



# Natural hybridization between pen shell species: *Pinna rudis* and the critically endangered *Pinna nobilis* may explain parasite resistance in *P. nobilis*

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## Abstract

Recently, *Pinna nobilis* pen shells population in Mediterranean Sea has plummeted due to a Mass Mortality Event caused by an haplosporidian parasite. In consequence, this bivalve species has been included in the IUCN Red List as “Critically Endangered”. In the current scenario, several works are in progress to protect *P. nobilis* from extinction, being identification of hybrids (*P. nobilis* x *P. rudis*) among survivors extremely important for the conservation of the species. Morphological characteristics and molecular analyses were used to identify putative hybrids. A total of 10 individuals of each species (*P. nobilis* and *P. rudis*) and 3 doubtful individuals were considered in this study. The putative hybrids showed shell morphology and mantle coloration intermingled exhibiting both *P. nobilis* and *P. rudis* traits. Moreover, the analyses of 1150 bp of the 28S gene showed 9 diagnostic sites between *P. rudis* and *P. nobilis*, whereas hybrids showed both parental diagnostic alleles at the diagnostic loci. Regarding the multilocus genotypes from the 8 microsatellite markers, the segregation of two *Pinna* species was clearly detected on the PCoA plot and the 3 hybrids showed intermediate positions. This is the first study evidencing the existence of hybrids *P. nobilis* x *P. rudis*, providing molecular methodology for a proper identification of new hybrids. Further studies testing systematically all parasite-resisting isolated *P. nobilis* should be undertaken to determine if the resistance is resulting from introgression of *P. rudis* into *P. nobilis* genome and identifying aspects related to resistance.

**Keywords** Hybrid · Pinnidae · Bivalves · Critically endangered species · Molecular identification · Mediterranean Sea

## Introduction

The family Pinnidae Leach 1819 are filter-feeder marine bivalve molluscs known as “pen shells” with a long triangular shape and fragile shells with the pointed end anchored in the substrate using the byssus threads. This is one of the few bivalve families, where all members are large in size, exceptionally up to 120 cm (*Pinna nobilis*) [1]. This family used to include three genera, but recent phylogenetic studies rather divided this family in two genera (*Pinna* and *Atrina*,

[2]; with *Streptopinna* nested within *Pinna*) with approximately 50 species described worldwide [3]. In the Mediterranean Sea, 2 species on the genus *Pinna* can be found: *Pinna nobilis* Linnaeus, 1758; and *P. rudis* Linnaeus, 1758. *Pinna nobilis* is the largest Mediterranean bivalve and one of the biggest worldwide [4] that is endemic to the Mediterranean Sea.

Recently, *P. nobilis* population in Spain has plummeted, causing concern and a status change from “Vulnerable” category to “Critically Endangered” with a serious extinction risk (Orden TEC/1078/2018); and has been included in the IUCN Red List as “Critically Endangered” [5]. This is due to a Mass Mortality Event (MME) that affected *P. nobilis* populations starting in early autumn 2016 with extremely high mortality levels and which is still ongoing (reaching up to 100% at monitored populations, [5, 6]. In the mean time, the congeneric species *P. rudis* is not affected by this MME [7] and remains distributed in patches throughout the Mediterranean Sea and Atlantic Ocean [8, 9]. *Pinna rudis*

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is included in Annex II of the Bern Convention as strictly protected and the Barcelona Convention as threatened or endangered marine species. Although the priority habitats are different for both species, they coexist in certain places and circumstances since both species share a wide bathymetric overlap [10–12]; and are closely related in the phylogeny of Pinnidae [2].

External features based on shell morphological traits, mantle characteristics and shell size highlight that there might be hybrid specimens, such as 3 individuals and several empty shells spotted at the Cabrera MPA since 2011, pointing to a possible hybridization of *P. nobilis* and *P. rudis*. Hybridization among species is a common process in natural systems and is viewed as a stimulating force in evolution [13], with contrasting outcomes [14]. Hybridization can lead to the collapse of species barriers [15], or to the formation of new species [16]. The frequent occurrence of interspecific hybridization in several groups of animals is indicative of its key role in animal evolutionary history mostly by increasing their adaptability to environmental change [17], through hybrid vigor or heterosis or simply by adding new combinations of traits that offer a strong advantage over the parents [18].

