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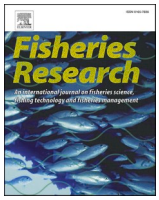

Full length article

Uncovering neutral and adaptive genomic differentiation among European perch with brackish water and freshwater origin in the western Baltic Sea region

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ABSTRACT

Environmental variation across the range of wild species can lead to local adaptations. The Baltic Sea was formed when the Fenno-Scandian ice sheet retreated around 12 thousand years ago, creating a new brackish water habitat colonised by both marine and freshwater fish species. The European perch (*Perca fluviatilis*) is a predatory freshwater fish with a large geographical distribution across Eurasia, where it inhabits a wide range of environmental niches. In the Baltic Sea region it has even developed a specialised brackish water perch variant that can tolerate environmental salinity levels, which are lethal to freshwater perch. However, very little is known about the underlying mechanisms facilitating the colonisation and adaptation of perch to the Baltic Sea. Here, we use Genotyping-By-Sequencing data from six freshwater and six brackish water localities to disclose the evolutionary relationship between freshwater and brackish water perch. Our results show that the brackish water perch occurs in multiple distinct genetic clusters. We find that gene flow between brackish water perch with full access to the sea likely led to lower levels of differentiation and higher diversity than in freshwater perch. Selection analyses suggest that genomic adaptation played a role in the colonisation of the Baltic Sea and that the top three regions under selection harbour salinity tolerance genes. We complete by discussing the implications of our findings for management of brackish water perch in the western Baltic sea.

1. Introduction

Environmental heterogeneity plays a key role in shaping intraspecific variation in wild species (McDonald and Ayala, 1974). Novel habitats can offer new opportunities and challenges to populations in a subsection of the species distribution range, thus promoting local adaptation and increased levels of differentiation (Langerhans et al., 2013). Hence, intraspecific variation in morphology, behaviour and physiological capabilities is often correlated with environmental variation and can over time lead to the formation of separate ecotypes (Foote et al., 2016; Skovrind et al., 2016). While phenotypic plasticity can be a vector towards adaptation (Radersma et al., 2020), genomic diversification is often the key mechanism governing the formation of ecotypes (Feder et al., 2012; Foote et al., 2016; Seehausen et al., 2014). When populations inhabit separate niches and no longer interbreed, genetic drift and/or selection will affect them separately and increase the differentiation between them across the genome and in particular in genes underpinning local adaptation.

Almost all teleost species have internal osmotic pressures corresponding to salinities of 9–12 parts per thousand (ppt) (Brett, 1979), but

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have evolved to live in the stable salinity environment of either freshwater (around 0 ppt) or marine habitats (around 35 ppt). Even subtle variations in internal osmotic pressure can compromise their physiological well-being and be lethal (Brauner et al., 1992; Christensen et al., 2019; Lutz, 1972). To counterbalance adverse effects of alternated internal salinity, fish osmoregulate (Larsen et al., 2014). Although fish are the most diverse vertebrate taxa in the world (Magurran et al., 2011), relatively few species are capable of both osmoregulating at ambient salinities below their internal osmotic pressure and ambient salinities above their internal osmotic pressure (Evans, 1984). Hence, teleost fish species diversity is generally low in intermediate and fluctuating salinities found in brackish water estuaries, compared to adjacent fresh and marine habitats (Remane, 1934; Whitfield et al., 2012). However, since the biological productivity of estuaries is high, species or populations that adapt to this challenging environment can benefit from high food availability and low interspecific competition.

The Baltic Sea is the world's largest estuary at 400,000 km^2 and characterised by a marked salinity gradient from nearly freshwater (<0.5 ppt) in the north to fully marine (35 ppt) as it merges with Kattegat and the North Sea(Leppäranta and Myrberg, 2009; Weckström et al., 2017). In the southwestern Baltic Sea, the environmental salinity fluctuates between 7 and 22 ppt making it a challenging habitat requiring both osmoregulation below and above iso-osmotic levels (hyper and hypo-osmoregulation, respectively) (Weckström et al., 2017). In this region, a few species of fish adapted to freshwater (stenohaline freshwater fish) have populations or groups of populations that can tolerate brackish water and utilise the greater resources in the brackish environment, e.g. ide (*Leuciscus idus*) (Skovrind et al., 2016), northern pike (*Esox lucius*) (Jacobsen et al., 2017) and perch (*Perca fluviatilis*) (Christensen et al., 2021; Skovrind et al., 2013).

Perch, also called European perch or Eurasian perch, is a freshwater predatory fish species distributed across the Eurasian continent where it inhabits a wide variety of habitats. Perch form shoals, usually together with equally sized individuals and feed on invertebrates and smaller fish. Given the right conditions, it grows to more than 2 kg and 50 cm. In freshwater, perch has an important ecological role as predator with a significant top-down regulating effect on the ecosystem (Jeppesen et al., 2000; Ljunggren et al., 2010). While the ecological role of perch is not well studied in brackish water, it predates the invasive round goby (*Neogobius melanostomus*) in the Baltic Sea (Liversage et al., 2017). Perch is an important species in the recreational and commercial fisheries, and a substantial part of commercial landings of perch in the Baltic Sea region comes from brackish water stocks. However, landings of perch in the Baltic Sea have declined dramatically in the last few decades, probably owing to overfishing and pollution (Ådjers et al., 2006; Ljunggren et al., 2010). Perch that inhabit brackish water and freshwater differ in a number of ways. Like all stenohaline freshwater fish, perch have over millions of years evolved to ambient salinities lower than their internal osmotic pressure, and keep it stable (homeostasis) by secreting diluted urine and taking up specific ions from the environment (hyper-osmoregulation) (Larsen et al., 2014). However, perch populations inhabiting brackish water can also maintain homeostasis at salinities higher than their internal osmotic pressure (Christensen et al., 2017), likely through gastro-intestinal uptake of imbibed ambient water, and excretion of excess ions (hypo-osmoregulation) (Larsen et al., 2014), something that freshwater perch can not. As a result of the different physiological capabilities, the two variants show different maximum salinity tolerances; ~10 ppt in freshwater perch and ~17.5 ppt in brackish water perch (Christensen et al., 2019). While most perch populations spend all life stages in freshwater, brackish water populations inhabit the brackish water during the warmer months and exhibit homing to their natal streams for spawning in freshwater in the fall. In locations where there are protected bays and spawning substrate (Westerbom et al., 2023), they can also spawn in rivers or stream deltas with salinities up to 9.6 ppt (Skovrind et al., 2013). Egg hatching experiments have suggested that eggs with brackish water origin can hatch up to 12 ppt (Christensen et al., 2016). While the different responses to salinity in brackish water and freshwater perch is documented, the underlying mechanism is yet to be disclosed. Potential mechanisms could be maternal effects or simple acclimatisation where the increased salinity tolerance is induced by the environment itself as it has been suggested for ruffe (Gymnocephalus cernua) (Albert et al., 2006). Alternatively, it could be selection acting on alleles advantageous in the brackish water environment as seen in marine fishes adapting to freshwater (Velotta et al., 2022).

