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Full-length hybrid transcriptome of the olfactory rosette in Senegalese sole (*Solea senegalensis*): an essential genomic resource for improving reproduction on farms

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Abstract

Senegalese sole is a promising European aquaculture species whose main challenge is that captive-born males (F1) are unable to reproduce in farms, hindering breeding programs. Chemical communication through the olfactory system is hypothesized to stem this issue. Although significant advancement in genomic resources has been made recently, scarce information exists on the genomic basis of olfaction, a special sensory system for demersal species like flatfish, which could play a prominent role in reproduction, social and environmental interactions. A full-length transcriptome of the olfactory rosettes including females, males, juveniles and adults, of both F1 and wild origins, was generated at the isoform-level by combining Oxford Nanopore long-read and Illumina short-read sequencing. A total of 20,670 transcripts actively expressed were identified: 13,941 known transcripts, 5,758 novel transcripts from known genes, and 971 from novel genes. Given the important role of olfaction in reproductive behaviour, we comparatively examined the expression and functional enrichment of the olfactory receptor gene families (OlfC, OR, ORA, and TAAR). Our comprehensive olfactory transcriptome of Senegalese sole provides a foundation for delving into the functional basis of this complex organ in teleost and flatfish. Furthermore, it provides a valuable resource for addressing reproductive management challenges in Senegalese sole aquaculture.

Keywords: Senegalese sole; hybrid transcriptome; olfactory rosette; olfactory receptor gene families.

1. Introduction

Olfaction is a key sense for fish survival, as it plays a crucial role in fundamental behaviours such as reproduction, food detection, and interactions with other individuals and the surrounding environment in a broad sense.[1–4] Chemoreception through olfaction occurs within the olfactory organ, a paired structure known as the olfactory rosette, characterized by its multilamellar and rosette-shaped structure.[5] At cellular level, the olfactory epithelium comprises a complex arrangement of nonneuronal cells (supporting, basal and glandular cells) interspersed with olfactory sensory neurons (OSNs), including ciliated, microvillous, crypt, kappe and pear cell-types, the last two only described in zebrafish.[6–10] Although OSNs seem to express a single receptor gene,[11,12] fish olfactory epithelium needs more in depth investigation which cover its wide diversity, especially regarding intraspecific communication and its relevance for reproduction.

The olfactory receptors expressed in OSNs can detect a wide variety of semiochemical compounds, including pheromones.[13] Although significant progress has been made in olfactory receptor deorphanization in the past decade,[14–18] further research is still needed to elucidate the species-specific molecules that stimulate the different olfactory receptors, especially in nonmodel species.[12,19] OSNs generate electrical signals in response to chemical stimuli that are then transmitted through their neuronal projections, ultimately converging to form the olfactory nerve, which reaches the brain for the first synapse at the level of the olfactory bulbs, the primary integrating centre of olfactory information.[2,20]

Four main olfactory multigene families responsible of chemoperception have been described in teleost: olfactory receptors class C (OlfC), related to the mammalian vomeronasal receptor V2R; odorant receptors (OR); olfactory receptors class A (ORA), related to the mammalian vomeronasal receptor V1R; and trace amine-associated receptors (TAAR).[21–25] Collectively, these families comprise the olfactory gene repertoire, with a wide variation in gene number among fish species.[26,27] Furthermore, a positive correlation has been shown between the structural complexity of the olfactory rosette

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and the size of the olfactory repertoire.[27] While important research has been conducted on the olfactory gene repertoire, most studies have focused on model species such as zebrafish[21,22,28,29] and scarce information is available for other teleost, especially for aquaculture species. Thus, understanding this complex sensory system is a first step towards identifying potential pheromone-related socio-sexual behaviours. Altogether, this field of research provides valuable information for optimizing reproductive strategies and behavioural management in aquaculture.[30]

Flatfish (Pleuronectiformes) constitute a diverse taxonomic group adapted to demersal lifestyle, including some species of great commercial value worldwide.[31–33] The development of genomic resources applicable to aquaculture breeding programs or sustainable fisheries management have greatly increased in the last decade.[34–36] The adaptation to a benthic environment, involving low light radiation and a sediment-rich seabed, made olfaction a critical and highly specialized sense for flatfish.[37]

Senegalese sole (*Solea senegalensis*) is an emerging flatfish aquaculture species in Europe with great market value, which shows high growth and larval survival rates.[38] However, some bottlenecks, such as reproduction issues in captivity, still curtail the expansion of its production. Specifically, males born in farms (F1) present a behavioural dysfunction, being unable to court the females and carry out the subsequent fertilization of eggs.[39–42] Additionally, F1 males produce viable gametes but in lower volume than their wild counterparts.[43,44] Consequently, the production of *S. senegalensis* relies on the capture of wild males that are acclimated to captive conditions and utilized as breeders.[40,45] Thus, reproduction makes up one of the main constraints for developing breeding programs in this species, which has prompted diverse investigation approaches, including feeding,[46,47] gamete production,[44,48,49] testes methylation and transcriptome profiles,[50] reproductive behaviour,[40,41,51,52] hormonal treatments,[53–56] photoperiod rhythms,[39,57,58] environmental enrichment,[59] and chemical communication through olfaction.[60,61] While these studies contributed to a better understanding of the mechanisms controlling gonad maturation and reproduction in *S. senegalensis*, an explanation for the failure of courtship in F1 males has not been found yet. It has been hypothesized that chemical communication may underlie courtship failure and that environmental differences operating across life stages in farms *vs* wild may contribute to the low performance of F1 males.[39–41] Thus, studying the role of the olfactory system in courtship might provide crucial insights into the reproductive behaviour issues of F1 males.