Regarding the mass mortality event of the endemic *P. nobilis*, it is crucial to identify the putative hybrids that might have survived the parasite infestation in the Mediterranean, since few survival individuals manifested combined traits of both *P. nobilis* and *P. rudis*. Clear morphological and molecular evidence for hybridization among the two studied species (i.e. *P. nobilis* and *P. rudis*), as well as the development of genetic tools for detecting hybrids, could provide insight into the prevalence of hybridization among *P. nobilis* and *P. rudis* and the conditions under which it may occur. Moreover - with the current scenario of extinction in process - the proper identification of survivors of *P. nobilis* is highly important. Therefore, this study aimed to assess the hybrid status of three putative *P. nobilis* and *P. rudis* hybrids by using a combination of taxonomic traits and molecular markers. The outcome will be essential in order to understand and assess the current status of *P. nobilis* populations and the survivals, for future conservation strategies.

## Material and methods

### Sampling and morphological characteristics and determination

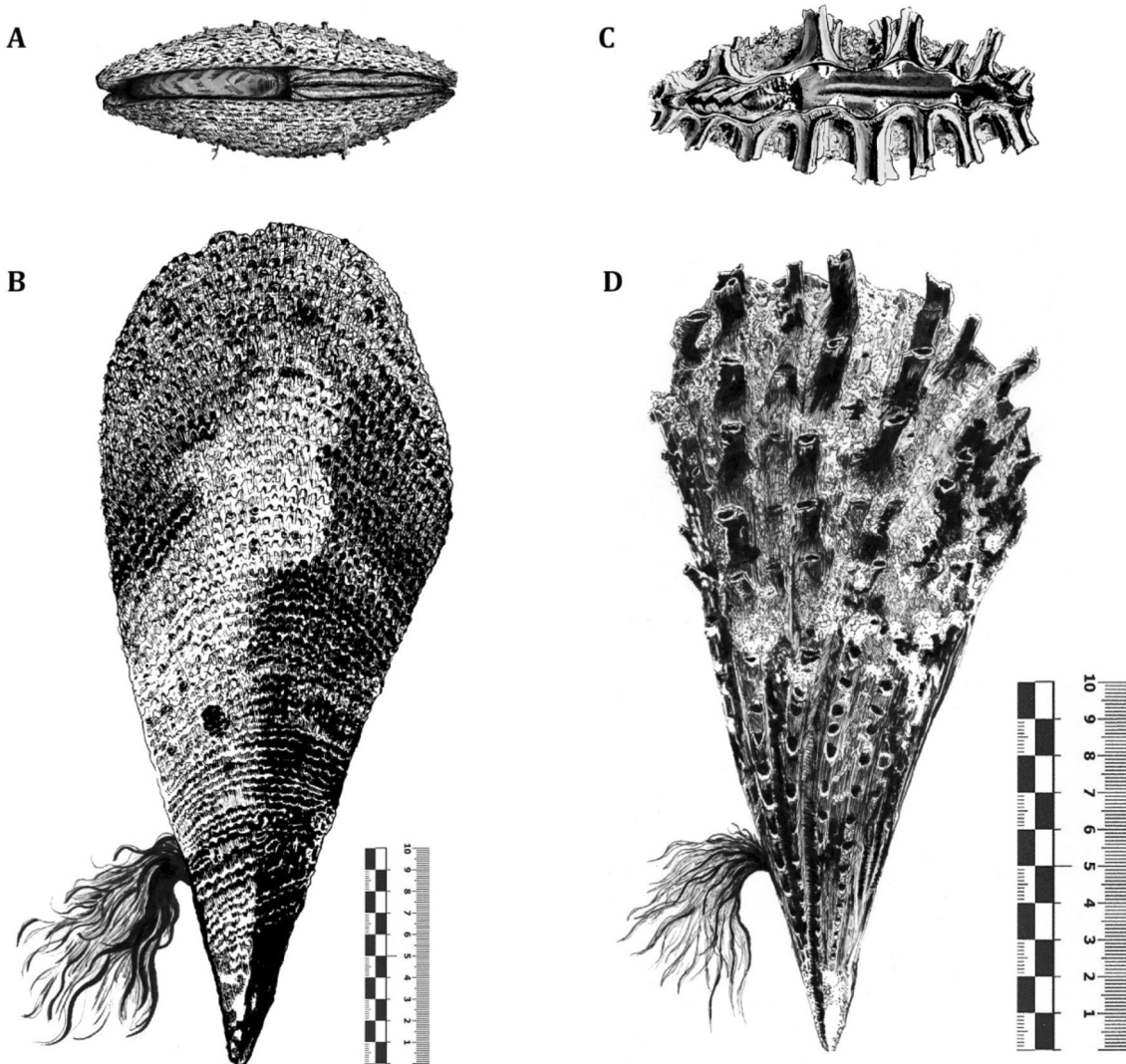
Samples of *P. nobilis* and *P. rudis* were collected in Cabrera National Park (Fig. 1) since densities and distributions of both species are well known derived from several years of monitoring [10, 11, 19]. A small portion of the mantle tissue of each individual was collected with tweezers (non-lethal