Through the analysis of Genotype-By-Sequencing (GBS) (Elshire et al., 2011) data of 190 individuals from six brackish water and six freshwater perch populations in the southwestern Baltic Sea region (Fig. 1), we provide novel insights into the evolution of brackish water and freshwater perch. Specifically, we infer the genomic population structure of these populations and identify genomic regions under selection in the brackish water perch, which could explain the increased ability to thrive in its unique brackish water habitat.

2. Methods

2.1. Sampling

A total of 190 fin-clip samples were collected between September 2013 and April 2014 at 12 localities (Fig. 1c); six freshwater localities and six brackish water localities (Table 1). The brackish water localities, which all harbour anadromous perch, fell into two categories; (i) perch with full access to the brackish water of the southwest Baltic Sea (NAK-B, KAR-B and ISH-B); and (ii) perch inhabiting a brackish water fjord, but isolated from other brackish water perch populations by high saline marine water (RAN-B, ROS-B and KET-B). The number of samples per population ranged from 10 to 30. All samples were stored in 96–99% ethanol at − 18 °C. The fish were individually numbered and adult individuals were preserved at the National History Museum of Denmark as part of their collections (See Table S1 for ID numbers). A summary of the sample localities are presented in Table 1. Permission for scientific fishing was provided by the Danish Ministry for Food, Agriculture and Fishery (journal no. 2009–02530–23088).

2.2. Data generation

Genomic DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The extracts were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). To check for molecular integrity, an aliquot of each DNA extract was run on a 1% agarose gel against a 1-kb ladder. We sent 190 extracts that passed our filters (minimum DNA concentration of 10 ng/μL and average fragment size above 20 kb) to the Institute for Genomics Diversity at Cornell University, where the GBS method was applied (Elshire et al., 2011). Samples were sent in two 96-well plates, each including a negative control. Once at Cornell, the DNA extracts were treated with the restriction enzyme EcoT22I before library preparation. All libraries had appropriate concentration, fragment size distribution and minimal adapter dimers, thus passing quality control. The libraries of each plate were pooled separately and sequenced on the HiSeq 2000 (Illumina, San Diego, CA) using a single-end 100 bp technology.

2.3. GBS data processing, filtering and mapping

The raw sequencing GBS data was demultiplexed using GBSX v1.3 (Herten et al., 2015) allowing for one mismatch in the barcodes (-mb 1) and one mismatch in the enzyme cut-site (-me 1) while retaining common sequencing adapters (-ca false). Chimeric GBS reads were identified as discussed in Pacheco et al. (2020). In short, these reads occur when reads from two or more biological cut-sites are merged into a single artificial read. Thus, chimeric reads were defined as reads with more

