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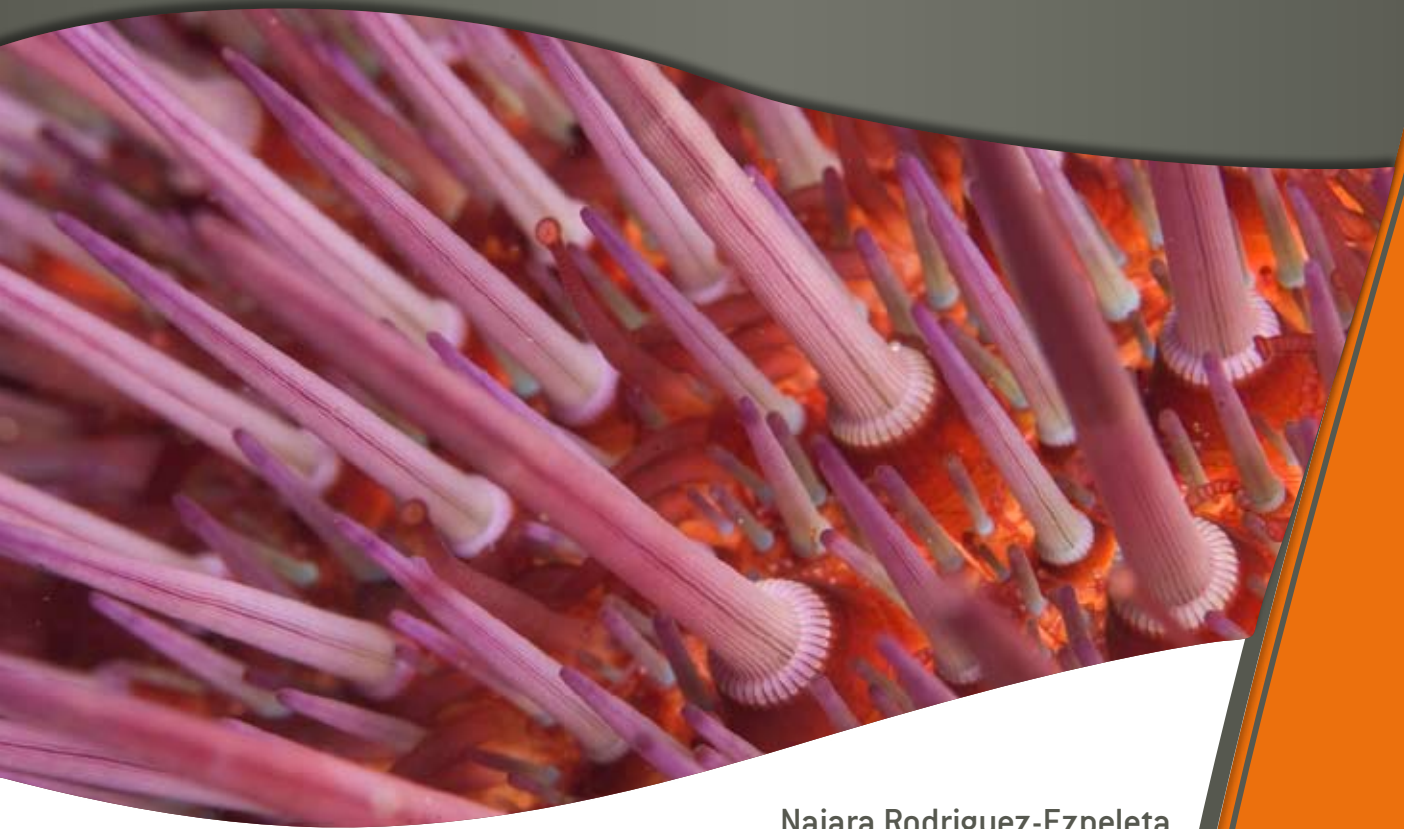
20(6) Effect of fish sampling and tissue storage conditions in DNA quality: considerations for genomic studies



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Effect of fish sampling and tissue storage conditions in DNA quality: considerations for genomic studies

Naiara Rodriguez-Ezpeleta*, Iñaki Mendibil, Paula Álvarez and Unai Cotano

Abstract

Population genomics experiments of non-model organisms, such as most commercial fish, rely on isolating good integrity DNA from the study subjects. Yet, this task is not trivial as, when an organism dies, its DNA starts breaking down in small pieces. This process is called DNA degradation and can fortunately be stopped or slowed down by preserving the samples in cold and/or dry conditions. Here, we have assessed the effect of tissue type, storage time, preserving conditions and post-mortem interval in fish DNA integrity. From the four factors tested, post-mortem interval is the one that most drastically affects DNA integrity, tissues sampled after 24 hours of death yielding partial or totally degraded DNA. In order to preserve DNA integrity, we provide some recommendations to be considered when sampling fish tissue for genetic analysis.

Resumen

Los estudios de genética de poblaciones en peces de interés comercial dependen de la obtención de ADN de buena calidad. Esta tarea no es fácil ya que, cuando un organismo muere, su material genético comienza a romperse en fragmentos pequeños en un proceso llamado degradación. Este proceso no reversible puede afortunadamente detenerse preservando las muestras en condiciones de frío o desecación. En este trabajo, se ha estudiado el efecto del tipo de tejido, tiempo y condiciones de almacenamiento, y tiempo post-mortem en la integridad del ADN. De los cuatro factores evaluados, el tiempo post-mortem es el que más drásticamente afecta a la integridad del ADN, obteniéndose del tejido muestreado después de 24h un ADN parcial o totalmente degradado. Proporcionamos una serie de recomendaciones a considerar durante actividades de muestreo para optimizar la integridad del ADN obtenido de muestras de peces de interés comercial.

