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ORIGINAL ARTICLE

Otolith microchemistry combined with genetics reveal patterns of straying and population connectivity in anadromous brown trout (*Salmo trutta*)

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Abstract

Salmonids are well known for their natal homing behaviour, meaning they return to breed in the same area where they originated. However, not all individuals return to their natal breeding grounds—a behavioural trait known as straying. The prevalence of straying is difficult to explore and therefore quantitative estimates for straying are seldom reported. In this study, otolith microchemistry and genetics were combined to investigate patterns of straying over ecological and evolutionary time, respectively, between neighbouring rivers flowing into Mariager fjord, Denmark. Otolith microchemistry was used to determine the river of origin for sea trout (*Salmo trutta*) upon their return to freshwater and 288 SNP markers were used to determine genetic structure among the rivers in the fjord. In this system, where the distance between rivers is short, otolith microchemistry achieved 80% accuracy in assigning juvenile brown trout to their natal river, thus allowing us to determine that approximately 43% of the adult sea trout had returned to non-natal rivers to spawn, with a similar proportion of strayers and natal homers in all of the rivers. Genetic analysis further supported that there was substantial gene flow among individuals originating from different rivers, indicating that sea trout in Mariager fjord make up one population. The findings obtained from otolith microchemistry and genetics complement each other and provide further evidence that sea trout in this system migrate to non-natal rivers and spawn there, which consequently affects the genetic structure of the population.

KEYWORDS
dispersal, gene flow, natal homing, phenotypic plasticity, salmonids

1 | INTRODUCTION

The movement of fish between populations may have varying levels of impact on both the donor and recipient population by affecting population dynamics, demographics and genetic structure (Bett et al., 2017; Bowler & Benton, 2005; Schtickzelle & Quinn, 2007). Therefore, assessing population connectivity and structure by tracking individuals' movements as well as determining genetic differentiation among populations over evolutionary timescales is essential in understanding the factors that affect population

sustainability. This is especially relevant in the Anthropocene, when many populations are increasingly vulnerable to negative effects caused by climate and habitat changes and other anthropogenic factors (Last et al., 2011).

Otolith microchemistry and genetics have proven to be effective tools for investigating population structure and connectivity in fishes (Bekkevold et al., 2020; Collins et al., 2013; Heidemann et al., 2012). Otolith microchemistry provides the possibility to infer information on migratory patterns (Brennan et al., 2015; Sturrock, Wikert, et al., 2015; Taal et al., 2014;), habitat use (Ciepiela & Walters, 2019; Phillis et al., 2018; Volk et al., 2010) and the origin of fish among marine, freshwater and diadromous populations (Chen et al., 2020; Heidemann et al., 2012; Matetski et al., 2022). Otoliths are particularly well suited to track migrations among aquatic ecosystems, as they grow continuously and incorporate chemical differences among water sources over the lifetime of an individual (Campana, 1999; Svedäng et al., 2010; Tabouret et al., 2010). The capability of otolith microchemistry to discriminate populations is contingent on the assumption that individuals that originate from different habitats differ in their chemical composition of the otolith (Brennan et al., 2015; Campana et al., 1994; Chang & Geffen, 2013). While the physico-chemical properties of water affect otolith microchemistry (Brown & Severin, 2009; Macdonald & Crook, 2010), other factors, for example, physiology (Sturrock, Hunter, et al., 2015) and ontogeny (Walther et al., 2010) are also involved. Therefore, the extent to which otolith microchemistry differs between populations varies among different species and systems (Chang & Geffen, 2013), resulting in varying levels of discrimination (Collins et al., 2013; Morales-Nin et al., 2022; Turcotte & Shrimpton, 2020).

While otolith microchemistry allows us to follow the movements of individuals over their life cycle, genetic marker analysis ('genetics')—a central tool in studies of population connectivity—offers the opportunity to disentangle patterns of population structure over an evolutionary time (Bekkevold et al., 2020; Massa-Gallucci et al., 2010; Waples & Gaggiotti, 2006). Although, genetics can also be applied to identify immigrants from demographically isolated populations (Bekkevold et al., 2004; Masson et al., 2018), in species that are characterized by larger population sizes and high dispersal, identifying immigrants is often not possible due to weak genetic differentiation among populations. Nevertheless, genetics provide valuable insights into evolutionary forces, including population size and patterns of selection, which may affect population structure. Consequently, combining both otolith microchemistry and genetics through an interdisciplinary approach could provide a resolution for population connectivity and structuring over varying timescales.

Salmonids are well known for their natal homing behaviour, meaning they may return to spawn in the same area where they hatched (Jonsson & Jonsson, 2014; Keefer & Caudill, 2014). It is an evolutionarily important mechanism through which individuals increase their likelihood of finding suitable habitats and mates during the breeding season (Keefer & Caudill, 2014). It is also a mechanism through which inter-population genetic structuring takes place, and local adaptations develop (Hendry et al., 2004; Peterson et al., 2014). Consequently, in salmonids, the spatial-scale of population boundaries is often defined by the rivers or tributaries individuals originate from or return to spawn, because dispersal and overall connectivity among rivers are expected to be low (see Miettinen et al., 2021 and references herein). The prevalence of natal homing may however vary significantly between species of salmonids, as well as between populations of the same species (Ayllon et al., 2006; King et al., 2016; Östergren et al., 2012; Quinn, 1993). For example, among anadromous brown trout (*Salmo trutta*), also referred to as sea trout, straying rates have been previously documented to vary between 1.6 and 55% among wild populations (Jonsson & Jonsson, 2014; Källo, Birnie-Gauvin, Baktoft, et al., 2023). However, quantitative estimates on the rate of straying are scarce, limiting a complete assessment of the prevalence of straying in different geographical regions, and its potential impacts on population dynamics. Furthermore, as straying may (or may not) be accompanied with gene flow, the implications of straying may vary between populations (Dionne et al., 2008; Mobley et al., 2019).