The arrival of new long-read sequencing technologies makes it feasible to obtain highly contiguous confident genomes,[62,63] but also a much better characterization of transcriptomes at the isoform-level by combining short- and long-read sequencing using hybrid bioinformatic approaches.[64–66] The ability of the olfactory system to detect a huge variety of compounds suggests a complex underlying gene family repertoire, as well as its resolution at the isoform level, especially in species with a demersal lifestyle like flatfish.[37]

The recent highly contiguous and annotated chromosome-level genome assembly of *S. senegalensis*[67,68] represents an essential resource for genomic exploration, including insights into genes associated with olfactory perception. However, the gene repertoire and transcriptome underlying the *S. senegalensis* olfactory organ remains scarce and, to our knowledge, only a transcriptomic profile of the olfactory organ comparing the ocular-side rosette of males F1 *vs* males captured in the wild has been reported.[60] Among the identified differentially expressed genes, some were either olfactory receptor genes or genes related to reproduction.

In the present study, a comprehensive and consistent isoform-level *S. senegalensis* olfactory transcriptome was generated by performing a hybrid sequencing approach, combining Illumina short-read and Oxford Nanopore long-read sequencing (ONT) technologies. Specifically, we conducted a transcriptomic comprehensive characterization of the olfactory rosettes of juvenile and adult individuals with different life-histories (female and male, F1 and wild origin), all acclimated to the same culture environment. We identified novel genes and vast transcript diversity, which were compared with previous RNA-seq data from the olfactory organ of *S. senegalensis*, significantly improving the available genomic information of this species. Our study represents the foundation for a confident investigation of the genetic mechanisms underlying the olfactory function in *S. senegalensis*, aiming to better understand its putative impact on the reproductive failure of F1 males, critical for the application of breeding programs in this species.

2. Materials and methods

2.1. Animal sampling and experimental design

A total of 15 animals were employed to characterize the transcriptome of the olfactory rosettes of *S. senegalensis*, six 10-mo-old juveniles and nine 27-mo-old adults. All fish were maintained indoors in tanks at the same standard water temperature and feeding conditions of the usual production protocol.[69] The main effort was put on long-read sequencing considering its higher resolution for reconstructing the full transcriptome, so 12 F1 individuals were used for this purpose: three juvenile males, three juvenile females, three adult males, and three adult females. Additionally, we took advantage of a previous single-nuclei RNA-seq dataset on the sole olfactory rosette, including three adult individuals acclimated to farm (F1 male, wild male, and wild female),[70] to refine our olfactory transcriptome.

All animal procedures complied with the ethical regulations of the University of Santiago de Compostela responsible of the study, and the “Instituto Español de Oceanografía de Santander” and the company Stolt Sea Farm, which provided the animals for the study, in accordance with EU guidelines (86/609/EU). Experimental protocols were approved by the competent authority on animal welfare under the authorization number: 2022GCELCE105202. All fish were sacrificed by decapitation and immediately the two olfactory organs of each fish were dissected and preserved in liquid nitrogen, to be then stored at −80 °C until their use. Olfactory organs consist of a rosette-shaped paired structure located in the rostral part of the head, rostromedially to the eyes and close to the jaw. The olfactory rosettes are placed inside an olfactory chamber, enclosed by cartilage and coated by abundant connective tissue and mucous (Fig. 1). The ocular- and blind-side olfactory chambers have direct communication with the environment through two nostrils that regulate the water-flow into the chamber. Both rosettes were dissected after accessing the olfactory chambers through an incision between the two nostrils.

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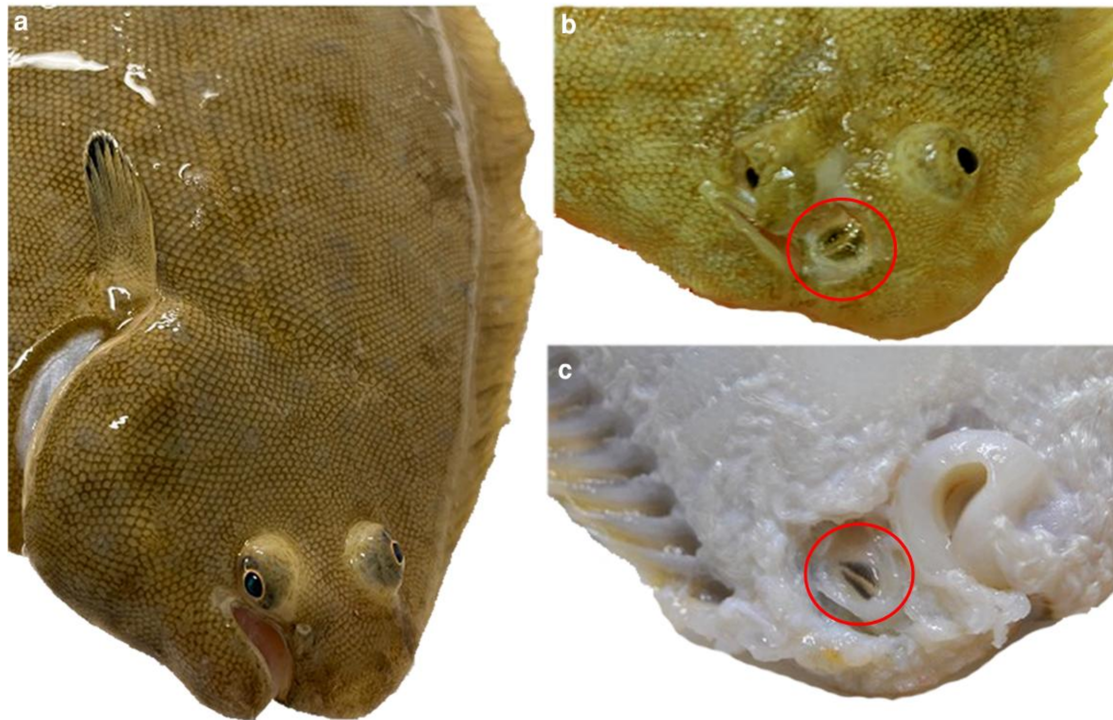