Introduction

DNA analysis is becoming important in fisheries research. In particular, the study of the genetic structure of fish populations by means of polymorphic DNA markers has proven critical in defining management units, in setting priorities for conservation, and in understanding the effects of climate change (Nielsen *et al.*, 2009). Polymorphisms are natural variations in DNA that have no adverse effects on the individual and occur with high frequency in the general population. A polymorphism involves two or more variants of a particular DNA sequence. The most common types of polymorphisms used in population genetics are microsatellites and single nucleotide polymorphisms (SNPs). Microsatellites are repeating DNA sequences that occur in variable number (*e.g.* one individual contains two repetitions of one DNA stretch – ACAC, whereas another one contains five – ACACACACAC), and SNPs are variations occurring at a single nucleotide position (*e.g.* one individual contains the nucleotide sequence AATCCG whereas another one contains AATTCG – there is a SNP in position four of the sequence) (Hartl and Clark, 2007).

The discovery of microsatellites and SNPs in a given species has typically involved i) sequencing genomic regions of interest from multiple individuals to find potentially polymorphic sites, ii) developing genotyping assays to measure variation of each potential marker, and iii) proving of the assays in a screening population before full deployment across a large number of individuals. This process is both time consuming and expensive, and usually results in the generation of very few (tens) working markers (Duran *et al.*, 2009; Slate *et al.*, 2009). This approach is therefore prohibitive for population genomics, which consists on analyzing a large number of markers to study genome-wide variation within and among populations in time and space (Nielsen *et al.*, 2009). Alternatively, high-throughput sequencing technologies offer the possibility of discovering, sequencing and genotyping thousands of markers in tens to hundreds of individuals in a single step. Approaches such as genotyping by sequencing (GBS) and restriction site-associated DNA sequencing (RAD-seq) consist on cutting the DNA with restriction enzymes and on sequencing a common subset of the generated fragments in all individuals to be analyzed (reviewed in (Davey *et*

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al., 2011)). In contrast to the genotyping of known markers, which can be performed on partially degraded (fragmented) DNA samples, the *de novo* genome-wide marker discovery assays require large quantities of high quality unfragmented DNA as starting material (Etter *et al.*, 2011).

DNA degradation starts occurring at the moment an organism dies, when cell membranes break down. This allows entrance of bacteria and other threats to the cell and release of DNAses, enzymes that sequentially cut single nucleotides from DNA until the molecule is so short that cannot longer be called DNA. The speed of the DNA degradation process depends on many factors, and can be slowed down by keeping the samples cold and/or dry or by using preservative agents that prevent DNAse activity. Refrigeration, by storage at – 20 ºC, -80 ºC or in liquid nitrogen (-196 ºC), and desiccation, by drying at 70 ºC or placement in silica desiccant, impede degradation by microbes and enzymes, which require temperatures above 0 ºC and the presence of water to be active (Michaud and Foran, 2011). Commonly used preservative agents not only dehydrate the sample by permeating the tissue and displacing water, but have also other properties that prevent DNA degradation; for example, ethanol precipitates enzymes and kills bacteria and fungi, DESS (dimethyl sulfoxide combined with NaCl and ethylenediaminetetraacetic acid) chelates metals on which enzyme activity depends (Dawson *et al.*, 1998), and the commercial RNA stabilizing solution RNAlater™ (Ambion, TX, USA) inhibits the action of enzymes by co-precipitating them with the nucleic acids. A number of studies have been performed comparing different methods of sample storage to prevent DNA degradation (e.g. (Willliams, 2007; Omar *et al.*, 2009; Michaud and Foran, 2011; Eschbach, 2012)).

The aforementioned methods stop or slow down degradation, but do not revert it. That is, if the DNA is already partially or totally degraded, applying any of these methods will simply prevent any further degradation, but will not make the DNA return to its original integer state. Thus, regardless of the method of choice for sample preservation, tissue storage in appropriate conditions has to be applied as close as possible to the time of death of the organism, when DNA starts degrading. This is often a challenge when collecting individuals from natural populations, where sampling takes place in difficult conditions, and there may be a delay between organism death and tissue storage. As opposed to the study of the effect of different preservation methods, few studies have focused on the effect of post-mortem interval on DNA integrity (Michaud and Foran, 2011), and, surprisingly, none has focused on commercial fish, whose genomic studies are particularly sensitive to this issue due to sampling constraints.

Sampling of commercial fish for DNA analysis is challenging. In best case scenario, qualified scientific personnel perform the sampling onboard, but even then conditions are not ideal. First, lack of clean and sterile surfaces as well as working in tough weather conditions can prevent meticulous manipulation of the samples; second, a large number of fish are generally caught at the same time, meaning that, even if caught alive, sampling will be performed soon after death only in some individuals. Oftentimes, sampling is the result of collaborative efforts among different institutions, some of which are not directly involved in DNA analysis and, therefore, are not aware of the strictness required during the sampling process. In other cases, sampling onboard is not possible, and fish are collected when they reach port, often without knowledge of the conditions in which they have been stored in the boat.

It is widely accepted that fisheries research will largely benefit from newly available high-throughput sequencing techniques for polymorphism marker discovery and genotyping (Nielsen *et al.*, 2009). Yet, the application of these techniques requires often difficult to obtain high quality DNA. The aim of this study is to mimic real life situations of fish sampling and tissue storage in order to evaluate the effect of retarded sampling and preserving conditions, and to determine the borderline circumstances in which the required DNA quality for population studies cannot be assured.

Material and Methods

Fish material collection and preservation

The first set of samples is part of a project aiming to study mackerel population structure, and consists of individuals collected in Canada, Crete, the Bay of Biscay and Galicia. Whole fish from Canada and Crete were collected at their arrival to port and shipped to AZTI frozen in a DHL Thermobox®, although the samples from Crete arrived unfrozen for unknown reasons. Samples from the Bay of Biscay and Galicia were collected by AZTI on board and muscle tissue was kept on ethanol. The second set of samples consists of bluefin tuna samples samples collected by AZTI AZTI during landings of baitboats and trap fisheries (courtesy of Haritz Arrizabalaga, Nicolas Goñi and Igaratza Fraile). These samples arrived in ethanol 96% at room temperature to the laboratory.