sampling method), and stored in small plastic bags by scuba diving. Back from the dive, samples were immediately preserved in 96% ethanol and stored at room temperature. Due to status as an endangered and protected species, sampling of *P. nobilis* and *P. rudis* was carried out under permission of competent authorities of “Servei de Protecció d’Espècies” and “Cabrera National Park,” both of Conselleria de Medi Ambient, Agricultura i Pesca” (Govern de les Illes Balears)”. A total of 10 biopsies of each species were considered for this study. It should be noticed that *P. nobilis* biopsies were collected prior the pandemic since no individuals are remaining presently in the area of Cabrera. Additionally, biopsies from 3 doubtful individuals were also collected. The putative hybrids exhibited morphological characteristics intermediate between the two species that hindered taxonomic classification. Thus, morphological features that distinguish these two congeneric species were quantified for the putative hybrid individuals. The morphological examinations were performed following the examination procedures described by [1]. These authors state that *P. nobilis* can be hardly confused with any other pinnid; smaller specimens are at once distinguished by crowded, distorted small spines. Juveniles have many small, very closely spaced spines and no other pinnid has such a spine formation. But, adult of *P. nobilis* can be confused with large *P. rudis* (in the Mediterranean Sea). *P. rudis* has large, widely spaced spines on well marked 5–10 radial ribs even as adults. Putative hybrids can be confused with *P. nobilis* or *P. rudis* depending on external characteristics expressed.

### Molecular analyses

Total genomic DNA was extracted from mantle tissue using a QIAextractor robot (Qiagen, Hilden, Germany). We focused genetic analysis in sequencing nuclear DNA that is inherited by both parents and therefore likely to show hybridization from two parents of different species. In this objective, it should be noticed that while mtDNA fragment such as CO1 are usually in species barcoding, the mtDNA, maternally inherited only cannot show evidence of F1 hybridization. We selected two types of markers; a 3 fragment of the 28S nuclear gene and a set of 8 microsatellites. Drawings made by Pelopantón.