In Mariager fjord, Denmark, significant levels of straying (12–55%) have been previously documented among sea trout originating from four different rivers using passive integrated transponder (PIT) telemetry (Källo, Baktoft, Birnie-Gauvin, et al., 2022; Källo, Baktoft, Kristensen, et al., 2022; Källo, Birnie-Gauvin, Baktoft, et al., 2023). However, as telemetry is not a feasible solution in many systems, the aim of this study was to test whether otolith microchemistry and genetic methods can be used to assess population connectivity (via straying and/or gene flow) among anadromous brown trout originating from neighbouring rivers flowing into the same marine system. More precisely, otolith microchemistry was used to assign mature adult sea trout collected in freshwater to their river of origin, thus quantifying the number of spawners that had strayed and genetic analysis (SNP analyses) was used to infer information on gene flow among the rivers in the fjord and determine patterns of reproductively successful straying over an evolutionary time.

2 | METHODS

2.1 | Study area

Juvenile and adult brown trout were sampled from four rivers: Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk. All rivers flow into Mariager fjord, Denmark, which is situated in Northern Jutland, and flows to the Kattegat (Figure 1). The fjord is about 40 km long, 2 km wide and has a maximum depth of 30 m. The surface water salinity varies from around 12‰ in the inner part of the fjord, to >20‰ in the outer part of the fjord (Fallesen et al., 2000).

2.2 | Sample collection and preparation for microchemistry analysis

Juvenile brown trout were collected in March 2020 and 2021 via electrofishing, at one or multiple stations in each river (Figure 1), with

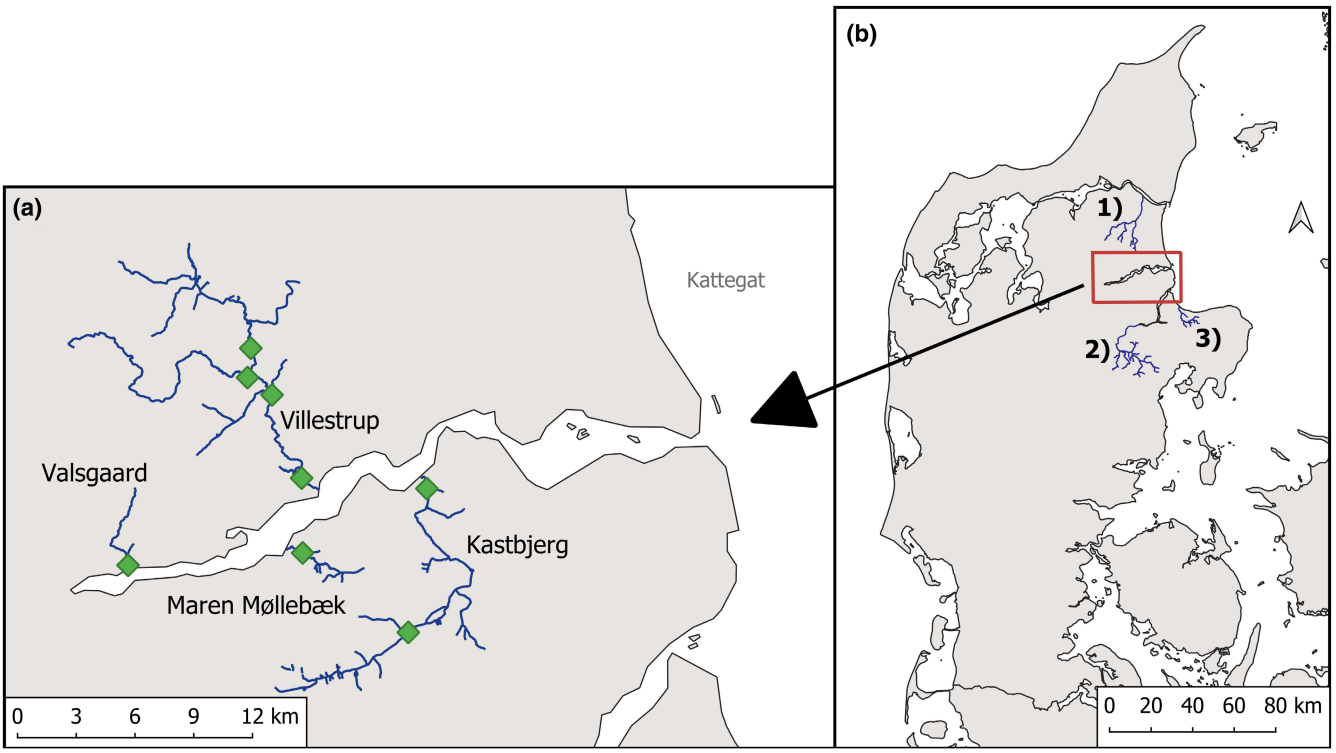

FIGURE 1 Juvenile and adult brown trout were collected from Rivers Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk (a) to identify strayers and determine gene flow among the rivers. We further estimated genetic differentiation as a proxy for gene flow between individuals from the rivers of Mariager fjord (red rectangle) and the neighbouring rivers Lindenborg (1), Lilleaa (2) and Hevring (3), which are situated outside of Mariager fjord (b). Green diamonds mark the sampling stations where juvenile brown trout were collected in 2020 and 2021 (a).

TABLE 1 Sample sizes and total lengths (cm) of juvenile brown trout collected in rivers Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk for otolith microchemistry and genetics analysis, divided by river and year of sampling.

River	Number of juveniles included in otolith microchemistry analysis (2020/2021)	Number of juveniles included in the genetic analysis (2020/2021)	Length ± SD of juveniles collected for analysis
Villestrup	74 (53/21)	30 (10/20)	9.4 ± 2.3
Kastbjerg	55 (33/22)	30 (10/20)	10.9 ± 2.6
Valsgaard	34 (20/14)	20 (10/10)	9.4 ± 2.2
Maren Møllebæk	30 (15/15)	24 (10/14)	9.7 ± 3.3
Total	193 (121/72)	104 (40/64)	9.9 ± 2.6

approximately 20 individuals randomly collected at each location per year (Table 1). After being caught, juvenile brown trout were immediately euthanized via an overdose of benzocaine (Sigma Chemical Co.). All juveniles were of a size consistent with fish that have never been to sea (Table 1), so we assume these fish had hatched in the river of capture. Adult sea trout were caught via electrofishing in each of the four rivers during the spawning season in November–December 2020 and immediately euthanized upon capture through a blow to the head. The sampling scheme for adult sea trout aimed for a 1:1 sex ratio, which was roughly achieved for all of the rivers, besides Maren Møllebæk, where too few males were caught.