Fig. 1. Macroscopic topography of the *S. senegalensis* olfactory rosettes. a) Ocular side, where both eyes are located, and the nostrils are visible closer to the upper jaw. b) Ocular side after removing the skin that covers the olfactory chamber. Ocular-side olfactory rosette (circle) is strongly pigmented and surrounded by mucus. c) Blind side after removing the skin. Blind-side rosette (circle) with its black pigmentation.

2.2. Nanopore long-read sequencing

Total RNA extraction of ocular- and blind-side rosettes for long-read sequencing was performed with TRIZOL Reagent (Life Technologies) according to manufacturer's instructions. RNA quantity and quality were evaluated in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.) and in a Bioanalyser 2100 (Agilent Technologies, "RNA integrity number" [RIN]). The 24 samples (12 ocular-side olfactory rosettes and 12 blind-side olfactory rosettes) were evenly pooled into a single sample (RIN = 9.6). The pool was delivered to Novogene (Cambridge, UK) for ONT library construction and long-read sequencing. The ONT raw reads were filtered with Nanofilt (https://github.com/wdecoster/nanofilt), and then aligned against the reference transcriptome of *S. senegalensis* (GCA_919967415.2) with minimap2,[71] applying the parameters established for ONT sequencing.

2.3. Illumina short-read sequencing

Olfactory rosettes' Illumina sequences were obtained from nuclei RNA.[70] Briefly, nuclei were extracted,[72] libraries were built in a 10× Chromium, and Illumina 150 bp paired-end sequencing was performed in a NovaSeq 6000 at the Novogene Platform. The fasta files from raw sequencing were checked using FASTQC quality control. Reads from the six samples were aligned against the reference *S. senegalensis* genome (GCA_919967415.2) using STAR v2.7.9,[73] with default parameters.

2.4. Olfactory transcriptome characterization

For the olfactory transcriptome assembly, we combined ONT long-read and Illumina short-read sequencing datasets. *S. senegalensis* individuals from different origins (F1 and wild), sexes (males and females), and developmental stages (juveniles and adults) were gathered to achieve the broadest transcriptomic information as possible from diverse life-history specimens. This sequencing approach was expected to generate a comprehensive olfactory transcriptome at the isoform-level for the species.

Long-read data has become an essential tool for transcriptome reconstruction at the isoform level.[74] However, its integration with short-read data has demonstrated to be crucial for achieving a confident transcriptome reconstruction.[66,75] Therefore, the alignments used in our study from both sequencing techniques were merged by using the *–mix* option of StringTie v.2.2.1, since the hybrid dataset provides higher accuracy and sensitivity for gene and isoform identification.[65] The available genome annotation of *S. senegalensis* (GCA_919967415.2) was used as input. The resulting StringTie output comprised a GTF file containing information of the identified transcripts based on sequencing reads grouped by genomic coordinates. Transcripts were correlated, when possible, with known transcripts included in the annotation files of the *S. senegalensis* genome with their corresponding coding genes.

Several filtering steps were applied to the StringTie output to obtain a consistent dataset to be included in the *S. senegalensis* olfactory transcriptome. First, we performed a structural classification analysis of our transcripts by using SQANTI3 v.5.2.1 (https://github.com/ConesaLab/SQANTI3),[76] with default parameters. SQANTI3 provides a structural category and subcategory for each transcript based on how the reads match the available information in the annotated genome. The following categories were retrieved: (i) full splice match (FSM), an identical match to a known transcript in the genome annotation; (ii) incomplete splice match (ISM), all exons

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match to a known transcript, although some exons are missing at the ends; (iii) novel in catalogue (NIC), a novel transcript with a new combination of known exons; (iv) novel not in catalogue (NNC), novel transcripts containing at least one previously unknown exon; (v) intergenic, transcripts mapping to an intergenic region in the reference genome; (vi) genic, transcripts that overlap both known introns and exons in the reference genome; (vii) antisense, transcripts matching to the antisense strand of an annotated gene in the reference genome; and (viii) fusion, transcripts covering two different annotated genes.

Based on this categorization, we retained all FSM transcripts, while the rest of the transcripts were thoroughly evaluated before being included in the olfactory transcriptome of *S. senegalensis*. The filtering pipeline was applied to the entire StringTie output dataset to check that FSM transcripts were real transcripts that successfully passed the established filters.