A portion of 1150 base pairs (bp) of the 28S nuclear gene was amplified in 3 fragments (A, B, C) using primers from [20, 21] for fragment C as well as primers that we newly designed for fragment A and B: A: 28S\_Pinna\_F (5'-GGG AAGAGCCCAGCACCGAAT-3') and Pinna\_Rint (5'-GCC GTTTCACGTACTCTTGAAGTTC-3'); and B: Pinna\_Fint (5'-GAGTCCGATAGCGGACAAGTAC-3') and 28S\_Pinna\_R (5'-CCGACCGATTCCGCACTGAC-3'). Fragment C, which is after a long gap between *P. rudis* and *P. nobilis*, was amplified using the primers 28Sa (5'-GAC CCG TCT



**Fig. 1** Illustrations of *Pinna nobilis* and *Pinna rudis* based on shells collected from the study area (Cabrera National Park, Spain). (a) top view of *P. nobilis*, (b) lateral view of *P. nobilis*, (c) top view of *P. rudis*; and (d) lateral view of *P. rudis*. According to Schultz & Huber [2] *P. nobilis* can be hardly confused with any other pinnid; smaller specimens are at once distinguished by their crowded, distorted small

spines. Juveniles have many small, very closely spaced spines and no other pinnid has such a spine formation. But, adult of *P. nobilis* can be confused with large *P. rudis* (in the Mediterranean Sea). *P. rudis* has large, widely spaced spines on well marked 5–10 radial ribs even as adults. Putative hybrids can be confused with *P. nobilis* or *P. rudis* depending on external characteristics expressed

TGA AAC ACG GA-3') and 28srd5b (5'-CCA CAG CGC CAG TTC TGC TTA C-3'). Sequences were amplified in 25  $\mu$ l comprising: 2.5  $\mu$ l of buffer 10x + MgCl<sub>2</sub>, 2.5  $\mu$ l of dNTP, 0.1  $\mu$ l of TAQ, 16.175  $\mu$ l of sigma water, 1  $\mu$ l of each primer and 2  $\mu$ l of DNA (concentration of 5 ng/ $\mu$ l). PCR cycling parameters were as follows: a first denaturation step at 95 °C for 2 min, followed by 35 cycles of: 30 s denaturation at 95 °C, 30 s optimal annealing at 62 °C – 64 °C, 60 s elongation at 72 °C, and a final elongation step of 5 min at 72 °C.

Additionally, a total of 8 microsatellite markers were amplified following PCR cycling protocols and mixture adapted from [22]. From the microsatellites published by

[22] 14 markers amplified in *P. rudis*, however 6 of them were removed in the present study since they revealed problems in the scoring of alleles or presented high frequency of null alleles. Therefore, only the 8 markers that amplified clearly and gave robust results were kept: 11847, 15,415, 15,584, 4482, 5017, 3.2, 12,107 and 6980. PCR products were verified on 1% agarose gels and were sent for genotyping to a private company (GenoScreen, Lille, France). Microsatellite genotypes were scored using GeneMapper (Applied Biosystems). The software Microchecker [23] was used to search for scoring error, null alleles and large allele drop-out. The multilocus genotypic dataset was then analyzed using GenAlEx 6.503 [24]. A Principal Coordinates

Analysis (PCoA) was performed in GenAlEx. Bayesian clustering was performed using the software Structure [25]. The most likely number of groups (K) was searched by testing K = 1 to K = 5, with an initial burn-in period of 150,000 iterations followed by 100,000 recorded iterations and 10 iterations per K values. The most likely number of clusters present in this dataset was then determined using the Evanno's  $\Delta K$  approach [26], computed with Structure Harvester online [27].

## Results

The putative hybrids showed shell morphology and mantle coloration intermingled exhibiting both *P. nobilis* and *P. rudis* traits (Table 1) with slight differences among them. Regarding Hybrid 1, visually this individual could not be classified as *P. nobilis* or *P. rudis* based on the external appearance of the shell as it had intermediate taxonomic characters of both species (Fig. 2). This individual presented around 18 radial ribs in the valves with the presence of small tubular spines in the basis. Regarding mantle and gills, since this individual lives in a sciaphilic environment system (sea cave at 34.5 m depth), the mantle lacks of particular coloration being very homogeneous. The maximum shell width was 23.9 cm. Similarly, Hybrid 2, cannot be classified visually as *P. nobilis* or *P. rudis* based on the external appearance of the shell as it has also intermediate taxonomic characters of both species (Fig. 2). This individual presented around 12–13 radial ribs in the valves with the presence of medium tubular spines. Mantle and gills were similar to that of *P. rudis*. The maximum

