The observed and expected heterozygosity of the individual loci ranged from $H_O = 0.346$ (PLM5, PI-F) and $H_E = 0.752$ (PLM5, PI-F) to $H_O = 0.815$ (PLM4, PI-15) and $H_E = 0.964$ (PLM4, PI-15) (Table S1). The microsatellite loci had a polymorphic information content mean value of 0.897 (SD = 0.068), and the observed and expected heterozygosities had average values of 0.574 (SD = 0.189) and 0.898 (SD = 0.065) respectively (Table S1). Testing these markers with Microchecker 2.2.3 and FreeNA showed that the heterozygote deficiency could be due to null alleles, among other factors (Table S1).

Microsatellite analysis showed that significant differences in the genetic variation levels between the wild and restored localities were not found ($P > 0.05$) (Table 2). Differential patterns were not detected in terms of the presence of private alleles among those localities (Table 2). All markers showed significant deviations from Hardy–Weinberg equilibrium due to significant heterozygote deficits (mean $F_{IS} = 0.343$; $P < 0.05$) (Table 2). None of the wild or restored localities in Asturias have revealed probable recent bottleneck events ($P > 0.05$). However, Candás (Ca) showed the highest R_{xy} value (0.018) (Table 2).

The F_{ST} statistics found in this study indicated population differentiation (significant global $F_{ST} = 0.026$, $P < 0.05$) (Figure 2). There were three populations (Calonge (Mediterranean), Candás, and El Porto) with significant differentiation values in the F_{ST} pairwise comparisons (Figure 2). The results of the DAPC (genetic differentiation) showed high overlap except for the Mediterranean population (Calonge), which was clearly differentiated from the rest (Figure 3c; stress value: 0.0856). The Bayesian structural analysis resulted in a highly probable population structure with three clusters (Evanno's $k = 3$; likelihood: $\Delta K = 13.2388$). The cluster assignment probabilities revealed equal distributions of these three clusters in the wild and restocked samples (Figure 3c). The results of both the DAPC