First, we screened the StringTie v.2.2.1 transcript list obtained with RepeatMasker v.4.1.2[77] using the bony fish (*Actinopterygii*) database, to identify putative repetitive elements (RE) in our transcripts. The identification of interspersed repeats representing transposable elements (TEs) within the coding sequences (CDS) was analysed by cross-checking these data with the output from Transdecoder v.5.7.1[78] utilizing default parameters. Transdecoder predicts CDS by homology search against Pfam 33.0 to detect open reading frames (ORFs) with a minimum length of 100 amino acids. Subsequently, using an in-house Perl script, transcripts overlapping TEs more than 5% to the complete CDS were removed, but transcripts with trinucleotide simple repeats were kept in our dataset.

Once a consistent list of olfactory transcripts was obtained, we returned to the SQANTI3 structural annotation for in-depth analysis of the potential functionality of each transcript. SQANTI3 subcategories provide additional information for each transcript including exon number, coding region and presence of introns, among other data. Noncoding transcripts were removed. Furthermore, antisense, fusion, and genic categories (vi, vii, and viii), were considered to not represent real olfactory transcripts, and thus, were discarded. Accordingly, only transcripts composed of mature RNAs expected to contain complete CDS and lacking introns were retained. Thus, the transcripts included within the subcategories 'intron retention', 'mono-exon by intron retention', and 'mono-exon' were removed. The latter was retained within the FSM category only when mono-exonic transcripts overlapped a mono-exonic known transcript. Finally, the mono-exon transcripts placed at intergenic regions were retained.

The FSM category was fundamental for *S. senegalensis* olfactory transcriptome reconstruction. The case where a transcript matched partially a reference isoform, was included within the ISM category. We filtered the ISM category by performing a pairwise comparison of our list with all FSM transcripts. When the transcript overlapped the FSM transcript of an annotated gene, we considered the ISM transcript of the pair pertaining to the FSM isoform. Consequently, ISM transcripts matching an existing FSM transcript of the same gene were not considered, since missing exons may be related to incomplete long-read sequencing. Another case within ISM category was when a transcript represented a novel isoform in which fewer exons than those described in the canonical FSM transcript (including all exons) were detected. In that case, these ISM transcripts were considered as novel and retained.

Moving into the categories composed of novel genes, the same strategy was followed for NIC and NNC, retaining novel transcripts that contained a novel combination of exons 'combination_of_known_splicesites', or one previously unknown exon 'at_least_one_novel_splicesite'. While these subcategories were retained, the mono-exonic transcripts and those exhibiting introns were discarded. Intergenic transcripts, located in genomic regions where no information about coding regions existed in the reference genome were kept, representing novel genes.

2.5. Quantification of gene expression in the olfactory rosette

Normalized transcripts expression in the rosette was obtained as the number of transcripts per million of reads (TPM) provided by the StringTie output. There is lack of consensus regarding the TPM threshold, mostly depending on the sequencing technology employed, the specific tissue analysed and the gene expression level.[79–81] We decided to use a TPM > 0.3 to consider a transcript as expressed in the olfactory rosette above the background noise considering the low expression levels of olfactory receptors[82–85] and that a high proportion of long-read sequences represented the whole transcript length (~2 kb the canonical transcript on average). This threshold could be similar to the 5 TPM usually used for short-read RNA-seq. The pipeline followed for the present study is shown in Fig. 2.

2.6. Olfactory transcriptome comparison

We took advantage of the publicly available Illumina short-read RNA-seq data from a previous study[60] describing the transcriptomic profiles of the ocular-side olfactory organ in three wild and three F1 *S. senegalensis* males to compare and enrich the olfactory transcriptome here constructed and to critically analyse the steps followed in our pipeline. To do so, we downloaded the RNA-seq raw reads for all six samples available on the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) database, under accession number PRJNA319182. Adapter sequences and low-quality bases were trimmed using Trimmomatic v0.39[86] with standard parameters, and the resulting high-quality reads were pseudoaligned to the *S. senegalensis* reference transcriptome (GCA_919967415.2) using kallisto v0.48.0,[87] with default settings. Transcript abundance was measured based on TPM value provided by kallisto. A threshold of TPM > 5 in at least two replicates per condition (wild, F1) was set up to define expressed transcripts following a usual criterion for short-read RNA-seq.[88,89] The list of expressed transcripts was then compared to those present in our olfactory transcriptome, and those transcripts not detected in our transcriptome were further examined to investigate if they could have been filtered out at any step of our pipeline.

2.7. Olfactory receptor gene expression

To check the expression differences between the four olfactory receptor gene families (OlfC, OR, ORA, and TAAR) in our transcriptome, we applied a Kruskal–Wallis rank sum test. Post-hoc pairwise comparisons between gene families were performed using Dunn's test with Bonferroni correction. All statistical analyses were conducted in RStudio using 'stats' and 'dunn.test' packages.