Anchovies and sardines were collected alive from a seawater cage located in Mutriku (Bizkaia) maintained by AZTI Tecnalia as part of a study to assess the adaptation of individuals to captivity. The sampling schedule was designed to simulate realistic scenario of fish sampling. Two sampling efforts were performed. Both were designed to test post-mortem interval, preservative and post-mortem interval. Additionally, the first sampling effort tested the effect of tissue type (fin or muscle), and the second tested for the effect of species (anchovy or sardine). During the second sampling effort only storage at room temperature was tested and an additional preservative, DESS, was included.

In the first sampling effort, about one square centimeter fin and muscle tissue samples were taken from four subsets of 24 anchovies each. Tissues from the first subset were excised within one minute after death, whereas tissues from the other subsets were excised after letting the anchovies stand for 2 and 24 hours on ice and for 5 days in the refrigerator (4 ºC), respectively. Samples were stored without preservative at -20 ºC, in ethanol 96% at -20 ºC, in ethanol 96% at room temperature and in RNAlater™ at room temperature. Ethanol was changed after 24h. These samples remained in storage 30 and 60 days before DNA extraction. A total of 192 DNA extractions (three replicates of each combination of factors) from different tissue types stored at different conditions during variable times were performed (Table 1).

In the second sampling effort, about one square centimeter muscle tissue sample was taken from two anchovies and two sardines within a minute of death. The same individuals were let stand for 4h on ice and for 4 days in the refrigerator (4 ºC) before subsequent tissue excisions. Samples were stored in DESS (20% DMSO, 0.25 M disodium-EDTA, and NaCl to saturation, pH 7.5 (Seutin *et al.*, 1991)) and in ethanol 96%, both at room temperature. No ethanol replacement was performed in this case. These samples remained in storage 30 and 120 days before DNA extraction. A total of 48 DNA extractions (two replicates of each combination of factors) from muscle tissue stored at room temperature in ethanol or DESS during variable times were performed (Table 2).

DNA extraction and quantity, purity and integrity evaluation

All DNA extractions were performed using the Wizard® Genomic DNA Purification kit (Promega, WI, USA) following manufacturer´s instructions for "Isolating Genomic DNA from Tissue Culture Cells and Animal Tissue". The starting material was about 20 mg of tissue and after extraction, all samples were suspended on an equal volume of Milli-Q water. DNA quantity (ng/µl) and purity (260/280 and 260/230 absorbance ratios) were evaluated on the Nanodrop® ND-1000 (Thermo Scientific) system. Absorbance ratios are used to assess protein contamination in the DNA sample; in a pure sample, the 260/280 ratio is usually higher than 1.8 and the 260/230 ratio is usually higher than 1.5 (ThermoScientific, 2010). DNA integrity was assessed by electrophoresis, migrating about 100 ng of GelRed™-stained DNA on an agarose 1.0% gel. When applying an electric field, the negatively charged DNA migrates towards the positive pole through the agarose matrix, shorter molecules migrating faster than the longer ones. Thus, integer unfragmented DNA usually appears as a compact, high-molecular-weight band with no low-molecular-weight smears. Based on the gel images, numbers from 0 (full degradation) to 10 (full integrity) were assigned to each of the samples (modified from (Salgado *et al.*, 2007)). Different treatments were compared and statistical significance was assessed with the Wilcoxon rank sum test and signed rank test for unpaired and paired samples, respectively.

Results

Real examples

The integrity of the DNA obtained from the mackerel and Bluefin tuna samples is variable (Figure 1). Mackerel samples from Canada and Galicia yield DNA of excellent integrity, whereas the DNA extracted from the samples from Crete and the Bay of Biscay is highly degraded. Bluefin tuna samples taken from fish caught using traps yield DNA of better integrity than the ones having spent few days refrigerated on board.

Table 1. Number of DNA samples obtained during the first sampling per *post morten* and storage time, and preservative and tissue type.

		-20		Ethanol -20		Ethanol RT		RNAlater RT		
		Fin	Muscle	Fin	Muscle	Fin	Muscle	Fin	Muscle	
Sampling	Storage time									
<1 hour	*30 days*	3	3	3	3	3	3	3	3	48
	60 days	3	3	3	3	3	3	3	3	
2 hours	*30 days*	3	3	3	3	3	3	3	3	48
	60 days	3	3	3	3	3	3	3	3	
24 hours	*30 days*	3	3	3	3	3	3	3	3	48
	60 days	3	3	3	3	3	3	3	3	
5 days	*30 days*	3	3	3	3	3	3	3	3	48
	60 days	3	3	3	3	3	3	3	3	
Total		48		48		48		48		**192**

RT: Room temperature

Table 2. Number of DNA samples obtained during the second sampling per *post morten* and storage time, and preservative and species.

		Ethanol		DESS		
		Anchovy	Sardine	Anchovy	Sardine	
Sampling	*Storage time*					
<1 hour	*30 days*	2	2	2	2	16
	120 days	2	2	2	2	
4 hours	*30 days*	2	2	2	2	16
	120 days	2	2	2	2	
4 days	*30 days*	2	2	2	2	16
	120 days	2	2	2	2	
Total		24		24		**48**

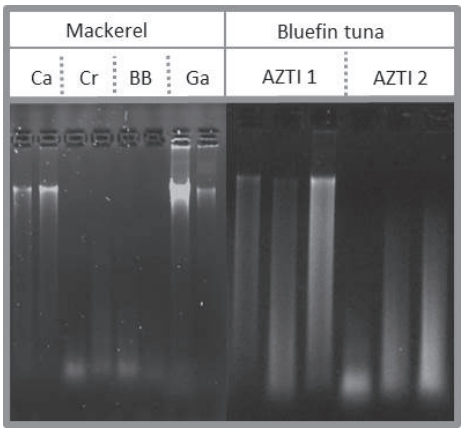

Figure 1. DNA integrity of example samples. Agarose gel electrophoresis image of DNA extracted from tissue samples arrived at AZTI at different conditions. Each lane corresponds to one sample. For mackerel Ca: Canada, Cr: Crete, BB: Bay of Biscay, Ga: Galicia. For Bluefin tuna: AZTI 1: samples collected by AZTI from fish caught using traps, AZTI 2: samples collected by AZTI at their arrival to port after being stored refrigerated in a boat for several weeks.

