Extracting a century of preserved molecular and population demographic data from archived otoliths in the endangered European eel (*Anguilla anguilla* L.)

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ABSTRACT

Archived otolith collections represent an invaluable source of information to study demographic and genetic changes in commercially important fish populations. Studies combining both approaches are however rare and reliable extraction of molecular and population demographic data from the same collection of otoliths has never been assessed in the endangered European eel (*Anguilla anguilla* L.). Here we evaluate various DNA extraction protocols to compare DNA yield, microsatellite amplification success, genotype integrity and precision of age determination for eel otoliths that have been archived for 4 weeks, and for 28 and 48 years. Our results show a high amplification success and an equal genotype integrity for DNA fragments extracted from both recently sampled otoliths and high quality reference DNA tissue. Although historical samples yielded low amounts of DNA, PCR amplification was successful and genotyping reliable for short fragments, but decreased significantly with PCR fragment size. None of the extraction protocols caused physical damage to the otoliths and precision of age determination was high for both treated and untreated otoliths. Hence, the methodology can be applied as a standard for the further joint analysis of past demographic and genetic changes during the last century in the highly exploiting European eel and in other fish requiring urgent conservation measures.

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1. Introduction

Over the past decade, technological improvements for extracting information from archived otolith collections yielded novel insights about demography and population structure to better understand the evolutionary consequences of anthropogenic pressure in wild fish populations (Nielsen and Hansen, 2008). Besides the increasing reliability in aging techniques, it is now feasible to use dried tissue on the rough surface of archived otoliths as a unique source of DNA (Hutchinson et al., 1999). Hence, otoliths of freshwater, anadromous and marine fishes have been analyzed genetically, resulting in several studies contrasting historical with present-day genetic diversity and evaluating the temporal stability of allele frequencies to estimate historical effective population sizes (Nielsen et al., 1997; Hoarau et al., 2005; Poulsen et al., 2006). Nevertheless, the full potential of retrospective information available in historical collections remains underutilized. The joint analysis of past demographic and genetic data lodged in the otoliths holds great potential to assess the evolutionary consequences of natural and human-induced changes on the demography, connectivity and adaptive potential of commercially important fish species (Heath et al., 2007; Nielsen and Hansen, 2008).

Extracting DNA from archived otoliths is a challenging task as the DNA is often degraded and therefore more difficult to analyze than modern high quality DNA (Leonard, 2008). Further, DNA extraction from otoliths typically involves incubation of the otolith into a lysis solution containing compounds potentially harmful to its physical structure. Otolith characteristics (shape, thickness, CaCO3 composition, opacity and transparency) are species-specific and no consensus method applicable for all fish species could be found yet (Heath et al., 2007; Cuveliers et al., 2009; Therkildsen et al., 2010). Since historical otolith collections are limited and fragile, it is of critical importance to test properly how multiple research applications can be combined on the target species without damaging the archived material.

The target species of this study is the European eel *Anguilla anguilla* (L.), a fish with a catadromous life-strategy making it completely dependent on exogenous selective pressures in both the oceanic and the continental environment (Maes and Volckaert, 2007). The species has experienced a sharp decline in both recruitment (Dekker, 2000) and stock (Dekker, 2003) levels and is now listed as critically endangered on the IUCN Red List of Threatened Species (IUCN, 2010). A management framework for the recovery of the European eel was established in 2007 by the Council of the European Union (European Commission, 2007) aiming at increasing the spawner escapement to 40% of its pristine situation. Evaluating this goal, however, is a complex task given the drastic lack of information.
regarding demographic and genetic history through the latest century of exploitation.

An important aspect to enable the investigation of life history traits and population demography of fish is the accuracy and precision in age estimation (Lin and Tzeng, 2009). However, age determination of eels has long been the subject of much debate about the ideal technique and relatively few validation studies have been performed. So far, reliable interpretation is often hampered by the vast variation in growth and maturation rates throughout the range of the species (Cullen and McCarthy, 2003). Luckily, both otolith preparation and age validation techniques have recently been standardized for eel to increase reliability and comparability among European fisheries institutes (ICES, 2009).