Total length was measured for all fish (to the closest cm) and adult sea trout gonads were visually inspected to ensure maturity. All trout were sampled for genetic analysis, by cutting the adipose fin and storing it in 96% ethanol. Otolith pairs (*sagitta*) were removed for all trout with tweezers, cleaned, dried and stored in Eppendorf vials. Prior to otolith microchemistry analysis, one otolith per fish was chosen at random, embedded in two-part epoxy (Epofix; Struers), and ground in transversal plane using abrasive papers with grit size P800-P2400 (Struers) until the core of the otolith was visible. All otoliths were polished with abrasive paper of grit size p4000 (Struers) and finally glued to a glass slide using superglue.

2.3 | Otolith microchemistry analysis

Otolith microchemistry analysis for trace elements was conducted at the department of Geoscience at Aarhus University by

Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICPMS) using a Resonetics 193 nm laser coupled to an Agilent 7900 Quadrupole instrument. Concentrations of ^{88}Sr, ^{43}Ca, ^{55}Mn, ^{24}Mg, ^{66}Zn, ^{138}Ba and ^{208}Pb were measured through a transect of consecutive spots of 60 μm in diameter along the longest growth axis of the otolith with laser energy set at 80 mJ, pulse frequency at 10 Hz and 90 s data acquisition times. Background levels were measured for 30 s before and after each spot analysis. Juvenile otolith transects were set from the edge of the core until the edge of the otoliths. Adult otolith transects were set from the edge of the core until the presumed freshwater exit, based on visual inspection of growth bands. LA-ICPMS data were processed with the open-source Python package *LAtools* (Branson et al., 2019). The software automatically removed instrument artefacts (despiking), subtracted background, normalized raw intensities to the internal standard (^{43}Ca) and computed element/Ca ratios in mol/mol for samples using two-point calibration curves based on NIST612 and NIST610 glasses analysed during the run. NIST standards were analysed every three to four otoliths to monitor for instrumental drift. To determine the river of origin of adult sea trout, the otolith section containing the freshwater stage was determined, based on an increase in Sr/Ca and concurrent decrease in Ba/Ca values, along the otolith microchemistry transect (Macdonald & Crook, 2010).

In addition to trace element analysis, strontium isotope ratios (^{87}Sr/^{86}Sr) were measured at the University of California-Davis Interdisciplinary Center for Plasma Mass Spectrometry, using a solid state Nd:YAG 213-nm laser (Elemental Scientific Lasers, UP213) coupled to a (Nu Ametek) Plasma HR Multiple-Collection Inductively Coupled Plasma Mass Spectrometer (LA-MC-ICP-MS), following established protocols (Willmes et al., 2021). Briefly, all otoliths were ablated from the edge of the core until the ventral edge (juveniles) or presumed first freshwater exit (adults), using a beam diameter of 40 μm, moving 5 μm/s, at 10 Hz frequency and a fluence of 4–6 J/cm^2. Primary data handling was conducted using the *IsoFishR* package for R (Willmes et al., 2018), with which we applied a normalization for mass bias, ^{87}Rb interference correction and on-peak subtraction for ^{86}Kr. Outliers were removed based on a 20-point moving interquartile range (IQR) criterion. Accuracy and reproducibility of the LA-MC-ICP-MS were evaluated using an otolith isotopic reference material from a white seabass (*Atractoscion nobilis*) collected offshore of Baja California, which yielded a mean ^{87}Sr/^{86}Sr value of 0.70912 ± 0.00013 ($n = 72 \pm 2\sigma$) in good agreement with the global average ^{87}Sr/^{86}Sr value of modern seawater of 0.70918 (Veizer et al., 1999). For all otoliths, element/Ca and ^{87}Sr/^{86}Sr ratios were averaged over the freshwater phase of each individual.

2.4 | Genetic marker analyses

A subsample of juveniles chosen for otolith microchemistry analysis was selected for genetic analysis (Table 1) to investigate genetic structuring among the rivers in Mariager fjord (Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk) and in comparison with the adjacent rivers outside of the fjord (Lindenborg, Lilleaa and Hevring; Figure 1). To further examine the temporal stability of genotype data, we extracted genotype data from a previous study (Bekkevold et al., 2020) for 34 adult sea trout sampled from Villestrup in 2011, which corresponds to approximately three generations prior to those sampled here. Temporal data were not available for the other three rivers within Mariager fjord.

Genetic analyses of juvenile trout sampled in this study were based on the analysis of 288 SNP markers selected from a genome-wide SNP panel analysed for trout populations spanning large parts of Northern Europe (Bekkevold et al., 2020). The 288 SNPs were selected to maximize resolution among local Danish trout populations (Bekkevold, D., Knutsen, H., Hemmer-Hansen, J., Sodeland, M., Höjesjö, K., Bleeker, K., Aarestrup, K., Skov, C. and Nielsen, E.E., unpublished data). DNA was extracted from fin clips and genotyping was performed using methods described in Bekkevold et al. (2021).

