PRYING GENOMES

Providing a molecular foundation for sustainable oyster restoration

Sophie Valk

Propositions

1. Acknowledging intraspecific genetic variation is essential for effective and sustainable species conservation (this thesis).
2. Hermaphrodite oysters are a rule rather than an exception and as such do not reflect an intermediate stage (this thesis).
3. Supporting natural recovery is a more sustainable marine restoration approach than species reintroduction.
4. Obtaining a PhD degree incentivizes individual performance at the expense of collaborative science.
5. When driven by intrinsic motivation and passion, the pursuit of knowledge becomes more rewarding than the scientific outcome.
6. The historical importance of fisheries to the Dutch economy and culture warrants greater representation in education.
7. Nature restoration initiatives in offshore wind farms require critical scientific assessment to avoid greenwashing.

Propositions belonging to the thesis, entitled

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PRYING GENOMES

Providing a molecular foundation for sustainable oyster restoration

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Voor Silas en Matilde

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Glossary

Biogenic reef: a three-dimensional structure made from aquatic living organisms that provides substrate utilized by other species.

Collinearity: the preservation of the linear order of genetic variants or genes along a chromosome (Secomandi et al., 2025).

Core genome: The pool of variants and genes common to all the studied genomes of a given species.

Closed pangenome: A pangenome that contains little to no variants or genes unique to any of the studied genomes of a given species.

Dispensable genome: The pool of variants and genes that are not unique to all the studied genomes of a given species (core genome).

Ecosystem: A (a)biotic environment where organisms interact.

Ecosystem service: a service provided by a natural ecosystem that is beneficial (to humans).

Epigenetics: mitotically and/or meiotically heritable changes in gene expression that do not result from alterations in gene sequence but from (chemical) modifications to DNA, RNA, or proteins (Chapelle & Silvestre, 2022).

Epimutation: A heritable mutation in gene regulation or expression that results from the gain or loss of an epigenetic mark or modification.

Evolutionary dynamics: the dynamics that shape changes in populations and individuals over time that can be driven by mutation and selection.

Functional genome annotation: the assigning of functions to encoded proteins that help to understand gene functions and predict their physiological effects (Schell et al., 2025).

Gene flow: the exchange of genetic material between individuals, populations, or species caused by any form of population connectivity (Slatkin, 1985).

Genetic drift: random fluctuations in allele frequencies within a population across generations, often caused by mutations (Ohta, 1982).

Genetic load: a reduction in fitness caused by the accumulation of harmful, often recessive, deleterious variants (Launey & Hedgecock, 2001).

Genetics: the study of genes and their inheritance.

Genome: The complete set of genetic material found in an organism.

Genome assembly: the computational effort to collate, align, and merge an organism's DNA to reconstruct the original genome, consisting of full-length chromosome sequences (Baker, 2012).

Genomics: the study of genomes, including gene interactions, functions, and their evolution.

Genomics: the collection of genome sequences and captured variation within across individuals, populations, and species (Schell et al., 2025).

Hardy-Weinberg equilibrium (hwe): the theory that states that allele frequencies remain constant over time in the absence of evolutionary influences (including genetic drift, random mutation, natural selection, or gene flow) in a population.

Identity by descent (IBD): quantifies inheritance from a common ancestor by the probability that two individuals share the same allele at a given locus.

Inbreeding: increased homozygosity caused by the mating of closely related individuals.

Inbreeding depression: reduced fitness as a consequence of inbreeding.

Intraspecific genetic diversity: the genetic variation between individuals or populations from the same species.

Inversion: A segment of DNA that is in reversed orientation compared to a reference.

Kinship: genetic relatedness, the proportion of shared alleles between individuals.

Life-history characteristics: represent traits that describe a species' reproduction, growth, and survival.

Linkage disequilibrium: the non-random association of alleles at different loci in a population.

Linkage disequilibrium pruning: bioinformatical data processing that removes highly correlated variants that are in linkage disequilibrium.

Linkage map: a genetic map that displays the positions of a species' known genes or genetic markers by recombination frequency.

Local adaptation: the development of enhanced fitness in specific environmental conditions associated with genetic variants or mutations, driven by natural selection.

Maladaptation: a condition of suboptimal fitness that can include inbreeding depression, evolutionary traps, and a mismatch of traits to environmental conditions.

Mapping: the alignment of DNA sequences to a reference sequence, often a genome assembly.

Metabarcoding: a PCR-based molecular approach that amplifies and sequences target nucleotide fragments that can be identified by matching against a reference database.

Metagenomics: a PCR-free molecular approach that relies on sequencing of genomic DNA from a sample extract, from which species can be simultanously identified by matching obtained sequences against nucleotide databases.

Metatranscriptomics: the detection of expressed genes, most often in the form of RNA transcripts, from an environmental sample (Sutherland et al., 2023).

Open pangenome: A pangenome that contains a high number of variants or genes unique to any of the studied genomes of a given species.

Pangenome: a collection of genetic variation across individuals or populations of a species, often captured in so-called pangenome graphs obtained from raw sequences, sequence alignments, or assemblies.

Phenotypic plasticity: the ability of a single genotype to produce various phenotypes in response to variable environments.

Population genomics: the study of genomic diversity across populations to understand concepts of evolution and population dynamics (Hohenlohe et al., 2021).

Population demographics: describe the structure and dynamics of a population and include processes such as age, sex ratio, and population density (Montero-Serra et al., 2018).

Phasing: the process of determining which genetic variants are inherited together on the same chromosome.

Recruitment: the contribution of new individuals to a population through reproduction.

Reference genome: a contiguous and accurate genome assembly that is representative of a species.

Relatedness: quantifies genetic similarity by determining the set of shared alleles between two individuals.

Reproductive cohorts: a subset of individuals produced from the same reproductive event.

Resilience: the capacity of a natural ecosystem to recover from disturbances and return back to an equilibrium state.

Restoration: assisting the recovery of a species or ecosystem.

Translocation: the (often reciprocal) movement of individuals or populations from one location to another.

Spat: settled juvenile oysters that have permanently attached to a substrate.

Abbreviations

5hmC: 5-hydroxymethylcytosine

5mC: 5-methylcytosine

ddPCR: digital-droplet PCR

DML: Differentially methylated locus

DMG: Differentially methylated gene

DMR: Differentially methylated region

eDNA: environmental DNA

eNA: environmental Nucleic Acids

eRNA: environmental RNA

epi-eDNA: epigenetic patterns derived from eDNA

F: Female

FAIR: Findable, Accessible, Interoperable, Reusable

FDR: False discovery rate

GES: Good Environmental Status

GO: Gene Ontology

HBS: Hermaphrodite both sexes equally present

HPF: Hermaphrodite predominantly female

HPM: Hermaphrodite predominantly male

IBD: Identity by descent

KEGG: Kyoto Encyclopedia of Genes and Genomes

M: Male

NGS: Next Generation Sequencing

OWF: Offshore wind farm

PCR: Polymerase chain reaction

qPCR: quantitative PCR

RT-PCR: Real-Time PCR, also known as qPCR

SNP: Single nucleotide polymorphism

SV: Structural variant

TGS: Third Generation Sequencing

CHAPTER 1

General Introduction

Sophie Valk

The ecological importance of oyster habitats

The North Sea is one of the most productive and exploited marine ecosystems worldwide, subjected to numerous industries such as fishing, energy production, shipping, and tourism (Emeis et al., 2015). Where large areas of seabed were historically covered with hard substrate, consisting of shellfish reefs, peat-rich sediments, and erratic rocks, the North Sea floors are nowadays depleted from structure by centuries of bottom-trawling and dredging (Clark & Frid, 2001; Dickey-Collas et al., 2010; Engelhard et al., 2014; Moullec et al., 2021; Olsen, 1883). Oyster beds alone covered approximately 21,000 km² of the southern North Sea, but it is estimated that over 80% of former native shellfish reefs have since been lost (Olsen, 1883; Pogoda, 2019). This loss of benthic structures and habitat complexity resulted in reduced overall biodiversity, diminished productivity of benthic ecosystems, and the collapse of several commercially and ecologically important fish species, accompanied by shifts in trophic interactions (Dickey-Collas et al., 2010; Jennings et al., 2001; Kaiser et al., 2006; Saygu et al., 2025; Sguotti et al., 2016, 2022).

Following the widespread degradation of benthic ecosystems, the ecological relevance of hard substrates in marine environments is increasingly acknowledged, particularly for their contribution to overall biodiversity (Pogoda et al., 2019). Shellfish beds act as biogenic reefs that support diverse benthic and epibenthic fauna such as algae, invertebrates, and fish (Smyth & Roberts, 2010; Thomas et al., 2022). As such, there is a strong global incentive to protect or restore these habitats and corresponding biodiversity. In Europe, habitat restoration has become an important objective in marine nature conservation within the framework of the Oslo-Paris Commission (OSPAR Commission, 2021b). According to the guidelines of the EU Habitats Directive, we should: "protect and preserve the marine environment, prevent its deterioration or, where practicable, restore marine ecosystems in areas where they have been adversely affected" (European Parliament and Council of the European Union, 2017). More recently, shellfish reefs were identified as one of the key marine habitats under the EU Nature Restoration Regulation, which requires the restoration of degraded marine habitats (European Parliament and Council of the European Union, 2024).

The European flat oyster, *Ostrea edulis* (Linnaeus, 1758), represents one of the key species included in the objectives of multiple habitat restoration initiatives, such as the OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR Commission, 2021a), the EU Flora-Fauna-Habitat Directive (FFH Directive) (Habitats Directive, 1992), and the Marine Strategy Framework Directive (MSFD) (European Parliament and Council of the European Union, 2017).

The flat oyster plays a pivotal role in marine ecosystems as it generates biogenic shellfish reefs which provide opportunities for a wide range of marine organisms to settle, hide, forage, and reproduce (Smyth & Roberts, 2010; Thomas et al., 2022). As such, oyster reefs sustain overall ecosystem biodiversity and can be considered a "keystone" species (Bond, 1994; Coen et al., 2007). In addition to their pivotal contribution to sustaining biodiversity, oyster reefs provide coastal protection through facilitating wave attenuation and stabilization of sediment (Thomas et al., 2022). Furthermore, oysters improve water quality and enhance benthic-pelagic coupling by filter-feeding on phytoplanktonic algae, thereby also capturing other suspended particles (Coen et al., 2007; Fabra et al., 2025; Lee et al., 2020; Thomas et al., 2022). Together, these ecosystem services illustrate the crucial role that oysters play in shaping benthic ecosystems and associated communities (Coen et al., 2007; Rodríguez-Perez et al., 2019; Smaal et al., 2019). In addition to its ecological relevance, the flat oyster has historically been considered a delicacy and currently holds a market price that exceeds that of the non-native Pacific oyster (*Magallana gigas*) by three to five times, underscoring its economic importance (Frongia et al., 2023).



Figure 1 | Piscatorial atlas of the North Sea illustrating the distribution of *O. edulis* populations along the northwestern European coastline in 1883 (Olsen, 1883).

Ecology of the flat oyster

The European flat oyster exhibits a wide natural distribution ranging from the North Sea and European Atlantic coast to the southern waters of the Mediterranean and Black Sea (Alves Monteiro et al., 2024; Stagličić et al., 2020). It inhabits both intertidal and subtidal coastal waters, ranging from a few tens to 50 meters in depth, but is occasionally found in deeper waters (Bennema et al., 2020; Kerckhof et al., 2018; Pogoda, 2019). These native oysters once formed extensive and abundant reefs along the European coastline (Fig. 1; Olsen, 1883). Within the family Ostreidae, *O. edulis* belongs to the subfamily Ostreinae and is most closely related to *O. angasi*, native to Australia, *O. chilensis*, native to Chile and New Zealand, and *O. lurida*, native to the west coast of the United States (Pritchard et al., 2015; Salvi et al., 2014).

Reproduction

The flat oyster is a sequential hermaphrodite that can reverse sex throughout its life. Hermaphroditism represents a sexual reproduction strategy whereby both male and female gametes are produced during an organism's lifetime, either simultaneously or sequentially (Leonard, 2006). Simultaneous hermaproditism is thought to evolve in species with a low probability of mating events, whereas sequential hermaphroditism represents a plastic strategy that allows species to optimize reproductive output by alternating sex across various life-stages (Leonard, 2006). Sequential hermaphroditism typically arises when one sex achieves higher fecundity at an early life stage while the other sex benefits a fitness advantage at an older life stage, a trade-off often associated with size or age (Leonard, 2006).

Sequential hermaphroditism can be bidirectional and is classified as either protogynous or protandrous. Protogyny involves initial maturation as a female, followed by sex reversal to male reproduction, and occurs frequently in fish where large males can monopolize mating events (Avise & Mank, 2009; Oldfield, 2005). Protandry, in contrast, involves initial maturation as a male, followed by a switch to female reproduction, and is suggested to occur in species where female fitness exceeds male fitness at a later life stage (Leonard, 2006; Warner, 1975). The majority of sequentially hermaphroditic invertebrates, including marine bivalves, adopt a protandrous reproductive strategy (Leonard, 2006; Picard et al., 2021; Subramoniam, 2018). The flat oyster is generally considered a protandrous sequential hermaphrodite, although, one-year old reproducing females have been observed (Dodd et al., 1937), illustrating the plasticity of this life-history trait.

Gametogenesis takes place in the gonadal ducts (Fig. 2A), and although both male and female gametes may be produced within the same follicle, asynchronous gametogenesis is thought to minimize the potential of self-fertilization in oysters (Da Silva et al., 2009). Female ovocytes are transported to the pallial cavity once they have completed maturation, where they await fertilization. Males release spermatozeugmata, a cluster of spermatozoa heads with flagella, into the environment (Fig. 2B). Following disassociation of these spermatozeugmata, free-swimming spermatozoa are released (Fig. 2C; Suquet et al., 2018). During water filtration by the female oyster, these spermatozoa enter the females' pallial cavity, where they fertilize matured ovocytes (Fig. 2D).

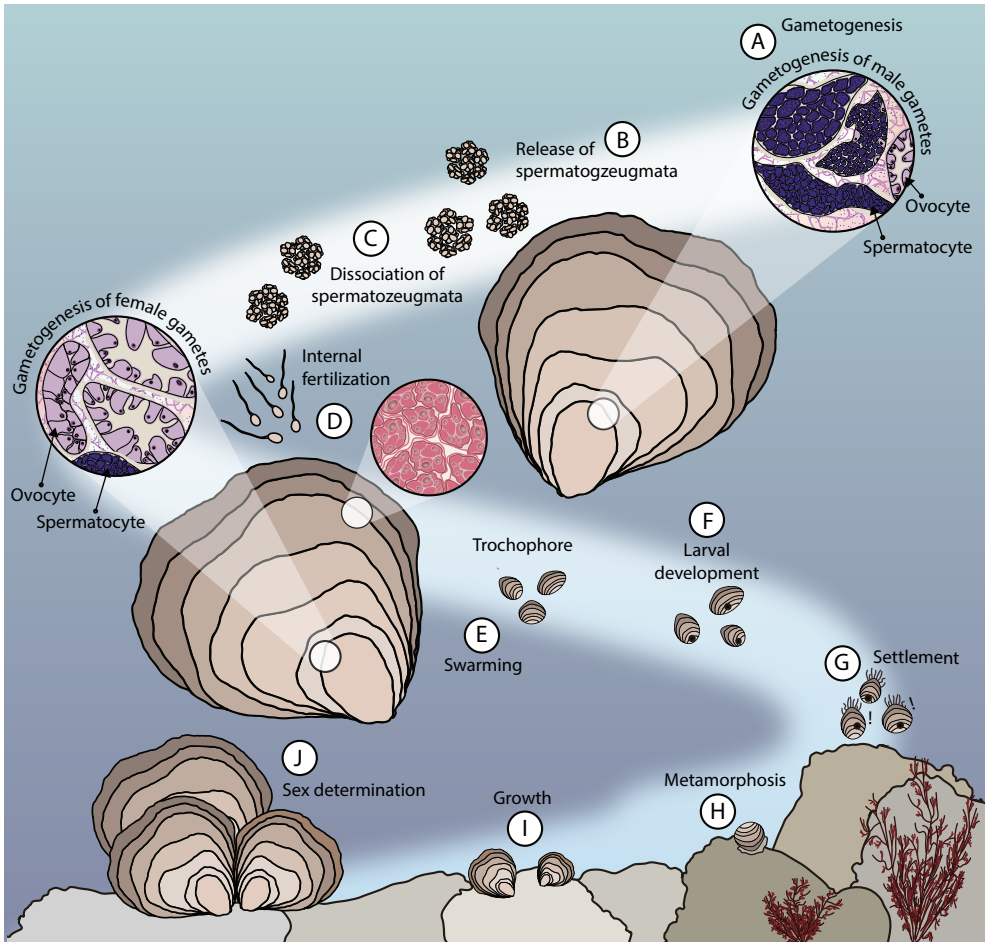


Figure 2 | Illustration of the reproductive cycle of *Ostrea edulis*.

Life cycle

The flat oyster adopts a brooding strategy, involving high parental investment (Ó Foighil & Taylor, 2000). After fertilization, the female broods the eggs for about 7-10 days and releases them into the environment as trochophore larvae, a process referred to as "swarming" (Fig. 2E; Korringa, 1940; Rodriguez-Perez et al., 2020). Hereafter, the larvae feed on phytoplankton to complete development from "D-stage" into an "early umbo", followed by the "umbo" stage (Rodríguez-Perez et al., 2020). The larvae are about 280-320 µm in size when they have developed a functional foot organ and their eyespot has undergone full pigmentation, from this stage onwards the larvae are referred to as "pediveliger" (Fig. 2F). Although the exact function of the eyespot remains elusive, larvae of other oyster species have been shown to actively move towards light, providing evidence for their phototactic competence (Cole, 1938; Kim et al., 2024; Wheeler et al., 2017). Pediveliger larvae are thought to select suitable substrates by examining surfaces with their foot organ, showing preferences for particular substrate structures and compounds, often calcium-rich materials such as oyster shells, which promotes self-recruitment within reefs (Fig. 2G; Christianen et al., 2018; Kamermans et al., 2025; Rodriguez-Perez et al., 2019; Rodriguez-Perez et al., 2020; Ter Hofstede et al., 2024). Once a larva has selected a suitable substrate, the glands in the foot secrete compounds that allow for permanent cementation to its selected substrate, referred to as settlement (Cranfield, 1973b, 1973a). After undergoing metamorphosis several larval organs, including the foot and eyespot, functionally disappear (Fig. 2H; Cole, 1938). Upon permanent attachment to a substrate, referred to as "spat fall", the oyster spat continues to grow (Fig. 2I) and typically reaches sexual maturity at two or three years of age (Fig. 2J), although some individuals have been observed already reproducing after only one year (Dodd et al., 1937; Korringa, 1952).

Anthropogenic stressors and flat oyster population demise

A brief history of oysters in human culture

Beyond its ecological significance, the European flat oyster holds substantial cultural value and has a long history of human consumption and trade. The earliest archaeological evidence of human consumption of *O. edulis* dates back to around 5,900 B.C. during the Late Mesolithic, where flat oyster shells were found in kitchen middens (Larsen et al., 2018; Lewis et al., 2016). Later, oysters were consumed by the ancient Greeks, reflected in the famous expression that they are "hard to open, but easy to eat", although the extent of their dietary contribution remains uncertain (Andrews, 1948). In contrast, during the Roman empire, oysters represented the most

popular consumed shellfish species (Déry, 1997). By 301 C.E, oysters were charged twice the price of sea urchins and mussels, reflecting their high economic and culinary value at the time (Andrews, 1948). The Romans developed efficient methos for transporting and preserving live oysters to inland regions, further increasing their economic significance. The most famous shipping route enabled the transport of live oysters from southwestern France to Rome, and oyster shells have been found at Roman army stations in Germany and Switzerland (Andrews, 1948). In addition to inland transport, the development of lakes and ponds ensured year-round availability of high-quality oysters by enabling the outgrowth of oysters collected from the wild (Polanco, 2023). Through innovations such as the use of poles and ropes, along with the development of warm-water ponds, the Romans established the foundation for cultivating a wide variety of oyster shapes and qualities, many of which are still employed today (Polanco, 2023). After the time of the Roman Empire, oyster fishing and cultivation continued extensively, supported by the use of wooden rowing boats that were later equipped with sails (Menzel, 2018). Oyster fishing persisted throughout the Middle Ages and the Renaissance, and became industrialized with the introduction of fishing trawlers using steam engines. From the 18th century onwards, humans began actively culturing flat oysters by successfully completing their full life cycle in captivity (Heral, 1989).

Tragedy of the commons

Industrialization revolutionized the shipping industry, with steam fishing trawlers rapidly replacing wooden sailing vessels (Engelhard, 2008). These machine-driven vessels resulted in enhanced accessibility of remote fishing sites as well as intensified exploitation of oyster reefs. Already at the time, there was high opposition against the use of bottom-trawlers, as they were accused of destroying the spat and habitats of ecologically valuable shellfish and fish species (Engelhard, 2008). Alongside industrial improvements in infrastructure that increased the inland market for oysters, these anthropogenic developments soon became unsustainable, with observed population decreases being reported from the mid-1800s onward (Engelhard, 2008; Zu Ermgassen et al., 2012).

The collective collapse of oyster stocks quickly spread throughout Europe. In France, landings exceeded more than 100 million oysters annually at the beginning of the 19th century. But around the 1860s more than two-thirds of the originally harvested beds had been completely lost. At the start of the 19th century, upon recognition of population declines, the Wadden Sea oyster fisheries were among the first to be regulated (Berghahn & Ruth, 2005). However, the already degraded state of oyster populations prevented recovery, and by the 1950s the flat oyster was considered extinct in the entire Wadden Sea. Flat oyster stock declines in the UK followed similar

trends. By the mid-19th century, the British Government recognized difficulties in meeting high landing demands, with annual oyster consumption exceeding over 700 million oysters in London alone (Neild, 1995). Within less than a century, national production declined to approximately 3 million oysters per year (Gercken & Schmidt, 2014). The Netherlands experienced a slight delay in stock collapse, where annual production reached its peak at 30 million oysters before it collapsed after a severe winter in the early 1960s (Drinkwaard, 1998). After this collapse, local populations were replenished with oysters from Norway, the UK, France, Ireland, and Scotland (Thurstan et al., 2013). As such, the Firth of Forth, Scotland, represented a crucial oyster stock used for replenishing foreign populations (Thurstan et al., 2013). However, the continued exploitation of this area as a source population, combined with increasing national production demands, resulted in the collapse of Scotland's most profitable oyster fishery within a period of just 60 years. These few examples illustrate the drastic decline of native oyster populations throughout Europe. Consequently, *O. edulis* is considered functionally extinct in several areas, including the southern North Sea (Kerckhof et al., 2018; Pogoda, 2019).

Upon the introduction and spread of diseases

The emergence of two virulent pathogens further accelerated the ongoing collapse of *O. edulis* populations. In 1968, the protistan parasite *Marteilia refringens* (Perkins 1976) caused high mortalities among French populations and the parasite rapidly spread to distant oyster beds via international translocations (Alderman, 1979; Longshaw et al., 2001). Transmission of *M. refringens* between oysters happens indirectly and is thought to involve a calanoid copepod as intermediate host.

In 1979, *Bonamia ostreae*, was first detected in France (Pichot et al., 1979). The haplosporidian parasite is believed to have been accidentally introduced via the translocation of infected oysters from California to Europe. After its rapid spread northwards, *B. ostreae* reached Dutch oysters by 1980, with mortality rates exceeding 80% (van Banning, 1991). *B. ostreae* exhibits a parasitic life-cycle and can be directly transmitted between oysters (Flannery et al., 2016). The parasite primarily infects hemocytes, with advanced stages of infection associated with hemocyte disruption and lysis (Morga et al., 2009). Although infections are reported in juvenile oysters, mortality due to bonamiosis occurs predominantly in adult oysters aged two years or older (Cáceres-Martínez et al., 1995). More recently, the potential for developing tolerance or genetic resistance to the disease is explored (Kamermans et al., 2023; Sambade et al., 2022; Sas et al., 2020). Although *B. ostreae* currently receives increased attention due to its direct transmission and high induced mortality, *M. refringens* also remains a widespread threat across European oyster populations, and both pathogens continue to pose significant risks to oyster stocks (Mérou et al., 2023).

Introducing non-native oysters

To mitigate the economic losses resulting from the decline of flat oyster populations, the Portuguese oyster (*Crassostrea angulata*), native to Taiwan and introduced to Portugal in 1866, was intensively cultivated in France in the late 1960s (Boudry et al., 1998). However, this species soon experienced high mortality rates, prompting oyster farmers to replace it with another non-native species, the Pacific oyster (*Magallana gigas*). *M. gigas*, native to Japan and subjected to previous translocations along the Atlantic coast of North America, was initially believed to be incapable of reproducing in relatively cold European waters (Troost, 2010). Against all expectations, the non-native oyster spread rapidly within a few years. Although concerns were raised regarding the invasive potential of this rapidly establishing species, attempts to control *M. gigas* populations proved unsuccessful. Extensive spatfall was observed in the late 1970s and as the non-native species became more abundant, Dutch oyster farmers began cultivating *M. gigas* alongside *O. edulis* (Troost, 2010).

Ecological consequences of overexploiting oyster reefs

The decline of oyster reefs has not only impacted *O. edulis* as a species, but also initiated cascading effects throughout associated ecosystems (Smyth & Roberts, 2010; Thurstan et al., 2013, 2024). Oyster reefs typically sustain over a 100 different marine species, and their structural complexity positively influences species richness, thereby contributing to overall ecosystem biodiversity and resilience (Bracewell et al., 2018; Oliver et al., 2015; Rapport, 1998; Smyth & Roberts, 2010; Zu Ermgassen et al., 2025). The broad-scale collapse of these reefs has led to substantial changes in community composition and biotic interactions, resulting in a markedly degraded ecological state that represents a "shifted baseline" compared to historically described oyster reef ecosystems (Pogoda et al., 2019; Zu Ermgassen et al., 2025). These broader ecological consequences underscore the urgent need for intervention. In particular, under emerging climate change-related pressures, including rising sea surface temperatures and ocean acidification, maintaining ecosystem resilience is crucial. Accordingly, restoration efforts are increasingly recognized as requiring not only the recovery of oyster populations for species conservation, but also the rehabilitation of reef habitats and associated biodiversity historically supported by these biogenic reefs (Beck et al., 2011; Pogoda et al., 2019; Pouvreau et al., 2023).

Oyster restoration

Since the early 2000s, oyster restoration has emerged as an active practice aimed at reviving *O. edulis* populations, driven by both their ecological and commercial value (Pogoda, 2019). Today, native oyster restoration projects are practiced in at least 14 European countries (Projects – NORA, 2026). Existing restoration measures can be broadly divided into passive and active restoration approaches. Passive oyster restoration focusses on re-establishing oyster populations by facilitating settlement and reef accretion, typically through the introduction of settlement substrates (Kamermans et al., 2025; ter Hofstede et al., 2024). This approach does not involve direct oyster supplementation but instead supports natural recruitment and recovery. A range of substrates and structures have been investigated to identify suitable settlement surfaces for *O. edulis* larvae (Colsoul et al., 2020; Kamermans et al., 2025). Based on these findings, substrates with preferred structural characteristics or materials are deployed as artificial settlement substrates at local restoration sites (Kamermans et al., 2025; Klunder et al., 2020; Potet et al., 2021).

In contrast, active oyster restoration involves the direct supplementation of depleted populations or the establishment of new ones through the introduction of oysters. Active restoration can be achieved via three approaches: (i) the release of (cultured) larvae, (ii) the deployment of spat, and (iii) the translocation of adult oysters, either from local or foreign sources (Lallias et al., 2010a). While the culturing of oysters for restoration purposes is gaining momentum, challenges in production and skewed-sex ratio's often hamper the upscaling of hatchery output (Colsoul et al., 2020; Ermgassen et al., 2023). Consequently, current restoration efforts mostly rely on the translocation of spat or adult oysters collected from wild source populations (Bos et al., 2023; Kerckhof et al., 2018; Pogoda, 2019).

Translocation entails the intentional movement of a species from its native occurrence to areas where it has become depleted or locally extinct. However, such interventions are not without risks. Despite being implemented for restoration purposes, translocations of *O. edulis* over the past decades have inadvertently caused substantial consequences, including the introduction of non-native species and pathogens (Bromley et al., 2016). For instance, the translocation of *Crassostrea virginica* to Europe also introduced the non-native slipper limpet *Crepidula fornicata* and the oyster drill *Urosalpinx cinerea*, both of which further contributed to the decline of *O. edulis* (Bromley et al., 2016). Similarly, the reintroduction of *O. edulis* from the US facilitated the spread of the invasive parasite *B. ostreae* (van Banning, 1979).

Genetic implications of oyster restoration

Beyond the introduction of non-native species, translocations can also pose risks to the genetic integrity of restored populations (Šegvić-Bubić et al., 2020). Reciprocal movements of oysters between regions may contribute to the homogenization of genetic diversity, potentially weakening or delaying the development of local adaptation or resilience across genetically diverged populations (Bromley et al., 2016; Lallias et al., 2010a). In addition, restoration approaches such as the propagation of hatchery-produced oysters, may present other challenges for maintaining genetic diversity. Oyster reproduction often follows a "sweepstakes" pattern of reproductive success, in which only a small number of parents contribute to the broodstock population (Hedgecock et al., 2007). Consequently, limited reproductive output and small broodstock sizes may reduce genetic diversity, potentially compromising population resilience (Hughes et al., 2008; Šegvić-Bubić et al., 2020). This issue raises particular concerns in hatcheries, where broodstock sizes are typically much smaller than those occurring in wild populations (Diaz-Almela et al., 2004; Lallias et al., 2010b).

Genetic diversity, defined as the variability of genetic information within a population or species, is positively correlated with species health and resilience (Aguirre & Marshall, 2012; Vásquez et al., 2023). Hence, genetically diverse populations have greater potential to respond or adapt to environmental disturbances (e.g. pathogens or extreme weather events) (Hughes et al., 2008). Within a population, higher genetic diversity can enhance productivity, growth, and stability, while positively influencing ecological interactions and ecosystem processes at broader scales (Vásquez et al., 2023). Understanding genetic population structure and diversity is key to developing effective restoration strategies, as genetically distinct populations may require separate management and locally tailored approaches. However, genetic diversity has rarely been assessed or incorporated into *O. edulis* restoration efforts to date, raising concerns about its preservation.

Molecular insights into *O. edulis* ecology and population structure

The sequencing revolution in the genetic characterization of *O. edulis*

From the end of the 20th century onwards, coinciding with the initiation of the first restoration projects, the relevance of genetic insights for understanding and managing *O. edulis* populations began to be increasingly recognized (Pouvreau et al., 2023). Early studies mainly examined genetic variation using techniques such as allozyme genotyping or PCR-based fragment length polymorphism analysis, generally revealing low levels of genetic diversity (Lallias et al., 2007; Saavedra et al., 1993; Saavedra & Guerra, 1996). Although some evidence for isolation-by-distance was observed between European and Mediterranean populations, genetic markers largely failed to detect fine-scale population structure across European populations (Launey et al., 2002; Saavedra et al., 1993). High connectivity in marine ecosystems, often lacking physical barriers to gene flow, combined with the extensive history of *O. edulis* translocations, were identified as the primary drivers of the relatively low genetic structure observed across populations.

In the early 2000s, advances in sequencing technologies enabled the genotyping of short mitochondrial sequences and microsatellites, facilitating the development of the first genetic linkage maps, reflecting a graphical presentation of co-inherited genetic marks or genes and their position along a chromosome (Lallias et al., 2007; Launey et al., 2002; Sobolewska & Beaumont, 2005; Taris et al., 2009). The advent of a second generation of sequencing technologies, also referred to as next-generation sequencing (NGS), substantially increased sequencing throughput and reduced associated costs, allowing the characterization of genome-wide variation by targeting single-nucleotide polymorphisms (SNPs) (Heather & Chain, 2016). The development of commercially available high-throughput SNP arrays further expanded access to genotyping resources and enhanced the characterization of genetic variation across *O. edulis* populations. To date, at least three SNP arrays have been developed, differing in the total number and type of markers: two focus on general genetic characterization from neutral markers (Gutierrez et al., 2017; Lapègue et al., 2014), while the third includes SNPs specifically associated with detecting resistance against *B. ostreae* (Sambade et al., 2022; Vera et al., 2019). Collectively, whole-genome SNP genotyping has revealed the presence of population substructure among European *O. edulis* populations. In addition, comparative analysis of mitochondrial genomes confirmed these phylogeographic patterns and allowed for resolving evolutionary processes shaping historical population structure by using DNA from museum specimens (Ewers et al., 2025; Hayer et al., 2021).

Further advances in sequencing technologies, with NGS platforms giving way to third-generation sequencing (TGS), have enabled whole-genome characterization by sequencing longer stretches of DNA. These approaches have revealed microgeographic population structure in *O. edulis* populations, for example indicating subtle differences in genetic diversity and heterozygosity among Scandinavian oysters (Alves Monteiro et al., 2024). Additionally, where SNP markers had already suggested the presence of large genomic regions of linkage disequilibrium, reflecting the non-random association between alleles at different loci, whole-genome sequencing confirmed these patterns and revealed three chromosomal inversions in the flat oyster genome (Alves Monteiro et al., 2024; Lapègue et al., 2023; Vera et al., 2019). Furthermore, advances in TGS platforms have resulted in the current availability of four different reference assemblies of the *O. edulis* genome (Adkins et al., 2023; Boutet et al., 2022; Gundappa et al., 2022; Li et al., 2023).

SNPs have long been regarded as the most common and predominant type of heritable genetic variation (Morin et al., 2004). However, TGS platforms, which allow the sequencing of whole nucleotide molecules, have revealed the widespread presence of other types of genetic variation across the animal kingdom, collectively referred to as structural variation (SV) (Wellenreuther et al., 2019). SVs encompass genetic variation that can vary in orientation, genomic location, or copy number among individuals or populations. The contribution of SVs to shaping intraspecific genetic diversity is increasingly recognized (Ho et al., 2020; Pokrovac & Pezer, 2022). Particularly inversions, a type of SV in which segments of DNA are in reversed orientation, are thought to play a significant role in adaptive evolution and speciation (Stuart et al., 2026; Wellenreuther et al., 2019). Such inversions often disrupt collinearity between chromosomes and limit recombination in heterozygote individuals (Berdan et al., 2023). Nearby alleles affecting fitness traits can be co-transmitted with inversions, potentially driving genetic divergence and local adaptation, which in some cases can result in speciation (Schwander et al., 2014; Thompson & Jiggins, 2014; Wellenreuther et al., 2025). In the flat oyster, three chromosomal inversions have been identified, exhibiting substantial variation in genotype frequencies among European populations (Alves Monteiro et al., 2024; Sambade et al., 2022, 2025b). Several studies suggest a correlation between one of these inversions and genetic resistance to *B. ostreae* (Kamermans et al., 2023; Sambade et al., 2026; Vera et al., 2019). Beyond the identification of these inversions, however, the broader SV landscape of *O. edulis* remains largely unexplored and may contain important insights into evolutionary and population dynamics, with potential implications for the species' conservation and restoration.

Previous studies on the genetic characterization of *O. edulis* have predominantly focused on single-nucleotide variation, often across broad geographic scales along the European coastline. While these studies have provided valuable insights into large-scale population structure, they offer a limited perspective on a microgeographic scale, which may constrain the effectiveness of local restoration efforts. Moreover, additional and equally relevant sources of genetic variation, such as SVs, may provide deeper insights into population dynamics and demographics. To support the conservation and restoration of *O. edulis* under current and future environmental conditions, it is essential to adopt an integrative approach that enables the comprehensive characterization of intraspecific genetic variation across multiple biological layers, capturing variation at both within- and between-population scales.

Beyond genetics

Phenotypic plasticity describes the ability of a single genotype to produce different phenotypes in response to environmental stimuli (DeWitt & Scheiner, 2004; West-Eberhard, 1989). This mechanism occurs broadly across the tree of life and enables organisms to respond to environmental change and cope with external stressors by regulating differential gene expression across environments (Borges, 2009; Roff, 1999). A well-known example is found in Arctic charr (*Salvelinus alpinus*), where environmental differences across Icelandic lakes have diverged two distinct morphotypes: a smaller benthic morphotype that primarily feeds on benthic invertebrates, and a larger pelagic morphotype that preys on fish and zooplankton (Küttner et al., 2014).

There is increasing evidence that phenotypic plasticity is driven by molecular mechanisms collectively referred to as epigenetics (Duncan et al., 2014; Ecker et al., 2018; Vogt, 2022). Epigenetics are defined as mitotically heritable changes in gene activity that do not rely on alterations of the underlying DNA sequence. These mechanisms enable a more nuanced regulation of genetic information by influencing (post-)transcriptional processes (Gibney & Nolan, 2010; Jaenisch & Bird, 2003). Recent developments in sequencing and bioinformatic analyses have improved our understanding of various epigenetic mechanisms, including DNA methylation (Jones & Takai, 2001), non-coding RNAs (Li, 2021), and histone modification (Murrell et al., 2005). Among these mechanisms, DNA methylation is the most extensively studied epigenetic mark, leading to a comprehensive understanding of its role in regulating phenotypic plasticity, particularly in eukaryotes (Nasrullah et al., 2022; Zemach et al., 2010).

DNA methylation involves the covalent transfer of a methyl group (-CH3) to a DNA molecule. In animals, methylation marks occur primarily at CpG dinucleotides and are commonly found in promoters, gene bodies, and repetitive regions (Jones, 2012; Schübeler, 2015). The dynamic interplay between DNA methylation and its passive

or active removal (demethylation) can influence gene regulation in several ways. For example, methylation in promoter regions is often associated with transcriptional repression or gene silencing (Turker, 2002), while methylation of transposable elements can limit their activity by suppressed mobility (Zhou et al., 2020). In contrast, methylation within gene bodies has been linked to increased transcriptional flexibility (Ecker et al., 2018; Roberts & Gavery, 2012). While in vertebrates DNA methylation is largely concentrated at CpG-rich regions near promoters, commonly referred to as CpG islands, invertebrates generally display a more mosaic-like pattern of DNA methylation across gene bodies (Sarda et al., 2012; Suzuki et al., 2007). This genome-wide distribution of gene-body methylation has been suggested to facilitate phenotypic plasticity by enabling transcriptional flexibility (Roberts & Gavery, 2012). Changes in gene-body methylation can influence alternative splicing, for example by promoting exon skipping or the use of alternative transcription start sites (Suzuki et al., 2007). Accordingly, elucidating how such molecular mechanisms are associated with facilitating phenotypic plasticity and local adaptation is essential in the context of species conservation and restoration in changing environments.

Phenotypic plasticity and sex reversal

In most vertebrates, sex is determined genetically through clearly differentiated sex chromosomes, a system known as genetic sex determination (GSD). In many invertebrates, sex determination is influenced by environmental stimuli, commonly referred to as environmental sex determination (ESD) (Hedrick & Hedgecock, 2010). While previous studies have shown that sex determination and reversal are at least partly under genetic control in some oyster species, the apparent absence of sex chromosomes implies limited genetic contribution to sex determination (Adzigbli et al., 2019; Hedrick & Hedgecock, 2010).

Several abiotic factors, including temperature, food availability, photoperiod, and acidification, are known to induce sex reversal in oysters (Da Silva et al., 2009; Joyce et al., 2013; Sun et al., 2023; Yusa, 2007). Many of these environmental stimuli alter DNA methylation patterns in gonadal tissue, facilitating gonad development and gametogenesis into distinct sexes (Sun et al., 2022, 2023, 2024; Venkataraman et al., 2022). Accordingly, epigenetic mechanisms are thought to play a pivotal role in regulating sex determination in response to environmental change (Sun et al., 2022, 2024; Zhang et al., 2018). However, most studies have focused on characterizing the relationship between epigenetics and sexual plasticity in the Pacific oyster, a widely recognized model organism. In contrast, the flat oyster exhibits a different reproductive strategy, with females brooding their eggs internally (spermcasting), rather than releasing both male and female gametes into the environment (broadcasting). Additionally, the proportion of hermaphrodites is considerably higher

in spermcasting species compared to broadcast spawners (Hassan et al., 2018). These inherent differences in reproductive strategy suggest that sex-associated epigenetic mechanisms in *O. edulis* may differ fundamentally from those in *M. gigas*, with distinct regulatory mechanisms potentially underlying the expression of diverged life-history traits (Shpigel, 1989). Furthermore, studies of sex-associated DNA methylation have largely been confined to gonadal tissue, leaving potential associations with sex reversal in somatic tissues unexplored.

While ESD provides flexibility in the onset of gametogenesis and gamete release which can enhance overall population fecundity, it may also disrupt synchronicity during gametogenesis and spawning (Da Silva et al., 2009). Asynchronous spawning can further amplify sweepstakes reproductive success, generating high variability in genetic diversity among reproductive cohorts and potentially reducing overall diversity or increasing the risk of inbreeding (Hedgecock et al., 2007; Taris et al., 2009). Skewed sex-ratios, which often result in male-biased populations, can limit female gene flow and further exacerbate the loss of genetic diversity (Diaz-Almela et al., 2004). These risks are particularly pronounced in hatchery-based production of *O. edulis*, raising concern given that reductions in genetic diversity have been widely reported across aquaculture systems for various species (Fang et al., 2021).

To mitigate these risks, hatchery programs would benefit from tools that enable reliable sex identification and allow balancing of sex ratios in broodstock oysters, thereby facilitating more effective mixing of breeding populations and maintenance of genetic diversity while optimizing production. However, current methods for sex identification either require lethal sampling of gonadal tissue or rely on costly techniques such as magnetic resonance imaging (Da Silva et al., 2009; Davenel et al., 2010). Consequently, there is a strong need for more affordable and accessible approaches to sex determination. Potential links between epigenetic mechanisms and sex reversal in somatic tissues may offer promising alternatives by enabling molecular-based methods for sex identification.

In this context, understanding the mechanisms underlying sex reversal and their epigenetic regulation is particularly relevant for restoration and aquaculture programs. Insights into reproductive plasticity may contribute to improved broodstock management and enhanced aquaculture production. Furthermore, elucidating the relationship between epigenetic processes and sex reversal could facilitate the development of sex-specific molecular markers, potentially forming the basis for non-lethal approaches to sex identification.

The application of molecular techniques in ecosystem monitoring and restoration

The abovementioned paragraphs illustrate the broad range of molecular techniques employed to assess genetic diversity in *O. edulis.* However, as previously mentioned, oyster restoration does not solely focus on species conservation but also aims to restore habitat structure and associated biological communities (Bennema et al., 2020; Colsoul et al., 2021; Thomas et al., 2022). In recent years, marine monitoring approaches have shifted from primarily morphological methods to integrative frameworks that combine both morphological and molecular techniques (Doorenspleet et al., 2025; Rodríguez-Ezpeleta et al., 2021a, 2021b). The advent of high-throughput sequencing technologies has enabled the assessment of biodiversity through environmental DNA (eDNA), derived from genetic material released by organisms into their surroundings (Blackman et al., 2024; Nagler et al., 2022; Suren et al., 2024). In addition to molecular approaches used to assess genetic diversity within oyster populations, broader biodiversity assessments are essential for determining restoration effectiveness. In the North Sea, a wide range of studies have applied both traditional and molecular approaches to monitoring marine biodiversity (Cornelis et al., 2024; Doorenspleet et al., 2025; Kristensen et al., 2026). However, a comprehensive overview of currently used and emerging techniques is lacking. Furthermore, the integration of molecular methods into standardized monitoring frameworks is still limited, and practical strategies for their implementation remain underdeveloped.

Therefore, to understand how molecular tools can be adopted in monitoring programs to support active management and restoration of North Sea ecosystems, a comprehensive overview of available and emerging techniques is required.

Knowledge gaps and research objectives

Genetic insights in evolutionary dynamics and population structure can reveal patterns of genetic divergence and local adaptation, underscoring the importance of integrating genetic information into restoration strategies to improve their effectiveness at local scales. However, genetic characterization of *O. edulis* has predominantly focused on single-nucleotide variation across broad geographic scales. While these studies have provided valuable insights into population dynamics, they offer limited resolution of microgeographic population structure. As such, local populations remain insufficiently characterized, potentially limiting the optimization of restoration efforts at local scales.

In addition, other sources of genomic and epigenomic variation that shape intraspecific diversity remain largely unexplored. For instance, despite growing recognition of the importance of structural genomic variation and its association with local adaptation, such variation remains uncharacterized in *O. edulis*. Similarly, while epigenetic mechanisms may regulate phenotypically plastic traits, such as sex reversal, they remain poorly studied. Together, these knowledge gaps highlight a limited understanding of molecular variation across multiple biological layers in *O. edulis*. Addressing these gaps is essential for improving our understanding of intraspecific diversity and supporting sustainable restoration strategies at local scales. In addition, despite the importance of molecular findings for understanding evolutionary processes and population demographics, these insights are rarely incorporated into restoration practices. Insufficient integration of genetic information can inadvertently reduce genetic diversity, raising concerns about the long-term sustainability of restored populations.

Furthermore, the effectiveness of oyster restoration is additionally determined by the composition of associated species supported by the biogenic reefs. The advent of high-throughput sequencing and bioinformatic analyses has enabled the assessment of biodiversity trough molecular approaches, particularly by targeting eDNA. However, current and emerging molecular tools are rarely integrated into standardized monitoring frameworks. There is a pressing need for a comprehensive overview of the molecular tools available for assessing broader ecosystem biodiversity and for understanding how these tools can be effectively incorporated into routine monitoring programs to support effective management and restoration of North Sea ecosystems.

This thesis addresses these knowledge gaps by characterizing intraspecific genetic and epigenetic variation among *O. edulis* individuals and populations. Based on this work, recommendations are provided for integrating molecular insights into applied restoration and monitoring strategies. Accordingly, the overarching aim of this thesis is:

To comprehensively characterize the intraspecific genetic and epigenetic diversity of the European flat oyster to support sustainable restoration efforts.

This thesis primarily adopts a fundamental approach, aiming to elucidate the genetic and epigenetic characteristics of *O. edulis*, while simultaneously linking these molecular insights to applied strategies for sustainable restoration and management of the flat oyster and their biogenic reef habitats. To achieve this objective, the following research questions were posed:

i) How does genomic variation contribute to patterns of intraspecific diversity within and among O. edulis populations in a restoration context?

ii) How are epigenetic patterns associated with sex reversal in O. edulis, and how can these insights support the development of non-lethal approaches for sex identification?

iii) In what ways can molecular approaches be applied to support ecosystem-scale biodiversity monitoring and restoration management in the North Sea?

Thesis outline

The work described in this thesis employs various molecular approaches to explore the intraspecific (epi)genetic architecture of *O. edulis*, examining multiple layers of variation within biological and evolutionary contexts, thereby providing a molecular foundation for sustainable oyster restoration.

In **Chapter 2** the genetic connectivity among Dutch wild populations is compared with translocated oysters from foreign sources and hatchery-produced oysters, both of which are currently used in local restoration initiatives. This work provides insights into genetic diversity across the Dutch restoration seascape and highlights key genetic considerations for future restoration strategies.

In addition to single-nucleotide genetic variation, structural variants (SV) represent an important source of genetic diversity. Therefore, **Chapter 3** provides a comprehensive characterization of the SV landscape across the *O. edulis* genome, examines its influence on population structure, and assesses the contribution of repetitive elements to genetic variance.

Beyond genetic variation, elucidating how epigenetic mechanisms shape population demographics by regulating phenotypically plastic traits, such as sex reversal, is essential for the effective restoration of *O. edulis*. This is addressed in **Chapter 4** where DNA methylomes from gill tissue were compared across distinct, histologically determined, sex phenotypes, including hermaphroditic, male, and female oysters. This chapter comprehensively characterizes sex-specific epigenetic patterns and their potential regulatory associations with sex reversal by employing a long-read sequencing approach.

Subsequently, in **Chapter 5**, a machine learning approach is adopted, with models trained on sex-specific DNA methylation to identify epigenetic sex markers, potentially paving the way for non-lethal sex identification. The accuracy of sex classification based on these markers is then evaluated across multiple sex phenotypes using an alternative approach that combines bisulfite conversion with amplicon sequencing.

To inform on the integration of molecular insights into applied restoration and conservation, the use of current and emerging molecular approaches for studying biodiversity and ecosystem dynamics in the North Sea is examined in **Chapter 6**. Limitations affecting their incorporation into standardized monitoring practices are discussed, and potential areas for improvement are identified. By addressing key challenges, this chapter offers recommendations to facilitate the integration of molecular tools into routine monitoring, ultimately supporting effective management and restoration of North Sea ecosystems.

Finally, the general discussion, **Chapter 7**, addresses the overarching objective of this thesis in light of the findings from the research chapters. An integrative roadmap is presented to guide the incorporation of molecular insights into applied restoration, supporting the monitoring, management, and recovery of *O. edulis* and their biogenic reef habitats at local sales. This roadmap provides recommendations for molecular monitoring of both genetic diversity in oyster populations and broader ecosystem biodiversity, while also highlighting key avenues for future research.

CHAPTER 2

Insights into a native oyster restoration seascape

Genetic divergence between wild, foreign, and cultured *Ostrea edulis* populations

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Abstract

The European flat oyster (*Ostrea edulis*) is suffering population declines and the species is considered functionally extinct in southeastern parts of the North Sea. To conserve the flat oyster and protect their biogenic habitats, (inter)national restoration projects are implemented. In the Netherlands, this has resulted in a restoration seascape where foreign oysters, from Ireland or Norway, and cultured native oysters are used for restocking. However, their genetic connectivity to wild native oysters in the Netherlands remains unknown. Therefore, genetic population structure and connectivity between wild, foreign, and cultured *O. edulis* populations were investigated. Genotyping of 390 individuals, using a medium-density SNP array, demonstrated that foreign oysters from Ireland and Norway were genetically distinct from wild native oysters, questioning their suitability for restoration purposes. Cultured native oysters showed minimal genetic divergence from wild native oysters, however, exhibited higher kinship. As such, restoration of wild oyster populations by restocking with foreign or cultured native oysters requires careful consideration. Moreover, the recent establishment of two wild populations provided evidence for natural recolonization. Minimal founder effects suggest a rapid genetic recovery from a bottleneck, emphasizing the species' capacity for genetic resilience. Collectively, our findings highlight the importance of integrating genetic monitoring in flat oyster restoration, providing valuable insights into the risks and opportunities associated with the translocation of non-native and cultured oysters for restoration purposes.

Introduction

Biogenic reefs sustain biodiversity worldwide by providing habitats utilized by various species (Kazanidis et al., 2022; Thomsen et al., 2022). Habitat destruction, overfishing, and poor water quality, along with many other anthropogenic stressors, form serious threats to biogenic reefs resulting in the worldwide decline of reef communities (De'ath et al., 2012; Ponti et al., 2021). Recently, the impact of reef degradation on global biodiversity has received substantial attention and restoration efforts are implemented on both national and international scales to restore these habitats (Bos et al., 2023; Pogoda et al., 2019; Pouvreau et al., 2023).

The European flat oyster (*Ostrea edulis*) is considered a keystone species that provides numerous ecosystem services such as water filtration, benthic-pelagic coupling, and wave attenuation (Coen et al., 2007; Rodríguez-Perez et al., 2019; Smyth & Roberts, 2010). Additionally, oyster reefs are recognized for their contribution to marine biodiversity due to the structurally complex habitats they provide, as their biogenic reefs offer foraging, refuge, and reproduction grounds to numerous economically relevant species (Gilby et al., 2018; Grabowski et al., 2022; Humphries & La Peyre, 2015). The flat oyster was once very abundant in European waters (Olsen, 1883; Sander et al., 2021). However, since the early 20th century, populations have been decimated from overfishing and the emergence of various parasites (Berthe et al., 2004; Engelsma et al., 2014; Pogoda, 2019; Pouvreau et al., 2023; Sander et al., 2021). As a result, the flat oyster is considered functionally extinct in many parts of the southeastern North Sea (Beck et al., 2011; Engelsma et al., 2010; Pouvreau et al., 2023).

Over the past decade, restoration programs have been implemented to restore oyster reef ecosystems throughout Europe (Bos et al., 2023; Colsoul et al., 2020; Gilby et al., 2018; Grabowski et al., 2022; Humphries & La Peyre, 2015; Kamermans et al., 2018, 2025). Generally, restocking of flat oyster populations as part of restoration relies on two main strategies: 1) restocking with locally (native) cultured or wild oysters, and 2) translocation of wild or cultured oysters from elsewhere (foreign) (Bos et al., 2023; Colsoul et al., 2020; Ermgassen et al., 2023; Pouvreau et al., 2023; Zu Ermgassen et al., 2023). Notably, the genetic impact and potential risks of both strategies are not well understood and rarely monitored. The flat oyster is a brooding species with sweepstake reproductive success, limiting recruitment (Diaz-Almela et al., 2004; Hedgecock et al., 2007; Lallias et al., 2010b). Additionally, skewed sex-ratios are common in wild populations, where reduced female gene flow is frequently observed (Diaz-Almela et al., 2004; Kamphausen et al., 2011; Lallias et al., 2010b). This limited reproductive output and broodstock contribution can decrease genetic diversity, posing a threat to a population's resilience (Hughes et al., 2008). In aquaculture settings, where broodstock sizes are typically smaller than in natural populations, there is an increased

risk of loss of genetic diversity (Šegvić-Bubić et al., 2020). Therefore, the use of cultured oysters for restoration can form a high risk to the genetic diversity of remnant wild populations. As such, genetic monitoring is essential for maintaining genetic diversity and should be integrated in restoration efforts. However, genetic diversity is often overlooked as a representative variable for assessing population resilience and health. For instance, the handbook for European native oyster restoration lacks recommendations on genetic monitoring (Zu Ermgassen et al., 2021).

Alternatively to the propagation of cultured native oysters, restoration efforts also involve restocking degraded flat oyster populations with translocated individuals from foreign regions (Bos et al., 2023). Flat oysters have a history of extensive management and exploitation, where evidence of translocations trace all the way back to the Mesolithic (Lewis et al., 2016; Thurstan et al., 2024). Recently, translocating oysters has become increasingly restricted due to the widespread prevalence of the parasite *Bonamia ostreae* (Sas et al., 2020). Since its introduction into European waters in the early 1970s, the parasite has persistently caused high mortality rates among flat oyster populations (Engelsma et al., 2010; Lallias et al., 2008). To prevent any further spread of the parasite, the translocation of oysters from infected areas to non-infected areas is strongly discouraged (Pogoda et al., 2019). In the Netherlands, all known remaining wild populations are currently infected with *B. ostreae* (Engelsma et al., 2010). Consequently, to kick-start local flat oyster reef recovery, *Bonamia*-free oysters from Norway and Ireland are used in national restoration programs (Bos et al., 2023). While the physiological performance of these foreign oysters is routinely monitored, their genetic divergence from the remnant wild native Dutch populations remains unknown (Bos et al., 2023).

To date, the genetic structure of Dutch oyster populations remains understudied and insights into the genetic effect of restoration practices are lacking. Although previous studies have highlighted the presence of phylogeographical structuring in Atlantic and Mediterranean oysters, knowledge on the genetic diversity and connectivity in Dutch oyster populations remains scarce, as earlier studies included only two sampling sites from the Netherlands (Alves Monteiro et al., 2024; Hayer et al., 2021; Lapègue et al., 2023; Sambade et al., 2022; Vera et al., 2016). Recently, two additional wild *O. edulis* populations were discovered along the southwestern coast of the Netherlands (Christianen et al., 2018; Kardinaal et al., 2021). One of these populations is located in the Maasvlakte, a heavily exploited port in the Rotterdam area that was only recently constructed (Kardinaal et al., 2021). The rapid establishment of these two populations is indicative of the adaptive and regenerative potential of the species. However, their vast emergence also raises concerns about genetic diversity, as rapid population expansions could imply a potential genetic founder effect with reduced genetic variation (Launey et al., 2001). In order to maintain genetic diversity and resilience

in wild and restored populations in the Netherlands, a better understanding of the current genetic diversity and phylogeographic population structure is needed (Zu Ermgassen et al., 2023).

To enhance our understanding of genetic diversity in the broader context of ongoing restoration efforts, we investigated the genetic population structure and connectivity between wild, foreign, and cultured oysters. Using a medium-density marker array, comprising of ~14K SNPs, 390 oysters originating from nine study sites in the Netherlands were genotyped. As such, we aimed to provide insights into the genetic diversity within the context of local flat oyster restoration, particularly emphasizing the role of natural recolonization and translocation of foreign and cultured oysters.

Materials & Methods

Sample collection & DNA extraction

Flat oysters were collected from a total of nine study sites, representing four wild native populations, two wild foreign populations, and three cultured native populations (Table 1; Fig. 1A). The four wild native populations are the only "naturally functioning" remaining oyster beds in the Netherlands, and are located in the southwest of the Netherlands: lake Grevelingen (GR), the Oosterschelde estuary (OS), the Voordelta area in the North Sea (VD), and the Maasvlakte port of Rotterdam (MA). Although the flat oyster populations in lake Grevelingen and Oosterschelde are originally wild populations, they are heavily exploited and managed, where spat collection and harvesting of consumption-size oysters frequently occurs (Maathuis et al., 2020). The Voordelta and Maasvlakte represent recently established natural populations and have remained relatively undisturbed, likely owing to their recent discovery (Christianen et al., 2018; Kardinaal et al., 2021).

Until recently, flat oysters were considered extinct in the Wadden Sea area. However, in 2017, a handful of oysters were discovered in the Dutch Wadden Sea. To investigate their potential for re-establishment, a culturing experiment was conducted at the Royal Netherlands Institute for Sea Research (NIOZ) in 2018, using 38 oysters as broodstock population (Jacobs et al., 2020). The progeny of this broodstock population were used in the present study to represent the remnant Wadden Sea population. Although derived from wild-origin oysters rather than a true hatchery strain, these individuals are hereafter referred to as "cultured native" (Table 1).

Cultured native oysters were produced in commercial hatcheries (H1 and H2) in the Netherlands, and represent the offspring of oysters originating from the Oosterschelde (OS). For the H1 population, spat was produced from combining reproductive cohorts produced by two broodstock batches, each comprising 100 oysters. For H2, spat was produced by pooling reproductive cohorts produced by 12 distinct broodstock batches, each consisting of 15 oysters. A previous study estimated parental contributions for the WS and H2 population, determining that 16 individuals contributed to reproduction in each population (Sambade et al., 2022). The number of contributing parents in the H1 population remains unknown.

The wild foreign oysters from Ireland (IR) were collected in Tralee Bay, and Norwegian oysters (NW) were collected in Hafrsfjord, Norway. These two populations are currently the only Bonamia-free sources used in Dutch restoration projects, as such they were included in this study (Bos et al., 2023).

For each oyster, genomic DNA was extracted from gill tissue using the DNeasy Blood and Tissue kit (Qiagen), following the manufacturer's instructions. Quality of extracted DNA was visualized using agarose gels and quantity was checked using the Qubit HS assay (Thermofisher Scientific, Waltham, MA, USA).

Table 1 | Oyster sampling site characteristics, sampling sizes (N), and status of origin. For native cultured oysters from H1 and H2, Latitudes and Longitudes (DD) refer to the geographical location where the broodstock oysters originate from.

Sampling	Geographic Location	Latitude	Longitude	N	Status
GR	Grevelingen, Netherlands	51.76245	3.96400	62	Wild native
OS	Oosterschelde, Netherlands	51.63766	3.83037	45	Wild native
VD	Voordelta, Netherlands	51.78583	3.85917	43	Wild native
MA	Maasvlakte, Netherlands	51.96022	4.00970	44	Wild native
WS	Wadden Sea	53.17750	4.88611	31	Cultured native
H1	Hatchery 1 (Stichting Zeeschelp)	51.63766	3.83037	51	Cultured native
H2	Hatchery 2 (Roem van Yerseke)	51.63766	3.83037	32	Cultured native
IR	Tralee Bay, Ireland	52.29001	-9.92999	37	Wild foreign

SNP genotyping & quality filtering

Genomic DNA extracts were genotyped using a combined-species medium-density Affymetrix Axiom array covering 14,950 SNPs for *O. edulis* (Gutierrez et al., 2017). Genotyping was carried out on the GENTYANE platform of INRAE Clermont-Ferrand. Raw data (CEL format) was imported into Axiom Analysis Suite (v2.0.035, Affymetrix) for quality control and genotype calling. Samples with a dish quality control (DQC) < 0.78,

and/or call rate (QC) < 0.94 threshold were discarded following the "Best Practices Workflow", recommended by Affymetrix (Gutierrez et al., 2017). After filtering, 10,407 variants were retained. In total, 390 individuals representing nine study sites were successfully genotyped and passed all filtering thresholds. Genotyped markers were mapped to the *O. edulis* reference genome xbOstEdul1.1 (NCBI RefSeq Assembly: GCF_947568905.1) (Adkins et al., 2023).

Chromosomal inversion genotypes

To explore signatures of structural variation, notably putative large chromosomal inversions, principal component analysis (PCA) was performed on the initial 10,407 variants from the Total panel (Table 2). PCAs and association analyses indicated the presence of three large genomic regions causing a segregation of samples that did not correlate with geographical population structure (Supplementary Fig. S1 & S2). The majority of SNPs associated with this structure were located on the chromosomes NC_079167.1, NC_079169.1, and NC_079172.1 (Supplementary Fig. S3). Similar stretches of structural variation (SV) were reported in prior studies (Alves Monteiro et al., 2024; Lapègue et al., 2023). To confirm whether SVs found in this study correspond to previously reported SVs, the three genomic regions were mapped to two other publicly available reference genomes: Oe-Roscoff_1 (RefSeq:GCA_024362745.1; Boutet et al., 2022) and OEROSLIN_1.1 (RefSeq:GCF_023158985.2; Gundappa et al., 2022), using minimap2 v2.28-r1209 (Li, 2021) (Supplementary Table S1). As discussed in previous literature, the three genomic stretches of structural variation are suggestive of chromosomal inversions, which represent genomic DNA fragments of reversed orientation. To genotype inversion frequencies, SNPs were recoded to numeric allele counts using -recode A in PLINK. For each individual, the mean genotype across all SNPs located within a chromosomal inversion was calculated. Individuals were then assigned to genotype classes according to the following thresholds: mean genotype <0.5 for homozygous reference (0/0), mean genotype between 0.5 and 1.5 for heterozygotes (0/1 or 1/0), and mean genotype >1.5 for homozygous inversion (1/1). Genotype frequencies were then calculated as the average frequency across all individuals for each of the three chromosomal inversions.

SNP pruning

Because SVs can overshadow local geographical population structure, all loci located within the three chromosomal inversion regions were omitted in subsequent analyses (Vendrami et al., 2019). Variants were pruned to study spatial population structure without bias of correlated variants. Pruning was performed in PLINK using --indep-pairwise 50 5 0.5, representing a window of 50 SNPs, a step size of 5 SNPs, and an LD threshold value of 0.5, resulting in a total of 8,900 variants. Another 497 variants deviated from Hardy-Weinberg equilibrium (hwe; p-value < 0.01), and were extracted

from the dataset. This resulted in a final set of 8,403 variants for downstream analyses (Pruned panel; Table 2). Association analyses showed that variants associated with the three genomic inversions were successfully removed (Supplementary Fig. S4).

Genetic population structure and connectivity

From the Pruned panel, population structure across all nine study sites was investigated by conducting principal component analysis (PCA) and admixture analysis, using PLINK v1.90b7.2 and ADMIXTURE v1.3 respectively (Alexander et al., 2009; Chang et al., 2015; Steiß et al., 2012). To identify the optimal number of clusters, fivefold cross-validation was performed using the --cv flag in ADMIXTURE.