Otoliths represent a rather limited source of DNA since a maximum of two sagittal otoliths can be collected and archived (Heath et al., 2007). Eel otoliths are further extremely small compared to other bony fishes, providing a limited amount of dried tissue on the surface. Low copy number of template DNA in combination with degradation after years of storage can result in potential DNA contamination, poor amplification success, (large) allelic dropout and false alleles (Pompanon et al., 2005). The use of short fragment markers in combination with high laboratory standards may prevent these artefacts and is needed to guarantee reliable genetic results (Jakobsdottir et al., 2006). Virtually all studies employing archived fish samples for genetic research have analyzed microsatellites (SSRs) due to their high statistical power and many versatile applications (Selkoe and Toonen, 2006; Nielsen and Hansen, 2008). Additionally, SSR markers are ideal to assess the loss of genetic diversity over a certain time period of population decrease, as allelic loss is irreversible and identifiable with dedicated analyses (e.g. Piry et al., 1999). In contrast, novel markers such as Single Nucleotide Polymorphisms (SNPs), although requiring only minimal fragment sizes of 50–100 bp, are mostly taxon specific and lack the latter applicability due to their bi-allelic nature and their development on contemporary samples (only detecting present day polymorphisms). Microsatellite markers derived from Expressed Sequence Tags (EST-SSRs) are highly transferable between taxa and further allow a link to the functional characterization of a particular locus and a rapid in silico development from readily available genomic resources in many species (Bouck and Vision, 2007). Finally, grouping microsatellites of various fragment sizes in multiplex reactions is a cost-efficient strategy to genotype multiple loci while minimizing the use of template DNA.

To evaluate the reliability of combined molecular and population demographic analyses on archived eel otoliths, the present study compares DNA yield, microsatellite amplification success and precision of age determination for otoliths originating from different sampling periods and treated with various DNA extraction protocols. We also investigate the integrity of otolith DNA by verifying the genotype coherence with high quality DNA from finclips of the same individual.

2. Methods

2.1. Samples

Historical eel otoliths were obtained from an extensive collection preserved at the Institute for Marine Resources and Ecosystem Studies of The Netherlands (Wageningen IMARES). For more than a century otolith samples of European eel have been collected from Lake IJsselmeer (called Zuiderzee before the dam construction in 1932) and stored dry in paper envelopes. The samples selected for this study consisted of yellow eel otolith pairs from September 1960 (52°49’N 5°18’E; N=20) and September 1980 (52°50’N 5°15’E; N=20). These yellow eels were caught with a beam trawl (1960) and an electrified trawl (1980) and total body length ranged from 13.6 to 26.3 cm in 1960 and from 16.2 to 30.3 cm in 1980. To examine whether various DNA extraction protocols have any effect on aging reliability, from each pair of otoliths one control otolith was directly sent to the Institute of Freshwater Research (Drottningholm, Sweden) for embedding and age reading, while the other one was used for DNA extraction before the age was determined. To evaluate the integrity of otolith DNA in comparison to other tissues, additional contemporary otolith and finclip samples were obtained from yellow eels collected at Lake IJsselmeer with an electrified trawl in November 2008 (52°44’N 5°26’E; N=40). Sampled otoliths were air-dried in individual paper envelopes for four weeks prior to DNA extraction and finclips were stored in 100% ethanol.