Effect of tissue type, preservative, storage time and post-mortem interval in DNA quality

No obvious differences in DNA integrity due to preservative, tissue type or storage time can be seen (Figure 2). There is however a clear tendency to degradation as the post-mortem interval increases, being the DNA extracted from tissues collected within a minute of death of full integrity, and the one extracted from tissues collected after 5 days almost fully degraded in most cases. In order to quantitatively assess variations in DNA quantity, purity and integrity due to the different factors tested, we compared DNA concentration (in ng/µl), 260/280 and 260/230 absorbance ratios, and our numeric representation of DNA integrity of the samples grouped according to preservative, tissue, days of storage and days post-mortem (Figure 3).

The use of fin or muscle tissue has no significant effect in DNA quality, whereas longer storage times decrease DNA purity as evidenced by a significant decrease of the 260/280 and 260/230 ratios ($P < 9x10^{-8}$ and $P < 0.005$, respectively). Obtained DNA concentration is significantly higher in samples stored at -20 ºC without any preservative ($P < 0.04$, $P < 7x10^{-4}$ and $P < 9x10^{-5}$ when compared with ethanol -20 ºC, ethanol at room temperature

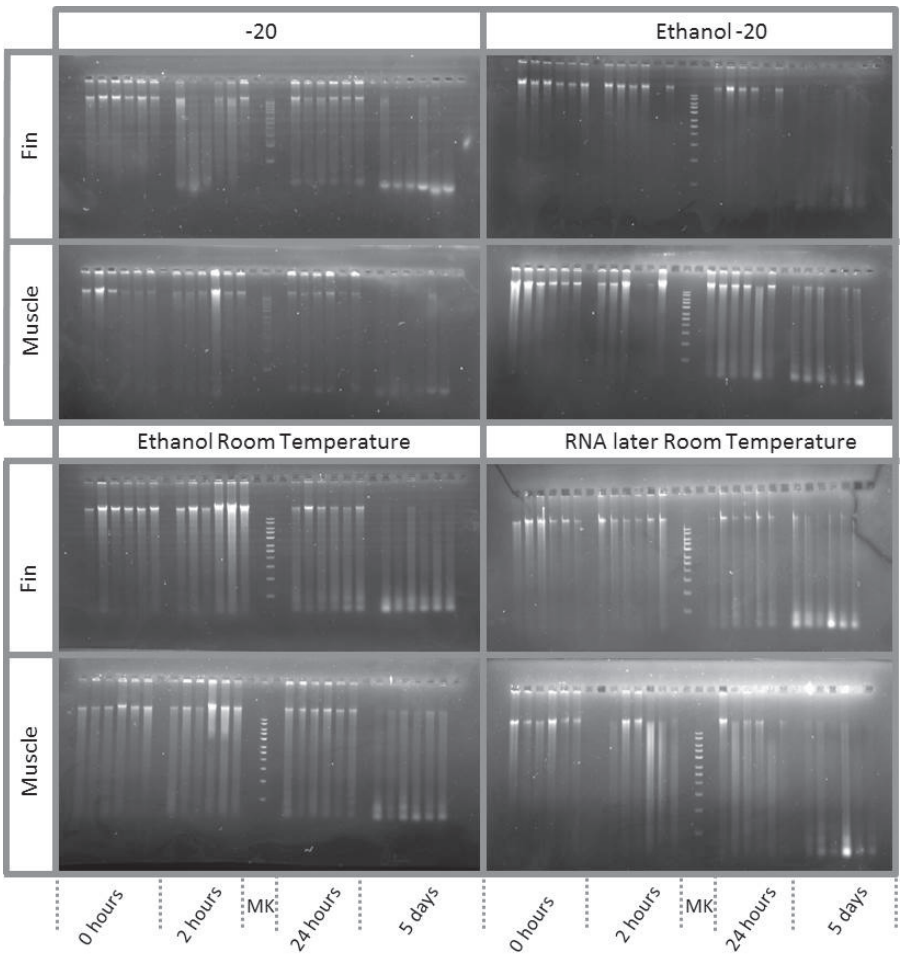

Figure 2. DNA integrity of the anchovy samples collected during the first sampling effort. Agarose gel electrophoresis image of samples are grouped in 6 according to combinations of tissue type, preservative and postmortem time. Within each 6, the three on the left were stored for 30 days, whereas the three on the right were stored for 60 days. MK denotes 1Kb ladder DNA used for reference.