To gain insight into general population descriptive statistics, minor allele frequencies (MAF), levels of heterozygosity, nucleotide diversity (π), and Tajima's D were calculated after pruning, using vcftools v0.1.16 (Danecek et al., 2011). Two different measures of genome-wide diversity were estimated: nucleotide diversity (π), which quantifies the average number of pairwise differences among individuals; and Tajima's D, which is based on the estimations of the average number of nucleotide differences and the number of segregating (polymorphic) sites (Tajima, 1989). Levels of nucleotide diversity (π) and Tajima's D were calculated over windows of size 10,000 bp, averaged over all individuals per study site and compared between study sites. Observed heterozygosity – He (O) - was calculated as the fraction of heterozygotes (1-observed homozygotes) from the total number of individuals. MAF and Fis were calculated using -freq in PLINK.

To study kinship within and between study sites, pairwise relatedness (phi) was calculated using --relatedness2 in vcftools. Identity by descent (IBD) was estimated from genome-wide identity by state (IBS) for each pair of individuals, based on the average proportion of shared alleles and expressed as the proportion IBD (Pi_hat), using --genome in PLINK.

PCA, admixture analysis, and estimations of relatedness across all nine study sites revealed that native cultured oysters (WS, H1, and H2) contained a high proportion of related individuals. Highly related individuals can dominate apparent population substructures (Vendrami et al., 2019). Thus, to better study population structure between wild native (GR, OS, VD, and MA) and foreign populations (IR and NW), cultured native oysters (WS, H1, and H2) were omitted from the dataset, resulting in the "Pruned wild panel" of 9,293 variants (Table 2). For this selective dataset, PCA, admixture analysis, and pairwise Fst values were calculated, where pairwise Fst values were computed between all individuals using PLINK -Fst and --within-pop.

To study genetic divergence across all five native oyster populations (GR, OS, VD, MA, and WS), descriptive statistics (e.g. Fis, He, IBD, and relatedness), PCA, admixture

analysis, and IBD network graphs were studied from the Pruned native panel (8,403 variants; Table 2). Because the WS oysters represent the offspring of one reproductive batch of the native Wadden Sea population, and comprised a high proportion of related individuals, which may overshadow phylogeographic population structure, data was subsampled by omitting related individuals, using --rel-cutoff 0.10 in PLINK. After removal of related individuals, only 6 individuals were retained for the WS population.

Non-parametric Kruskal-Wallis tests and subsequent Dunn's post hoc tests were used to compare mean genetic descriptive statistics between populations, using a Bonferroni correction for multiple testing. Plots were generated in Python using matplotlib and seaborn (Hunter, 2007; Waskom, 2021).

Results

Structural genome variation

A total of 390 oysters were successfully genotyped, with a total of 10,407 variants passing the quality filtering thresholds for DQC and QC (Total panel; Table 2). Principal component analysis (PCA) revealed clustering biased by structural variation (SV) rather than based on spatial population structure (Supplementary Fig. S1). Association analysis revealed three genomic stretches of outlier loci along the first three PC axes (Supplementary Fig. S2). These three regions overlap with parts of chromosomes NC_079167.1, NC_079169.1, and NC_079172.1, and are indicative of chromosomal inversions. Similar occurrence of SVs was detected in earlier studies covering different *O. edulis* populations and different genotyping methods (Alves Monteiro et al., 2024; Sambade et al., 2022). The inversion-associated SNPs identified in this study, using the XbOstEdul1.1 reference genome, were mapped against two other publicly available reference genomes for *O. edulis*: OEROSLIN_1.1 (Gundappa et al., 2022) and Oe-Roscoff_1 (Boutet et al., 2022). The three inversion regions on chromosomes NC_079167.1, NC_079169.1, and NC_079172.1 mapped to scaffolds 2, 5, and 8 for OEROSLIN_1.1, and scaffolds 5, 4, and 8 for Oe_Roscoff_1 respectively (Supplementary Table S1 (Boutet et al., 2022; Gundappa et al., 2022).

Estimations of inversion genotype frequencies across populations revealed that the occurrence of homozygous inversion genotypes (1/1) was generally lower for NC_079169.1 and NC_079172.1 than for NC_079167.1 (Supplementary Fig. S5). The genotype frequencies exhibited a geographical trend along the chromosomes NC_079167.1 and NC_079172.1. For the inversion on chromosome NC_079167.1, NW and WS exhibited the lowest frequencies of inversion homozygotes (1/1), whereas the most southwestern population (IR) displayed the highest frequency of inversion homozygotes (Supplementary Fig. S5A). A similar, but less pronounced north to south

cline was visible along chromosome NC_079172.1, where only the MA, H1, IR, VD, and OS populations contained a small proportion of inversion homozygotes (1/1) (Supplementary Fig. S5C).

Because inversion-associated SNPs can dominate local geographical population structure, all SNPs that fell within these three chromosomal inversion regions were omitted from subsequent analyses. In total, 765 SNPs associated with inversions located on chromosomes NC_079167.1, NC_079169.1, and NC_079172.1 were removed (Table 2). Subsequent association analysis with the first 4 PC axes revealed all SNPs associated with the three genomic inversions were omitted (Supplementary Fig. S4).

Table 2 | Overview of the four SNP panels used for population structure analyses of nine wild, foreign, and cultured *Ostrea edulis* study sites (GR, OS, VD, MA, WS, H1, H2, IR, and NW). With number of SNPs and number of individuals (N) indicated per panel.

SNP Panel	SNPs	N	Details
Total panel	10,407	390	The total set of variants
Pruned panel	8,403	390	Pruned variants for all individuals from all nine study sites (GR, OS, VD, MA, WS, H1, H2, IR, and NW)
Pruned wild panel	9,293	277	Pruned variants for individuals from wild native (GR, OS, VD, and MA) and wild foreign study sites (IR and NW)
Pruned native panel	8,403	199	Pruned variants for individuals from native study sites (GR, OS, VD, MA, and WS)

Genetic population structure of wild, foreign, and cultured oysters

Pruning of SNPs that were not in hwe resulted in a Pruned panel containing 8,403 SNPs that were used to assess the overall genetic population structure across all nine study sites (Table 2; Fig. 1A). The first principal component separated H2 from all other study sites (Fig. 1B), while PC2 separated IR from the main cluster, which included GR, OS, VD, MA, WS, H1, and NW. Within this main cluster, WS and NW demonstrated finer-scale clustering.

Admixture analysis resulted in similar genetic population structure as observed from the PCA (Fig. 1C; Supplementary Fig. S6). Cross-validation analysis identified 6 clusters as the optimal number, with GR, OS, VD, MA, and H1 showing similar genetic cluster assignments (Supplementary Fig. S7). Both wild foreign study sites (IR and NW) displayed genetic divergence from wild native oysters, and showed predominant assignment to distinct genetic clusters, C3 and C5 respectively. WS and H2 displayed high within-population variability in terms of genetic cluster contribution, which is likely due to elevated kinship between individuals. Individuals from H1 displayed a population structure comparable to that of wild native oysters.

Average estimations of nucleotide diversity did not differ significantly between

populations (Supplementary Fig. S8A). Estimations of Tajima's D were slightly higher for oysters from H2 compared to all other populations, albeit not significant (Supplementary Fig. S8B).

Table 3 | Overview of study sites and descriptive population genetic estimates: MAF, He (O) and He (E), and Fis, for the Pruned dataset (including 8,403 variants). MAF, minor allele frequency; He (O), observed heterozygosity; He (E), estimated heterozygosity; Fis, Inbreeding coefficient. For MAF, He (O), He (E), and Fis lowercase letters denote statistically significant differences ($p < 0.05$) based on the non-parametric Kruskal-Wallis test, followed by a post-hoc Dunn test.

Sampling Site	Geographic Location	MAF	He (O)	He (E)	Fis
GR	Grevelingen, Netherlands	0.1955^{ac}	0.2767^{a}	0.27801^{abc}	0.004548^{a}
OS	Oosterschelde, Netherlands	0.1958^{ac}	0.2774^{a}	0.27805^{ab}	0.002459^{a}
MA	Maasvlakte, Netherlands	0.1953^{ac}	0.2767^{a}	0.27795^{abd}	0.004462^{a}
VD	Voordelta, Netherlands	0.1946^{ac}	0.2770^{a}	0.27794^{abc}	0.003148^{a}
WS	Wadden Sea	0.1949^{bc}	0.2769^{a}	0.27794^{ac}	0.003624^{a}
H1	Hatchery 1 (Stichting Zeeschelp)	0.1954^{abc}	0.2771^{a}	0.27808^{b}	0.003477^{a}
H2	Hatchery 2 (Roem van Yerseke)	0.1932^{b}	0.2767^{a}	0.27780^{c}	0.004121^{a}
IR	Tralee Bay, Ireland	0.2015^{ac}	0.2796^{a}	0.27780^{cd}	-0.006551^{a}
NW	Hafrsfjord, Norway	0.1955abc	0.2703^{b}	0.27780^{abc}	0.027699^{b}

When comparing descriptive genetic statistics across populatoins, estimates of average minor allele frequency (MAF) were similar between all nine populations (mean 0.196±0.151; Table 3). Observed heterozygosity - He (O) - was significantly lowest for NW, and highest for IR, albeit not significant (Fig. 1D; Table 3). Estimations of expected heterozygosity - He (E) - were highly similar across populations (Table 3; Supplementary Fig. S9). The inbreeding coefficient (Fis) was significantly highest for NW (mean 0.006±0.23; Fig. 1E; Table 3).

In terms of kinship, values of within-population relatedness were significantly higher for WS, H2, and IR, with H2 exhibiting the highest values of within-population kinship estimates (Supplementary Fig. S10). The elevated relatedness within H2 may explain the cryptic within-population structure observed along PC1 in the PCA (Fig.1B; Supplementary Fig. S10A). Within-population IBD estimates were significantly higher for both WS, H2, IR, and NW, where H2 displayed highest values (Supplementary Fig. S10B).

Population structure as explained by the IBD network graph was in concordance with findings from the PCA and admixture analysis, where oysters from IR and NW clustered separately (Fig. 1F). Analysis of inter-population connectivity by pairwise comparisons of IBD estimates indicated that IR and NW exhibit comparable levels of genetic connectivity with Dutch wild native oyster populations (GR, OS, VD, and MA; Fig. 1F; Supplementary Fig. S11).

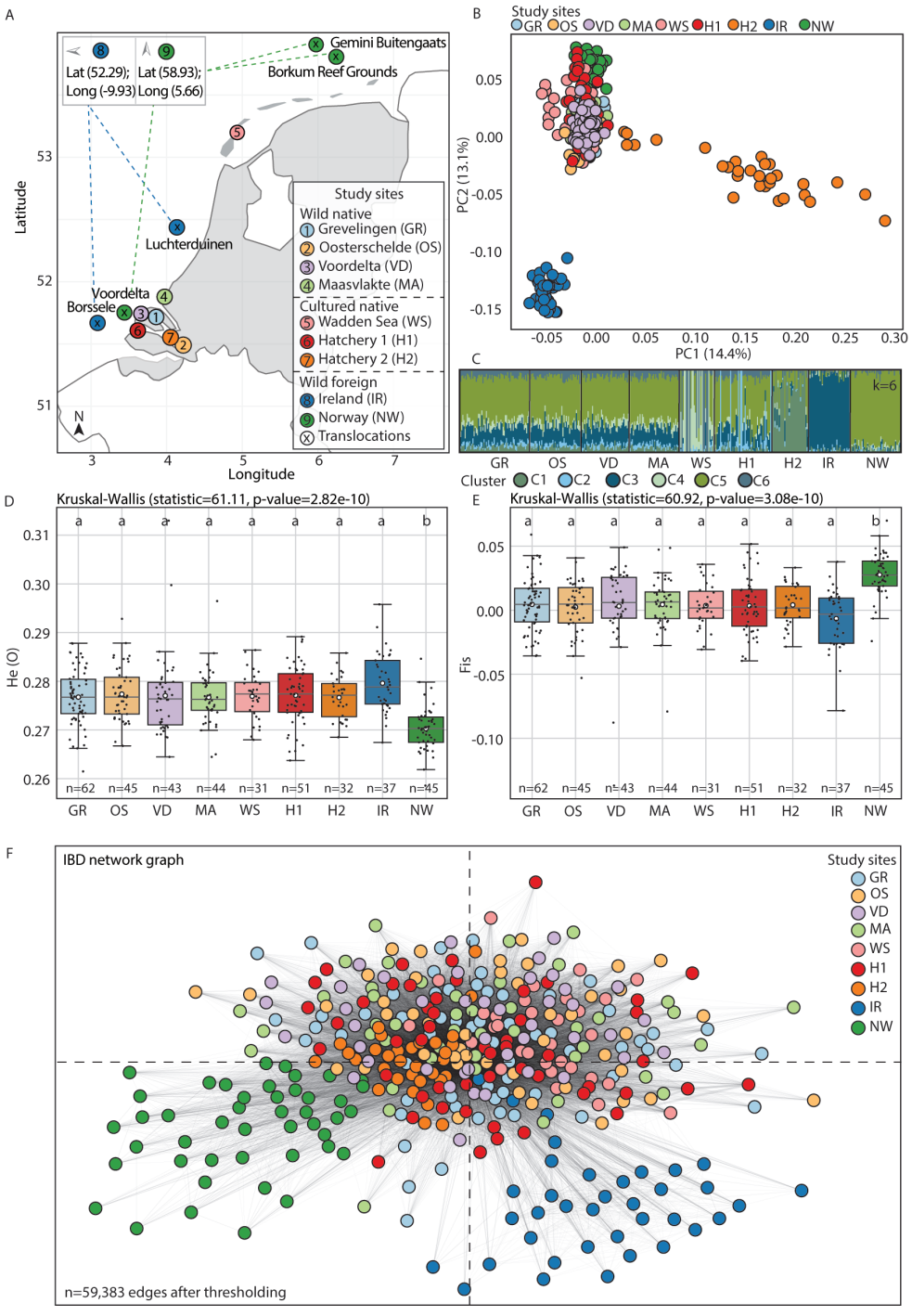


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Figure 1 | Genetic population structure including all nine study sites, based on 8,403 pruned variants (Pruned panel). **(A)** Geographical map with colored dots representing the nine study sites. Dashed lines refer to the four deployment locations where wild foreign oysters originating from IR (Blue) or NW (Green) have been translocated to. **(B)** First two axes of Principal Component Analysis (PCA). **(C)** Admixture analysis where each individual is represented by a vertical line, blocks represent different study sites, and colors represent 6 different genetic clusters (k=6). The optimal number of clusters was determined by fivefold cross-validation. **(D)** Observed heterozygosity - He (O). **(E)** Inbreeding coefficient (Fis). In both boxplots the white dots represent the mean value. Different lowercase letters denote statistically significant differences ($p < 0.05$) for values of He (O) and Fis between study sites. **(F)** IBD network graph based on pairwise IBD estimates between all individuals (n=59,383 edges after thresholding).

Genetic similarity between wild native and wild foreign oysters

To study genetic similarity between wild native and wild foreign study sites, cultured native oysters (WS, H1, and H2) were excluded from the dataset, resulting in the Pruned wild panel, including 9,293 variants (Table 2). The PCA revealed distinct clustering of IR and NW from wild native oysters (GR, OS, VD, and MA; Fig. 2A).

Overall, comparisons of pairwise genetic differentiation estimates (Fst) indicated low levels of genetic differentiation in allele frequencies between wild native and wild foreign study sites (mean 0.017±0.007; Fig. 2B). Among all comparisons, the highest pairwise Fst value were observed between the wild foreign sites IR and NW (Fst 0.0324). Admixture analysis of wild native and wild foreign study sites revealed similar population structure as the PCA, where wild foreign oysters (IR and NW) were assigned to distinct genetic clusters compared to wild native oysters (GR, OS, VD, and MA), which were predominantly assigned to a single genetic cluster (Fig. 2C).

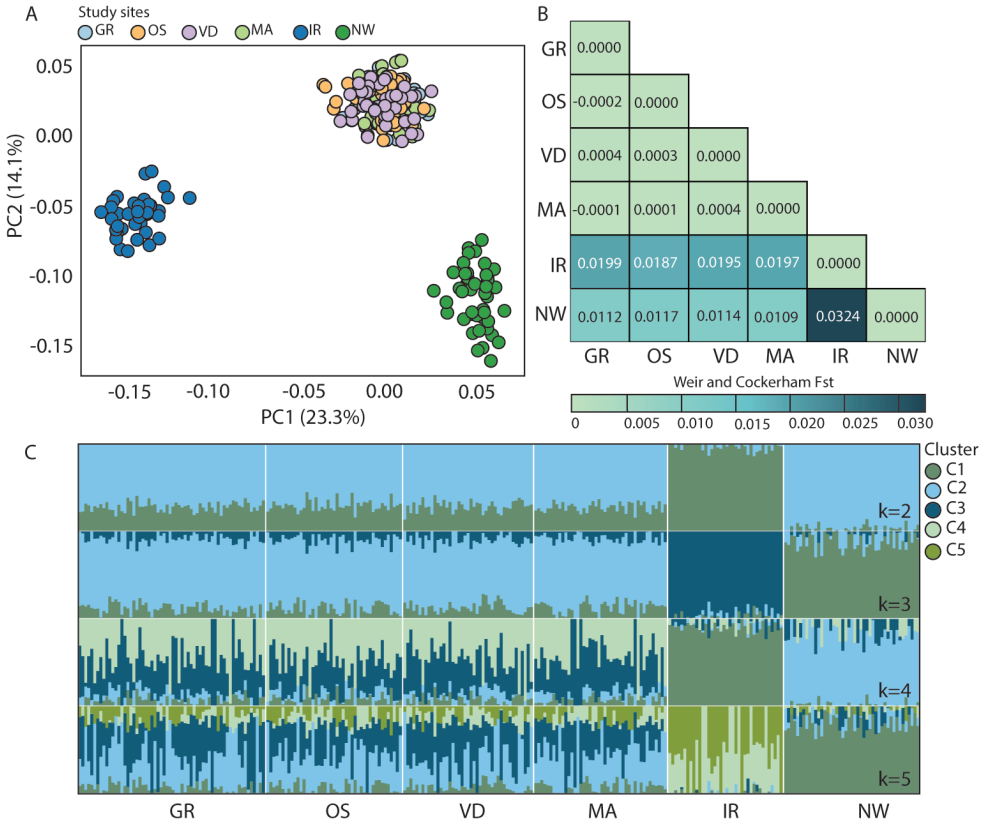


Figure 2 | Genetic population structure of wild and foreign populations, based on 9,293 pruned variants from the Pruned wild panel. **(A)** First two axes of Principal Component Analysis (PCA). **(B)** Heatmap of Weir and Cockerham pairwise Fst values. **(C)** Admixture analysis (k=2-5), where each individual is represented by a vertical line. Fivefold cross-validation analysis identified 2 clusters as the optimal number of clusters (Supplementary Fig. S12). The horizontal blocks represent the six study sites (GR, OS, VD, MA, IR, and NW). Each color represents a different genetic cluster (C1-C5).

Population structure and connectivity among native oysters

As patterns of phylogeographical population structure can get overshadowed wen populations contain a high proportion of closely related individuals, higlhy0related individuals were excluded from the dataset to allow for studying population structure across Dutch native oyster sites (Vendrami et al., 2019). This resulted in the Pruned native panel, including 8,403 variants for 199 individuals rom GR, OS, VD, MA, and WS (Table 2).

PCA revealed that all native oysters formed a single uniform genetic cluster (Fig. 3A). Similarly, IBD network analysis showed all five native oyster sites contributed to a uniform population (Fig. 3B). Generally, both estimates of inbreeding coefficient (Fis: mean -0.001±0.023) and observed heterozygosity (He(O): mean 0.277±0.006) were similar across all five native populations and did not differ significantly (Supplementary Fig. S13). Although not statistically significant, analysis of within-population relatedness and IBD revealed that the more recently established populations, VD and MA, contained a larger proportion of closely related individuals, visible as outliers from the boxplots (Fig. 3C & D). These elevated values of kinship observed individuals from VD and MA could be indicative of a founder effect. WS oysters exhibited significantly higher values of relatedness, although based on a small sample size (n=6).

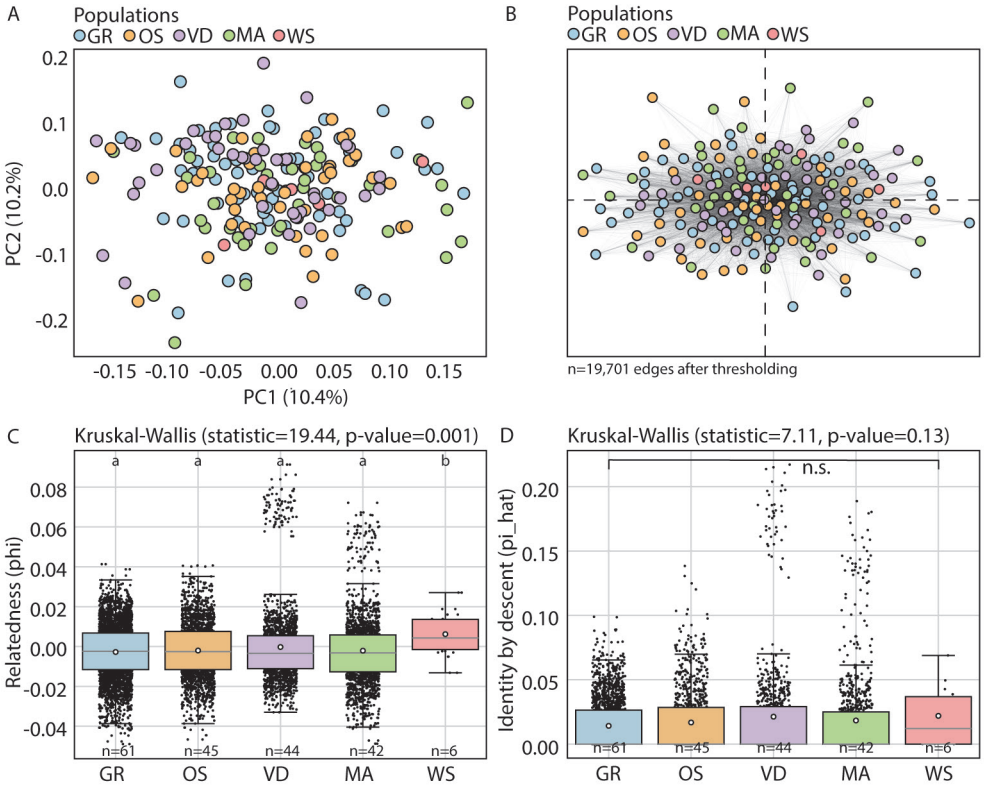


Figure 3 | Genetic population structure of native sampling sites (GR, OS, VD, MA, and WS), using the Pruned native panel with 8,403 variants. **(A)** First two axes of Principal Component Analysis (PCA). **(B)** IBD network graph based on pairwise IBD estimates between all individuals (n=19,701 edges after thresholding). **(C)** Within-population relatedness (phi). **(D)** Within-population pairwise IBD (pi_hat). For boxplots in C and D, white dots represent population mean values. Different lowercase letters denote statistically significant differences ($p < 0.05$) for values of relatedness and IBD between study sites, non-significant differences are denoted by "n.s.".

Discussion

Understanding the genetic population structure of *O. edulis* is vital for its conservation and restoration (Zu Ermgassen et al., 2023). Given ongoing restoration efforts and the intensified management of populations, genetic monitoring is critical to preserve high levels of genetic diversity and prevent inbreeding. Here, we aimed to study the genetic population structure and similarity between wild, foreign, and cultured oysters within the Dutch restoration seascape of an ecologically relevant but intensively exploited oyster species.

Genetic divergence between native and foreign oyster populations

Using a medium-density SNP array, we characterized genetic diversity across nine study sites of wild, foreign, and cultured flat oysters within the Netherlands. Our findings show that despite the numerous translocations of oysters across Europe, a substantial phylogeographical population structure remains present, which is in accordance with previous findings (Alves Monteiro et al., 2024; Lapègue et al., 2023). Foreign Irish (IR) oysters predominantly contributed to a different genetic cluster compared to all other populations, whereas wild native oysters represented a uniform population in the Netherlands. These genetic differences were also highlighted by previous studies on European *O. edulis* populations (Lapègue et al., 2023; Sambade et al., 2022). In a study of Lapègue et al (2022), the genetic differentiation found between North Sea and Atlantic populations is argued to be caused by the English Channel, potentially representing a physical barrier of gene flow (Lapègue et al., 2023). Similar to Irish oysters, Norwegian oysters exhibited genetic divergence from wild native Dutch oysters. Interestingly, the observed reduced heterozygosity and weak signatures of inbreeding have not been reported in previous studies (Alves Monteiro et al., 2024; Lapègue et al., 2023). Norwegian oysters that have been used as a source for restocking in Dutch restoration projects originate from the Hafrsfjord (Bos et al., 2023). The Hafrsfjord is a fjord system in the southern part of Norway, with a narrow opening and relatively little connection to open water (Mortensen et al., 2024). Reduced gene flow in fjord systems is frequently observed in marine species, and could explain the observed decrease in heterozygosity (Breistein et al., 2022; Hansen et al., 2007; Johannesson et al., 2018). In contrast to our findings, a study of Alves Monteiro et al. (2024) revealed that Norwegian oysters from Hafrsfjord clustered together with native oysters from Grevelingen and the Wadden Sea (Alves Monteiro et al., 2024). This discrepancy may be attributed to differences in the sampling sizes of the Dutch and Norwegian populations between the two studies.

Collectively, the reduced heterozygosity and elevated levels of inbreeding observed in foreign populations suggest that their inclusion in future restoration initiatives should be carefully evaluated, emphasizing the necessity of ongoing genetic monitoring.

It should be emphasized that the sampling in this study represents a single temporal snapshot, as sampling was not conducted across multiple years. This limited temporal scope may underestimate genetic diversity within these populations. A more comprehensive analysis incorporating larger sample sizes and multi-year sampling is required to more accurately characterize patterns of genetic resilience and temporal stability.

Opportunities and challenges of utilizing cultured oysters for restoration

Cultured native oysters (H1 and H2) showed high genetic similarity with wild native oysters and displayed comparable levels of genetic diversity. Given the potential for local adaptation to environmental conditions, restoration initiatives advocate the use of native oysters for local restoration practices (Vera et al., 2016; Zu Ermgassen et al., 2020). The prevalence of *Bonamia ostreae* in Dutch populations represents a major issue in native restocking for restoration purposes, as translocating infected oysters to non-infected areas is highly discouraged to prevent further spread of the parasite (Kamermans et al., 2023; Zu Ermgassen et al., 2023). Interestingly, Dutch wild oysters are thought to have developed enhanced resilience against the pathogen's virulence, with some studies reporting genomic signatures indicative of resistance (Kamermans et al., 2023; Sambade et al., 2022; Vera et al., 2019). To this extent, culturing locally-adapted native oysters might pose a favorable alternative to the translocation of foreign oysters that remain naïve to a *Bonamia* infection (Lapègue et al., 2023; Mortensen et al., 2024). Currently, non-destructive screening methods are applied to identify disease-free broodstock for culturing by hatcheries, and genetic approaches for the selection of *Bonamia*-resistant genes are under development (Kamermans et al., 2023; Vera et al., 2019). However, selective breeding can potentially decrease genetic diversity and thereby increase the risk of inbreeding depression (Charlesworth & Willis, 2009; Hollenbeck & Johnston, 2018). Therefore, there is an urgent need for combining genetic selection for resistance against *B. ostreae* with the development of a breeding program that aims to preserve genetic diversity.

Although cultured populations displayed high genetic similarity to wild native oysters, both cultured oysters from H1 and H2 exhibited elevated levels of kinship relative to wild populations. The individuals from H1 and H2 represent the offspring of wild-caught oysters originating from the Oosterschelde (OS), but were produced from only a few reproductive cohorts with broodstock sizes of a maximum of 200 oysters. Flat oysters generally display large variation in reproductive output and sex-ratios are often male-

biased (Da Silva et al., 2009; Davenel et al., 2010; Diaz-Almela et al., 2004; Hedgecock et al., 2007; Kamphausen et al., 2011). As such, a single reproductive cohort may be sourced from only one or a few females and can show considerable variation in the number of contributing males (Diaz-Almela et al., 2004; Lallias et al., 2010b). Due to this sweepstake reproductive success, levels of kinship between individuals from one reproductive cohort can be high, but can also vary greatly between reproductive cohorts or hatcheries, as was observed in this study (Hedgecock et al., 2007; Lallias et al., 2010b; Naciri-Graven et al., 2000; Vera et al., 2016). Accordingly, high levels of kinship within a population can lead to a reduction of genetic diversity and eventually induce inbreeding in future generations (Charlesworth & Willis, 2009; Lallias et al., 2010b, 2010a). In turn, elevated levels of inbreeding can have negative implications on the performance or resilience of a population, especially in stressful circumstances (Camara et al., 2008; Fang et al., 2021; Fang & Li, 2023). Practices aimed to preserve genetic diversity remain mostly undocumented and can differ between hatcheries. Increasing broodstock size and combining multiple reproductive cohorts are expected to reduce kinship among individuals and preserve genetic diversity, enhancing the suitability of cultured oysters for restoration (Lallias et al., 2010a; Sambade et al., 2022). However, these expectations require further validation with increased sample sizes and the inclusion of multiple reproductive cohorts. Consequently, the use of cultured oysters for restocking wild populations as a restoration strategy should be considered with care. Integrating genetic monitoring with the development and implementation of a standardized breeding program will help ensure that sufficient genetic diversity is maintained in future restoration efforts (Lallias et al., 2010a).

Founder effect in recently established wild flat oyster populations

Comparing genetic connectivity across native oyster populations (GR, OS, VD, MA, and WS) revealed that all five sampling sites comprise a uniform population. Generally, average levels of genetic diversity (He (O): ~0.2774; He (E): ~0.2771) were similar to those reported by Sambade et al. (2022). The elevated kinship observed among individuals of both Voordelta (VD) and Maasvlakte (MA) populations is indicative of a founder effect caused by a recent recolonization event, which confirms the recent establishment of both populations (Christianen et al., 2018; Kardinaal et al., 2021; Szűcs et al., 2017). The relatively low proportion of highly-related individuals in both populations is suggestive of swift genetic recovery and a rapid post-founder population expansion. Rapid genetic recovery can be stimulated by high gene flow and limited genetic divergence between populations (Dupont et al., 2003; Roman, 2006). An important lesson learnt from these recent establishments is that flat oysters show capable of natural regeneration and population expansion. Therefore, facilitating the natural recolonization of populations may be preferable to translocating foreign oysters, as genetic diversity, local adaptation, and potential resilience or resistance

against *B. ostreae* can be retained. However, natural recolonization of native oysters may contribute to the further spread of the parasite and implementation of such a restoration strategy requires careful consideration.

Genetic connectivity between Wadden Sea oysters and other native oyster populations

Population structure analysis across the five native oyster populations (GR, OS, VD, MA, and WS) revealed that WS oysters contribute to the same genetic cluster as the other four native populations (GR, OS, VD, and MA). In contrast to our findings, genetic divergence between oysters from the Wadden Sea and Grevelingen populations was previously reported by Alves Monteiro et al. (2024). It is important to note that our study was based on a relatively small sample size and. More critically, the Wadden Sea population comprised numerous closely related individuals, as it consists of the progeny of one reproductive cohort representing wild native oysters originating from the Wadden Sea. Inclusion of these related individuals in genetic studies can mask potential population structure and should therefore be interpreted with caution (Vendrami et al., 2019). To minimize the confounding effect of kinship on underlying population structure, we decided to subsample the dataset by excluding all highly-related individuals, at the cost of further reducing the sample size. The inclusion of highly-related individuals in the dataset of Alves Monteiro et al. (2025), may account for the genetic divergence they observed between the WS population and other native Dutch oyster populations.

Flat oysters are thought to have gone extinct in the Wadden Sea area around 1930 (Berghahn & Ruth, 2005; Ewers et al., 2025). The extreme dynamic conditions that characterize this shallow, tidal inlet system are suggested to have played a major role in their local decimation (Ewers et al., 2025). Phylogeographic reconstruction based on DNA isolated from shell remains of the historic Wadden Sea population revealed genetic divergence with present-day European populations (Ewers et al., 2025; Hayer et al., 2021). These studies hypothesize that local adaptation to historic Wadden Sea conditions may have been maladaptive to current Wadden Sea environmental conditions, which potentially contributed to the demise of Wadden Sea flat oysters (Ewers et al., 2025; Hayer et al., 2021). Interestingly, since the 1950s, flat oysters are occasionally found in different areas of the Wadden Sea (Berghahn & Ruth, 2005; Sambade et al., 2022; van der Have et al., 2018). Although our findings are based on a limited sample size for the remnant WS population, the findings indicate panmictic clustering of all native Dutch populations. This connectivity suggests ongoing gene flow between the Wadden Sea and other native Dutch oyster populations. It remains unclear whether the present-day Wadden Sea oysters analyzed in this study represent remnants of a historical relict population (Ewers et al., 2025), or whether they have

more recently recolonized the Wadden Sea through natural larval dispersal or via human-mediated translocations. To clarify the genetic population structure of these remnant oysters, more comprehensive and representative sampling is required.

Structural variation

Mollusks are known for their complex genomes and exceptionally high levels of structural variation (Calcino et al., 2021). Structural variation can contribute to population differentiation or reveal signatures of selection or local adaptation (Wellenreuther & Bernatchez, 2018). In *O. edulis*, the occurrence of chromosomal inversions were repeatedly reported, and the three major inversions characterized by previous studies were also detected in the present study (Alves Monteiro et al., 2024; Lapègue et al., 2023; Sambade et al., 2022). Large chromosomal inversions can disrupt recombination in heterozygote individuals, which can facilitate local adaptation or sometimes lead to speciation due to limited gene flow between populations (Berdan et al., 2023). For instance, the genomic inversion on chromosome 8 (XbOstEdul1.1: NC_079172.1) has been associated with resistance against *B. ostreae* (Sambade et al., 2022; Vera et al., 2019). The genotype frequencies of this chromosomal inversion exhibited a geographical pattern across the nine study sites included in this study, suggesting signatures of selection driving inversion polymorphism. This geographical pattern was also reported by Alves Monteiro et al. (2024). However, the medium-density SNP array used in this study lacks the genetic resolution needed to detect smaller inversions or other types of structural variation. Moreover, structural variation can dominate potential genetic population structures. As characterizing genetic population structure and connectivity between wild, foreign, and cultured populations was the objective of this study, SNPs associated with the three chromosomal inversions were excluded from the dataset. Further investigation into the role of structural variants in facilitating local adaptation would be valuable, but will require a higher-resolution genomic approach, such as whole-genome sequencing (Alves Monteiro et al., 2024).

The findings of this study enhance our understanding of local genetic population structure and can inform the selection of suitable populations for local effective restoration. However, significant genetic differences do not necessarily translate directly into biological relevance. It is crucial to emphasize that the relationship between genotype and phenotype is central to understanding a species' adaptive potential. Interpreting patterns of genetic divergence becomes most meaningful when linked to local adaptation and physiological performance. Therefore, future research should prioritize genotype-phenotype studies by integrating genetic, physiological, and ecological data to better understand the implications of genetic divergence and apply this knowledge to improve restoration practices.

Conclusion

In this study, we analyzed genetic diversity and connectivity of *Ostrea edulis*, representing wild, foreign, and cultured populations that shape the restoration seascape in the Netherlands. Foreign oysters from Ireland and Norway, translocated to Dutch waters for restoration, displayed genetic divergence from Dutch wild native oysters. Cultured native oysters, produced in hatcheries, are genetically similar to wild native oysters and may retain signatures of local adaptation. In combination with potential pathogen resilience or resistance, culturing native oysters poses a favorable restoration strategy. However, elevated levels of kinship in cultured populations highlight the urgent need for a defined breeding program with clear guidelines for retaining genetic diversity in the future. Further, wild native oysters comprise of a rather uniform population, where increased kinship suggests a weak founder effect in the recent natural establishment of Voordelta and Maasvlakte populations. In the context of flat oyster restoration, careful considerations should be taken regarding the restocking of wild populations with translocated or cultured individuals. Altogether, this study provides a genetic baseline for future flat oyster restoration and stresses the importance of monitoring genetic diversity of an intensively exploited keystone species.

Data availability statement

Genotyping data was made available on SEANOE (10.17882/108288).

Declaration of competing interest

The authors declare no conflicts of interest

Funding

The research was funded by the Dutch Ministry of Agriculture, Food Quality, Fisheries and Nature (project BO-43-116.01-003).

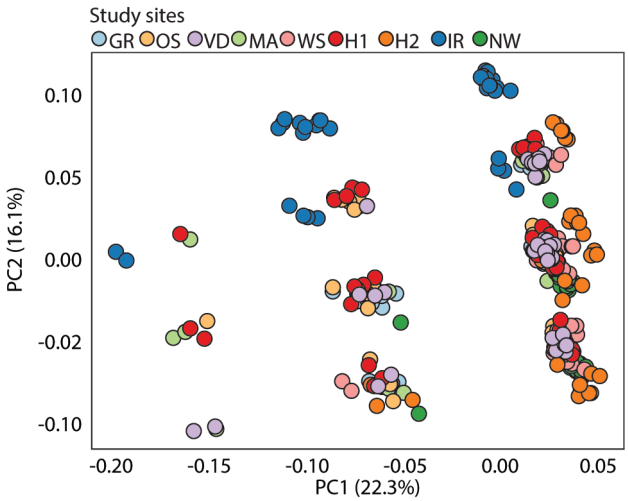
Acknowledgements

We are very grateful to Marco Dubbeldam from Stichting Zeeschelp, Nienke Bakker from Roem van Yerseke, and Katja Philippart from NIOZ for providing us with cultured oysters from their hatcheries. We would like to thank Marc Engelsma, Michal Voorbergen-Laarman, and Betty van Gelderen, from Wageningen Bioveterinary Research, for their support in sample collection of oysters from lake Grevelingen and the Oosterschelde. Further, we would like to thank Marjolein Kelder and Renate Olie, from De Rijke Noordzee, for their support in sample collection of Irish and Norwegian oysters, and thank Joost Bergsma of Waardenburg Ecology, for his support in collecting oysters from Maasvlakte and Voordelta. Additionally, we would like to acknowledge Jennifer Chin, from Wageningen Marine Research, for her assistance in DNA extraction. We further wish to thank Sylvie Lapègue and Pierre Boudry, from Ifremer, for their suggestions and advice in selecting a genotyping platform, providing samples for testing reproducibility of the genotyping assay, and to acknowledge the support from Ifremer for genotyping of the samples. The research was funded by the Dutch Ministry of Agriculture, Food Quality, Fisheries and Nature (project BO-43-116.01-003).

Supplementary information

Supplementary Table S1 | Mapping characteristics of genomic stretches of structural variation (SV) identified in *Ostrea edulis* using reference genome: XbOstEdul1.1 (Adkins et al., 2023). SVs were mapped against two other publicly available reference genomes: OEROSLIN_1.1 (Gundappa et al., 2022) and Oe_Roscoff_1 (Boutet et al., 2022). The green, blue, and orange colors in the first column refer to the inversion arrangement as indicated by Sambade et al., 2025a.

Query chromosome	Query Assembly	Chromosome Reference	Reference Sequence ID	Reference genome
NC_079167.1	GCA_023158985.1	scaffold2	NW_026039113.1	OEROSLIN_1.1
NC_079169.1	GCA_023158985.1	scaffold5	NW_026039446.1	OEROSLIN_1.1
NC_079172.1	GCA_023158985.1	scaffold8	NW_026039779.1	OEROSLIN_1.1
NC_079167.1	GCA_024362745.1	scaffold5	CM044539.1	Oe_Roscoff_1
NC_079169.1	GCA_024362745.1	scaffold4	CM044538.1	Oe_Roscoff_1
NC_079172.1	GCA_024362745.1	scaffold8	CM044542.1	Oe_Roscoff_1



Supplementary Figure S1 | First two principal components (PCs) of the Principal Component Analysis (PCA) of 10,407 non-pruned genome-wide SNPs for all nine study sites.

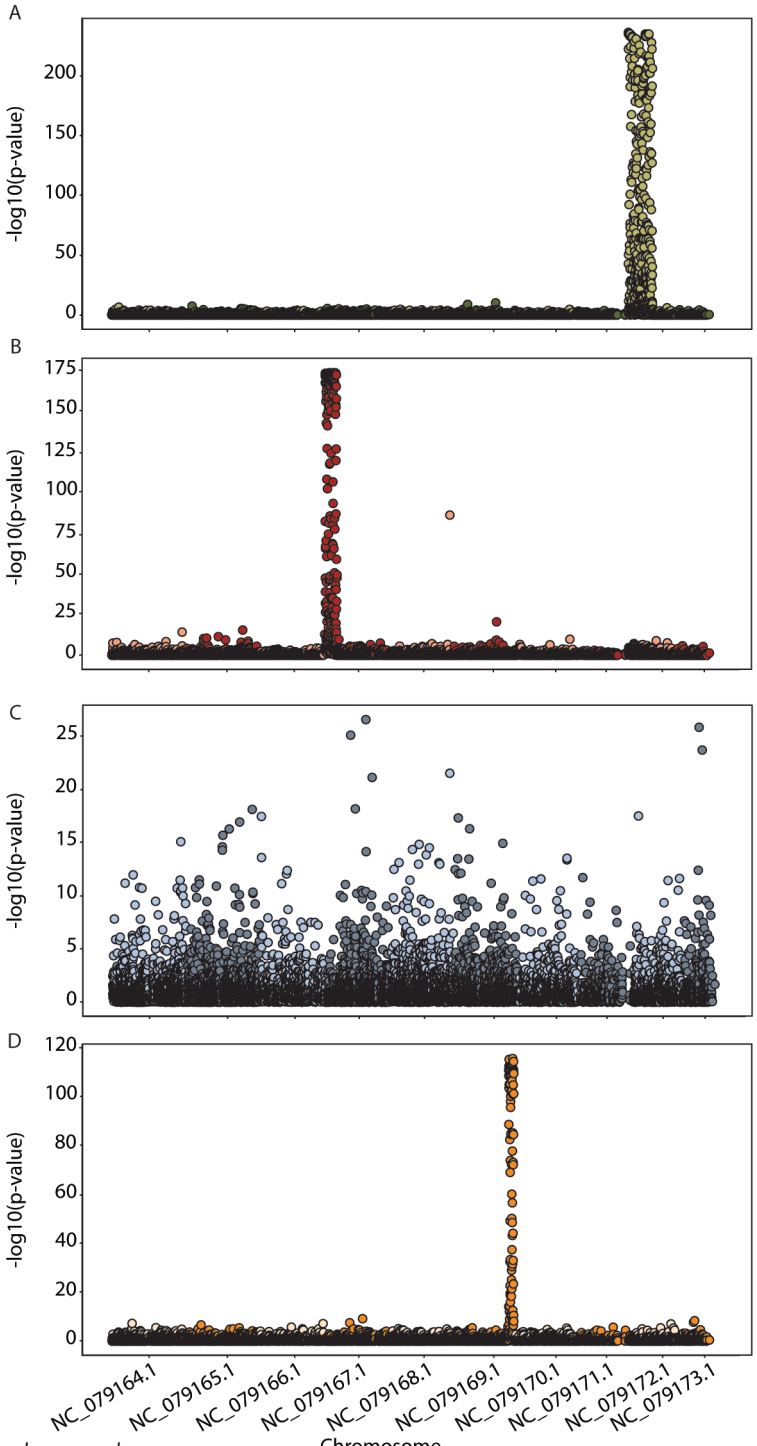
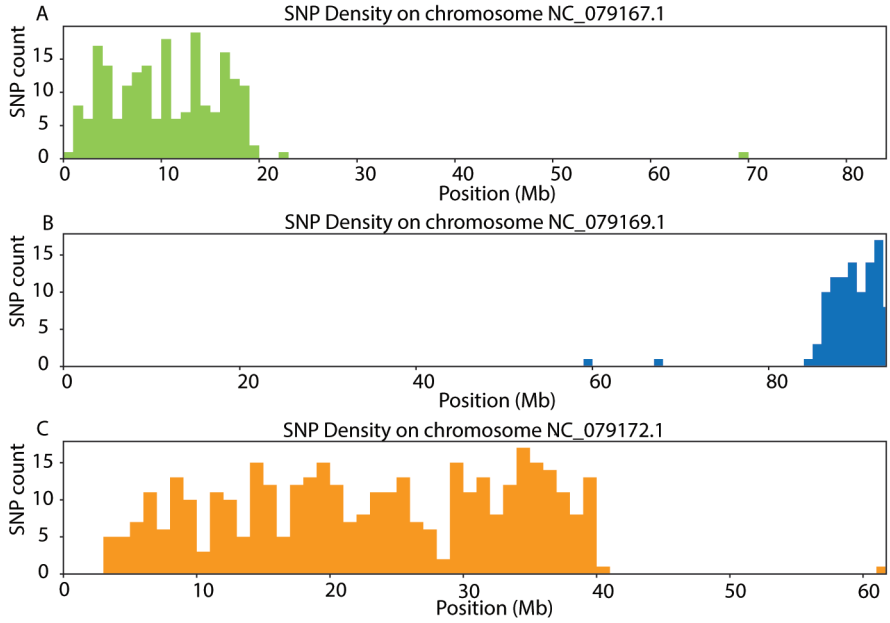


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Supplementary Figure S2 | Manhattan plots showing the association of genome-wide SNPs with the first four principal components (PCs) based on 10,407 SNPs from the Total panel. **(A)** PC1, **(B)** PC2, **(C)** PC3, and (**D)** PC4. Individual chromosomes are identified by the alternate dark and light coloring, with SNPs plotted as individual points based on their genomic position within each chromosome.



Supplementary Figure S3 | Histogram plot of number of SNPs along their genomic position. All SNPs depicted are significantly associated with the first four principal components and located on the three genomic inversions on chromosomes **(A)** NC_079167.1, **(B)** NC_079169.1, and **(C)** NC_079172.1. The green, blue, and orange colors refer to the inversion arrangement as indicated by Sambade et al. (2025a).

Supplementary Figure S4 | Manhattan plots showing the association of genome-wide SNPs with the first four principal components (PCs) based on 8,403 SNPs from the Pruned panel. **(A)** PC1, **(B)** PC2, **(C)** PC3, and **(D)** PC4. Individual chromosomes are identified by the alternate dark and light coloring, with SNPs plotted as individual points based on their genomic position within each chromosome.

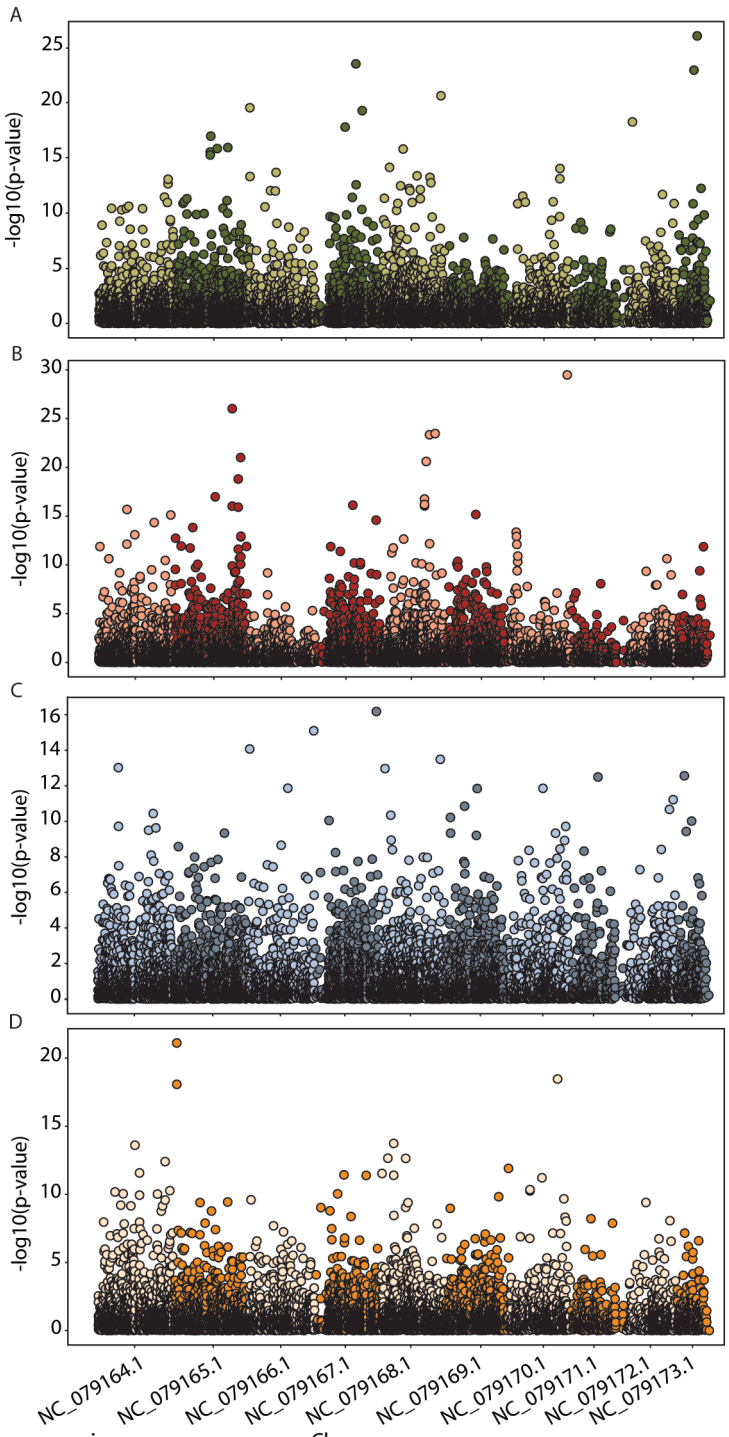
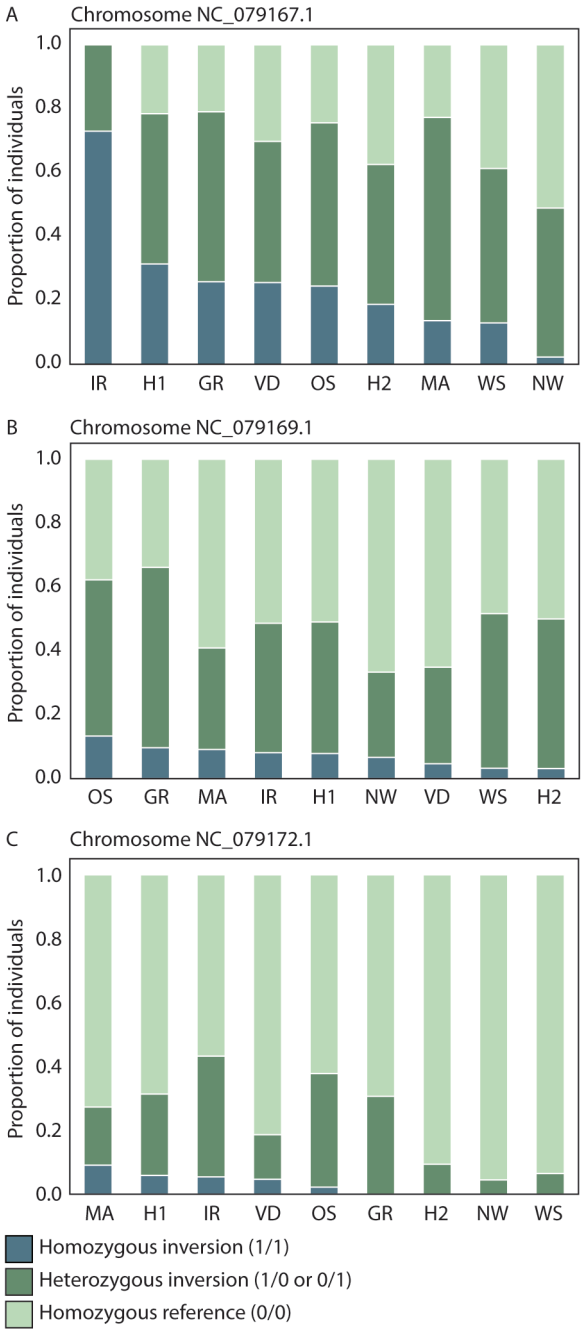
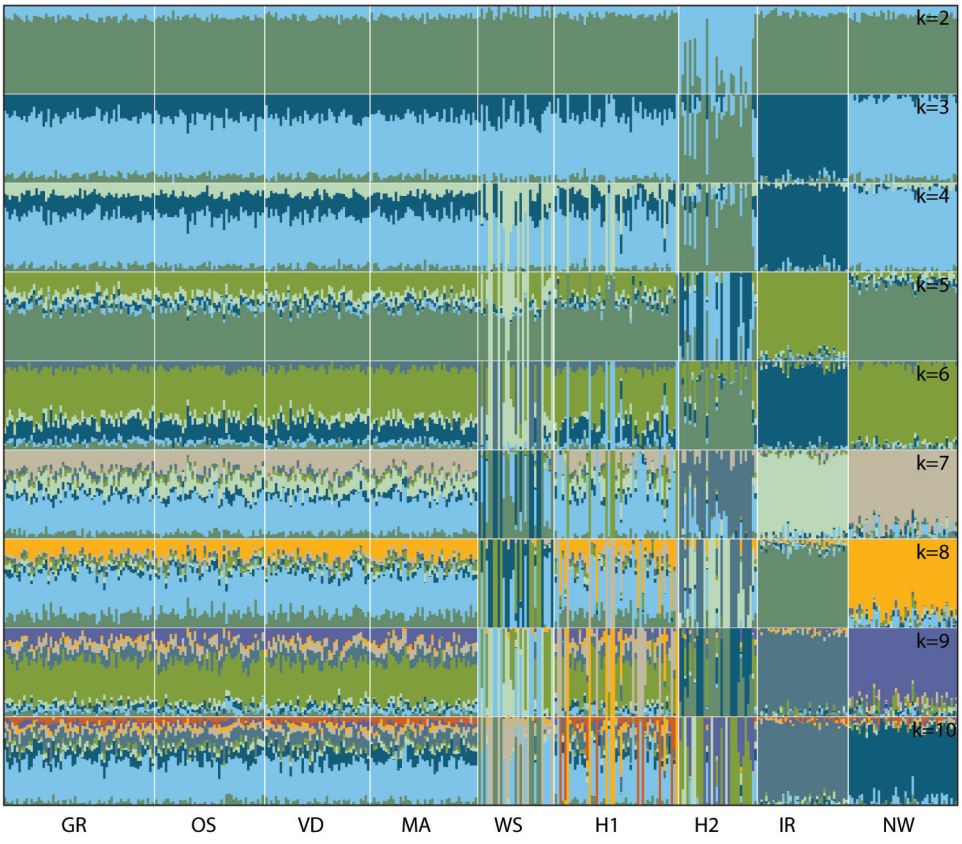


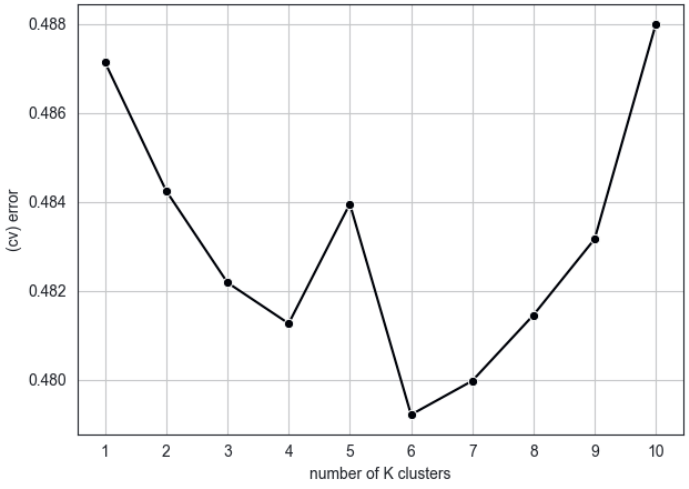
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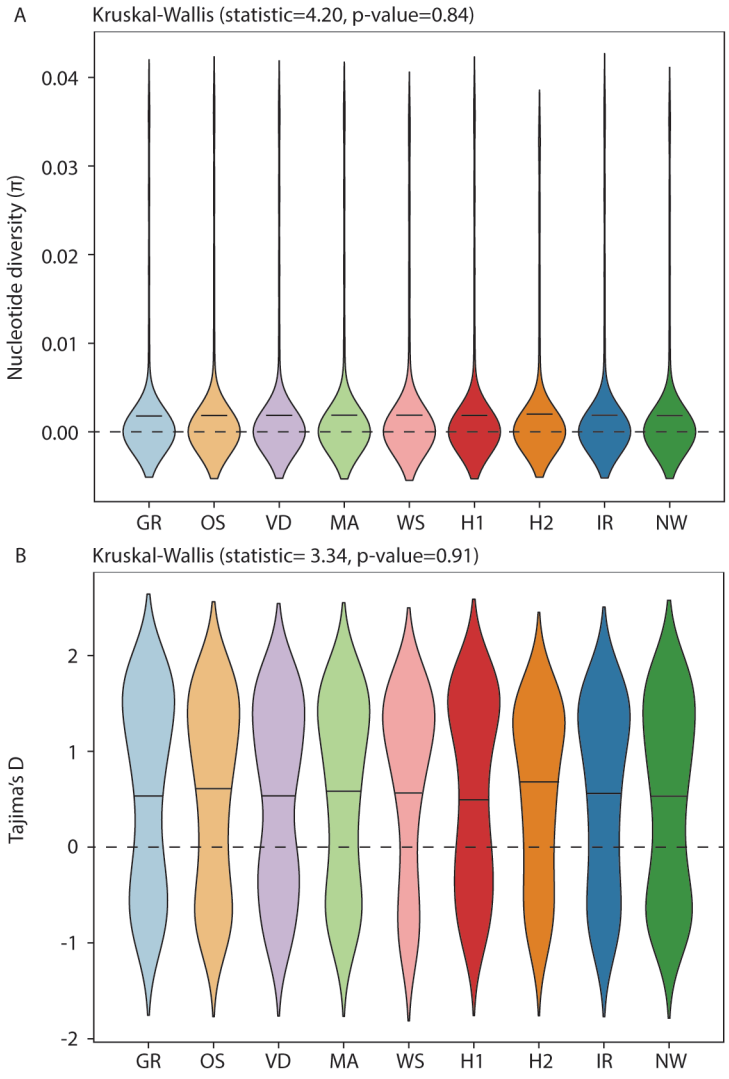
Supplementary Figure S5 | Inversion genotype frequencies of the three chromosomal inversions on chromosomes **(A)** NC_079167.1, **(B)** NC_079169.1, and **(C)** NC_079172.1. Study sites are arranged in the order of decreasing frequency of inversion homozygotes (1/1).



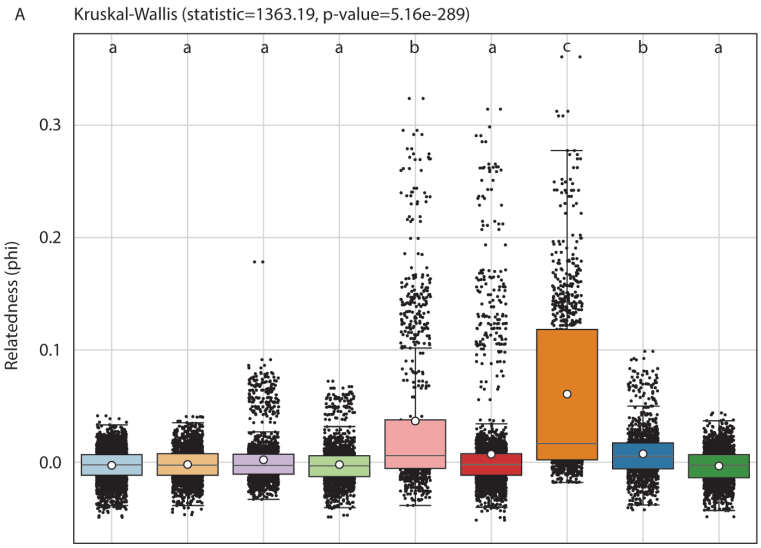
Supplementary Figure S6 | Admixture analysis across nine study sites based on 8,403 variants of the Pruned panel, with k=2-10 clusters.



Supplementary Figure S7 | Five-fold cross-validation error values for admixture clusters k=2-10, based on 8,403 variants of the Pruned panel.



Supplementary Figure S8 | Genome-wide genetic diversity calculated over 10Kb windows across 8,403 variants (Pruned panel) for all nine study sites. **(A)** Nucleotide diversity (π). **(B)** Tajima's D. Black bars within violin plots represent average values for each study site.



Supplementary Figure S9 | Genome-wide expected heterozygosity He (E) based on 8,403 variants from the Pruned panel for all nine study sites. White dots represent average values per study site. Different lowercase letters denote statistically significant differences ($p < 0.05$) for values of He (E) between study sites.

Supplementary Figure S10 | Kinship analysis based on 8,403 variants from the Pruned panel for all nine study sites. **(A)** Within-population relatedness (phi). **(B)** Within-population identity by descent (pi_hat). White dots represent average values per study site. Different lowercase letters denote statistically significant differences ($p < 0.05$) for values of Relatedness (phi) and Identity by descent (pi_hat) between study sites. The dashed lines represent IBD values for full-sibling (pi_hat = 0.5) and half-sibling (pi_hat = 0.25) kinship.

