



Life Pinna

LIFE20 NAT/IT/001122

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*Conservation and re-stocking of the Pinna nobilis
in the western Mediterranean and Adriatic sea*

C.2: Report on the genetic make-up of survival specimens

C.2.1: Report on the genetic make-up of survival specimens



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Deliverable name	Report on the genetic make-up of survival specimens
Status and Version	
Action contributing to the deliverable	Genetic variation analyses of <i>Pinna nobilis</i> surviving individuals and comparison with the past genetic make-up of the species with the aim to better understand how new restocked population can evolve
AC/Task responsible	Prof. Marco Casu, Prof. Daria Sanna
Other contributors	Dr. Fabio Scarpa, Dr. Ilenia Azzena, Dr. Chiara Locci

Molecular characterization of surviving individuals of *Pinna nobilis*

During the year 2022, a genetic characterization, based on the mitochondrial molecular marker that can be used to compare data from different studies (the Cytochrome c Oxidase subunit I gene, COI), was performed on the population of *Pinna nobilis* from Friuli Venezia Giulia (Miramare, Gulf of Trieste), which was originally chosen as donor area, and also on a further, still surviving, population from Tunisia (Kerkennah). In the case that the species completely disappears from western Mediterranean during the LIFE-PINNA activities, this latter Tunisian population could represent a source of funder individuals for restocking activities.

Furthermore, during the year 2023 (June-September) the population from Friuli Venezia Giulia was also characterized using a nuclear highly variable marker, the microsatellites. This analysis was requested by the Italian Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) to obtain a fine genetic assessment of this potentially donor population. Microsatellite analyses would allow to better infer on the genetic variability of Friuli Venezia Giulia population by comparing the obtained data with those from the previous studies belonging to the unique populations of *Pinna nobilis* already genotyped for microsatellites (Balearic Islands, Spain by González-Wangüemert et al. 2015 and Banyuls, Gulf of Lion, France by Peyran et al. 2020, 2022).

However, during August and September 2023, the death of *Pinna nobilis* individuals from the Venetian Lagoon (Veneto) population before their transplantation in the Ligurian restocking area of Capo Mortola, allowed us to perform a genetic characterization by means of both mitochondrial (COI) and nuclear (microsatellites) molecular markers for 11 adults and a few of their larvae and 1 sample of pseudo feces.

Collecting molecular data also for Venetian Lagoon (Veneto) population is useful as it has been chosen as new alternative donor population.

In this context, mitochondrial and microsatellite analyses, even if performed on a reduced number of individuals (less than 10), may be useful for:

- Assessing the current level of mitochondrial genetic variability in the Venetian Lagoon population. This will allow comparison with data obtained for the same population in the previous study by the researchers of the University of Sassari (Sanna et al. 2013), thus revealing any potential changes over time.

- Comparing the microsatellite results for the Venetian Lagoon population with those from Friuli Venezia Giulia, Spain, and France. This comparative analysis can depict similarities and differences in genetic structure across these populations.

Mitochondrial characterization of individuals from the Friuli Venezia Giulia and Tunisia

Twenty samples belonging to individuals of the surviving population of Friuli Venezia Giulia (from Miramare) were genotyped with mitochondrial markers during the first year of activities. The twofold aim of the analyses was to evaluate the diachronic evolution of the genetic variability of this population before and after *Pinna nobilis* mass mortality and evaluate the level of genetic homogeneity between the allelic variants currently present in this population of Friuli Venezia Giulia and those that were found, in the past years, in the populations of western Mediterranean before their extinction as a consequence of the mass mortality. Molecular data corresponding to populations that went extinct in the past years, come from previous research (Katsares et al. 2008, Rabaoui et al. 2011, Sanna et al. 2013, Sanna et al. 2014, Wesselmann et al. 2018) and from old-stored samples which were recently analysed in the laboratories of the University of Sassari.