2.2. DNA extraction

DNA was extracted from dry tissue surrounding the otolith, using two DNA extraction protocols. The first protocol (named protocol A) of Cuveliers et al. (2009) was originally described by Hutchinson et al. (1999), but the concentrations of EDTA and SDS were respectively lowered to 1 mM and 0.5% in order to reduce otolith damage. The second extraction protocol (named protocol B) was a commonly used commercial DNA extraction kit (NucleoSpin Tissue, Macherey-Nagel), applied according to the manufacturer’s instructions. Batches of five historical otoliths were selected for each combination of sampling year, extraction protocol and incubation time (1 or 3 h). Batches of 20 contemporary otoliths were selected for both extraction protocols with a single incubation time of 1 h (see Table 1 for batch code definitions). Otoliths were partitioned along the different batches such that various eel body length size classes were equally represented. After removing the otoliths from the lysis suspension, they were rinsed with distilled water and dried in a fresh paper

Table 1

<table>
<thead>
<tr>
<th>Batch code</th>
<th>DNA source</th>
<th>Sampling year</th>
<th>Extraction protocol</th>
<th>Incubation time (h)</th>
<th>Sample size</th>
<th>DNA concentration (ng μL⁻¹)</th>
<th>Amplification success rate (%)</th>
<th>Genotypic error rate (%)</th>
<th>Percentage of agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60A1</td>
<td>Otolith</td>
<td>1960</td>
<td>A</td>
<td>1</td>
<td>5</td>
<td>0.17±0.07</td>
<td>58.94±17.78</td>
<td>97.50 (8)</td>
<td>100.00 (10)</td>
</tr>
<tr>
<td>60A2</td>
<td>Otolith</td>
<td>1960</td>
<td>A</td>
<td>3</td>
<td>5</td>
<td>0.17±0.04</td>
<td>69.09 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>60B1</td>
<td>Otolith</td>
<td>1960</td>
<td>B</td>
<td>1</td>
<td>5</td>
<td>0.16±0.07</td>
<td>67.27 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>60B2</td>
<td>Otolith</td>
<td>1960</td>
<td>B</td>
<td>3</td>
<td>5</td>
<td>0.13±0.03</td>
<td>74.55 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>60A3</td>
<td>Otolith</td>
<td>1960</td>
<td>A</td>
<td>3</td>
<td>5</td>
<td>0.16±0.06</td>
<td>70.91 (55)</td>
<td>87.50 (8)</td>
<td>100.00 (10)</td>
</tr>
<tr>
<td>80A1</td>
<td>Otolith</td>
<td>1980</td>
<td>A</td>
<td>1</td>
<td>5</td>
<td>0.16±0.07</td>
<td>74.55 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>80A2</td>
<td>Otolith</td>
<td>1980</td>
<td>A</td>
<td>3</td>
<td>5</td>
<td>0.16±0.06</td>
<td>74.55 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>80B1</td>
<td>Otolith</td>
<td>1980</td>
<td>B</td>
<td>1</td>
<td>5</td>
<td>0.16±0.07</td>
<td>74.55 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>80B2</td>
<td>Otolith</td>
<td>1980</td>
<td>B</td>
<td>3</td>
<td>5</td>
<td>0.16±0.06</td>
<td>74.55 (55)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
<tr>
<td>08A1</td>
<td>Otolith</td>
<td>2008</td>
<td>A</td>
<td>1</td>
<td>20</td>
<td>0.33±0.47</td>
<td>96.64 (220)</td>
<td>0.69 (434)</td>
<td>75.00 (8)</td>
</tr>
<tr>
<td>08B1</td>
<td>Otolith</td>
<td>2008</td>
<td>B</td>
<td>1</td>
<td>20</td>
<td>0.28±0.36</td>
<td>97.73 (220)</td>
<td>1.17 (428)</td>
<td>75.00 (8)</td>
</tr>
<tr>
<td>08B2</td>
<td>Finclip</td>
<td>2008</td>
<td>B</td>
<td>6</td>
<td>40</td>
<td>56.94±17.78</td>
<td>99.55 (440)</td>
<td>100.00 (8)</td>
<td>100.00 (8)</td>
</tr>
</tbody>
</table>


* For each averaged variable the number of cases is mentioned between parenthesis.
envelope for later embedding and age reading. Contemporary finclip DNA was extracted following protocol B with a standard lysis incubation time of 6 h to dissolve the tissue completely (Table 1). Purified DNA was resuspended in 50 μL (for otolith DNA) or 200 μL (for finclip DNA) of ultrapure water (protocol A) or elution buffer (protocol B). To avoid contamination of historical DNA samples, all DNA extractions, DNA quantifications and subsequent preparations of PCR reactions, were completed under a laminar flow on a separate floor from the laboratory where PCR products were analyzed. All equipment was placed 30 min under UV–light prior to use. Negative controls (N = 32) were added at every step from extraction to genotyping to check for cross- and aerosol contamination (Hoarau et al., 2005).

