Faculty of Science

Conservation genetics of Small-spotted catshark

-and an evaluation of DNA sampling technics on elasmobranchs

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Content

1. Sammanfattning

Populationer av broskfiskar minskar världen över och för att motverka detta är bevarandeprogram en god början. Metoder och protokoll för detta borde testas på en icke hotad modellorganism innan de appliceras på en mer utsatt art.

Den småfläckiga rödhajen (*Scyliorhinus canicula)* betraktas som en stabil art men en nedgång i antal individer i svenska vatten har noterats. För att bemöta denna nedgång så har det publika havsakvariet Havets Hus i Lysekil sedan 2002 startat upp ett bevarandeprogram där de föder upp samt släpper ut småfläckig rödhaj varje år. För att bevara genetisk diversitet hos de frisläppta hajarna måste man introducera lämpliga föräldrar till uppfödningsprogrammet. Detta görs genom att välja föräldrar som inte är nära släkt men som ändå kommer från samma genetiska population.

I denna studie gjorde vi genetiska analyser med mitokondriella och nukleära markörer för att jämföra hajar från franska och svenska vatten som hålls i akvarium och testade provtagningsmetoder för broskfiskar. Vårt resultat visar på att de troligtvis kommer från samma population vilket indikeras av en låg genetisk populationsuppdelning (F_{ST}= 0,0239). De är därmed lämpliga för parning inom bevarandeprogrammet. Vi testade olika provtagningsmetoder (vävnad, blod och användnings av tops) för att reducera stressen som hajarna får under provtagningen. Våra resultat visad att även den mindre invasiva metoden med tops fungerar för att få fram DNA.

2. Abstract

Elasmobranchs populations are decreasing around the world and to take action against this conservation programs are a good start. Methods and protocol should be tested on a non-threatened model organism before applied to more vulnerable species.

The small-spotted catshark (*Scyliorhinus canicula)* is generally considered to be a stable species but has seen a decline in number of individuals in Swedish waters. To meet this decline the public marine aquarium Havets Hus in Lysekil, Sweden, has started a conservation program (in 2002) in which they breed and releases small-spotted catsharks every year. To conserve genetic diversity among the released sharks suitable parents must be introduced into the breeding program. This is done by choosing parents that are not close relatives but still within the same genetic population.

In this study we performed genetic analyzes using mitochondrial and nuclear markers to compare available sharks in aquariums from French and Swedish waters and also to try out methods for other elasmobranchs. Our results indicates (F_{ST}= 0.0239) that they probably are part of the same breeding population and are suitable for mating. To reduce the amount of stress during sampling for the sharks, we also tried out different sampling methods (tissue, blood and swab samples). We found out that less invasive one (swab) also works for DNA sampling.

Keywords: *Scyliorhinus canicula*, conservation, genetics, DNA-sampling.

3. Introduction

Around the world the elasmobranchs (sharks and rays) are generally declining due to a number of factors. They are caught as bycatch, fished for recreational or commercial use. This in combination with their relative long generation time and often few offspring has a devastating effect on many species of elasmobranchs (Shepherd et al., 2005). One way of helping these sharks and rays are with conservatory programs. But to start up such a program there must first be knowledge of how to do it best before applying it to a vulnerable species. The best way is then to use a stable species as a model organism to try out methods and protocol.

The small-spotted catshark (*Scyliorhinus canicula, Linneus, 1758)* is the most abundant catshark in the northeast Atlantic and occurs on the continental shelf and continental slope, down to 400 meters, from Norway, down to Senegal and in the Mediterranean Sea (Ellis & Shackley, 1997). *Scyliorhinus.*canicula is often used as a model organism. The species is listed as "Least Concern" on IUCNs red list and no conservatory actions have been taken (IUCN Red List, 2014). Globally the small-spotted catshark population is estimated to be stable but in some areas local depletion have been seen (Kousteni et al., 2014) which could lead to a decline in genetic difference within the species if it is made up of local populations. An earlier study showed genetic differences between *S.canicula* from the Atlantic and the Mediterranean Sea, indicating different populations within the species but also some differences within the populations. The population genetic structures were found using mitochondrial and microsatellite markers (Gubili et al., 2014).

Havets Hus in Lysekil, Sweden, is breeding small-spotted catsharks on a small scale in a conservatory program, because of its decline in Swedish water (Havets Hus). For a program like this to keep going, some problems, such as inbreeding, needs to be countered to maintain the genetic diversity and produce healthy offspring for the future (Boscari et al., 2014). The parents should be of the same breeding population but not closely related. Local populations could be problematic for a breeding program due to the difficulty of finding enough parents that are not of a kin (Keller & Waller, 2002).