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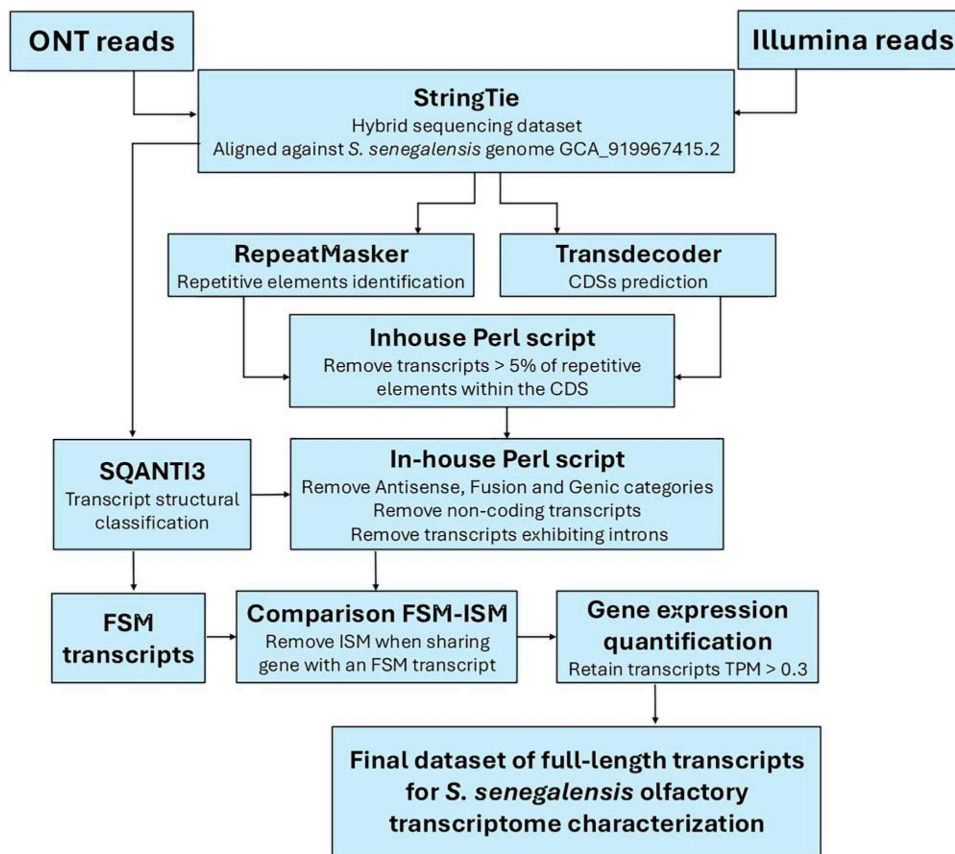

Fig. 2. Overview of the pipeline for the olfactory transcriptome characterization. FSM, full-splice match; ISM, incomplete splice match; TPM, transcripts per million of reads.

We performed hypergeometric tests to identify overrepresented gene ontology (GO) biological functions within each olfactory receptor gene family taking as reference the whole olfactory transcriptome using the 'GOfuncR' package in RStudio. A family-wise error rate (FWER) was applied, and genes associated to biological functions with FWER_overrep ≤ 0.05 were considered enriched.

3. Results and discussion

3.1. Olfactory transcriptome characterization

The chromosome-level genome assembly of *S. senegalensis*[67,68] has provided an invaluable resource for functional genomics evaluation of olfaction-related traits in this promising aquaculture species.[38] The olfactory rosette, responsible for chemical communication, has been recognized as an essential organ for courtship and reproduction.[4,28,90,91] However, to our knowledge, only a transcriptomic profile study explored its putative role on the reproduction failure of farm (F1) males by comparing the transcriptome of olfactory rosettes of animals from wild and F1 origin.[60] Additionally, in this study, a large differentially expressed gene repertoire was identified, an expected outcome given the markedly different environmental conditions in the wild and on farms. Indeed, to properly address the reproductive issue, the comparison between wild fish acclimated to farm environment and F1 fish would be necessary to identify the functional signals underlying communication failure. The essential point for this analysis is to generate a high-quality reference olfactory transcriptome, especially considering the poor transcriptomic annotation of this complex sensorial organ in fish.[37,92] Such transcriptome was the primary goal of our study, and it was feasible through a long-read and short-read hybrid approach, essential to characterize the genes and isoforms involved in the olfactory function, particularly the olfactory receptor genes. Previous transcriptomic characterization through long-read sequencing techniques have been tackled in several tissues in flatfish.[93] Other studies have characterized the olfactory transcriptome in teleost through short-read sequencing techniques[60,94–97] but to our knowledge, this is the first olfactory transcriptome constructed through a hybrid sequencing approach in any fish species.

A total of 23.7 million (M) reads averaging 1,366 base pairs (bp) were generated through ONT long-read sequencing in our study from a single pool of ocular- and blind-side olfactory rosettes including individuals of both sexes across various developmental stages. Of these, 98% mapped to the *S. senegalensis* reference genome. Illumina 150 bp pair-end reads from ocular- and blind-side rosettes of three individuals with different life-histories ranged between 246 and 540 M reads, 76.1% on average mapping to the reference genome (range: 71.8% to 82.3% reads per sample).

Our approach involved merging long- and short-read sequencing datasets to obtain a hybrid assembly using StringTie.[65,75] The inclusion of the Illumina short-read sequencing enriched the biological material for the analysis and improved the accuracy of exon and intron identification, reducing the errors inherent to ONT long-read sequencing, which allowed a more precise transcriptome characterization at the isoform level.[66] Although there are differences in third-

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generation sequencing techniques, both PacBio and ONT long-read sequencing are known to be suitable for transcriptomic analysis, especially when combined with the accurate Illumina sequencing.[66,98] The StringTie output dataset rendered a total of 36,743 genes and 70,470 transcripts. Among them, 13,092 genes encoding 15,652 transcripts (22% of the total) were categorized as FSM by SQANTI3, representing a full match to the known transcriptome in the reference genome of the species.[68]

All transcripts, including the FSM category, were further analysed following an in-house pipeline based on previous results employing diverse sequencing techniques and bioinformatic tools in different taxonomic groups.[99] SQANTI3 software greatly aided in the process of filtering transcripts from our dataset to obtain the final olfactory transcriptome since it provides accurate transcript structural annotation.[76,100] Transdecoder, which identified the best candidate ORF for each transcript, aided to predict the complete CDS. Among the initial set of 36,743 genes, Transdecoder identified ORFs with more than 100 codons in 21,552 genes encoding 51,807 transcripts. Thus, these transcripts were predicted to contain a complete CDS, being capable of producing functional proteins.