2.3. DNA yield

DNA yield was determined with a fluorescent dye-binding assay using PicoGreen dsDNA (Invitrogen) as a quantification reagent and 10 μL of otolith DNA extract. The measurements were performed according to the manufacturer’s instruction using an Infinite M200 micro plate reader with a detection limit of 20 pg dsDNA in a 200 μL assay volume (TECAN Trading AG). Individual sample concentrations were calculated based on the RFU (relative fluorescence units) values.

2.4. Molecular analysis

Nuclear DNA amplification was performed using a multiplex reaction for 11 EST-linked microsatellite loci (Pujolar et al., 2009), grouped in three non-overlapping size ranges: 86–142 bp (small), 153–226 bp (medium) and 239–271 bp (large) (Table 2). Amplifications were performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems) using the QIAGEN Multiplex PCR Kit. The reaction volume of 10 μL contained 1 μL of template DNA, 5 μL QIAGEN Multiplex PCR Master Mix, between 0.05 μL and 0.20 μL of 5.0 μM forward and reverse primer solutions (according to prior optimization) and ultrapure water. PCR conditions consisted of an initial denaturation step of 15 min at 95 °C, 26 (142 bp) or 35 (260 bp) cycles of 30 s at 95 °C, 90 s at 57 °C and 60 s at 72 °C, and a final elongation step of 30 min at 60 °C. PCR fragments were sized on an automatic capillary sequencer ABI 3130 AVANT (Applied Biosystems) according to a Liz500 (50–500 bp) marker and genotypes were obtained using GENEMAPPER version 4.0 (Applied Biosystems). Each DNA sample was amplified twice and amplification success was scored 1 if a reliable consensus genotype could be determined within these two attempts, 0 if not. Amplification success rate was calculated as the percentage of positive values across batches and loci. Samples of contemporary otolith and finclip DNA were genotyped independently and both genotypes were considered as replicates to calculate the genotypic error rate per locus for each extraction protocol (Pompanon et al., 2005).

2.5. Age determination and otolith size

The age of both otoliths (treated and untreated) was determined for all historical samples (N = 80). Each otolith was embedded convex side up in thermoplastic quartz cement (No. 70C, Lakeside Brand) on an individual microscopic slide to obtain a sagittal view. The otolith was ground with a series of wet-grinding papers (600, 800 and 1200 grade) under a constant supply of water until the primordium was exposed. The preparation was etched with a 1% solution of concentrated HCl and colored with neutral red (following Svedang et al., 1998). All otoliths were renumbered and mixed randomly before age determination by two independent readers under an optical microscope with transmitted light, following the recommendations of the WKAREA manual (ICES, 2009). Otoliths were digitally photographed and otolith diameter was measured along the longest axis with an image processing software (GIMP 2.6.7, GNU General Public License).

2.6. Statistical analysis

For the contemporary otolith and finclip samples, a t-test was used to test for differences in DNA yield between both extraction protocols. The statistical significance of the categorical variables (extraction protocol, lysis time and sampling year) in relation to the DNA yield of historical samples, was tested with a factorial ANOVA. To fulfill the normality assumption, the log10 value of the response variable (DNA yield) was used in both tests. Additionally, correlation between DNA yield and otolith size was examined using a Pearson correlation test.