Mitochondrial DNA (mtDNA) is maternally inherited and has a slower mutation rate than microsatellites. Microsatellites are non-coding, small, repetitive sequences in the nuclear DNA with high mutation rate. The information acquired through looking at mtDNA, with a low mutation rate, gives an evolutionary perspective and only pieces of the whole picture. Microsatellites, with its high mutation rate, gives higher resolution of information and are used to study more recent information such as relatedness and population history on a more individual scale (Zhang & Godfrey, 2002).

In this study, we performed genetic analyses on available small-spotted catsharks from French and Swedish waters to compare how genetically different these two populations are to see if they are part of the same genetic population, with possible breeding intentions in mind. We also tried out different sampling methods to see if less invasive ones could generate good results, which could potentially decrease the amount of stress the animals receive in future samplings. This work could contribute to guidelines for future sampling and conservation programs among sharks and rays.

4. Hypothesis

With consideration to previous articles about the small-spotted catshark this study aimed to test the hypothesis that our two populations are not genetically different from each other.

5. Material and Methods

5.1 Sample collection and storage

Samples were collected from *S.canicula* in different ways at Universeum (Gothenburg, Sweden), Sjöfartsmuseet Akvariet (Gothenburg, Sweden) and Havets Hus (Lysekil, Sweden) as a part of a checkup by a veterinarian. The sharks from Universeum were a mix of individuals from Sweden and France (Roscoff). Sample collection methods were: tissue sample, blood sample, by the use of a swab (Sterile Rayon Tipped Applicators, Puritan, Maine USA) (anally, orally or on the skin) and tissue sample from egg capsules. The samples were stored in 2 ml sampling tubes in a freezer at -24°C. Tissue was stored in ethanol and blood was stored on a filter paper if the sampling yielded low amount of blood. The bigger catsharks were sedated during sampling.

5.2 DNA extraction

DNA was extracted mainly using 100 ml QuickExtract™(epicenter®, Madison, WI USA) with the following protocol: incubation of 25 minutes at 65°C followed by 2 minutes at 95°C. Few tissue samples were extracted using E.Z.N.A.® Tissue DNA kit and a few (n=2) blood samples were extracted with E.Z.N.A.® Blood DNA mini kit (Omega bio-tek, Norcross, GA 30071 USA), both using standard protocol. Extracted DNA was stored in 1.5 ml Eppendorf tubes in a freezer at -24°C.

5.3 Mitochondrial and microsatellite primers

For amplifying mitochondrial DNA fragment the primer pairs Fish1 and Fish2 were used (Kousteni et al., 2014). These primers pairs are fish specific and target the cytochrome c oxidase 1 (CO1) region.

For amplifying nuclear DNA we used hypervariable region markers, or microsatellite markers. These are listed in Tabel 1.

Table 1 – Microsatellites used in this study showing locus, primer sequence for forward and reverse, annealing temperature (TA) and published allelic size range (base pair).

Locus	Primers (5´-3´)	TA (°C)	Size (bp)
Scan03	F: TGG ATA ATT GAC AGA ATC GGC R: TGC GAT TGT AAT AAT GGA CAC A	55	170–180
Scan04	F: ACC AAA GAT CAA GCC AGG AA R: TGT GAA TGG TGC GAT GTT TT	55	237–247
Scan05	F: ACC GAT TCA AGG ATC ACG AG R: ACC ATC AGT AAA GGC AAC CG	55	176–184
Scan06	F: GGC AGT GAT TGC ATT CTT GA R: CAG AAA CTG TGC AGA AAT CAC A	55	204–224
Scan09	F: GAG AAT TGT TTC CCA GTG GC R: ACC TGC CCA GCT CTT GAG TA	55	114–116
Scan13	F: CGC CCT CCC CTA AAA TAG AC R: TGA ACA CAA GCG AAC GAA AC	55	174–188
Scan14	F: AAC CAT CCT CCG CAA ATA AA R: GAA CAG TGC CCC AAG TTC AT	55	277–284

5.4 PCR amplification

A master mix for all samples was made in an 1.5 ml Eppendorf tube containing: both forward and reverse primers, VWR Red Taq DNA Polymerase 1:1 Master Mix (1.5 mM $MgCl_2$) and in some cases distilled water (to diluted when higher concentrated Taq x2 was used), as shown in the table below (Table 2),before each PCR amplification.

