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Evaluation of four stock discrimination methods to assign individuals from mixed-stock fisheries using genetically validated baseline samples

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ABSTRACT: For sustainable fisheries management, fish individuals in a mixing area need to be separated according to their stock affiliation. The assignment of individuals to one of the stocks requires reliable stock discrimination methods with high assignment accuracy. In the Baltic Sea, 2 genetically differentiated Atlantic cod *Gadus morhua* stocks, the western (WBC) and eastern Baltic cod (EBC), coexist in the Arkona Basin, inducing uncertainties in the stock assessments. Here, we evaluated a suite of non-molecular stock discrimination techniques (otolith shape analysis, stable isotope analysis on otolith nuclei, otolith readability and diameter of translucent zones [TZs]) on the same set of genetically validated Baltic cod baseline samples from the mixing area (Arkona Basin) and adjacent areas (Belt Sea, Øresund and Bornholm Basin). Otolith shape and stable oxygen isotope analyses showed the highest classification accuracies; between 80 and 84 % of cod individuals were correctly assigned to their respective stock of origin. Stable carbon isotope analysis, otolith readability and the diameter of the first 2 TZs yielded classification accuracies of only 52 to 61 %. Given the high assignment accuracy and the availability of archived otoliths, otolith shape and stable oxygen isotope analyses on otolith nuclei are powerful separation methods that allow for high-throughput quantification of present and past mixing proportions of Baltic cod stocks. This study provides the most comprehensive approach of genetically validated stock discrimination techniques currently available for Baltic cod, and evaluates the applicability and reliability of otolith-based methods for future research studies and for fisheries management purposes.

KEY WORDS: Stock separation · Baltic Sea · Single nucleotide polymorphisms · SNPs · Otolith shape analysis · Fourier analysis · Stable isotope analysis · Translucent zones · Readability

1. INTRODUCTION

The management of mixed-stock fisheries is challenging, as individual stocks may differ in growth, recruitment or exploitation rates. Moreover, adjacent fish stocks often display different intra- and interannual migratory patterns, so their relative contribution to mixed-stock catches may also vary considerably. Higher fishing pressure on a less-productive stock may lead to the overexploitation of a weaker stock (Ricker 1958, Waldman et al. 1997, Kell et al. 2004). Exploitation of mixed stocks is prone to unrealistic perceptions of stock status, and thus annual or regular quantification of the composition of mixed-stock fisheries is essential for sustainable fisheries management. Therefore, in mixed-stock fisheries situations, where substantial mixing is an issue, the development and application of reliable stock discrimination methods with high assignment accuracy are required (Cadrin et al. 2014).

The key challenge of stock discrimination techniques is to comprehensively cover the multidimensional nature of population units, considering spatial and temporal drivers of genetic and phenotypic diver-

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gence (Waples et al. 2008). These highly complex patterns can hardly be revealed by a single method, advocating interdisciplinary approaches of molecular and non-molecular methods (Begg & Waldman 1999, Stephenson et al. 2009, Cadrin et al. 2014).

Molecular discrimination methods that rely on genetic data to estimate the origin of unknown mixtures of fish usually use neutral markers that are not affected by environmental adaptation (Nielsen et al. 2009). Once specific markers are identified, genetic analysis is the preferred tool to unequivocally assign individual fish with unknown origin to the respective stock (Ward 2000, Waples et al. 2008) and to provide the basis for calibrating non-molecular stock discrimination methods.

Genetic approaches are still associated with high expenses and require tissue samples, which are generally only available from recent sampling. However, many fisheries research institutes maintain extensive otolith archives, which may allow a view back in time (Geffen et al. 2011). Therefore, alternative otolith-based stock discrimination techniques, such as otolith shape or stable isotope analyses, which can be calibrated with genetically validated baseline samples, are of particular interest because they can easily connect the present with the past (Campana 2005, Tanner et al. 2016, Afanasyev et al. 2017).

The commercially important fish species Atlantic cod *Gadus morhua* is managed as 2 distinct stocks in the Baltic Sea: one western stock (ICES subdivisions [SDs] 22–24) and one eastern stock (SDs 24–32; ICES 2019). Both stocks differ with respect to morphometrics and genetics (Berner & Vaske 1985, Müller 2002, Nielsen et al. 2003, Pocwierz-Kotus et al. 2015) and in spawning times and areas (Bleil et al. 2009). The western Baltic cod (WBC) spawn mainly in the Belt Sea and Øresund (SDs 22 and 23, respectively) from January to April ('spring spawners'); the eastern Baltic cod (EBC) spawn mainly east of Bornholm (SD 25) from June to September ('summer spawners'; Bleil et al. 2009). Historical tagging experiments suggest a habitat overlap between the 2 stocks, particularly in the Arkona Basin (SD 24, Berner 1967, Otterlind 1985), which may be caused by larval drift and feeding or spawning migrations (Aro 1989, Hinrichsen et al. 2009).

The mixing of the 2 cod stocks is still hampering our understanding of Baltic cod ecology, and has led to a series of stock discrimination studies. Historical stock separation approaches on Baltic cod stocks involved meristic and morphometric characteristics (Kändler 1944, Berner & Vaske 1985, Berner & Müller 1989), electrophoresis (Sick 1965, Jamieson & Otterlind 1971), parasite tags (Mellergaard & Lang 1999) and mark-recapture experiments (Aro 1989, Bagge & Steffensen 1989). To date, commonly used methods for discriminating Baltic cod stocks cover genetics (Nielsen et al. 2003, Pocwierz-Kotus et al. 2015, Hemmer-Hansen et al. 2019), chemical analyses (Deutsch & Berth 2006, Heidemann et al. 2012), otolith shape analysis (Paul et al. 2013, Hüssy et al. 2016a) and otolith readability (Stötera & Krumme 2016).

However, most stock discrimination studies on Baltic cod were conducted on samples taken in SDs 22 and 25, representing the main spawning habitats of WBC and EBC, respectively, but the methods were not applied on cod captured in the mixing area SD 24 (e.g. Deutsch & Berth 2006, Heidemann et al. 2012, Paul et al. 2013). Analytical approaches, which considered cod samples from SD 24 (e.g. Sick 1965, Berner & Müller 1989, Stötera & Krumme 2016), have not validated the stock affiliation of these samples, for instance through genetic assignment.

So far, only Hüssy et al. (2016a) have used more than 1 stock discrimination technique in their study to separate Baltic cod stocks from SDs 22 and 25 and the mixing area SD 24, by combining genetics and otolith shape analysis, albeit not all samples used in the study were genetically validated. Moreover, the length distributions and sample sizes of WBC and EBC samples of the baseline were unbalanced and showed only limited spatial coverage throughout the southern Baltic Sea, which may lead to biased assignment results.