2.5 | Otolith microchemistry data analysis

A random forest (RF) supervised machine learning algorithm, which has shown to provide high classification accuracy in similar studies (Mercier et al., 2011), was used to determine the origin of adult sea trout caught in rivers Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk based on otolith microchemistry data. Using package *randomForest* for R (Liaw & Wiener, 2002), a RF classifier was built using the juvenile otolith microchemistry data, and was subsequently used to assign spawning adult sea trout, whose origin was unknown, to their river of origin. The juvenile otolith microchemistry data were divided into training (75%) and validation data (25%), where the former was used to build the classifier and the latter to determine the overall classification accuracy (±SD) of the classifier and for each of the rivers. Each tree in the forest was constructed using a bootstrap aggregated dataset where at each node, three random variables were selected, and in total, 400 trees were constructed. To determine the deviance of the RF classifier, 500 RF classifiers, which used a random subsample of the training dataset, were built and subsequently tested on randomly subsampled validation dataset. All possible elemental combinations were tested using previously described RF classifier training and validation processes, with the elemental combination producing the highest classification accuracy chosen as the final model. The final elemental ratios used in the random forest classifier that produced the highest classification rate were as follows: ^{87}Sr/^{86}Sr, Sr/Ca, Ba/Ca, Mg/Ca and Mn/Ca.

To further determine how elemental ratios differ between rivers and years, element-specific generalized linear models (GLM) were applied on the juvenile otolith microchemistry dataset including the main effects of the variables. Each elemental model consisted of element/Ca or ^{87}Sr/^{86}Sr against river and year. This was followed by pairwise Tukey post hoc comparisons to determine which rivers differed from each other based on element/Ca or ^{87}Sr/^{86}Sr ratios.

Each RF classifier built ($n = 500$) was used to assign adult sea trout to their river of origin (i.e. each individual was assigned

500 times), allowing us to determine the within-individual variance in the assignment to their natal river, as well as the robustness of the results, where adult assignment data were subsequently used. Adult sea trout that were assigned to originate from a river other than the one they were collected in were categorized as strayers. The authors note that the design of this investigation allows to determine straying *from* and *into* a given river, which are both presented in the results section. To investigate whether there were differences in the proportion of strayers (that had migrated into a given river) and natal homers among adults collected in each of the rivers, a chi-squared test was applied to each assignment dataset produced by the RF classifier. To investigate the effect of total length, sex, river of origin and all possible two-way interactions on the likelihood of being a strayer, a Bernoulli-distributed GLM was used on each generated assignment dataset. To determine the importance of the interactions, the original models, including all main effects and interactions, were compared to a model where each of the interaction was removed one at a time. A ΔAIC below −2 was used as threshold for considering an interaction as important.

2.6 | Genetic data analysis

Genetic data were analysed to estimate sample-specific genetic differentiation among rivers in and outside of the fjord. Genotype data from extant neighbouring populations Lindenborg, Lilleaa and Hevring were obtained from Bekkevold et al. (2020). As the inclusion of siblings in analyses can bias genetic estimates (Hansen et al., 1997), functions from the *Demerelate* R package (Kraemer & Gerlach, 2017) were applied to genotype data, and the relatedness between individuals was estimated using the estimator from Wang (2002). Pairs of individuals showing relatedness coefficients of >0.4 were filtered to only include a single individual, with the expectation that this would filter out full siblings (see caveats in Wang, 2014). Pairwise estimates of genetic differentiation (Weir & Cockerham's F_{ST} estimator) were generated for all collections with the *genepop* R package (Rousset, 2008) and exact tests were used to test for statistical significance. River-specific collections in 2020 and 2021 were initially analysed individually, but pooled in subsequent analyses when exact tests indicated a lack of differentiation between the years. Pairwise p-values were corrected for multiple comparisons with *false discovery rate* (FDR) (Benjamini & Hochberg, 1995).

3 | RESULTS

3.1 | Determining a baseline otolith microchemistry fingerprint for each of the rivers

In total, 193 juvenile brown trout otoliths (Table 1), collected over 2 years, were analysed to obtain river-specific otolith microchemistry fingerprints. The average classification accuracy from the RF classifier based on juvenile otolith fingerprints was 80.4 ± 5.4% (Kappa coefficient of 72.4%), with the highest classification accuracies in rivers Valsgaard (94.6%) and Villestrup (87.8%), which are both located on the northern side of the fjord (Table 2). Most notably, 21.4% of individuals from river Kastbjerg were misclassified to originate from river Villestrup, while misclassification among the other rivers was significantly lower, between 0 and 10.0% (Table 2). The most important elemental ratios used to discriminate between the rivers were $^{87}Sr/^{86}Sr$ and Sr/Ca, with Mn/Ca, Ba/Ca and Mg/Ca contributing less.

The element/Ca and $^{87}Sr/^{86}Sr$ ratios included in the random forest classification tree were further analysed to investigate potential differences between rivers and years (Table 3). $^{87}Sr/^{86}Sr$ (Figure 2) and Sr/Ca differed significantly among rivers and years, Ba/Ca and Mn/Ca differed significantly among rivers and Mg/Ca differed only between years (Table 3). $^{87}Sr/^{86}Sr$ and Sr/Ca, which contributed the most to assigning individuals to their river of origin, differed in all pairwise comparisons, apart from Sr/Ca between rivers Villestrup and Kastbjerg (Figure 3). Ba/Ca differed among all the rivers except for Villestrup and Maren Møllebæk; Mn/Ca differed only among Kastbjerg and Maren Møllebæk, and Kastbjerg and Villestrup.

3.2 | Origin of adult sea trout and potential factors connected to patterns of straying

In total, 90 adult sea trout were collected from rivers Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk, and assigned to their river of origin using the RF classifier (Table 4). On average, 43 ± 2% ($n = 39 \pm 2$) of individuals were assigned to originate from a river other than the one they were collected in during spawning; meaning, they were estimated to have strayed (Table 5). There were no differences between the ratio of individuals that had strayed into a given river and natal homers in each of the rivers (Figure S1), with the average proportion of strayers ranging between 36% and 55% (Table 4).

TABLE 2 Cross-validation matrix of the random forest models indicating the level of incorrect classification ±SD (%), with exception of bold entries in diagonal, which represent the correct classification of juvenile brown trout to their natal river.