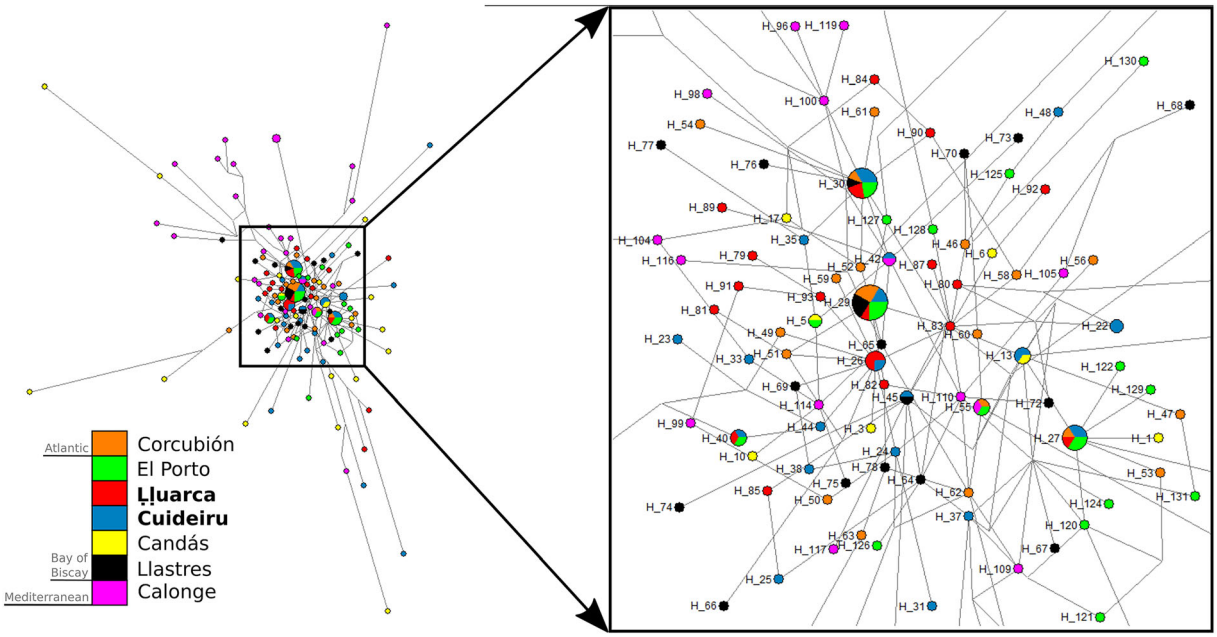

FIGURE 4 The mitochondrial haplotypes CytB network from *Paracentrotus lividus* localities sampled in this study in the Atlantic and Mediterranean areas (Galicia, Asturias, Catalonia). The network central area has been enlarged for a better visualization. The legend shows localities names (restored localities are in bold). Node sizes are proportional to the number of samples in which the haplotype was observed. Colour portions refer to the proportion of individuals in which the haplotype was present

analysis and the Bayesian structural analysis of the total number of individuals in this study showed high overlap except for the offspring (C_2015_O and P_2016_O) populations, indicating that the juvenile individuals used for supplementation were genetically different from the wild populations (Figure 3d; stress *r* value: 0.1110; Evanno's $k = 3$; likelihood: $\Delta K = 15.7886$).

3.3 | Parentage studies on hatchery and on restored localities

Genetic diversity data from broodstock and juveniles used by the RMRDNR for the population restoration strategy conducted in Asturias are shown in Table 3. Significant differences in the genetic variation levels between the breeder and offspring samples were found (Tables 3 and S2). The offspring showed significantly lower allelic richness values ($A_R = 8.582$) than the breeders ($A_R = 10.685$) in a global analysis ($P = 0.001$) (Table 3). Furthermore, the global offspring values of H_E (0.839; $P = 0.033$) and F_{IS} (0.275; $P = 0.024$) were significantly lower than those of the breeders, while the F_{ST} (0.073; $P = 0.033$) and relatedness values *R* (0.110; $P = 0.021$) were significantly higher (Table 3). The estimates of effective population sizes N_e obtained for both the breeders used for restoration actions and the siblings of the offspring released in the restored areas showed that the effective number was extremely low, especially in the case of the siblings of the juveniles released, which ranged from 9 to 30 individuals (Table 3). High R_{xy} values were also found in the siblings of the offspring used to conduct the different restoration actions. Among these, there was one value of $R_{xy} = 0.176$, which is between the expected values for the first cousins (0.125) and half siblings (0.250) (Table 3). Moreover, breeders, siblings of the offspring, and samples from the natural localities were genetically different ($P < 0.05$) (Table S2).

Table 4 presents the results of the parentage studies conducted in this study using the 92 available parents in captivity (91% of the 101 total parents involved), the simulated offspring (10,000 offspring), and the real descendants with known origin (250 offspring). First, the simulation results showed that the mean correct assignment for parent pair simulations would be 95% using Cervus' statistical support for relaxed conditions (80%) as the cut-off, whereas a range of 69–71% correct assignment would be reached using strict conditions (95%) for the Delta or LOD criteria (Table 4). The unassigned proportion in juvenile assignments was mostly due to the possible (real in this case) involvement of parents that were not sampled in the study. After that, a search for the real parents of the sample of 250 sibling offspring analysed in this study found 92% (230) and 90% (225) effective assignments using the relaxed conditions for the Delta and LOD cut-off values respectively (Table 4). As predicted by the simulations, the use of strict statistical confidence (95%) reported lower values of correct assignments (72% using Delta and 54% using LOD criteria) (Table 4).