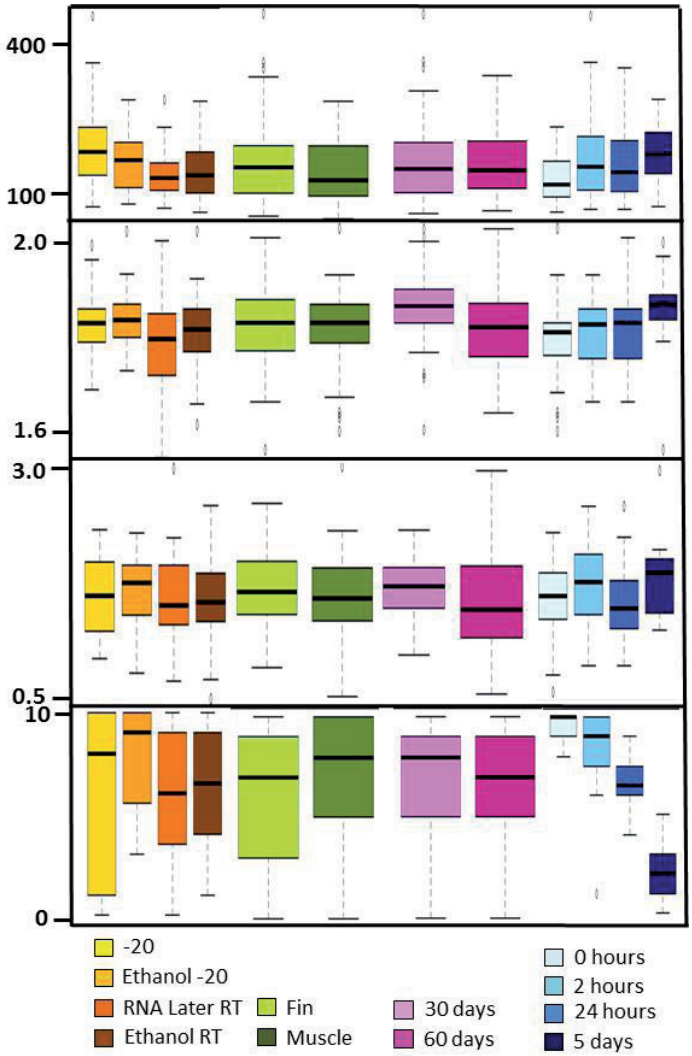

Figure 3. Differences in DNA quantity, purity and integrity due to the factors tested. Bloxplots (median, first and third quartile and standard deviation) for concentration (in ng/µl, first panel), 260/280 (second panel) and 260/230 (third panel) ratios and the numeric representation of DNA integrity (fourth panel) for the all 192 DNA samples grouped either by preservative, tissue, storage time or postmortem time.

and RNA later, respectively). Ethanol -20 ºC provides slightly higher DNA concentration than RNAlater ($P < 0.025$), whereas no significant differences are observed between ethanol at room temperature with respect to ethanol -20 ºC and RNAlater. DNA extracted from samples stored in ethanol -20 ºC has better integrity than the one obtained from samples stored in ethanol at room temperature and in RNAlater ($P < 0.0017$ and $P < 0.0104$, respectively). DNA purity does not differ between preservatives as in none of the comparisons the two ratios is significantly worse or better in one of the conditions.

post-mortem intervals have drastic effects in the DNA quality parameters measured. Integrity is significantly different in all pairs of comparisons, getting worse as the post-mortem interval increases ($P < 7x10^{-6}$ when comparing 0 versus 2h after death and $P < 7x10^{-16}$ in all other cases). Obtained DNA concentration is significantly lower in post-mortem interval 0 with respect to post-mortem intervals 2h, 24h and 5 days ($P < 0.013$ and $P < 0.04$, $P < 7x10^{-6}$, respectively). This difference should however not be taken into account since it is due to the fact that, due to the low concentration obtained in the first DNA exactions (post-mortem interval 0), the subsequent analyzed samples were let incubate longer during the tissue lysis step. In contrast to DNA integrity, which decreases with post-mortem interval, DNA purity increases, with 260/280 ratios significantly higher at 5 days post-mortem interval ($P < 2.6x10^{-7}$, $P < 1.1x10^{-4}$, $P < 0.0001$ when comparing with 0, 2 and 24h, respectively).

DNA integrity was assessed for all possible combination of pairs of conditions (Figure 4) in order to detect differences due to the combination of factors. Interestingly, although -20 ºC was the best preserving conditions when all samples are combined, preserving samples in ethanol at -20 ºC results in better DNA integrity for 5 days post-mortem intervals samples ($P < 4.1x10^{-5}$, $P < 0.00021$ and $P < 0.0004$ when compared to ethanol at room temperature and RNAlater respectively).

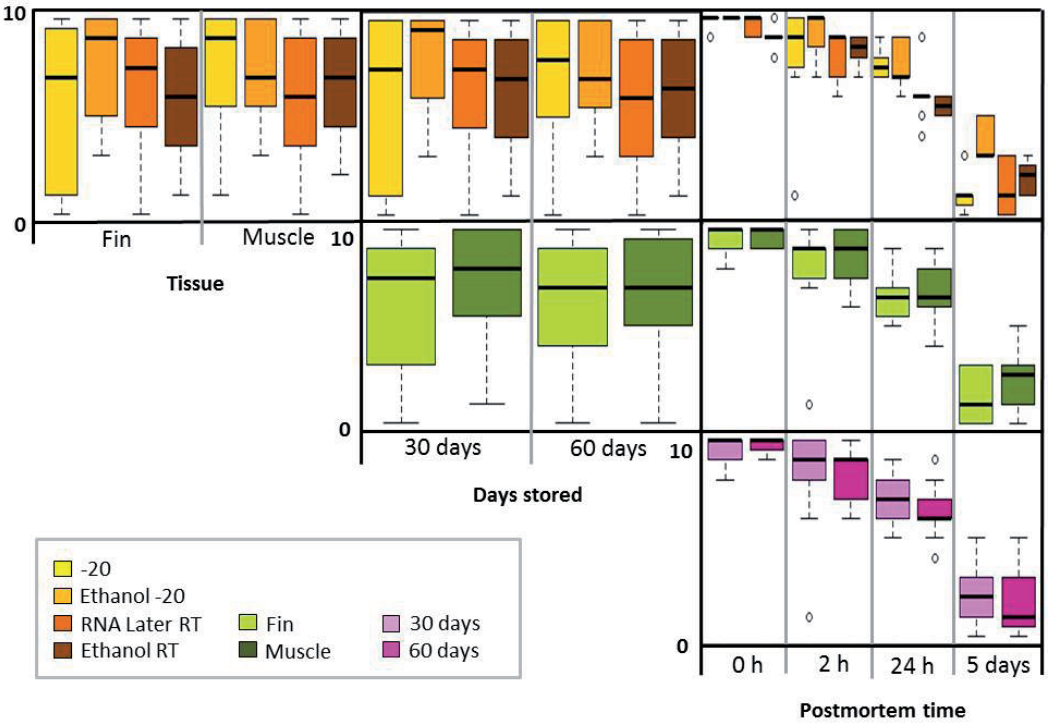

Figure 4. Differences in DNA integrity when factors are combined in pairs. Bloxplots (median, first and third quartile and standard deviation) for the numeric representation of DNA integrity for the all 192 DNA samples from the first sampling effort grouped by combinations of pairs of factors.