	RF-classified river of origin			
Known river of origin	**Villestrup**	**Kastbjerg**	**Valsgaard**	**Maren Møllebæk**
Villestrup	87.8 ± 7.7	10.0 ± 7.2	0	2.2 ± 3.2
Kastbjerg	21.4 ± 10.9	65.6 ± 12.9	7.2 ± 6.5	5.7 ± 6.2
Valsgaard	0	3.5 ± 7.6	94.6 ± 8.9	1.9 ± 4.9
Maren Møllebæk	9.0 ± 10.3	6.7 ± 9.1	9.7 ± 9.9	74.7 ± 15.2

TABLE 3 Result from the GLM analysis investigating differences in elemental ratios included in the RF classifier between rivers and years.

	Effect of river			Effect of year		
Element	df	*F*-statistic	*p*-value	df	*F*-statistic	*p*-value
$^{87}Sr/^{86}Sr$	3	225.9	**<.001**	1	7.4	**.007**
Sr/Ca	3	106.3	**<.001**	1	7.3	**.007**
Ba/Ca[a]	3	12.3	**<.001**	1	0.1	.7
Mg/Ca[a]	3	2.2	.09	1	5.3	**.02**
Mn/Ca[a]	3	5.8	**<.001**	1	0.8	.3

[a]These elements were log-transformed prior to the analysis to meet model assumption of normality. [b] Significant effects (p<.05) are indicated in bold.

Significant effects (p<.05) are indicated in bold.

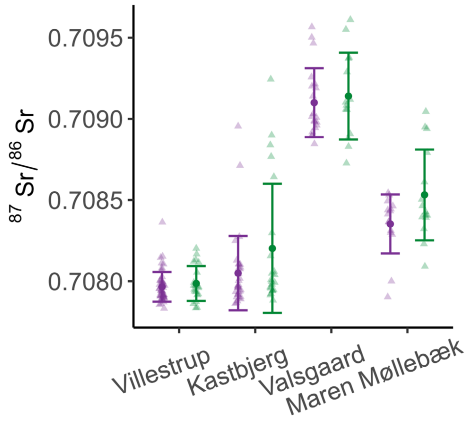

FIGURE 2 $^{87}Sr/^{86}Sr$ ratios measured from juvenile brown trout otoliths collected from different rivers flowing into Mariager fjord, in 2020 (purple) and 2021 (green). Points represent mean values, whiskers standard deviation and triangles measured $^{87}Sr/^{86}Sr$ values.

Proportionally, a larger number of adult sea trout had strayed into the larger rivers, Villestrup and Kastbjerg, compared to Valsgaard and Maren Møllebæk (Figure 4).

Several factors were linked to individual's likelihood to stray. There was a strong indication that length of adult sea trout upon return to freshwater was linked to individual likelihood of straying, although the patterns varied depending on the river of origin. In all assignment datasets, excluding the interaction between individual length and river of origin produced models where the ΔAIC was substantially lower than −2, which is a strong indication for the importance of the interaction between the aforementioned variables (Figure S2). The likelihood of straying decreased with individual length among individuals originating from river Villestrup, but increased with individual length among individuals originating from Kastbjerg and Valsgaard (Figure 5). Maren Møllebæk was excluded from the analysis due to the low number of individuals assigned as strayers originating from the river (Table 5). There was also some indication for the interaction between sex and length being linked to likelihood of straying, since 43% of the assignment datasets produced a ΔAIC lower than −2. However, this relationship may, at least partly, be driven by the misclassification of individuals originating from river Villestrup to river Kastbjerg (Figure S3). Further, even among RF assignment datasets, where the interaction between sex and length was considered to be important, the wide overlapping confidence intervals suggest at most inconclusive results (Figure S3). The interaction of sex and river of origin was not linked to individual likelihood of straying (Figure S2).

3.3 | Genetic estimates of differentiation

Tests for related individuals within the samples identified two pairs of fish from Maren Møllebæk with relatedness >0.4, resulting in the removal of one of the individuals from each of the pairs (in total $n=2$) from further analyses. No other collection showed evidence of closely related individuals. Based on tests for population differentiation, all Mariager Fjord collections showed statistically significant differentiation from all neighbouring populations (Hevring, Lilleaa, Lindenborg), and no differentiation among collections within Mariager Fjord (Table 6). This is indicative of prevalent gene flow among rivers within Mariager Fjord, and restricted exchange between Mariager Fjord and neighbouring rivers outside of the fjord. Comparing samples from Villestrup collected approximately 10 years apart also showed a lack of genetic differentiation, although a single within-fjord comparison (rivers Maren-Møllebæk and Villestrup in 2011) showed weak, but statistically significant, differentiation at $p<.05$ (Table 6).

4 | DISCUSSION

The movement of individuals between populations can have varying levels of impact to population demography and recruitment, and consequently population sustainability (Bett et al., 2017; Bowler & Benton, 2005). In this study, an interdisciplinary approach of combining otolith microchemistry with genetics was used to estimate the degree of straying and gene flow among sea trout originating from four rivers flowing into the same fjord system. Otolith microchemistry gives the opportunity to determine the patterns of connectivity over the course of an individual's life, while genetics can document these patterns over evolutionary time.

To determine the origin of adult mature sea trout captured in freshwater during spawning season, river-specific baseline otolith

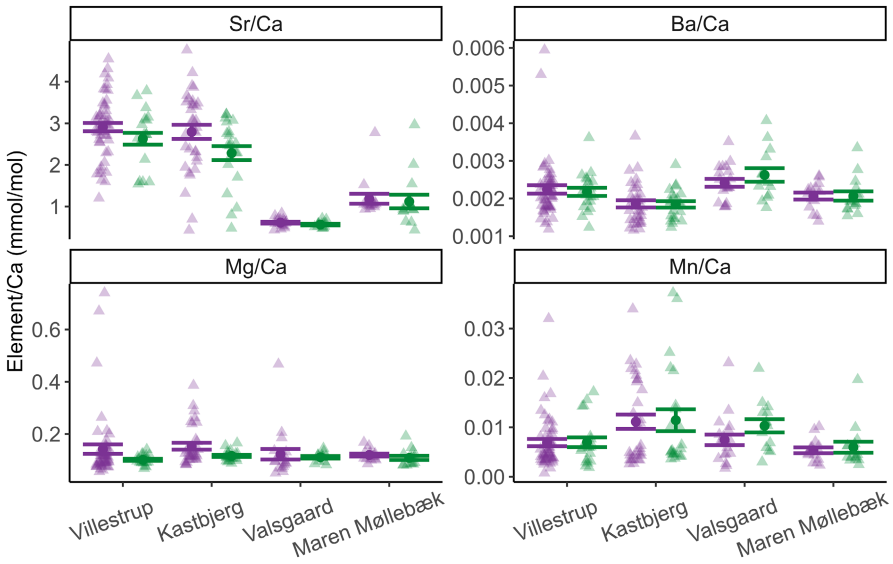

FIGURE 3 Element/Ca ratios measured from juvenile brown trout otoliths collected from different rivers flowing into Mariager fjord, in 2020 (purple) and 2021 (green). Points represent mean values, whiskers standard deviation and triangles measured element/Ca values.