RepeatMasker made it feasible to identify RE in the *S. senegalensis* transcriptome and to remove suspicious transcripts containing a significant portion of REs within the coding regions.[77] TEs have been demonstrated to be highly variable between species and to play a significant role in genome evolution,[101] being involved in gene expression[102,103] or genome reorganization.[104,105] A remarkable variation in TE content has been reported in teleost, reflecting a positive correlation with genome size.[106] RepeatMasker detected 14.71% REs in our transcripts, mainly involving retroelements and DNA transposons (12.57%) (Table 1). This value is similar to that reported in other fish transcriptomes,[107] and within the wide range reported across teleost (from 1.6% to 28%).[108,109] As expected, the percentage of REs in the transcriptome was lower than that reported in the whole genome[110] considering the functional constraints of coding regions. We cross-checked the REs list retrieved from our sequencing data with the Transdecoder output to identify those coding regions with >5% RE overlapping with ORFs in our transcripts for an additional filtering. After this step, a total of 36,703 transcripts encoded by 18,325 genes were retained, averaging ~2 transcripts per locus.

Subsequently, we took advantage of the SQANTI3 categorization and discarded 2,342 transcripts included in the antisense, fusion and genic categories, and further removed 1,568 transcripts classified as noncoding by SQANTI3 (Table 2).

Then, we explored the subcategories assigned to each of the 32,793 coding transcripts from which SQANTI3 provided information of exon and intron distributions, and checked how these transcripts matched the available information in the reference genome (Table 3). Transcripts that matched to a mono-exonic transcript in the genome were included in the 'mono-exonic' subcategory of FSM and thus, retained. Conversely, if a mono-exonic transcript matched a reference multiexonic transcript, it was discarded due to missing information, except for mono-exonic transcripts within the intergenic category, for which no previous information existed.

The maturation of mRNA involves several processes, including splicing, where noncoding introns are removed from primary mRNAs. However, intron retention has sometimes been demonstrated to influence the regulation of gene expression,[111,112] and a certain proportion of immature mRNAs including introns may be identified.[113] We found that 22% of our transcripts still included introns. We cannot conclude whether these transcripts containing introns play a significant role in the regulation of gene expression or just represent immature mRNAs.[114] Thus, we removed 7,421 transcripts subcategorized as 'intron retention' and 'mono-exon by intron retention'.

At this point of the filtering process, our *S. senegalensis* olfactory transcriptome consisted of five structural categories according to their correspondence with the available genome information. FSM, representing a perfect match to a known transcript, included 85.60% transcripts classified as 'reference match'. The rest of FSM transcripts included slight variations, either missing 3′ end or 5′ end, or both. The explanation behind the variability between transcript boundaries may be associated with the poor annotation of the UTR regions in the species. In the same way, ISM transcripts were associated by SQANTI3 with a known transcript in the genome, although some exon(s) were missing compared to the reference isoform. Special attention was paid to this category, since ISM transcripts could represent novel transcripts with fewer exons than the canonical FSM. The pairwise comparison between FSM and ISM revealed a total of 1,191 pairs sharing the same associated gene and transcript. Following a conservative criterion, we assumed that those ISM corresponded to the same FSM transcript. Still, we retained the remaining 1,663 ISM transcripts that were associated with a single gene lacking an FSM transcript in our dataset. These transcripts need to be further explored to know whether they represent novel transcripts with a novel exon combination, or whether they are artefacts of ONT sequencing.

Table 1. Percentage of RE overlapping the *S. senegalensis* olfactory transcripts using RepeatMasker.

Type	% in the transcriptome	Type	% in the transcriptome
Retroelements	**5.68**	**DNA transposons**	**6.52**
SINES:	**0.43**	hobo-Activator	2.86
Penelope	0.06	Tc1-IS630-Pogo	0.47
LINES:	**1.86**	PiggyBac	0.08
L2/CR1/Rex	1.01	Tourist/Harbinger	0.36
R1/LOA/Jockey	0.04	Other	0.03
R2/R4/NeSL	0.02	Rolling-circles	1.12
RTE/Bov-B	0.10		
L1/CIN4	0.48	**Unclassified**	**0.37**
LTR elements:	**3.39**		
BEL/Pao	0.53		
Ty1/Copia	0.01	Small RNA:	**0.33**
Gypsy/DIRS1	2.26	Satellites:	**0.30**
Retroviral	0.26	Simple repeats:	**1.51**
Total	**14.71%**		

Table 2. Structural categories in the olfactory transcriptome of *S. senegalensis*.