Table 2 - Amount of Taq DNA Polymerase 1:1 or Taq DNA Polymerase x2, sterile water, forward primer, reverse primer added to the master mix for each targeted mitochondrial sequence or microsatellite loci and amount of DNA-extract added to the 0.2 ml tubes

	Taq DNA polymerase 1:1 (µl)	Taq DNA polymerase X2 (µl)	Sterile water(µl)	Forward primer (µl)	Reverse primer (µl)	Extracted DNA (µl)
Fish1	8.5	-	-	0.33	0.33	0.5
Fish2	25	-	-	1	1	0.5
Scan03	-	12.5	11	0.5	0.5	0.5
Scan04, 05, 06, 09, 13 and 14	25	-	-	1	1	1

Master mix was added into 0.2 ml tubes in PCR-strips of 8 together with template DNA. The strips were vortexed and centrifuged before placed in an Arktik Thermal Cycler (Thermo Fisher Scientific Oy, Vantaa, Finland) for PCR amplification.
The following settings were used for Fish 1 and Fish2: Initial denaturation for 2 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 45 s and extension at 72°C for 45 s, before final extension step at 72°C for 10 min (Kousteni et al.,.
The following settings were used for microsatellites (Scan): Initial denaturation for 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, before a final extension step at 72°C for 10 min (Griffiths et al., 2011).
The PCR-strips were stored in a freezer at 24°C after amplification.

5.5 DNA visualization
After PCR amplification, the DNA was visualized on 50ml of 1% agarose gel. 2 µl GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA USA) was added to the gel so the DNA would light up under UV-light.

5.6 DNA purification, mitochondrial DNA sequencing and microsatellite genotyping
8 µl of the PCR products amplified with the Fish1 and Fish2 primer pairs respectively was purified with 2µl X4000 Exonuclease-1 (Thermo Scientific) in the Arktik Thermal Cycler with the following settings: 37°C for 45 min, 75°C for 15 min. 5 µl of the purified product and 5 µl of forward or reverse primer was added to an Eppendorf tube and sent to GATC Biotech for sequencing.

The microsatellite DNA for each individual was divided into multiplex 1, containing 6 µl of amplified DNA from Scan05, Scan06 and Scan13, and multiplex 2, containing 4 µl of amplified DNA from Scan03, Scan04, Scan09 and Scan14, so that the amount of tubes to send away was significantly reduced. If the DNA concentration in Scan04 and Scan09 differed, by visual estimation, then the higher one was diluted to roughly the same concentration with sterile water before added to the multiplex due to them having the same color. Each multiplex was added to an Eppendorf tube and sent to Eurofins Genomics, for genotyping.

5.7 Statistics

Mitochondrial data was imported and analyzed in Geneiouse 8.0.5.
Microsatellite data was analyzed in Genepop version 4.2 to receive the expected heterozygosity, F_{ST}-value and p-value with the following settings.
Option 3 with Genic differentiation: For all pairs of population, Dememorization number: 1000, Number of batches: 100 and Numbers of iterations per batch: 1000 gave us p-value per locus and across all loci.
Option 5 with Diploid settings generated the expected heterozygosity (H_E).
For the F-statistic values, Option 6 was used with following settings: Allele identity: For all population pairs, Estimation ploidy: Diploid, Minimum distance between samples to be taken in account for regression: 0.0001, Number of permutations for Mantel test: 1000.

6. Results

6.1 Mitochondrial data

The results from the mitochondrial analysis, using sequences from this study and downloaded sequences from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) are shown in Figure 1.