TABLE 4 Number of adult sea trout sampled in each of the rivers (grouped by sex), their length (min–max) and the number of individuals assigned to have strayed from/into a given river.

River	Number of fish sampled (male/female)	Average length in cm (min–max)	Number of sea trout assigned as strayers ± SD (%; male/female)[a]	Total number of sea trout ±SD (%) assigned to have originated from a given river[b]	Total number of strayers ±SD (%) assigned to have originated from a given river[c]
Villestrup	22 (13/9)	59 (51–72)	8 ± 1 (36%; 4/4)	31 ± 3 (34%)	17 ± 2 (44%)
Kastbjerg	28 (15/13)	49 (32–64)	11 ± 2 (39%; 4/7)	30 ± 3 (33%)	13 ± 3 (33%)
Valsgaard	20 (10/10)	38 (26–51)	9 ± 1 (45%; 5/4)	19 ± 1 (21%)	8 ± 0 (20%)
Maren Møllebæk	20 (5/15)	45 (28–61)	11 ± 1 (55%; 3/8)	10 ± 2 (11%)	1 ± 1 (3%)
Total	90 (43/47)	48 (26–72)	39 ± 2 (43%; 16/23)	90	39

[a]Percentage is calculated based on the number of fish collected in the given river.
[b]Percentage is calculated based on the total number of adults sampled.
[c]Percentage is calculated based on the total number of strayer.

TABLE 5 Average number ± SD of mature adult sea trout based on their assigned river of origin (rows) and the river of destination (columns), along with the total number of individuals assigned to either group.

	River of destination				
River of origin	Villestrup	Kastbjerg	Valsgaard	Maren Møllebæk	Total
Villestrup	13.8 ± 1	10 .0 ± 2	3.8 ± 1	3.6 ± 1	31
Kastbjerg	7.0 ± 1	17.0 ± 2	4.6 ± 1	1.6 ± 1	30
Valsgaard	1.0 ± 0	1.0 ± 0	11.2 ± 1	5.8 ± 0	19
Maren Møllebæk	0.1 ± 0	0	0.5 ± 1	9.0 ± 1	10
Total	22	28	20	20	

microchemistry fingerprints were determined for each river in Mariager fjord using juvenile brown trout otoliths. Juvenile brown trout otolith elemental ratios were used to build a Random Forest classifier, which was subsequently used to assign spawning adult sea trout to their river of origin. The classification accuracy of the Random Forest classifier, which included $^{87}Sr/^{86}Sr$, Sr/Ca, Ba/Ca, Mg/Ca and Mn/Ca, was 80%, which is a high accuracy for classifying individuals to their river of origin, resembling results from previous studies. For example, in otolith microchemistry studies among Pacific salmonids, a classification accuracy between 89 and 100% (Maguffee et al., 2019; Turcotte & Shrimpton, 2020) has been documented, while studies on brown trout have reported accuracies between 73% and 93% (Matetski et al., 2022; Mikheev et al., 2021). Our study therefore provides further evidence that otolith microchemistry can be used to differentiate juvenile trout originating from different rivers. Remarkably, the differences in otolith microchemistry between juvenile trout originating from different rivers in Mariager fjord were detectable over small spatial

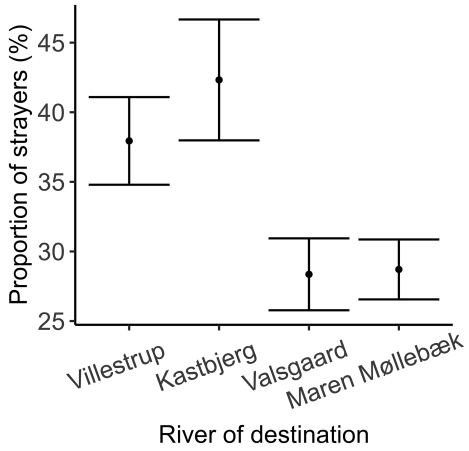

FIGURE 4 Estimated proportion of strayers (%) based on each of the assignment datasets ($n = 500$) depending on whether strayers migrated into rivers Villestrup, Kastbjerg, Valsgaard and Maren Møllebæk, independent where they had originated from. The point and the whiskers represent the mean and the standard deviation respectively.

scales, as the distance between the mouths of the rivers in this system ranged between 4 and 16 km.