The microsatellite-based parentage analysis conducted for the restored localities included the available breeders from the broodstocks in use by the hatchery and the 200 juveniles recaptured from Lluarca and Cuideiru. Based on the use of strict Delta assignment conditions (95% confidence), the results showed that

TABLE 4 Parentage assignments results in *Paracentrotus lividus* after genetic analysis using eight microsatellites in the hatchery and in the restored localities in Asturias, Bay of Biscay

			Delta			LOD		
	Locality	Level	Critical Delta	Simulation (%)	Observed assignment rate (%)	Critical LOD	Simulation (%)	Observed assignment rate (%)
Hatchery offspring with known origin ($n = 250$; 47 sires, 54 dams)	Hatchery (Castropol, Asturias)	Strict (95%)	2.69	71	72	10.25	69	54
		Relaxed (80%)	0.32	95	92	3.81	95	90
Juveniles from restored areas ($n = 200$; 42 sires, 43 dams)	Lluarca, Asturias	Strict (95%)	3.00	72	2	10.47	70	0
		Relaxed (80%)	0.37	94	29	3.88	94	5
	Cuideiru, Asturias	Strict (95%)	3.04	66	5	10.67	63	0
		Relaxed (80%)	0.61	89	31	4.80	90	5

seven out of the 200 individuals analysed (global 3.5%: 2% in Lluarca and 5% in Cuideiru) were of hatchery origin (Table 4). The relaxed Delta conditions for assignments (80%) revealed that approximately 30% of the offspring were of probable hatchery origin (Table 4). The use of LOD cut-off values as criteria using strict assignments (95%) did not reveal hatchery origin; however, using a relaxed confidence level (80%) revealed that, globally, 5% of the offspring (five offspring in Lluarca and five in Cuideiru) were of hatchery origin (Table 4).

4 | DISCUSSION

Implementing realistic conservation goals and promising management strategies in marine environments is a difficult challenge. Many factors are involved in determining whether recovery efforts for marine ecosystems or species have been successful. The major drivers include the reduction of human impacts (especially exploitation, habitat loss, and pollution) and the promotion of favourable life histories, environmental conditions, community awareness, and legal protection and enforcement of management plans (Lotze et al., 2011).

In recent decades, there have been a number of attempts to recover species and ecosystems with high social, cultural and economic relevance in Asturias (north-west Spain). Habitat restoration and massive juvenile reintroductions have been performed since the 1980s for sympatric Atlantic salmon *Salmo salar* L. and brown trout *Salmo trutta* L. populations in Asturian rivers using allochthonous and autochthonous individuals (Blanco et al., 2005; Horreo et al., 2011; Horreo et al., 2012). Introducing allochthonous individuals had a negligible effect, whereas promoting access to upstream spawning sites and improving habitats were the most efficient measures for increasing population sizes in Asturian rivers (Horreo et al., 2011). In addition, the autochthonous groove carpet shell *Ruditapes decussatus* has also been the target of a regional enhancement programme designed to promote sustainable clam fisheries. However, the seeds obtained in hatcheries for supplementation campaigns did not represent the wild gene pools well, and reductions of effective breeding numbers relative to the actual number of breeders were as large as 65%, due to unequal parental contributions and family variances (Borrell et al., 2014).

In addition to species exploitation, the northern Atlantic is facing another challenge: temperature increases at more than 0.5 °C per decade (Taboada & Anadón, 2012) and range changes have been observed for various intertidal species (e.g. Herbert et al., 2007; Hawkins et al., 2008) and subtidal communities (e.g. Voerman, Llera & Rico, 2013). Ocean warming due to climate change can have significant effects on the composition and structure of marine communities, including changes in the ranges of species distribution (IPCC, 2019). Although the range of temperatures that *P. lividus* tolerates is wide (approximately 8–28 °C; Boudouresque & Verlaque, 2020), studies in the easternmost area of the Mediterranean Sea have reported massive deaths due to numerous heat waves (Yeruham et al., 2015). Recently, it has been proposed that the retreat of kelp forests (intrinsically linked to sea urchin populations) on the coast of Asturias has occurred as a result of the increased frequency and intensity of marine heat waves, a product of climate change (Oliver et al., 2018; Izquierdo, 2019). Although the temperature of the Cantabrian Sea is not as high as that of the Mediterranean, it could be the case that the heat waves that reduced the abundance of *Laminaria* spp. also affected the abundance of sea urchins. 'Natural' macroalgae restoration or reintroduction of specimens without population manipulation has not always been successful (Layton et al., 2020); thus, it seems that the best strategy for the restoration of these ecosystems in this area is the selection of seedlings to promote repopulation based on thermal tolerance (J. M. Rico et al., unpublished results). Therefore, attempts to reintroduce sea urchins into the Cantabrian coasts could help restore interspecific ecosystem interactions.