Otolith shape analysis accounts for stock-specific differences in the morphometric outline of otoliths, and is a common otolith-based stock discrimination method in fisheries science (Campana & Casselman 1993, Cadrin et al. 2014, Afanasyev et al. 2017). For Baltic cod, it has been shown that otolith shape differs significantly when comparing samples from SDs 22 and 25 (Paul et al. 2013, Hüssy et al. 2016a).

Another common stock discrimination method is the stable isotope analysis of otoliths, as the chemical composition of otoliths reflects the water chemistry of the habitat in which the fish lived (Coyle 1998, Cadrin et al. 2014). The main spawning sites of WBC and EBC display distinct hydrographical conditions, which were demonstrated by different stable oxygen isotope values in cod otoliths sampled in SDs 22 and 25 (Deutsch & Berth 2006). The composition of stable carbon isotopes in otoliths has not yet been successfully used to discriminate cod stocks in the Baltic Sea, but regional isotopic characteristics were found in cod otoliths from North Atlantic waters (Weidman & Millner 2000, Jamieson et al. 2004).

Another potential discrimination method relies on otolith readability, which refers to the quality of annual ring patterns in otoliths, formed by opaque and translucent zones (TZs).

For Baltic cod, the incidence of 'readable' and 'unreadable' otoliths differed strongly between areas SDs 22, 24 and 25 (Kändler 1944, Berner 1968, Stötera & Krumme 2016), suggesting stock-specific patterns in otolith readability of WBC and EBC.

A fourth potential discrimination method is the use of the diameter of the first TZ. Spring spawning of WBC and summer spawning of EBC may affect the timing of the first TZ formation in otoliths, resulting in size differences of TZs between cod stocks (Bagge & Steffensen 1989). We are not aware of a study that has considered the diameter of TZs in otoliths to discriminate between WBC and EBC, but e.g. Clausen et al. (2007) have successfully used the size of the first TZ to separate Atlantic herring *Clupea harengus* stocks.

Presently, the Baltic cod stock assessment relies on otolith shape analyses (ICES 2019), but alternative stock discrimination methods have not been thoroughly assessed.

In fact, no study on Baltic cod has yet conducted a comprehensive comparison of multiple promising separation techniques in a single study using genetically validated baseline samples.

In this study, we apply a holistic approach of 4 otolith-based stock discrimination methods (otolith shape analysis, stable oxygen and carbon isotope analyses on otolith nuclei, otolith readability and diameter of first 2 TZs) on the same set of genetically validated Baltic cod baseline samples, caught in the mixing area SD 24 (Arkona Basin) and in adjacent SDs 22 (Belt Sea), 23 (Øresund) and 25 (Bornholm Basin).

This study aims at (1) investigating the applicability of different non-molecular stock discrimination methods for separating individual WBC and EBC, particularly when samples originate from the mixing area, and (2) identifying reliable methods with high assignment accuracy for discriminating Baltic cod stocks applicable in future research studies and for fisheries management purposes.

2. MATERIALS AND METHODS

2.1. Sampling

Between September 2015 and July 2016, samples of cod *Gadus morhua* were taken throughout the southern Baltic Sea from commercial, survey and recreational catches (Fig. 1, Table S1 in the Supplement at www.int-res.com/articles/suppl/m627p125_supp.pdf).

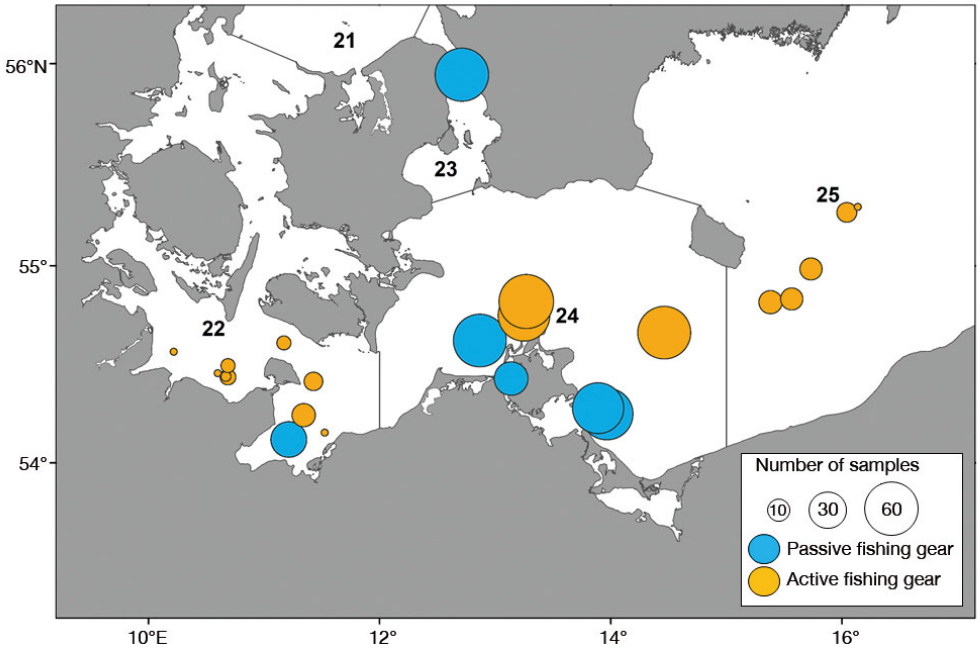

Fig. 1. Distribution of sampling locations of cod specimens within the southern Baltic Sea including ICES subdivisions 22 (Belt Sea), 23 (Øresund), 24 (Arkona Basin) and 25 (Bornholm Basin), separated by active (bottom trawl) and passive fishing gear (gill net, fishing rod)

Fish were measured (total length, cm), and sex and maturity stage were determined (Tomkiewicz et al. 2002). The sagittal otoliths were removed and stored individually in a dry place until further processing. For genetic analysis, a piece of gill or muscle tissue was taken and stored in 2 ml plastic tubes filled with absolute ethanol in a freezer at –80°C.

