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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microsatellite amplification success, genotype integrity and precision of age determination for eel otoliths that have been archived for 4 weeks, and for 28 and 48 years.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 exploited European eel and in other fish requiring urgent conservation measures.

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, $CaCO_3$ 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

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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
Batch code, DNA source, sampling year, extraction protocol, incubation time and sample size of the various otolith batches. Also listed are average DNA concentration (±S.D.), amplification success rate (average across all loci), genotypic error rate (average across all loci) and percentage of agreement between the age of treated and untreated otoliths (averaged across both readers).

Batch code	DNA source	Sampling year	Extraction protocol	Incubation time (h)	Sample size	DNA concentration (ng μL^{-1})	Amplification success rate (%)[a]	Genotypic error rate (%)[a]	Percentage of agreement (%)[a]
60A1	Otolith	1960	A	1	5	0.17 ± 0.07	60.00 (55)		75.00 (8)
60A3	Otolith	1960	A	3	5	0.17 ± 0.04	69.09 (55)		100.00 (10)
60B1	Otolith	1960	B	1	5	0.10 ± 0.04	67.27 (55)		100.00 (8)
60B3	Otolith	1960	B	3	5	0.12 ± 0.03	74.55 (55)		100.00 (8)
80A1	Otolith	1980	A	1	5	0.16 ± 0.07	74.55 (55)		100.00 (8)
80A3	Otolith	1980	A	3	5	0.16 ± 0.06	67.27 (55)		60.00 (10)
80B1	Otolith	1980	B	1	5	0.09 ± 0.02	70.91 (55)		87.50 (8)
80B3	Otolith	1980	B	3	5	0.09 ± 0.02	74.55 (55)		87.50 (8)
08A1	Otolith	2008	A	1	20	0.33 ± 0.47	98.64 (220)	0.69 (434)	
08B1	Otolith	2008	B	1	20	0.28 ± 0.36	97.73 (220)	1.17 (428)	
08B6	Finclip	2008	B	6	40	58.94 ± 17.78	99.55 (440)		

Extraction protocol A = Hutchinson et al. (1999) modified by Cuveliers et al. (2009). Extraction protocol B = NucleoSpin Tissue (Macherey-Nagel).
[a] For each averaged variable the number of cases is mentioned between parenthesis.