The diachronic genetic analyses performed for the Friuli Venezia Giulia population were carried out by comparing two groups of samples from the Gulf of Trieste which were collected in 2018 before the mass mortality that involved *Pinna nobilis* in the Adriatic Sea, and in 2022 during the activities of LIFE-PINNA project.

Noteworthy, the samples from the Gulf of Trieste, which were collected before the mass mortality in 2018, were analysed by UNISS in 2021 during the mandatory preparatory action which anticipated the beginning of LIFE-PINNA project. Indeed, as requested by ISPRA to release the permission to move individuals of *Pinna nobilis* from the Adriatic to the western Mediterranean restocking sites, it was necessary to ascertain the occurrence of a genetic homogeneity among these populations before mass mortality.

Furthermore, as above reported, 3 samples from a still surviving eastern Tunisian population (Kerkennah) were also analysed just to evaluate the genetic variation (and its similarity with western Mediterranean) of a population that could be used as a donor in the unfortunate case that the donor populations from Adriatic go extinct during the activities of the project.

Results

Results obtained for the Adriatic population of Friuli Venezia Giulia, pointed out that many of the haplotypes that are currently present in this population were also present in western Mediterranean

before the mass mortality with particular reference to the Sardinian site of Asinara (see Fig. 1 for details). The occurrence of such a relevant haplotype sharing between Adriatic and western Mediterranean further corroborates the possibility that the restocking activities planned for LIFE-PINNA project would originate a new population poorly affected by genetic drift with a genetic variability similar to that one which was present in the restocking areas of western Mediterranean before mass mortality.

Furthermore, results evidenced that the genetic variation and the mitochondrial haplotypes which are currently present in the potentially donor population of Friuli Venezia Giulia are identical to those that were present in the same population before mass mortality.

This finding suggests that, over the past few years, no significant evolutionary forces have acted on this population. Consequently, it could be considered a strong candidate for a donor population in restocking activities within the western Mediterranean. This approach would minimize the impact of genetic drift or genetic pollution on newly established populations.

Results obtained by the analyses of Tunisian individuals (see Fig. 2 for details), evidenced that the haplotypes found in the coast of Kerkennah correspond to those which were the most common in western Mediterranean before the mass mortality of *Pinna nobilis*. Such an interesting and surprising (in previous studies Tunisian samples grouped with eastern Mediterranean populations, Sanna et al. 2013) finding suggests that, if necessary, the restocking activities foreseen by LIFE-PINNA project could be performed even using this Tunisian population as a donor. However, further analyses on an enlarged number of individuals from the Kerkennah population would be necessary to confirm the putative haplotype sharing between past western Mediterranean populations and currently surviving Tunisian Kerkennah population.