Effect of preservative at room temperature, storage time and post-mortem interval in DNA quality

A decrease in DNA integrity due to an increase in postmortem and storage times can be observed (Figure 5). Within 30 days of storage, all post-mortem interval show some amount of integer DNA, albeit in considerable less proportion in samples stored 4 days after death. Within 120 days of storage, DNA integrity remains intact in samples stored within a minute after death; however, some of the samples stored 4 hours after death and all those stored 4 days after death show total degradation of the DNA (Figure 6).

The preservative used has no significant effects in DNA quantity and integrity, but purity 260/280 ratios are significantly higher for samples stored in ethanol versus DESS ($P < 0.0006$). An increase of storage time significantly decreases DNA integrity ($P < 0.0024$) and purity ($P < 0.0093$ for the 260/280 ratio). As in the previous case, DNA integrity significantly decreases with post-mortem interval, being the worst integrity obtained when samples are taken after 4 days of death ($P < 0.0004$ and $P < 2.6x10^{-7}$ when compared with 4 and 0 hours, respectively).

DNA integrity was assessed for all possible combination of pairs of conditions (Figure 7). The decrease on DNA integrity due to storage time is more drastic in samples collected 4 days after death ($P < 0.0007$), and sardine samples give significantly lower DNA integrity when comparing samples at 4h post-mortem interval($P < 0.0127$).

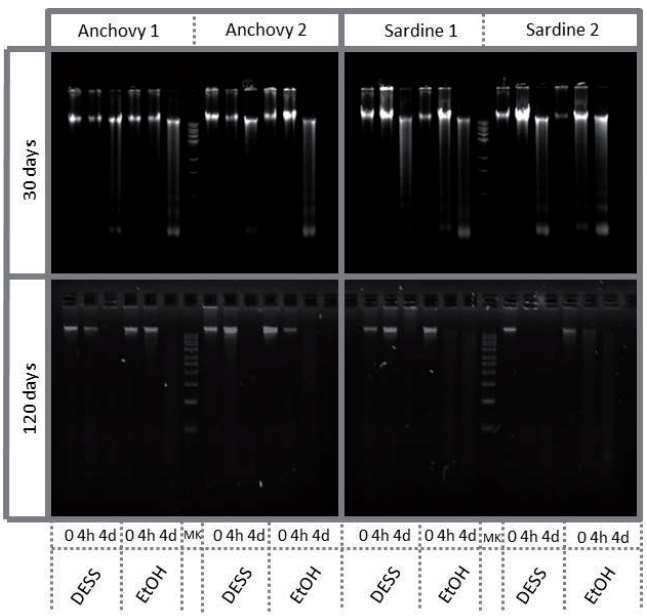

Figure 5. DNA integrity of the anchovy and sardine samples collected during the second sampling effort. Agarose gel electrophoresis image of samples are grouped in according to combinations species, storage time and preservative and sorted within groups according to post-mortem interval. MK denotes 1Kb ladder DNA used for reference.

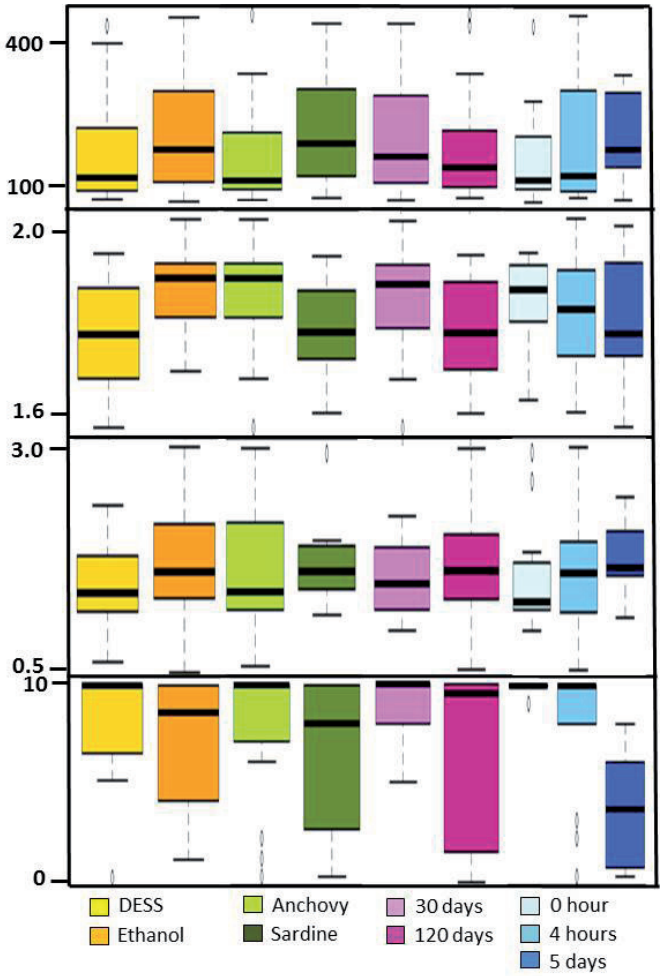

Figure 6. Differences in DNA quantity, purity and integrity due to the factors tested. Bloxplots (median, first and third quartile and standard deviation) for concentration (in ng/µl, first panel), 260/280 (second panel) and 260/230 (third panel) ratios and the numeric representation of DNA integrity (fourth panel) for the all 48 DNA samples grouped either by preservative, species, storage time or postmortem time.