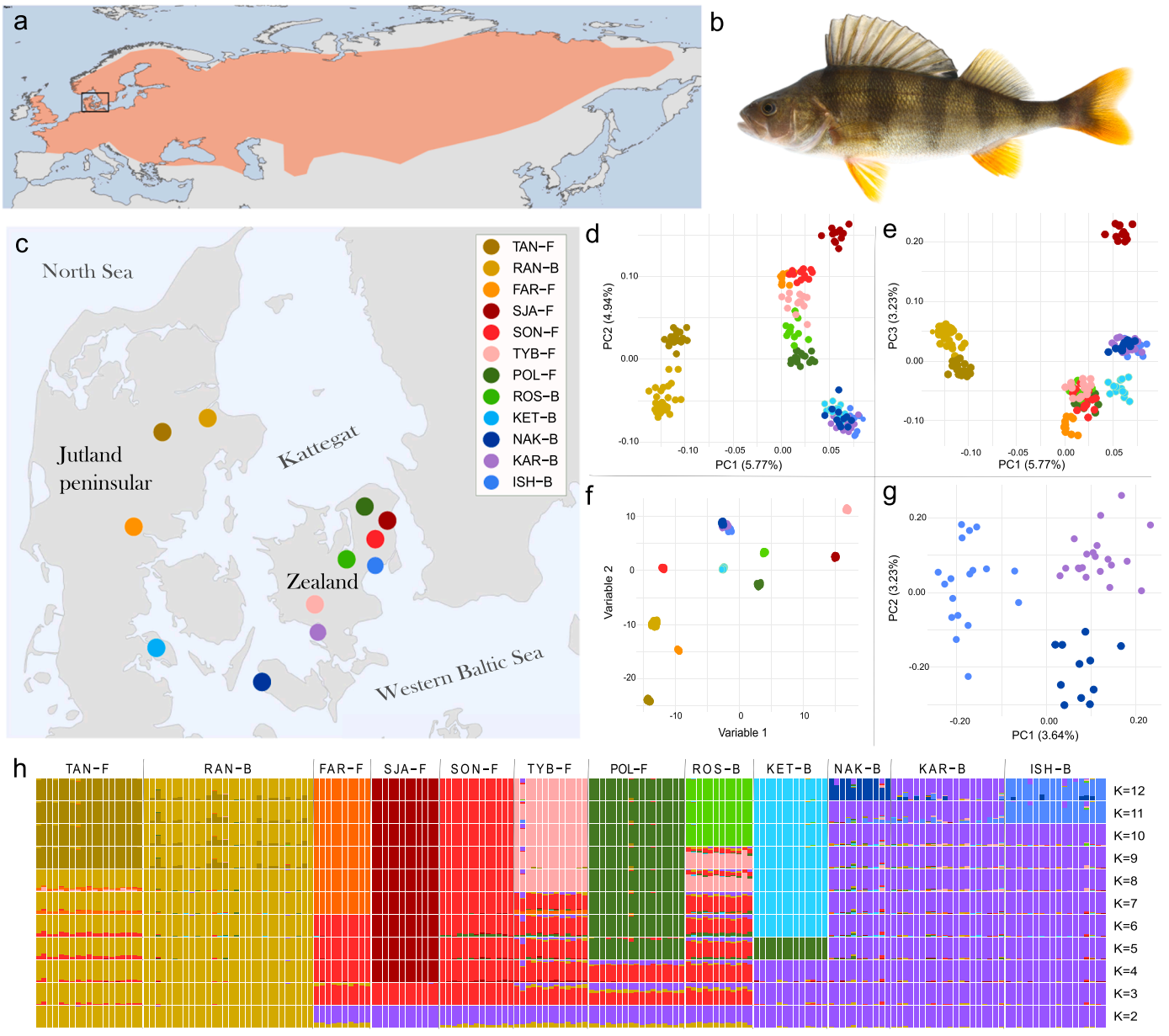

Fig. 1. Population structure of perch in the southwestern Baltic Sea region. **a)** Native distribution of perch *Perca fluviatilis*. **b)** A 24.5 cm European perch from Køge harbour on the east coast of Zealand December 20th 2022 (photo by Henrik Carl). **c)** Brackish water and freshwater sample sites included in the present study of Baltic Sea perch. -B indicates brackish water and -F indicates freshwater localities. **d-g)** Principal Component Analyses. Panels **d** and **e** are the PCAngsd results for PC1-PC2 and PC1-PC3, respectively. Panel **f** is the UMAP analyses, which used the PCAngsd results as input. **g)** Principal Component Analysis of brackish water perch from NAK-B, KAR-B and ISH-B. **h)** Ancestry proportions estimated with NGSadmix. Vertical bars represent single individuals. Different colours indicate the estimated ancestry proportions in each individual.

than one cut-site that mapped to two or more noncontiguous regions in the reference genome. Chimeric reads could bias our coverage statistics, so they were excluded using a script available at: https://github.com/g-pacheco/PerchGenomics/wiki/5.-Filtering-For-Chimeric-Reads.

Processing of demultiplexed sequencing reads and mapping were performed with the Paleomix pipeline 1.2.12 (Schubert et al., 2014). First, Illumina adapter sequences were trimmed from read ends with adapterremoval v2.3.0 (Schubert et al., 2016) applying default settings, except for a minimum read length of 30 bp (–minlength 30) a minimum base quality of 15 (–minquality 15) and a maximum of of 40 Ns per read (–maxns: 40). Second, processed reads were mapped to the GENO_P-fluv_1.0 European perch reference genome (NCBI accession: GCA_010015445.1 (Ozerov et al., 2018) with BWA (Li and Durbin, 2009) applying the mem algorithm, with a minimum mapping quality of 20 (–MinQuality 20), filtering PCR duplicates (–FilterUnmappedReads yes). The mapping was restricted to the 24 nuclear chromosomes in the genome alignment thus excluding additional unplaced scaffolds. Next, within Paleomix, reads that mapped to multiple positions or had mapping quality scores lower than 20 were removed with samtools v1.9 (Li et al., 2009), sequence duplicates were removed using the MarkDuplicates function in Picard v2.18.26 (Broad Institute, 2016), and finally indels were realigned with gatk v3.8.1 (McKenna et al., 2010). The Paleomix.yaml files can be found in the GitHub page (https://github.com/g-pacheco/PerchGenomics).

Analyses were further restricted to the fraction of the genome theoretically available to the GBS method. To determine this fraction, we performed an in-silico digestion on the reference genome assembly with the same enzyme used in the GBS protocol (EcoT22I) using BioSeq

Table 1
European perch sample localities.

Abbreviation	Locality name	Sample size	Environment	Latitude	longitude	Characteristics
TAN-F	Tange Sø	19	Fresh water	56.32858	9.58544	Artificial lake established in the early 20th century. Surface are: 5.4 km^2. Mean depth: 3 m. Drains east into Randers Fjord (RAN-B).
RAN-B	Randers Fjord	30	Brackish	56.47218	10.20451	Fjord downstream of Gudenåen river, Connected to Tange Sø (TAN-F). Perch population isolated by high salinities.
FAR-F	Fårup Sø	10	Fresh water	55.73158	9.39451	Natural lake. Surface area: 1.0 km^2. Mean depth: 5.6 m. Drains east into Vejle Fjord.
SJA-F	Sjælsø	12	Fresh water	55.86633	12.43903	Natural lake. Surface area: 2.9 km^2. Mean depth: 2.7 m. Drains east into Øresund.
SON-F	Sønder Sø	13	Fresh water	55.77652	12.35170	Natural lake. Surface area: 1.2 km^2. Mean depth: 3.3 m. Drains west into the central part of Roskilde Fjord.
TYB-F	Tystrup-Bavelse Sø	13	Fresh water	55.33723	11.62058	Set of natural lakes connected by a 50-meter stream. Surface area: 7.7 km^2. Mean depth: 8.2 m. Drains into Karrebæk Fjord (KAR-B)
POL-F	Pøle Å	18	Fresh water	55.99437	12.22543	Stream. Drains west into Arresø, which was a fjord until the middle of the 18th century.
ROS-B	Roskilde Fjord	12	Brackish	55.67265	12.01448	Fjord. Connected to Kattegat. Perch population inhabits only the southern part. Perch population isolated by high salinities.
KET-B	kettinge Nor	13	Brackish	54.97014	9.86534	Fjord. Connected to southwestern Baltic Sea. Perch population isolated by high salinities.
NAK-B	Nakskov Inderfjord	12	Brackish	54.82582	11.14329	Fjord. Connected to southwestern Baltic Sea in a region where salinities are within the tolerance of Brackish water perch.
KAR-B	Karrebæk Fjord	20	Brackish	55.20862	11.71302	Fjord. Connected to southwestern Baltic Sea in a region with salinities within the tolerance of Brackish water perch
ISH-B	Ishøj Havn	18	Brackish	55.60949	12.38105	Stream delta/artificial lagoon. Connected to southwestern Baltic Sea in a region where salinities are within the tolerance of Brackish water perch.

v/1.11 (Cock et al., 2009), and considered only the regions spanning 93 bp downstream and upstream each locus. Loci located less than 93 bp apart from each other, were merged into single-locus loci. The scripts used to achieve this can be found at the GitHub page (https://github.com/g-pacheco/PerchGenomics/wiki/4.-Creation-of-Mapping-Targets).

Samples were excluded if they had low amounts of data. To identify these samples, we created a presence/absence matrix for all loci, where loci covered by a minimum of three reads were scored as present and loci covered by fewer than three reads were scored as absence. Due to the magnitude of the matrix, we clustered the loci (k-means with K = 300 clusters). The resulting matrix was plotted as a heatmap with the samples hierarchically clustered by employing the R package pheatmap (Kolde, 2012). This heatmap (Fig. S1) was visually inspected, and samples that clustered with the negative controls were excluded from further analyses.