The effects of DNA extraction protocol, lysis time and sampling year on the amplification success of historical DNA fragments, were analyzed for each marker separately with a generalized linear model. The correlation between amplification success rate (both per locus and overall) and mean allele size was examined using a Pearson correlation test. Locus-specific deviations from Hardy–Weinberg equilibrium were calculated in each sample group (based on sampling year and DNA source: 1960 otoliths, 1980 otoliths, 2008 otoliths and 2008 finclips) with Wright’s inbreeding coefficient FIS according to Weir and Cockerman (1984) using GENETIX version 4.05 (Belkhir et al., 1996–2004). Finally, an ANCOVA was used to test whether mean allele size and sample group affected FIS-values.

To evaluate the quality of aging, the percentage of agreement between age determinations of two independent readers was

| Locus specific characteristics, amplification success rate and genotypic error rate per locus. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus name | Accession number | Repeat motif | Range (bp) | Average size (bp) | Group size | Alleles | Amplification success rate (%) | Genotypic error rate (%) |
| AANC202 | EU884524 | (CA)9 | 86–88 | 88 | Small | 2 | 100.0 | 100.0 | 100.0 | 100.0 | 0.00 (40) | 0.00 (40) |
| AAN35N10 | EU884514 | (TA)15 | 88–96 | 92 | Small | 3 | 100.0 | 100.0 | 100.0 | 100.0 | 0.00 (40) | 0.00 (40) |
| AAN26N13 | EU884512 | (CT)13 | 87–111 | 99 | Small | 12 | 90.0 | 100.0 | 100.0 | 95.0 | 100.0 | 0.00 (40) | 0.00 (40) |
| AAN33D15 | EU884513 | (TG)11 | 98–142 | 114 | Small | 15 | 85.0 | 85.0 | 95.0 | 95.0 | 100.0 | 0.00 (38) | 0.00 (38) |
| AAN24L24 | EU884511 | (TG)10 | 153–171 | 167 | Medium | 5 | 60.0 | 65.0 | 90.0 | 100.0 | 95.0 | 5.56 (36) | 5.56 (38) |
| AANCT070 | EU884523 | (AC)13 | 166–176 | 170 | Medium | 6 | 100.0 | 100.0 | 100.0 | 100.0 | 95.0 | 100.0 | 0.00 (38) | 0.00 (38) |
| AAN24A99 | EU884510 | (GA)17 | 168–226 | 198 | Medium | 28 | 35.0 | 75.0 | 100.0 | 90.0 | 100.0 | 0.00 (40) | 0.00 (36) |
| AANCT712 | EU884528 | (ATC)13 | 239–257 | 248 | Large | 7 | 90.0 | 75.0 | 100.0 | 95.0 | 100.0 | 0.00 (40) | 0.00 (38) |
| AAN22D01 | EU884507 | (ACA)8 | 254–263 | 260 | Large | 4 | 35.0 | 25.0 | 100.0 | 100.0 | 100.0 | 2.50 (40) | 2.50 (40) |
| AAN44B14 | EU884519 | (CA)11 | 253–271 | 263 | Large | 8 | 15.0 | 35.0 | 100.0 | 100.0 | 100.0 | 0.00 (40) | 0.00 (40) |
| AANC446 | EU884527 | (AATC)8 | 258–270 | 266 | Large | 4 | 35.0 | 30.0 | 100.0 | 100.0 | 100.0 | 0.00 (40) | 0.00 (40) |

*Average across four batches.

*See Table 1 for definition of batch codes.

*The number of compared genotypes is mentioned between parenthesis.
calculated. The quality of aging was also evaluated with the average coefficient of variation (CV) (Chang, 1982), as this parameter adjusts for the absolute age of the fish and is widely used in both past and recent studies to estimate imprecision (Kimura and Lyons, 1991; Campana et al., 1995; Power et al., 2006). To evaluate the potential harmful effect of various DNA extraction protocols applied prior to age determination, the percentage of agreement and CV between treated and untreated otolith age determinations (i.e. L and R otolith within one otolith pair and within one reader) were calculated for each batch and averaged across both readers. The difference between CV values of between reader and between treatment comparisons was evaluated with a nonparametrical Mann-Whitney U test.