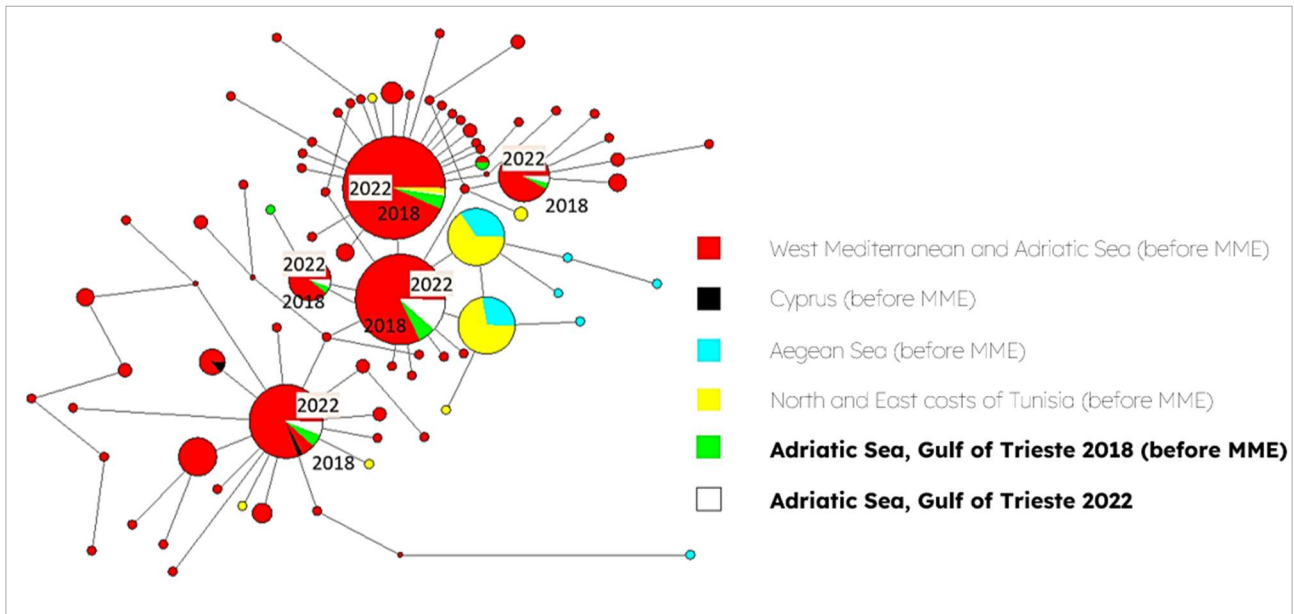


Figure 1: The graph shows the network analysis focused on all the samples currently present in the literature and on those analysed recently and belonging to the population of the Gulf of Trieste sampled in 2018 and 2022. A network represents the relationships among lineages and the possible evolutionary paths that occur among haplotypes. Each sequence included in the analysed dataset corresponds to a circular spot on the network; the diameter of a spot is proportional to the number of individuals that share the same lineage and colours within the spots are representative of the geographical areas to which the individuals who share a lineage belong. The more a spot is large the more the lineage is frequent. The branches of the network represent the nucleotide mutations that differentiate two sequences. The length of the branches is proportional to the number of mutations that occur between two lineages.

In the graph the sequences from Friuli Venezia Giulia are analysed, along with sequences belonging to individuals collected before the MME, using the mitochondrial COI gene as a molecular marker. The presence within the same spot of colours representative of the Friuli Venezia Giulia populations collected in 2018 (green) and 2022 (white) evidences that these individuals share the same mitochondrial haplotypes. Such a finding suggests that there is genetic homogeneity between individuals sampled before and after the mass mortality of *Pinna nobilis*.