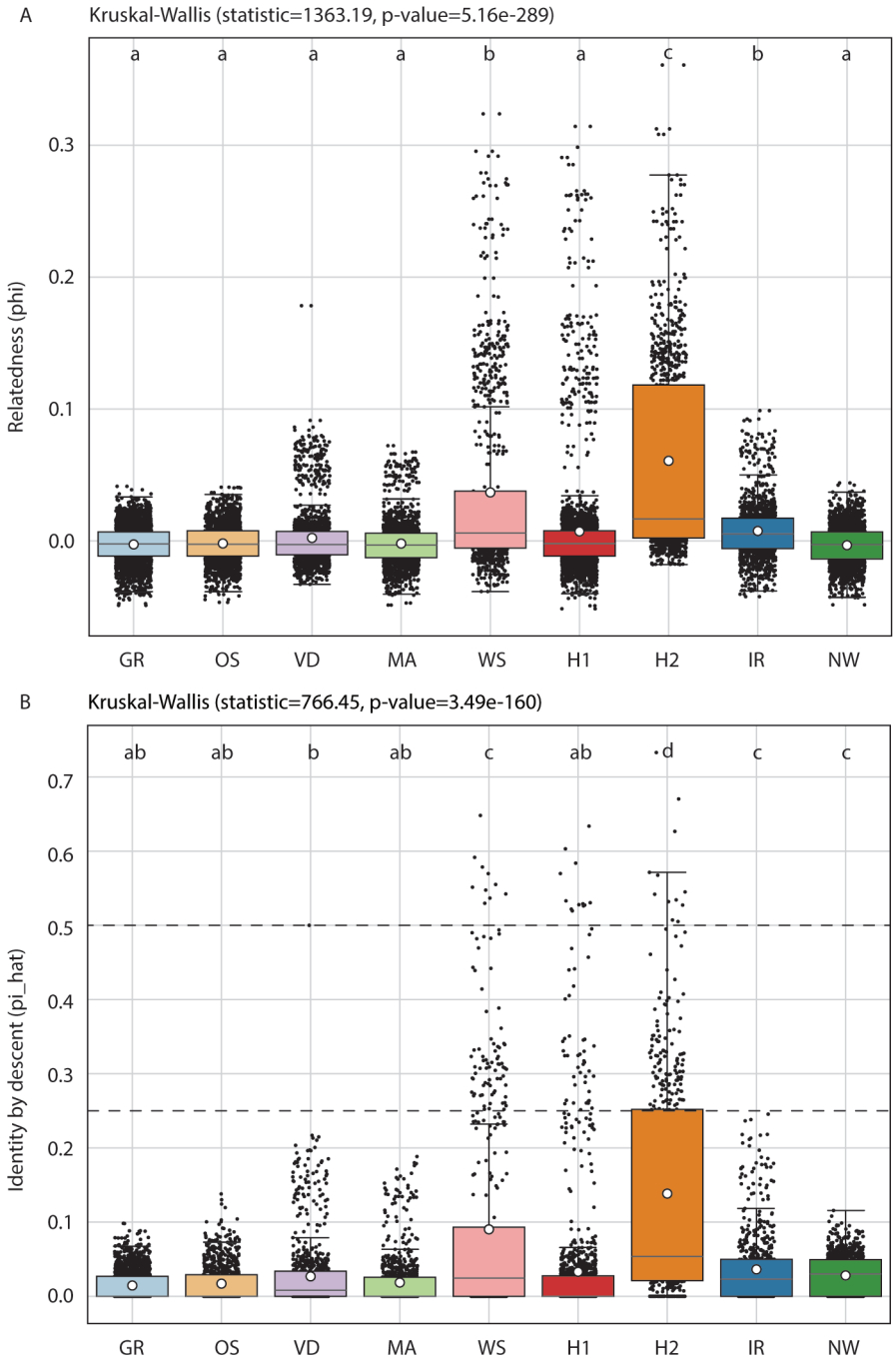
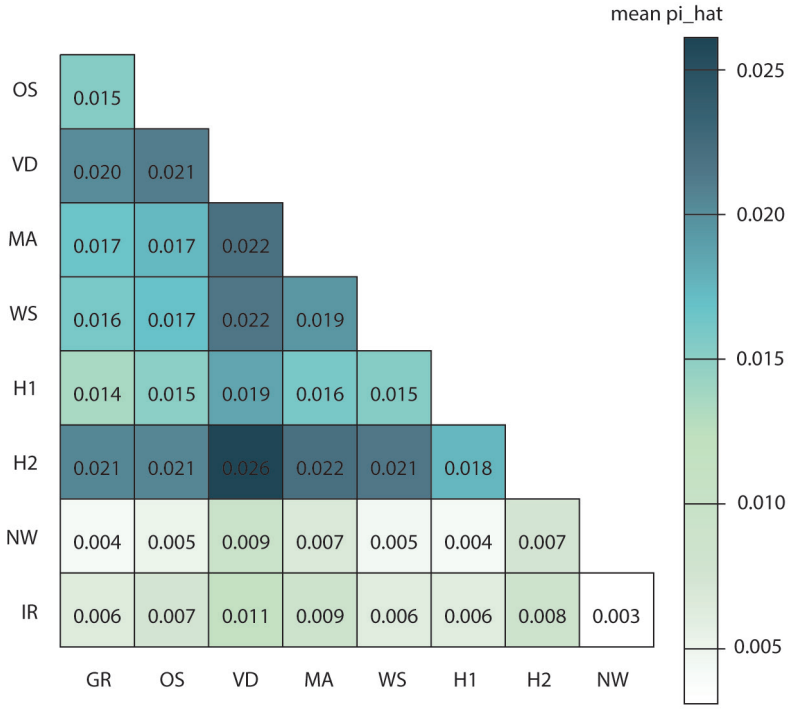
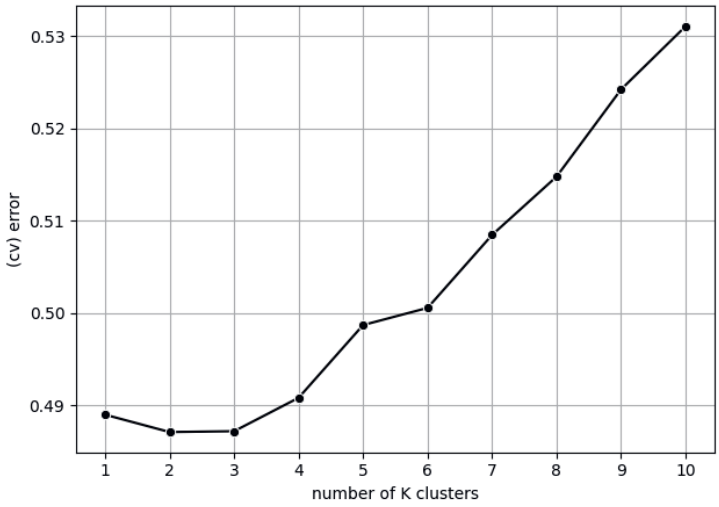


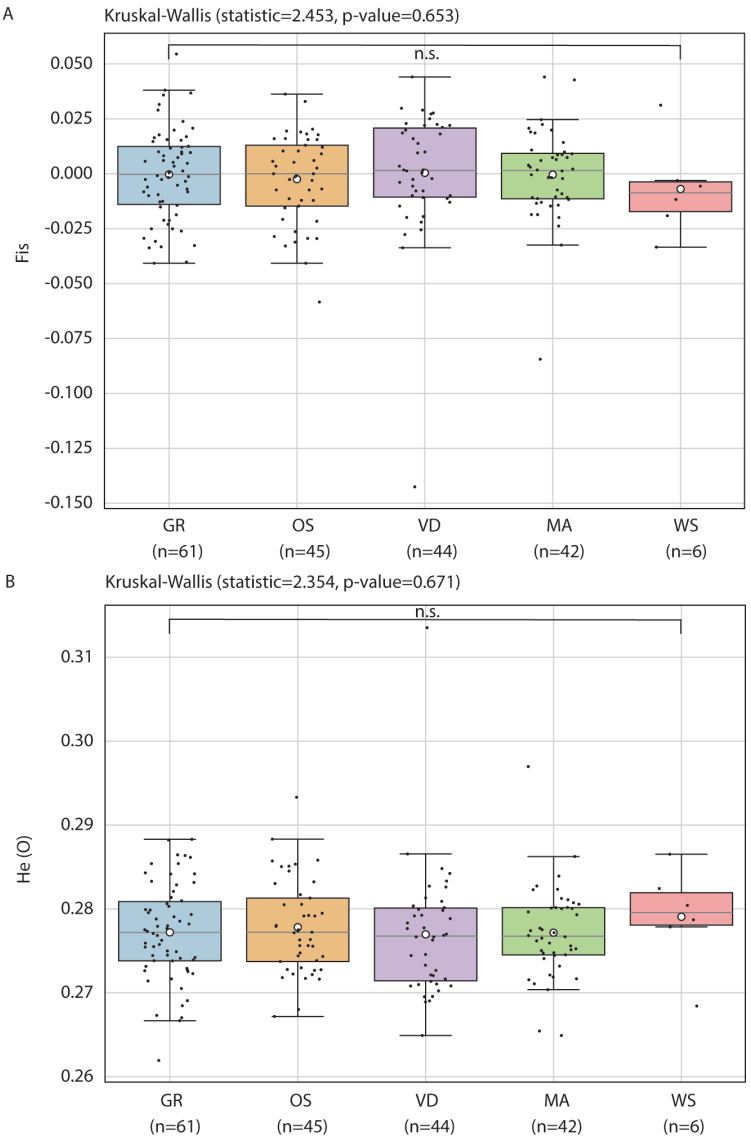
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Supplementary Figure S11 | Inter-population connectivity by pairwise comparisons of identity by descent (IBD) values (pi_hat) across populations.



Supplementary Figure S12 | Fivefold cross-validation error values for admixture clusters k=2-10, based on 9,293 variants of the Pruned wild panel.



Supplementary Figure S13 | Genetic diversity on 8,403 variants from the Pruned native panel across the five native study sites. **(A)** Inbreeding coefficient Fis). **(B)** Observed heterozygosity. White dots represent average values per study site. Non-significant ($p < 0.05$) differences between values of Inbreeding Coefficient (Fis) and Observed Heterozygosity - He (O) - across populations are indicated as "n.s.".

CHAPTER 3

The structural variation landscape of the flat oyster genome

Sophie Valk, Hendrik-Jan Megens, Reindert Nijland, & Manu Kumar Gundappa

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DOI: https://doi.org/10.1016/j.ygeno.2026.111274

Abstract

Structural variants (SV), ranging in size from a few base pairs to entire chromosomes, are important contributors to population structure and evolutionary processes, such as local adaptation or speciation. Despite their impact on gene regulatory mechanisms, SVs remain uncharacterized in non-model organisms due to high sequencing costs and bioinformatic challenges. This work presents a genome-wide characterization of SVs in the European flat oyster (*Ostrea edulis*), an ecologically and economically important bivalve, using long-read sequencing data from 35 individuals sampled in the Netherlands. A broad spectrum of SV types and sizes were identified, with many overlapping repetitive elements, highlighting their contribution to within-population genetic structure. Analysis of haplotype divergence across three large inversions revealed signatures of recombination suppression in heterozygote individuals. Together, these findings provide an overview of the SV landscape in *O. edulis* and highlight the potential role of structural variation in shaping genomic diversity, underscoring its relevance for oyster restoration efforts.

Introduction

Characterizing intraspecific genetic variation stands at the core of understanding evolutionary processes, as genetic insights in population dynamics can reveal patterns of local adaptation or speciation (Haltiner et al., 2024; Hays et al., 2021; Hirase et al., 2021; Muir et al., 2020; Sanford & Kelly, 2011; Segovia et al., 2017). With the worldwide threat of biodiversity loss, effective species conservation and restoration rely crucially on further elucidating such genetic diversity and evolutionary processes (Bending the Curve of Biodiversity Loss, 2020; Schwartz et al., 2007; Wold et al., 2021).

Genetic diversity can be quantified on various levels, ranging from sequence-level markers such as single-nucleotide polymorphisms (SNPs) and microsatellites (short tandem repeats) (Al-Breiki et al., 2018; Pujolar et al., 2011), to genomic stretches of structural variation (SVs) (Pokrovac & Pezer, 2022). SVs encompass genetic variants other than single-nucleotide variants that differ in copy number, orientation, or genomic location among individuals or populations (Wellenreuther et al., 2019). Structural variants can be balanced, representing an exchange of genetic material, such as an inversion or translocation within or between chromosomes (Wellenreuther et al., 2019). In contrast, unbalanced SVs involve the loss or gain of genetic material through deletions, insertions, or duplications. SVs are widely spread across both plant and animal genomes (Pokrovac & Pezer, 2022), and typically arise through mechanisms of DNA recombination, replication, and repair (Weischenfeldt et al., 2013). SVs most often range in size from a few bp to extreme lengths covering entire chromosomes (Mérot et al., 2023). However, their detectable size range is primarily determined by methodological constraints, with most SV callers reliably detecting SVs between 50 bp to a few kilobases in length (Stuart et al., 2026).

Genetic rearrangements from SVs can disrupt regulatory mechanisms and consequently exert substantial functional effect on an organism's phenotype (Ho et al., 2020; Weischenfeldt et al., 2013). Such effects primarily arise through alterations in gene regulation and RNA splicing (Damián et al., 2023; Lee et al., 2012; Weischenfeldt et al., 2013; Xu et al., 2022). A wide variety of SVs have been associated with various severe disease phenotypes in humans (Lupski, 2015; Prakash et al., 2016), including Alzheimer's disease (Weischenfeldt et al., 2013). It is therefore not surprising that the most extensively characterized SVs are those identified in humans, while in other organisms functionally characterized SV databases are still minimal (Pokrovac & Pezer, 2022). However, beyond their association with disease phenotypes, the regulatory potential of SVs facilitates genetic adaptation to environmental conditions and may ultimately drive evolutionary divergence or even speciation (Berdan et al., 2024; Feulner & De-Kayne, 2017; Zhang et al., 2021). This underscores the importance of studying SVs across a broad range of organisms. Despite their adaptive potential

and generally larger contribution to genetic variation than SNPs (Mérot et al., 2023; Wellenreuther et al., 2019), characterizing SVs remains challenging in non-model organisms as bioinformatics tools are predominantly developed for model systems (Ho et al., 2020; Schell et al., 2025). Consequently, SVs remain comparatively understudied in non-model organisms (Catanach et al., 2019; Recuerda & Campagna, 2024; Schell et al., 2025; Wellenreuther et al., 2019).

Recent advances in sequencing technologies, together with rapidly declining costs, have made sequencing more accessible to a broader scientific community, thereby intensifying research on SVs over the past few years (Ho et al., 2020; Pokrovac & Pezer, 2022; Wold et al., 2021). These efforts have yielded numerous insights, including a clearer understanding of the functional impact of chromosomal inversions (Berdan et al., 2023; Wellenreuther et al., 2025; Wellenreuther & Bernatchez, 2018). Chromosomal inversions, a type of SV characterized by the reversed sequence orientation of a chromosome segment, can disrupt collinearity between chromosomes and often impair recombination during meiosis in inversion heterozygotes (Jay et al., 2024; Wellenreuther et al., 2025; Wellenreuther & Bernatchez, 2018). This suppressed recombination, which may also occur in other classes of structural variation, can induce co-transmission of nearby alleles that might have adaptive potential or selective advantage, creating so-called supergenes (Jay et al., 2024; Schwander et al., 2014). By generating fitness differences among genotypes, these favorable allele sets can drive speciation through reproductive isolation between coexisting subpopulations (Wellenreuther & Bernatchez, 2018). As such, an enhanced understanding of the establishment and maintenance of such structural variant polymorphisms associated with suppressed recombination can provide valuable insights into evolutionary processes, with important implications for species conservation and restoration.

Beyond inversions, transposable elements (TEs), defined as DNA or RNA sequences that can move around in the genome, also contribute significantly to structural variation (Almojil et al., 2021). Although environmental stimuli can influence TE activity and contribute to intraspecific genetic diversity (Casacuberta & González, 2013; Schrader & Schmitz, 2019; Stapley et al., 2015), the poor annotation of repeat databases still limits robust TE characterization, particularly in non-model organisms (Bell et al., 2022; Loreto et al., 2023).

Despite the prevailing hypothesis that high population connectivity in marine environments results in the absence of population structure and local adaptation (Lamichhaney et al., 2012), advances in genotyping techniques have revealed pervasive genetic variation and emphasized the role of SVs in shaping microgeographic population structure across a wide range of marine organisms (Cariolato et al., 2025; Meyer et al., 2024; Robert et al., 2025; Rumberger et al., 2025; Sanford & Kelly, 2011).

Mollusks, in particular, are a phylum characterized by substantial genetic variation, with pronounced population differentiation and local adaptation reported for many species (Calcino et al., 2021; Cariolato et al., 2025; Dupont et al., 2003; Sambade et al., 2022). This suggests that mollusks provide important systems for studying the role of SVs in driving evolutionary divergence and local adaptation.

The European flat oyster (*Ostrea edulis*) is a bivalve exhibiting a wide natural distribution, ranging from the cold waters of the northern North Sea, to inhabiting warmer regions of the Mediterranean and Black Sea (Aydin & Biltekin, 2020; Bromley et al., 2016; Olsen, 1883; Stagličić et al., 2020). Since the arrival of the haplosporidian parasite Bonamia Ostreae in the late 1970s, the species has experienced vast declines in population size (Culloty et al., 1999; Engelsma et al., 2010; Pichot et al., 1979). Corroborated by continuous overfishing and habitat degradation, these stressors have resulted in its local functional demise in various coastal regions (Beck et al., 2011; Engelsma et al., 2010; Ewers et al., 2025; Sander et al., 2021; Thurstan et al., 2024). Consequently, the flat oyster has become a key target of both national and international restoration efforts (Kamermans et al., 2025; Pouvreau et al., 2023; Zu Ermgassen et al., 2023), resulting in increased interest in the genetic characterization of the species (Alves Monteiro et al., 2024; Boutet et al., 2022; Gundappa et al., 2022; Lapègue et al., 2023; Robert et al., 2025). More recently, three chromosomal inversions were identified, displaying substantial variation in genotype frequencies across European populations (Alves Monteiro et al., 2024; Sambade et al., 2022, 2025a). Various studies have suggested that selective pressures contribute to the establishment and maintenance of these inversions (Alves Monteiro et al., 2024; Lapègue et al., 2023; Sambade et al., 2022), with one inversion notably associated with genetic tolerance to the parasite B. ostreae (Kamermans et al., 2023; Sambade et al., 2022). Despite these discoveries, the genome-wide landscape of SVs remains uncharacterized in the flat oyster. In this study, we address this gap by characterizing SVs in a comparative analysis across 35 individuals from a single population, using whole-genome long-read nanopore sequencing. Our findings highlight the importance of structural variation in shaping population structure and adaptive potential.

Methods

Sample collection

Oysters were collected by dredging in Grevelingenmeer, the Netherlands (53°30'40"N, 08°06'20" E), during April and September 2023, and April 2024. Individuals of similar shell length (74.1 ± 7.45 mm) were selected for gill tissue collection, which was preserved on RNAlater or snap-frozen using liquid nitrogen, and stored at -80 °C until further use. Oysters were collected in the context of the national monitoring for mollusk diseases in the Netherlands (Project no: WOT-01-002-032).

DNA extraction and sequencing

Genomic DNA was extracted from gill tissue using the Qiagen Gentra Kit for genomic DNA or Monarch HMW DNA extraction kit for tissue, according to the manufacturer's instructions (Qiagen catalog no. 158667; NEB catalog no. T3060).

Samples were multiplexed according to the native barcoding protocol (SQK-NBD114-24), and subsequently sequenced using the Oxford Nanopore Platform, following the manufacturer's guidelines for library preparation (ONT, Oxford, UK). This long-read data has been previously used in a different study that aimed to characterize DNA methylation patterns across individuals of different sex phenotypes. For a more detailed description of DNA extraction and sequencing, we refer Valk et al. (2025).

Raw read processing

Raw sequenced reads (POD5) were basecalled using Dorado v0.4.1 (Oxford Nanopore Technologies, 2022/2025) and basecalling model dna_r10.4.1_e8.2_400bps_sup@4.2.0, filtering for a minimal read quality of 7 (Fig. 1A). After basecalling, the reads were demultiplexed using the demux function of Dorado. The reads were mapped to reference genome xbOstEdul1.1 (NCBI RefSeq Assembly: GCF_947568905.1) (Adkins et al., 2023), using minimap2 v2.28-r1209 (Li, 2021). Mapped BAM files were then filtered for high quality reads (mapping quality of Q60), after which all unmapped reads or secondary alignments were removed using Samtools v1.17 (Danecek et al., 2021). Following filtering, BAM files were sorted and indexed using Samtools. Mapped reads are available from the European Nucleotide Archive (ENA: https://www.ebi.ac.uk/ena/browser/home), under study ERP168521 and accession number PREJB85091.

Structural Variant calling

Structural variants were identified from nanopore long-read data using a stepwise workflow (Fig. 1). Briefly, SVs were first independently detected for each of the 35 individuals using three SV callers: Sniffles2, SVIM, and cuteSV. Following SV calling, SVs were merged across callers and subsequently genotyped in all 35 individuals. The SV calling and merging workflow was adapted from https://github.com/LaurieLecomte/SVs_long_reads. Detailed usage and parameter settings for each SV caller are described below.

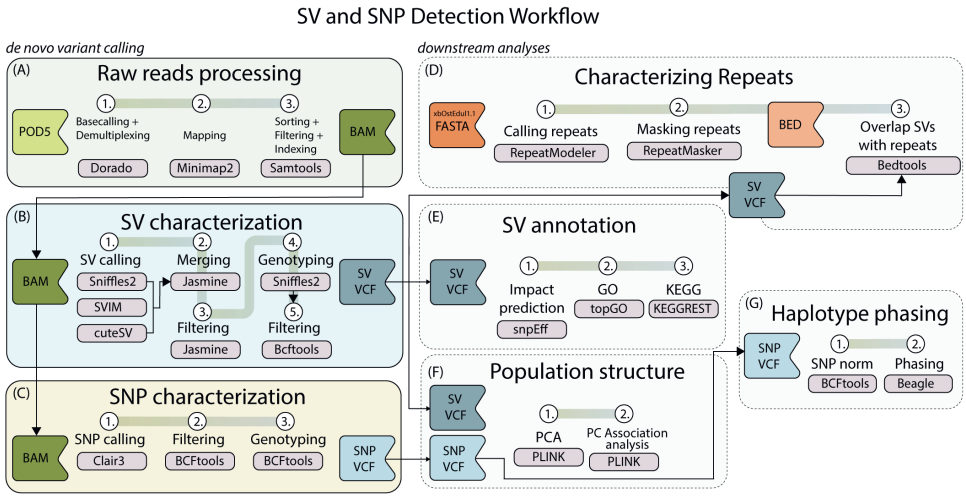


Figure 1 | Workflow for detecting, processing, and analyzing structural variants (SVs) and single-nucleotide polymorphisms (SNPs) from long-read nanopore sequencing data in the *O. edulis* genome. Panels A-C illustrate the workflow for de novo variant calling, while panels D-G visualize downstream analyses of the identified variants. **(A)** Raw-read processing. **(B)** SV calling and characterization. **(C)** SNP calling and characterization. **(D)** Repeat detection and characterization using the XbOstEdul1.1 reference genome assembly. **(E)** SV impact prediction and annotation. **(F)** Population structure analysis using SV and SNP datasets. **(G)** SNP-based haplotype phasing.

Sniffles

Sniffles v2.6.2 was used with default parameters (Smolka et al., 2024), retaining read names ("--output-rnames") and combining variants across sequence loadings within each BAM file of every individual ("--output-rnames" and "--combine-consensus") (Fig. 1B). Variants called by Sniffles were filtered for "PASS" and "PRECISE" to retain high-confidence variants with confidently resolved breakpoints. SVs positioned in unplaced scaffolds were discarded using bcftools v1.17 (Danecek et al., 2021).

Because SV callers differ in assigning and distinguishing insertions and duplications, duplications were temporarily converted to insertions during within-caller merging across individuals to ensure consistent clustering across tools (Heller & Vingron, 2019),

using "--dups_to_ins" and "--preprocess_only" parameters with Jasmine v1.1.5 (Kirsche et al., 2023). SV breakpoints were polished through refining with Iris v1.0.5 (Kirsche et al., 2023), using parameters "--keep_long_variants" and "--also_deletions". After refining, all SVs were merged across the 35 individuals using Jasmine with parameters: "--ignore_strand" to merge variants across both strands, "--mutual_distance" to require two variants to be within the same distance threshold of each other, "--allow_intrasample" to merge SVs from across different sequence loadings within one individual, and "--output_genotypes". After merging and refining, temporarily converted duplications were restored using Jasmine "--dups_to_ins" and "--postprocess_only", and SVs were subsequently filtered to retain only variants labelled as "PASS" and "PRECISE".

SVIM

SVIM v2.0.0 (Heller & Vingron, 2019) was used to call variants with the alignment function and parameters: "--insertion_sequences" to retain variant sequences along with the variant's characteristics, "--read_names" to retain read names, "max_consensus_length=5000" to set the maximum size of calling insertion sequences at 5000 bp, "interspersed_duplications_as_insertions" to temporarily convert duplications to insertions (Fig. 1B). After SV calling using SVIM, SVs were filtered for "PASS" and "PRECISE" using bcftools, and SVs positioned in unplaced scaffolds were discarded.

To allow for consistent merging across tools at a later stage, duplications were temporarily converted to insertions using Jasmine ("--dups_to_ins" and "--preprocess_only"), after which SVs were refined using iris with default parameters, "--keep_long_variants", and "--also_deletions" to retain long variants as well as deletions. Similarly to merging SVs called by Sniffles, SVs called by SVIM were merged across all 35 individuals using Jasmine with default parameters and "--ignore_strand" to ignore strand ordination, "--mutual_distance" to require equal distance thresholds for two SVs, "--allow_intrasample" to enable merging across sequence loadings within each individual, "--output_genotypes", and "--sample_names" to retain sample information. After merging, insertions were converted back to duplications using Jasmine "--dups_to_ins" and "--postprocess_only", and SVs were filtered to retain only variants labelled as "PASS" and "PRECISE".

cuteSV

Similar to calling SVs with Sniffles and SVIM, calling SVs with cuteSV v2.1.1 (Jiang et al., 2020), was performed with default parameters, using mapped BAMs as input file and the reference genome (Fig. 1B). After calling, variants were filtered for "PASS" and "PRECISE" using bcftools, and any variant positioned in an unplaced scaffold was removed. Prior to refining breakpoint areas using Iris, duplications were temporarily converted to insertions using Jasmine with parameters "--dups_to_ins"

and "--preprocess_only". Iris was then used to refine SVs with parameters "--keep_long_variants" and "--also_deletions".

After refining, SVs called by cuteSV were merged across all 35 individuals using Jasmine with parameters "--ignore_strand", "--mututal_distance", "--allow_intrasample", "--output_genotypes", and "--sample-names". Temporarily converted duplications were reverted using jasmine with parameters "--dups_to_ins" and "--postprocess_only".

SV merging, filtering, and genotyping

After SV calling with three different callers, SVs were merged across the three callers using Jasmine with parameters "--ignore_strand" to merge SVs regardless of strand ordination, "--ignore_merged_inputs" to allow merging across the three callers, "--normalize_type" to enable merging of SVs that represent the same underlying event but may still differ in SV type annotation across callers (Fig. 1B). Genotypes were retained using "--output_genotypes", and within-sample merging across callers was enabled with "--allow_intrasample". Distances between SVs were evaluated using mutual distance "--mutual_distance" to account for discrepancies in breakpoint detection and SV type assignment across the three different callers, "--max_dist_linear" was set to 0.1, such that SVs were merged only if their breakpoints differed by no more than 10% of the variant length. Lastly, SVs were filtered for a minimal SV length of 50 bp using bcftools, and SVs were retained only if supported by all three callers. Inversion detection from long-read sequencing remains comparatively challenging, as inversions typically do not reflect changes in sequence coverage and must instead be identified by breakpoint-aware tools (Hanlon et al., 2022) Therefore, filtering criteria for inversions were relaxed, and inversions were retained when supported by at least two callers. After filtering, SVs were genotyped in all 35 individuals using Sniffles2 "--genotype-vcf".

To assess the distribution of SVs across genomic features, positions of coding sequences, introns, exons, 3'UTRs, 5'UTRs, promoters, and intergenic regions were extracted from the reference genome annotation using makeTxDbFromGFF from GenomicFeatures (Lawrence et al., 2013). To test for significant enrichment of the four SV types (deletions, insertions, duplications, & inversions) in these genomic features, regioneR (Gel et al., 2017) was used to compare the observed number of SVs overlapping each feature with the expected number based on SV count and length, using 1,000 permutations.

SV annotation

To predict the genetic impact of structural variants, SVs were annotated using SNPeff v5.3a (Fig. 1E) (Cingolani et al., 2012). Annotation required generating a custom SnpEff database for *O. edulis*. This database was built from snpEff.jar build, using the

reference genome sequence (genomic.fna), the corresponding genome annotation file (genomic.gtf), and annotated coding sequences (cds_from_genomic.fna), all obtained from the reference genome (xbOstEdul1.1; NCBI RefSeq Assembly: GCF_947568905.1) and associated FTP repository from NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/). Following custom database generation, SVs were annotated with SnpEff, and variants predicted to have a high functional impact (classified as "HIGH"), were retained for downstream analyses.

High-impact variants were analyzed for functional enrichment through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. GO enrichment was performed using topGO in Bioconductor (Alexa & Rahnenführer, 2017). Gene IDs were annotated with GO terms for Biological Processes (BP), Cellular Components (CC), and Molecular Functions (MF) using the Gene Annotation File (GAF) obtained from the reference genome. Enrichment was assessed with Fisher's exact test and p-values were adjusted for multiple testing by applying a Benjamini-Hochberg correction. GO terms were considered significantly enriched at an adjusted $p < 0.05$.

For a more detailed analysis of specifically enriched pathways, KEGG enrichment was performed using KEGGREST in R (Tenenbaum, 2017). Enrichment pathways were retrieved for O. edulis using the organism's code "oed" in keggLink, and enrichment analysis was conducted with the enrichKEGG function from clusterProfiler (Yu et al., 2012). P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure to control for a false discovery rate (FDR).

Modeling and masking repeats

To characterize repetitive elements among SVs, RepeatModeler and RepeatMasker were used from the Dfam consortium (Fig. 1D) (Wheeler et al., 2012). To generate a de novo custom repeats database, the reference genome was indexed using BuildDatabase, and repeats were identified with RepeatModeler, including the "-LTRstruct" option to improve detection of long terminal repeat elements.

A second repeat library was retrieved from the available Dfam database (dfam39_full.0.h5.fa) (Wheeler et al., 2012), using the "--ancestors" and "--descendants" options restricted to Bivalvia and the option "--include-class-in-name" to retain repeat classification in the output.

Both repeat libraries were concatenated to form a comprehensive repeat database, which was then used to mask repeats in the reference genome. Structural variants were subsequently overlapped with annotated repeats using bedtools v2.29.1 intersect (Quinlan & Hall, 2010), allowing identification of SVs.

To capture active repeats in the *O. edulis* genome, high abundance SVs of equal length (275-295 bp and 315-335 bp) were extracted as a case study. Insertion sequences were extracted directly obtained from the ALT field of the VCF, while deletion sequences were retrieved from the reference genome using bedtools getfasta after converting VCF to BED format. Sequences were clustered using CD-HIT v4.8.1 (Li & Godzik, 2006), at 97% identity with default parameters to identify highly active repeat sequence families in the *O. edulis* genome. Clusters containing the largest number of sequences were selected, and representative sequences for each cluster were extracted using subseq from seqtk v1.3-r106 (Li, 2012/2012). Representative sequences were blasted against a local NCBI nt database (core_nt, Jan-2025) using blastn from Nucleotide BLAST v2.15.0+ (Camacho & Madden, 2025), retaining up to five hits per query sequence. The repeat consensus sequences were aligned using MAFFT v7.526, and percentage identity was computed for all pairwise alignments between repeat consensus sequences (Katoh & Standley, 2013).

SNP calling

Single nucleotide polymorphisms (SNPs) were jointly called across 35 individuals using Clair3 v1.2.0 (Zheng et al., 2022), with mapped BAM files that were pre-filtered for a minimum mapping quality of 20 used as input, and the genotyping model r1041_e82_400bps_sup_v500 (Fig. 1C). To retain high-confidence SNPs, calls were filtered for the "PASS" flag, and a minimal Phred score of 20, corresponding to a ≤1% probability of the variant being incorrect. Only SNPs with a read depth equal to or greater than the median read depth across all SNPs for the respective individual were retained for downstream analysis. SNPs were merged across individuals using bcftools merge -m all, and subsequently genotyped with bcftools pileup and call, using the reference genome xbOstEdul1.1 (Adkins et al., 2023).

Population structure analysis

Prior to population structure analyses, SVs were stringently filtered for requiring a minor allele frequency (MAF) of 0.1, to reduce the inclusion of false-positive genotypes and retain a high-confidence SV dataset. Furthermore, SVs were filtered on missing genotypes by retaining variants for which at least 90% of the individuals contained genotype information (--geno 0.9) (Fig. 1F). Population substructure from genome-wide SVs among the 35 individuals was then assessed using principal component analysis (PCA) implemented from PLINK v1.90b7.2 (Chang et al., 2015).

In addition to studying population substructure using SVs, population substructure among individuals was also assessed for SNPs called from Clair3. Similarly, SNPs were filtered using a minimum MAF threshold of 0.1 and a missing genotype threshold of 0.9 (--geno 0.9), whereby SNPs containing genotype information for less than 90% of the

individuals were omitted for subsequent analysis.

Principal Component (PC) association analyses were conducted using PLINK v1.90b7.2 to identify which variants contributed to the variation observed along the PC axes. These analyses indicated that population structure was primarily driven by variants located within three previously identified chromosomal inversions: NC_079167.1, NC_079169.1, and NC_079172.1. To illustrate the contribution of these inversions to population structure, PCA plots were colored according to chromosomal inversion genotype.

To assess the potential presence of a correlation between SV and SNP density across genomic regions, a generalized linear model (GLM) with a semi-Poisson distribution was fitted to binned (per Mb) variant densities using statsmodels (Skipper & Josef, 2011/2010) in Python. SNP count per bin was used as the predictor variable, and SV count per bin was the response variable. Standard residuals were calculated from the model and used to plot the differences in SV and SNP density over binned windows (1 Mb) across the genome. For consistency and comparability of SV and SNP density estimates across the genome, identical filtering thresholds were applied to both variant classes, including a MAF threshold of 0.1 and missing genotype threshold of 0.9.

Haplotype phasing and genetic divergence

Chromosomal inversions are commonly associated with reduced or suppressed recombination, and patterns of haplotype divergence within inversion regions can therefore provide insight into their evolutionary dynamics (Berdan et al., 2023; Sambade et al., 2025a). Because PCA results indicated that population structure was primarily driven by SVs and SNPs located within three known inversions on chromosomes NC_079167.1, NC_079169.1, and NC_079172.1, patterns of haplotype divergence were further investigated within the inversion boundaries. To this end, SNPs were haplotype phased using the following procedure (Fig. 1G): First, SNPs were extracted from the inversion regions (500,000-16,620,000 on NC_079167.1, 86,500,000-93,000,000 on NC_079169.1, and 34,100,000-40,000,000 on NC_079172.1) (Sambade et al., 2025a), using bcftools. Subsequently, variants were normalized using bcftools norm -m +any to retain biallelic SNPs only, after which duplicate variants were removed using bcftools norm -d. Finally, the selected SNPs were haplotype phased using Beagle 5.5 (Browning et al., 2021). Pairwise haplotype divergence for inversion genotypes was calculated by hierarchical clustering of haplotypes using the UPGMA method, based on the pairwise Hamming distance from all SNPs within each inversion.

All plots were generated in ggplot2 v3.5.2 (Wickham, 2016) in R and Matplotlib v3.10.1 (Hunter, 2007) in Python.

A complete overview of all tools and their versions used in this study, along with a more detailed description of their function, is provided in the supplementary material (Supplementary Table S1).

Results

SV characterization

SVs were detected from long-read nanopore sequencing data of 35 oyster individuals (mean sequencing depth: 13.5 ± 4.2x), using three callers: Sniffles2, SVIM, and cuteSV. Sniffles2 identified 747,853 SVs, while cuteSV identified 1,512,512 SVs, and SVIM identified 2,272,194 SVs. Merging SVs across these three callers and filtering for different parameters led to retaining a total of 288,756 variants (mean length 591.79 bp, median length 169 bp), representing a retention rate of 11.1% from the total number of merged SVs across the three callers. Of this final set of filtered SVs, 171,424 were deletions (59.4%, mean length 641.64 bp, median length 170 bp), 116,090 insertions (40.2%, mean length 465.71 bp; median length 166 bp), 730 inversions (0.3%, mean length 3983.71 bp, median length 928 bp), and 512 duplications (0.2%, mean length 7,650.63 bp, median length 2,577.5 bp). Overall, SVs covered a total length of 170,881,901 bp, accounting for 19.1% of the entire length of the reference genome (GCF_947568905.1; 894.7 Mb excluding 42 unplaced scaffolds). Most SVs exhibited a length < 1 kb (87.3%; mean length 235 bp, median length 144 bp, Fig. 2A), whereas only a small proportion of the SVs were longer than 5 kb (1.5%). The ideogram shows that SVs are distributed more or less evenly across the genome, with an average density of one SV per 3,092 bp (323.37 SVs per Mb; Fig. 2B).

Insertions contributed most substantially to intronic regions (Fig. 2C). The distribution of SVs across genomic features was most similar between deletions and duplications, both primarily overlapping introns. In contrast, inversions displayed a distinct pattern, with a higher proportion positioned in genic regions, including coding sequences, exons, 3'UTRs, and 5'UTRs.

Enrichment analysis revealed that deletions were significantly depleted across all genomic features compared with the genomic background (Fig. 2D). Similarly, insertions predominantly exhibited depletion in genic regions, including exons, 3'UTRs, and 5'UTRs, while displaying weak but significant enrichment for intronic regions. Inversions exhibited weak, non-significant enrichment in 5'UTRs, but were strongly and significantly depleted in coding sequences, exons, and introns. Duplications displayed a largely neutral enrichment profile, characterized by weak but significant depletion in exons, and strong significant depletion in 3'UTRs. Overall, structural variants exhibit pronounced depletion in genomic features compared to the genomic background.

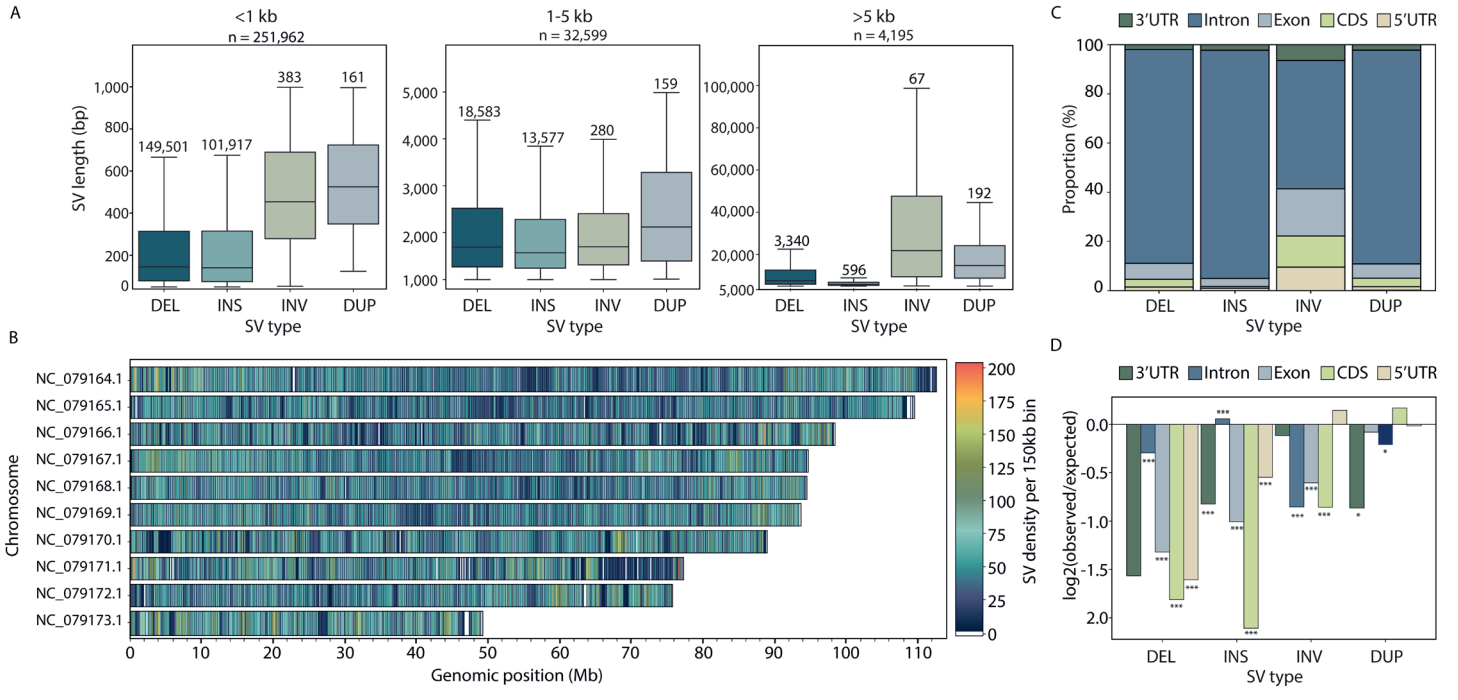


Figure 2 | Genome-wide landscape of structural variants (288,756 SVs) mapped across the European flat oyster genome. **(A)** Boxplots for all SV types (deletions, insertions, inversions, and duplications), split into three length classes: < 1 kb, 1,000 bp to 5,000 bp, and > 5,000 bp. **(B)** Ideogram displaying SV density per 150 kb intervals along the ten chromosomes. **(C)** SV proportional distribution across genomic features. **(D)** Observed/Expected ratio of SV occurrences across genomic features with asterisks denoting different significance thresholds from enrichment analysis (*p < 0.05, **p < 0.01, ***p < 0.001).

SV impact and annotation

Genetic variant annotation and prediction of SV effects on genes were performed using snpEff (Fig 3A). A total of 278,780 SVs (96.5%) were assigned a "MODIFIER" impact, indicating variants that are unlikely to affect gene function, typically occurring in non-coding regions such as introns, untranslated regions (UTRs), or intergenic sequences, and are therefore considered non-disruptive. Additionally, 7,770 SVs (2.7%) were classified as "HIGH" impact, representing variants predicted to strongly disrupt gene function. The remaining SVs were categorized as "MODERATE" or "LOW" impact, reflecting variants that may alter gene function to a lesser extent than variants categorized as "HIGH". Predicted high-impact SVs most frequently overlapped the effect categories "splice region variant", "intron variant", "frameshift variant", "feature ablation", and "splice acceptor variant" (Fig. 3B).

Functional enrichment analysis of genes overlapping high-impact SVs revealed significant enrichment for biological function related processes associated with compound binding and cellular metabolism pathways (Fig. 3C). The most significantly enriched GO terms, considering P-value, gene ratio, and gene count, included GO:0034641, Cellular nitrogen compound metabolic, GO:1901360, Organic cyclic compound metabolic, GO:0046483, Heterocycle metabolic process, GO:0006725, Cellular aromatic compound metabolic, and GO:0006139, Nucleobase-containing compound, among others. Additionally, several DNA repair- and recombination associated processes were significantly enriched, including GO:0006310, DNA recombination, GO:0051276, Chromosome organization, GO:0006260, DNA replication, and GO:0006302, Double-strand break repair. Regarding cellular components, processes associated with non-membrane bounded organelles (e.g. GO:0005622, Intracellular anatomical structure, GO:0005737, cytoplasm, GO:0005634, nucleus, GO:0043232, Intracellular non-membrane-bounded, and GO0043228, Non-membrane-bounded-organelle) were the most enriched, whereas for molecular function, pathways associated with cyclic compound binding (e.g. GO:0005488, binding, GO:0097159, organic cyclic compound binding, GO:01901363, Heterocyclic compound binding, and GO:0003676, Nucleic acid binding) were most significantly enriched (Supplementary Fig. S1).

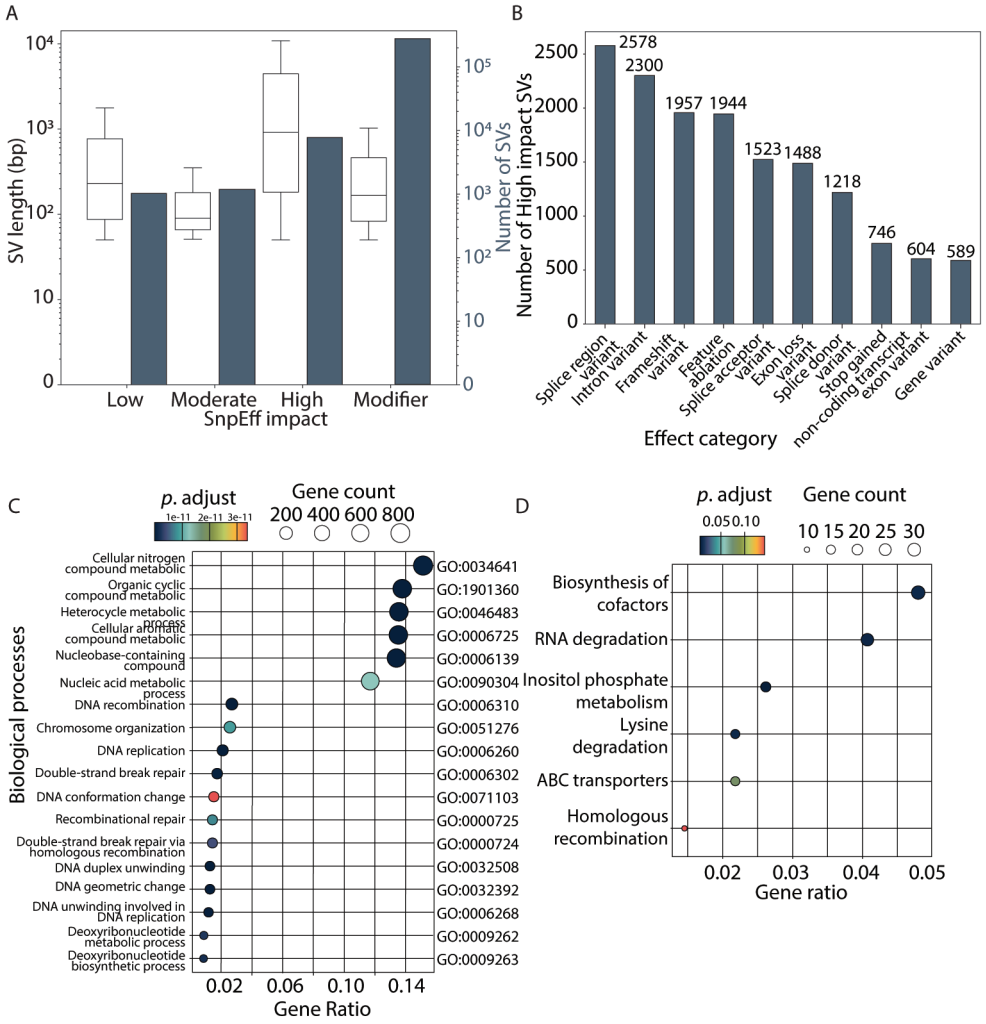


Figure 3 | Annotation of structural variants (SVs). **(A)** Boxplot showing the length distribution of SVs across predicted impact categories based on snpEff annotation (modifier, low, moderate, or high; left y-axis), bar plot displaying the number of SVs in each impact category (right y-axis). **(B)** Bar graph displaying the top 10 predicted effect categories overlapping annotated high-impact SVs. **(C)** GO enrichment analysis for Biological Processes associated with genes overlapping high-impact SVs. **(D)** KEGG enrichment analysis of genes overlapping high-impact SVS.

A complete overview of all enriched GO terms for each enrichment category is provided in the Supplementary material (Supplementary Tables S2-S4). Pathway enrichment analysis identified six significantly enriched KEGG pathways, of which the most significantly enriched included Biosynthesis of cofactors, RNA degradation, Inositol phosphate metabolism and Lysine degradation (Fig. 3D). In addition, less significantly enriched KEGG pathways included ABC transporters and Homologous recombination.

Repeat characterization and annotation

Repeat annotation of the *O. edulis* genome (assembly: GCF_947568905.1; total length of 894.8 Mb), using RepeatModeler and RepeatMasker, revealed that 58.2% (521.1 Mb) of total genomic length was masked as repetitive sequence. Retroelements represented the largest annotated repeat class, occupying 15.4% of the genome (137.3 Mb), with long interspersed nuclear elements (LINEs) representing the most abundant repeat family within retroelements (8.2%), followed by long terminal repeat (LTR) elements (3.83%), Penelope elements (2.3%), and short interspersed nuclear elements (SINEs; 1.1%). DNA transposons constituted an additional 3.5% of the genome (31.6 Mb). Together, retroelements and DNA transposons accounted for 18.9% of the genome, representing the majority of annotated TEs. Rolling-circle transposons were rare, accounting for only 0.04% of the genomic sequence. A substantial proportion of repetitive sequences could not be assigned to a known TE class and remained unclassified (37.7%; 337.1 Mb).

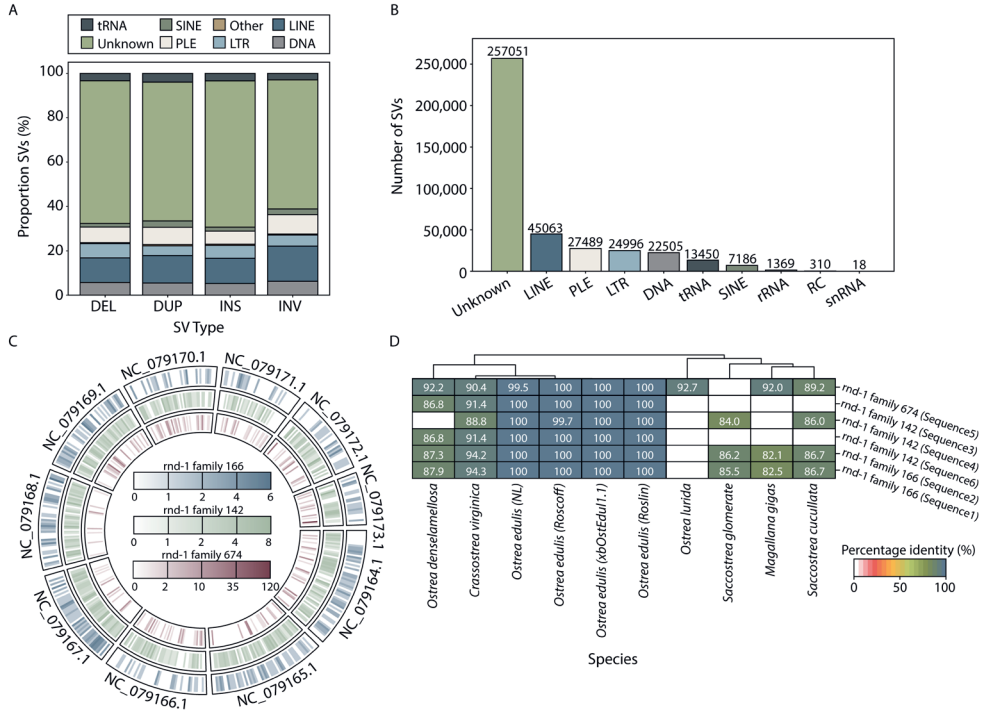


Figure 4 | Characterization of structural variants (SVs) overlapping repeat elements in *O. edulis* genome. **(A)** Proportion of SV types (insertions, deletions, duplications, and inversions) overlapping repeat elements. **(B)** Number of SVs overlapping different repeat classes. **(C)** Circos plot illustrating the genome-wide distribution and density of three repeat-element families across the ten chromosomes of the *O. edulis* xbOstEdul1.1 reference genome assembly. **(D)** Heatmap displaying percentage identity from BLAST analysis, where six consensus sequences overlapping three distinct repeat-element families identified from SVs in *O. edulis* were blasted against the four reference genomes for *O. edulis* and closely-related species within the Ostreideae family.

To characterize structurally variable repetitive sequences and identify highly active transposable elements (TEs), SVs were overlapped with repeat sequences. In total, 58.1% of SVs (167,959 of total 288,756 SVs) overlapped with 399,437 repeat elements. Among the SVs overlapping repeat elements, 108,818 were deletions (64.8%), 58,087 insertions (34.6%), 705 inversions (0.5%), and 349 duplications (0.2%; Fig. 4A). From the total number of SVs overlapping repetitive sequences, 167,959 SVs contributed to TEs (52.71%). The largest fraction of SVs overlapping these repeats were classified as "unknown" (131,920 SVs; 78.5%) (Fig. 4B). The second most abundant repeat class was represented by LINEs (30,095 SVs; 17.9%), followed by PLE (18,414 SVs; 11.0%), and LTRs (18,253; 10.9%). A lower number of SVs overlapped with DNA transposons (16,044 SVs; 9.6%). Followed by tRNAs (10,271 SVs; 6.1%) and SINEs (5,719 SVs; 3.4%). The least frequent repeat classes that SVs overlapped with were ribosomal RNAs (1,239 SVs; < 1%), RCs (192 SV; < 1%), and small nuclear RNAs (9 SVs; <1%).

SV length distribution histograms revealed two prominent peaks in the number of deletions and insertions within the 280-340 bp size range, likely corresponding to repetitive sequences structurally active in the genome (Supplementary Fig. S2). To identify the repeats underlying these peaks, SV-associated sequences within this size range were extracted and compared against the *O. edulis* custom repeat database generated in this study. This analysis showed that the most abundant sequences in this size range were consistently annotated as unknown repeat classes. Despite their unknown classification, these repeats exhibited a broad genomic distribution across the *O. edulis* genome (Fig. 4C). BLAST searches of the repeat sequences against the NCBI RNA RefSeq database for *O. edulis* revealed near-identical matches (> 95% identity) to multiple predicted *O. edulis* transcripts from unrelated genes (e.g. *histone deacetylase 6-like, protein Fe65 homolog, sodium/glucose cotransporter 4-like, beta-1,3-galactosyltransferase 5-like,* or *putative inhibitor of apoptosis*). This indicates that the repeat sequences occur broadly across transcribed genomic regions rather than representing a gene-specific sequence. Inspection of genomic feature locations in the Integrative Genomics Viewer (IGV) in NCBI revealed that the repeats predominantly occur in the untranslated regions (UTRs), which likely explains their frequent presence in RNA RefSeq datasets.

Subsequent BLAST searches against other available reference assemblies of *O. edulis* (all four available assemblies), and closely related species, including Ostrea denselamellosa, *Ostrea lurida, Saccostrea cucullata, Saccostrea glomerata, Crassostrea virginica,* and *Magallana gigas* yielded the highest-confidence matches to *O. edulis* genome assemblies (Fig. 4D). The next highest percentage identities were observed in *C. virginica* and *O. denselamellosa*, with identities ranging 86.8 – 92.4%. Blasting against *O. lurida* only resulted in one hit with consensus sequence 5, suggesting this may reflect a reference genome assembly issue rather than true absence of the

repeat family in this species. The repeat sequences displayed decreasing sequence similarity in more distantly related oyster genera, whereby *S. glomerata, M. gigas*, and *S. cucullata* exhibited lower percentage identities.

Pairwise alignments of all repeat sequences revealed consensus sequences 4 and 6 (rnd-1 family 142) were most similar (98.5% identity), whereas consensus sequences 3 was more divergent from sequences 4 and 6 (59.8% and 59.4% identity respectively; Supplementary Figure S3). Consensus sequences 1 and 2 (rnd-1 family 166) exhibited moderate similarity (60.1%). Consensus sequence 5 (rnd-1-family 674) was highly divergent from the other sequences. This variability in percentage identity among repeat sequences, both within the same repeat family ad across closely related bivalve species, reflects the high activity and polymorphic nature of these repeats.

Genetic population structure from genome-wide SVs and SNPs

Principal component analyses (PCA) were performed to investigate population structure inferred from genome-wide SVs. The first two principal components (PC1 and PC2) captured the largest proportion of variation (17.3% and 13.1%, respectively) (Fig. 5A). Subsequent PC association analysis revealed a strong clustering of SVs associated with PC2 within a large genomic region on chromosome NC_079167.1 (Fig. 5A; Supplementary Fig. S4). Additional clustering along PC3 was associated with another large genomic region on chromosome NC_079169.1 (Fig. 5A; Supplementary Fig. S5). Previous studies have reported three large chromosomal inversions in *O. edulis* on chromosomes NC_069167.1, NC_079169.1, and NC_079172.1 (Alves Monteiro et al., 2024; Sambade et al., 2022, 2025a). In contrast to the substantial contribution to population structure of SVs located within inversions on chromosomes NC_079167.1 and NC_079169.1, SVs located within the inversion on NC_079172.1 did not display any clear association with population structure inferred from genome-wide variants (Fig. 5A).

To asses how population structure inferred from SNPs compared with that inferred from SVs, genome-wide SNPs were de novo called from the long-read nanopore data. SNP calling using Clair3 resulted in a total identification of 23,483,156 SNPs, corresponding to an average density of one SNP per 38 bp across the genome (26,298.5 SNPs per Mb). PCA revealed that population structure inferred from genome-wide SNPs was primarily explained by PC1 (14.0%) (Fig. 5A). PC association analysis further showed that SNPs located within the inversions on chromosomes NC_079167.1 and NC_079172.1 substantially contributed to the variation explained along PC1 (Supplementary Fig. S6). PCAs based on genome-wide variants excluding the three inversion regions revealed clustering of individuals into two distinct groups along PC1 and PC2 in both SV and SNP datasets (Supplementary Fig. S7), indicating this structure persists beyond the inversion regions.

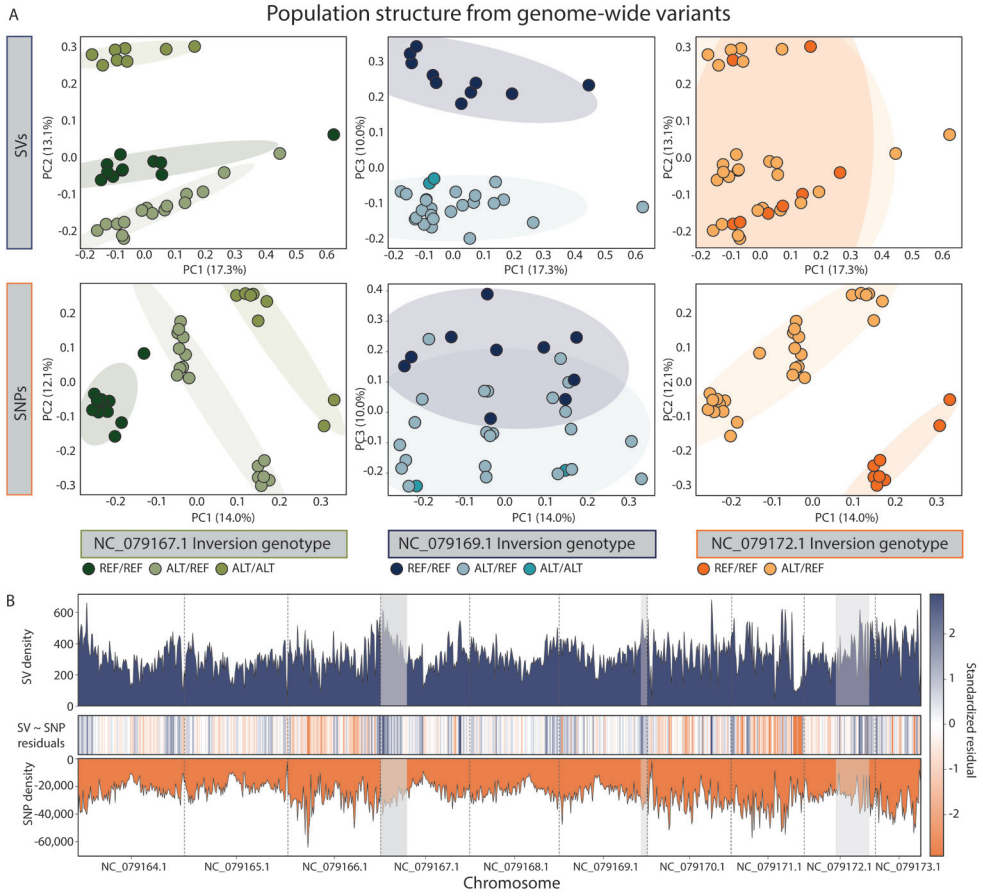


Figure 5 | Genome-wide genetic population structure from single nucleotide and structural genetic variation across 35 *O. edulis* individuals. **(A)** Principal component analysis (PCA) displaying within-population structure inferred from genome-wide SVs (upper) and SNPs (lower). Individuals are colored according to chromosomal inversion genotypes on chromosomes NC_079167.1 (green), NC_079169.1 (blue), and NC_079172.1 (orange), while the data from the PCA itself is based on genome-wide variants. Ellipses represent 95% confidence intervals. **(B)** SV (upper) and SNP (lower) densities in 1 Mb genomic windows, with vertical panels indicating the 10 chromosomes. The central heatmap shows standardized residuals from a quasi-Poisson generalized linear model (GLM) predicting SV density based on SNP density per genomic bin. Positive residuals (blue) indicate higher-than-expected SV density, whereas negative residuals (orange) reflect lower-than-expected SV density. Grey windows represent chromosomal inversions on chromosomes NC_079167.1, NC_079169.1, and NC_079172.1, with boundaries defined by Sambade et al. (2025a).

After filtering for missing genotypes and applying a MAF threshold of 0.1, the SV dataset retained a total of 247,040 variants, while the SNP dataset retained 13,556,523 polymorphic sites. Although SV density strongly correlated with SNP density (generalized linear model: $z = 120.2$, $p < 0.001$), visualization of the model's residuals revealed substantial variation in both SNP and SV densities across the genome, with certain regions showing an overrepresentation of SVs relative to SNPs (Fig. 5B). Across the genome, SVs occurred at lower densities (mean 323.4 SVs per Mb) compared to SNPs (mean 26,296.9 SNPs per Mb). Mean SV densities within the three inversions were 412, 471, and 358 SVs per Mb, respectively, whereas the background genome (excluding inversion regions) had an average SV density of 316 SVs per Mb. Mean SNP densities within the three inversions were 28,833, 32,761, and 26,480 SNPs per Mb, respectively, with the background genome (excluding inversion regions) showing an average density of 26,399 SNPs per Mb. This indicates that both SV and SNP densities are elevated in inversion regions compared with the genomic background.

Haplotype divergence across chromosomal inversion haplotypes

Because chromosomal inversions can reduce or suppress recombination, they may contribute to genetic differentiation among individuals within populations (Berdan et al., 2023; Wellenreuther et al., 2025). After identifying that population structure inferred from genome-wide SVs and SNPs was largely associated with variants located within three chromosomal inversions, we further investigated genetic divergence among inversion-associated haplotypes to evaluate potential signatures of reduced recombination.

Haplotype phasing revealed three distinct haplotypes for the inversion on chromosome NC_079167.1. The largest haplotype cluster (n = 32) displayed a mosaic-like pattern of relative genetic divergence, with the highest divergence observed between the two haplotypes of a single individual, consistent with a heterozygous genotype for the inversion polymorphism (Fig. 6A). Analysis of mean pairwise allele distance and genetic divergence (Dxy) across haplotype clusters further confirmed that this cluster corresponds to inversion heterozygotes (Supplementary Fig. S8A, S9, & S12A).

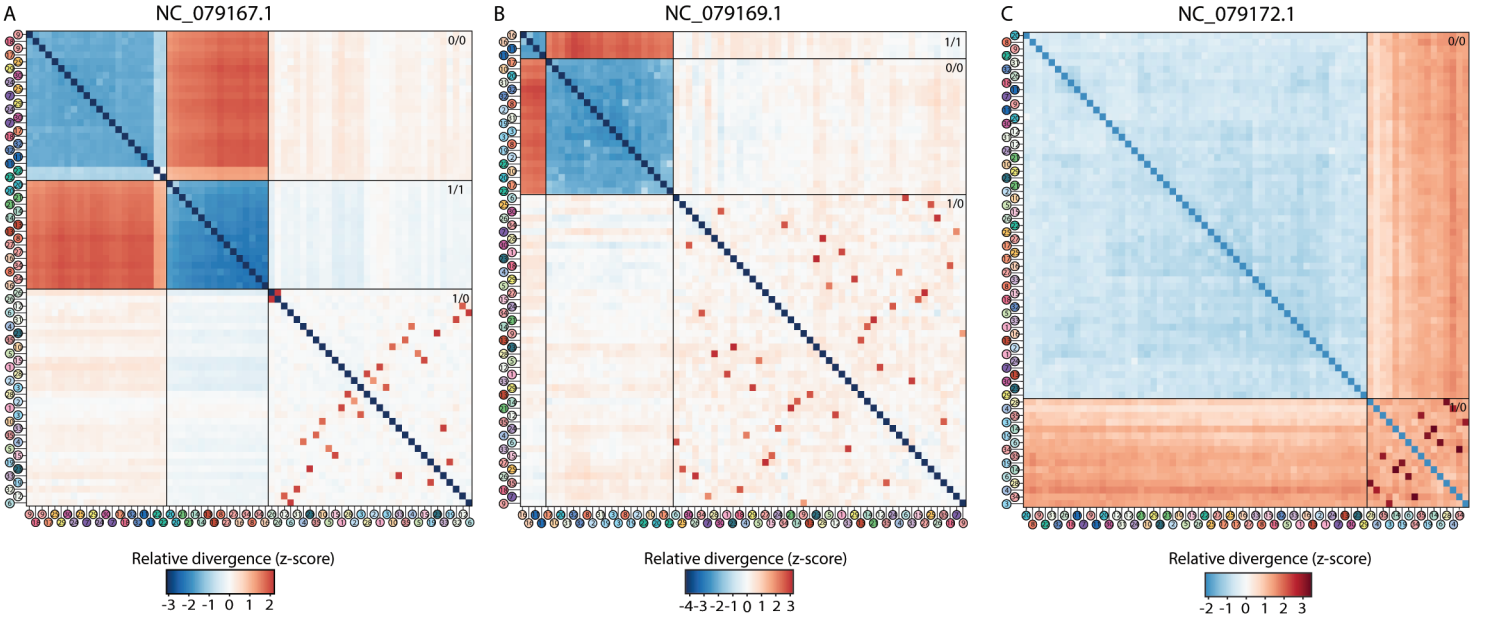


Figure 6 | Characterization of chromosomal inversion haplotypes. (A-C) Pairwise relative divergence (z-score) between haplotypes, calculated from hierarchical clustering based on pairwise Hamming distances from haplotype-phased SNP data. Analyses were performed for three genomic regions containing chromosomal inversions: 500,000-16,620,000 bp on NC_079167.1 **(A)**, 86,500,000-93,000,000 bp on NC_079169.1 **(B)**, and 34,100,000-40,000,000 bp on NC_079172.1 **(C)**.