shell width was 18.8 cm width, and this individual was found in a *Posidonia oceanica* seagrass meadow at 12.8 m depth. Regarding Hybrid 3, visually this individual had valves very similar to the species *P. nobilis*, but the mantle presented stains characteristic of the species *P. rudis*. Moreover, this individual presented 13 and 15 radial ribs respectively at each valve (Fig. 2). The maximum shell width was 17.5 cm, and this individual was also encountered in *P. oceanica* seagrass meadow at 11.5 m depth.

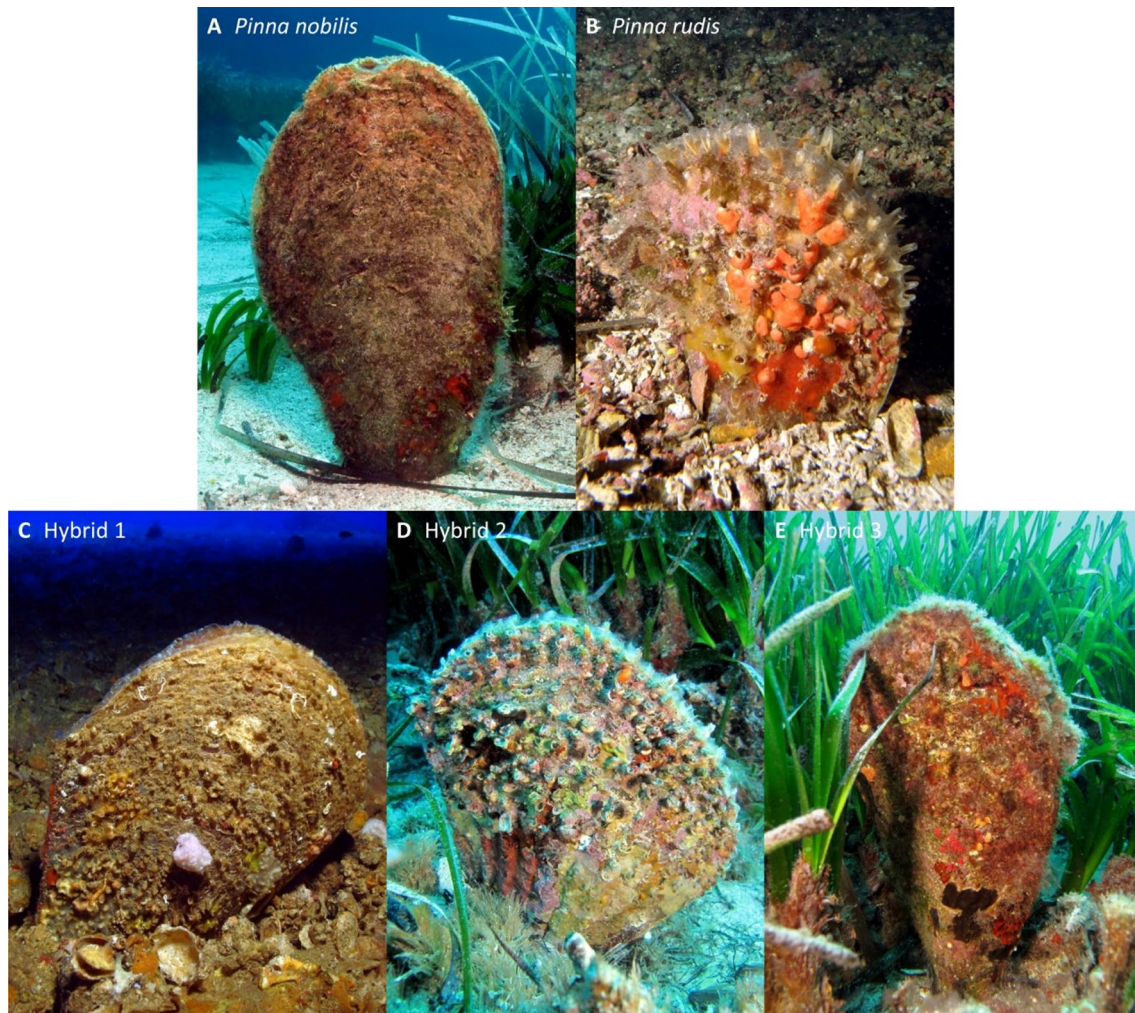
The analyses of 1150 bp of the 28S gene showed 9 diagnostic sites between *P. rudis* and *P. nobilis*. The 3 individuals showing intermediate morphological features were revealed as hybrids (Table 1). They showed both parental diagnostic alleles at 5 of the diagnostic loci (heterozygous sites). However, due to an insertion/deletion event, the three hybrids showed shifted alleles creating unreadable sequences for a portion of 230 bp over the full 1150 bp length (including 4 diagnostic sites between *P. rudis* and *P. nobilis*).