2.2. Genetic assignment of baseline samples

Baseline samples of Baltic cod (n = 519) from SDs 22, 23, 24 and 25 were genetically identified and unambiguously assigned to the WBC or EBC stock (samples are a subset of samples used by Weist et al. 2019). In brief, DNA was extracted from gill or muscle tissue using the Invisorb® Spin DNA Extraction Kit (Stratec Molecular). Samples were then genotyped using a 'minimum' panel of 20 diagnostic nuclear single nucleotide polymorphism (SNP) markers designed to differentiate between individuals of the WBC and EBC stock and a second ('full') panel, which was extended by 18 adaptive SNPs within regions putatively under selection (38 SNPs in total). To confirm the assignment power of both SNP panels (minimum and full), we used the programme GeneClass2 (Piry et al. 2004) to assign our baseline individuals to the most likely reference population (WBC or EBC). Assignment scores were calculated based on genotype likelihoods (thereby following Rannala & Mountain 1997). Individual assignment was done based on a principal component analysis (Smartpca) implemented in the EIGENSOFT software (v5.0, Patterson et al. 2006) using the option 'lsqproject' to improve handling of missing genotype data. The parameter 'poplistname' was set to infer eigenvectors using only individuals from a subset of all sampled populations, and then project individuals to be assigned onto those eigenvectors. Spawning individuals (Table S1, maturity stages 5 and 6) from the Belt Sea (SD 22) and the Bornholm Basin (SD 25) were used as reference samples for the WBC and EBC stock, respectively. Samples were used as baseline samples for the otolith analyses only if the individual assignment was congruent for both SNP panels.

2.3. Otolith-based stock discrimination methods

The 4 otolith-based stock discrimination approaches are presented in order of otolith processing. All otoliths used in this study originated from cod, which were genetically validated.

2.3.1. Otolith shape analysis

Prior to imaging, otoliths were cleaned with a brush and scanned for damages and hyaline or crystalline structures. Images of entire otoliths (n = 507) were taken with a stereomicroscope (SZX10, Olympus) equipped with a digital microscope camera (Axiocam 105 color, Zeiss), using transmitted light with an exposure time of 36 ms, a resolution of 3.5 megapixels and a magnification of 1.25× or 1×, depending on otolith size. For imaging, otoliths were horizontally orientated with the convex side facing up. Preferably the right otolith was chosen; otherwise, the image was flipped afterwards.

Subsequent otolith shape analyses on high-contrast images (Fig. S1 in the Supplement) were conducted using the ShapeR package (v0.1.5, Libungan & Pálsson 2015) in the R environment (v3.6.0, R Core Team 2019). Images were transformed into grey scale and were binarized using a threshold pixel value of 0.2. An additional contour smoothing with 100 iterations was performed to eliminate high-frequency pixel noise. Otolith shapes were quantified using normalized elliptical Fourier descriptors (EFD) proposed by Kuhl and Giardina (1982). The precision of the reconstruction of shapes was assessed by calculating the Fourier power (Lord et al. 2012) of the first 30 harmonics. The first 12 harmonics (giving 48 shape coefficients) reached 99% of cumulated power percentage, and were chosen to describe the shape variations of cod otoliths. The first 3 coefficients were used for standardization of otoliths with regard to size, rotation and starting point (48 – 3 = 45).

Detected contours, which were drawn on top of the original otolith images, were visually verified, and the shape coefficients were manually scanned for outliers. In our study, all contours were correctly detected, thus all otolith shapes were included in the analysis.

As fish length may influence shape descriptors (Lleonart et al. 2000), the effect of fish length on each stock-specific shape coefficient was investigated. Because no interaction between coefficients and fish length could be detected, all elliptical Fourier descriptors were used in the shape analysis (n = 45).

2.3.2. Otolith readability

After imaging, otoliths of genetically validated specimens were embedded in epoxy resin (GTS Polyester casting resin, Voss Chemie, 35–40% Styrol and a MEKP hardener) and hardened for 1 wk. Otolith blocks were sawed along the otolith nucleus

into 0.5 mm thick slices with a wet abrasive cut-off machine (Brilliant 250, ATM). Sliced otoliths (n = 517) were pasted on glass slides and digitalised (SZX10 [Olympus] equipped with Axiocam 105 color [Zeiss]) using transmitted light with an exposure time of 46 ms, a resolution of 3.0 megapixels and a magnification of 3.2× or 2.5×. A scale was added on each image, depending on the magnification used.

Otolith images were categorised according to the readability of annual ring patterns in otoliths, formed by opaque and TZs, following the description by Stötera & Krumme (2016): 'readable' for a well-defined ring structure with clear demarcations between otolith zones, 'uncertain' showing poorly defined annual rings with vague demarcations and 'unreadable' for otoliths without defined ring structures showing no demarcations between otolith zones. The quality of the demarcation of opaque and TZs was visually evaluated, and samples were assigned to 1 of the 3 readability categories (see Fig. S2 for examples).

2.3.3. Diameter of TZs

Measurements were performed on otolith images with 'readable' and 'uncertain' ring structures (n = 417 in total) using the image analysis program ImageJ (v1.50i, Schneider et al. 2012). After calibration of the scale, the diameter (in µm) of the first 2 TZs ('summer rings') was measured from the outer left edge to the outer right edge of each TZ (Fig. S3).

2.3.4. Stable isotope analysis

A subset of sliced otoliths (n = 50, Table S2) was selected to analyse the chemical composition of otolith nuclei. For this purpose, powder samples were milled out of the otolith nucleus with a computer-driven micro milling machine (MicroATX, New Wave Research). Milling tracks covered an area in the nucleus of 600 × 200 µm and a depth of 45 µm, representing the early life stage (i.e. hatching period) of each specimen. Powder samples of 20 to 80 µg were transferred into non-magnetic metal trays using clean needles, and stored individually in a dry place.

Stable oxygen and carbon isotope analyses were performed by the Leibniz Laboratory for Radiometric Dating and Stable Isotope Research at the University of Kiel, Germany. Values are reported relative to Vienna Pee Dee Belemnite standard as $\delta^{18}O$ and $\delta^{13}C$ (in ‰).

2.4. Data analysis

All statistical analyses and calculations were conducted in the R environment (v3.5.1, R Core Team 2019). A multivariate analysis of variance (MANOVA) and Tukey's multiple comparison tests were used to examine the effects of capture area (ICES subdivision), genetic stock affiliation, fish length and sex on the overall otolith shape using the Stats package (R Core Team 2019).

A multivariate generalized-linear model (GLM) with a negative binomial distribution was fitted to explain different patterns in otolith readability with capture area and genetic stock affiliation as fixed effects and sex as covariate applying the mvabund package (Wang et al. 2012).

Univariate GLMs with a Gaussian distribution were run to account for environmental and genetic effects on the diameter of TZs and on stable isotope values in otolith nuclei using the Stats package. Capture area and genetic stock affiliation were modelled as fixed effects, with sex as covariate. All univariate GLM effects were tested by analysis of deviance, and we assumed deviance change to be approximately χ^2-distributed. Wilcoxon rank sum test with p-value adjustment for multiple testing (Hochberg's method; Hochberg 1988, Chen et al. 2017) was used to explain significant effects detected by univariate tests.