For the chromosomal inversion on chromosome NC_079169.1, fewer haplotypes contributed to both the inverted and non-inverted clusters, with only two homozygous individuals comprising the smallest haplotype cluster (Fig. 6B). Similar to observations for NC_079167.1, analysis of mean pairwise allele distances and genetic divergence (Dxy) across haplotype clusters confirmed that the largest haplotype cluster (n = 46) corresponds to inversion heterozygotes (Supplementary Fig. S8B & S12B). Within the heterozygous cluster, the highest genetic divergence was observed between haplotypes from the same individual (Fig. 6C). Notably, within the inverted region, smaller genomic segments of high within-cluster genetic similarity differentiate the two homozygous haplotype clusters and coincide with regions of reduced genetic divergence within haplotype clusters, likely representing haploblocks (Supplementary Fig. S10).

Haplotype phasing of the inversion on NC_079172.1 revealed only two clearly defined clusters (Fig. 6C). The smaller cluster (n = 16) showed the highest relative genetic divergence between haplotypes within the same individual, likely representing inversion heterozygotes. This cluster also exhibited higher values of overall pairwise allele distances and genetic divergence (Dxy) among the haplotype clusters, comparable to those observed for heterozygotes for the other two inversions (Supplementary Fig. S8C & S12C). In contrast to NC_079167.1 and NC_079169.1, the larger cluster (n = 54) comprised homozygous individuals (Supplementary Figure S11).

To compare signatures of haplotype divergence across the three inversions, pairwise relative divergence within haplotype clusters was scaled to inversion size and compared among inversions (Supplementary Figure S13). No significant difference in haplotype divergence was observed between haplotype clusters 1 and 2 for the inversion on NC_079167.1. In contrast, genetic divergence within the heterozygous haplotype cluster 3 associated with the inversion on NC_079169.1 was significantly higher than that observed for the heterozygous cluster of the inversion on NC_079167.1 (cluster 3).

Discussion

Although mollusks are known to harbor extremely high levels of genetic diversity (Shi et al., 2014), structural variation remains poorly characterized across this clade. Recent developments in sequencing technologies and improved computational resources now enable more comprehensive characterization of structural genetic variation in non-model organisms, revealing extensive genomic diversity among bivalves (Alves Monteiro et al., 2024; Cariolato et al., 2025; Gundappa et al., 2022; Jiao et al., 2021).

This study provides the first comprehensive genomic structural variation landscape in the European flat oyster (*O. edulis*), an ecologically and economically important bivalve. By comparative analysis of 35 individuals we characterized how structural variants shape within-population genetic structure and explored the presence of active repeat elements in the *O. edulis* genome. We further assessed the differences in genetic population structure inferred from structural variants and single-nucleotide polymorphisms through comparative analysis. Finally, patterns of recombination and genetic divergence across three previously described chromosomal inversions were explored, using SNP-based haplotype phasing.

Characterizing the SV genomic landscape in the flat oyster

In this study, a total of 288,756 high-confident SVs were identified using whole genome nanopore sequencing, a number higher than those reported in previous studies of related bivalve species (Jiao et al., 2021; Jung et al., 2025). This discrepancy likely reflects differences in detection sensitivity between sequencing technologies, with long-read approaches providing improved genomic resolution and the ability to resolve complex or repetitive genomic regions (Iyer et al., 2024). Consistent with our findings, several recent studies employing long-read sequencing approaches have reported substantially higher numbers of SVs in comparison with short-read-based analyses (Cretu Stancu et al., 2017; Soto et al., 2020; Zhang et al., 2023). The European flat oyster genome proved to be highly polymorphic, with SVs accounting for 19.1% of the total genome assembly length. This proportion is comparable to levels reported in other oyster genomes (Jiao et al., 2021; Qi et al., 2021), and considerably higher than those described for fish (Jiao et al., 2025; Quah et al., 2025; Tigano et al., 2021). Similarly, a recent study on the Pacific oyster (*Magallana gigas*) reported that SVs accounted for >30% of the total genome (Jung et al., 2025).

Notably, the approach used in this study, retaining high-confident SVs that were consistently identified by all three callers, captured just 11% of the total number of SVs detected by any of the three callers. This highlights substantial inconsistencies among callers in detecting SVs from non-model organisms and underscores the need for further improvements in SV calling accuracy across tools (Mahmoud et al.,

2019). Additionally, the relatively low nanopore sequencing coverage (~13x) used for SV detection may have contributed to false-positive variant calls (Pei et al., 2024). Conversely, after stringent filtering, principal component and PC association analyses clearly revealed population structuring driven by known chromosomal inversions, thereby supporting the overall robustness and confidence of the identified SV dataset.

SV annotation and gene regulatory impact

The majority of SVs were located in intronic regions, whereas SVs exhibited pronounced depletion in coding regions (UTR3, exons, coding sequences, and UTR5), indicating strong purifying selection against deleterious variation. Such purifying selection appears to be highly effective in species with large effective population sizes, as was reported for several mollusks exhibiting highly polymorphic genomes (Gerdol et al., 2020; Jung et al., 2025; Qi et al., 2021).

Despite the high effective purifying selection against most structural variants, nearly 8,000 SVs were annotated as high-impact variants, underscoring the substantial contribution of SVs to gene regulation. Annotation of these variants indicated SVs primarily affect coding regions, mainly through inducing frameshifts. These findings are consistent with expectations for SVs classified as high-impact, as insertions or deletions of genomic segments can disrupt reading frames, abolish transcript structures, fuse adjacent genes, or introduce alternative stop or start sites (Demidov et al., 2024; Lee et al., 2012; Weischenfeldt et al., 2013). Enrichment analysis further demonstrated that genes overlapping high-impact SVs were significantly enriched in pathways related to cellular metabolism and mechanisms associated with DNA repair and recombination. Notably, enrichment of RNA degradation pathways points toward a potential role of SVs in post-transcriptional regulation. By impacting these pathways, SVs may facilitate adaptive regulatory flexibility. The contribution of post-transcriptional regulatory activity to phenotypic plasticity in oysters is only beginning to be explored, highlighting the need for a comprehensive investigation into the role of SVs in these regulatory processes (Clairet et al., 2025; Liang et al., 2025). Further, SVs affecting genes involved in inositol phosphate and lysine metabolism are potentially involved with facilitating responsiveness to environmental stimuli, as in other oysters both pathways were found associated with stress responses (Cao & Wang, 2017; Taji et al., 2006; Tu-Sekine & Kim, 2022). While enrichment analyses can highlight functional impacts of SVs, their reliability is constrained by the accuracy of genome annotation and so requires caution. Improved gene function prediction and expanded databases should enable more reliable estimates of the genetic impact of structural variation in the future.

The substantial role of highly active repeats in shaping structural variation

Transposable elements (TEs) represent repetitive sequences that can freely "move around" in the genome by (autonomous) insertion or deletion, contributing to population structure and genetic diversity in oysters (Jiao et al., 2021; Maside et al., 2005; Vera et al., 2015). To explore the relationship between SVs and repetitive sequences in *O. edulis*, a de novo repeat database was generated for the xbOstEdul1.1 reference genome assembly. This analysis showed that 58.2% of the genome consists of repetitive sequences, consistent with previous findings for bivalves (Martelossi et al., 2023). A substantial proportion of SVs was associated with annotated TEs, indicating that TEs substantially contribute to SV formation in *O. edulis*. Similar patterns have been observed in the Pacific oyster (Jung et al., 2025). Retrotransposons constituted the largest fraction of identified TEs, contrasting with earlier studies that identified DNA transposons as most abundant TEs (Vera et al., 2015). This discrepancy likely reflects methodological differences, as short-read sequencing can underestimate long repetitive elements (Bell et al., 2022).

Analysis of some highly frequent SVs revealed overlap with three repeat families of unknown function that appear to be highly active in the *O. edulis* genome. Given the relatively short lengths and widespread genomic distribution, these repeats likely represent non-autonomous transposable elements, such as SINEs. Their abundance and apparent activity in the *O. edulis* genome suggest that they may contribute to genetic diversity and local adaptation, consistent with evidence from other taxa linking active TEs to evolutionary adaptation (Almojil et al., 2021; Casacuberta & González, 2013; Martelossi et al., 2023; Stapley et al., 2015). However, many SVs overlapping repeats could not be classified, underscoring the need for improved repeat annotation in non-model organisms and further research into their role in bivalve evolutionary dynamics (Liu et al., 2021; Penso-Dolfin et al., 2020).

Complementary insights from genome-wide SVs and SNPs into population substructure

Genetic population structure is most often inferred from single-nucleotide polymorphisms (SNPs), identified through SNP genotyping arrays or short-read sequencing. Although these approaches provide a cost-effective means of assessing genome-wide intraspecific genetic diversity, they are inherently biased toward detecting point mutations and are therefore limited in their ability of de novo detection of larger-scale genomic variation (Wellenreuther et al., 2019). While strong patterns of linkage disequilibrium among SNPs can indirectly infer the presence of structural variants, such as chromosomal inversions (Kamermans et al., 2023; Lapègue et al., 2023; Sambade et al., 2022), the resolution required to characterize such variation in detail remains

limited. In contrast, long-read sequencing enables detection and analysis of structural variation across a broad range of sizes (Jiang et al., 2021; Pokrovac & Pezer, 2022; Scarano et al., 2024). Several studies even reported contrasting patterns of genetic variation inferred from SNPs and SVs (Stuart et al., 2023), highlighting the relevance of complementary analyses that integrate both sources of genetic variation. In this study, comparative analysis of population structure inferred from genome-wide SVs and SNPs revealed discordant patterns between the two approaches. Previous studies have identified three chromosomal inversions across chromosomes NC_079167.1, NC_079169.1, and NC_079172.1 in the *O. edulis* genome (Alves Monteiro et al., 2024; Sambade et al., 2022; Valk et al., 2026). Where population structure inferred from genome-wide variants was strongly influenced by the chromosomal inversion on NC_079167.1 for both SV and SNP datasets, SV-based population structure showed a stronger contribution by the inversion on chromosome NC_079169.1, whereas SNP-based population structure was predominantly determined by SNPs located within the inversion region of chromosome NC_079172.1. Haplotype phasing of SNPs across all three inversions revealed the absence of the inversion homozygote genotype among all 35 individuals. This absence may explain why the inversion on chromosome NC_079172.1 did not substantially influence population structure inferred from genome-wide SVs. Consistent with this observation, Kamermans et al. (2023) reported the rare occurrence of inversion homozygotes for NC_079172.1 in the "Grevelingen" population. Additionally, previous work has documented pronounced variation in genotype frequencies among populations, suggesting that selective pressures may drive both the maintenance and establishment of these inversion polymorphisms at varying frequencies, a processes referred to as balancing selection (Alves Monteiro et al., 2024; Lapègue et al., 2014; Sambade et al., 2022; Valk et al., 2026).

Haplotype divergence analyses of phased SNPs within inversion regions revealed high pairwise genetic divergence between haplogroups representing homozygotes for inversions on both NC_079167.1 and NC_079169.1. Notably, the elevated average genetic divergence among heterozygote haplotypes of inversion on chromosome NC_079169.1, suggests that this inversion may be older than the inversion on chromosome NC_079167.1, as genetic divergence is expected to accumulate over time in regions with suppressed recombination. A similar pattern of genetic divergence across haplogroups for the inversion on chromosome NC_079169.1 was reported in a previous study (Sambade et al., 2025a). If these inversions originated at different times, they may also have experienced different evolutionary histories and selective pressures, which could contribute to the observed variation in inversion genotype frequencies across European populations to date (Alves Monteiro et al., 2024; Sambade et al., 2025a; Valk et al., 2026).

Additionally, all three inversions consistently exhibited the highest levels of genetic divergence between heterozygous haplotypes, which is indicative of reduced or suppressed recombination within inversion regions. While these chromosomal inversions have been reported in previous studies, this work provides the first detailed assessment of haplotype-level divergence within these regions, revealing clear signatures of divergence patterns consistent with recombination suppression. Reduced recombination in inversion heterozygotes is a well-described phenomenon across taxa and may contribute to the accumulation of genetic differentiation and the early stages of reproductive isolation (Berdan et al., 2023; Meyer et al., 2024; Wellenreuther et al., 2025; Wellenreuther & Bernatchez, 2018). This highlights the importance of further investigating the evolutionary processes shaping these inversion genotypes across populations and their potential implications for conservation of *O. edulis*.

Interestingly, both SV and SNP densities were elevated in the inversion regions on chromosomes NC_079167.1 and NC_079169.1, relative to the genomic background, while the third inversion (on chromosome NC_079172.1) exhibited similar densities to the rest of the genome. The absence of elevated densities of structural or single-nucleotide genetic variation across the inversion region on chromosome NC_079172.1, may be reflected by the lack of homozygous genotype individuals for this inversion among the sequenced samples included in this study, highlighting the importance of representative sampling. In contract, the elevated densities of genetic variation observed in the other two inversion regions suggest that chromosomal inversions can act as large linkage disequilibrium blocks, serving as reservoirs of genetic variation and carrying distinct sets of variants (Battlay et al., 2023, 2025). Together with the highly active repeat elements identified in this study, representing an additional source of structural genetic variation, these findings point to ongoing genomic change and potentially represent balancing selection maintaining genetic variation across inversion genotypes. This interpretation is consistent with previous studies proposing that bivalves require rapid responses to environmental conditions due to their sessile lifestyle, potentially facilitated by mechanisms such as gene family expansion or genome duplication, both of which are suggested to contribute to the adaptive and evolutionary success of various bivalves (Modak et al., 2021; Regan et al., 2021). However, further research is required to identify the primary drivers of these highly polymorphic genomes. Collectively, this work underscores the relevance of future research aimed at elucidating how chromosomal inversions shape and maintain structural variant landscapes in evolutionary and conservation contexts.

Conclusion

This study presents the first whole-genome characterization of the structural variation landscape across the European flat oyster genome. We provide a comprehensive description of genomic distribution, types, and size ranges of SVs and discuss their potential functional implications. Intersection of SVs with de novo masked repetitive sequences highlighted the extensive contribution of repeat elements to structural variation. Additionally, patterns of population structure, recombination, and haplotype divergence were explored across three previously identified chromosomal inversions, leveraging the complementary insights provided by SVs and SNPs. Overall, the findings of this study reveal the extensive genomic variation established within the European flat oyster genome and underscore the value of incorporating structural variation data alongside single-nucleotide genetic variation to resolve fine-scale population structure and evolutionary dynamics in a marine species.

Data availability statement

Sequencing data used for SV and SNP calling were previously deposited in the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena/), as part of Valk et al. (2025), which examined sex-specific DNA methylation. This sequencing data is available under project number PRJEB85091, and accession number ERP168521.

SV and SNP datasets were made available in .VCF format on SEANOE (doi: 10.17882/112587).

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.

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Supplementary information

Supplementary Table S1 | Overview of all tools used in de novo calling of structural variation (SV) and single-nucleotide polymorphism (SNP) from Nanopore reads of 35 *Ostrea edulis* individuals.

Software	Version	Function	References
Sniffles2	2.6.2	Per-individual structural variant (SV)	(Smolka et al., 2024)
bcftools	1.17	VCF filtering/processing; merge SNPs; normalize SNPs; remove duplicates	(Danecek et al., 2021)
Jasmine	1.1.5	Convert duplications/insertions for cross-tool compatibility; merge SVs; harmonize SV types	(Kirsche et al., 2023)
Iris	1.0.5	Refine/polish SV breakpoints after initial calling	(Kirsche et al., 2023)
SVIM	2.0.0	Per-individual structural variant (SV)	(Heller & Vingron, 2019)
cuteSV	2.1.1	Per-individual structural variant (SV)	(Jiang et al., 2020)
GenomicFeatures	1.46.5	Extract genomic features from genome annotation file	(Lawrence et al., 2013)
regioneR	1.26.1	Genome location enrichment test	(Gel et al., 2017)
SnpEff	5.3a	Annotate SV effects	(Cingolani et al., 2012)
topGO	2.46.0	Gene Ontology enrichment analysis	(Alexa & Rahnenführer, 2017)
KEGGREST	1.34.0	Retrieve Kyoto Encyclopaedia of Genes and Genomes pathways	(Tenenbaum, 2017)
clusterProfiler	4.14.6	KEGG pathway enrichment analysis	(Yu et al., 2012)
BuildDatabase	2.0.7	Prepare genome for de novo repeat discovery	(Wheeler et al., 2012)
RepeatModeler	2.0.7	De novo repeat discovery	(Wheeler et al., 2012)
RepeatMasker	4.2.0	Annotate/mask repeats in the reference genome	(Wheeler et al., 2012)
bedtools	2.29.1	Intersect variant coordinates with annotated repeats	(Quinlan & Hall, 2010)
CD-HIT	4.8.1	Cluster sequences	(Li & Godzik, 2006)
seqtk	1.3-r106	Extract sequences	(Li, 2012/2012)
BLASTn	2.15.0+	Compare query sequences to reference database	(Camacho & Madden, 2025)
Clair3	1.2.0	Single-nucleotide polymorphism (SNP) calling	(Zheng et al., 2022)
PLINK	1.90b7.2	Downstream population structure analysis from variants	(Chang et al., 2015)
statsmodels	0.14.6	Statistical analyses	(Skipper & Josef, 2011/2010)
Beagle	5.5	Haplotype phasing	(Browning et al., 2021)
ggplot2	3.5.2	Plot generation in R	(Wickham, 2016)
Matplotlib	3.10.1	Plot generation in Python	(Hunter, 2007)
MAFFT	7.526	Aligning sequences	(Katoh & Standley, 2013)
minimap2	2.28-r1209	Mapping reads to reference	(Li, 2021)
Dorado	0.4.1	Demultiplexing; Basecalling	https://github.com/nanoporetech/dorado

Supplementary Table S2 | GO enrichment analysis of genes overlapping high-impact SVs significantly ($p < 0.05$) enriched for Biological Processes, sorted on adjusted p-value.

Term	Category	Count	p.adjust	logp_adjust	GeneRatio
GO:0006310, DNA recombination	BP	149	3.07E-21	20.512	0.027
GO:0006260, DNA replication	BP	116	1.13E-18	17.947	0.021
GO:0006139, nucleobase-containing compound	BP	741	3.09E-15	14.510	0.134
GO:0046483, heterocycle metabolic process	BP	750	1.91E-14	13.720	0.136
GO:0032392, DNA geometric change	BP	70	2.38E-14	13.623	0.013
GO:0032508, DNA duplex unwinding	BP	70	2.38E-14	13.623	0.013
GO:0006725, cellular aromatic compound metabolic	BP	748	3.19E-14	13.496	0.135
GO:1901360, organic cyclic compound metabolic	BP	762	3.24E-14	13.490	0.138
GO:0006268, DNA unwinding involved in DNA replication	BP	65	9.37E-14	13.028	0.012
GO:0034641, cellular nitrogen compound metabolic	BP	837	2.51E-13	12.600	0.151
GO:0006302, double-strand break repair	BP	96	2.51E-13	12.600	0.017
GO:0009263, deoxyribonucleotide biosynthetic process	BP	47	1.52E-12	11.818	0.008
GO:0009262, deoxyribonucleotide metabolic process	BP	48	2.32E-12	11.635	0.009
GO:0000724, double-strand break repair via homologous recombination	BP	79	4.24E-12	11.373	0.014
GO:0000725, recombinational repair	BP	79	7.52E-12	11.124	0.014
GO:0051276, chromosome organization	BP	142	8.91E-12	11.050	0.026
GO:0090304, nucleic acid metabolic process	BP	647	1.26E-11	10.900	0.117
GO:0071103, DNA conformation change	BP	84	3.37E-11	10.473	0.015

Supplementary Table S3 | GO enrichment analysis of genes overlapping high-impact SVs significantly ($p < 0.05$) enriched for Cellular Components, sorted on adjusted p-value.

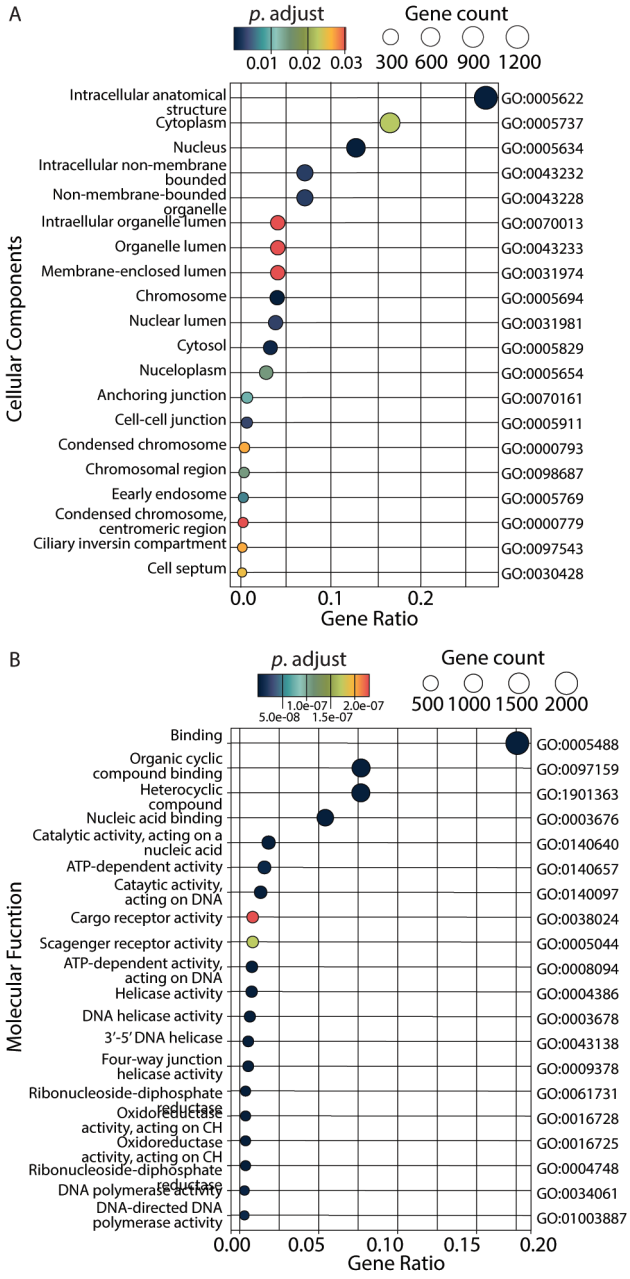
Term	Category	Count	p.adjust	logp_adjust	GeneRatio
GO:0005634, nucleus	CC	577	4.76E-07	6.322	0.128
GO:0005694, chromosome	CC	182	8.99E-07	6.046	0.040
GO:0005622, intracellular anatomical structure	CC	1229	4.1E-05	4.387	0.272
GO:0005829, cytosol	CC	148	0.000746	3.127	0.033
GO:0043228, non-membrane-bounded organelle	CC	321	0.002452	2.611	0.071
GO:0043232, intracellular non-membrane-bounded	CC	321	0.002452	2.611	0.071
GO:0031981, nuclear lumen	CC	174	0.002745	2.561	0.039
GO:0005911, cell-cell junction	CC	30	0.002988	2.525	0.007
GO:0005769, early endosome	CC	12	0.006417	2.193	0.003
GO:0070161, anchoring junction	CC	31	0.009751	2.011	0.007
GO:0098687, chromosomal region	CC	16	0.014441	1.840	0.004
GO:0005654, nucleoplasm	CC	127	0.014512	1.838	0.028
GO:0005737, cytoplasm	CC	748	0.022404	1.650	0.166
GO:0030428, cell septum	CC	6	0.025308	1.597	0.001
GO:0000793, condensed chromosome	CC	18	0.02682	1.572	0.004
GO:0097543, ciliary inversin compartment	CC	7	0.02682	1.572	0.002
GO:0000779, condensed chromosome, centromeric region	CC	11	0.03016	1.521	0.002
GO:0031974, membrane-enclosed lumen	CC	185	0.03016	1.521	0.041
GO:0043233, organelle lumen	CC	185	0.03016	1.521	0.041
GO:0070013, intracellular organelle lumen	CC	185	0.03016	1.521	0.041

Supplementary Table S4 | GO enrichment analysis of genes overlapping high-impact SVs significantly ($p < 0.05$) enriched for Molecular Function, sorted on adjusted p-value.

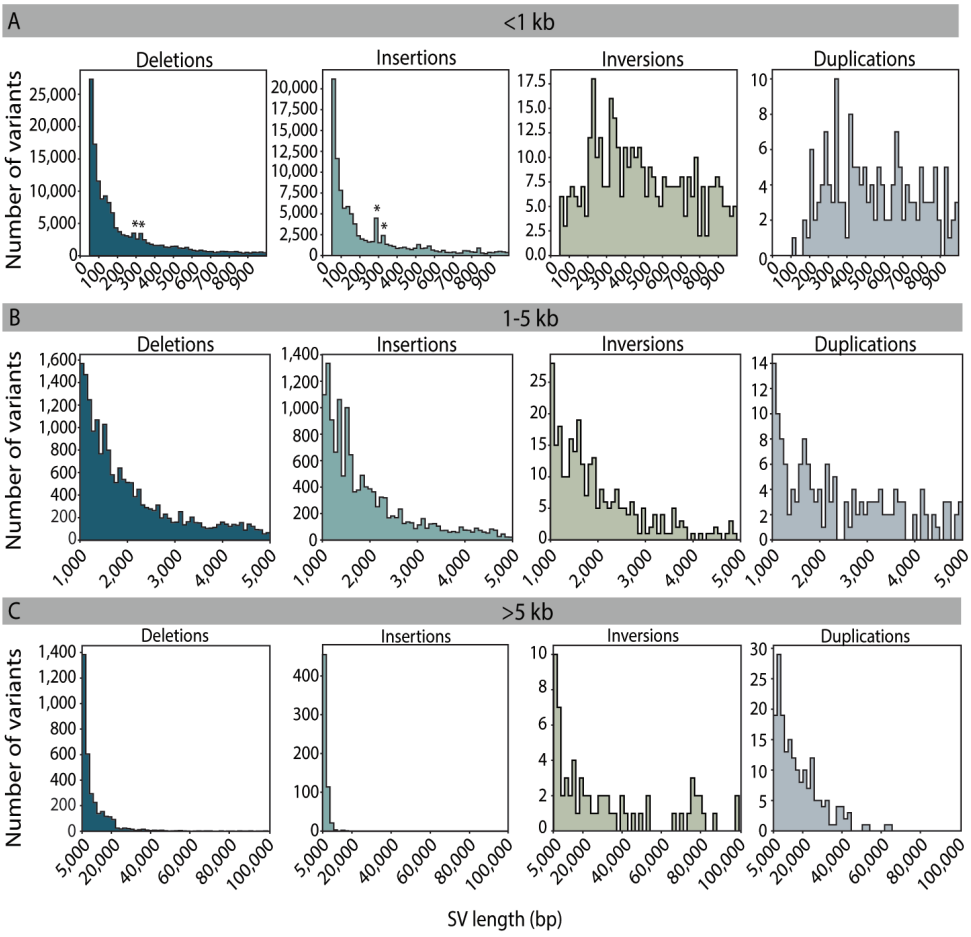
Term	Category	Count	p.adjust	logp_adjust	GeneRatio
GO:0140097, catalytic activity, acting on DNA	MF	165	6.38E-21	20.195	0.026
GO:0003676, nucleic acid binding	MF	676	6.38E-21	20.195	0.108
GO:0140640, catalytic activity, acting on a nucleic	MF	228	4.52E-18	17.344	0.037
GO:0009378, four-way junction helicase activity	MF	67	3.22E-14	13.493	0.011
GO:0043138, 3'-5' DNA helicase activity	MF	67	3.05E-13	12.516	0.011
GO:0004748, ribonucleoside-diphosphate reductase	MF	46	1.28E-12	11.892	0.007
GO:0016728, oxidoreductase activity, acting on CH	MF	46	1.28E-12	11.892	0.007
GO:0061731, ribonucleoside-diphosphate reductase	MF	46	1.28E-12	11.892	0.007
GO:0008094, ATP-dependent activity, acting on DNA	MF	96	1.31E-12	11.884	0.015
GO:0016725, oxidoreductase activity, acting on CH	MF	46	1.98E-12	11.704	0.007
GO:0004386, helicase activity	MF	94	3.48E-12	11.458	0.015
GO:0003678, DNA helicase activity	MF	80	3.48E-12	11.458	0.013
GO:0005488, binding	MF	2194	2.32E-11	10.635	0.352
GO:1901363, heterocyclic compound binding	MF	957	3.44E-11	10.463	0.153
GO:0097159, organic cyclic compound binding	MF	959	7.03E-11	10.153	0.154
GO:0034061, DNA polymerase activity	MF	38	4.09E-09	8.388	0.006
GO:0140657, ATP-dependent activity	MF	195	6.3E-09	8.201	0.031
GO:0003887, DNA-directed DNA polymerase activity	MF	37	6.44E-09	8.191	0.006
GO:0005044, scavenger receptor activity	MF	102	1.72E-07	6.763	0.016
GO:0038024, cargo receptor activity	MF	102	2.29E-07	6.640	0.016

Supplementary Table S5 | Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of genes overlapping high-impact SVs, sorted on ascending adjusted p-value.

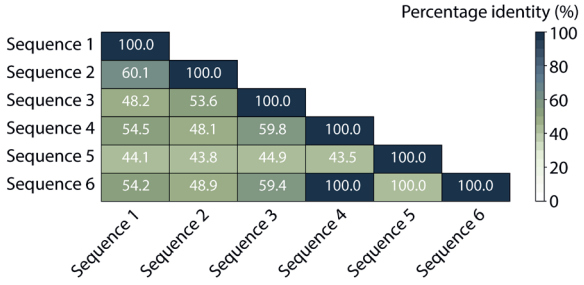
Term	Category	Count	p.adjust	logp_adjust	GeneRatio
Inositol phosphate metabolism	KEGG	18	0.007	2.179	0.026
RNA degradation	KEGG	28	0.010	2.012	0.041
Lysine degradation	KEGG	15	0.011	1.956	0.022
Biosynthesis of cofactors	KEGG	33	0.011	1.956	0.048
ABC transporters	KEGG	15	0.079	1.100	0.022
Homologous recombination	KEGG	10	0.143	0.846	0.014



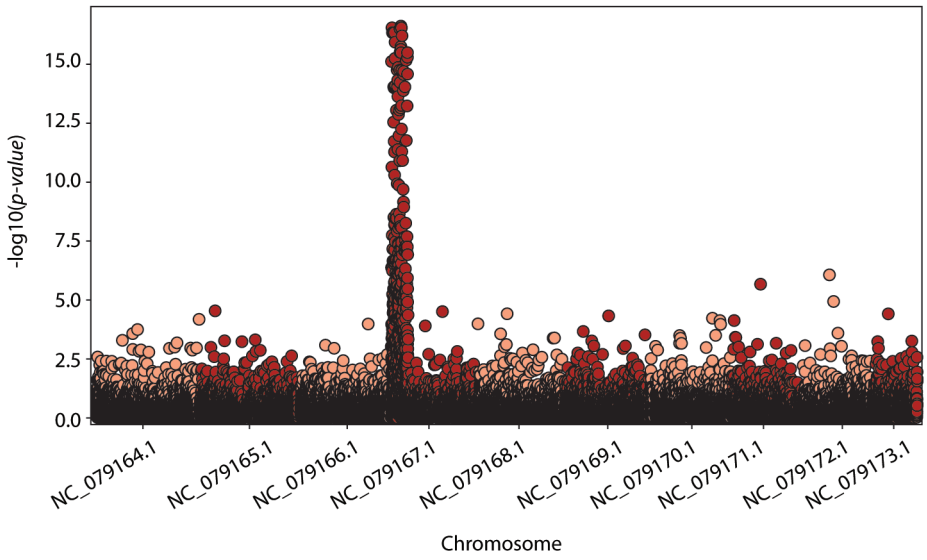
Supplementary Figure S1 | Gene Ontology (GO) enrichment analysis of genes overlapping high-impact SVs. **(A)** GO enrichment analysis of the Cellular Components category, displaying significantly enriched GO terms ($p < 0.05$) **(B)** GO enrichment analysis of the Molecular Function category, displaying significantly enriched GO terms ($p < 0.05$).



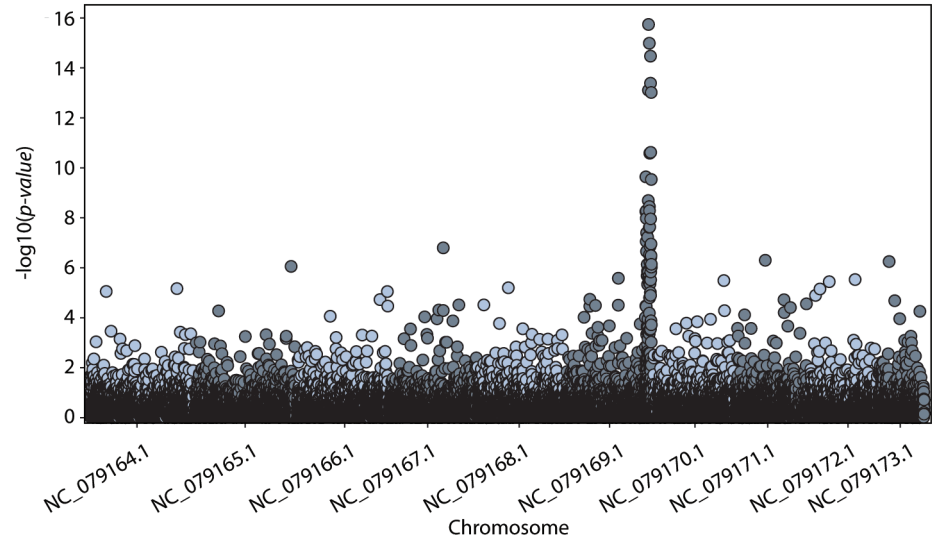
Supplementary Figure S2 | Length distribution histograms of structural variants (SVs) per SV type (insertion, duplication, deletion, and inversion) categorized into three length classes. **(A)** SVs < 1 kb. **(B)** SVs between 1 and 5 kb. **(C)** SVs > 5 kb. Asterisks indicate peaks in SV counts (insertions and deletions < 1 kb) that overlap with repetitive regions.



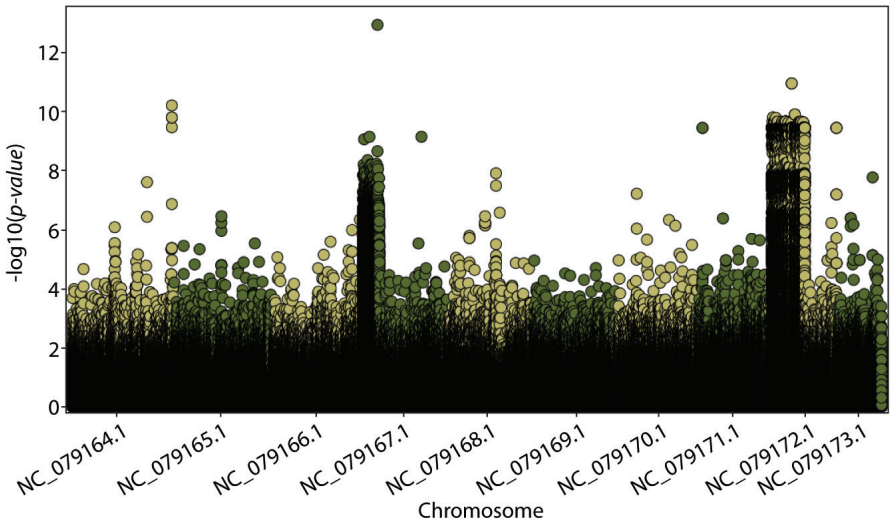
Supplementary Figure S3 | Heatmap displaying percentage identities derived from MAFFT pairwise alignments across consensus sequences from SVs overlapping three distinct repeat families.



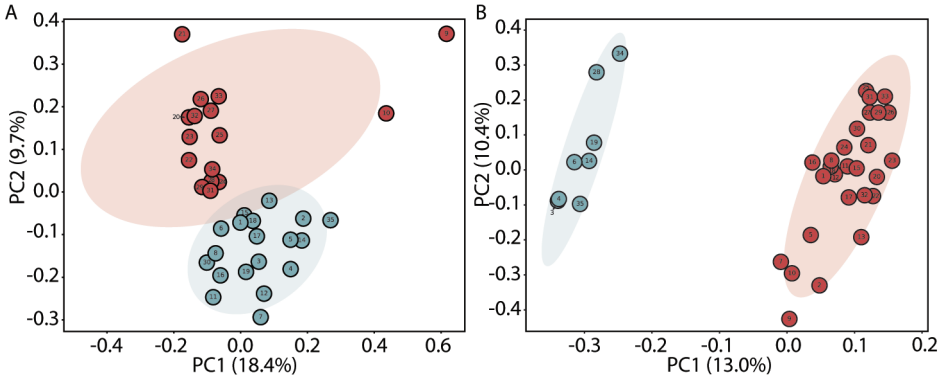
Supplementary Figure S4 | Principal component (PC) association analysis illustrating associations between genome-wide SVs and the second PC axis (PC2).



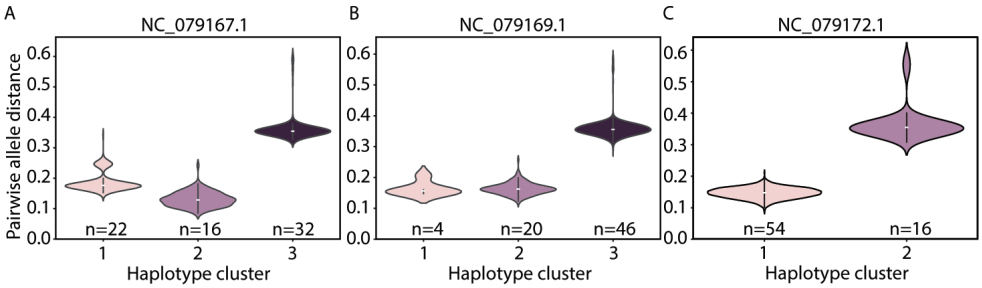
Supplementary Figure S5 | PC association analysis illustrating associations between genome-wide SVs and PC3.



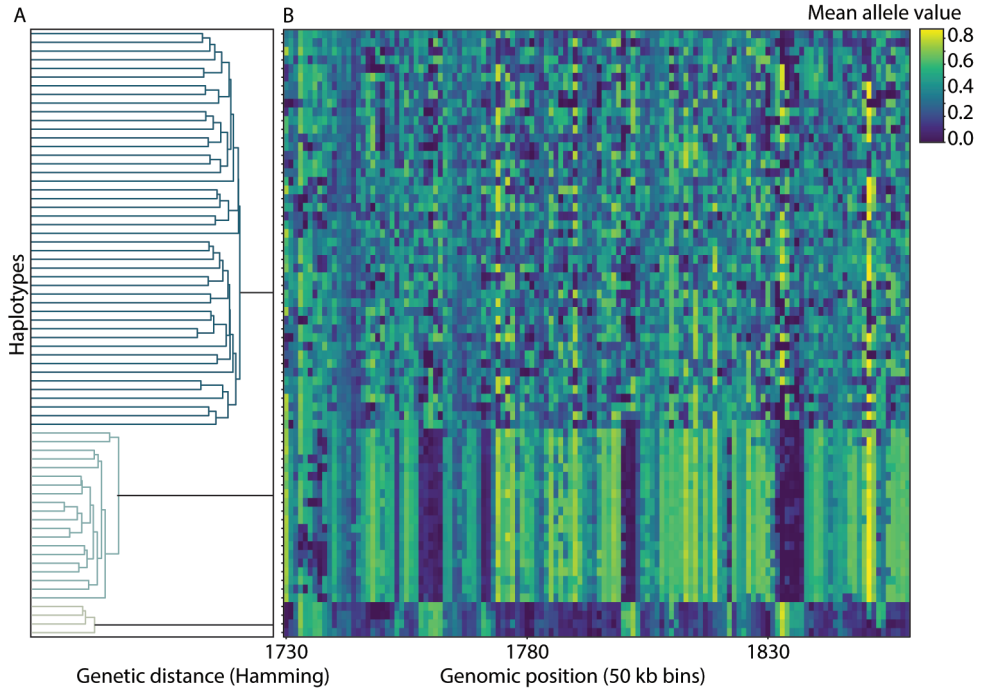
Supplementary Figure S6 | PC association analysis illustrating associations between genome-wide SNPs and PC1.



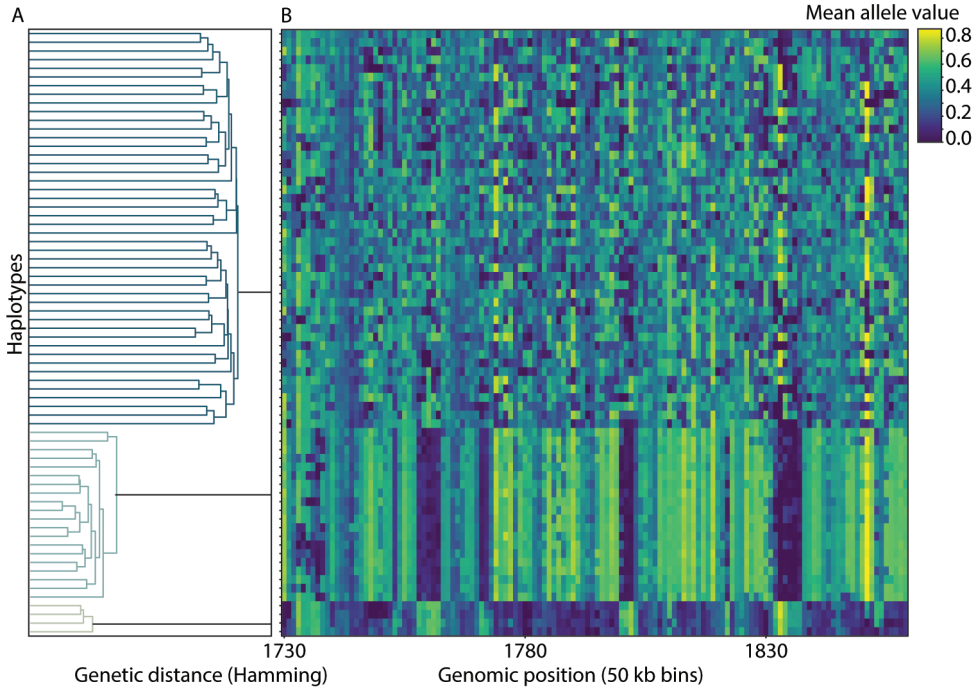
Supplementary Figure S7 | Population structure inferred from genome-wide variants, excluding variants overlapping the three known inversions (NC_079167.1, NC_079169.1, and NC_079172.1). **(A)** Principal component analysis (PCA) structure inferred from genome-wide structural variants (SVs). **(B)** PCA displaying population structure inferred from single-nucleotide polymorphisms (SNPs). Samples are colored according to k-means clustering (k=2) based on PC1 and PC2 coordinates. Ellipses represent 2 standard deviations from the clustered centroid.



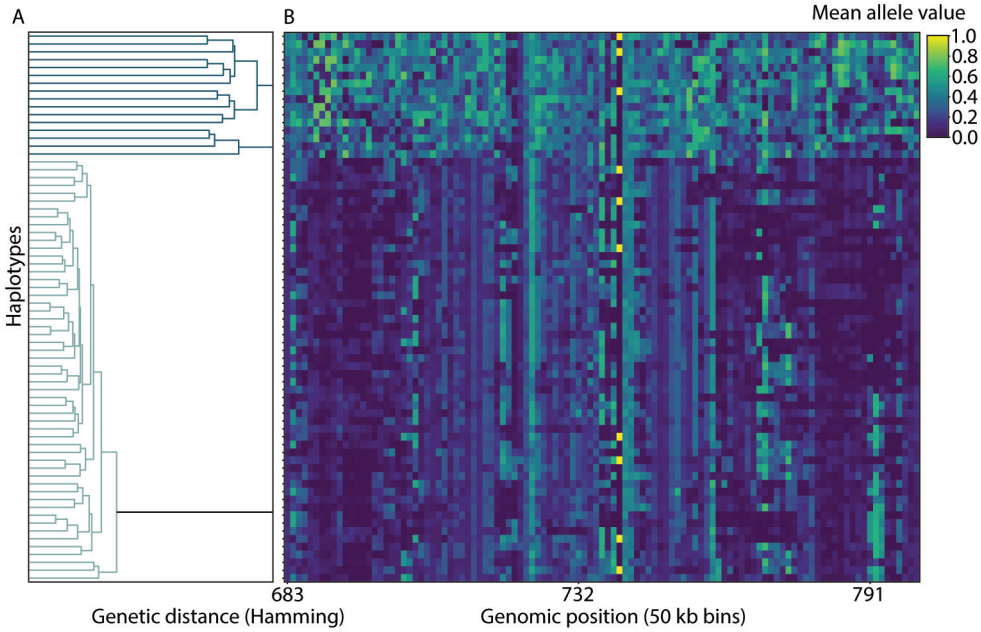
Supplementary Figure S8 | Mean pairwise allele distance for phased haplotype clusters inferred from SNPs called from long-read Nanopore data, on three chromosomal inversion regions. **(A)** Pairwise allele distance across three haplotype clusters of inversion region on chromosome NC_079167.1. **(B)** Pairwise allele distance across three haplotype clusters of inversion region on chromosome NC_079169.1. **(C)** Pairwise allele distance across two haplotype clusters of inversion region on chromosome NC_079172.1.



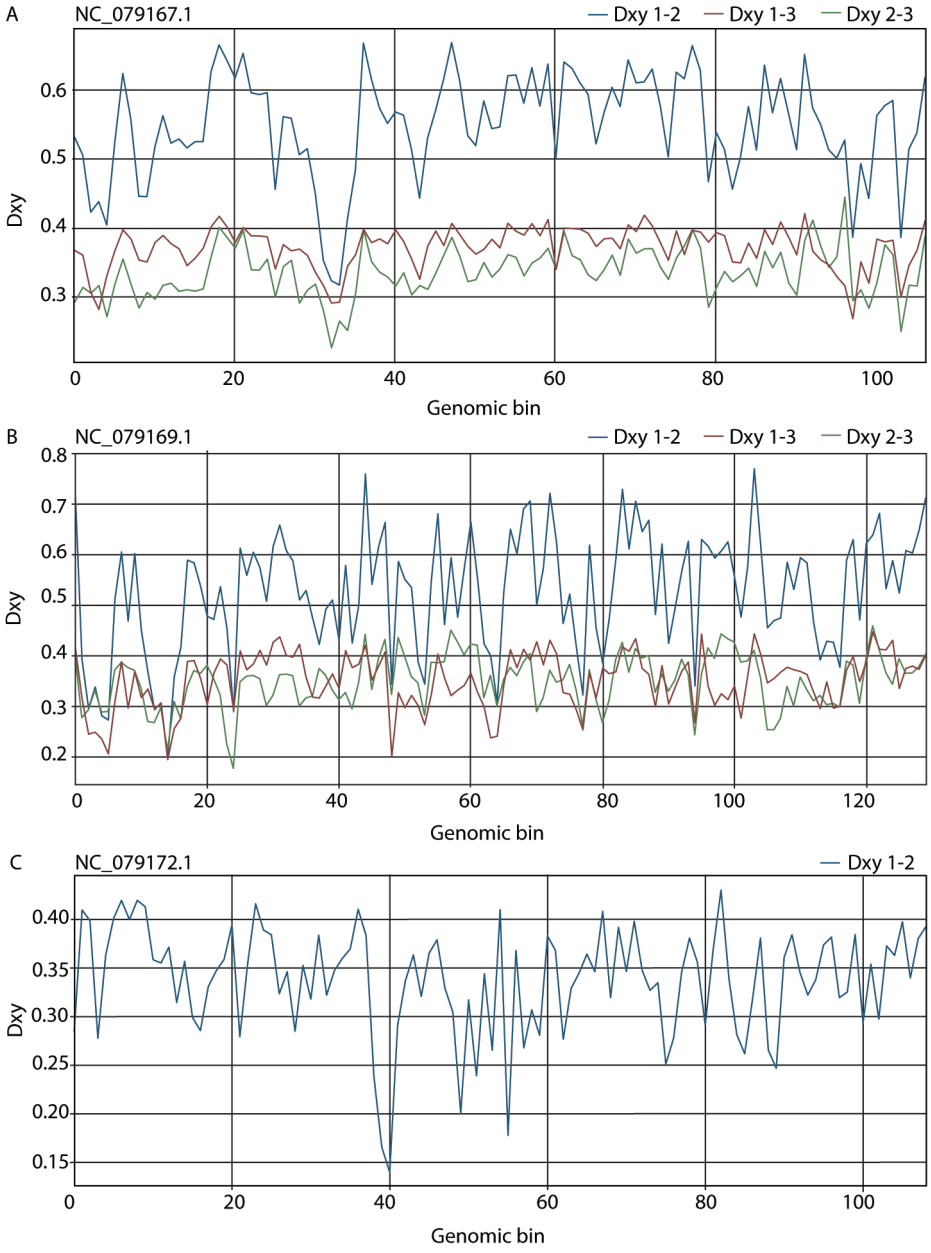
Supplementary Figure S9 | Pairwise genetic divergence across phased haplotypes inferred from SNPs called from long-read Nanopore reads located on the inversion region of chromosome NC_079167.1 (500,000-16,620,000 bp). **(A)** Dendrogram illustrating hierarchical clustering based on pairwise Hamming distances (proportion of differing SNPs) between haplotypes. **(B)** Heatmap displaying mean allele difference per haplotype across genomic bins of 150 kb.



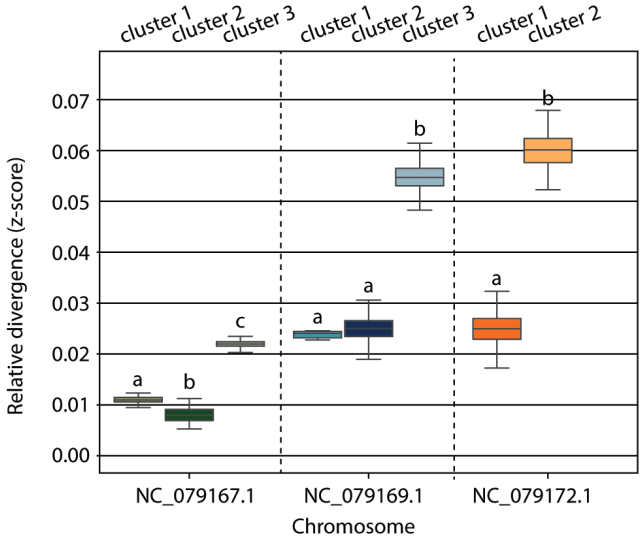
Supplementary Figure S10 | Pairwise genetic divergence across phased haplotypes inferred from SNPs called from long-read Nanopore reads located on the inversion region of chromosome NC_079169.1 (86,500,000-93,000,000 bp). **(A)** Dendrogram illustrating hierarchical clustering based on pairwise Hamming distances (proportion of differing SNPs) between haplotypes. **(B)** Heatmap displaying mean allele difference per haplotype across genomic bins of 50 kb.



Supplementary Figure S11 | Pairwise genetic divergence across phased haplotypes inferred from SNPs called from long-read Nanopore reads located on the inversion region of chromosome NC_079172.1 (34,100,000-40,000,000 bp). **(A)** Dendrogram illustrating hierarchical clustering based on pairwise Hamming distances (proportion of differing SNPs) between haplotypes. **(B)** Heatmap displaying mean allele difference per haplotype across genomic bins of 50 kb.



Supplementary Figure S12 | Pairwise genetic divergence (Dxy) across phased haplotype clusters within three inversions. Dxy represents the average number of nucleotide differences between haplotypes per genomic bin (50 kb). **(A)** Pairwise Dxy among three haplotype clusters in inversion NC_079167.1, computed across 150 kb genomic bins. Number of haplotypes per cluster: 1 = 22, 2 = 46, and 3 = 32. **(B)** Pairwise Dxy among three haplotype clusters in inversion NC_079169.1, computed across 50 kb genomic bins. Number of haplotypes per cluster: 1 = 4, 2 = 20, and 3 = 46. **(C)** Pairwise Dxy between two haplotype clusters in inversion NC_079172.1, computed across 50 kb genomic bins. Number of haplotypes per cluster: 1 = 16 and 2 = 54.



Supplementary Figure S13 | Boxplot displaying pairwise comparisons of relative haplotype divergence across haplotypes for three chromosomal inversion regions. Haplotypes were grouped into clusters using hierarchical clustering. Letters indicate significant differenced (p-value < 0.05) among pairwise comparisons of the average relative divergence between haplotype clusters within each chromosomal inversion (non-parametric Kruskal-Wallis test).

CHAPTER 4

Oysters in transition

Hermaphrodite oysters display unique DNA methylation patternsin gill tissue

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Abstract

European flat oysters (*Ostrea edulis*) are sequential hermaphrodites that alternate sex in response to environmental change. Epigenetics, including DNA methylation, are often involved in sex reversal through influencing gene transcription. Knowledge on the epigenetic mechanisms underlying sex reversal in hermaphrodite bivalves is limited to gonadal tissue and previous studies have only compared DNA methylomes of males and females. Therefore, the aim of this study is to assess whether sex-specific DNA methylation can be identified in somatic gill tissue of the flat oyster. By comparing whole-genome methylomes of 35 oysters of different sex phenotypes using nanopore sequencing, we demonstrate the presence of sex-specific DNA methylation patterns in somatic gill tissue. A total of 9,654 regions and 2,576 genes were differentially methylated between male, female, and hermaphrodite oysters. Functional analysis of differentially methylated genes indicated an association with energy homeostasis and metabolic processes, implying a remodeling of the energy balance. This study is the first to characterize DNA methylomes of hermaphrodite oysters, providing new insights into the epigenetic mechanisms underlying sex reversal in a sequential hermaphrodite invertebrate. Additionally, this study characterizes sex-specific DNA methylation in somatic gill tissue, paving the way for non-lethal sex identification using epigenetic biomarkers.

Introduction

Epigenetic mechanisms influence gene transcription without changing the DNA sequence itself (Jones, 2012). DNA methylation, the covalent transfer of a methyl group to a DNA nucleotide, is one of the most common epigenetic modifications in eukaryotes and is involved with various developmental and regulatory processes (Cheng et al., 2019; Okano et al., 1999). In vertebrates, DNA methylation occurs primarily on CpG islands where methylation of promoter regions often results in gene silencing (Klughammer et al., 2023; Sheldon et al., 2023). Contrasting to vertebrates, invertebrates acquire a more mosaic pattern of DNA methylation, mainly occurring on gene bodies (Sarda et al., 2012; Suzuki et al., 2007). Reduced gene body methylation has the potential to facilitate transcriptional opportunities by alternative splicing, for example through exon skipping or by providing access to alternative transcription start sites (Roberts & Gavery, 2012; Suzuki et al., 2007). Growth, development, and environmental conditions can initiate de novo DNA methylation or change its maintenance (Sheldon et al., 2023; Zhang et al., 2022). By influencing gene transcription, DNA methylation forms the crucial ingredient in facilitating phenotypic plasticity, whereby organisms can change their phenotype in response to changing environments (Roberts & Gavery, 2012). Understanding the epigenetic mechanisms that are at the core of the interplay between the environment and phenotypic plasticity is pivotal for understanding how species respond to or cope with environmental change.

One prevalent example of phenotypic plasticity is sex reversal, by which an animal is capable of changing its phenotypic sex without the need for a corroborative change in DNA. Sex reversal occurs in many lineages of the animal kingdom, such as in reptiles (Whiteley et al., 2021), amphibians (Akashi et al., 2024), fish (Casas & Saborido-Rey, 2021), and a wide variety of invertebrates (Adzigbli et al., 2019; Compton & Tu, 2022; Da Silva et al., 2009; Guerrero-Tortolero & Campos-Ramos, 2018; Proestou et al., 2024b). The plasticity and frequency with which animals can reverse sex are partly determined by the underlying sex determination mechanisms, which can be roughly divided into genetic sex determination (GSD) and environmental sex determination (ESD). The ability to reverse sex can be advantageous in highly dynamic environments, where sex-related differences in fitness may favor transition into one sex under specific environmental conditions (De Lisle & Bolnick, 2021; Senior et al., 2012; Yamaguchi & Iwasa, 2017). During sex reversal, epigenetic mechanisms play a pivotal role in facilitating sex differentiation by influencing gene expression (Liu et al., 2022; Roberts & Gavery, 2012; Shao et al., 2014; Todd et al., 2019).

Oysters represent excellent candidates for studying epigenetic mechanisms underlying sex reversal since they are sequential hermaphrodites that can repeatedly

alternate between male and female sex phenotypes (Da Silva et al., 2009; Davenel et al., 2010; Lee et al., 2024). A variety of environmental factors have been documented to initiate sex reversal in oysters by altering DNA methylation in gonads, examples of such environmental variables are acidification (Venkataraman et al., 2022) and starvation (Sun et al., 2023). Differential DNA methylation between phenotypic sexes, referred to as sex-specific DNA methylation, is suggested to lead to sex differentiation by influencing gene transcription (Broquard et al., 2021; Proestou et al., 2024b; Sun et al., 2022, 2023, 2024). Generally, energetically demanding or restricting environmental conditions lead to masculinization of gonadal tissue (Joyce et al., 2013; Sun et al., 2023). The association between DNA methylation and sex reversal has been primarily studied in gonad tissue, where sex-specific DNA methylation and gene expression are expected to have a direct relation to gametogenesis (Broquard et al., 2021; Zhang et al., 2018). In the Pacific oyster (*Magallana gigas*), differential methylation and expression of several genes are shown to be involved with sex differentiation in the gonads (Broquard et al., 2021; Sun et al., 2022, 2024; Yue et al., 2018). Interestingly, during the process of sex reversal, oysters often display intermediate sex stages where they simultaneously produce male and female reproductive tissue of different developmental stages (Da Silva et al., 2009). These individuals are characterized as hermaphrodites that display either male or female biased gonad tissue, or represent gonadal tissue containing both sexes with equal ratios (Da Silva et al., 2009; Diaz-Almela et al., 2004). The simultaneous occurrence of male, female, and hermaphrodite individuals within one population is suggested to represent a phenotypically plastic response to temporally dynamic environments (Broquard et al., 2020). Although sequential hermaphroditism is frequently observed in oysters and hermaphrodite individuals contribute largely to reproductive cohorts in wild populations (Broquard et al., 2020), previous studies have been exclusively comparing sex-specific DNA methylation between males and females (Feng et al., 2023; Sun et al., 2022, 2024). Hence, sex-specific DNA methylation of hermaphrodite individuals remains poorly understood (Broquard et al., 2020; Da Silva et al., 2009; Feng et al., 2023; Nicolini et al., 2023; Sun et al., 2024). However, understanding how DNA methylation may differ between male, female, and hermaphrodite individuals is crucial for understanding the role of DNA methylation during different stages of sex reversal in oysters (Nicolini et al., 2023).

Currently, sex reversal in oysters is understudied, since existing methods of sex-identification require sampling of gonad tissue and are therefore lethal. Notably, sex-specific DNA methylation is not restricted to gonadal tissue and accommodates sex-associated differences in somatic tissues (e.g. mantle and abductor muscle), and even whole-body methylomes of different invertebrates (Kvist et al., 2020; Proestou et al., 2024b; Teng et al., 2025). This highlights the relevance of characterizing

differential DNA methylation in somatic tissues between different sex phenotypes in oysters (Proestou et al., 2024a). Gill tissue, along with other somatic tissues, is often collected during biosecurity or monitoring surveys where the tissue can be sampled non-lethally, by using anesthetics (Engelsma et al., 2010; Kamermans et al., 2023). As such, identifying sex-specific methylation in somatic tissue could potentially pave the way for non-lethal alternatives of sex-identification.

Like many other bivalves, the European flat oyster (*Ostrea edulis*; Linnaeus, 1758) is a sequential hermaphrodite that can reverse sex within one reproductive season (Da Silva et al., 2009; Davenel et al., 2010). It plays a critical role in the ecosystem by sustaining biodiversity through generating biogenic reef habitats (Coen et al., 2007; Potet et al., 2021; Smyth & Roberts, 2010). The species is currently considered 'threatened' according to the Oslo-Paris Convention, due to ongoing habitat degradation, overfishing, and the spread of diseases (Helmer et al., 2019; OSPAR, 2006; van Banning, 1979). These population declines initiated restoration efforts to restock degraded populations with hatchery-produced oysters (Bos et al., 2023; Colsoul et al., 2020; Potet et al., 2021). However, male-biased skewed sex ratios limit reproductive output and are thought to hamper the upscaling of oyster culturing used for kick-starting oyster reef restoration (Diaz-Almela et al., 2004; Kamphausen et al., 2011). Identifying epigenetic mechanisms that underly sex reversal is crucial for a better understanding of the reproductive strategy of *O. edulis* and can facilitate in improving culturing strategies of the species (Joyce et al., 2013; Nicolini et al., 2023).

Therefore, this study aimed to assess whether sex-specific DNA methylation is present in somatic gill tissue, and to characterize the regional differences in sex-specific DNA methylation between male, female, and hermaphrodite flat oysters. By comparing gill methylomes using nanopore sequencing we show the presence of regional sex-specific DNA methylation across the genome. This study is the first to include DNA methylation of hermaphrodite individuals, providing new insights into the epigenetic mechanisms underlying sex reversal in a sequential hermaphrodite invertebrate. Additionally, this study highlights the potential of using somatic tissues to study sex reversal for non-lethal applications.

Materials and Methods

Sampling Site and Collection

In this study, DNA methylomes were obtained from whole-genome sequencing of gill tissue of 35 European flat oysters of different sex phenotypes. Oysters were collected by dredging in April and September 2023, and April 2024 in Grevelingenmeer, the Netherlands (53°30'40"N, 08°06'20" E). Individuals of similar shell length (74.1 ± 7.45 mm; Supplementary FiS1B) were selected and gill tissue was collected and preserved with RNAlater or snap-frozen using liquid nitrogen. All gill tissue samples were stored at -80 °C until further use. Oysters were collected in the context of the national monitoring for mollusk diseases in the Netherlands. Samples from gill, mantle, and digestive diverticulum tissue were tested for the presence of the haplosporidian parasite Bonamia ostreae, using species-specific real time PCR as described in Kamermans et al. (2023). Two oysters (nr. 3 and 17) tested positive for an infection of B. ostreae. We did not correct for this in our analysis, as other physiological differences between individuals were not assessed nor included in this study.

Histological sex identification

Cross sections of tissue representing both reproductive, respiratory, and digestive organs were embedded in paraffin. Sections (±5 µm) were stained with hematoxylin-eosin for histological observation. Each oyster was categorized a sex stage and gonad development index, according to the study of da Silva et al (2009) (Da Silva et al., 2009). The five sex stages are as follows: male (M), female (F), hermaphrodite predominantly male (HPM), hermaphrodite predominantly female (HPF), or hermaphrodite both sexes equally present (HBS). The gonad development index was considered as follows: Inactive or resting gonad (0), early gametogenesis (1), advanced gametogenesis (2), mature (3), partially spawned gonad (4), reabsorbing gonad (5). Oysters that displayed no clear gonad tissue were categorized to be in the inactive or resting gonad phase (0), and were not used for subsequent analysis. In total, 35 oysters were used for DNA methylome sequencing; 9 M, 8 F, 5 HPM, 9 HPF, and 4 HBS oysters. An overview of all histological images can be found in the supplementary material (Supplementary Figure S5).