Regarding the multilocus genotypes from the 8 microsatellite markers, the segregation of two *Pinna* species was clearly detected on the PCoA plot (Fig. 3). Importantly, the 3 putative hybrids showed intermediate positions on the PCoA plot, and were distinct from both parental species. Structure Harvester identified two genetic clusters (K = 2) as the most likely solution. Additionally, the Bayesian clustering showed an intermediate genetic make-up of the three putative hybrid individuals (Fig. 3). One of the specimens presented more alleles from *P. nobilis*, while the two other specimens were genetically closer to *P. rudis*. From the microsatellite analysis, hybrids 2 and 3 are grouped together aside from hybrid 1 and the STRUCTURE plotting also showing hybrids 2

**Table 1** Results from chromatograms in the 9 diagnostic sites in the 10 *P. nobilis*, 10 *P. rudis* and the 3 hybrid individuals. Sequence base positions correspond to the alignment with *P. rudis* sequence

Morpho-ecological characteristics	<i>Pinna nobilis</i>	<i>Pinna rudis</i>	Hybrid 1	Hybrid 2	Hybrid 3
Radial ribs	> 15 (smooth)	5–10	18	12–13	13–15
Mantle coloration	homogeneous	stains	uncoloured	stains as <i>P. rudis</i>	stains as <i>P. rudis</i>
Habitat	C,D,R,Sa,Se	C,D,R,Sa,Se	C	Se ( <i>P. oceanica</i> )	Se ( <i>P. oceanica</i> )
Depth (m)	4.2–46	4.7–35	34.5	12.8	11.5
Fragment	Base positions				
A	202	G	A	R	R
B	464	A	–	A & -	A & -
B	496	C	G	N	N
B	570	T	C	N	N
B	573	C	T	N	N
B	720	–	T	N	N
B	747	T	C	Y	Y
C	1048	T	G	K	K
C	1049	T	C	Y	Y

KJ366027. Morpho-ecological characteristics are also included: C: marine caves, D: detritic bottom, R: rock, Sa: sand; and Se: seagrass meadows



**Fig. 2** Pictures of *P. nobilis* (a) and *P. rudis* (b), and the three putative hybrids: (c) hybrid1, (d) hybrid 2 and (e) hybrid 3. All pictures have been taken in Cabrera National Park by Maite Vázquez-Luis

and 3 genetically closer to *P. rudis* while hybrid 1 is closer to *P. nobilis*.