The classification accuracy for assigning juvenile trout to their natal river was not uniform across rivers, as it varied between 66% and 95%. Individuals originating from the rivers situated on northern side of the fjord, Villestrup and Valsgaard, had a higher likelihood of being correctly classified to their natal river than individuals from rivers on the southern side, Kastbjerg and Maren Møllebæk. While overall high classification accuracy has been reported for different systems (Mikheev et al., 2021; Turcotte & Shrimpton, 2020), it has also been documented to be accompanied with high variability in classification accuracy for nearby rivers (Matetski et al., 2022). Surprisingly, individuals from the river Kastbjerg, which had the lowest classification accuracy, were most often misclassified to originate from river Villestrup, which is located on the opposite side of the fjord, indicating that geographical proximity is not the only factor affecting classification accuracy. While local bedrock is considered an important factor affecting the elemental composition of otoliths (Campana, 1999; Goldstein & Jacobsen, 1987), especially

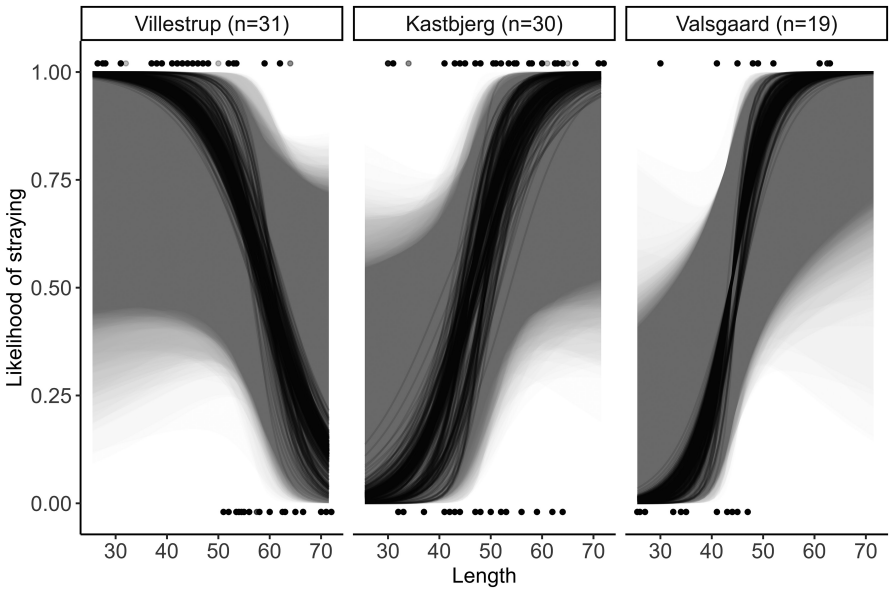

FIGURE 5 Modelled output of the likelihood of straying for spawning sea trout against length at capture, divided by river of origin. Individual likelihood of straying was dependent of length, with the patterns differing between the rivers. Maren Møllebæk was excluded from the analysis due to a low sample size. The lines and the shaded area represent the mean and 95% confidence intervals for each of the models. Model is conditional on sex = female. Points represent individual sea trout from each of the assignment datasets depending on whether they were assigned as strayers ('1') or natal homers ('0').

TABLE 6 Pairwise F_{ST} estimates (below diagonal) and FDR corrected p-values for differentiation among collections (above diagonal).

	Kastbjerg	Maren-Møllebæk	Valsgaard	Villestrup	Villestrup 2011	Hevring	Lilleaa	Lindenborg
Kastbjerg		.996	.996	.996	.996	<.0001	**<.0001**	**.0187**
Maren-Møllebæk	.004		.996	.583	**.017**	**<.0001**	**<.0001****	**<.0001**
Valsgaard	.001	.003		.996	.996	**<.0001**	**<.0001**	**.0072**
Villestrup	.001	.006	.001		.916	**<.0001**	**<.0001**	**.0001**
Villestrup 2011	.002	.009	.003	.002		<.0001	**<.0001**	**<.0001**
Hevring	.032	.036	.036	.028	.030		**<.0001**	**<.0001**
Lilleaa	.015	.021	.015	.014	.016	.042		**<.0001**
Lindenborg	.006	.016	.012	.012	.012	.032	.016	

Significant effects (p<.05) are indicated in bold.

concerning $^{87}Sr/^{86}Sr$ and Sr/Ca (Barnett-Johnson et al., 2010; Brown & Severin, 2009), other factors, for example presence of glaciated sediments (Frei & Frei, 2011) and fertilizers (Zieliński et al., 2016), which can affect $^{87}Sr/^{86}Sr$ and Sr/Ca values, may also have an effect in this system.

No single element had the ability to discriminate among all the rivers, supporting the importance of a multi-elemental approach. Among all the elements included in the classifier, $^{87}Sr/^{86}Sr$ and Sr/Ca contributed the most to classification accuracy, while Ba/Ca, Mn/Ca and Mg/Ca contributed less. The importance of $^{87}Sr/^{86}Sr$ and Sr/Ca has been documented in similar studies among different taxa originating from various systems (Heidemann et al., 2012; Matetski et al., 2022; Mikheev et al., 2021). During the analytical process, additional elements were measured from the otoliths (Pb and Zn), but did not improve, and in some instances negatively affected, the ability of the Random Forest classifier to assign individuals to their natal river, so they were excluded from the analysis.

4.1 | Assigning spawning adult sea trout to their natal rivers

Otolith microchemistry analysis estimated that 43% ± 2 of the adult sea trout that had returned to freshwater originated from another river than the one they were collected in, meaning they had strayed. This is a high estimate of straying in wild sea trout, as rates of straying between 1.6% and 35% has been previously documented in other systems (Jonsson & Jonsson, 2014; Masson et al., 2018). While, there is a level of uncertainty associated with the estimated proportion of strayers in this study due to possible misclassification of individuals, these results coincide with the findings from previous studies in this system, which documented straying rates between 12 and 55% using PIT telemetry (Källo, Baktoft, Kristensen, et al., 2022; Källo, Birnie-Gauvin, Baktoft, et al., 2023).

All the strayers in this study were collected in freshwater during the spawning season and were determined to be mature, indicating that these individuals had likely returned to the given river with the aim to spawn. However, some of them may have also returned to their natal river, or another non-natal river, prior to being captured in the non-natal river, as has previously been documented (Källo, Baktoft, Kristensen, et al., 2022; Källo, Birnie-Gauvin, Baktoft, et al., 2023). Unfortunately, such fine-scale movements using otolith microchemistry were not investigated in this study, as only the otolith sections corresponding to the juvenile stage in freshwater were analysed. It remains, however, unclear whether such patterns would be detectable if the entire cross section of the otolith would be analysed, as extended time in freshwater is required for the elements to be embedded in the otolith.