The data was further processed and filtered using ANGSD v0.935 (Korneliussen et al., 2014). To remove poor quality data, we removed anomalous reads (-remove_bads 1; SAM flag above 255), adjusted mapping quality for excessive mismatches (-C 50), removed reads with multiple best hits (-uniqueOnly 1) and performed BAQ computation (-baq 1). We also ignored bases with base qualities below 20 (-minQ 20), and removed reads with mapping qualities below 30 (-minMapQ 30). We excluded sites with more than five percent missing data across all samples (-minInd $((SampleNumber*95/100))). Sites with less than three reads per individual were called as missing data (-geno_minDepth 3). We calculated genotype likelihoods using the SAMtools approach (-GL 1) (Li et al., 2009), considered sites with a minimum genotype posterior probability of 95% (-postCutoff 0.95), and sites were only identified as variable if the minor allele was significantly different from zero with a p-value below 1e-6 (-SNP_pval 1e-6), according to a likelihood ratio test, using a chi-square distribution with one degree of freedom. To identify sites with excess coverage which we intended to remove as they could belong to paralogous regions, we used ANGSD to calculate the sequencing depth and created a density plot using ggplot2 (Wickham, 2016). After visually inspecting the global depth plot (Fig. S2), we set the maximum global depth at 190 times the number of individuals (-setMaxDepth $((SampleNumber*190))). Unless otherwise mentioned all subsequent analyses were performed using these filters.

2.4. Population genetic structure

The population genetic clustering of samples was estimated with PCAngsd (Meisner and Albrechtsen, 2018) under default mode and applying a minimum minor allele frequency of 0.006 (-minMaf 0.006), corresponding to a minimum of three copies of the minor allele in the dataset. Traditional principal component (PC) plots were made of PC1 against PC2 and PC1 against PC3. However, the covariance matrix generated by PCAngsd included 'n' number of dimensions (in our case 188). In order to evaluate the information stored in all these dimensions, we applied the dimensions reduction software UMAP, with default settings (Becht et al., 2018), which reduced the complexity of our 188-by-188 matrix to two dimensions making it possible to capture and visualise all dimensions in a single plot. To further investigate the relationship between closely related populations, we also performed a PCA analysis only including the brackish water perch from NAK-B, KAR-B and ISH-B, using the settings described above, except the minimum minor allele frequency, which was set to 0.025 (-minMaf 0.025), again corresponding to a minimum of three copies of the minor allele. To further estimate genetic clusters based on individual admixture proportions we used NGSadmix v3.2 (Skotte et al., 2013) for 2–12 ancestral populations (K-values), using default parameters, except for tolerance for convergence which was set to 1e-6 (-tol 1e-6), log likelihood difference in 50 iterations, which was set to 1e-3 (-tolLike50 1e-3) and maximum EM iterations which was set to 10,000 (-maxiter 10000). For each K-value, 100 replicates were applied and the replicate with the highest likelihood was used for subsequent plotting and interpretation.

2.5. Diversity, differentiation, geographic distance and salinity

ANGSD was used to compute the unfolded site allele frequency (SAF) of each population using the closely related North-american sister species, the yellow perch (*Perca flavescens*; accession number: SAMN10722690) as ancestral state. Else, we used the filters described above. The SAF was subsequently used to estimate the Site Frequency Spectrum (SFS) for each population and the two dimensional SFS (2dSFS) for each population pair using realSFS (Mas-Sandoval et al., 2022). The SFS was used to estimate the nucleotide diversity (π) of each population and the 2dSFS was used to estimate the pairwise differentiation (F_{ST}) among populations. To estimate the relation between geographic distance and genomic diversification (Wright, 1943), we plotted the F_{ST} values against the euclidean distance and waterway

distance, measured using Google Earth (available from https://earth.google.com/) for all locality pairs. Correlations were assessed for all localities, as well as brackish water pairs and freshwater pairs separately using a linear model. To assess the effect of salinity on perch genetic variation, we estimated the salinity levels that perch migrating to and from brackish water localities would have to endure. We did this by extracting the "migration salinity", which we defined as the environmental salinity of the sea immediately adjacent to the fjords or estuaries associated with the brackish water localities. All salinities were extracted once daily for the period 01–09–2013 to 31–08–2014 from the models created by MyOcean (accessible at: www.myocean.eu). Mean annual migration salinity was subsequently plotted against nucleotide diversity to estimate the effect of isolation by salinity on genetic diversity.

2.6. Putative regions under selection in brackish water perch

To identify regions of the genome showing signs of selection in the brackish water perch populations, which have increased salinity tolerance (Christensen et al., 2019), we calculated the Population Branch Statistics (PBS) in ANGSD (Korneliussen et al., 2014). PBS is a summary statistic that quantifies the genetic drift in one population, relative to two other populations, across the genome using a sliding window approach. The length of each branch of their corresponding three-population tree is estimated and windows with significantly longer branches indicate positive selection in the corresponding population. To increase the sample size, the PBS analysis was based on core populations belonging to the main genetic clusters that we identified (see results); Cluster A (RAN-B and TAN-F), Cluster B (SON-F and TYB-F), and Cluster C (NAK-B, KAR-B and ISH-B). The SFS was calculated for each of the groups using the same parameters used to calculate the summary statistics, with 50% missing data. The analysis was run with 50 kb overlapping windows in 25 kb steps. The generation time was set to 3.4 years, based on the median generation time of eight perch populations each estimated from the von Bertalanffy growth parameters published by Christensen et al. (2021) using the generation time estimation approach described in (Froese and Binohlan, 2000). Negative PBS values were plotted as zero. The eight populations included five with freshwater and three with brackish water origin; parameter details can be found in Table S2. As mutation rates are unavailable for the *Perca* genus, we applied a per generation mutation rate of $3.7 \times 10–8$ estimated for three-spined sticklebacks (*Gasterosteus aculeatus*) (Liu et al., 2016). To identify genes and their functions in the genomic regions most likely to be under positive selection, we aligned the annotated protein sequences from the reference genome to the NCBI database (Sayers et al., 2023) using blastP (Johnson et al., 2008). We included protein sequences from genes 100 kb either side of each window and used the result to search the Gene Ontology AmiGO2 database (Ashburner et al., 2000; Carbon et al., 2009; The Gene Ontology Consortium, 2021) for the corresponding genes and their molecular functions. Subsequently, literature searches were performed for all gene names in association with descriptive phrases "salinity tolerance", "salt tolerance" and "osmotic stress".

3. Results

3.1. Data summary

Each sample locality included between 10 and 30 samples (mean = 15.8). Locality details can be found in Table 1 and sample details can be found in Table S1. After inspecting the presence/absence heatmap (Fig. S1), the two blanks and two samples (NAK-B_09 and POL-F_15) that clustered with the control samples were excluded from further analyses. The dataset with all samples included 3722 variable sites, with an average distance of 252,478 bp. The dataset with Nak-B, KAR-B and ISH-B included 2829 variable sites with an average distance of 331,250 bp.