DNA extraction

Genomic DNA was extracted from oyster gill tissue using the Qiagen Gentra Kit for genomic DNA or Monarch HMW DNA extraction kit for tissue, according to the manufacturer's instructions (Qiagen catalog no. 158667; NEB catalog no. T3060). Sample input varied from 20 to 30 mg of frozen gill tissue and was grinded using liquid nitrogen. Long DNA (> 20 kb) was sheared using g-TUBES (Covaris, USA) in 1

minute centrifugation steps of 8, 10, 12, 14 (x1000G) respectively. Short reads were eliminated using the SRE XS PacBio kit, following the manufacturer's instructions (Pacific Biosciences, Menlo Park, CA, USA). DNA was quantified using the HS Qubit assay (Life Technologies, ThermoFisher Scientific). Quality and length of DNA were visualized using agarose gel electrophoresis.

Library preparation & sequencing

To prepare samples for whole-genome sequencing using the Oxford Nanopore Platform (ONT, Oxford, UK), short-read eliminated samples were used for end-prep and DNA repair, followed by barcode and adapter ligation according to the native barcoding protocol (SQK-NBD114-24). Up to a maximum of five oysters were simultaneously sequenced using PromethION R10.4.1 flowcells (FLO-PRO114M) on a PromethION-24 device, using MinKNOW software versions: 23.04.5 (July 2023), 23.07.12 (December 2023), 24.02.19 (May 2024), and 24.06.15 (December 2024). A minimum read quality threshold of 7 was applied.

Raw read processing

Raw sequenced reads (POD5) were basecalled using Dorado v0.4.1 (Oxford Nanopore Technologies, 2022/2025), allowing for simultaneous super accuracy basecalling (SUP) of canonical basepairs using model dna_r10.4.1_e8.2_400bps_sup@4.2.0, and 5mC modifications, using the –modified-bases 5mC flag for the detection of modified bases. The reads were mapped to the reference genome xbOstEdul1.1 (NCBI RefSeq Assembly: GCF_947568905.1 (Adkins et al., 2023)), with minimap2 (Li, 2021) as a built-in tool within the Dorado basecaller function. After mapping, the reads were demultiplexed using the demux function of Dorado. Mapped BAM files were then filtered on a minimal mapping quality of 60 and all unmapped reads or secondary alignments were removed using Samtools v1.17 (Danecek et al., 2021). After filtering, BAM files were sorted and indexed using Samtools. Descriptive statistics for BAM files were generated using NanoPack2 v1.12.0 (De Coster & Rademakers, 2023).

CpG calling

The pileup function of Modkit v0.2.1 (https://github.com/nanoporetech/modkit) was used to call CpGs from the mapped BAM files to generate BEDmethyl output files, using the xbOstEdul1.1 reference genome (Adkins et al., 2023). For some CpG sites more than half of the reads were identified as Ndel, indicating the read contains a deletion on this site compared to the reference genome. Reads where the number of Ndels was ≥ 60% of the total coverage for the corresponding CpG site were removed from the dataset.

Characterization of differentially methylated loci and regions

BEDmethyl files generated by Modkit were used as input for methylKit v.0.99.2 in R v.4.3.3 (Akalin et al., 2012; R Core Team, 2023). Methylation percentages were calculated for every CpG site by dividing the sum of methylated CpGs by the total number of CpGs covered. Methylation calls were filtered to a minimum required coverage of 3 reads per CpG site for all individuals. Methylation call distributions between samples were normalized using the normalizeCoverage function, to reduce the potential bias in varying coverage across individuals. For pairwise comparisons between sex phenotypes, data was reorganized to include only the individuals pertaining the selected sex phenotypes, using the reorganize function. CpG sites of individuals from two sex phenotypes were then merged using the unite function. To correct for a potential effect of maturation development stage on DNA methylation, maturation stage was added as a covariate.

Using a logistic regression, differentially methylated loci (DMLs) were identified for each pairwise comparison, using the calculateDiffMeth function in methylKit. P-values were adjusted for multiple testing using the Benjamin-Hochberg correction. DMLs were defined as loci with a minimal methylation difference of 20% between the two compared sex phenotypes, and an adjusted q-value < 0.01 (BH corrected). For every pairwise comparison, the number of hypermethylated and hypomethylated DMLs were determined whereby a hypermethylated DML was defined as a CpG site where the treatment group was more methylated than the control group. A hypomethylated DML was defined as a CpG locus where the treatment group was less methylated than the control group.

Differentially methylated regions (DMRs) were identified using a sliding-window approach of 200bp window slides and 100bp intervals, using the tileMethylCounts function from methylKit, with maturation stage added as a covariate. Only windows containing ≥ 5 CpG loci (cov.bases), a minimal difference in methylation percentage of 20%, and BH-adjusted q-value < 0.01 were considered as statistically significant DMRs. Hypermethylated DMRs were defined as DMRs where the treatment group was hypermethylated compared to the control group. Hypomethylated DMRs were defined as DMRs where the treatment group was hypomethylated compared to the control group. Differentially methylated loci and regions were visualized from BED files using IGV v2.19.1 (Thorvaldsdottir et al., 2013).

To identify the distribution of DMRs over genomic features (promoter, exon, intron, intergenic region), the position of genomic features was extracted from the reference genome using readTranscriptFeatures from methylKit. Subsequently DMRs were annotated to these genomic features using annotateWithGeneParts function from the genomation package (Akalin et al., 2015). Promoters were defined as regions

1000bp upstream or downstream of Transcription Start Sites (TSS). To test if there was an association between genomic location of DMRs and the genomic location of all CpGs with minimal coverage of 3 for all individuals, chi-square contingency pairwise tests were used.

Identifying differentially methylated genes

To identify the differentially methylated genes (DMGs), genes overlapping with DMRs, the DMRs within 100bp proximity of one another were first merged together. Subsequently, gene coordinates were extracted from the genome reference assembly annotation file: GCF_947568905.1_xbOstEdul1.1_genomic.gtf.gz (Adkins et al., 2023), using the gene function of GenomicFeatures (Lawrence et al., 2013). Genetic description and GeneID were added using getGenes from mygene (Adam Mark, 2017). Genes were considered statistically differentially methylated if they contained at least one DMR with minimal methylation percentage of 20% and BH-adjusted q-value < 0.01.

To analyze functional enrichment of annotated DMGs, Gene Ontology (GO) analysis was performed using TopGO in Bioconductor (Alexa & Rahnenführer, 2017). Using the Gene Annotation File (GAF) of the reference genome, GO terms were annotated to geneIDs and separated based on GO term categories: Biological Processes (BP), Cellular Components (CC), or Molecular Function (MF). Go terms with $p < 0.001$ were considered significantly enriched by DMR-overlapping genes. Heatmaps were generated using the pheatmap package in R (Kolde, 2018). The upset plot was generating using UpSetR (Conway et al., 2017).

Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed using the KEGGrest (Tenenbaum, 2017) package in R. Enrichment pathways were imported for Ostrea edulis (organism code: "oed"; RefSeq Assembly: GCF_947568905.1) using keggLink. Enrichment analysis was performed using the enrichKEGG function from the clusterProfiler package (Yu et al., 2012). A Benjamini-Hochberg adjustment of p-values was applied to correct for False Discovery Rate (FDR).

General statistical analysis

To check for differences in average shell length and average sequenced Gb per sex phenotype, one-way ANOVAs were performed using the stats package from R (R: The R Stats Package, n.d.). Deviations from normality and homogeneity of variance of the residuals were validated using Shapiro-Wilk's and Levene's test from the ¬car package v3.1.3 (Fox et al., 2001).

Results

Whole genome DNA methylomes obtained from gill tissue of flat oysters were compared between male, female, and hermaphrodite sex stages, with the aim of characterizing sex-specific DNA methylation. Oyster sex was validated using histology and five sex stages were distinguished: male (M), female (F), hermaphrodite predominantly male (HPM), hermaphrodite predominantly female (HPF), and hermaphrodite both sexes equally present (HBS). In total, whole-genome sequencing of 35 flat oyster individuals resulted in 111 million reads, with an average of 3.2 million reads per individual (Supplementary Table S1). Sequencing of all oysters was performed on 8 different 10.4 PromethION flow cells. Mapping of reads with minimal quality of 60 to the *O. edulis* reference genome assembly (XbOstEdul1.1), resulted in an average of 12.2 Gb per individual with read lengths varying between 1.1 - 14.6 kb and an average mapping percentage of 86.9% (Supplemental Table S1; submitted to ENA under accession number PRJEB85091). No significant difference in number of sequenced base pairs was observed between the five sex phenotypes (one-way ANOVA, F-statistic = 0.872, p-value=0.49; Supplementary Fig S1A). After CpG calling using modkit, between 10.4 and 30.5 million CpGs per individual, with a minimum coverage of 3, were used for downstream analysis.

General methylation patterns

Analysis of differentially methylated loci (DML) resulted in 555,259 CpG loci that were differentially methylated between any of the five sex phenotypes, with minimal coverage of 3 for all individuals. These CpGs were used as input for PCA analysis representing all sites that were differentially methylated between any two of the five sex phenotypes (Fig. 1A). PC1 (13.1% variance explained) displayed clear separation between the five sex phenotypes with males and females clustered together, and separated from the hermaphrodite sex phenotypes (HPM, HPF, and HBS).

Differentially methylated loci and regions between sex phenotypes

Characterization of DMLs between all five sex stages resulted in a total of 1,506 loci in which minimally two sex phenotypes showed significant differential methylation (BH corrected q-value < 0.01, ≥ 20% methylation difference; Supplementary Table S2). The pairwise comparison between HPM and HBS resulted in the highest number of DMLs (27,363 DMLs; 13,366 hypermethylated in HBS, and 13,997 hypermethylated in HPM; Supplementary Fig. S2A). Whereas 4,136 DMLs were identified between M-HPF, representing the lowest number of DMLs from all pairwise comparisons, followed by 4,252 DMLs identified between F-HPF (Supplementary Fig. S2A).

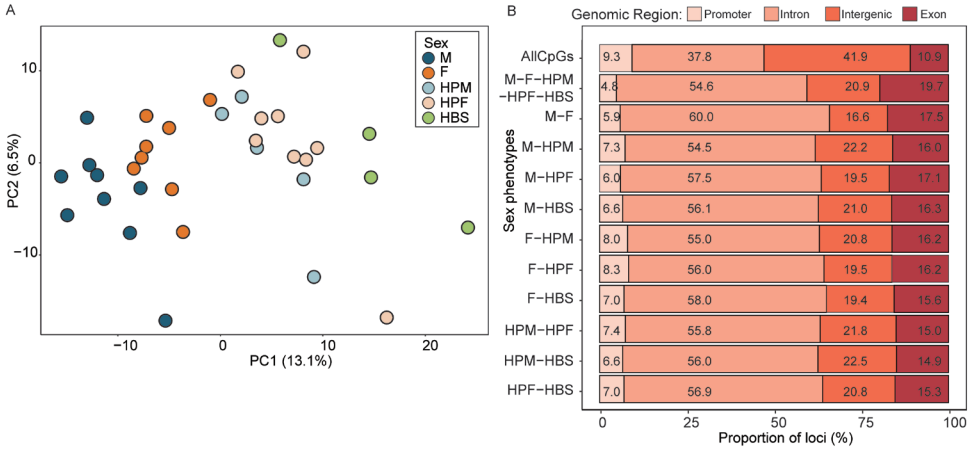


Figure 1 | Comparative analysis of differentially methylated loci (DMLs) between five sex phenotypes of flat oysters (M, F, HPM, HPF, and HBS). **(A)** Principal component analysis of all differentially methylated CpGs between any two of the five sex phenotypes (555,259 sites). **(B)** Distribution over genomic location of CpGs (minimal coverage of 3), DMLs identified from comparisons between any two of the five sex phenotypes, and DMLs from pairwise comparisons between the five sex phenotypes.

The majority of DMLs were located in introns (56.6%), followed by intergenic regions (20.4%), exons (16.0%), and promoters (7.0%; Fig. 1B). The genomic distribution of significant DMLs from pairwise comparisons between sex phenotypes differed significantly from the genomic distribution of all CpGs (minimal coverage of 3 for all individuals; Pearson's correlation: p-value < 0.05; Supplementary Table S3). Here, significant DMLs found between pairwise comparisons showed a positive association with intronic regions, and a negative association with intergenic regions.

Characterization of differentially methylated regions (DMRs) using a sliding-window approach resulted in a total of 21,160 significant DMRs from pairwise comparisons between any two of the five sex phenotypes (BH corrected $q < 0.01$, ≥ 20% methylation difference; Supplementary Table S4). Merging DMRs that were within 100 bp proximity of each other obtained a total of 9,651 DMRs that varied in length of 201 to 4001 base pairs (Supplementary Table S4). Generally, hermaphrodite sex phenotypes HPM and HBS exhibited higher numbers of DMRs than pairwise comparisons with or among males, females, or HPF. The highest number of DMRs was found between HPM and HBS (3,369 DMRs, 1,829 hypermethylated in HPM, and 1,540 hypermethylated in HBS; Supplementary Fig. S2B). The lowest number of DMRs was observed between females and HPF (164 DMRs; 84 hypermethylated in females, and 80 hypermethylated in HPF). Methylome comparison between males and females resulted in a total of 239 DMRs (125 hypermethylated in males, 114 hypermethylated in females). Two examples of DMRs between sex phenotypes have been visualized (Fig. 2A,B).

Gene annotation of differential methylation

Identifying differentially methylated genes (DMGs), defined as genes overlapping with DMRs, resulted in a total of 2,576 unique DMGs identified between pairwise comparisons of all five sex phenotypes (Fig. 2C). Of all these genes, 828 unique DMGs were identified between HPM and HBS. Males, HPM, and HBS showed to have the highest overlap in DMGs (Figure 2). Hierarchical clustering displayed that F-HPM, M-HPM, and HPM-HPF exhibited high similarity in identified DMGs, but clustered as a separate group with unique DMGs (Fig. 2D). The DMGs with highest difference in average methylation percentage between pairwise comparisons were: *MAM and LDL-receptor class A domain-containing protein 2-like* (LOC125683627; HPM-HBS), *UDP-GalNAc:beta-1,3-N-acetylgalactosaminyltransferase 2-like* (LOC125659768; F-HPM and F-HPM), *ATP-dependent DNA helicase Q5-like* (LOC125679612; M-HBS), and sacsin-like (LOC125679280; HPM-HBS; Table1). A complete overview of the top 10 most hypermethylated and hypomethylated DMGs identified for all pairwise comparisons can be found in the supplementary material (Supplementary Table S5).

In total, 125 genes were found to be differentially methylated between male and female oysters, of which 62 DMGs were hypermethylated in males, and 63 DMGs were hypermethylated in females (Supplementary Table S6). When compared to DMGs identified between other sex phenotypes, 15 DMGs were uniquely differentially methylated between males and females (Supplementary Table S6). DMGs with highest difference in methylation percentage between males and females were: *tRNA (guanine-N(7)-)-methyltransferase non-catalytic subunit wdr4-like* (LOC125645763), LOC125655458, and LOC125673983, where males were hypermethylated compared to females. DMGs with highest difference in average methylation percentage and females showing hypermethylation compared to males were: uncharacterized LOC125661226, uncharacterized LOC125661253, and *periodic tryptophan protein 1 homolog* (LOC125674140). Other DMGs uniquely identified between male and female oysters were: *cyclic AMP-dependent transcription factor ATF-2-like* (LOC125646345), *inhibitor of nuclear factor kappa-B kinase subunit alpha-like* (LOC125668035), *neuronal calcium sensor 2-like* (LOC125669759), *PR domain zinc finger protein 4-like* (LOC125680421), and *zinc finger protein 474-like* (LO130054705).

Gene ID	Gene Description	Groups	Chromosome	Length	ΔDNAm (%)
LOC125652448	*transport and Golgi organization protein 6 homolog*	M-F	NC_079164.1	1301	-53.4
LOC130051844	*ester hydrolase C11orf54 homolog*	M-F	NC_079164.1	301	37.3
LOC125645560	*proton-coupled zinc antiporter SLC30A2-like*	M-HPM	NC_079169.1	1701	-54.8
LOC130050362	*uncharacterized*	M-HPM	NC_079172.1	301	47.4
LOC125652448	*transport and Golgi organization protein 6 homolog*	M-HPF	NC_079164.1	401	-42.2
LOC130051844	*ester hydrolase C11orf54 homolog*	M-HPF	NC_079164.1	301	37.3
LOC125679612	*ATP-dependent DNA helicase Q5-like*	M-HBS	NC_079165.1	901	-60.3
LOC125670462	*protocadherin Fat 4-like*	M-HBS	NC_079167.1	201	52.1
LOC125659768	*UDP-GalNAc-beta-1,3-N-acetylgalactosaminyltransferase 2-like*	F-HPM	NC_079172.1	301	-50.8
LOC125655710	uncharacterized	F-HPM	NC_079170.1	201	61.9
LOC125675975	uncharacterized	F-HPF	NC_079166.1	301	-40.6
LOC125658752	*arsenite methyltransferase-like*	F-HPF	NC_079172.1	201	40.4
LOC125675975	uncharacterized	F-HBS	NC_079166.1	301	-50.5
LOC125679490	*putative polypeptide N-acetylgalactosaminyltransferase 10*	F-HBS	NC_079165.1	201	51.6
LOC125660846	*ATP-binding cassette sub-family B member 6-like*	HPM-HPF	NC_079171.1	301	-47.2
LOC125659768	*UDP-GalNAc:beta-1,3-N-acetylgalactosaminyltransferase 2-like*	HPM-HPF	NC_079172.1	201	56.2
LOC125679280	*sacsin-like*	HPM-HBS	NC_079165.1	1201	-62.5
LOC125683627	*MAM and LDL-receptor class A domain-containing protein 2-like*	HPM-HBS	NC_079169.1	601	62.7
LOC125681288	uncharacterized	HPF-HBS	NC_079164.1	201	-51.4
LOC125673062	uncharacterized	HPF-HBS	NC_079166.1	201	49.1

Table 1 | Overview of the top most hypermethylated and hypomethylated DMGs for each pairwise comparison across all five sex phenotypes (M, F, HPM, HPF, and HBS), with length of the DMG and average difference in DNA methylation % (ΔDNAm).

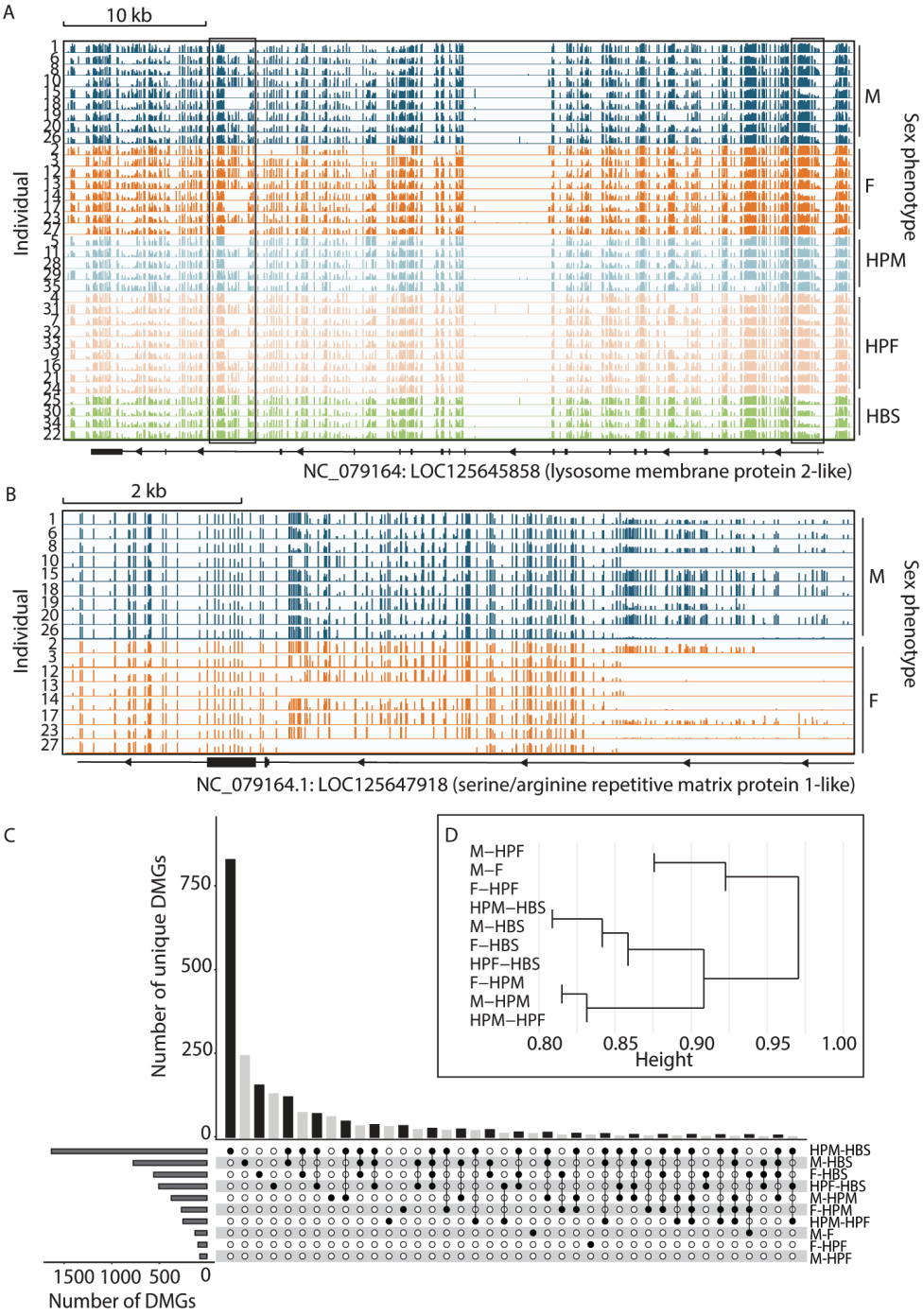


Figure 2 | Differentially methylated regions (DMRs) and genes (DMGs) from gill methylome comparisons between five sex phenotypes in flat oysters. *Legend continues on the next page.*

(A) DNA methylation profile of all 35 flat oyster individuals at the position of gene LOC125645858. Vertical bars represent methylation percentages per CpG site from 0 - 100%. Black boxes indicate DMR areas. **(B)** DNA methylation profile of all male and female oyster individuals at the position of gene LOC125647918. Vertical bars represent methylation percentages per CpG site from 0 - 100%. **(C)** Upset plot showing the number of overlapping and unique DMGs (n = 2,576) identified from pairwise comparisons between the five sex. **(D)** Hierarchical clustering of overlap in DMGs (n = 2,576) between the five sex phenotypes using Pearson's correlation coefficient.

Functional enrichment analyses

Analysis of difference in average methylation percentage over all DMRs within a DMGs resulted in 21 DMGs with a difference in average methylation percentage > 45%. DMGs with highest difference in average methylation percentage were *proton-coupled zinc antiporter SLC30A2-like* (LOC125645560; HPM-HBS), *MAM and LDL-receptor class A domain-containing protein 2-like* (LOC125683627; HPM-HBS), LOC125655710 (F-HPM), and *probable nuclear hormone receptor HR3* (LOC125661857; M-HPM & F-HPM), *ATP-dependent DNA helicase Q5-like* (LOC125679612; Me-HBS), and *protein ILRUN-like* (LOC125645979; M-HBS; Fig. 3B). Differential methylation between HPM-HBS was observed in the l*ysosome membrane protein 2-like gene* (LOC125645858), belonging to the superfamily of ABC transporters (Jiang et al., 2018).

Functional enrichment analysis of Gene Ontology (GO) terms of annotated DMGs resulted in 128 significant enriched GO terms for Biological Processes (BP; Fig. 3A), 45 enriched GO terms for Cellular Components (CC; Supplementary Figure S4), and 64 enriched GO terms for Molecular Function (MF; Supplementary Figure S5). GO terms for Biological Processes were related to neuronal activity and locomotion (e.g. GO0061564: axon development, GO0048666: neuron development, GO0040011: locomotion, and GO0006935: chemotaxis), protein targeting and localization (e.g. GO0006605: protein targeting and GO0015031: protein transport) and cellular and metabolic processes (e.g. GO0030030: cell projection organization, GO0051641: cellular localization, GO0009117: nucleotide metabolic process, and GOGO0006793: phosphorus metabolic process). Processes related to stress response were enriched in DMGs found between males and HBS (GO0043087: regulation of GTPase activity, GO0033554: cellular response to stress, and GO0042221: response to chemical"). Additionally, DMGs found between males and HPF were enriched in the "semaphorin-plexin signalling pathway" (GO0071526, GO1902285, GO1902287). Lastly, unique GO terms found between HPM-HPF were related to meiotic recombination (GO0140527, GO0007131, and GO0035825).



Figure 3 | Functional annotation of differentially methylated genes (DMGs) identified from pairwise comparisons of the five sex phenotypes. **(A)** Heatmap of Gene Ontology (GO) of DMGs (n = 2,576) enriched for Biological Processes (BP; n = 128). *Legend continues on the next page.*

BP were clustered hierarchically with Pearson's correlation coefficient. **(B)** Heatmap of DMGs with an average difference in methylation percentage > 45%. Positive differences in average methylation percentage indicate hypomethylation for the first group per pairwise comparison, negative differences in average methylation percentage indicate hypermethylation for the first group per pairwise comparison. **(C)** Scatter plot of enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways for DMGs identified between the five sex phenotypes.

Regarding CC, the largest group of DMGs showed enrichment for "intracellular anatomical structure" (GO0005622) and "organelle" (GO0043226; Supplementary Figure S3. DMGs enriched in MF were mostly related to pathways associated with nucleotide binding (e.g. GO0005488: binding, GO0005524: ATP binding, GO0030554: adenyl nucleotide binding, and GO0032559: adenyl ribonucleotide binding"; Supplementary Figure S4). Here, DMGs identified between HPM and HBS contributed mostly to these enriched pathways.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DMGs revealed 25 enriched pathways, of which 2 significant (padjust < 0.05; Figure 3C). The most significantly enriched pathways were involved in "Cytoskeleton in muscle cells" and "purine metabolism". Enriched pathways were mostly related to energy homeostasis due to their association with lipid, amino acid, and carbohydrate metabolism (e.g. "Purine metabolism", "Ascorbate and aldarate metabolism", "Beta-Alanine metabolism", "Inositol phosphate metabolism", and "Nicotinate and nicotinamide metabolism"). In addition to metabolism related pathways, DMGs from pairwise comparisons also showed enrichment for processes related to innate immunity (e.g. "ABC transporter" and "Peroxisome"; Figure3C).

Discussion

In invertebrates, DNA methylation is thought to contribute to phenotypic plasticity by increasing transcriptional variation in various ways (e.g. alternative splicing, increasing mutation rate) (Roberts & Gavery, 2012). The mosaic gene-body methylation is suggested to create transcriptional opportunities rather than regulating transcription mainly through (de)methylation in the promoter region (Roberts & Gavery, 2012). Sex reversal, a phenotypically plastic trait whereby animals can reverse sex in response to environmental stimuli, is associated with DNA methylation where sex-specific differences are thought to facilitate this reversal (Akashi et al., 2024; Collin, 2013; Piferrer, 2021; Shao et al., 2014; Sun et al., 2022; Venney et al., 2023; Wang et al., 2024a). Sex-specific DNA methylation has been widely reported in both vertebrates and invertebrates (Bock et al., 2022; Domingos et al., 2018; Glastad et al., 2016; Gombeau et al., 2016; Shao et al., 2014; Zhuang et al., 2020). In oysters, differential DNA methylation between male and female individuals is suggested to be associated with sex differentiation in the gonads, where epigenetic differences are linked to differential expression of genes involved in the sex determination pathway (Sun et

al., 2022, 2023, 2024). Although oysters are sequential hermaphrodites, knowledge on DNA methylation profiles of hermaphrodite individuals, undergoing a sex change, is limited. In this study, we aimed to characterize sex-specific DNA methylation of male, female and hermaphrodite European flat oysters (*O. edulis*), by comparing gill methylomes obtained through nanopore sequencing. In general, differential methylation occurred more frequently in intragenic regions (promoters, introns, and exons) compared to intergenic regions. These findings are consistent with results from other oyster species, where differential methylation is enriched in intergenic regions (Gavery & Roberts, 2013; Johnson et al., 2022; Johnson & Kelly, 2020). The most distinct DNA methylation profile was observed in hermaphrodite oysters, whereas males and females demonstrated more similar patterns of DNA methylation in their gills. Functional enrichment analysis indicated that differentially methylated genes (DMGs) were significantly enriched in pathways related to energy homeostasis and metabolism. Collectively, our data suggests that sequential hermaphrodites display an alternative state that represents the epigenetic reprogramming required for sex reversal. Additionally, the observed presence of sex-specific DNA methylation in gill tissue highlights the potential for non-lethal sex identification using somatic tissues.

Hermaphrodite individuals display an alternative epigenetic state in the gills

When oysters undergo sex reversal, gametogenesis of both sexes can occur within the same follicle (Da Silva et al., 2009). In these hermaphrodites, discrepancies in gamete development most often lead to asynchronous spawning of male and female gametes (Da Silva et al., 2009). In the gonads, the reversal of sex is accompanied by epigenetic reprogramming that facilitates cell growth, development, regulation, and restructuring, especially during early gametogenesis (Li et al., 2022b; Sun et al., 2024). In this study, whole-genome gill methylome comparisons between sex phenotypes demonstrated that hermaphrodites display the most distinct DNA methylation patterns when compared to males or females. Significantly enriched GO terms overlapping DMGs found between hermaphrodites and other sex phenotypes were related to neuronal activity and locomotion, and cell morphogenesis pathways. Additionally, the KEGG pathway related to "Cytoskeleton in muscle cells" was significantly enriched between hermaphrodite oysters. These findings suggest that hermaphrodite individuals undergoing sex reversal display an alternative state of epigenetic reprogramming when compared to males or females. It is possible that males and females, that have undergone the full process of sex reversal, represent an epigenetic state that matches the completion of sex reversal. Similarly, in a study of Sun et al. (2024), genome-wide DNA methylation patterns of gonadal tissue of Magallana gigas were accurately remodeled to the opposite sex after the completion of sex reversal (Sun et al., 2024).

In addition to the differential methylation patterns observed between male, female, and hermaphrodite individuals, numerous unique GO terms were identified between the different hermaphrodite sex phenotypes (HPM, HPF, and HBS). The majority of differentially methylated loci and regions (DMLs and DMRs) were identified between HPM and HBS. DMGs overlapping those regions showed significant enrichment for processes related to "cellular localization", "intracellular signal transduction", "phosphorylation" and "phosphate metabolism". The differences in annotated GO terms identified across hermaphrodite sex phenotypes imply that the direction of sex reversal is associated with differential DNA methylation. In *M. gigas*, a genome-wide increase in DNA methylation was observed when oysters underwent sex reversal female to male (Sun et al., 2024). While further elucidation of epigenetic reprogramming in hermaphrodite phenotypes is necessary to validate this, the highest number of DMLs and DMRs found between HPM-HBS suggests that the direction of sex reversal is associated with different epigenetic states. This supports the hypothesis that although sex reversal is bidirectional, patterns of epigenetic reprogramming may be different depending on the direction of a sex reversal event.

Sex-specific DNA methylation of gill tissue is related to energy homeostasis and metabolism

In this study, functional enrichment analysis of DMGs resulted in highest significant enrichment for GO-terms related to metabolic or energy homeostasis processes (e.g. ATP binding, nucleotide binding, hydrolase activity, DNA damage sensor activity). Similar to our findings, differential methylation and expression between pre-spawning and post-spawning oysters was enriched for metabolism and energy homeostasis pathways in gill tissue of *M. gigas* (Wang et al., 2024b). KEGG enrichment analysis of DMGs resulted in enriched pathways related to lipid, amino-acid, and carbohydrate metabolisms (e.g. "purine metabolism", "ascorbate and aldarate metabolism", "beta-Alanine metabolism", "nicotinate and nicotinamide metabolism"). The purine metabolism plays an essential role in energy supply, where it is involved in synthesis, degradation, and recycling of nucleic acids (Liang et al., 2023). In the mussel *Mytilus coruscus*, a reduction in energy consumption in the gills was observed after starvation exposure, where proteins related to purine metabolism and nicotinic and nicotinamide metabolism pathways were downregulated (Liang et al., 2023). Additionally, the inositol phosphate metabolism pathway, enriched in DMGs identified between M-HBS and HPM-HBS, contributes to metabolic signaling, and cellular reprogramming and growth in oysters (Tan et al., 2024; Tu-Sekine & Kim, 2022). Similarly, GO terms related to innate immunity, cell proliferation, and metabolic and developmental processes were enriched in transitionary transcripts of sex reversing wrasses (Todd et al., 2019). Gametogenesis requires excessive amounts of energy due to enhanced cell growth, cellular restructuring, and reprogramming (Frolov & Pankov, 1992; Hassan et al.,

2018). Therefore, it is likely that oysters undergoing sex reversal require a remodeling of the energy balance in all their tissues, reflecting processes of cell restructuring and rebuilding. The sex-specific differences in genes related to energy homeostasis and metabolism observed in this study, could be explained by a remodeling of the energy balance due to changes in energy requirement as a consequence of sex reversal.

Changes in immunity and stress response as a consequence of sex-specific DNA methylation

In addition to changes in DNA methylation of genes related to energy homeostasis and metabolism, DMGs found between the different sex phenotypes were also enriched in pathways related to immunity, stress, and toxin response. ATP-binding cassette (ABC) transporters play a key role in the defensive mechanism against pathogens in bivalves (Dong et al., 2024; Fang et al., 2022). Furthermore, peroxisomes are crucial in toxin response mechanisms (Li et al., 2022a). Lysosome membrane proteins are involved in the cellular defensive response and vital for the production of phagolysosomes (Zhang et al., 2006). Phagocytosis can have multiple functions in bivalves, namely enhanced innate immune response, or energy recycling by the reabsorption of damaged cells, which can be initiated by sex reversal (Brokordt et al., 2019). Upregulation of the phagocytosis pathway was observed in gill proteomes after starvation exposure in the mussel *M. coruscus* (Liang et al., 2023). One probable explanation of the observed differences in DNA methylation is that oysters of different sex phenotypes adopt different metabolic and immune response strategies depending on their energy requirements for reproduction. Notably, gametogenesis can lead to enhanced susceptibility to pathogens due to increased energetic demands required for reproduction (Huvet et al., 2010). During gametogenesis, reduced phagocytosis activity of hemocytes has also been reported in oysters (Delaporte et al., 2006). Similarly, immunity and stress response pathways were found to be enriched in differentially expressed genes identified between pre- and postspawning oysters (Wang et al., 2024b). Postspawning oysters are reported to experience a decrease in global immunity defense and become more vulnerable to chemical stress, due to energy loss after spawning (Li et al., 2010; Wang et al., 2024b). However, this study only assessed the differences in sex-specific DNA methylation, therefore, statements on how sex-specific DNA methylation in gill tissue affects functionality of these genes remains speculative and should be interpreted with caution.

Somatic sexual dimorphism as a consequence of DNA methylation differences between male and female oysters

Although comparison of DNA methylation between male and female oysters yielded the lowest number of DMRs and DMGs, 125 DMGs could still be identified of which 15 were uniquely differentially methylated between males and females. Most

of these unique DMGs were related to metabolic, signaling, or expression related processes. Guanine-N7-methyltransferases are involved in modifications of mRNA caps and required for mRNA processing and functioning (Trotman et al., 2017). Cyclic adenosine monophosphate (cAMP) is involved in regulating glycogen breakdown, and suggested to be related to the control of gonad maturation (Fabbri & Capuzzo, 2010). Another unique DMG found between male and female oysters was *inhibitor of nuclear factor kappa-B kinase* (*IKK*) *subunit alpha-like*. *IKKs* play a central role in cell signaling and are involved with innate immunity in oysters (Guo et al., 2015). Besides the unique DMGs identified between males and females, other DMGs with high difference in methylation percentage between male and female oysters are associated with pathways that regulate somatic sexual dimorphism in other animals. For example, the *serine/arginine repetitive matrix protein* (*SRRM*) gene was observed to be expressed in the gonads and has been allocated as a candidate sex-identification marker in a shrimp species (Garcia et al., 2024). In *Drosophila melanogaster*, *SRRM1* is involved in genitalia development and somatic sex determination (Fan et al., 2014; Qiu et al., 2019).

Additionally, the *Transport and Golgi organization protein 6 homolog* (*TANGO6*) gene is associated with mating behaviour in seahorses, where differential expression in somatic brain tissue was observed during mating (Mederos et al., 2022). In zebrafish, sex-specific DNA methylation was observed in genes associated with the arsenic metabolism, which was suggested to lead to sex-specific behavioral changes (Valles et al., 2020). Likewise, the arsenic metabolism appears to be gender-specific in rats (Muhetaer et al., 2022). Another interesting gene that was differentially methylated between males and females was *prostaglandin E synthase 3-like*. Prostaglandins are involved with ovary development and steroidogenesis in other bivalves (Liu et al., 2023). DMGs related to the semaphorin-plexin signaling pathway were solely enriched between M and HPF. Semaphorin proteins are involved in immune system regulation and are crucial for nervous system development (Messina & Giacobini, 2013). Additionally, semaphorin proteins were discovered to be involved in the development of neurons that secrete gonadotropin-releasing hormones in vertebrates, which are essential for reproductive development (Messina & Giacobini, 2013). The ECM-receptor interaction pathway was solely enriched between F-HPF. Extracellular matrix (ECM)-receptor interactions were shown to be enriched in skin transcriptomes of female fish compared to males upon exposure to sex steroids (Zhou et al., 2023). Lastly, *MAM and LDL-receptor class A domain-containing protein 2-like* gene is involved in ovarian development in clams (Liu et al., 2023). These examples indicate that while sex reversal mostly impacts gonadal tissue directly, somatic tissues are not left unchanged and sex reversal can induce sex-specific processes of cell restructuring or reprogramming in somatic tissues accordingly. Sexual dimorphism is

not as profound in oysters as in some other animals, however, indicators of sexual dimorphism are present (e.g. differences in growth or innate immunity) (Wang et al., 2024b). Based on the findings of this study, we hypothesize that differential DNA methylation between male and female oysters is associated with sexual dimorphism in somatic tissues as a consequence of sex reversal. However, understanding the link between sex-specific DNA methylation and sexual dimorphism requires further elucidation of the effects of sex-specific DNA methylation on gene functioning.

Tissue specificity of sex-specific DNA methylation in oyster gills

The occurrence of sex-specific DNA methylation has been reported in various animals and tissue types (Proestou et al., 2024b; Wan et al., 2016; Glastad et al., 2016; Laing et al., 2018; Legault, 2024). However, literature is inconsistent on the existence of tissue specificity for sex-specific DNA methylation as some studies clearly show altered sex-specific DNA methylation in different tissue types (Proestou et al., 2024b; Sun et al., 2022), whereas other studies demonstrate consistent sex-specific whole-body methylomes (Wan et al., 2016; Yuan et al., 2021). In a study of Teng et al. (2025), similar DMGs were identified in somatic muscle and gonadal tissue between male and female oysters. The *diacylglycerol kinase delta* (*Dgkd*) gene was consistently hypomethylated in somatic muscle and gonadal tissue of female oysters. Additionally, genes involved in Rho protein signal transduction were found to be consistently differentially methylated in both muscle and gonadal tissue (Teng et al., 2025). In oyster gonads, multiple genes (e.g. *Foxl2*, *Dmrt1*, or *Dgkd*), were discovered to be involved in sex differentiation, whereas these genes where not found to be differentially methylated in gill tissue in our study (Broquard et al., 2021; Sun et al., 2022, 2023, 2024; Teng et al., 2025). Additionally, DNA methylation in gonad tissue was often found to be hypermethylated among males compared to females (Sun et al., 2022, 2024; Teng et al., 2025). In our study, males or females did not show consistent hyper or hypomethylation for DMRs and DMGs. Although the enrichment for GO terms related to metabolism and energy homeostasis pathways do not overlap with DNA methylation patterns found for gonad tissue in other oysters, and may therefore be indicative of functional differences between tissues, a direct comparison of DNA methylation patterns between gill and gonadal tissue was not performed in this study and requires further elucidation.

In *Crassostrea virginica*, differences in sex-specific gene expression were identified between mantle and gonad transcriptomes (Proestou et al., 2024b). Here, differentially expressed genes found between male and female mantle tissue were associated with cell proliferation, apoptosis, and lipid transportation. Based on their findings, the authors suggest that sex-specific gene expression in mantle tissue has a specific function that could be potentially related to environmental sensing or signaling in *C.*

virginica (Proestou et al., 2024b). The observed enrichment in energy homeostasis and metabolism related pathways in sex-specific DMGs identified in this study could be indicative of functional processes related to sex reversal in the gills. However, to further elucidate potential functional differences in sex-specific DNA methylation of different tissues, more elaborate methylome comparisons between various tissues are required. Additionally, GO and KEGG enrichment analysis are both largely dependent on available gene function predictions, and so our discussion on the occurrence of functional sex-specific DNA methylation in the gills is limited to currently available reference genomes and gene annotations.

Causality dilemma of the role of somatic DNA methylation in sex reversal

Our findings clearly show the presence of sex-specific DNA methylation in gill tissue of *O. edulis*, however, it remains unknown where in the process of sex reversal this gill tissue DNA methylation may play a role. Sex steroids are known to not only influence sex differentiation in reproductive tissues, but can also affect cell fates in somatic tissues all throughout the body (Zhou et al., 2023). The concept of altered whole-body methylomes or transcriptomes in response to treatment with endocrine disrupting chemicals is widely observed in fish (Langston, 2020; Liu et al., 2015; Zheng et al., 2013). Interestingly, DNA methylation can also affect sex differentiation vice versa (Ribas et al., 2017). Based on this, it could be hypothesized that sex-specific DNA methylation of the gills occurs prior to gonadal sex differentiation, and plays a more active part in sex reversal. Environmental stressors like starvation have been demonstrated to affect energy homeostasis and metabolism pathways in the gills of bivalves through influencing gene expression (Sun et al., 2023; Wang et al., 2024b). Hence, it could be inferred that environmental stressors induce changes in gill DNA methylation, where a remodeling of the energy balance could subsequently induce sex differentiation in the gonads. In this case, gill DNA methylation would represent a more active player in sex reversal. Although gill and gonadal samples were collected simultaneously in this study, we do not know whether the phenotypic sex represented by the gonads is similarly represented by the DNA methylation state of the gills. Furthermore, to better understand differences in sex-specific DNA methylation between different tissues, the effect of sex-specific DNA methylation on gene expression and corresponding functionality should be elucidated.

Non-lethal sex identification using epigenetic markers

Sex identification is extremely relevant for understanding the process and dynamics of sex reversal in sequential hermaphrodites. Currently, sex identification in oysters is complicated by the dependency on gonadal tissue, of which sampling is lethal. With the use of anesthetics, some somatic tissues can be sampled non-lethally (e.g. abductor muscle, gill, and mantle tissue). Therefore, the use of molecular markers, that rely on somatic tissues for sex identification, provide a promising non-lethal alternative. Epigenetic markers are currently applied to assess sex and age, or response to pollutants in various marine species (Anastasiadi & Piferrer, 2020; Suarez-Ulloa et al., 2015; Valdivieso et al., 2023). In our study, comparison of gill methylomes between male and female oysters resulted in the characterization of 125 DMGs. Differential methylation on these genes could provide the basis for generating epigenetic markers that can assess the current sex phenotype of an oyster. Additionally, the distinct DNA methylation profile of hermaphrodite oysters would allow for a higher resolution of sex identification, whereby hermaphrodites could be distinguished from male or female individuals. Collectively, our findings indicate the potential of using somatic gill tissue for non-lethal sex identification utilizing epigenetic markers that can predict the current sex phenotype of an oyster.

Conclusion

In conclusion, this study is the first to characterize sex-specific DNA methylation in somatic gill tissue of a sequential hermaphrodite invertebrate. Our findings suggest that hermaphrodite oysters display distinct differences in gill DNA methylation patterns compared to male and female individuals. Annotation of differentially methylated genes resulted in significant enrichment of pathways associated with energy homeostasis, metabolism, and innate immunity. Collectively, our data suggests that sequential hermaphrodites display an alternative state that represents the epigenetic reprogramming required for sex reversal in a somatic tissue. Additionally, the characterized differentially methylated loci and regions between sex phenotypes from somatic gill tissue provide a basis for non-lethal alternatives to sex identification with epigenetic markers. Taken together, this study contributes to the understanding of epigenetic mechanisms underlying sex reversal in different sex phenotypes of the flat oyster, and shows the relevance of studying sex-specific DNA methylation in somatic tissues in relation to sex reversal.

Data availability statement

All processed sequencing data generated in this study (BAM format) have been submitted to the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena/) under project number PRJEB85091 with accession number ERP168521.

Declaration of competing interests

The authors declare that they have no competing interests

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Supplementary information

Supplementary Table S1 | Overview of all 35 sequenced flat oyster individuals with information on oyster sex and maturation stage, shell length, date and location (dd) of oyster collection, and sequencing results.

Oyster	Sex	Maturation	Latitude	Longitude	Collection date	Shell length (mm)	Mean read length	Mean read quality	Gb sequenced
1	M	2	51.735	3.970	17-4-2023	81.5	6631.3	16.0	10.3
2	F	2	51.735	3.970	17-4-2023	70.9	5519.6	15.5	5.8
3	F	2	51.735	3.970	17-4-2023	88.8	4780.8	15.4	8.1
4	HPF	3	51.735	3.970	17-4-2023	65.8	3387.4	15.0	7.0
5	HPM	2	51.735	3.970	17-4-2023	68.1	4830.4	15.2	6.7
6	M	2	51.735	3.970	17-4-2023	74.0	4183.1	16.0	14.7
7	HPF	3	51.735	3.970	17-4-2023	83.0	2945.2	15.0	10.5
8	M	2	51.735	3.970	17-4-2023	71.1	4767.2	16.0	13.5
9	HPF	2	51.735	3.970	17-4-2023	75.3	1130.6	14.2	5.4
10	M	3	51.735	3.970	17-4-2023	73.5	1375.1	14.2	8.1
11	HPM	3	51.769	3.952	26-9-2023	76.8	3413.5	15.0	19.4
12	F	2	51.769	3.952	26-9-2023	82.7	9082.3	15.3	7.6
13	F	3	51.769	3.952	26-9-2023	76.3	2531.3	14.2	10.4
14	F	2	51.769	3.952	26-9-2023	82.7	1659.0	14.2	10.7
15	M	3	51.769	3.952	26-9-2023	74.5	7192.5	15.5	9.6
16	HPF	3	51.791	3.908	15-4-2024	70.5	8249.2	16.1	12.5
17	F	2	51.791	3.908	15-4-2024	79.9	9769.0	16.0	8.5
18	M	2	51.791	3.908	15-4-2024	80.8	9149.8	16.0	9.3
19	M	2	51.791	3.908	15-4-2024	73.3	8948.8	16.0	9.9
20	M	1	51.791	3.908	15-4-2024	76.4	6216.8	16.5	14.4
21	HPF	3	51.791	3.908	15-4-2024	68.1	6628.6	16.4	17.0
22	HBS	2	51.791	3.908	15-4-2024	66.4	6727.3	16.3	14.6
23	F	2	51.791	3.908	15-4-2024	83.1	8436.0	16.4	13.0
24	HPF	2	51.791	3.908	15-4-2024	80.2	6925.8	16.4	13.9
25	HBS	4	51.769	3.952	26-9-2023	71.3	7642.9	16.5	14.6
26	M	4	51.769	3.952	26-9-2023	60.7	7205.6	16.5	18.4
27	F	2	51.791	3.908	15-4-2024	61.0	6006.9	16.3	16.9
28	HPM	1	51.791	3.908	15-4-2024	67.0	9940.7	16.4	15.2
29	HPM	1	51.791	3.908	15-4-2024	62.1	10062.4	16.1	15.3
30	HBS	1	51.791	3.908	15-4-2024	75.2	13392.4	16.1	13.0
31	HPF	2	51.791	3.908	15-4-2024	61.1	13582.8	16.5	15.3
32	HPF	1	51.791	3.908	15-4-2024	78.2	8000.2	16.4	20.0
33	HPF	2	51.791	3.908	15-4-2024	84.1	14563.8	16.5	14.6
34	HBS	2	51.735	3.970	17-4-2023	68.8	7945.4	15.9	13.3
35	HPM	2	51.735	3.970	17-4-2023	79.0	1468.9	14.0	8.6

Supplementary Table S2 | Overview of the number of DMLs identified from all pairwise comparisons between the five sex phenotypes (M, F, HPM, HPF, and HBS). DMLs are defined as CpGs with a minimal difference in average methylation percentage of 20% between compared groups, and BH-adjusted q-value<0.01.

Comparison	United CpGs	DMLs	Hypermethylated DMLs	Hypomethylated DMLs	Promoter (%)	Intron (%)	Intergenic (%)	Exon (%)
M - F - H P M - HPF-HBS	555259	1506			4.8	54.6	20.9	19.7
M-F	2489016	6294	2774	3520	5.9	60	16.6	17.5
M-HPM	4650324	12029	6370	5659	7.3	54.5	22.2	16
M-HPF	1601415	4136	1999	2137	6	57.5	19.5	17.1
M-HBS	7463705	16831	8668	8163	6.6	56.1	21	16.3
F-HPM	3471643	9352	5074	4278	8	55	20.8	16.2
F-HPF	1689997	4252	2300	1952	8.3	56	19.5	16.2
F-HBS	5239711	14337	8027	6310	7	58	19.4	15.6
HPM-HPF	3076409	7041	3355	3686	7.4	55.8	21.8	15
HPM-HBS	10451610	27363	13366	13997	6.6	56	22.5	14.9
HPF-HBS	4603541	11296	5729	5567	7	56.9	20.8	15.3
AllCpGs	555259				9.3	37.8	41.9	10.9

Supplementary Table S3 | Pearson's Chi-squared pairwise comparisons between proportions of genomic locations of significant DMLs compared with all CpGs with 3x coverage detected between all 5 sex phenotypes.

Pairwise Comparison	statistic	p-value
M-F	18.275	0.000386
M-HPM	10.284	0.0163
M-HPF	14.329	0.00249
M-HBS	12.041	0.007242
F-HPM	11.423	0.009646
F-HPF	12.797	0.005098
F-HBS	12.797	0.005098
HPM-HPF	10.669	0.01366
HPM-HBS	10.454	0.01508
HPF-HBS	12.016	0.007327
M-F-HPM-HPF-HBS	14.044	0.002846

Supplementary Table S4 | Overview of the number of differentially methylated regions (DMRs) identified from all pairwise comparisons between the five sex phenotypes (M, F, HPM, HPF, and HBS). DMRs are defined as regions of at least 200bp, containing ≥5 CpG loci, with a minimal difference in average methylation percentage of 20% between compared groups, and BH-adjusted q-value<0.01.

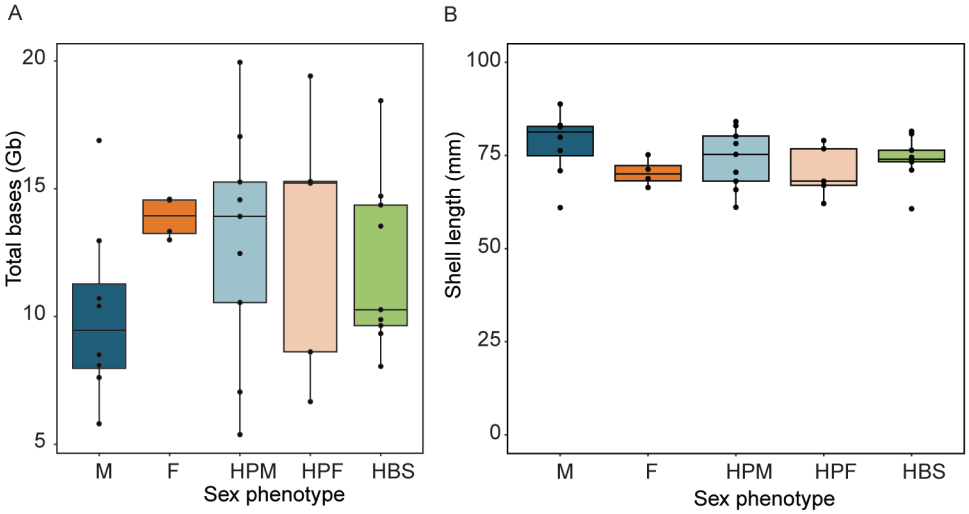
Group	Number of CpGs	number of DMRs	number of merged DMRs	Hyper-methylated DMRs	Hypo-methylated DMRs	Max Length
M-F	367030	491	239	114	125	1501
M-HPM	742143	1907	839	459	380	4001
M-HPF	218629	293	155	79	76	1301
M-HBS	1253820	3438	1617	791	826	2201
F-HPM	551888	1241	584	314	270	3201
F-HPF	236894	333	164	80	84	1401
F-HBS	868283	2507	1136	582	554	2601
HPM-HPF	468317	1026	521	243	278	1801
HPM-HBS	1810577	7769	3369	1540	1829	3601
HPF-HBS	733684	2155	1027	519	507	2201

Supplementary Table S5 | Overview of the top 10 hypermethylated and hypomethylated DMGs

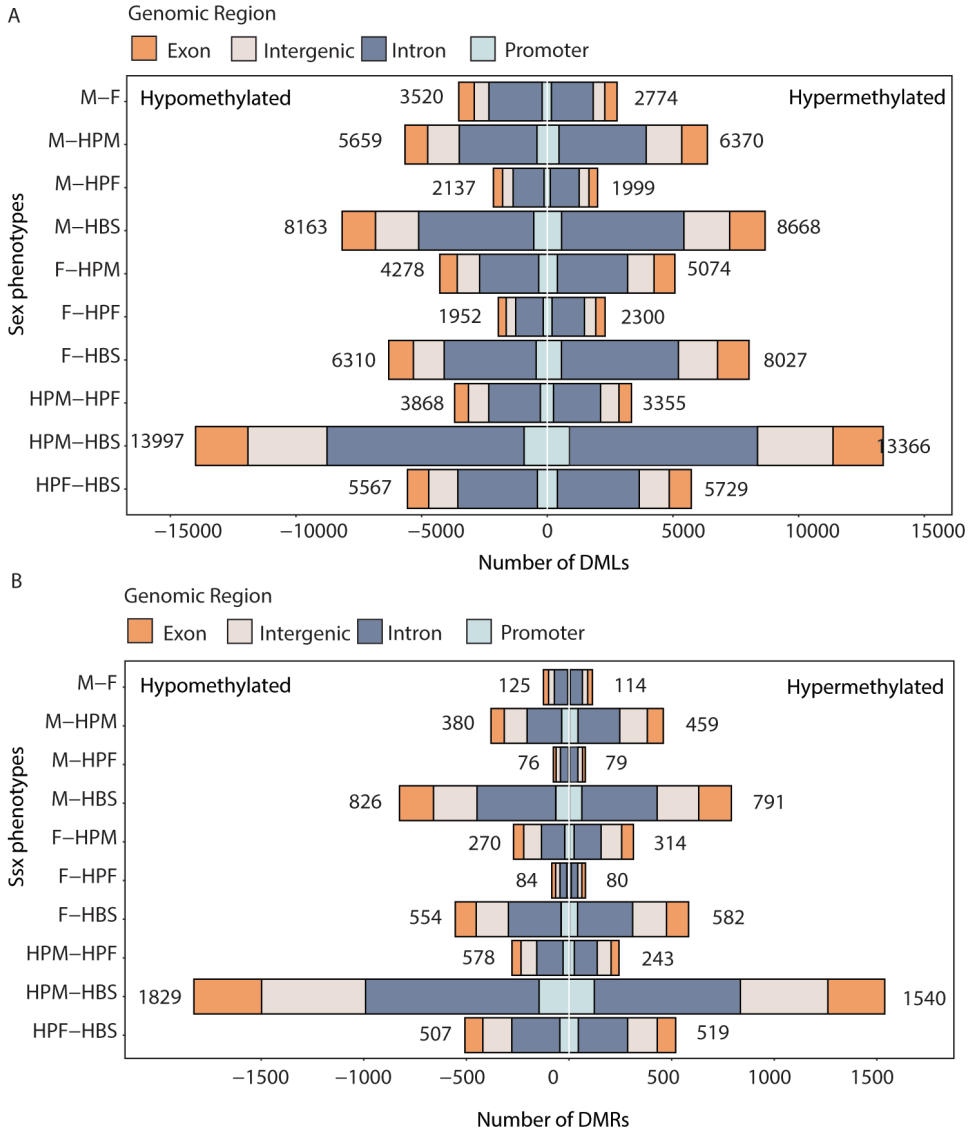
Owing to its size, Supplementary Table S5 is not included in this thesis but is available in Valk et al. (2025).

Supplementary Table S6 | Overview of uniquely identified DMGs between true male and female oyster individuals.

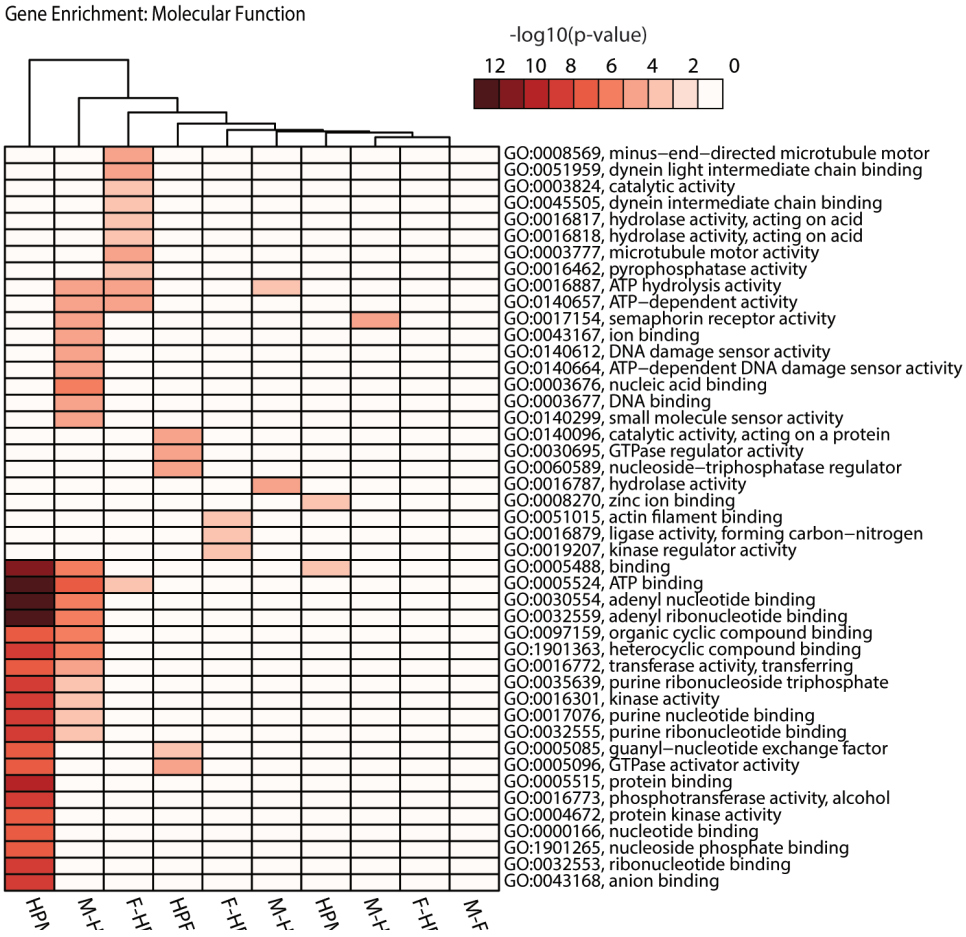
Gene ID	Chromosome	start	length DMR	Meth diff	q-value	Gene description
LOC125661226	NC_079171.1	57913900	201	24.5	7.04E-13	uncharacterized
LOC125661253	NC_079171.1	57913900	201	24.5	7.04E-13	uncharacterized
LOC125674140	NC_079166.1	14739300	201	23.1	1.27E-43	*periodic tryptophan protein 1 homolog*
LOC130054705	NC_079168.1	67558700	201	22.6	3.53E-10	*zinc finger protein 474-like*
LOC130047230	NC_079169.1	26497000	201	22.2	1.84E-13	uncharacterized
LOC125652893	NC_079168.1	3462000	301	21.6	4.99E-16	uncharacterized
LOC125668035	NC_079167.1	58554300	301	20.9	1.1E-12	*inhibitor of nuclear factor kappa-B kinase subunit alpha-like*
LOC130051962	NC_079165.1	89310600	201	20.5	0.001055	*uncharacterized*
LOC125680421	NC_079165.1	5093100	201	20.2	4.04E-11	*PR domain zinc finger protein 4-like*
LOC125669759	NC_079167.1	75657500	201	-20.8	2.08E-08	*neuronal calcium sensor 2-like*
LOC125646345	NC_079169.1	90786600	201	-21.1	1.81E-14	*cyclic AMP-dependent transcription factor ATF-2-like*
LOC125672047	NC_079167.1	32884200	201	-21.2	7.19E-10	*myocardin-related transcription factor B-like*
LOC125673983	NC_079164.1	92476700	501	-22.1	3.37E-14	uncharacterized
LOC125655458	NC_079173.1	1873400	201	-25.7	3.24E-78	uncharacterized
LOC125645763	NC_079169.1	71869100	301	-26.1	4.99E-15	*tRNA (guanine-N(7)-)-methyltransferase non-catalytic subunit wdr4-like*



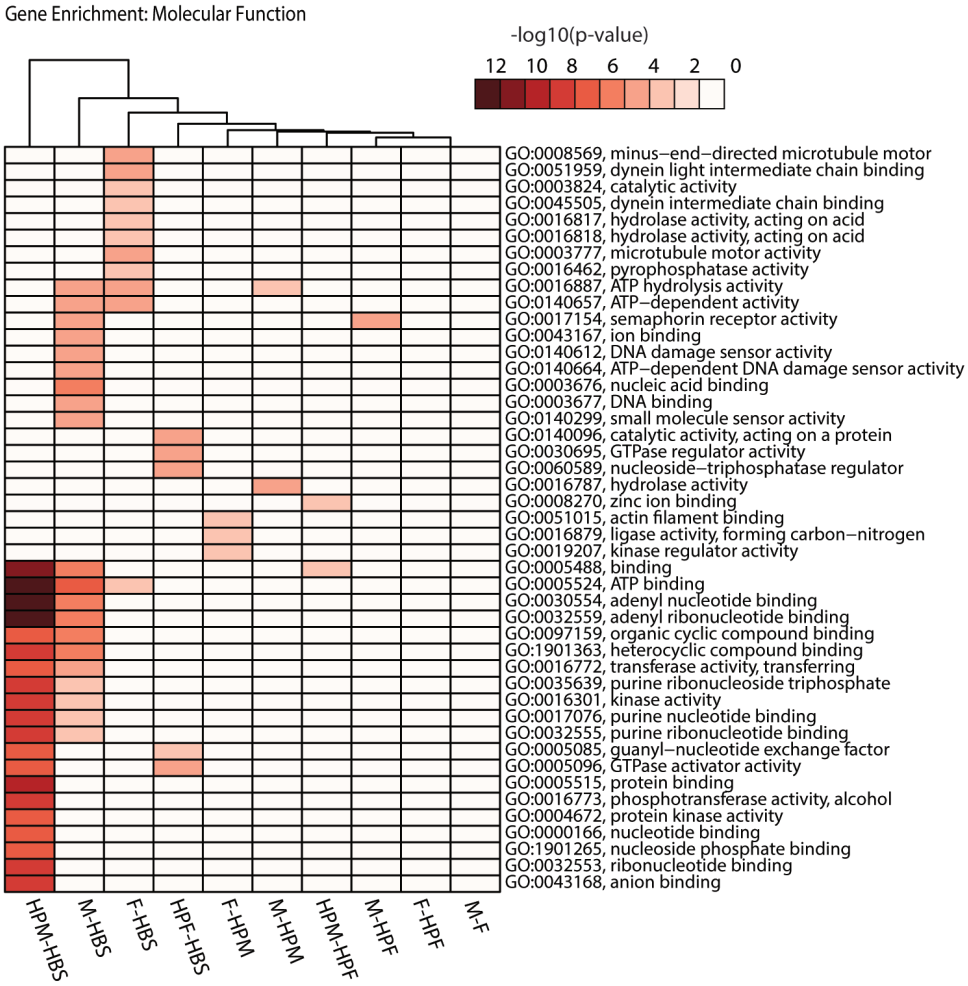
Supplementary Figure S1 | Oyster size. **(A)** Boxplot of shell length of flat oyster individuals per sex phenotype. **(B)** Boxplot of total number of sequenced base pairs (Gb) per flat oyster individual for each sex phenotype.