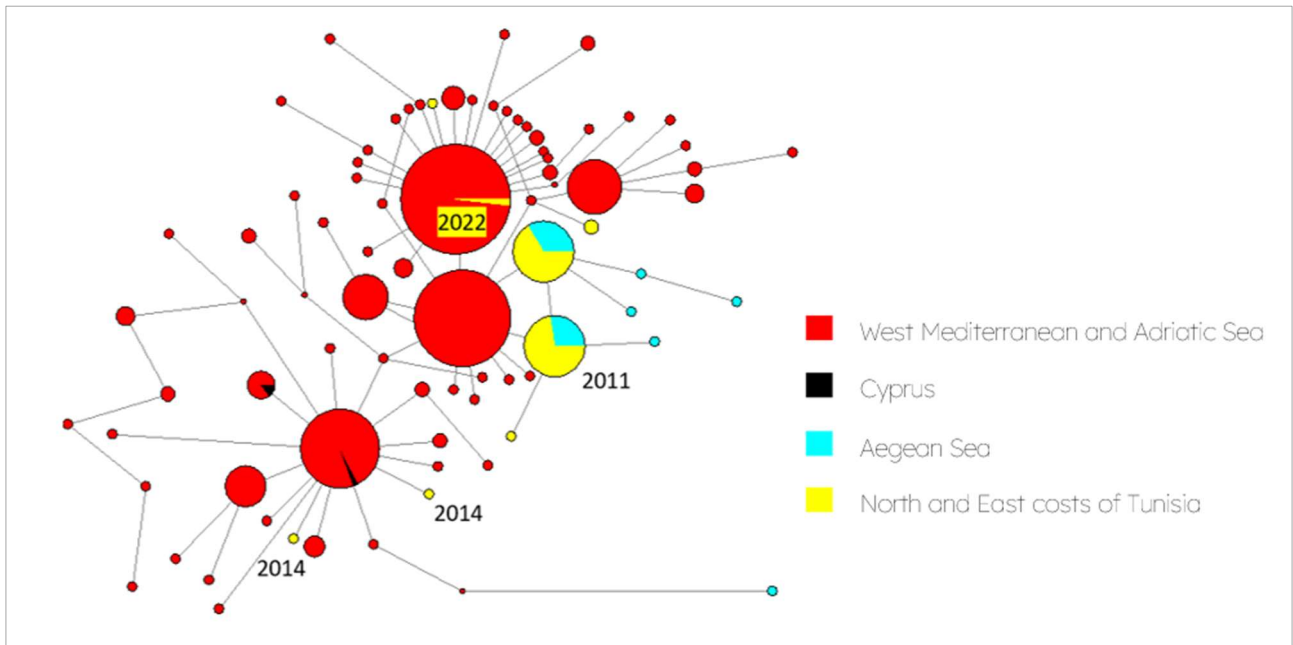


Figure 2: The graph shows the network analysis focused on all the samples currently present in the literature and on those analysed recently and belonging to the 3 Tunisian individuals from the Kerkennah population. All Tunisian individuals (collected in 2011, 2014 and in 2022) are coloured in yellow independently if they were sampled before or after the mass mortality of *Pinna nobilis*. The collection year is reported on the network. Results evidenced that all individuals collected in 2022 showed a mitochondrial lineage that was highly frequent in populations from western Mediterranean and Adriatic before mass mortality.

Mitochondrial characterization of individuals from the Venetian lagoon

During the analyses performed for the LIFE-PINNA project, it was possible to acquire 13 sequences corresponding to the mitochondrial COI gene belonging to samples of the Venetian lagoon population. In particular, 11 sequences belonged to adults, 1 to pseudofeces produced by the same individuals and 1 to a larva of one of these individuals.

These 13 sequences showed the presence of only 2 haplotypes/lineages corresponding to 3 polymorphic sites and characterized by a low level of haplotype diversity (see Tab. 1 for details).

Considering the whole dataset of 506 sequences from the whole Mediterranean which was obtained to evaluate the level of genetic homogeneity occurring between Venetian individuals collected in 2023 and past populations from western Mediterranean and Adriatic, the level of genetic variation depicted among the 13 new sequences represents the lowest values ever reported for *Pinna nobilis* populations (see Tab. 1 for details). This latter finding can be explained considering that a selective sweep might have selected the resistant individuals in the Venetian Lagoon during the mass mortality, thus reducing the number of mitochondrial lineages.

In particular 10 out of 13 of the new Venetian sequences correspond to the most widespread mitochondrial lineage which was/is present in the Mediterranean. Indeed, this lineage is present in 60% of the individuals included in the analysed dataset (506 sequences). In particular, it was reported in populations of the western Mediterranean, Adriatic and eastern Mediterranean (see Fig. 3 for details). The second haplotype, which was found among two individuals from the Venetian lagoon, corresponds to the second most widespread in the Mediterranean before and after the mass mortality, it is present in 13% of the individuals included within the dataset (see Fig. 3 for details).

These results suggest that the population of *Pinna nobilis* which is currently present in the Venetian lagoon is genetically homogeneous to the populations living in the western Mediterranean and the Adriatic before mass mortality. This important finding further supports the choice to include the surviving Venetian Lagoon population as a donor population within the present project. Consequently, this increases the possibility of creating new populations in the western Mediterranean that are genetically similar to extinct populations.

Please note that the present activity of mitochondrial characterization of Venetian individuals represents an ongoing process that must be completed in the next few months with the analysis (if possible) of a higher number of samples. For this reason, the results recently obtained must be considered as preliminary and the trend evidenced during the analyses should be confirmed after a set of complete analyses of genetic variation.