3.2. Population genetic structure

The PCA analyses separated perch by geography and salinity. The first principal component PC1 (5.77% variation) separated the two localities in northern Jutland from the remaining localities, while PC2 (4.97% variation) separated the brackish water perch from the freshwater perch (Fig. 1d). PC3 (3.23% variation) separated the KET-B locality from the other brackish water localities in western Baltic Sea (NAK-B, KAR-B and ISH-B) and separated the freshwater locality SJA-F from all other localities (Fig. 1e). When applying the UMAP dimension reduction method to the PCA data, all sample sites formed their own non-overlapping clusters, except for the three brackish water localities in southeastern Zealand (Fig. 1f). The analysis, which only included the three "open" brackish water localities (NAK-B, KAR-B and ISH-B) separated ISH-B from NAK-B, KAR-B on PC1, while NAK-B, KAR-B separated from each other on PC2 (Fig. 1g). Thus, each of these three brackish water localities in southeastern Zealand formed their own genetic clusters at a finer scale.

The individual admixture coefficients estimated for K= 2 to K= 12 supported the PCA and revealed further fine-scale population genetic structure among perch localities in the western Baltic Sea region (Fig. 1h). In the analyses of two ancestral populations (K=2) the two most northern localities on the Jutland peninsula (TAN-F and RAN-B) formed a separate cluster (Cluster A). At K= 3, the remaining populations were separated into two clusters, one (Cluster B), which included the remaining freshwater localities (FAR-F, SJA-F, SON-F, TYB-F, POL-F, and a single brackish water locality ROS-B), and the other (Cluster C), which included the remaining brackish water localities (KET-B, NAK-B, KAR-B and ISH-B). For K= 4 to K= 12, additional genetic structuring was detected with all 12 localities comprising their own genetic cluster at K= 12. Admixture (gene flow) was detected from TAN-F to RAN-B in Jutland and among the three brackish water populations NAK-B, KAR-B and ISH-B in southeastern Zealand.

3.3. Diversity, differentiation, geographic distance and salinity

The highest levels of nucleotide diversity (π) was found in the brackish water localities NAK-B, KAR-B and ISK-B, which had π estimates between 0.000360 and 0.000370 (Fig. 2a). Intermediate levels of diversity was observed in TYB-F and ROS-B with π estimates between 0.000339 and 0.000345 while the remaining localities (TAN-F, RAN-B, FAR-F, SJA-F, SON-F, POL-F and KET-B) had π estimates below 0.000307. The lowest F_{ST} values, which were found among the southeastern brackish water localities NAK-B, KAR-B and ISH-B, all ranged from 0.032 to 0.029 (Fig. 2b). All other F_{ST} values ranged between 0.100 and 0.278 with the highest values being found between FAR-F and KET-B. The IBD analyses revealed that there was very little correlation between genetic diversity (F_{ST}) and Euclidean geographic distance (Km) between localities (Fig. 3c). The weakest correlation was found when including only the freshwater localities (R^2 =0.002), and the strongest correlation was found when including only the brackish water localities (R^2 =0.308). The analysis of all localities revealed intermediate correlation (R^2 =0.108). When using the water way distances the correlations were equally low (R^2 =0.034 for the freshwater localities, R^2 =0.506 for the brackish localities and R^2 =0.132 for all samples). The environmental salinity data showed that perch migrating to and from the brackish water localities NAK-B, KAR-B and ISH-B would most of the year experience migration salinity levels lower than the 18 ppt physiological limit of perch, implying that immigration and emigration at these localities is not limited by salinity (Fig. 3a-b). In contrast, migrants to and from the KET-B and ROS-B localities would experience migration salinities above the physiological limits of 18 ppt approximately half of the days with ROS-B periodically experiencing much higher salinity levels (up to 30 ppt). The perch migrating to and from the RAN-B locality