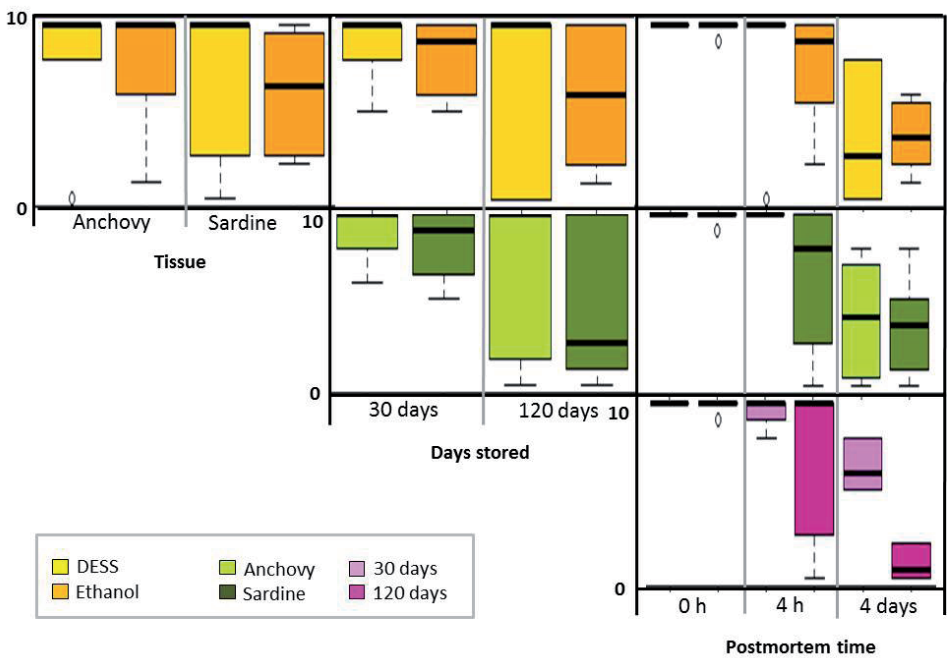

Figure 7. Differences in DNA integrity when factors are combined in pairs. Bloxplots (median, first and third quartile and standard deviation) for the numeric representation of DNA integrity for the all 48 DNA samples from the second sampling effort grouped by combinations of pairs of factors.

Discussion

Effect of tissue type

No significant differences in DNA quality are observed with respect to tissue sampled. Fin samples seem to consistently yield lower DNA integrities, although differences with respect to muscle are not statistically significant ($P > 0.05$). The choice of tissue type would therefore depend on other factors. For example, if killing the fish wants to be avoided, fin is preferred to muscle, as fin clips can be taken without harming the individuals (Wasko *et al.*, 2003). With respect to DNA extraction, fin digests faster and therefore speeds up the process by requiring less incubation time during the lysis step. On the other hand, muscle samples are easier to handle when preparing them for DNA extraction as they are more compact and therefore smaller pieces are enough to obtain the same tissue weight.

Effect of storage time

Longer storage times result in DNA of less purity; yet, at all storage times tested, most samples are within 260/280 and 260/230 ratios of good purity. Although DNA integrity is not disturbed by an increase in storage time during the first experiment (from 30 to 60 days), samples stored for 120 days during the second experiment show a significantly lower DNA integrity than those stored for 30 days. DNA of good integrity can be obtained from samples stored for months or even years (Nagy, 2010). For example the mackerel samples from Galicia (Figure 1) stored for six months before DNA extraction showed good DNA integrity, meaning that storage time is not the only explanation for the low DNA integrity obtained from anchovy and sardine samples after being stored for 120 days. A combination of the conditions in which these samples are stored and post-mortem intervals are the most likely explanation for these differences (see sections below).

Effect of preservative

Samples stored at -20 ºC without any preservative yield higher DNA quantity than samples stored with a liquid preservative. This might be due to the fact that the lysis buffer can easier penetrate the sample when no fixative agent has previously done so and therefore more efficiently release DNA. Yet, there was not a significant loss of DNA quantity in samples stored in ethanol, RNAlater and DESS, meaning that this difference in DNA concentration should not affect our choice of preserving conditions. DNA purity is significantly lower in samples stored in DESS than in those stored in ethanol. This is expected given the high concentration of ethylenediaminetetraacetic acid and salts of DESS (ThermoScientific, 2010). The presence of salts does not affect DNA integrity; however, it can affect downstream applications such as enzymatic reactions. Therefore, minimizing concentration of salts in the final DNA solution is recommended (Bessetti, 2007). In order to do so, a better previous cleaning of the sample with Milli-Q water should be performed. Nonetheless, during this step the DNA is exposed, and if not done quickly, its integrity could decrease.

Best DNA integrity is obtained from samples stored in ethanol at -20 ºC. This may be due to the fact that two preserving agents, cold and desiccation, are combined, significantly reducing the activity of enzymes and microorganism. None of the other three preservation methods clearly outperforms the others in what concerns DNA integrity, leaving method choice to be based on experimental design, field facilities, shipping constraints, and allowable cost. Although cheap and easy to apply, storing samples at -20 ºC without any preservative does have drawbacks. It requires the temperature of the sample to be kept low during the entire process, including sampling, storage, shipping, and handling in the laboratory, which is often not easy to achieve. For example, mackerel samples from Crete (Figure 1) where supposed to have arrived frozen at the laboratory, but, due to unknown reasons, arrived at unfrozen. In this particular case, the fact that the samples were defrost was known, but in other cases, even if samples arrive frozen to the laboratory, froze-thaw processes the researcher is unaware of can occur during transportation, making the causes of DNA degradation difficult to determine. Additionally, samples stored at -20 ºC without preservative need to be more carefully handled at the laboratory, where they cannot stand on the bench outside the fridge even for a few minutes like samples that contain preserving agents.