Cod individuals were classified to their stock of origin based on otolith shape coefficients, otolith readability, the diameter of TZs and stable isotope values using linear discriminant analysis (LDA) and leave-one-out cross-validation (Lachenbruch & Mickey 1968). Prior probabilities for LDA were set at 0.5 for both classification groups. Samples were assigned either to the WBC or EBC stock when estimated assignment probabilities were >50%. Classification accuracy of single and combined discrimination methods was calculated by comparing the results with the genetic assignment. Individuals were classified using the MASS package (Venables & Ripley 2010).

3. RESULTS

3.1. Genetic assignment of baseline samples

The minimum and the full panel of stock-specific genetic markers presented by Weist et al. (2019) allowed unambiguous discrimination between reference *Gadus morhua* samples from the Belt Sea (SD 22) and the Bornholm Basin (SD 25), representing the

genetically differentiated WBC and EBC stock, respectively (Fig. 2). In detail, the distributions of likelihood ratios were well separated for the genetic reference samples, corresponding to an assignment rate of 99.1% for both SNP panels (Fig. S4 in the Supplement). Only 1 spawning individual caught in SD 22 during summer was identified as EBC (Fig. 2).

All individuals from the Øresund (SD 23) were assigned to the WBC reference, whereas samples from the mixing area, the Arkona Basin (SD 24) were composed of both stocks: 41.8% were assigned to the WBC stock (mean length ± SD: 47.58 ± 8.20 cm, proportion of females: 60%, no spawning individuals), while 58.2% were assigned to the EBC stock (length: 43.93 ± 8.18 cm, proportion of females: 70%, spawning individuals: 7%).

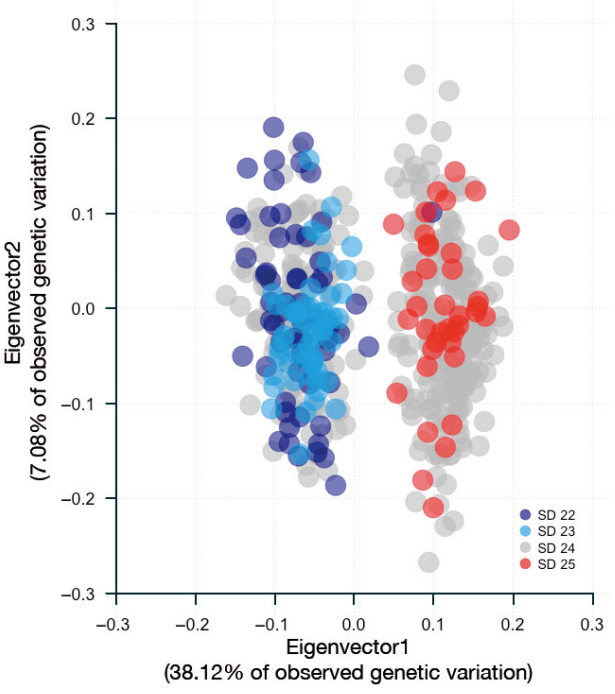

Fig. 2. Assignment of Baltic cod baseline samples according to their genetic structure based on a principal component analysis of genotypes using a minimal panel of 20 SNP markers. Colours indicate capture locations (ICES subdivision) of cod samples. Samples are a subset of samples used in Weist et al. (2019)

Table 1. Multivariate analysis of variance (MANOVA) of factors potentially affecting otolith shape, containing capture area (subdivision) and genetic stock affiliation (stock) as independent variables, fish length and sex as covariates. Significant ($p < 0.05$) values in **bold**; df: degrees of freedom, Num_df: numerator degrees of freedom, Den_df: denominator degrees of freedom

	df	*F*	Num_df	Den_df	p
Subdivision (SD)	3	3.66	135	1371	**<0.001**
Stock	1	8.87	45	455	**<0.001**
Length	1	8.80	45	455	**<0.001**
Sex	1	2.01	45	455	**<0.001**
SD × Stock	1	0.65	45	455	0.964

The minimum panel of diagnostic SNPs used by Weist et al. (2019) serves as a reliable tool to clearly assign Baltic cod individuals to their stock of origin, providing the basis for evaluating 4 alternative stock discrimination methods in this study.

3.2. Otolith-based stock discrimination methods

Results of the single stock discrimination approaches are presented in order of the highest individual classification success for samples originating from the mixing area SD 24. Different combinations of discrimination methods yielded either no or only minor overall improvements in assignment accuracy (Table S3). For some combinations, the sample size was strongly reduced, which induces uncertainties in the classification accuracy.

3.2.1. Otolith shape analysis

Shape variations could be detected between stocks (Table 1), particularly in the otolith length-width ratio, where the mean otolith width from the WBC stock was larger at the same standardised otolith length compared to those from the EBC stock (Fig. 3).

Additionally, the mean otolith shape varied with capture area (Table 1). Strongest differences in shape coefficients could be revealed between samples captured in SDs 22 and 25 (Tukey test, $p < 0.001$). The co-occurrence of both stocks in SD 24 was displayed in a non-significant interaction of capture area and genetic stock affiliation of cod samples (Table 1). Additional factors affecting otolith shape were fish length and sex (Table 1).

The stock assignment based on otolith shape coefficients resulted in the highest classification success compared to the classification accuracy of other otolith-based stock discrimination methods. In detail, 81.7% of the individuals from the mixing area (SD 24) and 83.2% of the cod samples originating from all capture areas (SD 22 to 25) were correctly assigned to their respective stock of origin (Table 2). Notably, the genetically identified EBC caught in SD 22 was also correctly assigned to the EBC stock based on its otolith shape. However, data adjustment according

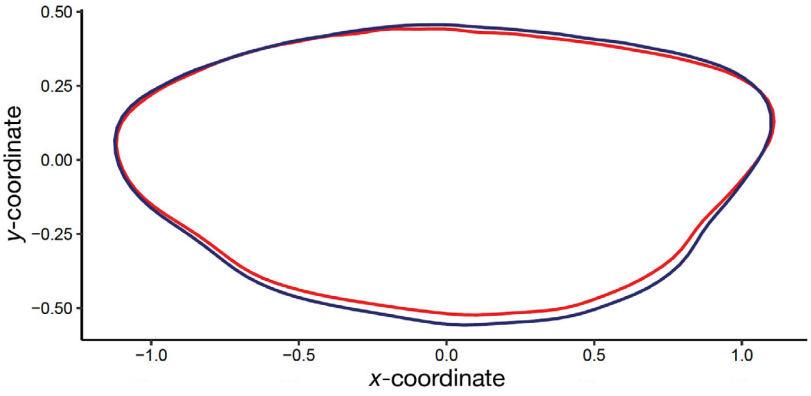

Fig. 3. Mean otolith shape of the western Baltic cod (blue) and eastern Baltic cod (red) stock based on the first 45 elliptical Fourier coefficients

to capture area, fish length or sex did not improve classification success.