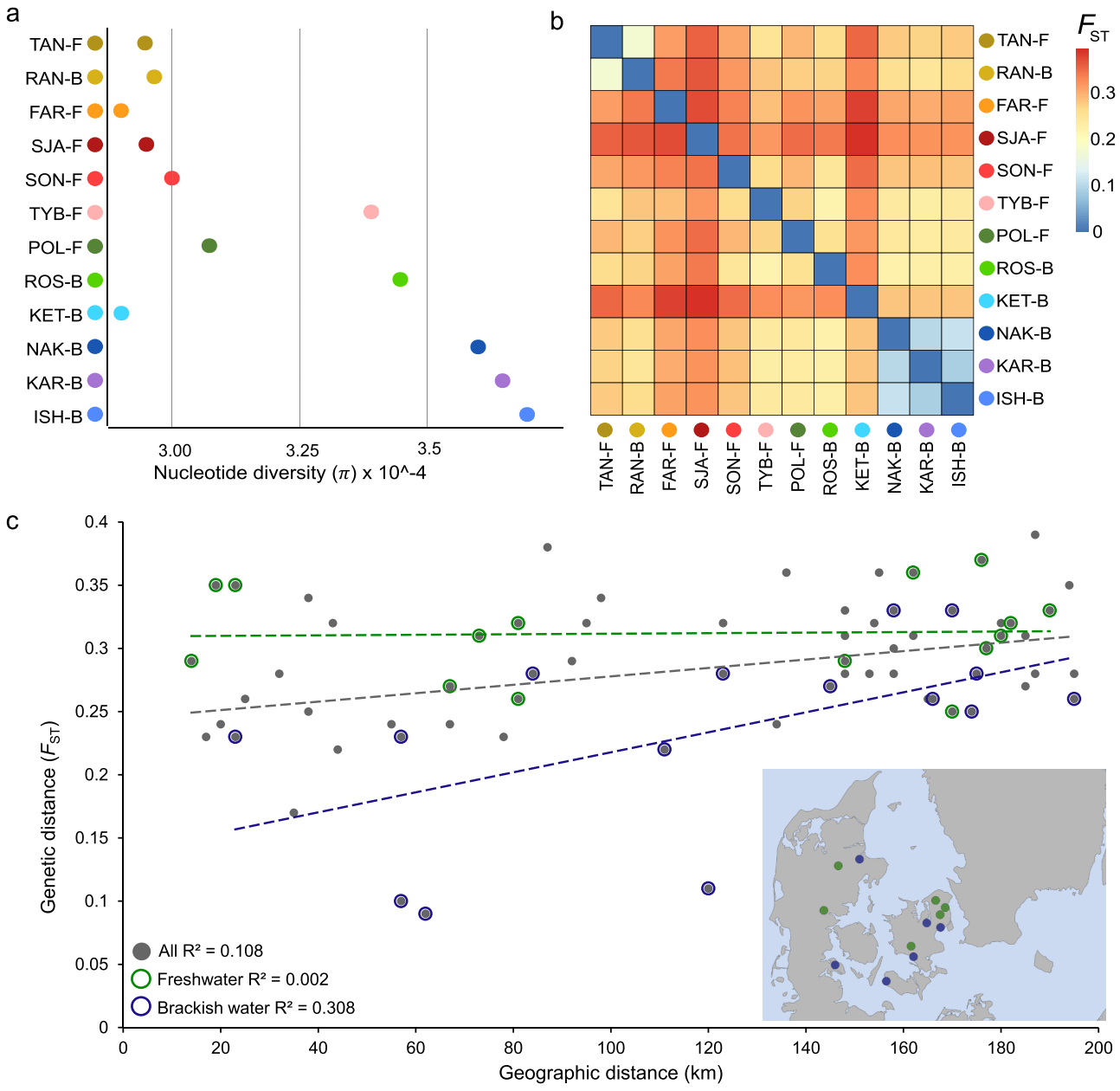

Fig. 2. Diversity and differentiation of perch in the western Baltic Sea region. **a)** Genomic nucleotide diversity (π). See Fig. 1 for locality names and geographic placement. -B=brackish; -F=freshwater. **b)** Population differentiation heatmap based on F_{ST} values. **c)** Isolation by distance. Linear correlations between genetic (F_{ST}) and geographic (Km) distance among perch populations in the western Baltic Sea region.

would experience higher salinities than any other locality with more than half of the days above 19.5 ppt. Overall, our results further showed that there was a strong negative correlation (R^2 =0.7132) between genetic diversity (π) and the salinity levels any putative migrants would have to endure (Fig. 3c).

3.4. Putative genomic regions under selection

In order to identify genomic regions under selection in brackish water perch, PBS analyses were performed on 26,165 windows with an average of 278 nucleotide sites per window (Fig. 4). The average genome wide PBS values for Clusters A, B and C were 0.019, 0.024 and 0.016, respectively. In Cluster C, comprised of the four brackish water localities (KET-B, NAK-B, KAR-B and ISH-B), the three genomic regions with the highest PBS values, which were all above 0.82, were found in chromosome 6 (range: 30650–30925 K), chromosome 9 (range: 32550–32825 K) and chromosome 18 (range: 28775–29050 K), each including seven, six and 12 protein coding genes, respectively (Fig. 4a-b; Table S3; Table S4).

4. Discussion

4.1. Patterns of genetic variation in the western Baltic Sea perch

Unravelling a species' population structure is a first step in attempting to understand the evolutionary processes that shaped the present day genetic variation among wild populations. Our data revealed that each sample locality constituted a separate genetic population and there was no evidence of substructure within populations. Isolation by geographical distance played a limited role in the genetic differentiation of populations. Rather, our results suggest that colonisation history, or potentially combined drift and migration, along with

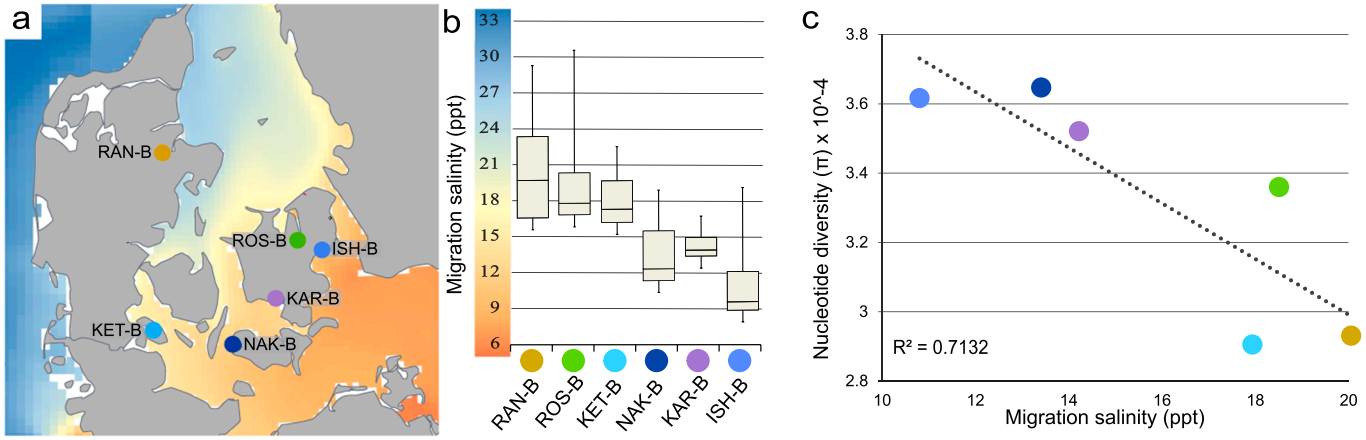

Fig. 3. Modelled environmental salinity of the western Baltic Sea and correlation with genetic diversity. **a)** Average annual environmental salinity based on one daily data point spanning from September 1st 2013 to August 30th 2014. **b)** Environmental salinity outside the stream/river/fjord associated with each brackish water population. **c)** Correlation between genetic diversity and salinity.

substantial salinity gradients have been the main underlying mechanism behind the observed variation found in modern perch populations in the western Baltic Sea region. Specifically, we found localities in northern Jutland (TAN-B and RAN-B) to form a separate genetic cluster (Cluster A) and be highly distinct from all other perch localities in our study. The other localities were split into two clusters; the first (Cluster B) comprised mainly of freshwater populations (FAR-F, SJA-F, TYB-F, POL-F), as well as a single isolated brackish water population (ROS-B). The second (Cluster C) included all southeastern brackish water localities with access to the western Baltic Sea (KET-B, NAK-B, KAR-B and ISH-B). Both cluster B and C were found on the Jutland peninsula and on the island of Zealand. Deep genetic splits among perch populations in northern Europe, as observed between cluster A and clusters B and C in our data, has previously been hypothesised to be a result of colonisation from different refugia after the retreat of the Fenno-Scandian ice sheet (Christensen et al., 2016; Nesbø et al., 1999; Toomey et al., 2020). However, the lack of reference populations from potential refugia in our dataset makes us unable to confirm this hypothesis. Alternatively, the differentiation pattern found in our data could also be the result of drift in cluster A combined with migration between RAN-B and TAN-F, which is supported by the presence of recent gene flow in our admixture analyses.