Understanding the historical and contemporary population genetic diversity patterns of the declining *P. lividus* population in the central southern area of the Bay of Biscay is a prerequisite before attempting any restoration action on this species. In terms of the sustainable management and conservation of natural populations, the correct identification of management units (MUs) is indeed essential (Palsbøll, Bérubé & Allendorf, 2007). Moreover, preserving and

managing species gene pools under the threat of climate change, potential diseases, and overexploitation requires all possible data about the demography and effective population sizes and the effects on the populations of evolutionary processes. Previous population genetics studies carried out on *P. lividus* throughout its distribution range have revealed that this species has two main genetic discontinuities between the Atlantic Ocean and the Mediterranean Sea (Duran et al., 2004; Maltagliati et al., 2010; Penant et al., 2013) and between the Adriatic Sea and the rest of the Mediterranean Sea (Maltagliati et al., 2010; Penant et al., 2013; Paterno et al., 2017). A recent study failed to find significant genetic differentiation between populations from Galicia, Asturias, and the Canary Islands (Spain) by sequencing a fragment of the mitochondrial cytochrome c oxidase subunit I to evaluate the possibility of restocking overexploited areas in the Canary Islands with sea urchins from the Spanish mainland (Tourón et al., 2018). In addition, using thousands of genome-wide markers, Carreras et al. (2020) detected so far unnoticed patterns of genetic structure among populations of *P. lividus* spanning most of its distribution range; and a gradient of differentiation following a longitudinal dimension, overlain by a major differentiation at the Atlantic–Mediterranean transition, was detected. During the last glacial period (115,000–11,700 years ago), the sea level in the Strait of Gibraltar was approximately 120 m shallower than at present, which reduced the water exchange and effectively isolated the Mediterranean Sea from the Atlantic Ocean (Mikolajewicz, 2011). This isolation and increased salinity due to the lower sea level caused the Atlantic and Mediterranean populations to evolve separately, which is probably reflected in the modern-day differences between them. It has been argued that secondary contacts between differentiated lineages after the glacial period might have contributed to the currently high haplotype and nucleotide diversity found in many marine species (Grant & Bowen, 1998).

Analyses of mitochondrial data showed that the *P. lividus* populations in Asturias (Bay of Biscay) could have originated as a single MU along with the Atlantic populations and experienced significant global population expansion in the past (e.g. after a bottleneck or a selective sweep) (Tajima, 1989; Ramos-Onsins & Rozas, 2002). The genetic diversity distribution pattern suggested three different haplogroups, although these groups differed from those previously reported by Maltagliati et al. (2010). These authors performed a similar analysis and reported three haplogroups: the western Mediterranean, eastern Mediterranean, and Atlantic haplogroups. When the data from the Bay of Biscay were included (in this work), the results suggested a unique haplogroup for the entire Mediterranean sample, a haplogroup for the Atlantic samples, and a third haplogroup for the Bay of Biscay samples. Thus, the Asturian populations might constitute a differentiated Bay of Biscay MU that is separate from that of the Atlantic populations. This new haplogroup and its possible associated MU could be the consequence of a genetic drift-associated process due to reductions in effective population numbers.