Ethanol at room temperature is an easy alternative as it is inexpensive, readily obtained and does not require refrigeration. The optimal ethanol concentration is 95-99% as traces of benzene in 100% ethanol can affect DNA preservation while 70% will lead to DNA degradation; as tissues are composed of water, in order to keep the right ethanol concentration (>70%), the ratio tissue volume ethanol should at least be 1:5 (Nagy, 2010). In this study, the effect of using different tissue/ethanol ratios was not tested as using an appropriate ratio is not considered a limiting factor during the sampling process. Additionally, in order to ensure a proper tissue/ethanol ratio, ethanol should be replaced within 24-48 hours when possible. Not replacing the ethanol is the most likely cause of DNA degradation in samples stored for 120 days, although no explanation could be found for the same effect observed in samples stored in DESS, as according to several studies, this preservative is adequate for long term storage (Dawson *et al.*, 1998; Michaud and Foran, 2011).

Effect of post-mortem interval

Unlike the other factors tested, which have slight or no effects in DNA integrity, post-mortem interval has dramatic consequences in DNA integrity. Within 24 hours post-mortem interval, DNA integrity is acceptable in most cases but after 4 days post-mortem interval, no DNA or DNA of very poor integrity remains. Four day post-mortem interval samples stored for 120 days in DESS or in ethanol at room temperature yield fully degraded DNA, meaning that when samples are partially degraded, preservation method and storage time are even more crucial than when samples are not degraded at the moment of storage. In our experiment, samples with 24h or less post-mortem intervals were kept on ice until the tissue was excised; it is nonetheless reasonable to think that in worse post-mortem conditions, as for example, on the deck in a hot summer day, more DNA degradation would occur within the same post-mortem interval.

The post-mortem interval has proven crucial in real examples. For example, Bluefin tuna caught using traps and sampled soon after death yield DNA of good integrity, although DNA sampled after being stored for days in a boat yield very low integrity DNA. The latter suggests that, in this case, the tissues were excised longtime after death, although there may be other factors, such as preservation conditions, affecting the integrity of the DNA obtained from these samples. In the mackerel samples used as examples of real cases, it was surprising to see that the DNA integrity of the fish collected in the Bay of Biscay yield lower DNA integrity than those collected in Galicia, as, according to the researchers who participate in the campaigns, in both cases, sampling and storage was performed in a similar way. A possible explanation for the differences could be the temperature the fish were exposed to between death and sampling, although it is impossible to determine the exact cause.

Considerations for genomic studies

Low integrity DNA may be sufficient for many genetic applications. For example, the DNA extracted from the mostly degraded mackerel samples in Figure 1 was successfully amplified on a polymerase chain reaction (PCR) for a > 120 nucleotide fragment (not shown). Genomic studies, especially those relying on restriction enzyme cutting coupled with random mechanical shearing of the DNA require however high integrity DNA. If not all, at least a fraction of the DNA should be intact, and when some amount of degradation is present, DNA concentration should be adjusted and additional tests performed in order to optimize the protocol for a semi-degraded sample (William Cresko, University of Oregon, personal communication). From the anchovy and sardine samples analyzed in this study, those collected at less than 24 hour post-mortem interval fulfill the DNA integrity requirements for a *de novo* polymorphism discovery study. The other samples show full or partial degradation, meaning that either they are not valid at all or require protocol tuning to be used. Therefore, when possible, full DNA integrity should be sought as a matter of priority for population genomics studies.

Outlook

DNA can be extracted from virtually any sample, including thousand year old ones (Millar *et al.*, 2008); nonetheless, some experiments not only require sufficient amount of DNA, but also good DNA integrity. Applications such as *de novo* polymorphism discovery and genotyping are particularly sensitive to DNA degradation. In this study, we have assessed the effect of tissue type, storage time, preservative and post-mortem interval in DNA integrity obtained from fish samples. Although a number of studies have been performed comparing different methods of sample storage to prevent DNA degradation (e.g. Willliams, 2007; Omar *et al.*, 2009; Michaud and Foran, 2011; Eschbach, 2012), only a few have considered post-mortem interval (Michaud and Foran, 2011), which we prove is the most crucial aspect to take into account. To our knowledge, this is the first study to compares the effect of post-mortem interval in the quality of DNA extracted from commercial fish.

Although muscle tissue is preferred, fin can be an appropriate alternative when no animal sacrifice is contemplated. Time elapsed between sampling and DNA extraction will largely depend on the experiment and, in some cases, DNA may need to be extracted from samples stored for a long time. If tissue preservation and sampling are appropriate, storage time should not be a factor to worry about, at least for short storage periods. Long term (> 1year) storage times require further investigation. Ethanol at -20 ºC is the best preserving method, although preservation in ethanol, RNAlater and DESS at room temperature may be good alternatives if refrigeration is not possible. Storage at -20 ºC without preservative is not recommended as DNA integrity will depend on the easy to break cold chain. Post-mortem interval is the most important factor to consider on which the success of a whole population genomics study could depend. Samples should be taken and stored in appropriate conditions as close as possible to the time of death. Given the cost of the genetic analyses involved in this kind of studies, the effort required in reducing post-mortem intervals is worthwhile.

Finally, it is important to mention that these considerations should be carefully explained to the personnel collecting fish samples for genomic analyses. They should be especially aware of the importance of post-mortem interval and of the conditions in which the fish are maintained between death and sampling. If possible, more samples than those required for the experiment should be taken, as DNA integrity may be good in some of them only.

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