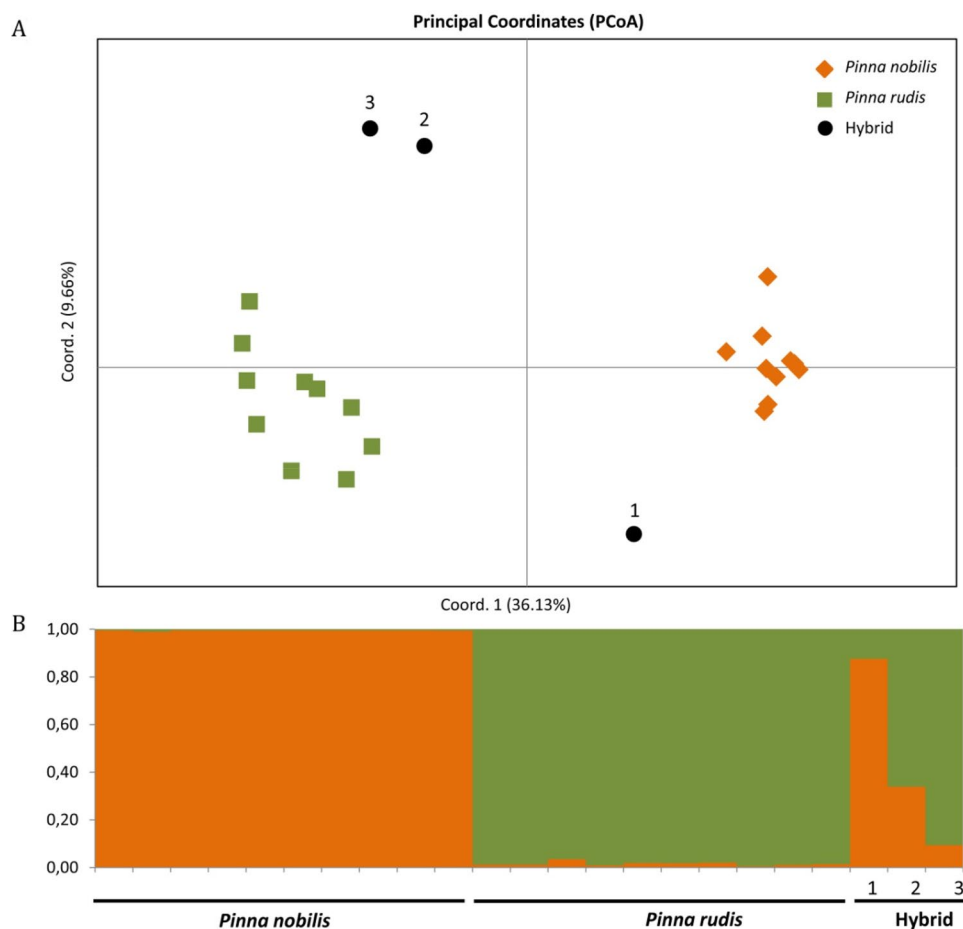
## Discussion

The present work demonstrated the first hybridization case for *P. rudis* and *P. nobilis* by using diagnostic molecular markers. Both parental species have a similar bathymetric distribution and are overlapping regarding habitat type [10, 11], which make hybridization possible. The prevalence of this hybridization in nature is still unknown; and despite these close associations, we are not aware of any previously reported cases of hybridization between these two species. Moreover, according to our field observations, the hybrids were also different from each other and in some cases were hard to distinguish from parent species. Hence, the genetic identification methods (28S

and microsatellites) are an essential tool for the hybrid individual validation. Both the mitochondrial and nuclear loci that we presented here clearly segregated the two species and will be helpful for future studies of pen shells conservation.

In the study area, we have observed in the last decade several cases of empty shells which seemed to be hybrids between *Pinna nobilis* and *P. rudis*, with valves showing characteristics of both species. In general, hybrids can be difficult to observe if they are morphologically similar to one of the purebred individuals living among them and if they consist of a very small fraction of the total population [28]. In the current frame of the mass mortality event of *P. nobilis*, which is bringing the species to the border of extinction, actions and recommendations for improvement of the survival of fan mussels has been identified [29]. Therefore, it is crucial to be able to properly identify *P. nobilis* resistant individuals. The correct taxonomic identification of resistant

**Fig. 3** Genetic results from 8 microsatellite markers: (a) PCoA based on multi-locus genotypes from 8 microsatellites explained 45.79% of variability; and (b) barplot of admixture values (q) from STRUCTURE showing the most likely number of groups: K=2. Orange bars indicate proportion of genome inherited from *P. nobilis* parents and green bars indicate proportion of genome inherited from *P. rudis* parents



individuals will indeed be essential for the recovery of the species.