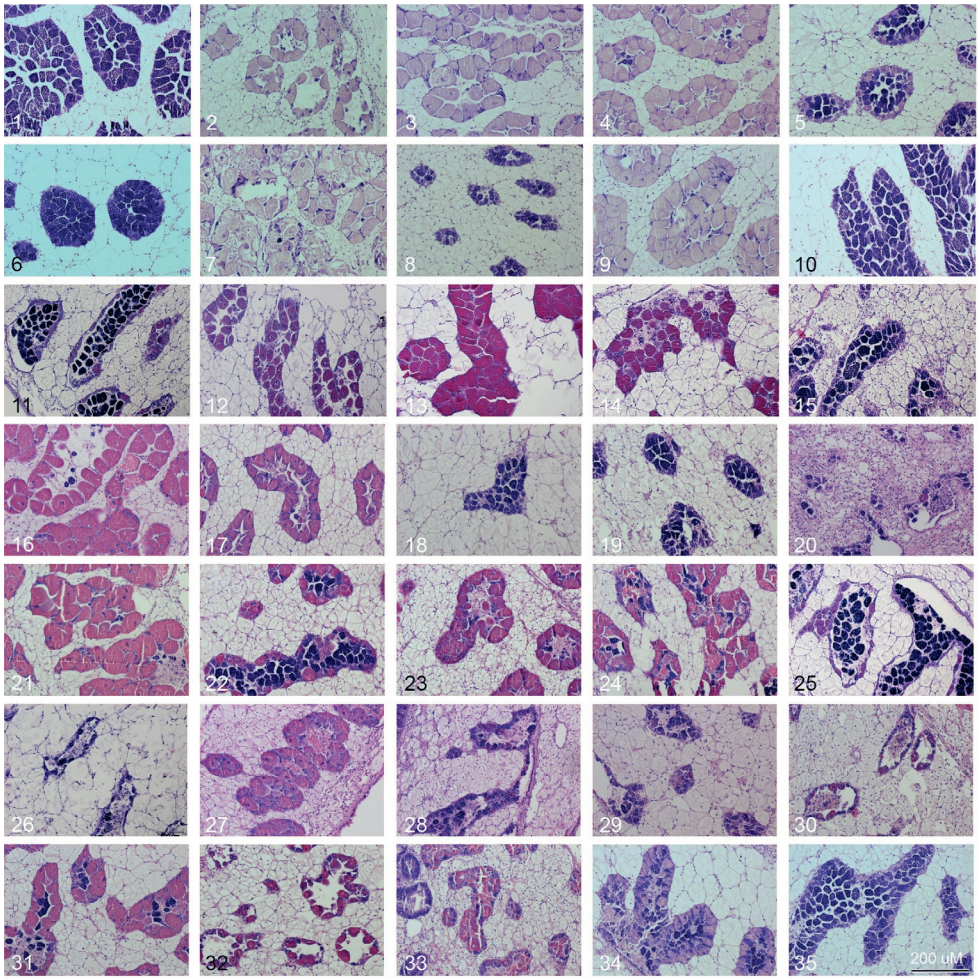
Supplementary Figure S2 | Differential methylation characterized for each pairwise comparison between the five sex phenotypes. **(A)**. Number and genomic distribution of DMLs identified for each pairwise comparison. **(B)** Number and genomic distribution of DMRs identified between each pairwise comparison.



Supplementary Figure S3 | Heatmap of Gene Ontology (GO) annotation of DMGs (n=2,576) significantly enriched for 45 Cellular Components (p<0.001). CC were clustered hierarchically with Pearson's correlation.



Supplementary Figure S4 | Heatmap of Gene Ontology (GO) annotation of DMGs (n=2,576) significantly enriched for 64 Molecular Functions ($p<0.001$). MF were clustered hierarchically with Pearson's correlation coefficient. The color scale represents log10(p-values) of Fisher's exact test.



Supplementary Figure S5 | Micrographs of histological sections of European flat oyster individuals showing the gonad area.

CHAPTER 5

Toward non-lethal sex identification in oysters

Developing epigenetic biomarkers of sex-specific DNA methylation

Sophie Valk, Pauline Kamermans, AlberTinka Murk, & Reindert Nijland

Abstract

Population demographics, such as sex and age, are essential for understanding species dynamics and informing conservation strategies. However, determining sex ratios can be particularly challenging in species lacking clear sexual dimorphism. In the European flat oyster, an ecologically and economically important, hermaphrodite bivalve, sex identification is further complicated by frequent sex reversal. Severe population declines, driven by ongoing disturbances including habitat degradation and prevalent diseases, continue to limit the species' recovery. As skewed sex-ratios can limit reproductive output and increase the risk of rapid loss of genetic diversity, aquacultural production for restoration purposes would greatly benefit from molecular sex identification methods.

This study aimed to identify epigenetic biomarkers that enable sex identification by assessing sex-specific DNA methylation in gill tissues. A combined approach of machine learning and amplicon sequencing was employed that resulted in a total of 14 epigenetic markers. Validation with samples of known sex phenotypes demonstrated that these markers could reliably classify hermaphrodite versus non-hermaphrodite oysters, and showed moderate performance in distinguishing male-biased from female-biased individuals. With further validation, these biomarkers could facilitate comprehensive studies of sex reversal mechanisms in living oysters. This work presents the first molecular approach of sex identification from gill tissue, with substantial opportunities for both aquaculture and the restoration and conservation of this sequential hermaphrodite bivalve.

Introduction

Hermaphroditism entails the simultaneous or sequential maturation of both male and female gonads during an organism's lifetime, and is widespread across teleost fishes and invertebrates (Warner, 1975). Sex reversal is considered selectively advantageous when one sex has fitness benefits at an early life stage, while the other sex gains higher fecundity or fertility later in life (Warner, 1975). Often, these fitness differences are associated with body size. In protogyny, individuals mature first as females and later reverse to males, a strategy common in several fish families where reproductive monopolization by males is determined in physical contests (Casas & Saborido-Rey, 2021; Goikoetxea et al., 2021; Oldfield, 2005). In contrast, protandry involves initial male maturation followed by reversal to female reproduction and occurs when female fertility exceeds that of males beyond a certain size. While protandry is less common among sex-reversing animals, it is widespread among invertebrates (Collin, 2013; Yusa, 2007).

Among the 45 bivalve superfamilies, at least 11 have been recorded to display hermaphroditic reproductive behaviour (Heller, 1993). All species belonging to the Ostreidae family are thought to be sequential hermaphrodites, although the distinction between simultaneous and sequential hermaphroditism is not always clear (Heller, 1993). For example, the European flat oyster, *Ostrea edulis*, is considered a sequential hermaphrodite, whereas the maturation of both male and female gametes can take place within the same follicle, implying simultaneous hermaphroditism (Da Silva et al., 2009). In *O. edulis*, sex reversal is primarily determined by environmental factors, such as temperature, food availability, and photoperiod (Cowan et al., 2025; Da Silva et al., 2009; Joyce et al., 2013). Fluctuations in biotic and abiotic conditions can lead to asynchronous maturation and spawning across individuals, resulting in highly variable sex ratios in wild populations (Da Silva et al., 2009; Davenel et al., 2010; Kamphausen et al., 2011; Lallias et al., 2010b).

More recently, *O. edulis* has received increased attention as it is a crucial ecosystem engineer that enhances local biodiversity by building biogenic reefs (Pogoda et al., 2019; Smyth & Roberts, 2010; Thomas et al., 2022, 2022). The species has suffered extreme population declines along its natural distribution range from the combined pressure of overfishing, habitat degradation, and several diseases (Pouvreau et al., 2023; Thurstan et al., 2024a, 2024b; Zu Ermgassen et al., 2025). Following flat oyster reef degradation, ecosystem community compositions exhibited shifting baselines or sometimes even faced full collapse (Helmer et al., 2019; Mérou et al., 2023; Thurstan et al., 2013). These ecological consequences have driven efforts toward both species conservation and ecosystem restoration (Gercken & Schmidt, 2014; Rodríguez-Perez et al., 2019; zu Ermgassen et al., 2021). Alongside the translocation of oysters from

source populations to supplement degraded oyster sites, aquaculture initiatives have been established for cultivation of the species (Colsoul et al., 2020; Lallias et al., 2010a; Zu Ermgassen et al., 2023). While oyster culturing provides opportunities to support both economic production and ecological restoration, hatchery-based propagation of oysters for ecological restoration may pose risks to the preservation of genetic diversity (Camara & Vadopalas, 2009). Broodstock populations in aquaculture are often smaller than those in natural systems, which may lead to reduced genetic variation in offspring (Šegvić-Bubić et al., 2020). Additionally, naturally occurring male-biased sex ratios may further reduce the effective population size, contributing to reduced genetic diversity in aquaculture (Diaz-Almela et al., 2004; Kamphausen et al., 2011; Lallias et al., 2010b; Zu Ermgassen et al., 2023). Together, these factors can decrease genetic variation at the population level and may limit the long-term sustainability of restoration initiatives that rely on restocking with hatchery-produced oysters (Baeza et al., 2010). As such, aquaculture would benefit from achieving balanced sex ratios to optimize production output while maintaining genetic diversity. Elucidating the mechanisms underlying sex reversal in *O. edulis* is therefore essential for managing effective population size and maintaining genetic diversity, both in aquaculture and in restoration contexts. However, sex identification methods currently rely on lethal or costly techniques and are therefore rarely applied (Da Silva et al., 2009; Davenel et al., 2010; Wilson & Simons, 1985). Such challenges in sex identification limit the upscaling of aquaculture production and hinder the study of sex reversal, underscoring the need for alternative approaches that enable non-lethal sex identification.

Recently, the role of epigenetic mechanisms in regulating sex reversal has received increased attention. By regulating (post-)transcriptional activity without requiring a change in the original DNA sequence, epigenetic mechanisms can facilitate phenotypic changes in response to environmental stimuli (Jones, 2012; Piferrer, 2013; Roberts & Gavery, 2012; Sarda et al., 2012). Among the most familiar epigenetic mechanisms are non-coding RNAs, DNA methylation, and histone modifications. In oysters, the association between DNA methylation and phenotypically plastic traits is well established for certain tissues, for example in sex reversal, where sex-specific DNA methylation regulates differential gene expression involved in gonad maturation and gametogenesis (Sun et al., 2024; Zhang et al., 2018). More recently, sex-specific DNA methylation patterns were identified in somatic gill tissue of *O. edulis*, suggesting a stronger association between somatic tissues and sex reversal than previously recognized (Valk et al., 2025). This finding poses opportunities for identifying epigenetic markers enabling non-lethal sex identification, as gill tissue can be sampled using anesthetics. Epigenetic biomarkers are already used in ecological studies, with most markers developed for age estimation in vertebrates (Anastasiadi & Piferrer, 2020; Balard et al., 2024; Belenguer et al., 2024; De Paoli-Iseppi et al., 2019).

In this study, we aimed to identify epigenetic markers for non-lethal sex identification in oysters. Using a machine learning approach, we selected several markers that were strongly correlated with sex phenotypes. Bisulfite conversion combined with amplicon sequencing provides an alternative method for quantifying DNA methylation at these target sites. This work presents the first attempt to develop epigenetic markers for non-lethal sex identification in oysters, providing a foundation for studying sex reversal in live individuals of this sequential hermaphrodite bivalve.

Materials and methods

Sample collection, DNA extraction & sequencing

Flat oysters were collected from the Grevelingenmeer, the Netherlands (53°30'40"N, 08°06'20" E), by dredging during the months April and September 2023, and April 2024. Oysters of similar shell length (74.1 ± 7.45 mm) were selected for gill tissue collection, which was preserved on RNAlater or snap-frozen using liquid nitrogen, after which they were stored at -80 °C until further use. Cross sections of tissue representing reproductive tissue were embedded in paraffin and stored until stained.

Genomic DNA was extracted from frozen gill tissue (~20 to 30 mg), using either the Qiagen Gentra Kit for genomic DNA or the Monarch HMW DNA extraction kit for tissue, following the manufacturer's instructions (Qiagen catalog no.158667; NEB catalog no. T3060). DNA quantity and quality were assessed using the HS Qubit assay (Life Technologies, ThermoFisher Scientific) and gel electrophoresis, respectively.

For whole-genome nanopore sequencing, DNA extracts were barcoded and multiplexed following the native barcoding protocol (SQK-NBD114-24) from Oxford Nanopore Technologies (ONT, Oxford, UK). Libraries were prepared, including end-prep, barcoding, and adaptation ligation, and sequenced on the ONT sequencing platform according to the manufacturer's guidelines. The long-read data were originally generated to characterize sex-specific DNA methylation across different sex phenotypes. For a more detailed description of DNA extraction, library preparation, and sequencing, we refer to Valk et al. (2025).

For histological sex identification, cross sections (±5 µm) of gonadal tissue embedded in paraffin were stained with haematoxylin-eosin. Oyster sex and gonad development state were identified, using the following sex stages: male (M), female (F), hermaphrodite predominantly male (HPM), hermaphrodite predominantly female (HPF), and hermaphrodite both sexes equally present (HBS), according to the guidelines of sex identification as described in Da Silva et al. (2009). The gonad development index included: inactive or resting gonad (0), early gametogenesis (1), advanced gametogenesis (2), mature (3), partially spawned gonad (4), and

reabsorbing gonad (5), following the classification of Da Silva et al. (2009). Oysters that displayed no clear gonad maturation and that were categorized to be in the "inactive or resting gonad" or "reabsorbing gonad" phase were excluded from sequencing and downstream analyses.

Bioinformatics

A detailed description of the complete workflow for oyster sex prediction using epigenetic markers is provided below, with all workflow steps illustrated in an integrative overview (Fig. 1).

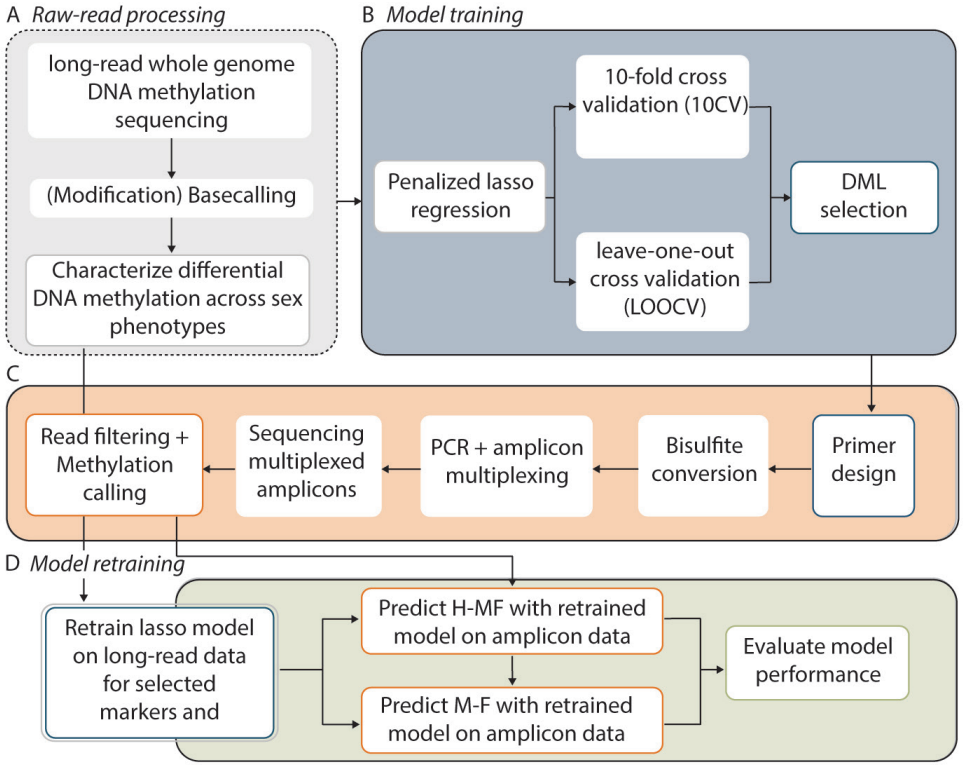


Figure 1 | Integrative overview of the workflow for sex prediction using machine learning models and a wet-lab assay for quantification of DNA methylation at target sites. **(A)** Bioinformatics pipeline, from raw nanopore long-read data to the characterization of differential DNA methylation between sex phenotypes. **(B)** Training of machine learning models to identify the most stable differentially methylated loci (DMLs) for sex prediction. **(C)** Workflow of the bisulfite-converted amplicon assay, including primer design, bisulfite conversion, PCR, and sequencing, for the quantification of DNA methylation at selected DMLs. **(D)** Retraining machine learning models using the selected DMLs and application of the model to web-lab assay data for sex prediction.

Determining differential methylation between sex phenotypes

As differentially methylated loci (DML) were previously identified, only a brief description of the bioinformatics pipeline for calling DMLs is provided below. For a more detailed description of raw read processes, see Valk et al. (2025).

Raw reads from 35 sequenced individuals were super accuracy base called using Dorado v0.4.1 with the model dna_r.10.4.1_e8.2_400bps_sup@4.2.0 and simultaneous modification calling via the -modified-bases 5mC flag. Base called reads were mapped against the xbOstEdul1.1 reference genome assembly (NCBI RefSeq Assembly: GCF_947568905.1; Adkins et al. 2023), using minimap2 (Li, 2021). Mapped reads with a minimum mapping quality <60, as well as unmapped or secondary alignments, were exclude using Samtools v1.17 (Danecek et al., 2021). DMLs were called from the mapped reads using Modkit v0.2.1 (https://github.com/nanoporetech/modkit). Sites with >60% Ndel coverage were removed, as this indicated potential deletions.

Identifying sex-specific DNA methylation



Differential DNA methylation across sex phenotypes was identified using methylKit v0.99.2 (Akalin et al., 2012). Methylation calls were first combined across all 35 individuals using the unite function and subsequently normalized and filtered to retain DMLs with a minimum coverage of three reads in all individuals. Gonad developmental stage was included as a covariate to account for potential effects of maturation on sex-specific methylation.

To reduce complexity in subsequent sex classification analyses, a two-step approach was applied in identifying differential methylation. First, differential methylation was assessed between hermaphrodites (H; n=18) and combined males/females (MF; n=17), using methylation percentages from all 35 sequenced individuals (H-MF comparison) and histologically identified sex phenotypes (Valk et al., 2025). In this step, the three hermaphrodite substages (HPM, HPF, and HBS) were pooled and treated as a single hermaphrodite group. Second, differential methylation between males (M; n=9) and females (F; n=8) was assessed separately (M-F comparison), using only the subset of male and female individuals. This two-step approach was motivated by the observation that hermaphrodite oysters exhibit a more distinct DNA methylation profile compared to males and females (Valk et al., 2025). By restricting the second analysis to male and female individuals only, the detection of sex-specific differential methylation was expected to improve as the influence of the distinct hermaphrodite methylation was minimized, thereby enhancing the identification of stable and predictive DMLs for downstream sex classification.

Differential methylation was determined by fitting a logistic regression model to methylation percentages using the methylDiff function from methylKit. To correct for multiple testing, a Benjamini-Hochberg (BH) false discovery rate p-value correction was applied. DMLs with an average methylation difference ≥20% and an adjusted q-value <0.01 were considered significant. The top 50 most significant DMLs were selected for downstream analysis. A principal component analysis (PCA) was performed to visualize the variation explained by these sites across sex phenotypes using the prcomp function from the stats package in R.

To assess the stability of DML selection, bootstrap resampling was applied with 100 iterations. For each bootstrap sample, a lasso logistic regression model was fitted using the glmnet package with cross-validation to determine the optimal regularization parameter λ. Selection frequencies across bootstrap iterations were calculated and features with higher selection frequencies were considered more stable predictors of sex classification.

Lasso regression model on sex identification

Machine learning models were trained to classify sex by distinguishing (i) hermaphrodites (H) from male/female (MF), and (ii) males (M) from females (F) (Fig. 1B). DMLs with near-zero variance across sex phenotypes were removed. Prior to model training, the methylation dataset was partitioned into a training dataset containing 70% of samples, and a testing dataset encompassing 30%, following the approach suggested by Piferrer and Anastasiadi (2023). Stratified sampling was applied to preserve relative proportions of sex phenotypes across the subsets.

Two separate sex classification machine learning models were trained using Lasso logistic regression implemented in the caret and glmnet packages in R (Friedman et al., 2008; Kuhn, 2007). Model performance was evaluated using two cross-validation strategies: (i) repeated 10-fold cross-validation with k=10 folds and 3 repeats (10CV), where the data is split into k-fold subsets and trained using k-1 folds for training and 1 fold for testing; and (ii) leave-one-out cross-validation (LOOCV) where the model is trained by alternatingly leaving one sample out. Performance was assessed by the following two performance metrics: the area under the cure (AUC) and receiver operating characteristic (ROC) curves using the pROC package in R, alongside confusion matrices, and performance metrics including accuracy, sensitivity, and specificity (Robin et al., 2010). Differences between performance curves from the two cross-validation approaches were evaluated using a DeLong test (Demler et al., 2012).

For both H-MF and M-F models, the ten DMLs with the highest bootstrap selection frequency were selected as candidate epigenetic biomarkers and subsequently used for primer design for the targeted bisulfite amplicon-sequencing assay.

Bisulfite amplicon sequencing of epigenetic markers

Primer design for bisulfite converted DNA

Reference genome sequences spanning 1500 bp around the target DMLs were extracted from the reference genome assembly and converted in silico to bisulfite-treated DNA. These sequences were subsequently used for primer design. Primers were designed specifically for bisulfite-converted DNA using MethPrimer (Li & Dahiya, 2002). Primers were designed to avoid overlap with CpG sites, and the target DML was positioned as close as possible to the center of the amplicon. Primer specificity was verified using NCBI BLAST against both the reference gnome and the bisulfite-converted reference genome. Primer sequences, amplicon lengths, and chromosomal positions of target DMLs are provided in Table1.

Primer pairs were first tested individually to assess amplification success using multiple tissue samples representing different sex phenotypes. Amplification success was confirmed by verifying the expected amplicon length using gel electrophoresis. After validation, two primer pools were created by combining primers targeting DMLs for the H-MF and M-F classifications

A control primer pair was used to generate an amplicon of 0% methylation. This fragment was subsequently treated with a CpG methyltransferase enzyme (M.SssI; M0226; New England Biolabs, USA) to produce a fully methylated (100%) control fragment. These methylation control fragments were included in subsequent sequencing to assess bisulfite conversion efficiency. The control fragment overlapped the amplicon amplified by primer pair NC64_9520705, used for M-F classification.

Table 2 | Primer details for all epigenetic makers. A total of 18 primer pairs were developed as epigenetic markers were designed targeting 9 DMLs for classifying Hermaphrodite – Male/Female, and 9 DMLs for classifying Male – Female. The last primer pair targets a control fragment used to validate the bisulfite conversion efficiency. Length indicates amplicon length in bp.

Primer pair	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Length
H - M/F			
NC079164.1_84756045	GTGAGATATTGGTTTGATGATATTAGTTAT	CACATTATCTCATTAATCAATATCATCCCAA	930
NC079166.1_80662189	GATTATAGATGGAGGAATTATGTATA	CATATCTAATATTATCCTTAAAAAAATC	1038
NC079164.1_99406849	TGTTTATTGTGTATTTTGAATTGTA	CTTCTATATTATACACTCCTCTACAAACTAA	1036
NC079171.1_60093832	GTAGTTAGTTTGTGTTAAAAGAGGTG	ACACCATATATACAAATAATCATAAATTC	318
NC079165.1_98521200	GATAATTTTTTTAATTTTGAATTTTGGATATTG	TCCATCAAACTCTACAAAATAAAAACA	238
NC079168.1_59842453	GTGATGAAAATTTAATTTAAAATATAGGATTATT	CAACTTCAAAATAAATATAAATCAAACTATAA	432
NC079167.1_30293075	GTTTTGTTTGTTATTGTTATAGGTTAGTTAG	CTAAATATTATAACAAAAAAATTAAACACAAATTC	298
NC079170.1_32052906	TTTTGTATTTTAAATTTTATTAGGTATGGA	CCTAATAAAATCCCAAACCAAACAATTTTC	311
NC079169.1_84115157	GATTGTTTTTAAATTATGTAAATTATGAGG	CTTTAACACTCTATTTTTCCTTTTAATTATTAC	178
M-F			
NC079166.1_91567046	GTTATATGTTATGTAAAGATAAATTGT	CTTATAATCCTATAACTACAATAATATAAC	1094
NC079164.1_78794301	TAGTGAGATAAGTTTAGGATTTTT	CTACTTATTATTTATATTACAATCAAATC	220
NC079164.1_9520705	TTAGAATTAGAGGTATTATGAGGATT	TTCATCTACCTAAAAATACTTTATAACAA	343
NC079165.1_50016212	TGATTTTAATGGGTAGAGGGTATAT	TAAACCTCTTATCTAACACAAAACTTATAA	255
NC079164.1_850955	GAGGATTACGTTGTGTTAGGTTTTT	CACAATCACATTAATCAATACTATAA	830
NC079171.1_58235261	TAGGTAAATGTTTAAAATAGAATATGAGTTAT	CATATAAAAACCTATTTAAACTAATAACATATC	425
NC079164.1_46967336	GATATTTTATTTATGTGTATTGAATGTATG	CCAACTCCTAAAATTAATCCTAAAA	235
NC079172.1_38159260	GATTAATTTAATTTATAAATATGATTGTATTGGTT	ACTAAATCCACTTAATAAATATAATATCAAA	326
NC079164.1_46968956	GTTTATATAGTTTTAATTATTGTGTATAT	CAAAAAAATTTCAATAAACATACTTAACC	775
Control Fragment			
NC079164.1_9520705	CTGTTCTCAATCTTTGTGGAGTC	CTTAATGCATAGTTATAATAGAATCTC	638

PCR, library preparation, and sequencing

To quantify DNA methylation across the selected epigenetic markers using the bisulfite-amplicon approach, a total of 24 individuals were sequenced, including 6 males (M), 4 females (F), and 14 hermaphrodites (H). Hermaphrodites were further classified as 3 HPM, 7 HPF, and 4 HBS. An overview of all samples, including phenotypic sex, size, length, and other relevant information, is provided in the supplementary material (Supplementary Table S1). Genomic DNA (500 ng per sample) was bisulfite converted using the Premium Bisulfite kit (C02030030; diagenode) following the manufacturer's instructions. Bisulfite-treated single-stranded DNA was quantified using DeNovix DS-7+ spectrophotometer (DeNovix).

PCR amplification was performed using approximately 50 ng bisulfite-converted DNA as input in a 10 µl reaction containing 5 µl MethylTaq Plus 2x Master Mix, 0.8 µl primer mix (10 µM), and 3,4 µl nuclease-free water. A touchdown PCR protocol was performed in a thermal cycler following the following conditions: initial denaturation at 95 °C for 5 minutes; followed by five cycles of 95 °C for 30 seconds, annealing for 30 seconds with 1 °C decrease per cycle from 55 °C until 50 °C, and extension at 72 °C for 80 seconds; followed by 35 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 80 seconds; with a final extension at 72 °C for 5 minutes. Amplification success was confirmed on agarose gel electrophoresis. PCRs for the H-MF and M-F classification were performed separately, and after confirmation of successful amplification were pooled for the same individual. All PCRs were performed in triplicate.

PCR products were purified using AMPure beads with an 80% ethanol wash and eluted in 15 µl nuclease-free water. Purified amplicons were prepared for native barcoding according to the manufacturer's instructions using the Ligation sequencing amplicons – Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies). Following end-preparation, individual native barcodes were ligated to each sample prior to pooling. Sequencing adapters were subsequently ligated to the pooled library, followed by a final clean-up using Short Fragment Buffer.

Sequencing of multiplexed amplicons was performed on a MinION device using an R10.4.1 flow cell (FLO-MIN114; Oxford Nanopore Technologies). Reads with a minimum quality score threshold of Q8 were retained for downstream analysis. The abovementioned workflow of primer design, bisulfite conversion, PCR, multiplexing, and sequencing was visually summarized as part of the infographic (Fig. 1C).

Basecalling and demultiplexing sequenced amplicons

Samples were demultiplexed using the demux function of Dorado, specifying the kit name (SQK-NBD114-96), and sequences were output as FASTQ files per barcode. After demultiplexing, reads were mapped with minimap2 v2.28-r1209 against the in

silico bisulfite-converted reference sequences that were also used for primer design (Li, 2021). Upon mapping, only primary aligned reads were retained in BAM format, after which reads were sorted and indexed using Samtools v1.20 (Danecek et al., 2021).

For each sequenced amplicon, forward primer sequences were first trimmed using Cutadapt v5.2 (Martin, 2011), requiring a minimum read length of 20bp after trimming and a maximum error rate of 0.3 for primer alignment. Reverse primers were subsequently trimmed using the same parameters. Trimmed reads were then remapped against the reference bisulfite-converted sequences to match the position of the target DMLs in the reference sequences.

Amplicon filtering and methylation calling

Methylation calls across all 18 epigenetic markers were obtained using Samtools mpileup. Methylation percentages were computed by considering only reads containing either a C or T at the target CpG site, and by calculating the proportion of C bases relative to the total number of C and T bases for each amplicon. Methylation data for epigenetic markers was only retained if at least ≥50% of the samples contained reads for the respective marker. Samples were retained for a given marker only if at least two replicates were successfully amplified, with a minimum of 20 reads per marker per replicate. If only one replicate was successfully amplified per oyster sample, the sample was omitted for that specific epigenetic marker. Methylation percentages inferred from the bisulfite-amplicon data were then compared with the long-read data that were originally used to train the sex classification models.

Model retraining and sex prediction

Based on the epigenetic markers successfully amplified in the bisulfite-amplicon assay, the two models (H-MF and M-F) were retrained using only these DMLs captured by the markers, while still using the long-read nanopore data as input for training and testing both models (Fig. 1E).

The retrained models were then applied to predict sex from the bisulfite-amplicon sequencing data, and prediction accuracy was assessed using confusion matrices.

All plots were generated in ggplot2 v3.5.2 (Wickham, 2016) in R and Matplotlib v3.10.1 (Hunter, 2007) in Python.

Results

Marker selection for H-MF classification

Differential DNA methylation between hermaphrodite (H) and male/female (MF) oysters identified 713 differentially methylated loci (DMLs) exhibiting significant methylation differences, with a minimum average methylation percentage of 20% (ΔDNAm ≥20) and a false discovery rate-adjusted q-value <0.001. The 50 most significant DMLs were selected for downstream analysis. Principal component analysis (PCA) with these 50 DMLs revealed clear clustering by phenotypic sex (PERMANOVA: $F = 8.42$, $p = 0.001$; Fig. 2A). Most of the variance was explained by the first two PC axes, with 31.1% explained by PC1 and 13.8% explained by PC2.

To prioritize strong contribution to sex classification along these axes, DMLs were ranked to their absolute magnitude of PC1 and PC2 loadings. Methylation percentages corresponding to these 50 DMLs were used as candidate predictors in a penalized logistic regression (LASSO) model to classify H-MF.

The two cross-validation approaches applied to the LASSO model: 10-fold cross-validation (10CV) and leave-one-out cross-validation (LOOCV) displayed comparable predictive performance (Supplementary Fig. S1A). Both models demonstrated high discriminatory power (10 CV AUC = 0.99; LOOCV AUC = 1), with no significant difference between the AUC values of both models (DeLong test: $p = 0.226$). Classification performance assessed by bootstrapping increased with the number of included DMLs and reached a maximum at 11 DMLs, after which performance plateaued, indicating that inclusion of additional loci did not improve prediction accuracy (Supplementary Fig. S1B).

For the 10CV approach, 16 DMLs contributed to classification between H and MF with absolute coefficient values > 0.001 (Fig. 2B). The top five DMLs with the largest absolute coefficients were hypomethylated in H relative to MF. Using LOOCV, 14 DMLs sites exhibited absolute coefficient values > 0.001, with the same five loci identified as the strongest contributors. Notably, 13 of the top contributing DMLs were shared between both cross-validation approaches. Although bootstrapping analysis showed that classification performance plateaued after 11 DMLs, all 13 DMLs identified by both cross-validation methods were retained for marker development because not all stable DMLs might meet the requirements for primer design.

Marker selection for M-F classification

Differential methylation analysis between males (M) and females (F) identified 5,949 significant DMLs (ΔDNAm ≥20 & q<0.01). PCA of the top 50 DMLs revealed clear clustering between M and F samples, with PC1 explaining 72.% and PC2 for 10.6% of

the total variance (PERMANOVA: F = 12.838, p = 0.001; Fig. 2C).

Similar to the classification model between H and MF, CpG sites were ranked based on absolute magnitude of loadings on PC1 and PC2. The corresponding methylation percentages were used as predictors in a penalized LASSO model for M-F classification. Model performance was slightly, but not significantly, better under LOOCV than 10CV (Supplementary Fig. S2A), with a higher AUC (10 CV AUC = 0.821; LOOCV AUC = 0.917), (DeLong test: p = 0.373; Supplementary Fig. S2B).

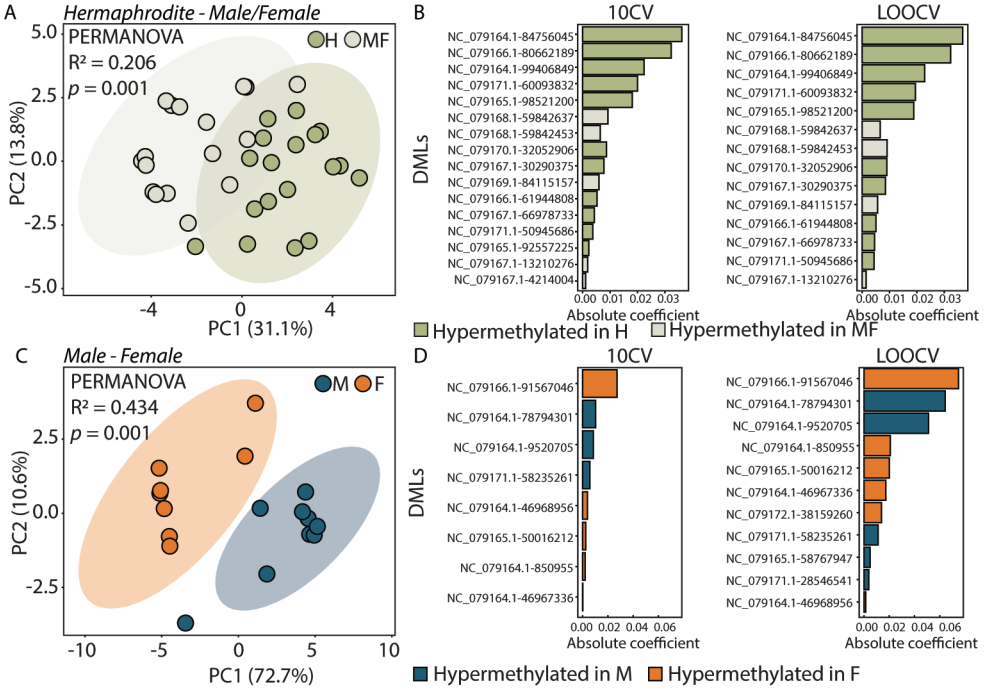


Figure 2 | The contribution of the 50 most significantly differentially methylated loci (DMLs) to clustering by phenotypic sex **(A)** Principal component analysis (PCA) between hermaphrodites (H) and males/females (MF). With **(B)** Top DMLs contributing to the H or MF classification using the penalized logistic regression (LASSO) model, ranked by absolute coefficient magnitude under 10-fold cross validation (10CV) and leave-one-out cross-validation (LOOCV). **(C)** PCA of the most significant DMLs between males (M) and females (F). **(D)** Top DMLs contributing to the M-F using the LASSO classification model, ranked by absolute coefficient magnitude under 10CV and LOOCV. The percentage variance explained by PC1 and PC2 is indicated on the axes in both PCA panels, and ellipses represent 95% confidence intervals.

Using 10CV, eight DMLs exhibited absolute coefficient values > 0.001, indicating strong contribution of these DMLs to sex classification (Fig. 2D). Under LOOCV, 11 DMLs exceeded this threshold. The eight DMLs identified in the 10CV model were also among the top contributors in the LOOCV model, although their relative contributions, quantified by absolute coefficient magnitude, differed between approaches.

For both the H-MF and M-F classification models, no significant differences in predictive performance were observed between 10CV and LOOCV. Moreover, the DMLs that exhibited highest contributions to each model, quantified by the absolute coefficient values, were largely shared between the two cross-validation approaches. Given the consistency in model performance and feature selection of both cross-validation approaches, the combined set of top-contributing DMLs were used to design primers for epigenetic markers.

Bisulfite-amplicon sequencing assay

Primer design yielded 18 primer pairs that successfully amplified the regions targeting the DMLs, hereafter referred to as epigenetic markers, with correct product lengths confirmed by gel electrophoresis. A total of 24 oyster gill tissue samples were bisulfite-converted, amplified in triplicate, and sequenced. Multiplexed sequencing of all amplicons produced 4.56 million reads passing the Q8 quality threshold.

After filtering the entire dataset of sequenced amplicons for a minimum of 20 reads per replicate, containing at least two replicates per sample, and retaining amplicons only if they had successfully amplified for at least 50% of all samples, a total of eight markers remained for H-MF (Fig. 3) and six for M-F (Fig. 4). Computing methylation percentages across replicates for all 14 markers and samples yielded an average standard deviation of 3.5% in methylation across replicates per sample (Supplementary Fig. S3 & S4).

To assess how accurately the bisulfite-amplicon assay captured DNA methylation levels, average methylation percentages obtained from this assay were compared with those obtained from long-read nanopore sequencing for each marker. Across the eight epigenetic markers used for H-MF classification, significant differences between methods were primarily observed among hermaphrodite oysters, specifically for the markers NC_079164.1-84756045, NC_079164.1-99406849, NC_079167.1-30290375, NC_079168.1-59842637, and NC_079171.1-60093832 (Fig. 3). Additionally, the marker NC_079164.1-99406849 displayed a significant difference in average methylation percentage between both methods in males (Wilcoxon rank-sum test: p-value < 0.05). Correlation analyses demonstrated positive associations between methylation percentages obtained from the bisulfite-amplicon assay and long-read sequencing across all eight markers (median Spearman correlation = 0.70; Supplementary Fig. S5).

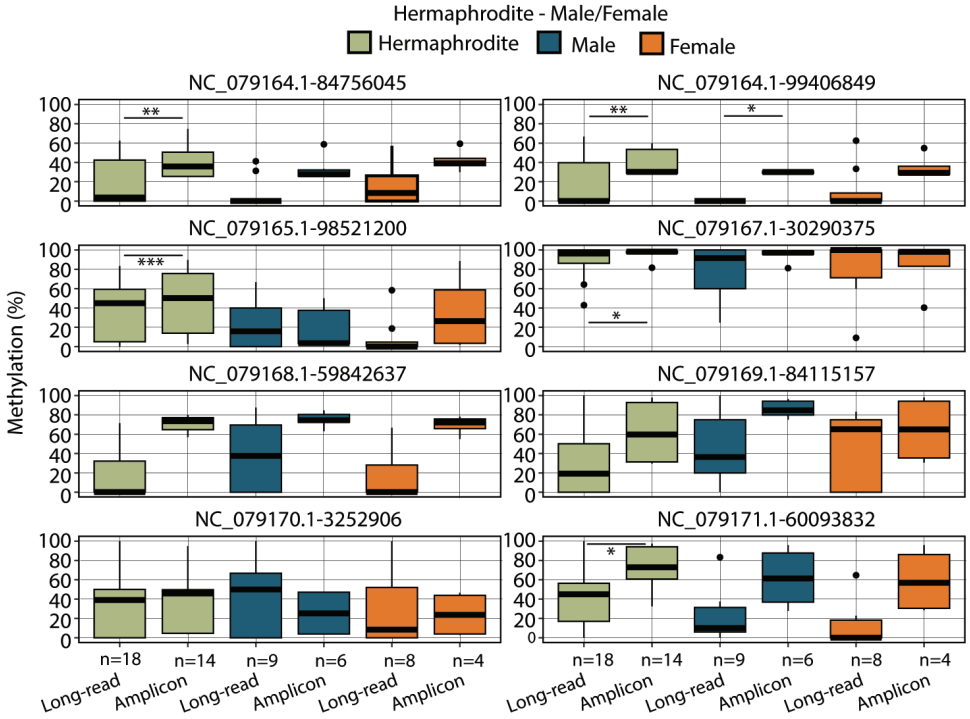


Figure 3 | DNA methylation percentages obtained by long-read nanopore sequencing (Long-read) and bisulfite-amplicon sequencing (Amplicon) across eight epigenetic markers used for H-MF sex classification. Methylation percentages are displayed per sex phenotype (H, M, and F), with sample sizes indicated per boxplot. Asterisks indicate significant differences in average methylation percentages between long-read and amplicon assays within each sex phenotype (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; non-parametric Wilcoxon rank-sum test).

Bland-Altman analysis indicated that the amplicon-based assay generally yielded higher DNA methylation estimates compared to the long-read sequencing approach (Supplementary Table S3; Supplementary Figure S6). The magnitude and statistical significance of this bias varied across features and sex phenotypes, with no consistent pattern of bias observed across sex phenotypes, suggesting that the observed discrepancies are primarily assay-dependent rather than driven by biological differences between sexes.

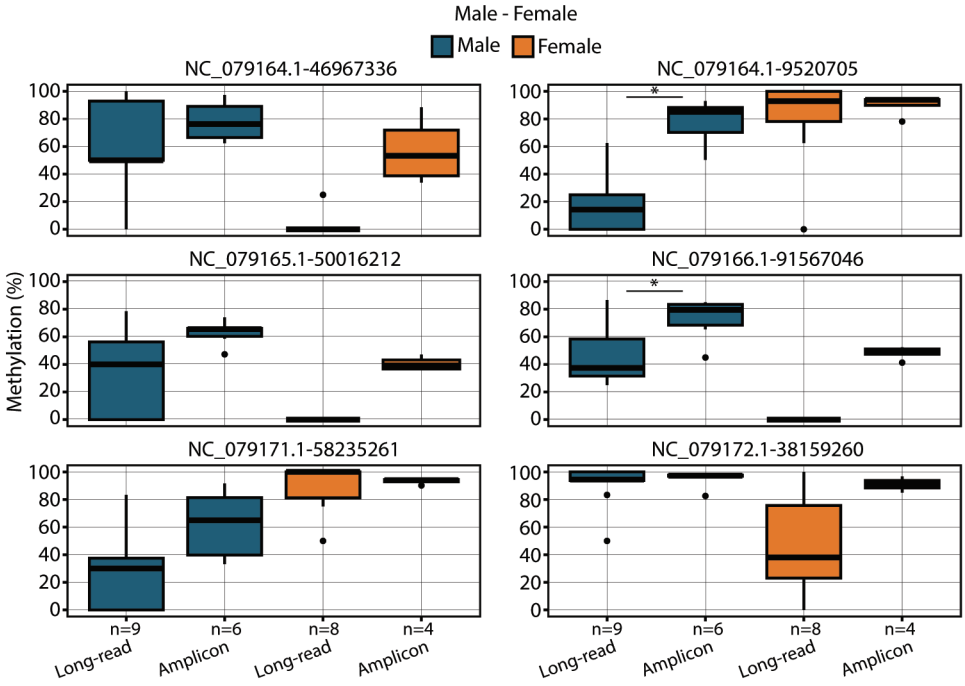


Figure 4 | DNA methylation percentages obtained by long-read nanopore sequencing (Long-read) and bisulfite-amplicon sequencing (Amplicon) across six epigenetic markers used for H-MF sex classification. Methylation percentages are displayed per sex phenotype (M and F), with sample sizes indicated per boxplot. Asterisks indicate significant differences in average methylation percentages between long-read and amplicon assays within each sex phenotype (*p < 0.05; non-parametric Wilcoxon rank-sum test).

Across the six markers used for M-F classification, significant differences in methylation percentages between the DNA methylation percentages obtained from long-read sequencing and amplicon sequencing were observed in males for markers NC079164.1-9520705 and NC079166.1_91567046 (Fig. 4). Positive correlations between methylation percentages obtained by both methods were observed in 5 markers (median Spearman correlation = 0.32; Supplementary Fig. S7). In contrast, the marker NC079166.1_91567046 displayed a strong negative correlation between DNA methylation percentages obtained from the amplicon assay and long-read sequencing (Spearman correlation = -0.71). Among all markers, only NC079171.1_58235261 was statistically significant for both methods in males. Bland-Altman analysis revealed that the amplicon assay generally yielded higher methylation values compared to the long-read sequencing approach (Supplementary Table S3; Supplementary Figure S8). Notably, the magnitude and direction of bias varied between markers and sex phenotypes, with stronger negative bias observed in males.

Sex prediction based on bisulfite-amplicon sequencing data

A new H-MF classification model was retrained with the same long-read data as was used for the original model, but using only the eight epigenetic markers that successfully amplified in the amplicon sequencing assay. Subsequently, application of this retrained model on selected features to predict sex classification from the amplicon data resulted in a prediction accuracy of 76.2% (95% CI: 58.3-91.8). Sensitivity for classifying hermaphrodites (H) was 83.3%, while specificity for classifying males/ females (MF) was 66.7%. Balanced accuracy was 75%, and Cohen's kappa indicated moderate agreement (k=0.51). Of the 21 individuals, 10 were correctly classified as H (true positives) and six as MF (true negatives) (Fig. 5A). Three MF individuals were incorrectly classified as H (false positives), and two H individuals were incorrectly classified as MF (false negatives).

To distinguish males from females, the retrained M-F classification model was applied to amplicon sequence data and resulted in a prediction accuracy of 60% (95%CI: 26.2-87.8). Sensitivity for classifying males (M) was 100%, while specificity classifying females (F) was 0%, meaning that the model misclassified all individuals as male and none as female. Balanced accuracy was 50%, and Cohen's Kappa indicated no agreement (k=0).

Integrated model for classification of male- and female-biased individuals

Further exploring male prediction probabilities from the M-F model used to predict sex from amplicon sequence data revealed that male-biased individuals (including M, HPM, and HBS) exhibited higher prediction probabilities than female-biased individuals (Supplementary Fig. S9). This observation suggested that the features selected to distinguish males from females might better differentiate male-biased and female-biased individuals, rather than males from females. Therefore, the model was retrained once more using the same selected features, but with classification of male-biased versus female-biased individuals.

Training an additional model using all successfully amplified target CpG features with 10-fold cross-validation resulted in a model prediction accuracy 71.0% (95%: 58.8-81.3). Sensitivity for classifying male-biased (Mb) individuals was 69.4%, whereas specificity for classifying female-biased (Fb) was 72.7%. Balanced accuracy was 71.1%, and Cohen's Kappa indicated moderate agreement (k=0.42). Mcnemar's test for marginal homogeneity across probabilities was not significant (p-value>0.05).

Applying this model to classify individuals into being male or female biased resulted in the accurate classification of 11 male-biased individuals and five individuals being correctly predicted as female-biased (Fig. 5B). Among the nine true female-biased

individuals, four were incorrectly classified as male-biased, suggesting that the model performed better at predicting male bias than female bias.

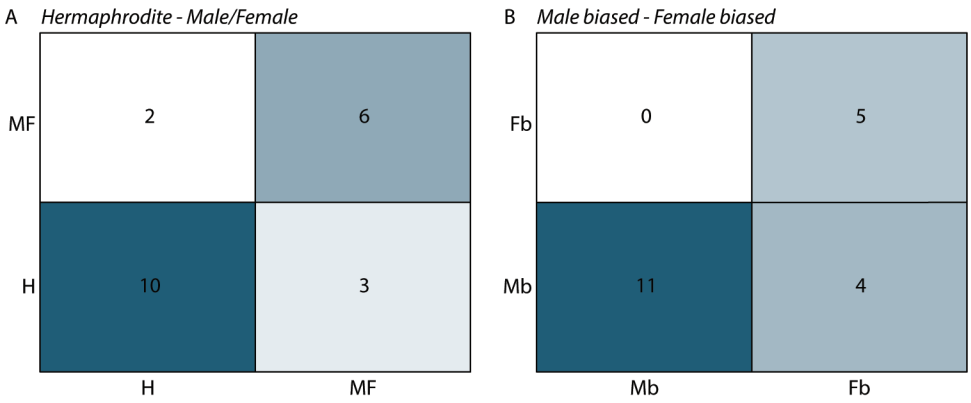


Figure 5 | Confusion matrices for predicting sex phenotypes using two classification models trained on DNA methylation from long-read sequencing data at selected epigenetic markers and applied to amplicon assay-derived methylation percentages. **(A)** True and false predictions for the classification of hermaphrodite (H) versus non-hermaphrodite (male-female, MF) oysters based on a LASSO model trained eight epigenetic markers for H-MF classification. **(B)** True and false predictions for the classification of male-biased (Mb) and female-biased (Fb) individuals using a LASSO model trained on 14 epigenetic markers from combined H-MF and M-F classifications.

Discussion

Understanding sex reversal in a sequential hermaphrodite is extremely relevant in an aquacultural context, as skewed sex ratios can reduce reproductive outcome and rapidly deplete genetic diversity, thereby increasing the risk of inbreeding (Diaz-Almela et al., 2004; Hedgecock et al., 2007; Hedgecock & Coykendall, 2007). As current approaches of sex identification are challenging and often lethal, there is an urgent need for non-lethal alternatives that enable sex identification of living oysters. Given the extensive sex-specific DNA methylation observed across the *O. edulis* epigenome (Valk et al., 2025), the present study aimed to identify epigenetic markers that can distinguish different phenotypic sex stages from analyzing gill tissue. Adopting a machine learning approach by training penalized-regression models on sex-specific DNA methylation across sex phenotypes, a total set of 14 markers were proposed. Of these markers, eight enabled the classification of hermaphrodite versus non-hermaphrodite oysters (model prediction accuracy: 76.2%). While six additional markers showed poor prediction accuracy in classifying males and females, integration of all 14 markers enabled distinguishing male-biased from female-biased individuals (model prediction accuracy: 71.0%), providing valuable opportunities forimplementation in aquaculture production of *O. edulis*.

Opportunities in high-throughput methods for targeted methylation quantification

Various high-throughput methods have been proposed for quantifying DNA methylation at target sites for the applications in epigenetic clocks or in assessing the association with other phenotypic traits (e.g. MeDIP-qPCR, MBS, or MSAP) (Li et al., 2017; Piferrer & Anastasiadi, 2023). The approach used in this study combines bisulfite treatment with a multiplex PCR assay followed by nanopore amplicon sequencing, enabling the simultaneous quantification of multiple epigenetic markers across many individuals. This targeted approach provides a cost-efficient alternative to whole genome methylation sequencing and generally requires lower DNA input quantities and quality. Comparison of DNA methylation estimates derived from the original whole-genome sequencing data and data from the amplicon-based sequencing assay revealed no significant differences in overall methylation levels, and positive Spearman correlations suggested high similarity between DNA methylation percentages obtained by both methods. Nevertheless, Bland-Altman comparisons between both methods revealed systematically higher methylation values obtained from the amplicon assay in almost all epigenetic markers. Such discrepancies may arise from technical differences in bisulfite conversion efficiency and DNA degradation, PCR amplification bias, or sequencing depth (Wreczycka et al., 2017). Despite the high differences in DNA methylation values obtained by both methods, the machine

learning models were capable of correctly classifying sex phenotypes with moderate accuracy (~70%), reinforcing the idea that sex identification from epigenetic markers is feasible in oysters. As such, it is likely that the machine learning models developed in this study rely more strongly on consistent relative differences in methylation values between sex phenotypes rather than on exact absolute methylation percentages. Following thorough and accurate validation of the 14 provided epigenetic markers and potential supplementation with additional markers, the development of a methylation chip could offer high-throughput and more consistent methylation quantification while minimizing the cost per sample.

Machine learning performance and the need for further validation

The application of two cross-validation methods in the machine learning workflow, resulted in identifying the same epigenetic markers to be most informative in sex classification, indicating the robustness of the proposed markers. However, training machine learning models on relatively low sample sizes increases the risk of overfitting (Piferrer & Anastasiadi, 2023). In the present study, this risk may have been further amplified by the relatively high number of DMLs used as input for training compared to the number of individuals representing each sex. Although the adopted penalized linear regression model partly mitigates the risk of overfitting by constraining model complexity, further validation using greater sample sizes will be important to assess the robustness and predictive performance of the proposed approach.

One limitation of the approach adopted in this study, is that the predictions from the trained machine learning models are constrained by the phenotypes included in the training dataset. In the present work, the models were trained using oysters with clearly defined reproductive phenotypes (males, females, and hermaphrodites). However, individual lacking such clear sexual characteristics, such as oysters in a "resting" or post-spawning state, were not included. Consequently, it remains uncertain how the current models based on the 14 provided epigenetic markers would classify such individuals, for example whether oysters with resting gonads would be predicted as hermaphrodites or non-hermaphrodites. Previous work in *M. gigas* has shown differential DNA methylation in gill tissue between pre- and post-spawning individuals (Wang et al., 2024). Expanding the marker panel to include such DMLs that capture the association with resting or post-spawning states will further expand the utility of the assay.

Once further validation across a broader range of reproductive stages has been conducted, such markers may have practical applications for broodstock management, as they can help identify the onset of reproductive activity or enable the exclusion of individuals that are not actively reproducing. Furthermore,

as both model training and testing of machine learning models were performed using oysters from a single population (Lake Grevelingen, the Netherlands), further research is needed to determine whether sex-associated DNA methylation patterns are conserved across populations exposed to different environments. For example, genome-wide DNA methylation revealed substantial epigenetic population structure among four populations of the Eastern oyster (*Crassostrea virginica*) (Johnson & Kelly, 2020). Therefore, validation using oysters from geographically distinct populations is required to assess the broader applicability of the provided epigenetic markers in sex identification.

Another key priority is to evaluate whether similar sex-associated methylation signatures are present in other somatic tissues. It has been suggested that sex-specific methylation patterns in somatic tissues reflect systemic epigenetic differences linked to gonadal sex determination (Sun et al., 2014; Teng et al., 2025; Valk et al., 2025). From this perspective, the presence of similar sex-associated patterns in other somatic tissues, such as mantle or intestine, could potentially enable non-invasive sex identification from environmental DNA (eDNA), for example originating from feces or gill mucous (Zhao et al., 2023). However, such applications remain speculative and require further study, particularly regarding the stability and preservation of epigenetic modifications of eDNA under environmental conditions.

Applicability for oyster aquaculture and restoration

The opportunity to classify oysters as hermaphrodites versus non-hermaphrodites provides relevant opportunities for oyster restoration and aquaculture, as it informs about the reproductive state of individuals. Hermaphrodite oysters simultaneously produce male and female gametes, with gametes of one sex typically more matured than those of the opposite sex (Da Silva et al., 2009). Consequently, hermaphroditism is frequently interpreted as a transitional reproductive phase, reflecting individuals that have not fully completed gametogenesis for one of both sexes, remaining in a continuous process of gonadal development (Harding et al., 2013). In the Pacific oyster (*Magallana gigas*), the highest proportion of hermaphrodites occurs at the beginning of the reproductive period, declining toward the end of the season as most individuals complete maturation (Lango-Reynoso et al., 2006). As such, a higher proportion of hermaphrodite oysters could reflect a population that is not yet ready to spawn.

Gametogenesis is known to be an energetically demanding process that can substantially influence metabolism and immune function (Li et al., 2010). Notably, the period following spawning is considered the most critical for oyster survival due to depleted energy resources and reduced immunity (Li et al., 2010). However, the implications of this vulnerable period for hermaphrodite oysters that continue the gametogenesis for the opposite sex after spawning remain unclear. In this context, the epigenetic markers identified in this study enable detecting hermaphroditic individuals and support the further study of physiological requirements in hermaphrodite oysters. Such knowledge is highly relevant for aquaculture, as adjusting husbandry conditions to the specific needs of different sex phenotypes may increase reproductive output. Moreover, given that gametes from true males or male-biased hermaphrodites are more competitive in survival than male gametes produced by female-biased hermaphrodites (Hassan et al., 2016), the classification of male- or female-biased individuals could enable strategic sorting and mixing of individuals based on their predicted sex, potentially enhancing overall reproductive output in an aquaculture context.

Conclusion

Taken together, this study provides 14 epigenetic markers capturing sex-specific DNA methylation in gill tissue, allowing for the classification of hermaphrodite versus non-hermaphrodite oysters and distinguishing between male-biased and female-biased individuals. While further validation across a larger sample size and geographically distinct populations is required to confirm the assay's robustness, the present study establishes the first steps toward developing a valuable tool for studying sex reversal in live oysters, thereby providing important opportunities for aquaculture and conservation of *O. edulis*.

Data availability statement

All data is available on request

Declaration of competing interest

All authors declare they have no competing interests.

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Supplementary information

Supplementary Table S1 | Overview of all 35 sequenced flat oyster individuals with information on shell length, date and location of oyster collection. Latitude and longitude are in decimal degrees (dd).

Oyster	Barcode	Sex	Stage	Latitude	Longitude	Date	Shell length (mm)
1	1	M	2	51.735	3.970	17-4-2023	81.5
2	2	F	2	51.735	3.970	17-4-2023	70.9
3	3	F	2	51.735	3.970	17-4-2023	88.8
4	4	HPF	3	51.735	3.970	17-4-2023	65.8
5	5	HPM	2	51.735	3.970	17-4-2023	68.1
6	6	M	2	51.735	3.970	17-4-2023	74.0
7	7	HPF	3	51.735	3.970	17-4-2023	83.0
8	8	M	2	51.735	3.970	17-4-2023	71.1
9	9	HPF	2	51.735	3.970	17-4-2023	75.3
10	10	M	3	51.735	3.970	17-4-2023	73.5
11	11	HPM	3	51.769	3.952	26-9-2023	76.8
12	12	F	2	51.769	3.952	26-9-2023	82.7
13	13	F	3	51.769	3.952	26-9-2023	76.3
14	14	F	2	51.769	3.952	26-9-2023	82.7
15	15	M	3	51.769	3.952	26-9-2023	74.5
16	16	HPF	3	51.791	3.908	15-4-2024	70.5
17	17	F	2	51.791	3.908	15-4-2024	79.9
18	18	M	2	51.791	3.908	15-4-2024	80.8
19	19	M	2	51.791	3.908	15-4-2024	73.3
20	20	M	1	51.791	3.908	15-4-2024	76.4
21	21	HPF	3	51.791	3.908	15-4-2024	68.1
22	22	HBS	2	51.791	3.908	15-4-2024	66.4
23	23	F	2	51.791	3.908	15-4-2024	83.1
24	24	HPF	2	51.791	3.908	15-4-2024	80.2
25	1	HBS	4	51.769	3.952	26-9-2023	71.3
26	2	M	4	51.769	3.952	26-9-2023	60.7
27	3	F	2	51.791	3.908	15-4-2024	61.0
28	4	HPM	1	51.791	3.908	15-4-2024	67.0
29	5	HPM	1	51.791	3.908	15-4-2024	62.1
30	6	HBS	1	51.791	3.908	15-4-2024	75.2
31	7	HPF	2	51.791	3.908	15-4-2024	61.1
32	8	HPF	1	51.791	3.908	15-4-2024	78.2
33	9	HPF	2	51.791	3.908	15-4-2024	84.1
34	10	HBS	2	51.735	3.970	17-4-2023	68.8
35	11	HPM	2	51.735	3.970	17-4-2023	79.0

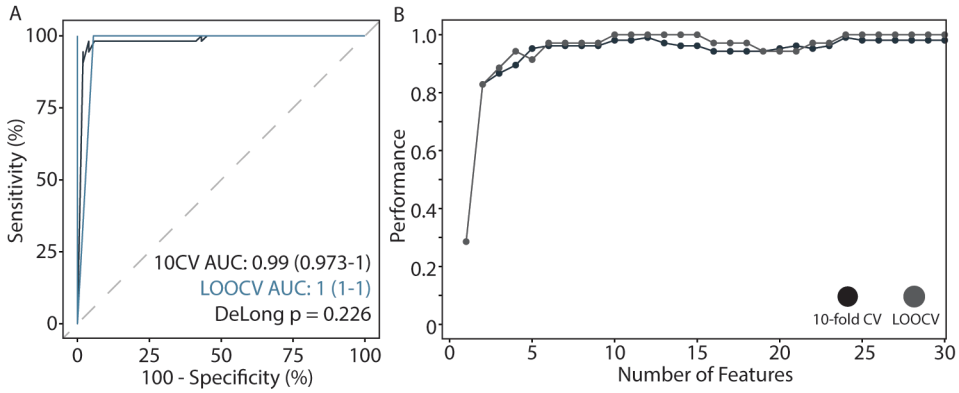
Supplementary Table S2 | Bland-Altman statistics comparing two methods of DNA methylation assessment (long-read sequencing vs amplicon assay) across eight markers for H-MF classification. Reported metrics include the mean and standard deviation of the difference (ΔDNAm) in methylation percentage between methods, limits of agreement (LOA), non-parametric Kruskal-Wallis p-values for bias testing, proportional bias (slope of linear regression), and associated p-values.