Microsatellite data revealed genetic heterogeneity. In this study, the Candás sample showed low observed heterozygosity values and high relatedness coefficient values among individuals. Moreover, Candás also presented the largest number of private alleles contributing to population differentiation. These findings suggest that the Candás population could be being sustained through local recruitment of related individuals. A local eddy has been previously reported in the area of the Cape Peñes (the area for the Candás sample) (Figure 1c), and it creates patches of recruits to be located east of the Cape Peñes due to different hydrodynamic conditions compared with those located both west and further east of that cape (Sánchez & Gil, 2000). In addition, Candás accounted for 51% of historical stony sea urchin landings between 2004 and 2018 (Dirección General de Pesca Marítima del Gobierno del Principado de Asturias, 2019). Reductions in genetic diversity in exploited species could be caused by selection. In this way, particular variants at specific loci could be selected to create regions in the genome with anomalous levels of diversity and/or to reduce genome-wide effective population size by increasing the variance in reproductive success among individuals (Pinsky & Palumbi, 2014). In addition, El Porto (the western locality within Asturias; Figure 1c) also revealed a genetically heterogeneous pattern with regard to the easternmost localities sampled here when using microsatellites. These genetic differences may be due to a few divergent individuals or genetic drift, which is a random process that can lead to large changes in populations over a short period caused by recurring small population sizes (Frankham, 1995).

Genetic monitoring can also help to answer relevant questions about restoration efforts. Are there truly genetic signals in the restored areas that point to a significant contribution of released individuals to the increment in stony sea urchins abundance observed by the RMRDNR technicians? Are the restoration processes influencing the genetic diversity patterns found in this work?

As previously stated, the localities of Punta Focicón (Lluarca) and Ensenada de La Arquina (Cuideiru) have been restored by the regional government with the aim of recovering past densities of *P. lividus*. Restocking using juvenile specimens cultivated in aquaculture facilities is usually done to restore populations of overexploited and endangered species to a level where they can once again provide regular, substantial yields (Bell et al., 2008). After completing the restocking, the RMRDNR reported an increase in stony sea urchin abundance in the restored localities (S. de la Uz, unpublished data). Although this finding may have been caused by a direct contribution of hatchery individuals to the *P. lividus* abundance, many other factors could have contributed to the observed increases of stony sea urchins in those localities. Adult translocation and recently enacted capture bans would favour the mobility and aggregation of *P. lividus* individuals. Sea urchin aggregation seems to increase survival rates due to a group defence mechanism against predators and waves, and such aggregations could promote the fertility of sea urchins and increase juvenile recruitment rates because of the protection of larger adult individuals (Ouréns, Naya & Freire, 2015). Unfortunately, genetic monitoring was not performed on the individuals translocated from Xixón (samples were not stored); therefore, the effect of translocation of adults on genetic diversity and other population genetic parameters could not be determined.

Stony sea urchins of hatchery origin were found when 100 juveniles from each of the two restored localities (Lluarca and Cuideiru) were randomly sampled. Using a strict level of confidence (95%), a global 3.5% of the total recaptured individuals originated from the hatchery. More relaxed confidence level conditions (i.e. 80%) suggested similar values (5%) when using the LOD scores or an even higher contribution of approximately 30% if Delta was chosen as the criterion. However, applying stringent criteria in parentage analysis significantly helps to minimize parentage assignment errors (Borrell et al., 2014). The findings obtained using stringent levels of confidence showed that, even with a presumably low impact, there was indeed some successful survival of the hatchery individuals released into the wild localities. These proportions are in the range of restorations of other marine invertebrate populations; for example, abalones (26%; Read et al., 2012) and clams (15%; Borrell et al., 2014). In the case of stony sea urchins, Couvray et al. (2015) obtained values between 3 and 12% with relaxed confidence conditions (80%) after releasing 250,000 sea urchin hatchery juveniles at each locality.