There are few studies on the biology and ecology of *P. rudis* and most of them have been focused on specific aspects, such as shell ornament, capacity of induced shell repair and the organisms associated to its shell, or its presence across the Mediterranean Sea [30–35]. Therefore, a knowledge gap on its biology and ecology exists as the reproductive aspects remain largely unknown. However, the hybridization with *P. nobilis* revealed in this study points out that at least the two species might share a reproductive window which both species releasing gametes to the water column either synchronically or at least within a partially overlapping period. *P. nobilis* reproduction has been studied pointing at one spawning season starting in May and peaking in June–July in shallow areas of Cabrera National Park [36].

The status of these 3 hybrids is still questionable since the 28S sequencing would suggest that those individuals are likely F1 hybrids while the microsatellite separating them in two groups with hybrids 2 and 3 genetically closer to *P. rudis* while hybrid 1 is closer to *P. nobilis*. In the meantime hybrids 2 and 3 were collected in *Posidonia oceanica* meadow mostly colonized by *P. nobilis* while hybrid 1 come

from a cave that is mainly inhabited by *P. rudis*, both observations being opposing the habitat association to the genetic relatedness. At this stage it is not possible to be conclusive on whether we have F1 hybrids or recombined hybrids with purebreds since the two genetic markers are not conclusive and also mismatch with the habitat preference. Therefore, it is unknown whether these hybrids are fertile or whether second generation hybrids can be produced. Such aspect will have to be further investigated as conservation plans may have to think on the possibility to hybridize the two species and making sure they will be able to reproduce after.

In the context of the actual pandemic, the important point to take into account is that hybrids seem to be resistant to *Haplosporidium pinnae*, such as *P. rudis* [7]. Therefore, genetic clues for resistance could be found within hybrids. In this sense, hybrids are showing an advantage against the pathogen, which is in accordance with the hypothesis of dominance and overdominance. The dominance hypothesis attributes the superiority of hybrids to the suppression of undesirable recessive alleles from one parent by dominant alleles from the other [37]; while the overdominance hypothesis attributes the heterozygote advantage to the survival of many alleles that are recessive

and harmful in homozygotes by certain combinations of alleles that can be obtained by crossing two inbred strains are advantageous in the heterozygote [38, 39]. In any case, hybrids are persisting through time given the advantage compared to *P. nobilis* species and further transcriptomic studies will help disentangle between dominance and overdominance processes as they have different consequences for gene expression profiling.

It must be taking into account that 3 individuals is a small number examined since hybrids are very rarely found. The population of *P. rudis* in Cabrera is unusual because there is a high concentration of *Pinna rudis* and in the meantime a high concentration of *Pinna nobilis* before the MME. Finding the two species in high concentration is not found in many places making hybridization rare. However, taking into account the actual situation we are facing with *Pinna nobilis*, this study is of great value for conservation of the species. This study demonstrated that hybridization among *P. nobilis* and *P. rudis* exists, now many individuals considered as “*Pinna nobilis* survivors” could be hybrids making serious mistakes with regard to the conservation of the species if the taxonomic status of individuals is incorrectly assigned. Moreover, molecular methodology for a proper identification of new hybrid cases is provided here. Further studies are needed to determine if these hybrid occurrences are isolated events or are part of an ongoing speciation process, they will also help clarify aspects related to resistance.

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## Compliance with ethical standard

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Ethical approval** Due to status as an endangered and protected species, sampling of *P. nobilis* and *P. rudis* was carried out under permission of competent authorities of “Servei de Protecció d’Espècies” and “Cabrera National Park,” both of Conselleria de Medi Ambient, Agricultura i Pesca” (Govern de les Illes Balears)”. Moreover the sampling was non-lethal.

**Informed consent** Not applicable in this study.

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