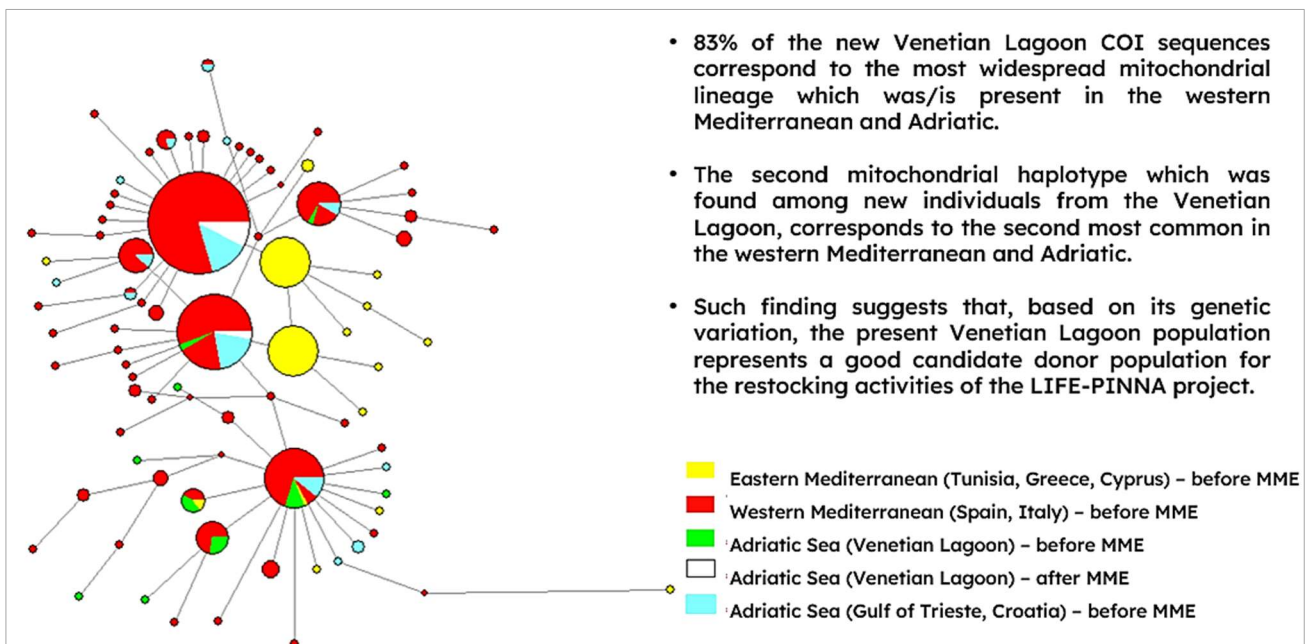


Figure 3: The graph shows the network analysis focused on all the samples currently present in the Venetian Lagoon and on those which were previous present in the same area (from Sanna et al. 2013).

Population	Sampling period	Size	Number of haplotypes	Polymorphic sites	Haplotype diversity	Nucleotide diversity
Gulf of Trieste 2022	After MME	20	7	6	0.727	0.004
Gulf of Trieste 2018	Before MME	18	7	7	0.817	0.006
Venetian lagoon 2023	After MME	12	2	3	0.303	0.003
Venetian lagoon 2011	Before MME	20	10	10	0.895	0.006
Kerkennah – Tunisia 2022	After MME	3	2	1	0.667	0.002
Tunisia coastlines 2011-2014	Before MME	50	7	7	0.621	0.003
Whole Mediterranean 2009-2023	Before and After MME	506	49	37	0.619	0.004

Table 1: The table reports the values of genetic divergence for the populations still living in the Mediterranean after the mass mortality event (MME) of *Pinna nobilis*. The population still present in the Venetian lagoon shows rates of nucleotide diversity comparable with those of the other populations still alive and also with that of the Venetian population living in the lagoon before MME. Such a finding is consistent with the normal trend of populations including individuals belonging to the same species. On the contrary, a considerably low level of haplotype diversity was evidenced for the population currently present in the Venetian lagoon. Such a value is almost half of those found for the other populations included in the table.