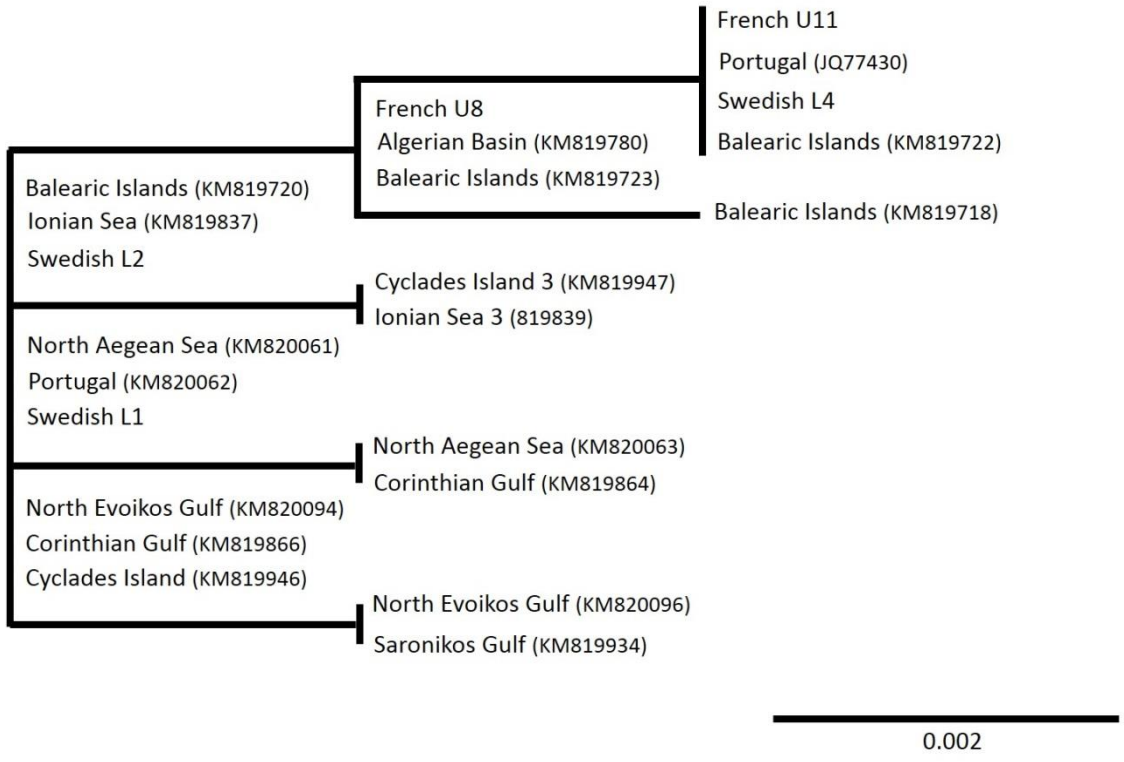

Figure 1 - Neighboring joining tree for mitochondrial sequences acquired with Fish1 primers. Swedish and French are from this study. All other data has been downloaded from Genbank. Genbank accession numbers for each downloaded sequence inside parenthesis. Legend shows percentage of the sequence that has mutated to the next step (here: one base pair/line).

6.2 Microsatellite data

A summarization of the values acquired using Genepop for the microsatellite loci are shown in Table 3.

Table 3 - Results for microsatellite genotyping showing number of alleles (N_a), observed heterozygosity (H_O), expected heterozygosity (H_E), differentiation between population (F_{ST}) and p-value per microsatellite locus.

	N_a	H_O	H_E	F_{ST}	P-value
Scan03	5	10	9.4815	0	0.47180
Scan04	3	4	6.1852	0.1436	0.07973
Scan05	3	10	11.2432	0	0.19911
Scan06	8	19	14.1892	0	0.95377
Scan09	3	2	1.9714	0	0.69328
Scan13	3	6	6.6207	0.0854	0.22229
Scan14	7	14	12.8276	0.0510	0.09270

Across all loci we got a F_{ST}-value of 0.0239 and p-value of 0.21.

6.3 Sampling Method

Successes rate of each sampling technic are listed in Table 4.

Table 4 – Shows the percentage of successfully amplified DNA per mtDNA gene and microsatellite loci based on sampling method.

	Fish1 (%)	Fish2 (%)	Scan03 (%)	Scan04 (%)	Scan05 (%)	Scan06 (%)	Scan09 (%)	Scan13 (%)	Scan14 (%)
Swab (5 samples)	100	20	20	20	100	100	100	40	40
Blod (11 samples)	100	18	82	91	100	100	91	91	91
Tissue (3 samples)	100	100	100	100	100	100	100	100	100

Out of 10 samples of egg capsules, one gave low amount DNA after extraction and was only visualized on gel after amplification by Fish1 primers.
Two swabs on skin samples were performed with no results.

7. Discussion

7.1 Genetics

According to a previous study (Gubili et al, 2014) the small-spotted catshark is divided into two major genetic population structures, one in the Atlantic and one in the Mediterranean Sea. So the hypothesis that we wanted to test was that there should not be a genetic difference between our French and Swedish populations as both these originates from the Atlantic.

The individuals in the two populations of catsharks are probably not close relatives with any individual in the other population as the geographical location from where they originate are far apart. But as the information about the species is limited there is yet a small possibility. In any case it should be a fresh addition of genetic material to the conservation program because of the breeding parents already in the program are probably genetically more similar.