3.2.2. Stable isotope analysis

Stable isotope analyses of otolith nuclei were successful for 48 of 50 samples (1 WBC from SD 24 and 1 EBC from SD 25 had to be removed from the dataset due to low signals of the mass spectrometer; Table S2). Stable oxygen isotope values ($\delta^{18}O$) ranged from −5.5 to 1.4 ‰ (mean ± StD: −3.2 ± 1.6 ‰) and varied with capture area. Values of $\delta^{18}O$ decreased eastwards from SD 22 and SD 23 over SD 24 to SD 25, showing significantly lower mean (±StD) values in SD 25 (−4.7 ± 0.5 ‰) than in SD 24 (−3.1 ± 1.2 ‰), SD 23 (−1.8 ± 1.8 ‰) and SD 22 (−2.7 ± 1.3 ‰, all Wilcoxon pairwise comparisons with $p < 0.001$; Fig. 4A, Table 3). Furthermore, $\delta^{18}O$ values differed between genetic stocks and were higher for otolith nuclei from WBC (mean ± StD: −2.3 ± 1.5 ‰) compared to EBC (mean ± StD: −4.3 ± 0.7 ‰; Fig. 4A, Table 3).

The assignment of cod individuals to their stock of origin based on stable $\delta^{18}O$ values resulted in a high classification accuracy of 79.5% for samples originating only from SD 24 and 84.2%, including samples from all capture areas (Table 2).

Stable carbon isotope values ($\delta^{13}C$) ranged from −6.0 to −1.9‰ (mean ± StD: −3.8 ± 1.0‰) and did not differ significantly between capture areas and between genetic stocks (Fig. 4B, Table 3). Hence, the overall classification success of cod individuals to their respective stock based on carbon isotope values was low (53.6 to 56.4%; Table 2).

3.2.3. Otolith readability

The distribution of readability categories of otoliths was highly correlated with capture area of cod samples (Table 4). The occurrence of readable otoliths with well-defined ring structures decreased from 39% in the west (SD 22) to 0% in the east (SD 25), whereas the proportion of unreadable otoliths with an undefined ring structure increased from 2% in SD 22 to 68% in SD 25 (Fig. 5). This pattern was also reflected in the distribution of readability categories between the 2 cod stocks (Table 4), comprising a relatively high proportion of readable otoliths (22%) and a relatively low proportion of unreadable otoliths (9%) for WBC, and a reversed result for EBC (4% readable otoliths, 29% unreadable otoliths; Fig. 5). However, the readability category 'uncertain' with poorly defined ring structures was most abundant, irrespective of capture area (SDs 22 to 24) and stock (Fig. 5), resulting in a low overall assignment accuracy of individuals to their respective stock of 57.7 to 60.0% (Table 2).

Table 2. Stock-specific and overall classification success (Mean) of genetically validated otolith samples based on single discrimination methods, including only samples from the mixing area (SD 24) and from all capture areas (SDs 22, 23, 24 and 25) using linear discriminant analysis. WBC: western Baltic cod, EBC: eastern Baltic cod, TZ: translucent zone

	SD 24					SD 22-25				
	WBC (%)	N	EBC (%)	N	**Mean (%)**	WBC (%)	N	EBC (%)	N	**Mean (%)**
Otolith shape analysis	80.1	146	83.2	202	**81.7**	83.3	264	83.1	243	**83.2**
Analysis of $\delta^{18}O$	71.4	7	87.5	8	**79.5**	77.8	27	90.5	21	**84.2**
$\delta^{13}C$	57.1	7	50.0	8	**53.6**	55.6	27	57.1	21	**56.4**
Otolith readability	93.3	150	22.1	208	**57.7**	90.7	268	29.3	249	**60.0**
Diameter of 1st TZ	57.9	140	45.3	161	**51.6**	63.8	243	46.6	174	**55.2**
2nd TZ	65.9	135	56.9	160	**61.4**	64.4	236	54.1	172	**59.3**

Table 3. GLMs containing stable isotope values ($\delta^{18}O$ and $\delta^{13}C$) as Gaussian response variable, capture area (SD) and genetic stock affiliation (stock) as fixed effects and sex as covariate. Significant values in **bold**; df: degrees of freedom, Dev: deviation, Res_df: residual degrees of freedom, ResDev: residual deviance

Response	$\delta^{18}O$					$\delta^{13}C$				
	df	Dev	Res_df	ResDev	p	df	Dev	Res_df	ResDev	p
Null			47	120.2				47	46.8	
SD	3	52.3	44	67.9	**<0.001**	3	2.0	44	44.8	0.577
Stock	1	6.1	43	61.8	**0.039**	1	2.3	43	42.6	0.134
Sex	1	1.6	42	60.2	0.285	1	0.3	42	42.2	0.570

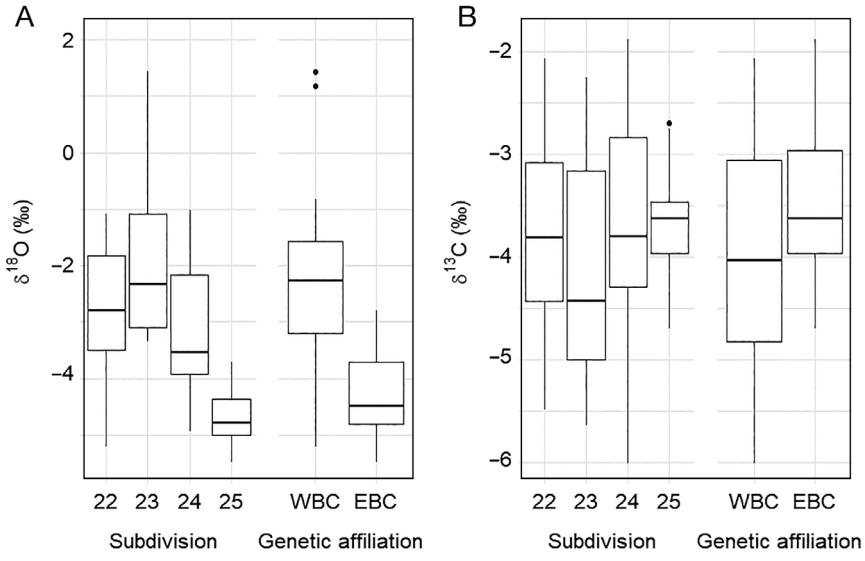

Fig. 4. Values (in ‰) of stable (A) oxygen and (B) carbon isotopes separated by capture area (ICES subdivision) and genetic affiliation of cod samples (WBC: western Baltic cod, EBC: eastern Baltic cod). The box represents the interquartile range (IQR) with the median (midline) and the first and third quantiles at the bottom and top of the box, respectively. Lower and upper whiskers are restricted to 1.5 × IQR, and black dots represent outliers

3.2.4. Diameter of TZs

The width of the first TZ differed strongly between capture areas (Table 4). Fish captured in SD 24 revealed the largest range of diameters of the first TZ from 1.1 to 4.0 mm (mean ± StD: 2.1 ± 0.6 mm), whereas cod samples from SD 22 showed a significantly smaller range of diameters from 1.4 to 3.0 mm (mean ± StD: 1.9 ± 0.3 mm, Wilcoxon pairwise comparisons: p = 0.018; Fig. 6A).