Microsatellite characterization of individuals from the Gulf of Trieste and Venetian lagoon

As above reported, starting from the year 2023 (June), individuals from the populations of Friuli Venezia Giulia and Venetian Lagoon (Veneto) underwent to a process of microsatellite characterization as requested by ISPRA. Indeed, as potential donor populations for the LIFE-PINNA project, they require further investigation to assess the level of genetic variation remaining among individuals after the mass mortality event. The aim is to assess the level of genetic variation for these two populations to further ensure to use donor population with a level of variability which may provide a pool of funders individuals with a high number of haplotypes.

However, it must be taken into consideration that, even if the levels of genetic variability in the donor populations would not be high enough to guarantee a limited risk of genetic drift in the new populations after restocking, the possibility of finding alternative donor populations with a genetic structure similar to that of the extinct populations in the western Mediterranean is low.

For this reason, due to the emergency conditions which is leading *Pinna nobilis* to the brink of extinction, even low levels of genetic variability within Friuli Venezia Giulia and Venetian lagoon (Veneto) donor populations should be considered acceptable for the restocking activities.

Starting from June 2003, the selection of the better panels of markers to be used and the standardization processes of the PCRs for the characterization of the microsatellites began. It

required a bibliographic search of all the microsatellites known up to now for the species (González-Wangüemert et al. 2015, 2019; Peyran et al. 2020, 2021, 2022).

The choice of the microsatellites that, according to previous studies showed to be the more informative was completed at the end of August 2023. During September 2023 the panels of microsatellites that were designed were tested on a low number of individuals before starting the whole process of characterization on 20 individuals from the Friuli Venezia Giulia and 12 (11 adults + 1 larvae) from the Venetian Lagoon.

The reading of microsatellite data and the bioinformatic analyses that must be performed to elaborate results, is a large ongoing process that will hopefully be completed in July 2024.

Results will be used to evaluate the internal level of genetic variability of populations from Gulf of Trieste (Friuli Venezia Giulia) and Venetian Lagoon and also to make a comparison between these northern Adriatic populations and those in western Mediterranean (France - Gulf of Lion, and Spain - Balearic Islands) for which microsatellite data are known (González-Wangüemert et al. 2015, 2019; Peyran et al. 2020, 2021, 2022).

Before evaluating the results that will be obtained, it should be taken into consideration that analyses performed using microsatellites are in general considered statistically supported and reliable if conducted on a number of individuals, hopefully of 50, necessarily above 25 or 30. However, in the context of the activities planned for the LIFE-PINNA project, even if non-lethal and minimally invasive, the sampling collection of a large number of individuals from donor populations would affect the wellness of individuals. Furthermore, for neither of the Adriatic donor populations a high number of individuals to be analysed is still present or available for sample collection.

For this reason, it is preferable to carry out analyses on a reduced number of individuals that, even if would not provide results that are statistically well supported, they could however provide a representative scenario on the genetic variability of the populations.

A particular attention must be also paid to the fact that the microsatellites which are available at the moment were designed based on populations of *Pinna nobilis* from Balearic Island (Spain) and Gulf of Lion (France). These latter populations are geographically distant from the Adriatic ones that require to be analysed during the LIFE-PINNA project. For this reason, problems of “misannealing” could affect the microsatellites PCRs performed on our samples, thus eventually compromising the fully success of analyses.

Methods for the selection of microsatellite markers

Preliminary evaluations of previous studies were carried out in the months of June and July 2023 for the choice of the best microsatellites set to be tested on Adriatic populations. The whole bulk of considerations is reported below.

Six papers used microsatellites markers to characterize different populations of *Pinna nobilis* in the western Mediterranean Sea: (González-Wangüemert et al. 2015, 2019; Wesselman et al. 2018; Peyran et al. 2020; 2021; 2022).