3. Results

3.1. DNA yield and otolith size

The DNA yield of contemporary otolith samples ranged from 0.05 ng μL⁻¹ to 2.00 ng μL⁻¹ and mean DNA yields were not significantly different between both extraction protocols (p = 0.918). The DNA yield of historical samples ranged from 0.05 ng μL⁻¹ to 0.27 ng μL⁻¹ with an average value of 0.13 ± 0.06 ng μL⁻¹. Neither sampling year (p = 0.188) or lysis time (p = 0.667) were identified as significant factors in explaining DNA yield variation. The extraction protocol, however, was a significant factor (p < 0.001). Mean DNA yield of historical batches were higher when protocol A was used (Table 1). There was no significant correlation between otolith size (1.24 ± 0.22 mm) and DNA concentration in the 1980 samples (p = 0.580), but in 1960 samples a significant correlation between both variables appeared (y = −0.015 + 0.131x, R² = 0.364, p = 0.006). The DNA yield of contemporary finclip samples was as expected much higher and ranged from 16.93 ng μL⁻¹ to 89.01 ng μL⁻¹.

3.2. Molecular analysis

The amplification success rate of DNA fragments from contemporary finclip and otolith samples was high for all loci (Table 2; Fig. 1) with a mean value of 98.64%, 97.73% and 99.55% for batches 08A1, 08B1 and 08B6 respectively (Table 1). The lack of a perfect amplification is in all three batches related to two or three individual samples with a failed amplification for one or more loci. The genotypic error per locus ranged from 0.00% to 5.56% (two mismatches in 36 genotypes of locus AAN44B14) (Table 2) and the mean error rate per locus was low for both protocol A (0.69%) and B (1.17%) (Table 1). All 40 historical extractions succeeded in amplifying for at least four of the 11 microsatellites with an average of eight loci amplifying per sample. No indications of contamination were observed. The average amplification success rate per batch ranged from 60.00 to 74.55% (Table 1). No significant effect of sampling year, extraction protocol or incubation time on amplification success of the various loci was found. The average amplification success rate per locus ranged from 15.00% for AAN44B14 (in 1960) to 100.00% for various smaller loci (in 1960 and 1980) (Table 2). The correlation between amplification success rate and mean fragment size was significant for every combination of sampling year, extraction protocol and incubation time (except for batch 60B3; p = 0.084) and highly significant for the overall amplification success rate in historical samples (y = 1.311–0.003x, R² = 0.580, p < 0.001) (Fig. 1).

A significant influence of mean allele size (p = 0.003), sample group (p = 0.032) and their interaction (p = 0.001) on the Fₛ value was found and equations were as follows: a positive correlation between Fₛ and average allele size was present for both historical samples of 1960 (y = −0.371 + 0.003x, R² = 0.296, p = 0.104) and 1980 (y = −0.482 + 0.005x, R² = 0.746, p < 0.001), while absent for the group of contemporary otolith (y = 0.194–0.001x, R² = 0.092, p = 0.364) and finclip samples (y = 0.186–0.001x, R² = 0.026, p = 0.637) (Fig. 2).

3.3. Age determination

The age distribution of historical samples (N = 74) ranged from one to six years (data not shown). When comparing ages between both readers, 86.48% generated identical age counts, 12.16% showed a maximum of one year discrepancy and one count (1.35%) showed a difference of two years of discrepancy (6 otoliths were not aged due to loss or damage during treatment). These discrepancies did not appear to be affected by age and size of the eels. Average CV between readers was calculated at 2.94%. When comparing treated and untreated otoliths of one otolith pair, the sections were in general physically similar and both easy interpretable (Fig. 3). The percentage of agreement between treated and untreated otoliths (average of both readers) ranged from 60.00% to 100.00% for the various batches (Table 1) with an average value of 86.93% across all batches. No systematic pattern appeared regarding extraction protocol or lysis time. The average CV between treated and untreated otoliths of 4.54% was found marginally higher than between readers (2.94%) and this difference was not significant (p = 0.978). The variation between both values was caused by only one otolith pair showing two disputable age rings present in one otolith while absent in the other.