Structural category	FSM	ISM	NIC	NNC	Intergenic	Total
Trascript count	16,701	4,178	5,506	6,677	1,299	34,361
Coding	15,653	4,119	5,431	6,619	971	32,793
Noncoding	1,048	59	75	58	328	1,568

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Table 3. Structural transcript categories and subcategories for olfactory transcripts of *S. senegalensis.*

	Structural category					
	FSM	ISM	NIC	NNC	Intergenic	Total
Filtered transcripts	15,653	1,663	1,696	4,062	971	**24,045**
Subcategory						
alternative_3end	567					
alternative_3end5end	456					
alternative_5end	821					
reference-match	13,403					
mono-exon	406				490	
3prime_fragment		790				
5prime_fragment		742				
internal_fragment		131				
combination_of_known_splicesites			1,696			
at_least_one_novel_splicesite				4,062		
multiexon					481	

Table 4. Summary statistics of the *S. senegalensis* olfactory transcriptome.

Olfactory transcriptome	
Gene count	14,917
Transcript count	20,670
Protein coding genes	14,708
lncRNA coding genes	209
Median transcript length (bp)	2,604
Mean transcript length (bp)	3,351
Exons/transcript	10.78
Transcripts/gene	1.39
Multiexonic transcripts (%)	96.02

For the examination of categories NIC and NNC composed of novel transcripts not annotated in the reference genome, we considered the scarce and puzzling annotation of the olfactory genes in teleost, and specifically in *S. senegalensis*. Both categories consisted of known genes with a novel isoform, either by a new combination of known splice-sites or by the appearance of a new splice-site. The NIC category included 1,700 transcripts consisting of new combinations of annotated exons, representing novel transcripts of a known gene. We found a larger number within the NNC category, where 4,103 novel transcripts that contained at least one previously unannotated exon were identified. These findings highlight the potential of long-read RNA sequencing for identifying novel transcripts, expanding our understanding of transcriptome complexity.[115]

The Intergenic category included novel transcripts placed in a genomic region with no previous annotated genes, which was possible by combining long-read with Illumina sequencing data using a broad sampling collection across different life-history fish. At this point, the *S. senegalensis* olfactory transcriptome consisted of 24,045 transcripts distributed in different categories and subcategories (Table 3).

3.2. Gene expression in the olfactory epithelium

Given the conservative filtering pipeline followed in our study, it can be assumed that we retained real transcripts with an active function in the olfactory rosette of *S. senegalensis*. Using a threshold of TPM > 0.3 from StringTie TPM values we identified a total of 20,670 full-length active transcripts encoded by 14,917 genes (1.39 isoforms per gene) in the *S. senegalensis* olfactory transcriptome (Table 4); 14,708 were protein coding genes and 209 were classified as long noncoding RNA (lncRNA) genes. Long-read sequencing has become the preferred approach for comprehensive characterization of lncRNAs,[116] a gene class that can modulate chromatin function, the assembly and function of nuclear bodies, the stability and translation of cytoplasmic mRNAs and that can interfere with signalling pathways.[117] A more detailed description for each transcript included in our olfactory transcriptome is presented in Table S1.

From the 14,917 genes constituting the olfactory transcriptome, 11,925 were annotated in the current genome of the species, whereas 2,021 had an Ensembl ID but no annotation. The remaining 971 were novel genes identified through our transcriptome characterization, and therefore, lacked Ensembl ID and annotation.

All in all, we retained 12,278 FSM transcripts, 1,663 ISM transcripts, 1,696 NIC transcripts, 4,062 NNC transcripts, and 971 Intergenic transcripts in the *S. senegalensis* olfactory transcriptome (Fig. 3a and b), 67.4% of them constituting known transcripts (FSM and ISM categories) included in the current genome of the species. The rest were novel transcripts (NIC, NNC, and Intergenic categories) that significantly improved the annotation of the species genome.

Distribution transcript length ranged from 303 to 90,883 bp, with a median of 2,604 bp and a mean of 3,351 bp, 38 transcripts being above 30 kb (Fig. 3c). Gene size ranged between 249 and 666,329 bp, with a median of 8,922 bp and a mean of 18,212 bp, 328 genes being above 100 kb. This represents a larger median gene length than the 7,566 bp reported for the whole genome.[68] Gene and transcript length are positively correlated, with a trend of increased length in highly conserved genes.[118,119] Furthermore, significant variations in gene length have been observed across tissues, with longer transcripts predominantly expressed in blood vessels, nerves, and brain, while shorter transcripts are more commonly found in skin and gonads.[120] The olfactory rosette is a complex highly vascularized organ with a significant composition of neural cells,[7] and therefore, it would be expected to express longer transcripts. Most transcripts (96%) were multiexonic, exhibiting a mean of 10.8 exons and a median of 8.0 exons per transcript. A total of 2,682 transcripts consisted of ≥20 exons, while 4 to 5 exons were the modal class in our dataset, including 2,902 transcripts (Fig. 3d).