The diameter of the second TZ varied with stock affiliation and had significantly larger mean (±StD) width for WBC (4.6 ± 0.6 mm) than EBC (4.3 ± 0.8 mm; Fig. 6B, Table 4). However, the range of diameters of the first and second TZs strongly overlapped between stocks, leading to low classification accuracy of individuals to their stock of origin between 51.6 to 61.4 % (Table 2).

4. DISCUSSION

We tested a suite of non-molecular techniques to unambiguously assign cod *Gadus morhua* individuals from the mixing area (SD 24) and adjacent subdivisions (SDs 22, 23 and 25) to their respective stock of origin based on genetically validated reference samples.

Table 4. Multivariate GLM containing readability categories of otoliths as negative binomial response variables and univariate GLMs containing diameter of first and second translucent zone (TZ) as Gaussian response variables, capture area (SD) and genetic stock affiliation (stock) as fixed effects, and sex as covariate. Significant values in **bold**; df: degrees of freedom, Dev: deviation, Res_df: residual degrees of freedom, ResDev: residual deviance

Response	Otolith readability				Diameter of first TZ					Diameter of second TZ				
	df	Dev	Res_df	p	df	Dev	Res_df	ResDev	p	df	Dev	Res_df	ResDev	p
Null			516				416	121.2				407	217.1	
SD	3	100.8	513	**0.001**	3	5.0	413	116.2	**<0.001**	3	3.3	404	213.9	0.086
Stock	1	27.2	512	**0.001**	1	0.4	412	115.8	0.239	1	14.8	403	199.1	**<0.001**
Sex	1	0.9	511	0.690	1	0.4	411	115.4	0.243	1	0.1	402	199.0	0.708
SD × Stock	2	3.9	510	0.084	1	0.8	410	114.6	0.082	1	1.2	401	197.9	0.125

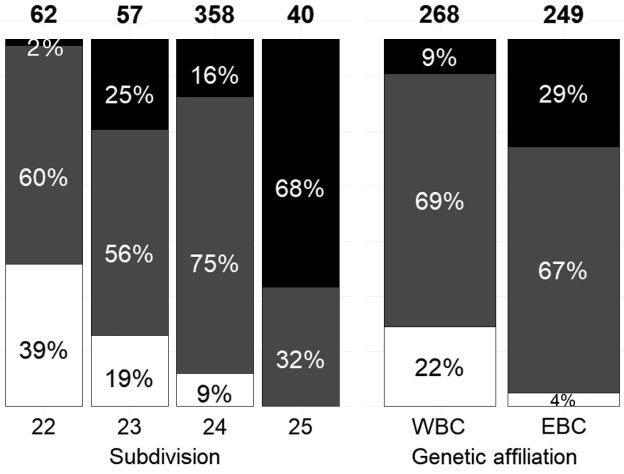

Fig. 5. Overall proportions of otolith readability categories (in %) separated by capture area (ICES subdivision) and genetic affiliation. Colours indicate readability categories (white: 'readable', grey: 'uncertain', black: 'unreadable'), and **bold** numbers above bars represent total sample size. Stock abbreviations as in Fig. 4

Most strikingly, otolith shape and stable oxygen isotope analyses yielded high classification accuracies, and thus provide a promising alternative to common molecular approaches (Table 5).

4.1. Genetic assignment as reference method

Using the smallest existing set of SNP markers for Baltic cod stock discrimination (Weist et al. 2019), cod reference samples from SDs 22 and 25 showed a clear separation pattern. Additionally, these stock-specific genetic markers allowed an unequivocal assignment of individuals with unknown origin to their respective stock, revealing co-occurrence of both cod stocks in SD 24 in recent times, which is also reflected in Müller (2002), Eero et al. (2012), Hüssy et al. (2016a) and Hemmer-Hansen et al. (2019). The genetically identified EBC caught in the area of the WBC (SD 22) and the genetic results from a recent study (Stroganov et al. 2018), indicate that mixing of both cod stocks to some extent also occurs west of SD 24, and may likely also occur east of SD 24. The co-existence of Baltic cod stocks in the Arkona Basin and adjacent subdivisions may be based on seasonal spawning and feeding migrations, larval drift or westward spill-over of EBC from the main distribution area SDs 25 to 24 (Aro 1989, Eero et al. 2012, Petereit et al. 2014), though Baltic cod mixing dynamics are still not well understood.

Genetic approaches using stock-specific markers are still time-consuming, cost-intensive and generally only applicable when tissue samples are available (Table 5). However, they allow the most reliable assignment of Baltic cod individuals to their respective stock, making them indispensable for the calibration of non-molecular approaches.

4.2. Otolith-based stock discrimination methods

4.2.1. Otolith shape analysis

Fish otoliths are formed during the egg stage and grow continuously throughout the fish's entire life (Campana & Casselman 1993). The shape of otoliths depends on the genetic background, the developmental stage of fish (e.g. body size or sex) and on environmental factors, such as water temperature or food composition (Cardinale et al. 2004, Hüssy 2008, Mille et al. 2016, Berg et al. 2018), which can lead to variations among fish populations (Campana & Casselman 1993, Stransky et al. 2008, Cadrin et al. 2014).