- González-Wangüemert et al. (2015) developed 10 microsatellites primers based on the analysis of 8 individuals from Mallorca (Balearic Islands, Spain).
- The same authors (González-Wangüemert et al. 2019) performed further genetic analyses on individuals from different locations within the Balearic Islands, using the 10 microsatellite loci reported in González-Wangüemert et al. (2015).

Among these loci, seven of them (Pn 2.1, Pn 3.4, Pn 3.3, Pn 3.6, Pn 4.2, occurring at six localities), Pn 4.3 (found in four localities), and Pn 3.5 (present in two localities) displayed significant deviations from Hardy-Weinberg Equilibriums. Upon analyzing all the samples, they concluded that some of the deficits in heterozygotes could be attributed to the presence of null alleles, although they confirmed that these deviations were not the result of genotyping errors.

Interestingly, three of the loci displaying significant heterozygote deficiency (Pn 3.4, Pn 3.6, Pn 4.2) also exhibited significant values for the presence of "null alleles." Despite this finding, the authors claimed that the inclusion or exclusion of these loci had no significant impact on population comparisons.

- In this context, Wesselman et al. (2018) used the whole group of 10 microsatellite loci provided by González-Wangüemert et al. (2015).
- Peyran et al. (2020) presented a study with the developing of a new set of 26 microsatellites markers based on the analysis of 13 individuals from Peyrefite (Banyuls, France). They tested both their set of microsatellites and the one from Gonzales et al. (2015).

After analyses, 3 loci from Gonzales et al. (2015) were eliminated (PN 2.1; PN 2.2; PN 4.2) due to the presence of an ambiguous pike profile for all the individuals analysed.

- In 2021, the same authors (Peyran et al. 2021) used the microsatellites which were validated in their previous study (Peyran et al. 2020) to analyse the genetic structure of *Pinna nobilis* across the Gulf of Lion, France.
- Based on these studies, we selected a subset of 10 microsatellites, which includes those from Gonzales et al. (2015) (those validated by Peyran et al. 2020) along with those from Peyran et al. (2020).

In particular, among the microsatellites proposed by Gonzales et al. (2015), we chose to include in our panels only those from which have been validated by Peyran et al. (2020) (PN. 3.2, PN 5.2, PN 4.3, and PN 3.5). Additionally, among the microsatellites proposed by Peyran et al. (2020), we chose 6 microsatellites based on our own considerations on annealing temperature (for the creation of functional panels) and expected/observed heterozygosity values.

Based on the considerations above reported, the final set of microsatellites that have been chosen by the researchers of the University of Sassari to be used to analyze the *Pinna nobilis* populations from Gulf of Trieste (Friuli Venezia Giulia) and Venetian Lagoon (Veneto) are reported in the table below with the corresponding annealing temperatures (Ta):

Panel	Locus	T a
1	11847; PN 3.2; 6980; 15415	53 °C
2	15584; 9902; 4482	55 °C
3	PN 5.2; PN 4.3; PN 3.5	61 °C

In August and September 2023, the designed panels of microsatellites were tested on some individuals belonging to the population of the Gulf of Trieste before proceeding with the characterization of the whole group of 30 samples.

During the process of methodological standardization, the samples used for the preliminary tests were amplified by standard PCR with pairs of non-fluorescent primers to evaluate the goodness of the PCR before performing PCR with fluorescent (and expensive) primers. The goodness of the PCR was roughly verified through gel electrophoresis and the PCR bands were sequenced to verify that the repeated motifs correspond to the microsatellites which were amplified. Indeed, the sequences obtained from preliminary PCRs products confirmed that the amplified microsatellites were those actually included in the set that was designed by the researchers of the University of Sassari.