The data received using mitochondrial DNA in combination with data from previous study of the small-spotted catshark was analyzed and visualized (Figure 1). Here we can see that our French and Swedish sharks share the same haplotype as other sharks originating from the Atlantic. But two of our sharks also have the central ancestral haplotype (Kousteni et al., 2014). This haplotype can be found both in the Atlantic and the Mediterranean Sea. It is from the central ancestral haplotype that the others are derived from as shown in figure 1. This can give an indication of some gene flow occurring between the two major genetic populations.

If any of our sharks have had a haplotype, which we did not find in this study, which is only found in the Mediterranean Sea then it could have raised suspicion that they are not suitable to introduce into a conservation program with sharks that have an Atlantic haplotype.

The microsatellite data (Tabel 3) strengthens our hypothesis that our French and Swedish populations are from the same genetic population as we received an F_{ST}-value of 0.0239 when all loci were compared. There are no definite guidelines of which values say that they are not different from each other but below 0.15 are often considered to be very low differentiation (Frankham et al., 2002). So our value of 0.0239 is very low in consideration which indicates that they are not different. We did get a p-value of 0.21 as compare to our chosen α-value of 0.05. We did not get a significant result from the microsatellite data but more an indication that our populations are from the same genetic population. With more samples and more microsatellite loci we could get a significant result.

Among the small-spotted catsharks that have been released outside of Havets Hus in Lysekil one has been found near the Norwegian coast (Helen Sköld, pers. comm.). This indicates that the catsharks are moving and are not stationary in local populations which strengthens our hypothesis.

When the French sharks reach maternity they could possibly be recruited into the conservation program because of the result generated from mitochondrial and microsatellites markers indicating that they are part of the same genetic population occurring along the Atlantic coast.

7.2 Sampling Method

There were differences in the sampling methods as Table 4 shows. As one could expect, tissue worked best, with a 100% success rate. When DNA was visualized from these samples you could also see that they had a higher concentration of the targeted DNA then the other samples. This is probably due to the fact that there are a lot of cells in the tissue which result in a lot of DNA.

Blood samples showed good results also, slightly less success rate than tissue but with a bigger sample size to back up the results. The blood samples were smaller and blood probably contains fewer cells with DNA than a tissue sample of the same size, which might contribute to the lower success rate. It could also be variance due to the relative small samples size of the two methods.

Samples taken by a swab, orally or anally, also gave positive results but in fewer cases then tissue and blood. Also when the positive samples were visualized on the gel, they all glowed with low intensity under the UV-light. This indicates that there was low amount of DNA present in these samples. The swab samples were re-extracted once or twice for some samples. When extracted only a part of the swab was cut off into the tube with the extraction liquid (QuickExtract™) and later extraction of the same swab yielded results when the first did not, indicating that the cells and DNA was sporadically distributed over the swab.

All of the swab samples was amplified and yielded DNA for at least one instance per targeted gene or microsatellite loci. This shows that the method is working but needs some optimization. Potential refining of the method could be to extract from the whole swab so that all available DNA will be used directly. The result from this could be sharper but comes with the price that there will be no backup from that sample.

There is a whole range of options when it comes to the PCR, the ration between DNA-extract, primers and Taq polymerase in the PCR-mix can be tweaked for better results. The settings of the Thermal Cycler can also change the outcome of the results by adding cycles and/or changing annealing temperature. This could be time consuming and regrettably there was no time for extensive optimization.

The negative results or the samples from egg capsules and swab on skin do not necessary say that these methods do not work. It is a small sample size for both methods, especially for swab on skin with only two samples. The methods need to be tested with more samples and for more microsatellite loci before it can be discarded. Particularly with one positive result from the eggs indicates that it might work with more testing and optimization.

If there was more time for this project we would also have tested the approach of environmental DNA (eDNA) as a sampling method. This relative new science where DNA is obtained directly from, for example, water samples is a non-invasive method (Lodge et al., 2012). If this is applicable to sharks and rays it would be an asset to the conservation programs.

8. Conclusion

To conclude this study we got results from mitochondrial markers that our two populations, French and Swedish, of sharks shared the same haplotype as sharks from the Atlantic. Genotyping of microsatellite loci gave us an indication that our two populations were not genetically different. This indicates that the French small-spotted catsharks can be introduced into the conservation program.

For the sampling methods the tissue samples worked best followed by blood then swab samples. But with some optimization of the swab technic better results could be generated. More time and samples are needed to both refine the methods and get a stronger significance of the genetic analyzes.

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No shark was harmed during this study.

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