In North Atlantic waters, the otolith shape has been successfully used to separate cod populations or ecotypes (Cardinale et al. 2004, Galley et al. 2006, Stransky et al. 2008, Bardarson et al. 2017). In the Baltic Sea, Paul et al. (2013) and Hüssy et al. (2016a) have

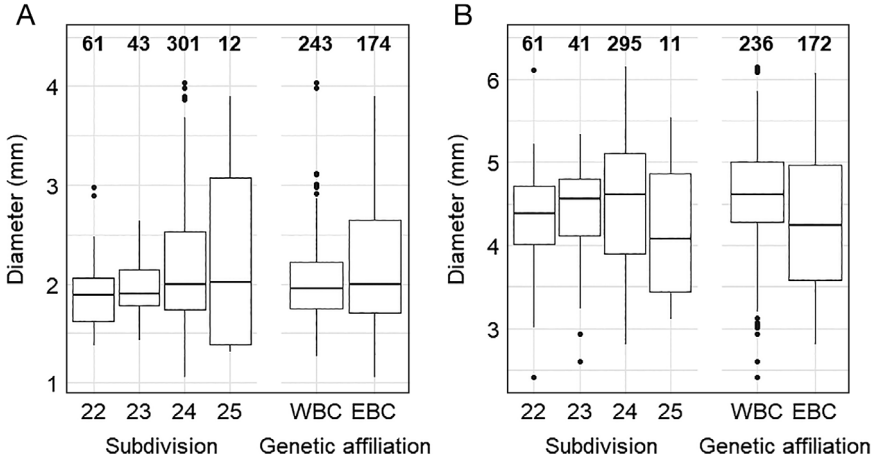

Fig. 6. Diameter (in mm) of (A) first and (B) second translucent zone separated by capture area (ICES subdivision) and genetic affiliation. **Bold** numbers above box plots represent total sample size. Stock abbreviations and definition of box plot components as in Fig. 4

Table 5. Overview of advantages and disadvantages of 1 molecular and 4 non-molecular stock discrimination methods to assign individual Baltic cod

Method	Type of material	Advantages	Disadvantages
Genetic analysis	Tissue, blood	• Highest assignment accuracy • No impact of environmental adaptation on neutral markers	• Molecular markers needed • Higher expenses • Time-consuming • Restricted tissue availability (mostly recent samples)
Otolith shape analysis	Entire otolith	• High assignment accuracy • Otolith availability in archives • Inexpensive • Fast and easy application (high-throughput)	• Not suitable for juvenile fish • Interaction of genetics and abiotic environmental factors (i.e. water temperature) still uncertain
Stable isotope analysis	Powdered otolith nucleus	• High assignment accuracy for $\delta^{18}O$ • Otolith availability in archives • Suitable for juvenile fish	• Low assignment accuracy for $\delta^{13}C$ • Higher expenses • Time-consuming • High impact of abiotic environmental factors (i.e. water temperature, salinity, DIC)
Otolith readability	Sliced otolith	• Otolith availability in archives • Inexpensive • Fast and easy application (high-throughput)	• Low assignment accuracy • Subjective interpretation • High impact of abiotic environmental factors (i.e. water temperature)
Diameter of TZs	Sliced otolith	• Otolith availability in archives • Inexpensive • Fast and easy application (high-throughput)	• Low assignment accuracy • Precise otolith sectioning process needed • Restricted reliability due to manual measurements • Not applicable to unreadable otoliths • High impact of abiotic environmental factors (i.e. water temperature)

used otolith shape analyses to discriminate between cod stocks with a classification accuracy of individuals between 21 and 100%, depending on fish length class. In our study, we were able to confirm the high discriminatory power of the otolith shape analysis, with a correct assignment of individual cod to their genetically validated stock of origin of 82 to 83%.

We found that the average otolith length-width ratio differed between cod stocks, with WBC having wider otoliths at the same standardised otolith length than EBC, which supports the findings of recent studies (Paul et al. 2013, Hüssy et al. 2016a). These stock-specific differences in otolith characteristics may be explained by the different relation of fish length and head length found between the 2 Baltic cod stocks (Berner & Vaske 1985, Berner & Müller 1989). As an alternative, observed differences may be due to the faster growth rate of the WBC cod, resulting in mean size differences up to 22 cm compared to the mean size of EBC within the same age class (Berner 1968, Bagge et al. 1994).

Even though our study showed a significant effect of fish length on otolith shape, single shape coefficients did not show a significant interaction with fish length. Hence, the use of different length classes for the stock assignment did not improve the classification success, which is contrary to other studies (e.g. Paul et al. 2013, Hüssy et al. 2016a). This result might be attributable to the well-balanced length distribution of WBC and EBC baseline samples used in this study, which do not require corrections for fish length.

Besides fish length effects, the otolith shape was significantly influenced by sex and capture area, confirming the results found in other studies (Lombarte & Lleonart 1993, Cardinale et al. 2004, Leguá et al. 2013). Sex differences in otolith shape are commonly explained by sex-specific somatic growth rates, physiology and metabolism (Campana & Casselman 1993, Begg & Brown 2000, Mille et al. 2016). Spatial effects on otolith shape were strongly related to the geographically segregated spawning areas of both cod stocks, showing strong habitat differences (Bleil et al. 2009). While the WBC stock spawns mainly in shallower waters (between 20 and 40 m water depth) in SDs 22 and 23 and benefits from well-oxygenated saltwater inflows from the Kattegat, the EBC stock spawns mainly in deeper basins (>50 m water depth) east of Bornholm (SDs 25 and 26) and encounters lower salinity and oxygen levels (von Dewitz et al. 2018).

The variability in otolith shapes highlights the importance of using genetically validated baseline sam-

ples, covering a wide range of fish length classes, capture areas, sampling years and both sexes to reflect genetic and environmental variations in the otolith shape.

Given the high assignment accuracy found in our study, the low costs and the easy application, this stock discrimination method can be considered an appropriate alternative to genetics (Table 5), suitable to assign not only recent cod samples from the mixing area and from adjacent areas but also historical samples from otolith archives to their respective stock.

4.2.2. Stable isotope analysis

Otoliths are composed of calcium carbonate containing trace elements and stable isotopes that are derived from ambient waters, reflecting environmental conditions the fish experienced during its lifetime (Campana et al. 1995, Høie et al. 2004). When stocks live in different habitats with divergent environments, they may be distinguishable by the chemical signatures retained in their otoliths (Begg & Waldman 1999). The chemical composition of the otolith nucleus reveals the environmental conditions during the early life phase of the fish (Campana et al. 2000). With regard to the specific hydrographical conditions of the spawning sites of the cod stocks (Berner 1967) and the site fidelity of juvenile cod (Grant & Brown 1998, Freitas et al. 2015), stable isotope analyses of otolith nuclei may be a potential discrimination approach for Baltic cod stocks, despite substantial mixing of adult cod.

In our study, we used stable oxygen ($\delta^{18}O$) and carbon ($\delta^{13}C$) isotopes to separate Baltic cod stocks. Stock-specific differences in the chemical composition of the otolith nucleus were only confirmed for stable oxygen isotopes, with significantly higher $\delta^{18}O$ values for WBC otoliths compared to EBC, which was also reported by Deutsch & Berth (2006). Stable oxygen isotopes are affected by water temperature (Høie et al. 2004), but salinity also plays an important role (Deutsch & Berth 2006), e.g. seawater shows $\delta^{18}O$ values around 0‰ (Craig & Gordon 1965), whereas freshwater has $\delta^{18}O$ values far below 0‰ (Kendall & Coplen 2001). In our study, the decrease in salinity from the western Baltic (SD 22) to the eastern Baltic (SD 25) was also reflected in the decrease in $\delta^{18}O$ values in the otoliths sampled along this gradient representing the main spawning areas of WBC and EBC.