An effective population size N_e of at least 50 individuals (sex ratio of 1:1) has long been recommended to avoid inbreeding depression in the short term (Franklin, 1980). However, the number of breeders used in the restoration actions conducted by the RMRDNR (mean number of spawning breeders by broodstock was 14.42) is much smaller than the recommended minimum breeder by stock numbers suggested by some authors, who mention numbers of broodstock several orders of magnitude higher (Tave, 1999; Agatsuma, 2013; Borrell et al., 2014). A high number of breeders is usually proposed to prevent random genetic drift as much as possible, which would have a greater influence than natural selection and could result in a decrease in the frequency of favourable alleles and an increase in deleterious alleles (Tave, 1999; Araki, Cooper & Blouin, 2007; Christie et al., 2012). Moreover, significant changes in genetic diversity patterns are expected when populations experience a Ryman–Laikre effect (Araki & Schmid, 2010; Waples, Hindar & Hard, 2012). The F_{ST} values (Table S2) DAPC and structure results (Figure 3d) for the microsatellite markers showed that the juvenile individuals used for population restoration were genetically different from the wild populations, which indicated a deficient representation of the wild gene pool in the broodstock because of the low number of individuals used as breeders. Other factors may have favoured some genotypes and/or family variances, such as the possible differential maturity of the broodstock (fertilization capacity), the physiological quality of the sperm and eggs at spawning in the hatchery (not all eggs or sperm spawned contributed to the next generation), or a different selection regime in the hatchery with respect to the wild environment (Borrell et al., 2014).

The genetic monitoring of the restored localities (Lluarca and Cuideiru) showed that they were not currently different from the other Asturian localities in terms of genetic diversity based on both genetic markers. There was no evidence of a significant decrease in genetic diversity in comparison with 'supposedly' non-restored localities; hence, there was no evidence of a Ryman–Laikre effect in the restored populations, although it cannot be ruled out. It has been reported that, after an initial rapid increase in genetic diversity, high proportions of hatchery-bred animals in wild populations could result in inbreeding, which could later generate a significant decline in the N_e of restored populations (Christie et al., 2012; Waples et al., 2016). Restored populations can be initially genetically similar to the original wild populations, although fitness losses due to interbreeding with hatchery individuals can be more harmful than a reduction in effective population size (Waples et al., 2016). Furthermore, there is also the possibility that (because only juveniles were sampled at the restocked localities, whereas adults were selected at the other localities) high mortality continues to occur from the sea urchin sizes sampled at the restored localities to adulthood, and that genetic diversity is reduced due to pruning of less optimal genotypes. The samples from Calonge (Mediterranean) and Corcubión (Atlantic Galicia) showed lower levels of allelic richness than the rest of the samples; however, whether these populations were subjected to restoration experiments or great harvesting pressure could not be determined.

In general, marine ecosystem restoration strategies are strongly supported by society, which emphasizes the right of future generations to satisfy their needs, just as their ancestors did, as rebuilding implies some form of pre-existing structure (Pitcher & Pauly, 1998; Sumaila, 2004). This interest is even more significant in the case of fishermen and other stakeholders, as their main livelihood is at risk with the disappearance of their target species (Pitcher & Pauly, 1998). In this way, marine ecosystem restoration strategies are rapidly growing in importance (Sumaila, 2004) as marine ecosystem degradation accelerates around the world caused by climate change, overfishing (Jackson et al., 2001), invasive species dispersal (Molnar et al., 2008), fertilizer runoff (Smith, Tilman & Nekola, 1999), plastic pollution (Derraik, 2002), ocean acidification (Doney et al., 2009), or general defaunation (McCauley et al., 2015), among others. Thus, an increasing number of people understand that marine ecosystems restorations can be one way to fight against rising anthropogenic effects. However, investments in such efforts by government agencies are still scarce and intermittent. When adopted, a restoration objective for resource management will encourage stakeholder consultation and consent and directly leverage traditional knowledge and history for a social objective (Pitcher & Pauly, 1998). New initiatives and projects have begun to be seriously considered in the Cantabrian Sea for restoring ecosystem webs and services (e.g. producing thermo-tolerant kelp restorations). Under this scenario, restoring stony sea urchin populations and their relevant ecological function could be part of a more general, ambitious strategy for mitigating anthropogenic-related changes in the marine environment and associated consequences for nature and for people in the Cantabrian Sea coastal areas.