Freshwater fish populations are often separated in lakes or drainages with very limited opportunities for gene flow, however the colonisation of the brackish water environment can enable migration, thus facilitating gene flow (Skovrind et al., 2016). In our study, the freshwater localities and isolated brackish water localities in Clusters A and B followed a characteristic genetic freshwater pattern (Ward et al., 1994), with lower levels of diversity and higher levels of differentiation. This pattern is most likely the result of genetic drift acting on smaller and/or isolated populations, which have had limited contact with other populations since the colonisation (Nei and Tajima, 1981). The population structure in Cluster C composed of brackish localities followed a different pattern, most likely driven by differences in environmental salinity. The southeastern brackish water localities (NAK-B, KAR-B and ISH-B) were closely related and had high levels of diversity. This could be because the sea surrounding the southeastern brackish water localities have environmental salinity within the tolerance of brackish water perch, thus allowing them to fully exploit the resources of the highly productive Baltic Sea and allow gene flow among them. In contrast, the brackish locality KET-B was more distantly related and had a low level of diversity, as gene flow likely is more restricted due to higher salinity levels, and perhaps deep waters (see e.g. Olsson et al., 2011).

An influx of migrants bringing novel alleles into or among the populations would also explain the elevated levels of diversity observed in NAK-B, KAR-B and ISH-B. The NGSadmix analyses suggest that admixed individuals are found in these three populations, supporting that gene flow plays a role in the high levels of diversity and lower differentiation. Another possible explanation for the high levels of diversity is that access to large marine habitats has allowed the southwestern Baltic Sea brackish water populations to maintain large populations, which would minimise the loss of diversity to genetic drift. The very close relatedness of NAK-B, KAR-B and ISH-B could also indicate recent divergence. However, high diversity and presence of recently admixed individuals suggests that gene flow is at least partly behind the observed population structure pattern in Cluster C.

Intriguingly, while NAK-B, KAR-B and ISH-B were grouped with the same genetic cluster and likely had substantial gene flow, we did detect substructure among them, suggesting that these southwestern Baltic brackish water perch exhibits site fidelity to their natal stream. This is in accordance with previous studies that found genetic differentiation among perch populations in the inner Baltic Sea sampled less than one kilometre apart (Bergek and Björklund, 2009), as well as genetic differentiation within a single lake (Bergek and Björklund, 2007). Other mechanisms maintaining the differentiation among perch populations have previously been suggested, including lower fitness of F1 hybrids (Behrmann-Godel and Gerlach, 2008) and a preference to their own population based on olfactory cues (Behrmann-Godel et al., 2006).

4.2. *Putative regions under selection in brackish water perch*

While the PBS method does not formally test whether the results deviate from neutral expectations, the three genomic regions with the strongest signals of selection all included genes linked to salinity tolerance in other organisms, suggesting that selection acted on genomic variation and possibly facilitated the colonisation of the fluctuating salinity brackish waters of the southwestern and western Baltic Sea. The strongest signal of selection was found in chromosome 18 where the analysis identified three proteins linked to salinity regulation; SLC22A2, MTHFR and IGF2/M6PR. The SLC22A2 protein transports organic cations across basolateral membranes and has previously been shown to be differentially expressed in Pacific spiny dogfish (*Squalus suckelyi*) exposed to different salinity levels (Cole, 2018). Also, organic cation transporter genes have previously been identified as under selection in brackish water pike in the Baltic Sea (Sunde et al., 2022). The MTHFR protein is a rate-limiting enzyme in the methyl cycle and is crucial for the formation of methionine (vitamin B9). A study of three-spined sticklebacks (*Gasterosteus aculeatus*) showed that MTHFR was expressed at significantly different levels in saltwater and freshwater exposed individuals. Vitamin B9 has even been demonstrated to mitigate