The $\delta^{13}C$ isotopic composition in fish otoliths is mainly influenced by dissolved inorganic carbon (DIC) of ambient seawater, but also by metabolic carbon derived from fish's diet (Schwarcz et al. 1998, Jamieson et al. 2004). In the present study and in Deutsch & Berth (2006), samples from the western Baltic (SDs 22 and 23) exhibited slightly lower $\delta^{13}C$ values in otoliths than samples from the eastern Baltic (SD 25), even though differences were not significant between capture areas or stocks. In contrast, recent studies showed a longitudinal and vertical decrease of $\delta^{13}C_{DIC}$ values in ambient waters of the Baltic Sea (Filipsson et al. 2017, Torniainen et al. 2017). A proper interpretation of $\delta^{13}C$ values in otoliths would require the quantification of the relative contribution of DIC and dietary carbon to observed otolith carbon.

Stable isotope analyses of cod otoliths have also been used in other marine areas to differentiate among sample sites, for instance, in the Northwest Atlantic (Campana et al. 1994), the Northeast Atlantic (Weidman & Millner 2000) and in the Northeast Pacific (for *Gadus macrocephalus*; Gao et al. 2005). Yet classification success was not reported or was stated as low with an estimated accuracy of only 30% (Campana et al. 1994). Contrary to otolith shape analysis, stable oxygen isotope analysis of otolith nuclei is time-consuming and cost-intensive, and the environmental effects are strong, but considering the high individual classification success of 80 to 84% we found in our study, this method can be deemed a suitable alternative discrimination method to genetics (Table 5) when tissue samples are not available.

4.2.3. Otolith readability

Although the complex mechanisms explaining the biomineralisation process of otoliths are still poorly understood, the quality of readability patterns in cod otoliths has been linked to water temperature (Neat et al. 2008, Millner et al. 2011, McQueen et al. 2018).

In the Baltic Sea, WBC are mainly exposed to higher temperatures in shallower waters, which possibly provoke well-defined ring patterns in otoliths, whereas EBC likely experience colder temperatures in deeper basins, often causing undefined ring structures (Dannevig 1956, Bagge & Steffensen 1989).

Early studies on Baltic cod already observed strong differences in the readability of otoliths (Kändler 1944, Berner 1968). The majority of otoliths from the WBC showed clearly defined translucent and opaque zones, while most of the otoliths from the Bornholm Basin had less defined ring structures (Bagge & Steffensen 1989, Hüssy 2010, Stötera & Krumme 2016), as reflected in our study.

However, Stötera & Krumme (2016) and the present study showed that cod from western Baltic areas and genetically assigned WBC also have 'unreadable' otoliths, whereas a small proportion of cod from eastern Baltic areas and genetically assigned EBC showed 'readable' otoliths. Most notably, the readability category 'uncertain' with poorly defined zones was most abundant in this study, irrespective of capture area and genetic affiliation. The occurrence of 'unreadable' otoliths in WBC and 'readable' otoliths in EBC in combination with the strong overlap of poorly defined annual rings in both stocks allow no reliable assignment of individuals to their stock, which was also reflected in a very low classification accuracy of 58 to 60%. In addition, otolith readability relies on a subjective interpretation and categorization of annual ring structures, and results can vary among age readers. Given the poor assignment accuracy found in our study and the strong environmental effects, otolith readability is not a suitable discrimination method for Baltic cod stocks (Table 5).

4.2.4. Diameter of TZs

The formation of TZs has been mainly linked to seawater temperature (Bagge & Steffensen 1989, Neat et al. 2008, Hüssy et al. 2009) and growth rate (Campana & Neilson 1985, Cadrin et al. 2014). Because the first TZ is formed between summer and late autumn (validated for WBC; McQueen et al. 2018), spring spawning WBC develops the first TZ in the first summer, whereas summer spawning EBC may develop the first TZ during the following summer, potentially leading to a larger first TZ for the EBC stock. However, several studies reported a prolonged spawning period for EBC from March to October (Wieland et al. 2000, Baranova et al. 2011, Stroganov et al. 2018), likely inducing a strong variability in the timing of the formation of the first TZ for EBC (Hüssy et al. 2016b). In this study, the largest variations in the diameter of the first TZ were found for EBC samples from SDs 24 and 25; thus, stock-specific differences in the width of the first TZ could not be detected. Diameter differences between stocks were only confirmed for the second TZ, with a significantly larger mean width for WBC compared to EBC. Irrespective of the variable timing of the first TZ in EBC, the faster growth rate of WBC (Bagge et al. 1994) may compensate for the timing effect of TZ formation, likely resulting in similar TZ diameters for both stocks or even leading to larger TZs in WBC.

However, the measured range of diameters strongly overlapped between stocks, resulting in a poor assignment accuracy of 59 to 61%. The method requires precise sectioning of the otolith nucleus, otherwise, the diameter of the TZs can vary with the position of the cut. Additionally, the diameter measurements are made manually, which can result in potential biases among users. The identification of the first TZ is known to be problematic because age readers often fail to distinguish between false annuli, or settlement checks, and true annuli, inducing uncertainties in the age reading (McQueen et al. 2018), particularly for the EBC stock (Hüssy et al. 2016b).

Since the discriminatory power between WBC and EBC was extremely low, the diameter of TZs is not a reliable stock separation method for Baltic cod (Table 5).

5. CONCLUSIONS

This study provides the most comprehensive set of stock discrimination techniques currently available for Baltic cod, applying 4 otolith-based methods on the same set of genetically validated Baltic cod samples. Otolith shape analysis and stable oxygen isotope ($\delta^{18}O$) analysis on otolith nuclei showed the highest assignment accuracies of cod individuals to their respective stock of origin, with a classification success between 80 and 84%. Genetic analysis with stock-specific markers is the preferred method for stock discrimination but generally requires fresh tissue samples. When only archived otoliths are available, otolith shape and stable oxygen isotope analyses of otolith nuclei are considered appropriate alternatives to genetics. These otolith-based approaches enable a high-throughput quantification of not only present but also past mixing proportions of Baltic cod stocks, which is of great importance for establishing meaningful reference points for mixed-stocks with historically fluctuating stock levels.

The identification of reliable stock discrimination methods is crucial for the sustainable management of mixed-stock fisheries. When comparing the performance of different stock discrimination approaches, an established method with a high assignment accuracy has to be used to validate the stock affiliation of the test samples, e.g. by genetic analysis. Reliability and applicability of discrimination methods may vary between mixed-stock fisheries and should therefore be tested on the same set of validated samples.

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