Epigenetic marker	Sex	Mean ΔDNAm	SD ΔDNAm	LOA upper	LOA lower	p-value	slope	Slope p-value
NC_079164.1-84756045	F	-16.47	21.49	25.66	-58.60	0.223	0.904	0.153
NC_079164.1-84756045	H	-22.82	13.05	2.77	-48.40	0.000	0.386	0.036
NC_079164.1-84756045	M	-26.11	5.14	-16.02	-36.19	0.000	0.261	0.085
NC_079164.1-99406849	F	-19.17	18.00	16.10	-54.45	0.123	0.801	0.007
NC_079164.1-99406849	H	-24.91	12.46	-0.48	-49.34	0.000	0.498	0.010
NC_079164.1-99406849	M	-29.64	1.78	-26.15	-33.13	0.000	-2.000	0.000
NC_079165.1-98521200	F	-16.45	15.71	14.34	-47.25	0.127	-0.405	0.119
NC_079165.1-98521200	H	-5.91	15.81	25.08	-36.90	0.185	0.003	0.984
NC_079165.1-98521200	M	-3.06	8.99	14.55	-20.68	0.442	-0.244	0.235
NC_079167.1-30290375	F	-6.73	16.37	25.35	-38.80	0.471	0.438	0.001
NC_079167.1-30290375	H	-13.20	18.28	22.62	-49.02	0.029	1.581	0.000
NC_079167.1-30290375	M	-11.12	25.59	39.04	-61.27	0.386	1.266	0.001
NC_079168.1-59842637	F	-53.74	15.38	-23.60	-83.88	0.006	0.728	0.384
NC_079168.1-59842637	H	-54.11	21.59	-11.79	-96.42	0.000	1.335	0.001
NC_079168.1-59842637	M	-35.66	38.70	40.19	-111.52	0.074	1.520	0.002
NC_079169.1-84115157	F	-29.22	6.03	-17.40	-41.04	0.002	0.138	0.121
NC_079169.1-84115157	H	-28.70	15.91	2.48	-59.89	0.000	0.187	0.157
NC_079169.1-84115157	M	-18.09	26.08	33.03	-69.20	0.150	1.293	0.027
NC_079170.1-32052906	F	2.92	8.92	20.40	-14.57	0.560	0.275	0.149
NC_079170.1-32052906	H	-4.76	12.58	19.89	-29.41	0.181	0.092	0.478

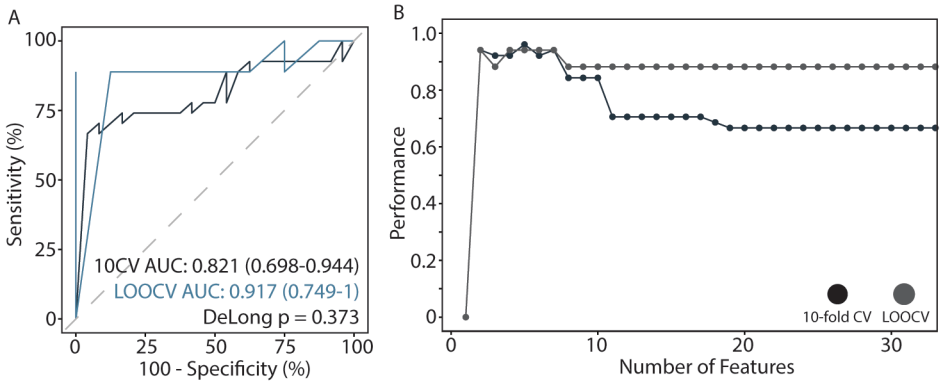
Epigenetic marker	Sex	Mean ΔDNAm	SD ΔDNAm	LOA upper	LOA lower	p-value	slope	Slope p-value
NC_079170.1-32052906	M	3.67	9.53	22.35	-15.00	0.388	0.307	0.014
NC_079171.1-60093832	F	-39.25	17.97	-4.02	-74.48	0.022	-0.138	0.758
NC_079171.1-60093832	H	-29.93	25.81	20.66	-80.52	0.001	0.541	0.069
NC_079171.1-60093832	M	-34.37	15.78	-3.45	-65.29	0.003	0.056	0.844

Supplementary Table S3 | Bland-Altman statistics comparing two methods of DNA methylation assessment (long-read sequencing vs amplicon assay) across eight markers for M-F classification. Reported metrics include the mean and standard deviation of the difference (ΔDNAm) in methylation percentage between methods, limits of agreement (LOA), t-test p-values for bias testing, proportional bias (slope of linear regression), and associated p-values.

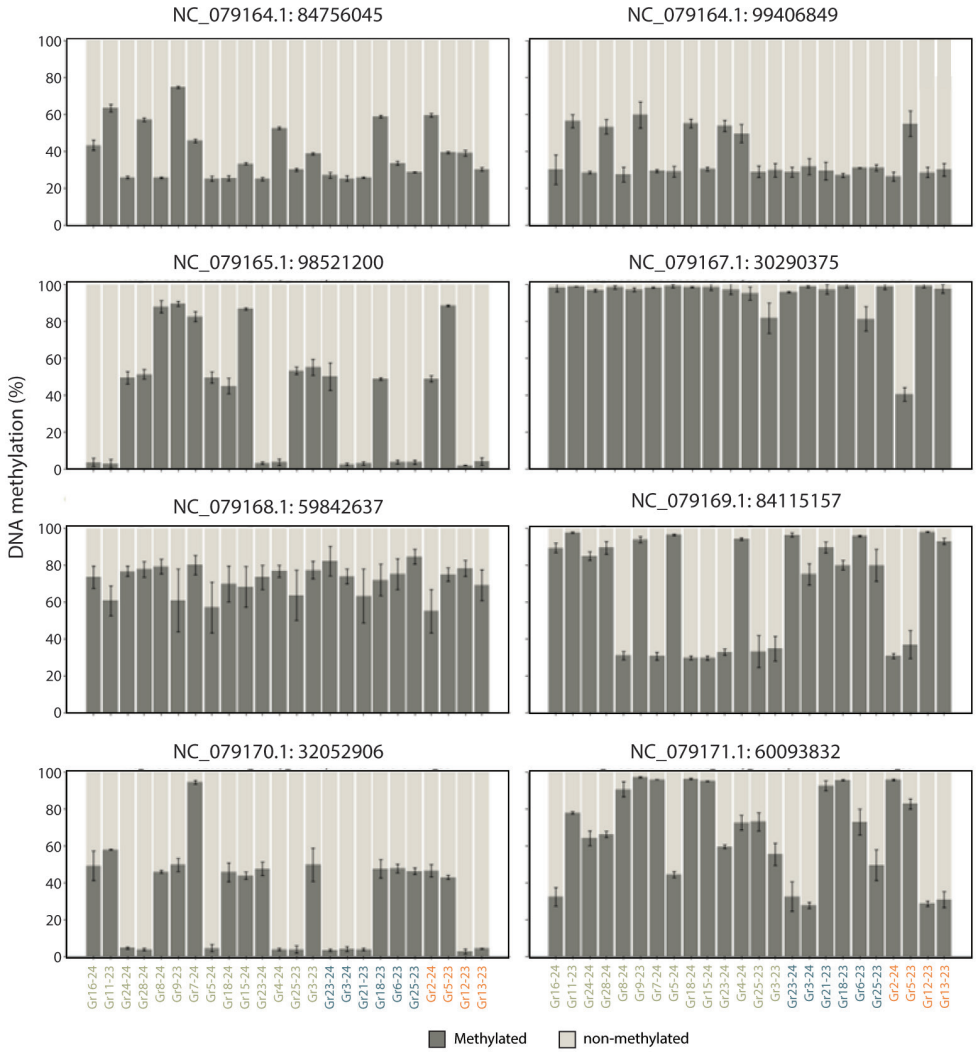
Epigenetic marker	Sex	Mean ΔDNAm	SD ΔDNAm	LOA upper	LOA lower	p-value	slope	slope p-value
NC_079164.1-46967336	F	-50.99	25.17	-1.66	-100.31	0.027	-1.009	0.387
NC_079164.1-46967336	M	-21.05	14.72	7.80	-49.89	0.017	0.273	0.603
NC_079164.1-9520705	F	-3.67	11.53	18.92	-26.26	0.569	0.803	0.127
NC_079164.1-9520705	M	-66.76	16.45	-34.52	-99.00	0.000	0.161	0.772
NC_079165.1-50016212	F	-40.68	4.59	-31.67	-49.68	0.000	-2.000	0.000
NC_079165.1-50016212	M	-15.28	22.16	28.16	-58.72	0.152	1.151	0.033
NC_079166.1-91567046	F	-48.46	4.93	-38.81	-58.12	0.000	-2.000	0.000
NC_079166.1-91567046	M	-40.20	19.63	-1.73	-78.67	0.004	-2.762	0.024
NC_079171.1-58235261	F	6.58	2.26	11.00	2.16	0.010	-2.000	0.000
NC_079171.1-58235261	M	-37.02	15.93	-5.79	-68.24	0.002	0.280	0.315
NC_079172.1-38159260	F	-45.00	46.52	46.19	-136.18	0.149	1.763	0.014
NC_079172.1-38159260	M	-9.13	12.87	16.10	-34.36	0.143	1.029	0.002



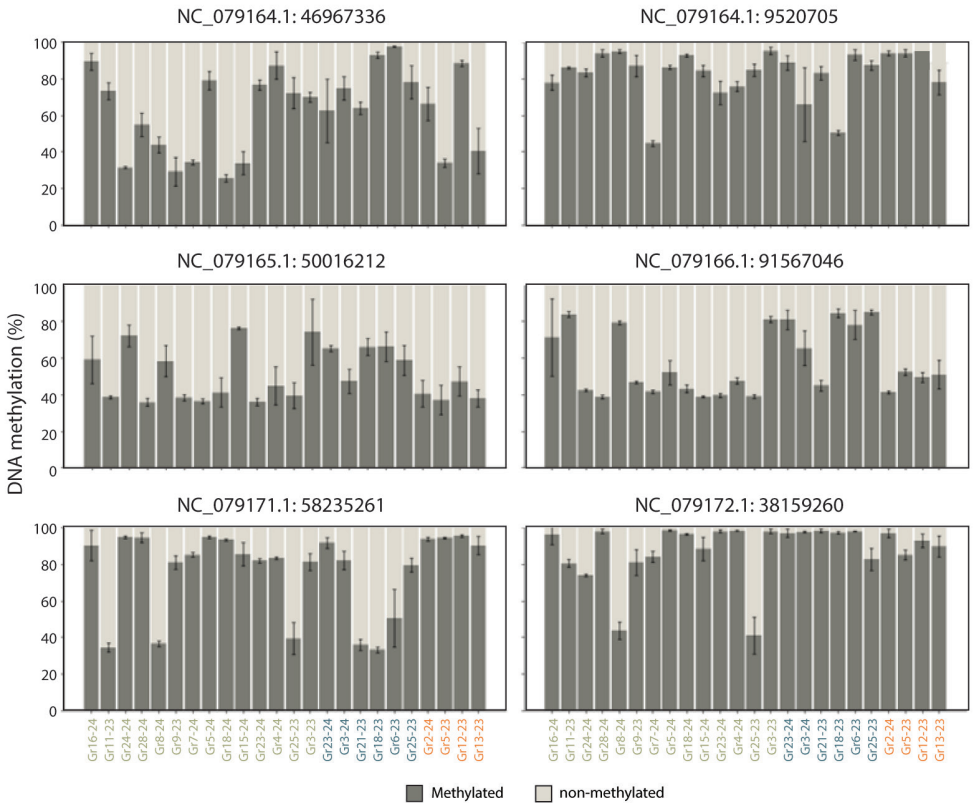
Supplementary Figure S1 | Evaluation of LASSO model performance for classifying H-MF. **(A)** Area under the receiver operating characteristic (AUROC) for both cross-validation approaches: 10CV and LOOCV. **(B)** Classification accuracy for H-MF classification plotted as a function of the cumulative number of the 30 top ranked, most stable DMLs for sex classification.



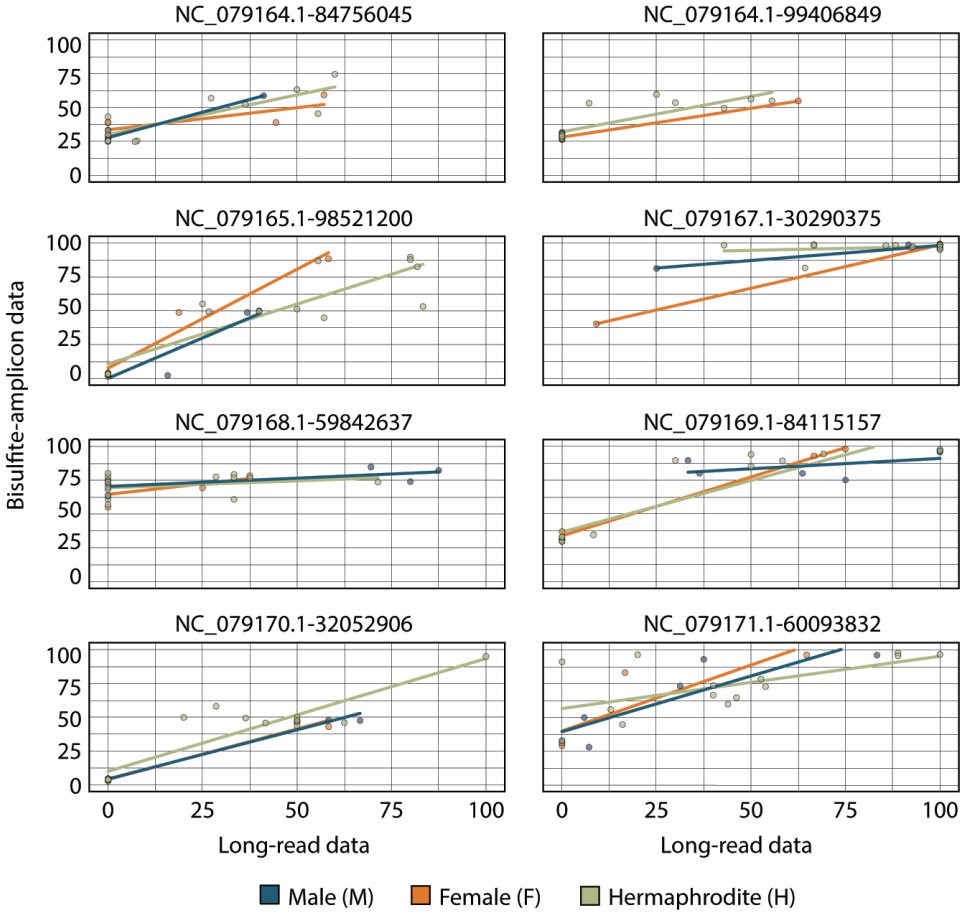
Supplementary Figure S2 | Evaluation of LASSO model performance for classifying M-F. **(A)** AUROC for both cross-validation approaches: 10CV and LOOCV. **(B)** Classification accuracy for H-MF classification plotted as a function of the cumulative number of the 30 top ranked, most stable DMLs for sex classification.



Supplementary Figure S3 | Stacked bar graphs visualizing the percentage DNA methylation for eight target DMLs for H-MF classification across 24 samples. Samples are sorted and colored on their phenotypic sex category, with H, M, and F arranged from left to right.

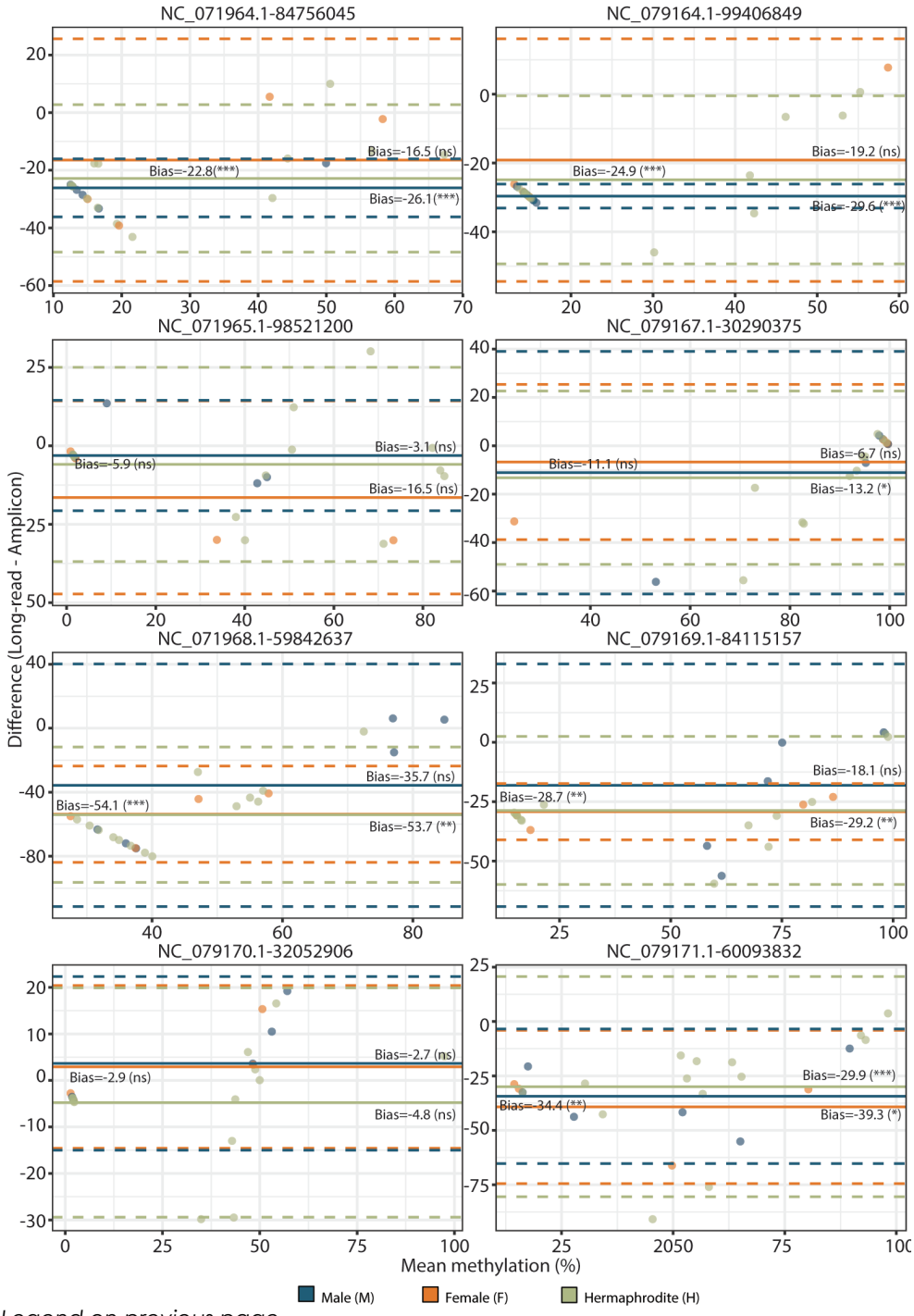


Supplementary Figure S4 | Stacked bar graphs visualizing the percentage DNA methylation for six DMLs for M-F classification across 24 samples. Samples are sorted and colored on their phenotypic sex category, with H, M, and F arranged from left to right.

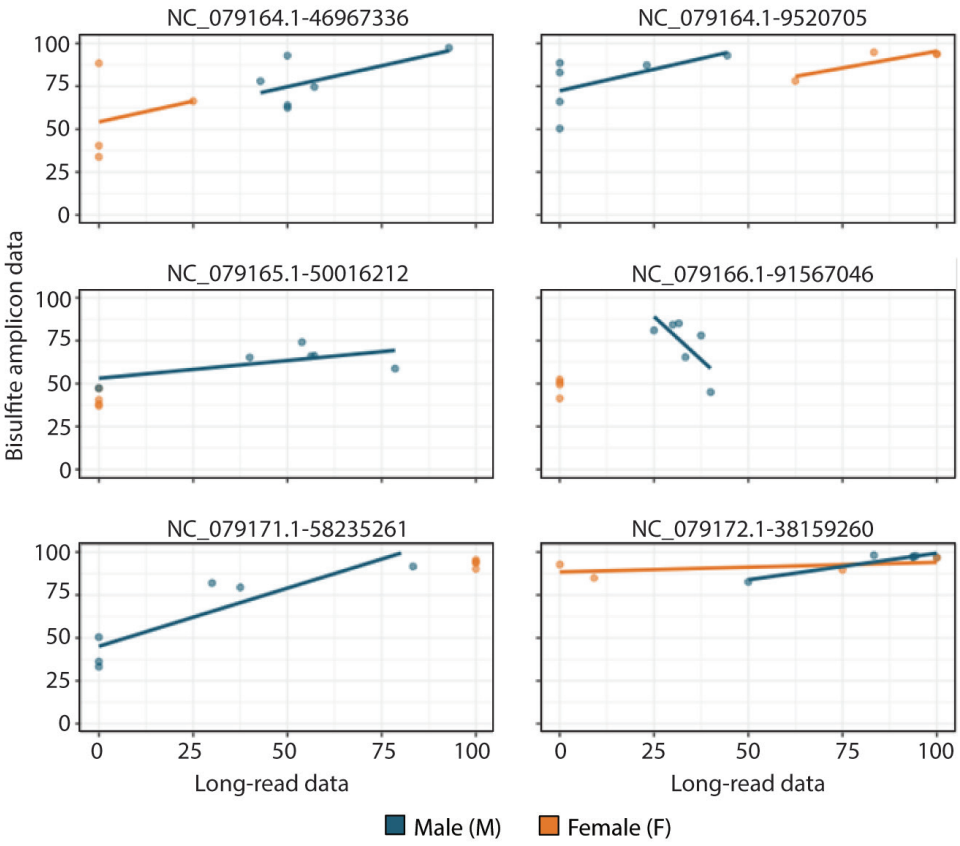


Supplementary Figure S5 | Spearman correlations between methylation percentages obtained from long-read sequencing (x-axis) and the amplicon assay (y-axis) across eight epigenetic markers for H-MF sex classification. Methylation percentages are compared between both methods for three different sex phenotypes separately (H, M, and F).

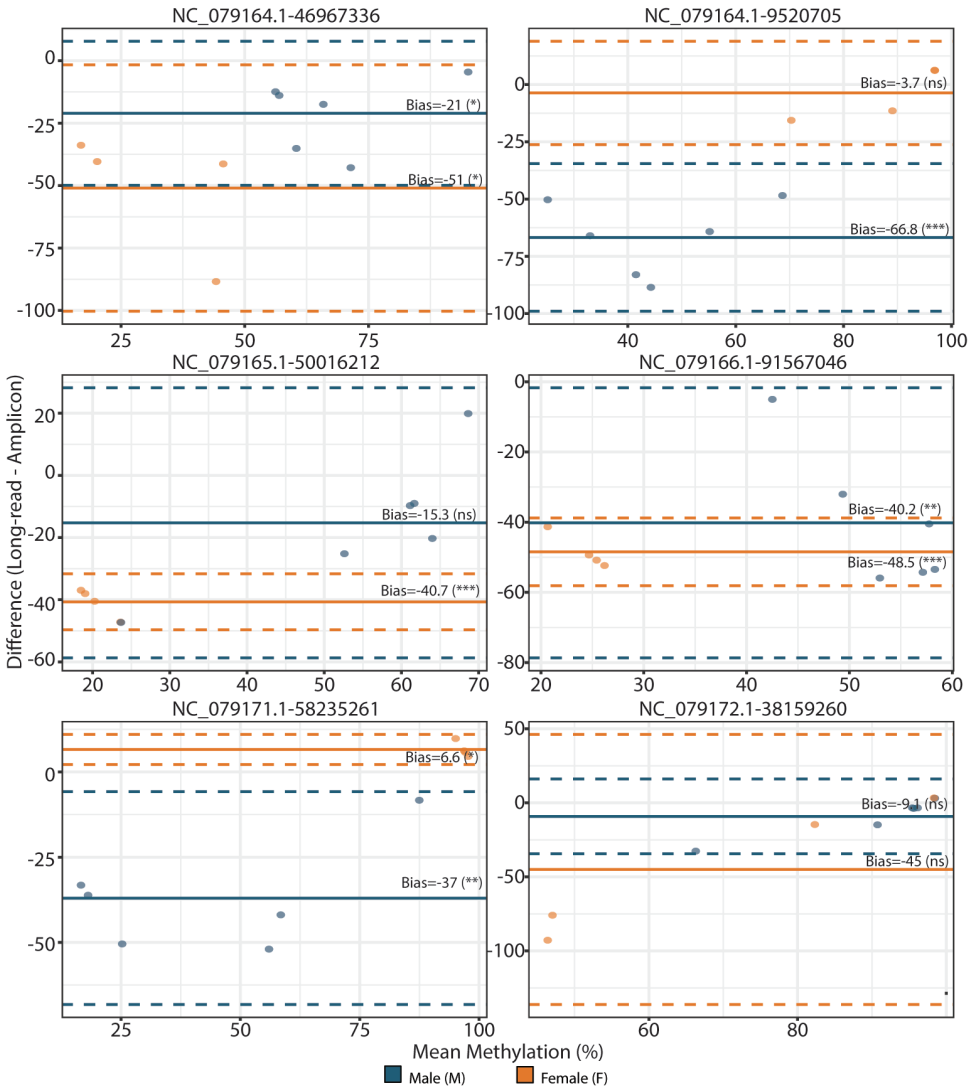
Supplementary Figure S6 | Bland-Altman analysis of DNA methylation measured by long-read sequencing versus amplicon assay across eight CpG features for H-MF classification. Each facet represents one epigenetic marker. Points display the difference between long-read sequencing and amplicon methylation plotted against the mean for each sample, colored by sex phenotype (H, M, or F). Solid horizontal lines indicate the mean difference (ΔD; bias) for each sex phenotype, and dashed lines represent the 95% limits of agreement (LOA).



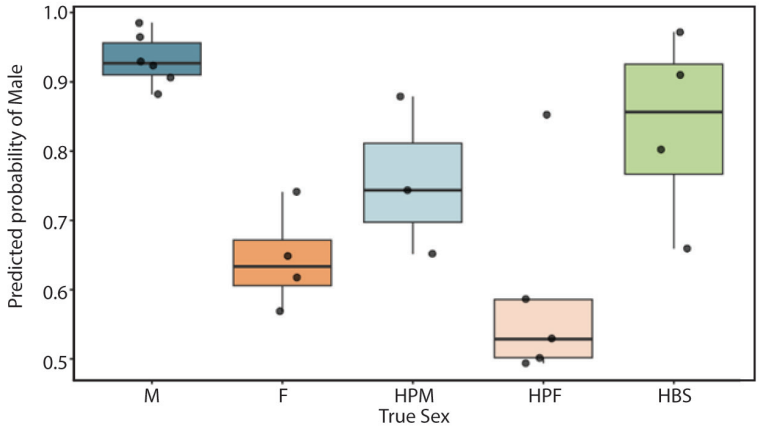
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Supplementary Figure S7 | Spearman correlations between methylation percentages obtained from long-read sequencing (x-axis) and the amplicon assay (y-axis) across six epigenetic markers for M-F sex classification. Methylation percentages are compared between both methods for two sex phenotypes separately (M and F).



Supplementary Figure S8 | Bland-Altman analysis of DNA methylation measured by long-read sequencing versus amplicon assay across six epigenetic markers for M-F classification. Each facet represents one epigenetic marker. Points display the difference between long-read sequencing and amplicon methylation plotted against the mean for each sample, colored by sex phenotype (M, or F). Solid horizontal lines indicate the mean difference (ΔD; bias) for each sex phenotype, and dashed lines represent the 95% limits of agreement (LOA).



Supplementary Figure S9 | Boxplot illustrating the predicted probability of a sample being male from an M-F classification model trained on DNA methylation obtained from long-read sequencing at six epigenetic markers and applied to amplicon assay-derived methylation data across five sex phenotypes (M, F, HPM, HPF, and HBS).

CHAPTER 6

Integrating molecular approaches for standardized monitoring of North Sea biodiversity and ecosystem dynamics

Sophie Valk*, Karlijn Doorenspleet*, Daniël van Berkel, Xantia van den Brink, Lara Jansen, Saskia Oosterbroek, Reindert Nijland, & Auriel Sumner-Hempel

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Abstract

The North Sea is among the largest and most productive shallow marine ecosystems, heavily influenced by historical and ongoing exploitation. Monitoring of North Sea biodiversity is vital for a better understanding of ecosystem responses to ongoing anthropogenic pressures and for informing effective management and conservation strategies. Molecular techniques provide a cost and time-efficient alternative to traditional biodiversity assessment methods and are increasingly employed in marine monitoring. In this narrative review we explore the current molecular-based monitoring approaches used to assess marine biodiversity in the North Sea, including species-specific assays, eDNA metabarcoding, and metagenomics, by highlighting their opportunities and areas for improvement. Furthermore, we address the potential for integrating novel DNA and RNA-based methods, such as epigenetics and metatranscriptomics, into standardized monitoring for marine conservation and restoration. We propose that further integration of a broader omics-based approach may enhance the overall understanding of ecosystem structure, function, and health. We then move beyond molecular tools and examine the challenges and opportunities associated with nucleotide reference databases, data curation, and data archiving. We further address how to improve the harmonization and standardization of marine biodiversity data. Finally, we provide three suggestions for integrating molecular methods in a standardized manner to enhance the assessment of North Sea ecosystem biodiversity, dynamics, and functioning.

Introduction

Biodiversity and conservation status of the North Sea

The North Sea is one of the world's largest and most productive shallow marine ecosystems, subject to historical and ongoing exploitation by multiple industries (e.g. fisheries, oil and gas drilling, renewable energy production, shipping, and tourism) (Emeis et al., 2015). Historically, this marine basin was covered with extensive hard substrates, such as shellfish reefs and rocks, which supported high levels of benthic and pelagic biodiversity (Olsen, 1883). However, many of these habitats have been degraded due to centuries of intensive bottom-trawling fisheries, targeting a wide variety of demersal fish, crustaceans, and bivalves (Dickey-Collas et al., 2010; Engelhard et al., 2014; ter Hofstede & Rijnsdorp, 2011). Bottom-trawling can have long-lasting adverse effects on ecosystem functioning by reducing habitat complexity, altering the benthic community composition, and diminishing the overall productivity of benthic ecosystems (Hiddink et al., 2011; Jennings et al., 2001; Kaiser et al., 2006).

Anthropogenic activities can also give rise to opportunities for the recovery of nature and biodiversity, as is the case with offshore wind farms (OWFs). OWFs create local no-take zones where fishing is prohibited. Additionally, the scour protection surrounding the turbine base provides a hard settlement substrate that facilitates growth of reef-building organisms, and is demonstrated to increase local biodiversity and recruitment of commercial fish species (Reubens et al., 2013a, 2013b, 2014; Scheidat et al., 2011). However, several studies also address the potential of OWFs, and associated epifaunal communities, serving as stepping stones for non-indigenous species (NIS) (De Mesel et al., 2015). The facilitated expansion of NIS may be further intensified by climate-related pressures, threatening North Sea ecosystem functioning (Beaugrand & Kirby, 2010; Dickey-Collas et al., 2010; Huserbråten et al., 2018). Although North Sea ecosystems have thus far demonstrated an ability to adapt to various stressors, the extent to which ongoing and intensifying pressures will alter ecosystem dynamics remains unclear (Brooks et al., 2017; Coates et al., 2015; Dickey-Collas et al., 2010; Wiltshire et al., 2008). Hence, effective biodiversity monitoring is crucial to improve our understanding of how various stressors and opportunities alter ecosystem processes. Consequently, monitoring of marine biodiversity will provide the scientific foundation for conservation and restoration strategies in a changing North Sea.

Conservation and restoration are increasingly being prioritized in countries bordering the North Sea, but nation-specific regulatory frameworks for marine management can be inconsistent or even conflicting between countries (Grip & Blomqvist, 2020; Kusters et al., 2025). An example is the Doggersbank, which is a sandbank in the North Sea that borders five countries with distinct regulatory frameworks in place for conservation of the shallow area (Mackelworth et al., 2019). At the European level,

several policy guidelines and frameworks are employed to guide the development and implementation of biodiversity monitoring systems for such transboundary marine areas. These include the United Nations guidelines for establishing national biodiversity monitoring programs, and the Marine Strategy Framework Directive (MSFD), which aims to protect the marine environment by achieving "good environmental status" (GES) (UNECE, 2023; Marine Environment - Environment - European Commission, 2025). In addition, guidelines have been formulated that focus specifically on habitat protection and restoration. For example, as part of the EU biodiversity strategy for 2030, biogenic reefs and their associated biodiversity are protected under the EU habitats directive (Biodiversity Strategy for 2030 - European Commission, 2025). Here, an interpretation manual was developed that more specifically explains which habitats, ranging from sand beds and reefs to sand dunes and grasslands, and their associated species require protection, restoration, and conservation (Interpretation Manual of EU Union Habitats - European Commission, 2023). This further emphasizes the need for routine biodiversity monitoring of ecosystems in the North Sea region to assess the effectiveness of restoration and conservation efforts, and improve them when necessary.

From morphological to molecular monitoring of North Sea marine biodiversity

Conventional monitoring approaches that are currently applied in biodiversity assessments heavily rely on morphological species identification, which is often invasive and time-consuming (Leray & Knowlton, 2016). Additionally, the lack of expertise on specific taxonomic groups can pose constraints when discriminating between cryptic species or upon identification of non-indigenous species (Danovaro et al., 2016; Duarte et al., 2021). Molecular based techniques, as an alternative to conventional taxonomic identification, have revolutionized biodiversity monitoring by greatly improving cost- and time efficiency, while often providing more accurate and reliable taxonomic classifications (Goodwin et al., 2017; Thompson & Thielen, 2023; Thomsen & Willerslev, 2015).

Molecular monitoring using environmental DNA (eDNA) is emerging as a powerful approach to assess biodiversity and ecosystem dynamics in the North Sea. With relatively little sampling effort, species can be identified from the genetic material that is released into the environment, from various sources (e.g. skin, mucous, scales, feces, gametes), or whole organisms such as bacteria and plankton (Holman et al., 2019; Rodríguez-Ezpeleta et al., 2021; Suarez-Bregua et al., 2022; Thomsen & Willerslev, 2015). Depending on the objective, monitoring using eDNA sampling can involve species-specific assays, to detect the presence of an individual target species, or metabarcoding, to characterize ecosystem community compositions and assess

broader ecosystem dynamics (Guri et al., 2024a; Ruppert et al., 2019). The possibility to use more in-depth analyses to study the composition of environmental nucleic acids (eNA), accompanied by (meta-)omics approaches, are presenting themselves as a valuable asset to the molecular toolbox (Garlapati et al., 2019; Littlefair et al., 2022; Miya, 2022). While these molecular methods hold promise for elaborate analysis of ecosystem dynamics, such as species interactions, functional responses to environmental change, and life-history characteristics, there are several challenges to be addressed prior to implementation in standardized monitoring of North Sea ecosystems (Bowers et al., 2021; Bunholi et al., 2023). Among those challenges are the methodological limitations that arise from sample filtration and preservation methods, which affect detection probability and comparability across replicates and studies. During water filtration, eNA molecule capture efficiency is strongly affected by filter pore size, membrane material, filtration volume, and preservation conservative (Bunholi et al., 2023; Majaneva et al., 2018). To address these limitations, recent studies have examined the use of natural eNA filters, such as sponges, which may offer an alternative as a passive eNA sampler, potentially expanding spatial coverage in species detection (Cai et al., 2022; Mariani et al., 2019; Weber et al., 2023).

Current opportunities for improvement include enhancing consistency and reproducibility between sampling efforts and combining tools used in molecular analyses (Bowers et al., 2021), advancing methods for quantifying species abundance (Gold et al., 2023), and expanding reference databases to enable species-level identification (van der Loos & Nijland, 2020). Furthermore, standardization of metadata organization across projects and countries is expected to facilitate more detailed comparisons among experiments (Fediajevaite et al., 2021). As such, continued efforts are needed to further incorporate, improve, and standardize emerging methods while improving reference databases and harmonizing data management. Additionally, advances in machine learning and artificial intelligence are expected to further improve automation and comparability in molecular North Sea monitoring efforts.

In this review, we explore the potential of the molecular toolbox to assess North Sea marine biodiversity and ecosystem dynamics. We summarize current and emerging molecular techniques, highlighting their application, not only for biodiversity assessment, but also for understanding broader ecosystem functioning and dynamics. We discuss the challenges that currently hamper the integration of these molecular methods into standardized marine monitoring, alongside the opportunities presented for future research to facilitate their incorporation. In addition, we address the role of genetic databases, as well as issues related to data curation and archiving which prevent consistent and harmonized marine biodiversity monitoring in a transboundary water basin. Finally, we make three recommendations to improve transparency and

facilitate reproducibility between molecular studies. While this review focuses on the North Sea as an illustrative case study, recommendations presented here are applicable to improve molecular biodiversity monitoring in other marine ecosystems.

Current molecular tools for marine biodiversity monitoring

Sampling in the North Sea is often challenging due to its complex hydrography and intensive anthropogenic use. Therefore, monitoring requires demanding field campaigns, which can be compromised in terms of sampling coverage and consistency. Monitoring approaches that use eDNA are promising alternatives to conventional tools as these methods require minimal sampling efforts and can greatly reduce time spent in the field (Kopp et al., 2023). Initially, these sampling efforts relied on molecular assays targeting species-specific occurrences, but community-based metabarcoding was soon adopted and showed the potential of using eDNA to study North Sea biodiversity (Miya, 2022). In this section, we summarize the current molecular techniques used for biodiversity assessments in the North Sea and highlight the advantages and limitations of these approaches (Fig. 1).

Species-specific assays

Over the past decades, molecular species detection from non-invasively collected environmental samples has emerged as an important approach for marine monitoring in the Nort Sea. The application of species-specific assays has enabled the detection of a wide variety of rare and non-native species in the North Sea from environmental samples, underscoring their potential to complement and enhance conventional monitoring approaches (Fig. 1A) (Engesmo et al., 2018; Keller et al., 2022; Morin et al., 2023; Shelton et al., 2022). Detecting the presence of a target species in environmental samples is most often carried out by real-time PCR, also known as quantitative PCR (qPCR), or by digital-droplet PCR (ddPCR) (Guri et al., 2024a). Currently, qPCR is the golden standard for species-specific detection because this method is highly sensitive to low DNA concentrations. Theoretically, species detection by qPCR relies on the presence of a single target DNA molecule, making it a suitable application for environmental samples that generally contain low concentrations of target DNA. Despite its high sensitivity, qPCR can be inhibited by contaminants originating from the environment, such as salts (Lance & Guan, 2020). The alternative approach, ddPCR, is less affected by PCR inhibition as DNA molecules are divided over individual oil droplets where amplification takes place separately within each droplet. The main advantage of both species-specific approaches is that they are quantitative, enabling not only the detection of a species' presence but also quantifying relative differences in occurrence across samples (Guri et al., 2024a). To this extent, species-

specific assays are widely applied in various contexts in the North Sea, from analysis of fish communities (Guri et al., 2024a; van Bleijswijk et al., 2020), to detecting cryptic and non-indigenous species (Laakmann et al., 2017; Mauger et al., 2021; Morin et al., 2023), to the detection of toxic microalgae, antimicrobial resistance in flatfish (Bourdonnais et al., 2024), and the monitoring of parasitic infections in aquaculture systems (Krolicka et al., 2022). Despite the high sensitivity of qPCR and ddPCR for DNA detection, both assays are inherently species-specific and therefore have limited applicability in comprehensive biodiversity assessments. Therefore, most biodiversity studies rely on broader molecular approaches that target all DNA fragments present within an environmental sample, an approach better described as eDNA metabarcoding.

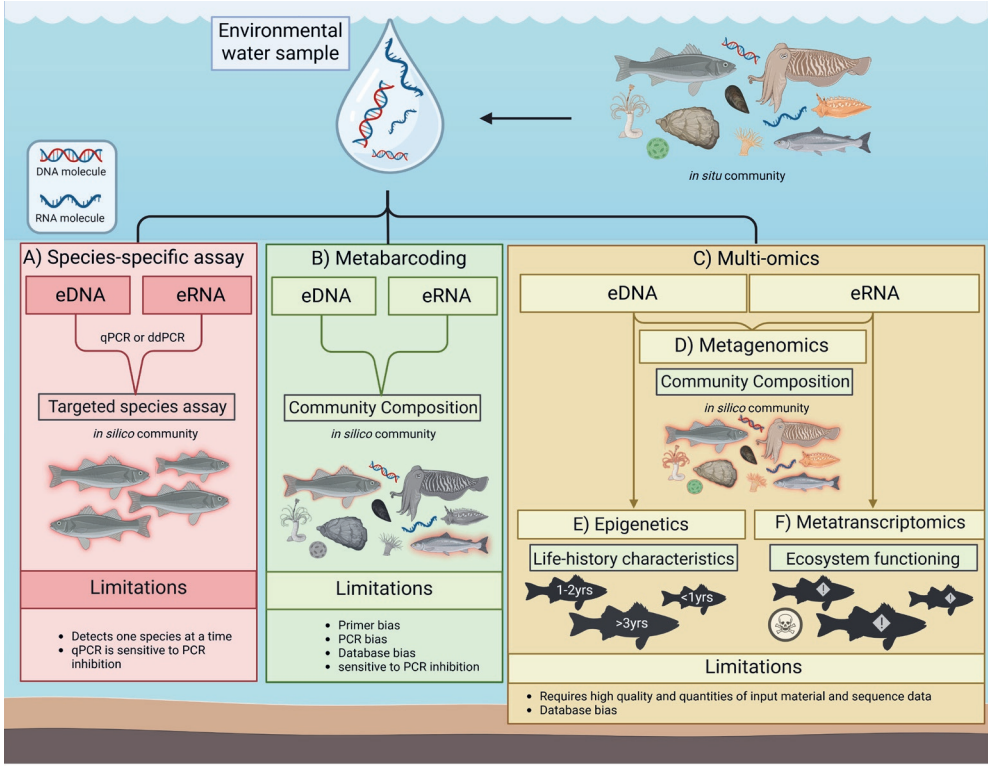


Figure 1 | Schematic overview of the types of molecular workflows used in assessing biodiversity and ecosystem dynamics from an environmental water sample. The figure illustrates the in situ biological community and the corresponding in silico communities generated by each workflow, focusing either on **(A)** specific species in species-specific assays, **(B)** groups of species or taxa through metabarcoding, **(C)** or the entire community or selected taxa using multi-omics approaches. This figure was created in BioRender.

Metabarcoding

Currently, metabarcoding is the most used molecular tool for identifying species compositions from DNA fragments in an eDNA sample (Cordier et al., 2019; Miya, 2022; Staehr et al., 2022; Zhang et al., 2020b). Metabarcoding, which is inherently coupled with next-generation sequencing (NGS), facilitates the simultaneous taxonomic identification of multiple species across numerous samples, providing an affordable and rapid alternative to conventional biodiversity assessment methods (Fig. 1B) (Ferreira et al., 2024; Scarano et al., 2024; Singer et al., 2019; van der Loos & Nijland, 2020). Depending on the objective, metabarcoding can be applied to various sample types, such as sediment, water, or bulk samples (Staehr et al., 2022; van den Bulcke et al., 2024; van der Loos & Nijland, 2020). For the purpose of this review, we only discuss metabarcoding of marine environmental samples.

In the North Sea ecosystem, metabarcoding is widely adopted to study marine biodiversity of different taxonomic groups, such as marine mammals (Boyse et al., 2024), fish (van Bleijswijk et al., 2020), crustaceans (Shum et al., 2023), phytoplankton (Martens et al., 2024), zooplankton (Ohnesorge et al., 2024), and meiofauna (Klunder et al., 2020). Beyond characterizing biodiversity, eDNA metabarcoding can monitor changes in community composition, detect shifts in interactions, reveal hidden trophic links, and assess the impacts of human activities, thereby providing insights into the integrity and functioning of ecological systems (Cordier et al., 2017; Käse et al., 2021; Li et al., 2024b; Ruppert et al., 2019). For example, eDNA metabarcoding has been used to examine connectivity patterns and co-occurrence networks along with establishing trophic interactions in the North Sea (Boyse et al., 2025). Further, eDNA metabarcoding applied to OWFs and oil platforms in the North Sea also demonstrated horizontal and vertical community patterns and revealed composition shifts of fish and invertebrate communities upon the introduction of artificial structures (Dukan et al., 2024; Cornelis et al., 2024; Mauffrey et al., 2021). Moreover, a comparison of species community composition between natural and artificial oyster reefs demonstrated similar occurrences of NIS on both reefs (Jansen et al., 2025). These examples illustrate the broadened scope provided by eDNA metabarcoding, which enhances our understanding of ecosystem dynamics and functioning.

Although metabarcoding is currently the most widely adopted molecular approach for assessing marine biodiversity, the method is subject to several biases and limitations. Biases may be introduced during laboratory processing such as primer choice, amplification efficiency, and stochasticity during PCR (Alberdi et al., 2019; Gold et al., 2023; Piñol et al., 2019; van der Loos & Nijland, 2020). Additionally, computational sequence processing may further limit reliable interpretation, (Hunter et al., 2017; Sigsgaard et al., 2020a; Suarez-Bregua et al., 2022; van der Loos & Nijland, 2020). Where

metabarcoding typically targets a universal genetic region that is conserved across taxa (e.g. COI, 18S, or 12S), the universality of the targeted region often compromises taxonomic resolution. This issue can be mitigated by using a multi-marker approach or by amplifying longer DNA fragments (Alberdi et al., 2018; Cordier et al., 2019; Doorenspleet et al., 2025a; Ferreira et al., 2024; Leray et al., 2013; Stoeck et al., 2010; Zhang et al., 2018). Without a clear relationship between (relative) read count and biomass, inferring species' abundance remains a significant challenge, limiting the use of eDNA in quantitative ecological assessments (Beng & Corlett, 2020; Garlapati et al., 2019; Guri et al., 2024a). Although various approaches have been proposed to improve quantitative accuracy in metabarcoding (Di Muri et al., 2020; Ershova et al., 2021; Guri et al., 2024b; Schenk et al., 2019), whether eDNA can ultimately provide reliable abundance estimates remains to be determined. Metabarcoding studies are further limited by the completeness of nucleotide reference databases, where the lack of species-specific sequences restricts species-level identification (Ferreira et al., 2024; van der Loos & Nijland, 2020; Zhang et al., 2020a).

Integrating metabarcoding into existing management frameworks can improve the capacity to detect, conserve, and restore species and habitats. Its wider adoption into international monitoring projects (e.g. GEANS and MARCO-BOLO) is already being extensively explored, and these projects have begun to demonstrate the feasibility of implementing metabarcoding as a standardized approach for routine biodiversity monitoring in the North Sea (GEANS, https://www.geans.eu/; MARCO-BOLO, 2025).

Metagenomics

Unlike targeted approaches that focus on specific taxa or genomic regions, metagenomic approaches encompass all available DNA nucleotides contained in a sample, thereby achieving broader taxonomic resolution (Fig. 1D) (Serite et al., 2023). As metagenomics relies on sequencing all DNA molecules from a sample, the approach omits potential primer and PCR biases (Callens et al., 2025; Crampton-Platt et al., 2016; Serite et al., 2023). The current application of metagenomics in studying North Sea biodiversity from eDNA samples shows considerable potential for achieving high taxonomic resolution while retaining a broad ecosystem-wide perspective (Bista et al., 2018; Doorenspleet et al., 2025b; Holman et al., 2025). Beyond facilitating marine biodiversity assessments, metagenomic approaches enable the analysis of population genetics (Crowley et al., 2025; Serivichyaswat et al., 2025). Notably, whereas the detection of viruses through metabarcoding is limited by the absence of universal genetic markers across viral taxa, metagenomic approaches provide valuable opportunities to characterize DNA-based viral communities by simultaneously identifying multiple genetic markers (Garin-Fernandez et al., 2018). The advent of third-generation sequencing techniques has enabled a more

comprehensive molecular characterization of DNA present within environmental samples, including the detection of base modifications, such as DNA methylation, alongside canonical sequences (Rang et al., 2018; Sigurpalsdottir et al., 2024). These epigenetic modifications provide the opportunity to analyze population otogeny or demographics (Anastasiadi & Piferrer, 2020; Wang et al., 2021).

While metagenomics has considerable advantages over metabarcoding, its incorporation into standardized monitoring is still in its infancy, as the approach exhibits notable limitations. As whole-genomic DNA is sequenced rather than a targeted amplified region, more data is required to ensure sufficient coverage for accurate taxonomic identification of all species in a sample (Manu & Umapathy, 2023). Further, analysis of metagenomics data requires a nucleotide reference database containing whole genome data, bioinformatic expertise, and substantial computing power (Garlapati et al., 2019; Holman et al., 2025). Although metagenomics circumvents quantification issues that arise in metabarcoding from primer or PCR biases, challenges in quantification of species abundance persist (e.g. due to copy number variation), emphasizing the need for further research to enable standardized implementation (Di Muri et al., 2020; Krehenwinkel et al., 2017; Serite et al., 2023). Nevertheless, approaches capable of simultaneously assessing biodiversity and relative biomass from environmental samples are currently under development (Callens et al., 2025). Consequently, expected continued reductions in sequencing costs and expansion of reference databases should further enhance the application of metagenomics for investigating marine biodiversity and ecosystem dynamics from environmental samples, and facilitate its integration into standardized ecosystem monitoring.

Emerging molecular tools and approaches for monitoring species dynamics

Ecosystem complexity, encompassing inter- and intraspecies interactions, or structural organization within and between species, plays a critical role in shaping ecosystem resilience, productivity, and overall stability (Farnsworth et al., 2012; Murgier et al., 2021; Tilman et al., 2014). While currently used molecular tools have proven useful to assess marine biodiversity, the potential to assess population status, species' demographics, or ecosystem health is generally limited. As molecular technologies advance beyond species-specific assays and metabarcoding, more research questions can be addressed by following a multi-omics based approach (Fig. 1C) (Fig. 1C; ("eDNA Approaches for Ecosystem Health Monitoring," 2025). Integration of these novel tools could offer new opportunities to enhance our understanding of North Sea ecosystem dynamics, functioning, and health. Here, we discuss several novel molecular tools for monitoring marine ecosystem dynamics and evaluate their potential for integration within current North Sea biodiversity monitoring frameworks.

Epigenetics

Epigenetics represent chemical modifications to DNA, RNA, chromatins, and histones that have the potential to influence gene regulatory mechanisms without changing the coding sequence (Jones, 2012). As such, these modifications are involved in important regulatory processes, such as growth, development, and reproduction (Duncan et al., 2014; Liu et al., 2022). Environmental conditions can modulate epigenetic programming, thereby providing the molecular machinery to facilitate phenotypic plasticity in a wide variety of organisms (Bogan & Yi, 2024; Bonasio, 2015; Roberts & Gavery, 2012). Patterns of DNA methylation, currently the most studied epigenetic mechanism, can vary in response to environmental conditions, such as changes in nutrition, temperature, and acidification (Bock et al., 2022; Eirin-Lopez & Putnam, 2019; Sun et al., 2023). Accordingly, within biodiversity research, the detection of such epigenetic changes or reprogramming can serve as a valuable indicator of environmental change (Fig. 1E) (Dang et al., 2023; Gombeau et al., 2016; Lee et al., 2020; Liu et al., 2023). For example, alterations in DNA methylation patterns facilitate thermal tolerance to rising temperatures in Acartia tonsa, an abundant copepod in the North Sea (Janssens et al., 2024). As DNA methylation changes often follow a predictable correlation with life-history characteristics, the detection of intraindividual and intraspecific differences in DNA methylation patterns can help to understand other aspects of population demography in changing environments (e.g. sex and reproductive behaviour, diseases, pathogen response, and species' welfare and resilience) (Belenguer et al., 2024; Brink et al., 2024; Ortega-Recalde et al., 2020; Saito et al., 2025; Sigsgaard et al., 2020b; Silliman et al., 2023; Valk et al., 2025; Viña-Feás et al., 2024).

In addition to studying epigenetic mechanisms from tissue samples, methylation profiles derived from environmental samples (epi-eDNA) show substantial potential in understanding population demographics in marine ecosystems (Balard et al., 2024; Sigsgaard et al., 2020b). For example, DNA methylation was proven a useful indicator for inferring the age of fish and invertebrates from eDNA samples (Ruiz et al., 2025; Zhao et al., 2023). However, despite early successes in using epi-eDNA, studying such modifications requires collecting well-preserved and large quantities of genomic DNA (Li & Tollefsbol, 2021). Moreover, it was shown that biases introduced by sample characteristics and methodology complicate comparability between studies (Balard et al., 2024; Rahman & Lozier, 2023; Shao et al., 2014; Trigg et al., 2022). As such, it is pivotal to expand our knowledge of epigenetic signals and markers in different species, life-stages, and environmental conditions to effectively integrate epigenetic and epi-eDNA data into biodiversity monitoring of North Sea ecosystems (Liu et al., 2025).

Environmental RNA and meta-transcriptomics

Beyond eDNA, environmental samples may also contain environmental RNA (eRNA), comprising a range of RNA types, including mRNA, tRNA, rRNA, and miRNA. Due to the higher instability of RNA, eRNA exhibits a faster degradation rate than eDNA and is therefore suggested to provide a more accurate representation of recent biodiversity (Giroux et al., 2022; Kagzi et al., 2022; Li et al., 2024a; Macher et al., 2024). Accordingly, eRNA detection is proposed to complement eDNA analyses and thereby improve the spatio-temporal resolution of biodiversity assays (Li et al., 2024a; Marshall et al., 2021; Miyata et al., 2022). An additional opportunity offered by eRNA in comparison to eDNA, is the possibility to differentiate between viable and non-viable organisms, improving pathogen detection sensitivity and biosecurity surveillance (Bass et al., 2023; Darling & Frederick, 2018; Mérou et al., 2020; Miyata et al., 2022). These applications highlight the potential of eRNA research in assessing ecosystem dynamics and health, and offer promising possibilities for their expansion into studying marine systems (Fig. 1F). The complementary application of eDNA and eRNA approaches, collectively referred to as eNA, in metabarcoding studies has been demonstrated to reduce false positives and subsequently provide a more accurate representation of overall biodiversity (Kagzi et al., 2023; Li et al., 2024a; Littlefair et al., 2022; Miyata et al., 2022, 2025). Consequently, the integrated use of eDNA and eRNA detection can expand the resolution of eNA-based monitoring and broaden our understanding of North Sea dynamics and other marine ecosystems (Fig. 1C).

In addition to its use in biodiversity monitoring through eRNA metabarcoding (Fig. 1B), eRNA-derived metatranscriptomic analysis, which targets transcriptional signals (Fig. 1F), provides the opportunity to assess species' ontogeny, population structure and dynamics, as well as organismal responses to environmental change (Hechler et al., 2023; Miyata et al., 2025; Parsley & Goldberg, 2024; Sutherland et al., 2023; Tsuri et al., 2021). Beyond biodiversity assessments, metatranscriptomics has the potential to enhance understanding of ecosystem functioning by identifying patterns of differential gene expression between individuals, species, or environments (He et al., 2025; Sutherland et al., 2023). For example, sequencing of all available transcripts from an environmental sample was demonstrated to elucidate stress responses in aquatic organisms (Hechler et al., 2023; Miyata et al., 2025). However, current applications of eRNA-based approaches are often applied to enclosed systems targeting a single species (Hechler et al., 2023; Miyata et al., 2025). A more comprehensive understanding of eRNA characteristics is required prior to its effective integration into biodiversity monitoring frameworks. Future research could explore eRNA degradation rates, shedding characteristics, or gene transcript quantities and variety in environmental samples (Giroux et al., 2022; Kagzi et al., 2022, 2023; Stevens & Parsley, 2023; Wood et al., 2020). Once these areas are better understood, eRNA

metabarcoding and metatranscriptomics can be integrated with eDNA analysis to expand our understanding of North Sea ecosystem functioning and health (Fig. 1).

Environmental nucleic acids (eNA)

Recently, biodiversity assessment approaches are emerging that collectively target environmental nucleic acids (eNA), encompassing all nucleic acids (both eDNA and eRNA) released by organisms into the environment (Littlefair et al., 2022; Wang et al., 2025). The applications of analyses targeting eNA reach far beyond biodiversity assessments, where for example, the detection of microbial activity can be utilized as an indicator of ecosystem health (Fig. 1C) (Cook et al., 2025). As such, eNA-based approaches represent the next step in molecular-based biodiversity monitoring by offering an integrated nucleic acid profile rather than utilizing eDNA or eRNA approaches separately. These developments demonstrate the advancement of omics-based tools to not only identify taxonomic diversity but also biological activity within marine ecosystems. However, similar to the limitations faced by single-molecule (DNA or RNA-based) approaches, biodiversity assessments relying on eNA are limited by variability in the amount of eNA released by different individuals and species, as well as by differences in eNA integrity, where eNA degradation varies under different environmental conditions (Kagzi et al., 2022, 2023; Littlefair et al., 2022). We refer to other reviews for a more complete overview of the current challenges that arise from eNA shedding, decay, and degradation in assessing species biodiversity (Collins et al., 2018; Kagzi et al., 2022, 2023; Lamb et al., 2022; Nagler et al., 2022; Wang et al., 2025).

With the ongoing improvement of sequencing technologies and bioinformatic analytical pipelines, it is only a matter of time before eDNA studies pave the way for broader eNA and omics-based approaches. In the meantime, further research is required to explore the complementary roles of eDNA and eRNA, and to understand how the integrated application of total eNAs can inform restoration and conservation efforts by elucidating ecosystem structure, stability, and function.

Data curation and standardization

Current challenges in database management

Nucleotide reference databases are a crucial component of most molecular workflows, however, challenges persist during accurate taxonomic assignment. Robust and accurate species identification requires a comprehensive and up-to-date database, including a broad range of taxa identified to the lowest taxonomic rank. Such reference databases may be public (Table 1), such as those under the umbrella of the International Nucleotide Sequence Database Collaboration (INSDC,

https://www.insdc.org/), for example, the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/browser/home), and the National Centre for Biotechnology Information database (NCBI, https://www.ncbi.nlm.nih.gov/). In contrast to public databases, which typically span broad taxonomic groups or geographic regions, local databases are more narrowly focused on specific areas, such the curated reference library for COI generated by GEANS (Christodoulou et al., 2025).

Despite the extensive taxonomic coverage of public repositories, the lack of curation, gaps in taxa representation, and irregular maintenance of these databases increases the risk of incomplete or erroneous taxonomic assignments (Hestetun et al., 2020, 2021; van der Loos & Nijland, 2020). Consequently, numerous studies have highlighted the need to expand public nucleotide databases, including clearer metadata requirements to ensure consistency and reproducibility (Alberdi et al., 2018, 2019; Bucklin et al., 2016; Duarte et al., 2020). To address some of these challenges, the EU project eDNAquaplan has been working collaboratively across EU and international research institutions and data repository leaders to harmonize metadata standards for eDNA metabarcoding datasets, with a focus on nucleotide database submissions (https://ednaquaplan.com/). In addition, databases belonging to INSDC have taken steps towards these requirements through the sharing of nucleotide data once uploaded to one of these three databases and creating resources for INSDC members, such as metadata structures and facilitating the submission of raw sequence reads. Local databases are typically more curated and, therefore, less likely to lead to erroneous species identification. However, non-indigenous, rare, or cryptic species are easily overlooked in local databases, thereby emphasizing the need to combine local and public databases for molecular biodiversity analysis (Duarte et al., 2020).

Furthermore, by maintaining "local" or private databases derived from publicly-funded research raises ethical as well as practical concerns regarding data equity, reproducibility and transparency. Limiting the sharing of such resources can impact international collaboration and the wider advancement of molecular knowledge, particularly in shared ecosystems such as the North Sea. Currently, there are several initiatives that aim to harmonize database curation and data sharing being implemented globally (e.g. FAANG, GEANS, OBON, and ELIXIR (FAANG Data Portal, https://data.faang.org; GEANS, https://www.geans.eu/; Samuel et al., 2021; Waterhouse et al., 2022). Beyond database accessibility, there are wider debates in the molecular ecology field over whether efforts should be made to transition away from targeted gene regions (e.g. COI, 12S, 18S) towards complete mitochondrial or whole-genome reference sequences (Dziedzic et al., 2023). This shift in direction could improve taxonomic resolution from eNA samples and facilitate a multi-target or target-free (multi-omics) approach to eDNA sequencing.

Table 1 | Summary of established open-access nucleotide reference databases, detailing taxonomic scope, molecule types and data types accepted for submission.

Database	Taxonomic scope	Target fragments	Data types accepted	Year	Reference
GenBank (NCBI)	All domains of life	Gene regions such as COI, 12S/16S/18S, ITS. Proteins, whole genomes	Nucleotide sequences, assemblies (inc. mitogenomes and whole genomes. Uses FASTQ, BAM/ CRAM or Genbank format.	1982	Sayers et al., 2025
SRA (NCBI)	All domains of life	Raw sequence data	Fastq, Fast5, BAM/ CRAM, HDF and HDF5	2007	Katz et al., 2022
European Nucleotide Archive (ENA)	All domains of life	Gene regions such as COI, 12S/16S/18S, ITS. Proteins, whole genomes	Raw reads (FASTQ, BAM/CRAM), assemblies, annotated sequences, metadata	1982	O'Cathail et al., 2025
DNA Data Bank of Japan (DDBJ)	All domains of life	Gene regions such as COI, 12S/16S/18S, ITS. Proteins, whole genomes	Raw reads (FASTQ, BAM/ CRAM), assemblies, annotated sequences, metadata	1987	Tateno & Gojobori, 1997
RefSeq (NCBI)	Curated representative sequences focused on model organisms	Genomes, transcripts, proteins (reference loci)	RefSeq data is derived from sequences curated from GenBank/ENA/ DDBJ.	2000	Pruitt, 2004
BOLD Systems (Barcode of Life Data System)	Broad eukaryotes (animals, plants, fungi)	Genetic markers only such as COI, rbcL and ITS	FASTA	2005	(Ratnasingham and Hebert 2007)
SILVA	Prokaryotes & eukaryotic rRNA	Ribosomal RNA markers only such as 16S, 18S and 23S rRNA (SSU/LSU)	FASTA, FASTA with metadata and ARB	2007	Quast et al., 2012
UNITE (fungal ITS)	ITS database predominantly Fungi	Ribosomal ITS region	UNITE data is curated from INSDC	2003	Abarenkov et al., 2024
PR2 (Protist Ribosomal Reference)	Protists / micro-eukaryotes	18S rRNA	Data extrapolated from genome assemblies on NCBI	2010	Krabberød et al., 2025

Table 2 | Summary of established open-access marine-focused nucleotide reference databases, detailing taxonomic scope, molecule types and data types accepted for submission.

Database	Taxonomic scope	Target fragments	Data types accepted	Year	Reference
AquaGene	Fish species	COI, CYTB and the nuclear genes myosin 6 (MYH6) and rhodopsin I (RHOD)	Does not accept public uploads	2013	AquaGene, n.d.
MarRef / MarDB / MarCat	Marine prokaryotes / metagenomes	Reference genomes	Uses genomes and metagenomes collated from ENA and NCBI	2017	Klemetsen et al., 2018
MARES (pipeline)	Marine eukaryotes	COI	Reproducible pipeline to retrieve sequences from GenBank and BOLD	2020	Arranz et al., 2020
Mare-MAGE (fish reference)	Marine fishes	Mitochondrial 12S rRNA (fish), COI	Sequences retrieved from GenBank, ENA, AquaGene and BOLD	2023	Kasmi et al., 2023
MetaZooGene Atlas (MZGdb)	Zooplankton, benthic invertebrates, fish, marine mammals and phytoplankton	COI, 12S, 16S, 18S and 28S	Sequences retrieved from GenBank and BOLD	2021	Christodoulou et al., 2025

Standardization and implementation of molecular data for routine North Sea marine monitoring

Molecular ecological methodologies, such as species-specific assays and eDNA metabarcoding, have become increasingly popular in recent years (Cornelis et al., 2024; Derycke et al., 2021; Moutinho et al., 2024). However, incorporation of these approaches into routine biodiversity monitoring has been hindered by a lack of standardization across sampling techniques, metadata collection, and subsequent inconsistencies in publications (Klymus et al., 2024; Shea et al., 2023; Veilleux et al., 2021). A clear overview on the reported use of monitoring methods demonstrated that over 20 different methods are used as part of monitoring programs in European regional sea conventions (Korpinen et al., 2022). Despite the spatial and temporal expansion of marine monitoring, the integration and harmonization across these methods remains uncoordinated. Recently, formal standards for eDNA-based approaches using metabarcoding were published as the "Minimum Information for reporting of Environmental Metabarcoding data" (MIEM), and is similar to the existing

guidelines for species-specific assays (Borchardt et al., 2021; Bustin et al., 2009; Hunter et al., 2017; Langlois et al., 2021; Loeza□Quintana et al., 2020; The dMIQE Group et al., 2020). However, subsequent reviews of similar guidelines found a lack of uptake and reporting in published articles (Bustin, 2024; Bustin et al., 2009; Bustin & Nolan, 2017). This inconsistency, compounded by heterogeneous or incomplete metadata curation in public nucleotide repositories, limits reproducibility and long-term data use (Gilbert et al., 2012; Pope et al., 2015). Some of these issues could be addressed by setting clear definitions to vocabulary, generating mandatory metadata fields, and creating consistent bioinformatic reporting protocols that align with MIEM and the FAIRe (Findable, Accessible, Interoperable, Reusable eDNA data) data principles. Achieving such objectives is essential for improving comparability of eDNA data across research institutes and countries and to ultimately facilitate integration with marine biodiversity frameworks, such as the MSFD and OSPAR monitoring frameworks.