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Fig. 1. Amplification success rate (± S.D.) in pooled samples of 2008 (●), 1980 (○) and 1960 (△) for three size groups of microsatellite fragments (short: 86–142 bp; medium: 153–226 bp; long: 239–270 bp).

Fig. 2. Relationship between Fₛ and average fragment length in four sample groups of 2008 finclips (●●●), 2008 otoliths (○○○), 1980 otoliths (□□□) and 1960 otoliths (△△△). See text for regression equations and parameters.
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4. Discussion

Otolith extraction protocols generally yielded low amounts of DNA in comparison to fresh tissue. The difference order of magnitude is the result of a limited amount of dried tissue on the small sized eel otoliths. Comparing contemporary and historical DNA amounts also shows the additional influence of DNA degradation on the yield in historical samples. Here, the amount of tissue lodged on the otolith surface was somewhat related to its size. Using identical extraction protocols for larger otoliths of Solea solea (average otolith size of 3.97 ± 0.46 mm; Cuveliers, pers. comm.), Cuveliers et al. (2009) obtained higher DNA yields (0.18 ± 0.09 ng μL⁻¹ to 2.42 ± 1.30 ng μL⁻¹) in comparison to the eel samples in this study. The nature of otolith preparation during their collection and cleaning before storage further defines the exact quantity of recoverable DNA from an otolith (Hutchinson et al., 1999). A significantly higher DNA yield was observed for historical eel otoliths treated with protocol A (Hutchinson et al., 1999) (modified by Cuveliers et al. (2009)) in comparison to protocol B (NucleoSpin Tissue, Magery-Nagel). This is no surprise, as in the discipline of ancient DNA, column-based methods are known to give lower yields due to the limited time for DNA to bind the silica matrix (Rohland and Hofreiter, 2007). No significant difference in yield among different lysis times could be observed, suggesting that DNA covering the dried otolith is quickly mobilized into the lysis suspension.

Although DNA yield was generally low, the PCR amplification success rate of contemporary otolith samples was high for the full range of fragments sizes in both the protocol A and B. The small variations in amplification success must therefore be related to locus specific effects, such as repeat motif, heterozygosity, primer annealing efficiency or secondary structures in the template DNA (Buchan et al., 2008). The high amplification success rate demonstrates the limited influence of low DNA yield when high laboratory standards are used. The genotypic error rates were generally very low and for eight loci all finclip and otolith genotypes were scored identically. Error rates in the other three loci were within acceptable levels and no systematic trends towards fragment size or extraction protocol were found. All mismatches were caused by missing alleles (allelic dropout) in samples originating from both tissue types and this proves the equal integrity of otolith and finclip DNA. Hence, we believe that after a testing procedure as done here, recently sampled and dried otoliths can serve as an alternative to ethanol preserved tissue in population genetic studies of other species, when DNA sampling was logically impossible or even not envisaged.

The PCR amplification success rate of the historical DNA samples was substantially lower than the contemporary samples. This difference is consistent with many studies reporting a reduced amplification when archived samples are used as DNA source (Hutchinson et al., 1999; Ruzzante et al., 2001; Hutchinson et al., 2003; Heath et al., 2007; Cuveliers et al., 2009; Therkildsen et al., 2010). A comparison of success rates between studies is however difficult because of interfering factors related to the species (otolith size and subsequent amount of preserved DNA), the archived collection (sample protocol, cleaning history and storage conditions; often not known) (Heath et al., 2007) and biochemical features (DNA type, PCR fragment size and primer annealing efficiency). In contrast to the effect on DNA yield, no significant effect of extraction protocol nor lysis time could be detected on the amplification success. Although DNA degradation from archived otoliths can increase with time through biological, physical and chemical factors affecting DNA quality (Wandeler et al., 2007), our study did not show a significant difference in amplification success between the 1960 and 1980 samples. This suggests DNA degradation occurred relatively rapidly after storage and remained very limited afterwards. Other authors already described the successful amplification of samples stored in paper bags (Nielsen et al., 1999), confirming the importance of good storage conditions (acid free paper bags kept under dry and cold conditions away from sunlight) of archived collections to perform historical genetic analyses (Pääbo et al., 2004).