The proportion of strayers was high in all of the rivers (between 36% and 55%), with no differences in the proportion of strayers (that had migrated into a given river) and natal homers among the rivers. This indicates that strayers make up a significant proportion of the spawning contingent in all of the rivers in Mariager fjord. There was also some indication that straying was more prominent towards the larger rivers Villestrup and Kastbjerg, which has also been documented previously in this system (Källo, Birnie-Gauvin, Baktoft, et al., 2023). While similar patterns of straying towards larger rivers have also been reported in other systems (Degerman et al., 2012; Unwin & Quinn, 1993), straying seems to occur predominantly towards rivers close to the natal river (Berg & Berg, 1987; Jonsson et al., 2003), disparate from the results of this study. However, as all the rivers in Mariager fjord are within the distance over which straying has been documented to commonly occur (60–80 km; Bekkevold et al., 2020; Jonsson et al., 2003), it may indicate that in close proximity to the natal river, other factors, such as river size and individual characteristics (e.g. total length), affect patterns of straying.

The likelihood of straying was linked to the length of adult sea trout upon return to freshwater, but patterns differed between rivers of origin. Longer individuals originating from Valsgaard and Kastbjerg were more likely to stray, while shorter individuals were more likely to stray if they originated from Villestrup. Nothing can be said about individuals originating from Maren Møllebæk due to a low number of strayers originating from the river being captured. While it is unclear which factors affect the documented patterns of straying, several hypotheses can be formulated, with the causes possibly differing among rivers. For example, in river Villestrup, where the spawning population is large (Birnie-Gauvin et al., 2018; Källo, Birnie-Gauvin, Jepsen, et al., 2023) and competition for mates and adequate spawning habitat is likely high, it may be more beneficial for smaller fish originating from Villestrup to stray to non-natal rivers, where they may have a better chance of spawning successfully. In contrast, for individuals originating from river Valsgaard, the decreased likelihood for larger individuals to return to their natal river may suggest that the size of the river limits the inclination for larger sea trout to return. River Valsgaard is a shallow river with relatively low flow (Källo, Birnie-Gauvin, Baktoft, et al., 2023), which may make it difficult for larger sea trout to enter it. However, this hypothesis likely does not apply to individuals originating from river Kastbjerg, where a similar pattern of larger individuals straying to a higher degree was documented, given that in size, it is more similar to river Villestrup than Valsgaard. Thus, it is unlikely that the size of the river is the factor affecting the documented pattern, with further research required to determine the true mechanisms behind these patterns.

We further investigated whether straying in this system was sex-biased, as has been previously documented in salmonids (Hamann & Kennedy, 2012; Turcotte & Shrimpton, 2020). While there was some evidence for the sex of the individual to have an effect on straying likelihood, possible bias stemming from misclassification and wide confidence intervals leave little to no evidence to support the notion that straying in this system is sex-biased. This is somewhat surprising, as male salmonids have often been documented to stray more (Hamann & Kennedy, 2012; Turcotte & Shrimpton, 2020), with differences in reproductive strategies suggested as a driver for this pattern (Hard & Heard, 1999), however not always (Unwin & Quinn, 1993). Despite the indication that males

and females stray proportionally to a similar extent in this system, it is likely that per capita there are more female strayers, as anadromy is more prevalent among female sea trout (Ferguson et al., 2019; Klemetsen et al., 2003). Having more female strayers would imply greater impacts of straying on population dynamics and recruitment in this system.

4.2 | Genetic structure of sea trout in Mariager fjord

The results of the genetic analysis indicate that individuals originating from different rivers in Mariager fjord likely make up a single breeding population and that this is consistent over generations. More specifically, genetic estimates of population differentiation were indistinguishable from zero, also at a decadal time scale within river, which is suggestive of gene flow among rivers within Mariager fjord. This further indicates that strayers migrating to non-natal rivers within the fjord are able to successfully spawn there. However, the identification and reproductive success of strayers cannot be determined with the applied genetic method, as straying of only a low number of individuals per generation will eradicate genetic signals of demographic structure (Waples & Gaggiotti, 2006). Alternatively, straying success could be evaluated using genetic tagging approaches (e.g. Beacham et al., 2021). In contrast, gene flow (successful reproductive straying) in Mariager fjord seems to be mainly confined to the rivers within the fjord, as shown by the clear genetic differentiation between collections from Mariager Fjord and those from the neighbouring rivers (river entrances separated by ca. 10–45 km). This finding supports the notion that (reproductively successful) straying is generally more prominent within local areas, and decreases in prevalence the further apart rivers are located. However, as mentioned above, the rivers investigated in this study (both inside and outside of the fjord) are within the distance over which straying has previously been documented (Bekkevold et al., 2020; Jonsson et al., 2003), perhaps indicating that the fjord acts as a barrier between the two areas, limiting gene flow. The latter may be partly also supported by the findings of del Villar-Guerra et al. (2014), who documented that a significant proportion of sea trout never leave Mariager fjord.

5 | CONCLUSION

In conclusion, otolith microchemistry combined with genetics provides an important tool for investigating population connectivity across the life of individuals and over evolutionary time. In this system, both methods indicated there was a substantial exchange of individuals and genetic material between the rivers. Further, this study contributes to the growing body of evidence that otolith microchemistry can differentiate between populations originating from different rivers, even at relatively small spatial scales. By comparing adult sea trout otolith fingerprints to juvenile river-specific otolith fingerprints, we were able to assign adult sea trout to their river of origin, and additionally confirm that strayers make up a significant proportion of the spawning population in all the rivers within the Mariager fjord system. We further identified individual length and river of origin as important factors affecting straying. A lack of genetic structuring between the rivers in Mariager fjord confirms that the strayers in this system are able to successfully spawn in non-natal rivers and that all the rivers make up a single population rather than separate, genetically distinct populations.

AUTHOR CONTRIBUTIONS

KK and KA contributed to the conception and the design of the study. KK carried out the fieldwork. KK, PG, CL and GB carried out the laboratory analysis. KK, HB and DB analysed the data. All authors contributed to the interpretations of the results and have given their final approval for the manuscript to be published.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be available from the authors upon reasonable request.

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