5 | CONSERVATION IMPLICATIONS

Numerous factors can cause the decline of a species, including harvesting pressure, predation, or various environmental causes, as well as the sum of all of these factors. Moreover, it is often not easy

to discern the origin and cause of this decline. When fisheries are properly managed, there are decreases in fishing pressure and increases in stock abundance, with some stocks reaching biologically sustainable levels, reflecting the role of fisheries managers and governments around the world that are willing to take strong action (Hilborn et al., 2020). The United Nations *Code of Conduct for Responsible Fisheries* states that, in adopting management measures, the 'best available scientific data should be used to assess the state of fishery resources' (Food and Agriculture Organization, 1995). However, most exploited stocks globally are classified as data poor (Costello et al., 2012) and their status, although highly uncertain, is generally considered to be worse than that of data-rich stocks (Worm & Branch, 2012). This is the first study to carry out genetic monitoring of *P. lividus* populations on the coast of Asturias, both of populations that were restored and of natural populations, laying the foundations for the threshold of scientific knowledge necessary for future management measures.

Previous studies on stony sea urchins in the central area of the Cantabrian Sea are very limited, and some of the available knowledge is to some extent inaccessible as it is in the form of private technical reports or old PhD dissertations. Having updated and publicly available scientific knowledge about the current state of this species' populations is still a clear necessity and could help inform rapid responses to possible emergencies arising from fluctuations in the density of these echinoderm populations. It is notable that approximately 15 years passed between the first and second population mapping events in which a decline of 44% in western populations was reported (de la Hoz et al., 1991; Álvarez-Raboso, 2006; Álvarez-Raboso, 2007). It cannot be the case that conservation management measures always come too late; so, despite (and precisely because) the fishery is currently closed, a conservation/restoration plan should be put in place. For this species, more data are needed on population dynamics and connectivity, and further studies are needed on gene flow. A previous study (Ouréns, Naya & Freire, 2015) mentions mismatches between biological, exploitation, and governance scales as well as ineffective management of *P. lividus* fisheries in Galicia. This study highlighted the probable existence of a differentiated Cantabrian Sea urchin gene pool caused by past and recent demographic events related to small population sizes and genetic drift. This finding represents a warning sign that *P. lividus* populations within the region may still be vulnerable and confirms the necessity and usefulness of the total capture bans established by the regional government in Asturias for the species. These year-round bans could help the recovery of a critical mass of stony sea urchins from which recruitment can become more effective. Oliva et al. (2016) highlighted the pivotal role that shelter —mainly from predation and provided by different ecological traits (related to the community, population, or physical structure)— plays in determining overexploitation limits in terms of both reproductive and recruitment success and refuge availability.

This work also confirms that released hatchery juveniles are able to survive in the wild, although at low rates. However, it is highly advisable to increase the number of breeders used to obtain juveniles when planning restoration actions to avoid impoverishing the gene pool in the Bay of Biscay's stony sea urchin populations. In addition, it is necessary to establish a monitoring programme for hatchery procedures to confirm that hatchery individuals truly represent the wild stony sea urchin gene pool and for early detection of possible effects on wild gene pools due to restoration efforts. Moreover, Calderón et al. (2012) reported patterns of a phenomenon known as genetic chaotic patchiness (see Eldon et al. (2016) for a review) in this species, so continued spatial and temporal monitoring is advisable. Finally, restoration using autochthonous individuals is the correct option. Currently, *P. lividus* is being harvested in other areas (Galicia and Portugal) for exporting to Asturias due to the great demand and strong consumption tradition. However, using allochthonous individuals in the restoration of the Bay of Biscay sea-urchin populations (even those coming from any other population of Atlantic origin) could negatively affect the genetic diversity of the wild populations because the new haplotypes could displace the autochthonous ones and affect their adaptability and fitness.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

Author elects to not share data.

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