Successful amplification (>80%) of historical DNA was mainly limited to short fragments of approximately 150 bp and this result is consistent with literature (Hutchinson et al., 2003; Heath et al., 2007). Archived DNA is generally degraded and the number of fragments containing complete target sequences logically decreases with increasing fragment size (Broquet et al., 2007; Nielsen and Hansen, 2008). Amplification of low quantity DNA samples is however no guarantee to obtain reliable genotypes (Taberlet et al., 1996). Low DNA yield can favor allelic dropouts (stochastic sampling), false alleles and contamination (Pompanon et al., 2005). A critical threshold value below which genotype errors could reach high proportions was defined earlier on as 56 pg (Taberlet et al., 1996) and 25 pg (Morin et al., 2001). The amount of historical DNA extracted in this study (a minimum of 54 pg μL⁻¹) can therefore be judged as sufficient. However, to genotype reliably (and to monitor sources of error), this level of DNA yield would still need a multiple tube approach (Taberlet et al., 1996; Pompanon et al., 2005). By only replicating each sample twice, it was not possible to calculate the rate of allelic dropout in our study. Hence, we evaluated genotype quality by comparing FIS values for various fragment sizes and sample groups (based on sampling year and DNA source) (Fig. 2). We assumed that biological and technical factors have similar influences on the FIS...
values of every sample group as eels in this study originate from the same geographical location and all samples were simultaneously treated in the laboratory. Therefore a deviating value of $F_{IS}$ corresponds to the absence of amplification of one of both alleles in heterozygous individuals. We graphically demonstrated that $F_{IS}$ values of smaller fragments are similar across various sample groups and this indicates the reliability of genotyping historical DNA fragments up to 150 bp. Historical fragments longer than 150 bp show a deviating $F_{IS}$ value with increasing fragment size in contrast to the contemporary samples, but we believe that genotyping can still be reliable when sufficient replicates are accomplished. The successful and reliable amplification of small fragments shows the importance of short microsatellite (Jakobsdottir et al., 2006) and for specific aims also short SNP (Morin et al., 2004) markers and their potential to contribute to long-term temporal genetic studies. Also novel genomic approaches using next generation sequencing for the full recovery of millions of short molecules can make use of archived otolith DNA (Hofreiter, 2008; Stiller et al., 2009).

The aging results show a high percentage of agreement and a low coefficient of variation between both readers, hence validating our methodology for preparation and interpretation of eel otoliths originating from relatively young individuals. No physical damage due to preceding DNA extraction could be observed and no systematic differences between various extraction protocols were found. The highly similar levels of inter-reader and within-pair (treated and untreated otolith) percentage of agreement and CV clearly reflect other factors than the extraction protocol being responsible for the small deviations in age interpretation. These deviations may depend on the general difficulty to age eel otoliths and the subsequent variation between readers and between left and right otolith interpretations (Heath et al., 2007).

Our study illustrates the great potential of combining molecular conservation and population demography analyses through extraction of DNA from archived and contemporary otoliths in combination with subsequent age determination (and derived parameters). Archived otoliths collected and stored under good conditions therefore can contribute to the study of evolutionary consequences of long-term changes in demography and connectivity, while opening a whole new way to estimate the potential loss of adaptive potential and resilience to selective pressure of harvested fish populations. Moreover, our results show the applicability of recently sampled otoliths as alternative tissue to extract DNA for genetic analyses. This may be of great importance to fisheries institutes performing only standardized sampling of otoliths and wanting to board on the fast moving ship of evolutionary enlightened fisheries management. For the endangered European eel, such combined analysis will help reconstruct the history of its decline and may highlight crucial changes in the population that lead to the current catastrophic population crash.

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