Consequently, a preliminary standardization process of the PCR with fluorescent primers on a small number of individuals belonging to the Gulf of Trieste and Venetian Lagoon was concluded in October 2023 (see Fig. 4). Therefore, the microsatellite characterization at the Sanger sequencer of all individuals available for Friuli Venezia Giulia and Veneto started in November 2023 and will be concluded in March 2024. After the molecular analyses of the microsatellite, the obtained data will be subjected to bioinformatic analyses. Overall, the preliminary results on the genetic variability of the *Pinna nobilis* populations of the Gulf of Trieste and the Venetian lagoon will be ready at the end of July 2024.

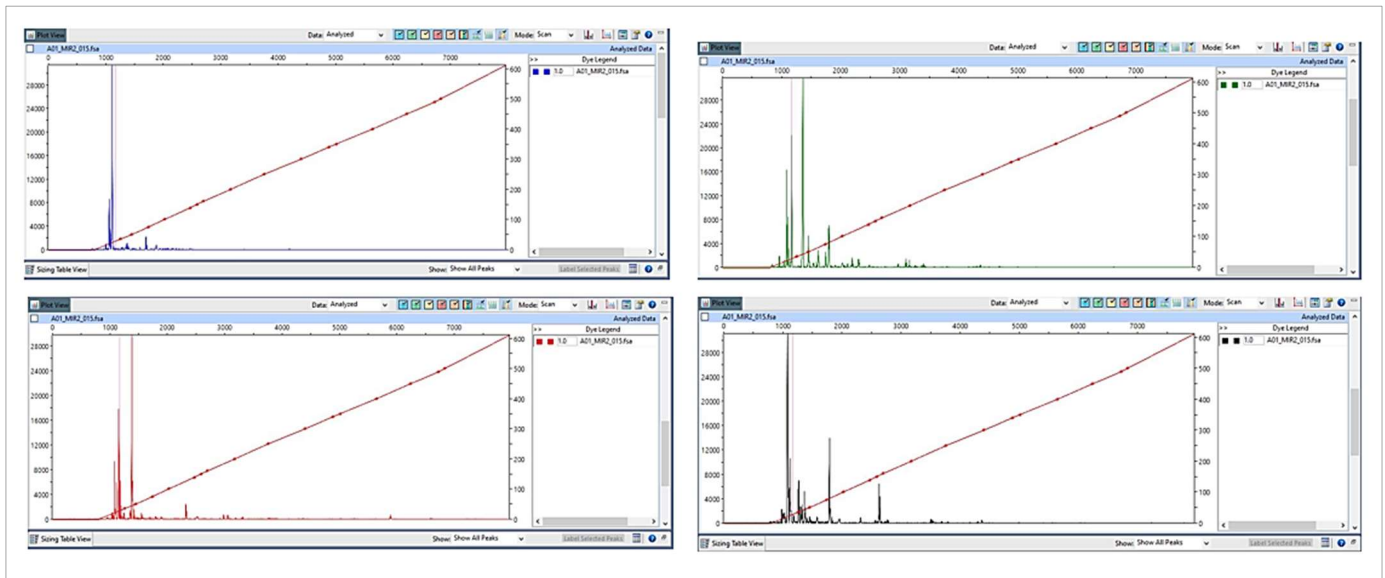


Figure 4: The figure shows some of the microsatellite peaks that were obtained at the Sanger sequencer characterization during the standardization process in 2023.

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Scientific papers published by the researchers the University of Sassari including studies involved with the topics of the action A.2.

In the following scientific paper, which is included in the present mid time report among the attachments, the researchers of the University of Sassari represent the principal investigators.

This study is focused on topics that overall correspond to the main objectives of the action A.2. of the present project.

- Daria Sanna, Ilenia Azzena, Chiara Locci, Pavel Ankon, Petar Kružić, Chiara Manfrin, Alberto Pallavicini, Saul Ciriaco, Marco Segarich, Edoardo Batistini, Fabio Scarpa, Marco Casu. **Reconstructing the Evolutionary History of *Pinna nobilis*: New Genetic Signal from the Past of a Species on the Brink of Extinction.** *Animals* **2024**, 14, 114. <https://doi.org/10.3390/ani14010114>