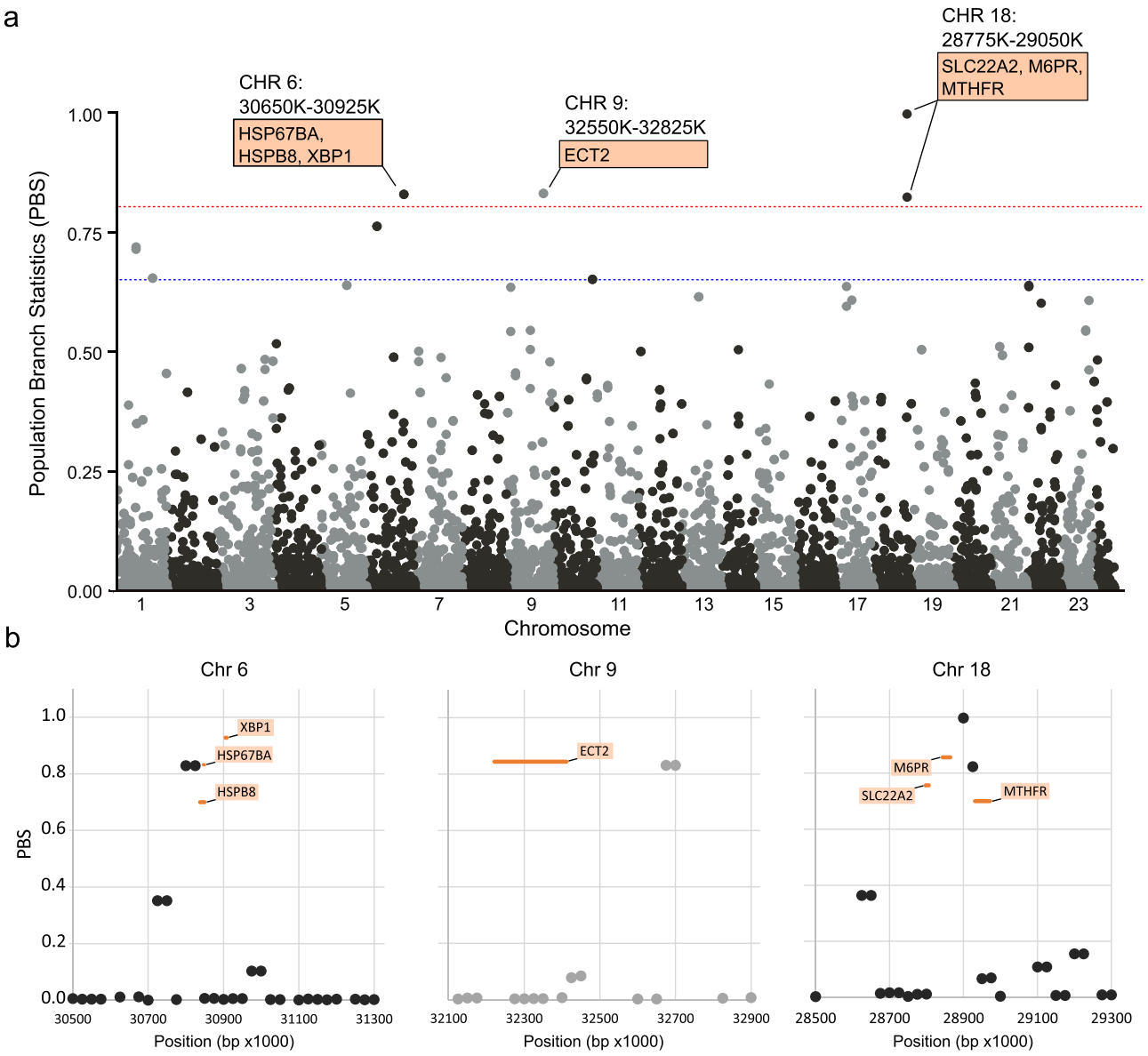

Fig. 4. Putative footprints of selection in brackish water perch in the western Baltic Sea (NAK-B, KAR-B and ISH-B). Identification of regions and genes under selection using Population Branch Statistics (PBS) performed with 50 kb windows in 25 kb steps. Grey and black points indicate results from uneven and even chromosome numbers, respectively. **a)**. Chromosomes 1–24, the blue and red horizontal lines represent the 99.5th and 99.9th percentile, respectively. **b)** The three regions with the strongest signs of selection located on chromosomes 6, 9 and 18. Genes with putative links to salinity adaptation are indicated in orange.

the effect of elevated salinity in barley (*Hordeum vulgare*) (Özmen and Tabur, 2020). IGF2/M6PR is a transmembrane protein (El-Shewy and Luttrell, 2009), which has been shown to be differentially expressed under different salinity regimes in several fish species, including Arctic charr (*Salvelinus alpinus*) and half smooth tongue sole (*Cynoglossus semilaevis*) (Li et al., 2020; Norman et al., 2011). The second strongest signal of selection was located in a region of chromosome 9, which included the ECT2 protein, playing a vital role in stabilising mRNA in the cytoplasm and bindinding to and promotes the transcription of N6–methyladenosine in a wide array of plant species subject to salinity stress, including cotton (*Gossypium* sp.), sweet sorghum (*Sorghum bicolor)*, sugar beet (*Beta vulgaris)* and arabidopsis (*Arabidopsis* sp.) (Cui et al., 2022; Wang et al., 2022; Zheng et al., 2021). The third strongest signal of selection was identified on chromosome 6 and included three salinity tolerance associated proteins (XBP1 and the two heat shock proteins HSPB8 and HSP67BA). XBP1 has been shown to be upregulated in red-eared slider turtles (*Trachemys scripta elegans*) exposed to 15 ppt salt water and suggested as a driver of adaptation to brackish water (Li et al., 2021). Heat shock proteins are involved in the response to osmotic stress (Deane and Woo, 2011; Sørensen et al., 2003) and have been shown to be expressed differently in European flounder (*Platichthys flesus)* translocated from marine to brackish water (Larsen et al., 2008). However, the resolution of GBS data is limited as it only captures genomic regions adjacent to cut sites and we are therefore not able to identify the specific genomic sites under selection, but only the general region. Thus, the signal of selection identified in our data is likely due to linkage between the GBS sites and the actual site or sites under selection.

Previous studies have shown that brackish water perch has a markedly increased salinity tolerance of at least 17.5 ppt achieved through an ability to hypo-osmoregulate that freshwater conspecifics have not (Christensen et al., 2019, 2017). The identification of salinity tolerance associated genes within each of the three genomic regions with the strongest signals of selection suggest that increased physiological abilities to cope with saline environments of brackish water perch is rooted in genomic adaptation and not a result of phenotypic plasticity alone. The Baltic Sea was formed relatively recently in evolutionary time, ~12

thousand years ago after the Pleistocene/Holocene transition when the Fenno-Scandian ice sheet retreated (Björck, 1995; Hall and van Boeckel, 2020). Thus, the adaptation to hypo-osmoregulation observed in our data likely arose within this period. Such rapid evolution has also been described in three-spined sticklebacks, which have adapted in parallel to freshwater habitats upon post-glacial isolation from marine environments (Hohenlohe et al., 2010). Our limited sample sizes only allowed for analyses of selection in the southeastern brackish water localities, but it is likely that similar adaptations are present in the isolated brackish water populations KET-B, RAN-B and ROS-B and likely elsewhere in the southwestern Baltic Sea. Future studies should seek to explore patterns of salinity tolerance and adaptations across multiple brackish and freshwater perch populations.

4.3. Implications for perch management

Brackish water perch populations in the Baltic Sea are currently under pressure from several abiotic and biotic factors, including human perturbations of the environment. During the autumn, when southwestern Baltic brackish water perch are returning to their natal streams, influxes of high-salinity sea water exceeding their physiological tolerance can lead to mass mortality among perch (Berg, 2012). There is also an increasing threat from cormorant (*Phalacrocorax carbo*) predation during the winter, when the brackish water perch are congregating (Salmi et al., 2014; Veneranta et al., 2020), and in some areas of the Baltic Sea there is egg predation from three-spined sticklebacks (Donadi et al., 2020). These pressures, combined with unregulated commercial and leisure harvesting during the summer, when the brackish water perch are in the marine environment, has led to greatly fluctuating population sizes of brackish water perch (Lindvig and Ebert, 2012). In addition, many streams in the southwestern Baltic Sea have floodgates installed to hinder flooding, which may deter the migration of perch and other migratory species of fish; in particular under future scenarios of climate change and rising sea levels. In our study region, particularly vulnerable populations may include ISK-B of only a few thousand individuals (Christensen et al., 2021), however for most populations there is no information of population sizes, which makes it difficult to identify at-risk populations. The genomic adaptations of southeastern brackish water populations indicate that they may not be easily rescued by migrants from upstream freshwater localities, but would have to await immigration from other brackish water localities. In isolated populations such as RAN-B, ROS-B and KET-B, local extinction would likely result in long-term disappearance of the brackish water ecotype making local nature management even more pertinent.

CRediT authorship contribution statement

MS, MTO and PRM conceived the study. MTPG, MTO and PRM provided funding. MS, MAK, PRM and HC collected the samples. MS, GP, EFC, THH and FGV performed the analyses. MS, GP, EFC and MTO drafted the manuscript with input from the remaining authors. All authors approved of the final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All resequencing data is publicly available at the European Nucleotide Archive (ENA), and can be accessed through the Project Number: PRJEB65985.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fishres.2023.106846.

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