Furthermore, an online repository that consists of molecular data complemented by ecological and geographical data would be an asset for biodiversity monitoring and analysis. To this end, the Global Biodiversity Information Facility (GBIF) has recently established the metabarcoding data toolkit (https://mdt.gbif.org/) to facilitate the standardized submission, curation, and integration of metabarcoding datasets with existing species occurrence records, thereby bridging the gap between molecular biodiversity data and traditional ecological observations (GBIF, https://www.gbif.org/; GBIF Secretariat, 2024). Another initiative that focuses on linking genetic sequence material to ecological metadata and geographical species occurrence databases (e.g. WORMS or OBIS), is the Genomic Observatories Metadatabase (Ocean Biodiversity Information System, 2022.; WoRMS - World Register of Marine Species, 2023; Riginos et al., 2020). To further promote data sharing, raw sequences that would otherwise be filtered out during bioinformatic workflows, could be archived in open-access repositories. This approach aligns with FAIR principles and would facilitate reproducibility, support the maintenance of bioinformatic pipelines, and improve future taxonomic assignment and database expansion.

Improving data processing and standardization for North Sea biodiversity assessment using machine learning

The integration of artificial intelligence and machine learning approaches into routine environmental biodiversity monitoring has the potential to facilitate faster and more accurate species identification, remote sample collection, and predictive modelling using environmental parameters (Ditria et al., 2022; Stefanni et al., 2022a). In the broader context of marine biodiversity, machine learning models have been applied to investigate the effects of abiotic environmental conditions on North Sea cod stocks (Kühn et al., 2021), map plankton distributions to define ecological niches (Plonus &

Floeter, 2024), and to assess the health of marine mammals (IJsseldijk et al., 2024). Machine learning is increasingly applied to monitor zooplankton (van Walraven et al., 2025), invertebrates (Sokolova et al., 2021), birds (Sommer et al., 2025), and pollution in the North Sea (Kaandorp et al., 2022), using automated species detection from high-resolution imagery and videos. For a complete overview of all the current applications of machine learning in marine ecology, we refer to the review of Rubbens et al., 2023. Another application of machine learning is facilitating continuous and automated sample collection based on forecasted environmental conditions, thereby minimizing labor and enabling access to remote areas, such as offshore wind farms (Stefanni et al., 2022b).

There are numerous possibilities for integrating machine learning tools into data collection, processing, and archiving, to facilitate biodiversity monitoring. For example, the utilization of neural network algorithms to curate molecular reference libraries has been explored and can greatly reduce the laborious efforts that come with manual curation (Fontes et al., 2024). Furthermore, the application of machine learning models for predicting the ecological quality status of marine environments, using eDNA metabarcoding data, has also been demonstrated (Cordier et al., 2017). This highlights machine learning as a promising tool to model complex ecological relationships that enhance ecological assessments. These models also show potential for organizing and autonomously maintaining large reference databases that integrate data from different sources (Stefanni et al., 2022b; Yang et al., 2024). Automated algorithms have the potential to assist in continuous monitoring and updating data records by cross-referencing changes in databases, ensuring data accuracy and reducing inconsistencies. This would reduce the likelihood of nomenclature errors as seen in current nucleotide reference databases, which often contain misidentifications and incomplete taxonomic coverage (Mugnai et al., 2021). Implementing automated systems requires agreement on standardized data formats and collaboration between host organizations of existing databases. A significant step toward integrating biodiversity data into single accessible platform was provided by GBIF (GBIF, https://www.gbif.org/). Moreover, an integrated framework that combines imaging, sound, habitat assessment, molecular data, and behavioral analysis into one autonomously managed database would significantly enhance biodiversity monitoring and our understanding of ecosystem function (Heberling et al., 2021; Stefanni et al., 2022b). Consequently, it is strongly recommended that researchers publish comprehensive metadata alongside environmental or molecular datasets to enrich data repositories and comply with the FAIRe principles (https://ednaquaplan.com/). Such complete datasets can be utilized in modelling approaches or incorporated into machine learning algorithms aimed at estimating ecosystem-related indices informing on ecosystem functioning, health, and overall stability.

The formulation of clear data-sharing guidelines, coupled with the support of machine learning in automated data curation and archiving, is expected to enhance the harmonization of marine datasets across experiments, thereby improving the understanding of marine ecosystem dynamics and biodiversity by increasing interpretability across studies.

Conclusion and recommendations

With the current intensity of anthropogenic exploitation in the North Sea, an accurate and more extensive understanding of ecosystem health and functioning is pivotal for management and conservation. Above, we have summarized current methods and highlighted novel molecular approaches that could be adopted into standardized monitoring frameworks for assessing the biodiversity and functioning of the North Sea. Upscaling and standardizing the utilization of these multi-omics tools for robust ecosystem biodiversity monitoring requires a collaborative approach among researchers, conservationists, and policymakers. This would not only require data sharing and integrating various monitoring technologies, but also active pursuance ensuring that these frameworks and guidelines are adhered to. This multifaceted approach will facilitate the identification of biodiversity dynamics, assess the impacts of environmental stressors, and inform management and conservation. Finally, we have highlighted how current limitations in reference databases, as well as challenges in data curation and archiving, can hamper harmonization across experiments. To this end, we provide three suggestions for integrating molecular methods in a standardized manner to improve assessment of ecosystem biodiversity, function, and structure in the dynamic North Sea:

i) Comprehensive archiving of metadata alongside biodiversity data facilitate integration across experiments and enhance the interoperability and reproducibility between molecular and conventional monitoring of marine biodiversity.

This approach, integrating molecular and conventional monitoring methods with comprehensive metadata curation, will improve understanding of ecosystem functioning by providing a multi-disciplinary perspective of different aspects and interactions within an ecosystem.

ii) Establish data-sharing guidelines to enable standardization and comparison between studies.

Once data collection and curation become more standardized, this will allow for comprehensive comparisons between studies, by using data collected through different approaches and on different scales.

iii) Make all molecular data publicly available to enable comprehensive analysis of ecosystem biodiversity and function.

Publishing all sequence data, instead of only the subset used for publication, is expected to improve understanding of ecosystem functioning by providing a more detailed image of molecular biodiversity in a spatial-temporal context.

Declaration of competing interest

The authors declare that they have no competing interests.

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CHAPTER 7

General Discussion

Sophie Valk

Main findings

Following extensive exploitation of the European flat oyster across its native range, restoration efforts have increasingly focused on reestablishing sustainable oyster reef habitats. Despite these efforts, most restoration projects fail to successfully integrate genetic considerations into their approaches. Limited integration of molecular insights raises concerns, as it may constrain the effectiveness of locally tailored restoration and jeopardize the preservation of genetic diversity. Therefore, **the aim of this thesis was to comprehensively characterize the intraspecific genetic and epigenetic architecture of *O. edulis***, providing a molecular foundation to support sustainable restoration efforts. Building on the work presented in the previous chapters, this final chapter summarizes the main findings, synthesizes concrete answers to the research questions posed in the general introduction (**Chapter 1**), and places the findings within a broader context of relevant literature. Key avenues for future research are highlighted, and an integrative roadmap is proposed, outlining guidelines for incorporating molecular insights into applied oyster restoration, thereby supporting effective management and restoration of *O. edulis* and their biogenic reef habitats in a local context.

The genetic characterization of *O. edulis* populations has expanded considerably in the last decades, with an increasing number of studies applying diverse genomic approaches (Alves Monteiro et al., 2024; Ewers et al., 2025; Gundappa et al., 2022; Lapègue et al., 2023). However, because most studies have focused on covering broad geographic scales, the characterization of small-scale genetic variation among local populations remains limited. In the context of local restoration efforts, the genetic diversity and connectivity of Dutch wild oyster populations were compared with foreign and locally cultured oysters, both of which are currently used in local restoration initiatives (**Chapter 2**). Genotyping of genome-wide single-nucleotide polymorphisms (SNPs) revealed that remnant wild Dutch populations comprise a panmictic cluster with high genetic connectivity. Moreover, two recently established wild populations displayed rapid genetic recovery from a bottleneck event, indicating the species' regenerative potential. In contrast, oysters from Ireland and Norway were genetically differentiated from Dutch populations. Although locally cultured oysters were genetically similar to wild oysters, hatchery-produced individuals exhibited elevated levels of kinship. These findings underscore the importance of integrating genetic insights into local restoration efforts and highlight the need for careful considerations in selecting restoration strategies to ensure preservation of genetic diversity.

Beyond single-nucleotide variation, structural variation (SV) was shown to represent an important and widespread component of genomic diversity across the *O. edulis*

genome (**Chapter 3**). Numerous SVs overlapped with repetitive elements, suggesting that highly active repeats contribute to generating structural genomic diversity. Variants within three previously identified chromosomal inversions substantially contributed to population structure, providing complementary insights from SV- and SNP-inferred analyses. Notably, elevated densities of both SVs and SNPs suggest that these inversions may act as reservoirs of genetic variation. Together, these findings emphasize the contribution of structural variation to evolutionary processes, and highlight the value of integrating both structural and single-nucleotide variation in the genetic characterization of *O. edulis*.

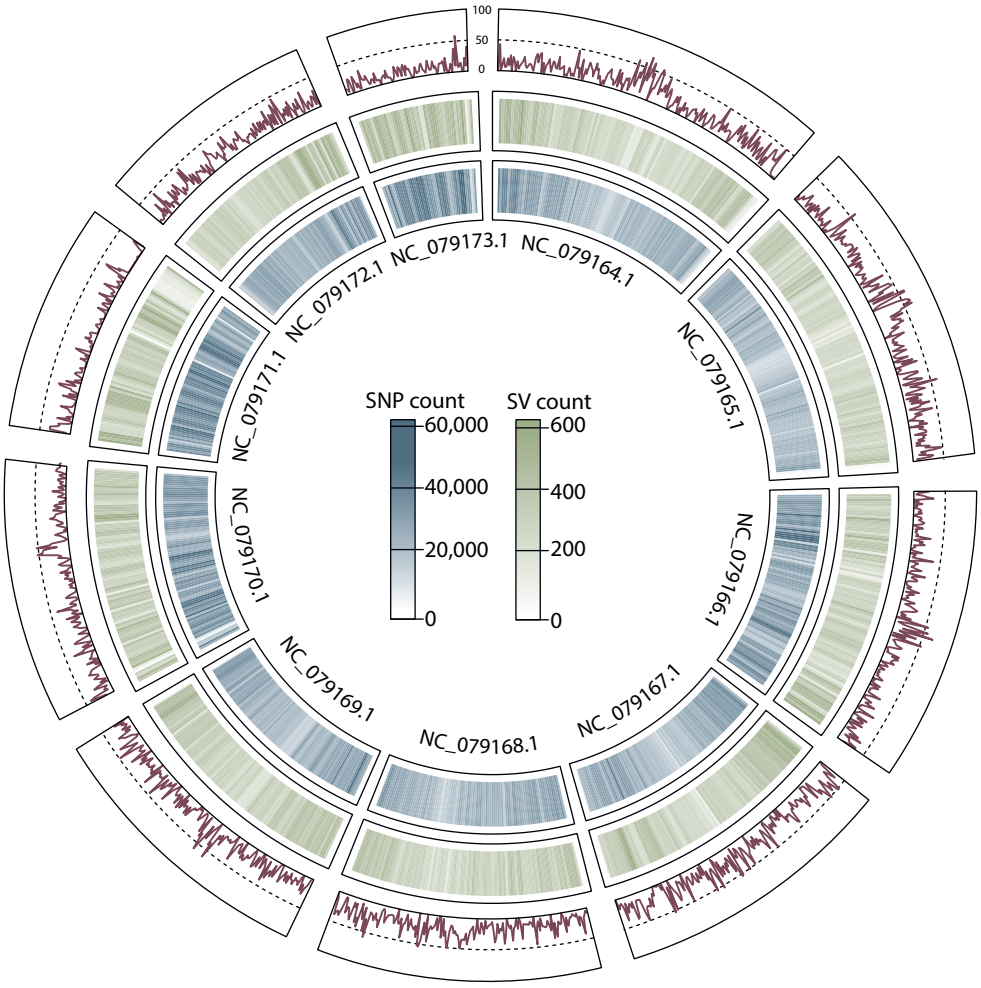
At the epigenetic level, comparisons of gill methylomes among distinct sex phenotypes revealed sex-specific DNA methylation patterns in *O. edulis* (**Chapter 4**). Hermaphrodite oysters displayed the most distinct DNA methylation profile, exhibiting the highest number of differentially methylated sites and regions relative to males and females. Over 9,000 differentially methylated regions (DMRs) were identified, overlapping at least 2,500 genes. Functional annotation of these genes indicated enrichment in pathways related to energy homeostasis and metabolism, suggesting that DNA methylation in gill tissue may be involved in epigenetic remodeling of energy balance during sex reversal.

Building on the characterization of these sex-specific epigenetic differences, a machine-learning approach was adopted to identify epigenetic markers that enable the molecular classification of sex phenotypes (**Chapter 5**). A total of 14 markers were selected, enabling the distinction of hermaphrodite versus non-hermaphroditic oysters, as well as differentiation between male-biased and female-biased individuals with moderate accuracy (~70%). These markers represent a molecular approach for sex identification in *O. edulis*, providing the first steps toward non-lethal alternatives.

Effective oyster restoration entails the reestablishment of biodiverse habitats in addition to the conservation of *O. edulis*. In a broader ecosystem context, biodiversity monitoring forms a crucial component for assessing the effectiveness of restoration efforts. Advances in sequencing technologies have revolutionized the study of North Sea marine biodiversity. However, the integration of these molecular approaches into standardized monitoring frameworks remains scarce. This chapter provides an overview of current techniques, and evaluates novel approaches for potential integration into routine monitoring (**Chapter 6**). Three recommendations are proposed to support the efficient and standardized integration of molecular methods that focus on (i) improving data archiving and curation, (ii) establishing data-sharing guidelines, and (iii) promoting public data availability to enable comprehensive analysis of ecosystem assessment parameters.

Collectively, the comprehensive characterization of the (epi)genetic architecture of *O. edulis* presented in this thesis is summarized in a circular plot, which integrates molecular variation data generated across the chapters of this thesis (Fig. 1).

Figure 1 | Circular plot summarizing molecular variation across 35 *O. edulis* individuals from the



Grevelingen, the Netherlands, displayed in 1Mb genomic bins. The inner blue layer displays genome-wide single nucleotide polymorphisms (SNPs), the middle green layer shows structural variants (SVs), and the outer red layer illustrates the average DNA methylation percentages across all individuals, values range from 0% to 100% methylation, with the dashed line indicating 50%.

In this section, the findings of the previous chapters are integrated to address the key

research questions outlined in the general introduction (Chapter 1).

RQ1: How does genomic variation contribute to patterns of intraspecific diversity within and among O. edulis populations in a restoration context?

The European flat oyster exhibits a wide geographical distribution ranging from Norway to the Black Sea (Alves Monteiro et al., 2024; Bergström et al., 2022; Cano & Rocamora, 1996; Robert et al., 2025), demonstrating the species' resilience and environmental tolerance across diverse environmental conditions. While high connectivity through the dispersal of motile larvae and a large effective population size (Ne) would suggest a panmictic population, numerous studies instead report the opposite: isolation-by-distance patterns, with genetic population structure even occurring at microgeographic scales (Alves Monteiro et al., 2024; Lapègue et al., 2023; Robert et al., 2025). On a finer spatial scale, genome-wide SNP genotyping revealed extensive genetic diversity and high population connectivity among Dutch wild oyster populations (**Chapter 2**). In addition, two recently established populations (Voordelta and Maasvlakte) demonstrate high regenerative potential, as evidenced by the rapid genetic recovery from a bottleneck event (**Chapter 2**). Furthermore, widespread structural variants contributed substantially to genetic within-population structure in a Dutch population, with many variants overlapping repeat elements (**Chapter 3**). Inversion polymorphisms contained elevated densities of both SVs and SNPs compared to the background genome, suggesting that structural variants play an important role in generating genomic variation (**Chapter 3**). These findings illustrate the highly polymorphic nature of the *O. edulis* genome and indicate considerable potential for local adaptation and resilience.

Notably, Dutch wild populations have persisted under high pathogen pressure from *B. ostreae* since the 1980s while maintaining high levels of genetic diversity, population growth, and recruitment (Engelsma et al., 2010). Collectively, the findings presented in this thesis suggest that, despite genetic risks associated with sweepstake reproductive success and potentially skewed sex-ratios, these populations remain highly genetically variable, likely facilitating their resilience to current environmental stressors, including pathogen exposure and anthropogenic disturbance. However, despite the currently high levels of genetic diversity, careful consideration is needed when designing restoration strategies to preserve this variation. Below, three key aspects of current restoration strategies are discussed with regard to their implications for the preservation of genetic diversity.

1. Sustainable restoration requires locally tailored restoration strategies and separate management of genetically diverged populations

Several studies suggest that local adaptation may underlie the observed phylogeographic population structure in *O. edulis* (Alves Monteiro et al. 2024; Lapègue et al. 2023). Population structure analysis on a local scale, revealed substantial genetic divergence between local remnant populations and translocated oysters originating from foreign regions (Norway and Ireland) (**Chapter 2**). This divergence suggests that the translocation of oysters adapted to their native environmental conditions may result in reduced survival and poor performance when introduced into non-native environments, potentially compromising restoration success. Similar patterns have been observed in the Olympia oyster (*Ostrea lurida*), where local adaptation occurs even between neighboring estuaries, and survival was highest in oysters of local origin (Bible & Sanford, 2016). Conversely, harvesting of foreign oysters that may perform poorly under non-native environmental conditions could further deplete source populations without effectively contributing to restoration success, thereby further exacerbating the decline of wild oyster populations. Accordingly, **when population genetic studies reveal limited or absent gene flow or connectivity between populations, these populations should be managed separately, with potential local adaptation explicitly considered in restoration planning.**

Based on the abovementioned findings, using wild Dutch oysters as source populations represents the most suitable restoration strategy for local efforts in the Netherlands, due to their potential adaptation to local environmental conditions and high genetic diversity. Nevertheless, restoration initiatives should be strategically designed and carefully implemented to avoid unnecessary exploitation of remaining wild population stocks. In particular, greater awareness is needed regarding the potential genetic consequences of anthropogenic interference, such as translocations or harvesting (Bromley et al., 2016). Although genetic diversity in wild populations is presently high, excessive harvesting for restoration purposes, combined with emerging environmental stressors such as rising sea surface temperatures or pathogens, could reduce resilience in these populations. As the only remaining native oyster populations in the Netherlands, they require cautious management.

Furthermore, although hatchery-produced populations may exhibit levels of genetic diversity similar to wild populations, aquaculture practices can rapidly reduce this diversity through increased kinship among cultured individuals (**Chapter 2**). Reduced genetic diversity in habitat-forming species is associated with diminished ecosystem resilience (Vásquez et al., 2023). Consequently, the use of cultured oysters to supplement degraded reefs may compromise the long-term sustainability of restoration efforts if genetic diversity is not carefully managed (Hedgecock & Coykendall, 2007; Lallias

et al., 2010a). Therefore, **there is a high need to develop a comprehensive breeding program with standardized guidelines that can be broadly adopted by hatcheries culturing** *O. edulis* **for restoration purposes.**

2. Challenges in biosecurity guidelines and the need to reassess disease status

A major bottleneck for local oyster restoration is posed by biosecurity guidelines which strongly discourage, or in some cases restrict, the translocation of oysters from regions infected with *B. ostreae* to non-infected areas (Ermgassen et al., 2023; Pogoda, 2019). *B. ostreae* is a widespread parasite that causes high mortality in *O. edulis* populations and has been a major driver of the species' dramatic population declines (Mérou et al., 2023). To enable the use of local oyster stocks, restoration efforts often rely on hatcheries to produce oysters free from *B. ostreae* infections (Kamermans et al., 2023; Sas et al., 2020). However, this requires costly pathogen screening of both broodstock and offspring, which currently limits the availability of local oysters and often necessitates the use of foreign stocks (Bos et al., 2023; Kamermans et al., 2023). As such, **while restrictions to the translocation of infected oysters are crucial to prevent further spread of pathogens, current legislation may inadvertently compromise restoration effectiveness by necessitating reliance on foreign oyster stocks.**

Moreover, the most recent assessments of *B. ostreae* infection status across European *O. edulis* populations date back to early 2020s, underscoring the need for an updated evaluation of parasite prevalence (Mérou et al., 2023; Sas et al., 2020). Despite known infected populations along the coastlines of the UK, the Netherlands, and Denmark, the North Sea is currently considered free of *B. ostreae* infection (Sas et al., 2020). This classification likely reflects limited detection rather than confirmed absence of the parasite (Engelsma et al., 2010; Mérou et al., 2023; Sas et al., 2020). Notably, a recent modelling study suggested that offshore restoration sites may still be at risk of future infection due to hydrodynamic connectivity with infected populations (Schmittmann et al., 2026). An updated assessment of *B. ostreae* prevalence would therefore greatly benefit restoration efforts by enabling the potential use of local oyster populations as source material in infected areas. This would circumvent current restrictions on the use of local stocks and avoid suboptimal restoration outcomes caused by the introduction of foreign oysters that may not be locally adapted.

3. Genetic risks in selective breeding of pathogen resistance

More recently, there has been a growing interest in using oysters that have developed genetic resistance to the prevalent pathogen *B. ostreae* for restoration purposes (Kamermans et al., 2023; Sas et al., 2020; Vera et al., 2019; Zu Ermgassen et al., 2023). Specifically, resistance has been associated with a chromosomal inversion on chromosome NC_079172.1, which could potentially be targeted in selective breeding programs (Sambade et al., 2025b, 2026). While increased resistance against this prevalent pathogen could greatly improve survival and is therefore relevant to the conservation of *O. edulis*, selectively breeding homozygous inversion genotypes to enhance resistance may pose risks to the preservation of overall genetic diversity, as increased homozygosity can rapidly reduce genetic variation (Launey & Hedgecock, 2001). Chromosomal inversions often cause impaired recombination in heterozygotes and may act as supergenes carrying co-inherited polymorphisms that can have both adaptive or maladaptive effects in different environmental conditions (Wellenreuther et al., 2025; Zhang et al., 2021). Such impaired recombination among heterozygotes was observed for all three known chromosomal inversions in the *O. edulis* genome (Chapter 3). As inversion genotypes become homozygous, recessive deleterious variants hidden within the inversion may be expressed alongside beneficial genes, referred to as genetic load (Launey & Hedgecock, 2001). Such deleterious alleles may become particularly harmful under stressful environmental conditions, as elevated levels of inbreeding reduce overall fitness (Bijlsma & Loeschcke, 2012; Plough & Hedgecock, 2011; Reed et al., 2012). Notably, the effectiveness of purifying selection, a mechanism that removes deleterious variants, is reduced in chromosomal inversions, further increasing the fitness risks of genetic load (Wellenreuther et al., 2025). Consequently, selective breeding of homozygote individuals could inadvertently comprise the species' resilience in an environmental context.

In *O. edulis*, developing resistance against *B. ostreae* appears to inflict a trade-off in metabolism and growth, and the immunity response in resistant animals is often only partial (Egerton et al., 2020; Morga et al., 2017). These fitness trade-offs may indicate the presence of deleterious variants, which could explain why this inversion has not reached fixation despite its association with enhanced resistance. For instance, Dutch oyster populations have been exposed to *B. ostreae* since 1980, yet the frequency of inversion homozygote genotypes for the resistance-associated inversion remains relatively low (**Chapter 2**). This low frequency of inversion homozygotes in Dutch oysters was also reported in earlier studies (Kamermans et al., 2023; Sambade et al., 2022). Notably, similar to inversion homozygotes, inversion heterozygotes were found to exhibit lower infection rates, potentially reflecting balancing selection driving the maintenance of heterozygote genotypes across populations (Sambade et al., 2026). Considering the elevated densities of both single nucleotide as well as structural

genetic variation observed within inversion regions, these chromosomal inversions may act as reservoirs of genetic variation (**Chapter 3**). The maintenance of such variation by balancing selection is consistent with patterns frequently observed in marine invertebrates characterized by large effective population sizes and high levels of genetic diversity (Gerdol et al., 2020; Hua et al., 2023).

Moreover, the highly polymorphic genomes observed across *O. edulis* individuals and populations (**Chapter 2** and **Chapter 3**), is suggestive of the species' capacity for local adaptation driven by standing genetic variation. Focusing primarily on the conservation of a single adaptive trait, such as pathogen resistance, risks overlooking the species' potential resilience to other current or future environmental challenges, including emerging diseases. **It is therefore imperative to adopt a long-term restoration strategy that prioritizes the preservation of genetic diversity and, with it, the adaptive potential of the species** (Derry et al., 2019). Maintaining this diversity is key to sustaining resilient oyster populations across the species' environmental distribution range under present and future environmental conditions. Consequently, selective breeding for pathogen resistance requires careful consideration, as it may compromise overall environmental resilience.

RQ2: How are epigenetic patterns associated with sex reversal in O. edulis, and how can these insights support the development of non-lethal approaches for sex identification?

Sex represents an important population demographic as balanced sex ratio's ensure preservation of genetic diversity by equal contribution of males and females to the genetic pool (Xu et al., 2019). In *O. edulis*, sex reversal is highly responsive to environmental stimuli, and abiotic factors such as photoperiod or elevated water temperatures can induce sex reversal regulated by epigenetic mechanisms (Joyce et al., 2013; Zapata-Restrepo et al., 2019). In large populations, asynchronous gametogenesis is thought to act as a plastic mechanism, allowing flexibility and resilience in reproductive output across spatiotemporally varying environments (Da Silva et al., 2009). However, in small populations, such as recently established or cultured populations, this asynchronous spawning often leads to male-biased sex ratios which can reduce reproductive output and increase the risk of genetic diversity loss (Diaz-Almela et al., 2004; Hedgecock et al., 2007; Kamphausen et al., 2011). This underscores the relevance of elucidating the epigenetic mechanisms underlying sex reversal in the context of species conservation.

As balanced sex-ratios contribute to the preservation of genetic diversity across generations, aquaculture production would benefit from reliable, non-lethal sex-identification methods that ensure balanced sex-ratios in broodstock populations (Xu et al., 2019). By training machine learning models on the identified sex-specific DNA

methylation patterns (**Chapter 4**), 14 epigenetic markers were identified that may enable non-lethal molecular sex identification (**Chapter 5**). The adopted machine learning approach demonstrated the ability to distinguish hermaphrodite oysters from non-hermaphrodites, and allowed for classifying male-biased and female-biased oysters. As these genetic markers capture DNA methylation differences associated with sex phenotypes in gill tissue, an assay targeting these markers could enable the study of sex reversal in live oysters via non-lethal gill sampling using anesthetics, similar to the protocol provided by Kamermans et al., (2023).

Determining the ratio of hermaphrodite to non-hermaphrodite oysters could provide an indication of the number of individuals currently undergoing gametogenesis (Da Silva et al., 2009), thereby serving as a proxy index for reproductive activity of a broodstock population. Such insights could provide valuable opportunities for the species' aquacultural production. Gametogenesis is an energetically demanding process due to enhanced cell growth and cellular restructuring (Frolov & Pankov, 1992; Hassan et al., 2018). Oysters undergoing gametogenesis are suggested to exhibit epigenetic reprogramming to meet the associated energetic requirements, which may temporarily reduce metabolic activity or immune responses (Todd et al., 2019). This pattern was also reflected by the differential DNA methylation observed in somatic gill tissue of hermaphroditic oysters, particularly affecting genes involved with regulating energy balance and innate immunity (**Chapter 4**). Accordingly, adjusting broodstock management to the physiological needs of individuals undergoing gametogenesis (e.g. adapting temperature or feeding regimes) could improve reproductive output.

Additional models also showed the potential for classifying male- and female-biased individuals with moderate accuracy (**Chapter 5**). Utilization of these markers may provide valuable insights into expected reproductive success and the estimated genetic diversity of offspring. For these reasons, further validation of the epigenetic markers across a wider range of sex phenotypes is highly relevant.

RQ3: In what ways can molecular approaches be applied to support ecosystem-scale biodiversity monitoring and restoration management in the North Sea?

Effective oyster restoration is not solely determined by the successful recovery of the flat oyster as a species, but is particularly motivated by the restoration of native biodiversity and associated ecosystem functioning and services (Beck et al., 2011; Colsoul et al., 2021; Ermgassen et al., 2023). Among the most important ecosystem services provided by oyster reefs is their capacity to support and sustain biodiversity (Smyth & Roberts, 2010; Thomas et al., 2022). Generally, biodiverse marine ecosystems are associated with increased biomass of commercially important fish species and enhanced ecosystem resilience (Gilby et al., 2018; Hughes et al., 2005; Humphries

& La Peyre, 2015). It is expected that insights in restored biodiversity can increase stakeholder inclusion and connection by illustrating the value of restoration efforts, as well as inform adaptive management to optimize ecosystem service return from restoration projects (Thomas et al., 2022; zu Ermgassen et al., 2021). Therefore, to evaluate restoration effectiveness, biodiversity monitoring should be prioritized.

The advent of molecular approaches has revolutionized biodiversity assessments by enabling the analysis of environmental DNA (eDNA), providing a promising alternative to morphological methods, which are time- and cost-intensive (Blackman et al., 2024). Furthermore, molecular approaches can elevate biodiversity assessments by enabling a more holistic evaluation of ecosystem functioning, capturing species interactions, and detecting environmental responsiveness (**Chapter 6**). For instance, metatranscriptomics allows the assessment of active biodiversity by distinguishing viable and non-viable organisms, providing a current snapshot of community composition (Bass et al., 2023; Miyata et al., 2022). In addition, molecular methods provide opportunities for monitoring pathogens, for instance the detection of B. ostreae from fecal samples (Regan et al., 2025). Beyond biodiversity, novel molecular approaches can reveal ecosystem functioning through the identification of stress markers (Hechler et al., 2023; Miyata et al., 2025), or capture DNA methylation patterns to infer population demographics, such as sex ratios (**Chapter 5**) or age (Zhao et al., 2023). As such, this multi-omics approach provides an integrative framework for assessing ecosystem dynamics and function.

The growing availability of molecular methods also introduces new challenges. Methodological differences among approaches can hamper comparability and integration across studies (Laamanen et al., 2025). In this thesis, several recommendations are proposed to facilitate the integration of molecular methods into standardized monitoring of ecosystem biodiversity, functioning, and structure (**Chapter 6**). These include: (i) comprehensive archiving of metadata to improve data curation, to facilitate integration across experiments, and to enhance the interoperability and reproducibility between molecular and conventional monitoring, (ii) establish data-sharing guidelines to enable standardization and comparison among studies, and (iii) make all molecular data publicly available to enable comprehensive analysis of ecosystem biodiversity and functioning. For oyster restoration specifically, such data-sharing guidelines are provided in the monitoring handbook (Zu Ermgassen et al., 2021).

Furthermore, advanced data modelling approaches, supported by artificial intelligence and machine learning, are expected to facilitate the integration of molecular and morphological datasets and improve interpretation of complex multi-omics data, thereby supporting their incorporation into standardized monitoring

frameworks (**Chapter 6**). Ecological models could also be applied to estimate ecosystem resilience, predict responses to future environmental conditions, and assess the expected success of restoration projects across spatial and temporal scales. **Overall, integrating molecular approaches into monitoring frameworks can substantially improve the resolution at which restoration success is evaluated and should be prioritized, thereby enabling detailed assessments of biodiversity, functioning and dynamics in North Sea ecosystems.**

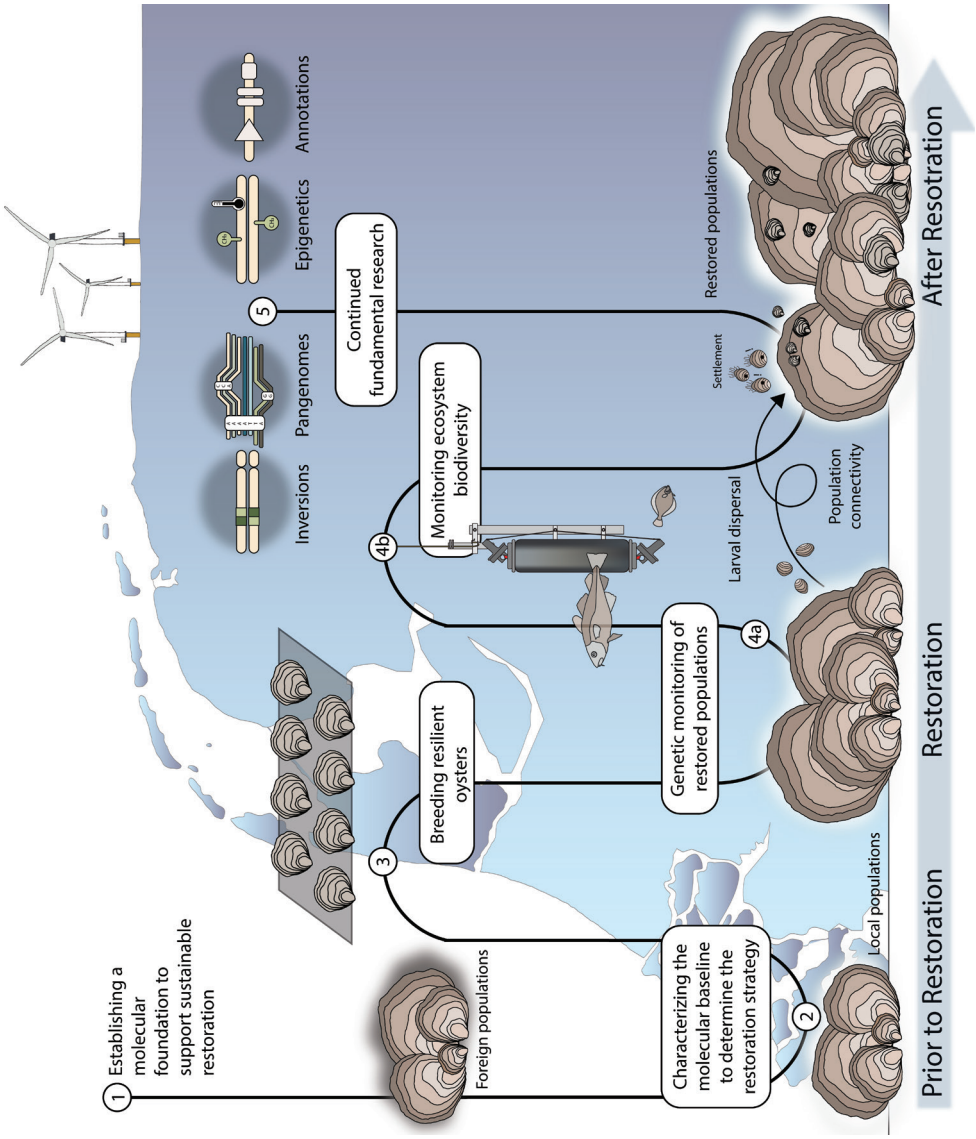


Figure 2 | An integrative roadmap presenting stepwise guidelines for incorporating molecular approaches to support sustainable native oyster restoration in future scenarios.

Guidelines for molecularly informed oyster restoration

1. Establishing a molecular foundation to support sustainable oyster restoration

The findings presented in this thesis reveal the extensive genomic and epigenomic variation present in *O. edulis*, reflecting the adaptive potential crucial for sustaining resilient populations under future environmental conditions (**Chapter 2-5**). However, observations of genetic divergence and elevated kinship raise concerns regarding the preservation of genetic diversity in various restoration strategies. To support resilient oyster populations and maintain genetic diversity, it is essential that molecular insights, such as those generated in this thesis, are more actively integrated into applied restoration efforts (**Chapter 6**). Building on these findings, an integrative roadmap is presented, in which all subsequent steps build upon the foundational first step: (1) Establishing a molecular foundation to support sustainable oyster restoration (Fig. 2.1). This first step has already been presented in the first part of the general discussion (**Chapter 7**), where the main findings of this thesis are synthesized in the context of relevant literature. From this foundation, subsequent steps follow logically, highlighting key priorities: (2) Characterizing the molecular baseline of local and source populations to determine the restoration strategy, (3) Breeding resilient oysters, (4) Assessing restoration effectiveness, and (5) Continuing fundamental research. Together, these priorities provide clear guidelines for the integration of molecular approaches into active and applied oyster restoration.

2. Characterizing the molecular baseline of local and source populations to determine the restoration strategy

Building on the molecular foundation established in this thesis (**Chapter 2-5**), **future restoration strategies should incorporate insights in genetic population structure to characterize the genetic background of both remnant and potential source populations** (Fig. 2.2). This approach is expected to help determine how restoration strategies can maximize restoration success (oyster survival and performance), while preserving genetic diversity and potential local adaptation. Practically, if source populations used in a restoration project have not yet been genotyped, efforts should be made to genotype them and assess their genetic similarity or divergence relative to the remaining local populations. Wherever feasible, restoration efforts should prioritize the use of local oyster populations as source stock due to their potential for local adaptation, provided they contain sufficient genetic variation comparable to wild populations. In the absence of remnant local stocks, source oysters should be selected from the nearest available populations or those exhibiting the minimal genetic divergence from the target restoration site.

3. Breeding resilient oysters

In a restoration context, the preservation of genetic diversity is crucial to sustain resilient populations and should be prioritized at all times, regardless of the restoration strategy employed (Engelhardt et al., 2014; Lallias et al., 2010a; Vásquez et al., 2023). However, the risk of genetic diversity loss is particularly apparent in aquacultural breeding, where broodstock sizes are typically much smaller than in natural populations (Hedgecock et al., 2007). This risk is further amplified by the sweepstakes reproductive success of flat oysters, in which only a small proportion of individuals disproportionally contribute to the next generation. Even in wild populations, the effective number of contributing parents can be as low as 10-20 individuals (Hedgecock et al., 2007). Additionally, skewed sex ratios, which are often male-biased, may further exacerbate the loss of genetic diversity (Diaz-Almela et al., 2004; Lallias et al., 2010b). Consistent with these established genetic risks in aquaculture-based production systems, cultured hatchery oysters we found to exhibit elevated levels of kinship, quantified through increased relatedness and identity-by-descent (**Chapter 2**). Although inbreeding coefficients were similar to those observed in wild populations, breeding of closely related individuals can rapidly lead to inbreeding within only a few generations. Given these genetic risks, several priorities are proposed to help preserve genetic diversity and environmental resilience in aquaculture practices used for oyster restoration (Fig. 2.3).

(i) Maintain a large broodstock population

Given the generally low number of effective parents in *O. edulis* populations, it is important to provide optimal conditions that maximize fertilization events (Hedgecock et al., 2007). Therefore, maintaining a large broodstock population, ideally around 100 oysters, is essential to preserve genetic diversity and limit inbreeding depression (Alves Monteiro et al., 2022; Frankham et al., 2014).

(ii) Accurately record reproductive activity and output

The accurate and comprehensive reporting of reproductive activity (e.g. which oysters reproduced as females, the number of larval releases during the reproductive period, or the quality of larvae in terms of size and swimming activity), along with a detailed reporting of the associated rearing conditions (e.g. water temperature, feeding regime, and day-night cycle) can provide valuable insights into patterns of reproductive behaviour and help identify efficient culturing practices (Wang, 2018). Additionally, documenting the origin of larvae or spat (e.g. broodstock population or reproductive cohort) used in restoration efforts may facilitate the tracking of kinship relationships among oysters derived from the same broodstock source across multiple restoration efforts (Hedgecock et al., 2007). Accurate documentation of breeding practices can inform sustainable restoration strategies and guide decisions on

broodstock renewal or the use of alternative hatchery sources to minimize kinship among oysters deployed across restoration efforts.

(iii) Mix larvae or spat from multiple reproductive batches

A study comparing kinship values in different reproductive cohorts from the same broodstock populations has shown that estimations of identity-by-descent can vary substantially between reproductive cohorts (Tonk et al., 2025). This variation is closely related to the number of contributing parents, which can differ substantially among reproductive events (Hedgecock et al., 2007; Lallias et al., 2010b). To minimize high kinship levels and preserve genetic diversity across restoration efforts, it is therefore recommended to mix reproductive batches from multiple parents or combine larval releases from different cohorts.

(iv) Genetic monitoring of broodstock and offspring

To maintain high genetic diversity in an aquacultural context, it is essential to monitor genetic diversity parameters in both broodstock and offspring populations over temporal scales (Colsoul et al., 2021; Diaz-Almela et al., 2004; Ermgassen et al., 2023). Recently, hatcheries routinely sacrifice a subset of broodstock and offspring oysters to screen for *B. ostreae* infections (Kamermans et al., 2023). Tissue samples collected for this purpose could concurrently be used for genetic analyses. As such, genetic monitoring can provide valuable insights into parentage dynamics and relatedness patterns, and enable the early detection of aquaculturally-induced genetic bottlenecks potentially leading to a loss of genetic diversity or inbreeding (Alves Monteiro et al., 2022; Hedgecock et al., 2007; Lallias et al., 2010b).

(v) Maintain a balanced sex ratio

Upon further validation and testing of the proposed epigenetic markers (**Chapter 5**), a standardized methodology should be developed for non-lethal sex identification. Such an assay could be widely applied in aquaculture to determine sex ratios in broodstock populations, help estimate the genetic impact of these ratios on the offspring, and facilitate targeted selection of specific sex phenotypes to balance sex ratios. Achieving a balanced male-to-female ratio ultimately helps preserve genetic diversity across generations (Colsoul et al., 2021; Joyce et al., 2013).

4. Assessing restoration effectiveness

One of the most straightforward steps following a restoration event is the evaluation of its effectiveness. Successful restoration can be quantified through various aspects, including the performance of the restored oysters, as well as the overall biodiversity and functioning of the restored ecosystem (zu Ermgassen et al., 2021). To properly evaluate these aspects, accurate monitoring assays should be implemented. This section discusses different approaches to evaluate restoration effectiveness and highlights how molecular monitoring methods can be incorporated to support the assessment of such effectiveness (Fig. 2.4).

a. Genetic monitoring of restored populations

Reciprocal translocations of genetically divergent populations may homogenize genetic population structure and inadvertently constrain the development of adaptation to local environmental conditions, including emerging pathogens (Bromley et al., 2016). Consequently, ongoing molecular monitoring of genetic population structure following restoration is pivotal for maintaining high genetic diversity. Implementing such monitoring also provides valuable insights into evolutionary dynamics, including detecting early shifts in inversion genotype frequencies or the occurrence of genetic bottlenecks, which can reveal cryptic environmental stressors that might otherwise remain undetected (Launey et al., 2001; Sambade et al., 2025a). Therefore, **it is recommended that genetic monitoring of restored oyster populations is conducted after restoration events to evaluate molecular consequences and assess restoration effectiveness in preserving genetic diversity** (Fig. 2.4a). One practical approach to achieve this would be to integrate genetic metrics into a revised version of the monitoring handbook, thereby directly incorporating genomic insights into restoration planning (zu Ermgassen et al., 2021). If genetic monitoring reveals a decline in diversity within restored populations, future restoration strategies should be reassessed to account for the potential genetic impacts of the respective restoration effort.

b. Monitoring ecosystem biodiversity

Sustainable oyster restoration would greatly benefit from high-resolution biodiversity assessments capable of quantifying the recovery of biodiversity and ecosystem complexity over the trajectory of a restoration event. Accordingly, it is crucial that molecular approaches are integrated into standardized monitoring of biogenic reef ecosystems to evaluate restoration effectiveness at the ecosystem scale. For oyster restoration in particular, **this entails assessing marine biodiversity during and after restoration events, ideally using a combination of complementary molecular and morphological approaches** (Fig. 2.4b).

5. Continuing fundamental research

The extensive (epi)genomic variation in *O. edulis* characterized in this thesis (**Chapter 2-5**) reflects the species' molecular plasticity and adaptive potential, underscoring the need for continued fundamental research alongside applied restoration to elucidate the mechanisms underlying its persistence under dynamic environmental conditions (Fig. 2.5). Below, several key avenues for fundamental research are highlighted that improve our understanding of the interplay between molecular architecture and environmental responsiveness in *O. edulis.*

Inversion polymorphisms

Inversion polymorphisms are associated with local adaptation in a wide range of organisms (Berdan et al., 2023; Wellenreuther et al., 2025; Zhang et al., 2021). In *O. edulis*, inversion genotype frequencies were highly polymorphic across European populations (**Chapter 2**), and contributed substantially to shaping genetic population structure of both single-nucleotide variation (**Chapter 2**) and structural variation (**Chapter 3**). A comprehensive understanding of their role in facilitating local adaptation would therefore be highly informative for future conservation efforts. This is particularly relevant as balancing selection may maintain different inversion genotype frequencies across *O. edulis*' geographical range which could warrant for separate management of local populations. Conducting a genome-wide association study (GWAS), ideally combined with a common-garden experiment in which oysters containing varying inversion genotype frequencies are exposed to different environmental conditions, could provide a deeper insight into the functional and phenotypic consequences of these inversions.

Pangenomes

In this thesis, different levels of genetic and epigenetic variation were characterized by mapping reads against the xbOstEdul1.1 reference genome assembly provided by Adkins et al. (2023) (**Chapter 4**). However, the primary focus on a single reference genome can cause a "reference bias" (Günther & Nettelblad, 2019), and risks to oversee potential genetic diversity across populations, as the lack of a variant in the reference genome can result in poor alignment of the mapped reads (Secomandi et al., 2025). Pangenomes provide the solution to this bias and can be defined as the collective genomic diversity across sequenced individuals or populations of a species. Pangenomes are characterized by a "core genome" containing variants and genes shared by all individuals or populations, whereas the "dispensable genome" represents variants or genes unique to a subset of individuals (Medini et al., 2005). By comprehensively capturing genomic diversity across a species' range, pangenomes enhance our understanding of how genomic variation underlies phenotypic

plasticity and environmental adaptation (Brito et al., 2016; McInerney et al., 2017). More recently, reporting of invertebrate pangenomes is gaining momentum and has revealed significant contribution of dispensable genes to the mussel pangenome, exceeding dispensable gene contribution in vertebrates by more than five times (Gerdol et al., 2020). The same study also reported on 25% of genes on the mussel genome to be subject to gene presence-absence variation (PAVs). PAVs often occur in regions enriched with transposable elements (TEs) (Bush et al., 2014), suggesting the key role TEs play in generating pangenomic variation. Active repeats were also shown to substantially contribute to genomic variation in the *O. edulis* genome (**Chapter 3**), further emphasizing the importance of studying genetic variation in a pangenomic context.

Given the genomic polymorphism and extensive hemizygosity in mollusks, generating pangenomes should be prioritized to enhance our understanding of evolutionary processes underlying genomic variation (Calcino et al., 2021). Such an initiative aligns closely with the goals of the Native Oyster Restoration Alliance (NORA), where the preservation of genetic diversity is highlighted as a key priority (Pogoda et al., 2019). The NORA genetics working group could lead this initiative, facilitating a new era of fundamental genetic research in *O. edulis*.

Beyond genetics and genomics

Beyond the scope of genomics, *O. edulis* displays substantial interindividual variation across the epigenome associated with different sex phenotypes (**Chapter 4**). Epigenetic mechanisms enable regulatory flexibility in gene transcription and thereby facilitate phenotypic plasticity as a response to environmental stimuli (Ecker et al., 2018; Roberts & Gavery, 2012; Vogt, 2022). These environmentally induced epigenetic variations allow organisms to endure stressful environmental conditions, providing a crucial coping mechanism in sessile marine invertebrates exposed to dynamic environments (Dang et al., 2023; Fallet et al., 2020; Männer et al., 2021; Sun et al., 2023).

Epigenetic variation is strictly genetically determined whereby specific genes encoding for several enzymes (e.g. DNA methyltransferases or ten-eleven translocation enzymes) and proteins (e.g. Polycomb protein complex) are mainly responsible for regulating epigenetic modifications (Lyko, 2018; Rey et al., 2020). However, methylated cytosines have the tendency to mutate into thymines with abnormal frequency, if not actively controlled through repair mechanisms (Bellacosa & Drohat, 2015; Bird, 1980). As such, methylation-rich areas can quickly aggregate mutations and consequently alter the genotype, hence the reciprocal interplay between genetics and epigenetics. Many studies have reported on epigenetic variation substantially exceeding genetic variation between populations (Chapelle & Silvestre, 2022; Johnson & Kelly, 2020;

Skinner et al., 2014). The higher spontaneous mutation rate of the epigenome along with the potential transgenerational inheritance of some epigenetic marks collectively support evidence indicating that epigenetic variation can be subject to natural selection and thereby provides a parallel trajectory contributing to evolution (Chapelle & Silvestre, 2022; Rondon et al., 2017). One study even provides evidence for accelerated environmental adaptation when selection primarily acts on epigenetic variation (Klironomos et al., 2013). Accordingly, there is substantial evidence that phenotypic plasticity and local adaptation do not solely rely on genetic variants but can also be shaped by environmentally induced epigenetic variation. Consequently, the observed substantial epigenomic interindividual variation (**Chapter 4**) warrants for generating an epi-pangenome. Assembling epigenetic variation across individuals could potentially reveal environmentally induced epimutations. For instance, comparing genome-wide DNA methylation among four populations of the Eastern oyster (*Crassostrea virginica*), revealed significant epigenetic population structure that was suggested to enhance phenotypic plasticity (Johnson & Kelly, 2020). Moreover, comparative analysis could identify core and dispensable methylation marks reflecting the epi-pangenome's openness, thereby providing insights into the conserved or flexible downstream regulation of traits along environmental clines (Zhong, 2016). As such, elucidating epigenomic variation across phenotypes or populations could provide deeper fundamental insights into phenotypic plasticity and evolutionary concepts, both of which are extremely relevant in a restoration and conservation context (Rey et al., 2020).

Functional annotation

One key challenge in linking genetic insights to local adaptation is the limited quality of genome annotations, or incomplete transcriptomics data from closely related species, particularly in non-model organisms (Lopez et al., 2019). Improving genome annotations and gene function prediction would greatly benefit genetic studies currently conducted in *O. edulis*. For instance, identifying the functions of genes located within chromosomal inversions could help clarify their adaptive potential. Therefore, more detailed and high-resolution genome annotations are essential to advance the fundamental understanding of evolutionary dynamics in this marine bivalve.

The non-model organism renaissance

Advances in sequencing technologies and bioinformatic analyses now enable high-resolution characterization of molecular variation, even in non-model organisms such as *O. edulis* (**Chapter 2-4**). While this study focused specifically on the native European flat oyster, the approaches employed and the insights into evolutionary dynamics are broadly applicable to other marine bivalves. Many bivalve species exhibit highly polymorphic genomes and large effective population sizes (Calcino et al., 2021; Gerdol et al., 2020). Additionally, particular life-history characteristics, such as sequential hermaphroditism, are widespread among bivalves (Feng et al., 2023; Nicolini et al., 2023; Picard et al., 2021). While each species has its own history of environmental challenges, broad descriptions of evolutionary patterns in one species can inform our understanding of similar processes in others, and as such guide restoration or conservation strategies on a broader ecosystem level.

Concluding remarks

Taken together, this thesis provides a molecular foundation to support the sustainable restoration of *O. edulis*. By integrating insights from population genomics, structural variation, and epigenetics, this work contributes to a more comprehensive understanding of the species' intraspecific genetic and epigenetic architecture. Elucidating such molecular insights is crucial for understanding evolutionary dynamics, ultimately supporting the development of sustainable and effective restoration strategies aimed at maintaining genetic diversity and ecological resilience on local scales.



APPENDICES

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SUMMARY

The European flat oyster (*Ostrea edulis*) is an ecologically and economically important bivalve that has been subjected to a long history of human exploitation. By forming biogenic reefs, *O. edulis* plays a key role in providing benthic habitat and thereby contributes to overall marine biodiversity. Rapid population declines from the beginning of the 20th century have incentivized restoration initiatives to restore flat oyster populations and their habitats. Since then, a growing number of studies focused on the genetic characterization of *O. edulis*, revealing phylogeographical population structure across its native distribution range. However, as outlined in **Chapter 1**, most genetic studies cover large spatial scales and therefore fail to capture fine-scale genetic population structure. Moreover, the majority of research has focused on single-nucleotide polymorphisms (SNPs), the most commonly studied form of genetic variation. Other sources of variation, such as structural variants (SVs), remain comparatively understudied despite their potential to reveal evolutionary dynamics relevant for restoration. This thesis therefore aims to fill this fundamental knowledge gap by elucidating intraspecific (epi)genetic diversity of *O. edulis* in a restoration context.

In **Chapter 2**, genetic population structure was assessed among local Dutch populations in the context of restoration efforts by genetically comparing foreign and locally cultured individuals with wild Dutch oysters. The remaining Dutch wild populations comprised a genetically diverse panmictic cluster with high gene flow. In contrast, foreign populations used as a source in Dutch restoration projects exhibited genetic divergence from wild populations. Although hatchery-produced oysters displayed high genetic similarity with Dutch wild oysters, elevated levels of kinship highlight the potential risk of inbreeding. Accordingly, future restoration strategies involving the supplementation of degraded reefs with translocated foreign or locally cultured oysters require careful consideration to optimize restoration effectiveness while preserving genetic diversity.

In addition to single-nucleotide variation, SVs represent an important source of genetic diversity, with recent advances in long-read sequencing and bioinformatic enabling their comprehensive characterization. In **Chapter 3**, the SV landscape of the *O. edulis* genome was characterized, demonstrating that SVs complement SNPs in capturing genetic diversity and population structure. In addition, overlap with repeat elements revealed the activity of repeats in generating genome-wide variation. Previously identified chromosomal inversions exhibited elevated densities of SVs and SNPs, suggesting that inversions act as reservoirs of genetic variation and may facilitate local adaptation. Overall, SVs play a key role in shaping genomic population structure, highlighting their relevance for the conservation of *O. edulis*.

Beyond genetic variation, epigenetic variation contributes to phenotypic plasticity by influencing (post-)transcriptional gene regulatory mechanisms. Sex reversal, one such plastic trait, is primarily driven by environmental factors and complicates culturing of *O. edulis* for restoration purposes. In **Chapter 4**, the association between epigenetic mechanisms and sex reversal was assessed by comparative analysis of genome-wide patterns of DNA methylation in gill tissue across sex phenotypes. This revealed hermaphrodite oysters displayed a distinct DNA methylation profile compared to male and female oysters, likely reflecting epigenetic remodeling involved with regulating energy homeostasis and metabolism following sex reversal.

Following characterization of sex-specific DNA methylation across sex phenotypes in **Chapter 4**, **Chapter 5** adopts a machine learning approach to select epigenetic markers for sex classification. The 14 proposed markers enabled classification of hermaphrodite versus non-hermaphrodite oysters, and distinguished male-biased from female-biased oysters with moderate accuracy. Upon further validation, these markers pave the way for studying sex reversal in live oysters, with important implications for culturing *O. edulis*.

Advances in sequencing approaches and bioinformatic pipelines have expanded the scope of molecular research, enabling assessment of ecosystem biodiversity and functioning in less invasive and time-consuming ways compared to morphological methods. **Chapter 6** explored how molecular approaches can be integrated into standardized monitoring frameworks. By taking an ecosystem-wide perspective, current molecular approaches for assessing North Sea biodiversity and ecosystem functioning were reviewed. Additionally, challenges and areas of improvement are discussed, providing a baseline understanding of the current state of molecular monitoring. Recommendations for integrating these tools into standardized marine monitoring programs of North Sea biodiversity are expected to support management and restoration of North Sea ecosystems.

In addition to the fundamental characterization of the (epi)genetic architecture of *O. edulis* in a restoration context (obtained in **Chapters 2** – **5**), it is essential that these molecular insights inform applied oyster restoration strategies to optimize restoration effectiveness while preserving genetic diversity. Therefore, the final chapter (**Chapter 7**) discussed how molecular insights into population structure and evolutionary dynamics can be integrated in active oyster restoration. An integrative roadmap is presented, offering practical guidelines for incorporating molecular insights into locally tailored restoration strategies to support the effective and sustainable restoration of *O. edulis* and their biogenic reef habitats.

Author contribution statement

Chapter 1

This chapter was written by SV and feedback was provided by AM, PK, and RN.

Chapter 2

SV, HJM, PK, and LT conceptualized the study. SV and LT performed DNA extractions and sample preparations, SV and HJM carried out downstream data analysis. The manuscript was written by SV and feedback was provided by HJM, PK, and LT. PK and SV acquired funding for the study.

Chapter 3

SV, HJM, and MKG conceived the study. SV and MKG developed the methodology and performed SV calling, while downstream analyses were carried out by SV under the supervision of MKG. SV wrote the original draft of the manuscript, which was reviewed and edited by HJM, RN, and MKG. All authors contributed to the interpretation of the results and approved the final manuscript.

Chapter 4

SV, HJM, and RN conceptualized the study. SV carried out the methodology and sequencing. Data analysis was performed by SV and HJM. Writing of the manuscript was done by SV, and reviewing and editing of the manuscript was carried out by SV, ME, HJM, PK, AM, and RN. RN was involved in supervision of the research. SV and PK acquired funding.

Chapter 5

SV and RN conceptualized the study, SV performed the laboratory work, conducted data analysis, and wrote the manuscript. PK, ATM, and RN revised the manuscript. All authors approved the final version of the manuscript.

Chapter 6

KD, ASH, and SV wrote the first draft of the manuscript. All authors contributed to writing and reviewing of the final manuscript.

Chapter 7

This chapter was written by SV and feedback from AM, PK, and RN was provided.

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It goes without saying that this PhD, both the thesis itself and the journey leading up to it, would not have been complete, or nearly as meaningful, without the support of the many people who accompanied me along the way. Rather than feeling as though they contributed to my PhD, it feels more like a collective effort, where I did not do this PhD alone, but with everyone. For that I am really grateful. With this in mind, I would like to dedicate the next few pages to expressing my gratitude to everyone (I sincerely hope that I am not forgetting anyone), who has contributed to this journey, academically, personally, or in any other way. To all of you, thank you.

Pauline, thank you for taking on the supervision of my PhD project when we did not really know each other yet. I feel we have grown much closer over time, and I really enjoy working with you. I am grateful for introducing me to the wider oyster (NORA) network, were you are rightfully regarded as the "Oyster Godmother". I am very proud of what we have achieved together in pushing for the inclusion of "genetics" in the Dutch oyster restoration network. It has been a real pleasure to share that effort with you, and I am looking forward to continue this work as proper colleagues at WMR.

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ABOUT THE AUTHOR

Marie Sophie Valk was born on 27 November 1998 in Zwolle, the Netherlands. From an early age, she was fascinated by the sea and its diverse marine life. In 2019, she obtained her BSc in Biology from Wageningen University, where she continued her studies with an MSc in Aquaculture and Marine Resource Management. During her master's program, she investigated the diet of herring and sprat using a DNA metabarcoding approach. This thesis project provided her with the opportunity to participate in several stow-net survey expeditions on the Wadden Sea. It also taught her that ecological questions could be addressed with molecular methods, sparking lasting interest in molecular ecology.

In March 2022, she began her PhD at the Marine Animal Ecology chair group of Wageningen University & Research. Although the project initially focused on resolving settlement behaviour in *Ostrea edulis* larvae, its direction quickly shifted towards a more fundamental investigation of intraspecific genomic diversity using molecular approaches. Through this work, she discovered a passion for uncovering the molecular mechanisms and architecture underlying phenotypic plasticity and adaptation in marine organisms facing environmental change. Her fascination with the marine environment continues to inspire her scientific curiosity and motivates her commitment to advancing nature conservation through both her current and future scientific career.



Sophie.valk.1998@gmail.com

SCIENTIFIC PUBLICATIONS

Valk, S., Engelsma, M., Megens, H.-J., Kamermans, P., Murk, A. J., and Nijland, R. 2025. Oysters in Transition: Hermaphrodite Oysters Display Unique DNA Methylation Patterns in Gill Tissue. *BMC Genomics* 26 (1): 567. https://doi.org/10.1186/s12864-025-11736-1.

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Kamermans, P., Anteau, F., Didderen, K., Ter Hofstede, R., Zhao, Y., Le Graet, A., Maas, D., Pouvreau, S., **Valk, S.**, Wijgerde, T., Zempleni, A., Kodger, T. E., & Murk, T. (2025). Novel settlement substrates for European flat oyster (*Ostrea edulis*) restoration. *Ecological Engineering, 212*, 107532. https://doi.org/10.1016/j.ecoleng.2025.107532

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Doorenspleet, K., van den Brink, X., **Valk, S.**, Coral, C., Coolen, J. W. P., Murk, A. J., & Nijland, R. (2026). The potential of eRNA to assess local vertebrate biodiversity in North Sea bivalve reefs. *BMC Marine Science*. In review.

Couperus, B., Nijland, R., Tulp, I., Van Berkel, D., & **Valk, S**. (2022). Dieet van jonge haring in het Eems-Dollard gebied in het najaar: Een verkennende studie door middel van DNA metabarcoding – nanopore sequencing. Wageningen Marine Research. https://doi.org/10.18174/580829

Tonk, L., Kamermans, P., **Valk, S**., & Megens, H.-J. (2025). Genetic characterization of hatchery produced Ostrea edulis and suitability for use in Dutch oyster restoration programs: BO-43-221-021 report NN Flat oyster production in hatcheries. Wageningen Marine Research. https://doi.org/10.18174/705709

TRAINING AND SUPERVISION PLAN

With the activities listed, the PhD candidate has met the educational requirements set by the Graduate School of Wageningen Institute of Animal Sciences (WIAS). One ECTS equals a study load of 28 hours.

The Basic Package (1.7 ECTS)

WIAS Introduction Day (2022)
Scientific Integrity course (2022)
Ethics in Animal Sciences course (2022)

Disciplinary Competences (13.8 ECTS)

Writing Research Proposal (2022)
University of Gothenburg: Unix applied to genomic data (2022)
University of Gothenburg: Analysis of next generation sequencing data (2022)
Advanced Statistics course Design of Experiments (2023)
Ecology and evolution of phenotypic plasticity (2024)
Python programming for PhDs (2025)

Professional Competences (10.4 ECTS)

Reviewing a scientific manuscript (2022)
Adobe Illustrator: Scientific Artwork & Info
Adobe Indesign: Scientific Publications & Layout (2025)
Scientific Publishing (2024)
Scientific Writing (2024)
Writing Grant Proposals (2024)
Efficient writing Strategies (2023)
Biodiversity Data Workshop: Boosting the impact of biodiversity data (2024)
NORA5 Student Group Session - organize session during conference (2023)
NORA6 Student Group Session - organize session during conference (2025)

Societal Relevance (1.8 ECTS)

Societal impact of your research (2024)
Presentation during NERA (Nederlandse Rifherstel Alliantie) netwerkdag (2024)

Presentation Skills (4 ECTS)

Een nieuwe basis voor platte oester herstel in de Noordzee, de Noordzeedagen, Wageningen, the Netherlands (2022)
Genetic diversity of European flat oytser populations by UMI-tagged amplicon sequencing, London Calling, London, the United Kingdom (2023)
Analysing epigenetic mechanisms of sex determination in flat oysters, NORA5, Middelburg, Netherlands (2023)
Sex-specific DNA methylation in European flat oysters, EPIMAR2025, Barcelona, Spain (2025)
Towards non-lethal sex identification in flat oysters, NORA6, Cartagena, Spain (2025)

Teaching Competences (6 ECTS)

Supervising 1 Bsc thesis student (2024)
Supervising 2 Msc thesis students (2024)
Teaching Molecular and Evolutionary Ecology (GEN20306) (2022-2023)
Teaching Advanced Molecular Ecology (MAE40306) (2024)

Total credits 37.7 ECTS

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