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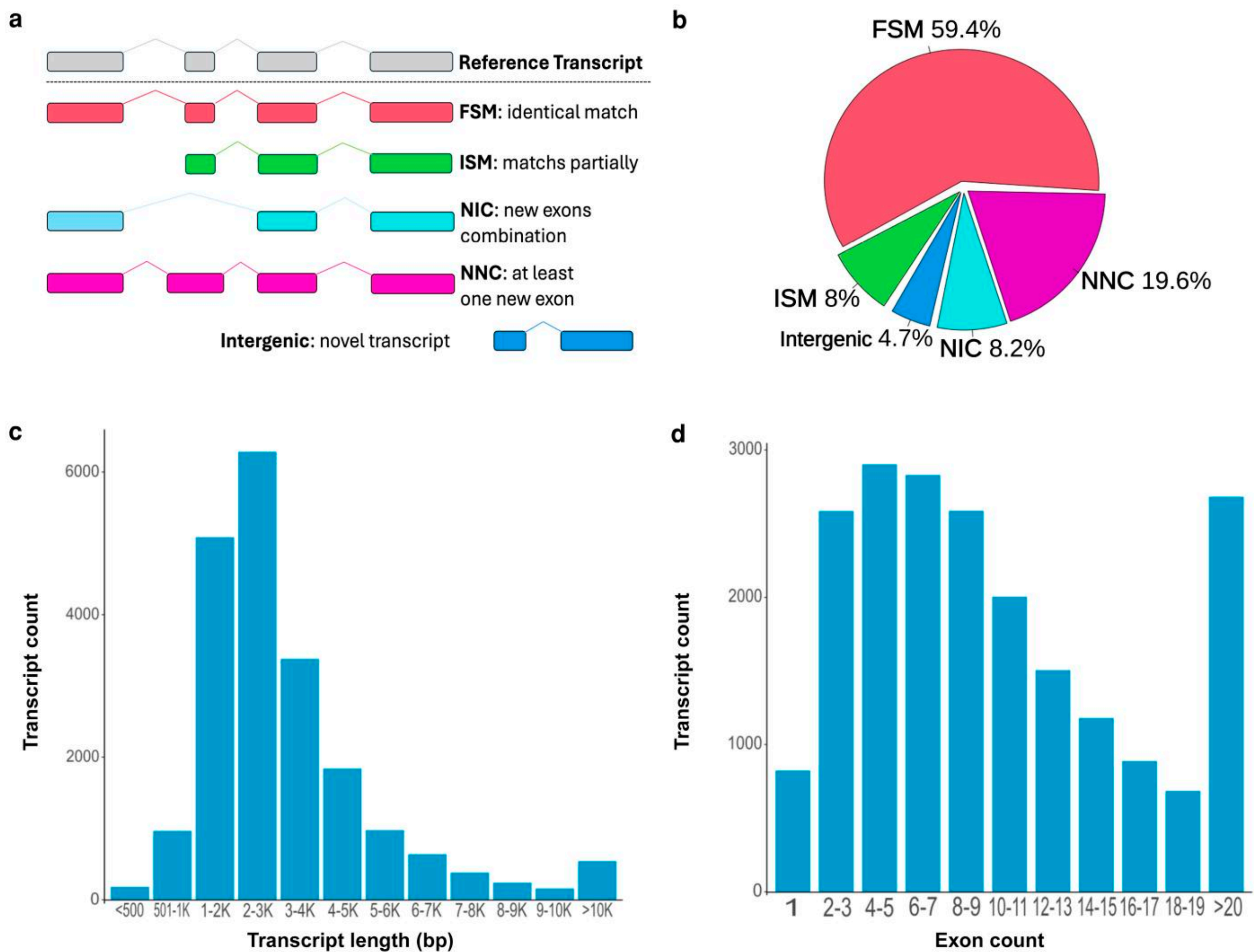

Fig. 3. Main features of the olfactory transcriptome of *S. senegalensis*. a) Structural categories based on the reference transcript genomic information. b) Distribution of structural categories: FSM, ISM, NIC, NNC, and intergenic. c and d) Transcript length and exon number distribution, respectively.

Expressed genes within the olfactory transcriptome, besides a unique StringTie code assigned, were associated, when possible, to an annotated gene in the reference genome. This rendered 13,946 known genes in the *S. senegalensis* olfactory transcriptome and the discovery of 971 novel genes, significantly enriching the current number of protein coding genes in Ensembl rapid release. Among the total olfactory transcript count, 13,941 (67.4%) were known full-length annotated transcripts in the genome, while 6,729 were identified as novel transcripts in our study. Among these, 5,758 transcripts (27.9%) were alternative splice variants encoded by known genes, whereas 971 (4.7%) were encoded by novel genes located on intergenic regions, not registered in the reference genome.

3.3. Comparison with previous short-read sequencing data

After quality trimming and pseudoalignment of the *S. senegalensis* olfactory rosette Illumina raw reads from public datasets[60] against the reference genome, we identified a total of 13,671 expressed transcripts (TPM > 5). Of these, 10,230 (74.8%) were present in our olfactory transcriptome, supporting the comprehensiveness and reliability of our assembly. Among the remaining 3,441 transcripts (25.2%), 228 (1.7% of the total) were not present in the initial StringTie output, indicating that they were not included in our dataset before filtering. This low percentage are likely explained by the different biological material and conditions used in our study and by Fatsini et al.[60] The remaining 3,213 transcripts were filtered out at different steps of our conservative pipeline: 494 based on their structural classification by SQANTI3 (fusion and genic categories removed), 1,705 by subcategory (intron retention, mono-exon), 293 classified as noncoding, 185 with TPM < 0.3, 517 after combining the results from Transdecoder and RepeatMasker (CDS > 5% RE), and 19 ISM transcripts that, upon comparison with FSM, were found to correspond to the same gene and transcript (Fig. 4).

To further assess the biological significance of these differences, we examined the annotation of the missing transcripts. Out of the 228 transcripts not identified by StringTie, 114 were annotated, and among them, two corresponded to olfactory receptor genes. Furthermore, among the 3,213 transcripts filtered out across the transcriptome characterization, 16 were encoded by 15 genes annotated as olfactory receptor genes, which highlights the delicate balance to ensuring the transcriptome reconstruction quality while discarding noise from contamination, sequencing artefacts or debris from the cell transcription machinery. Considering the key role of the

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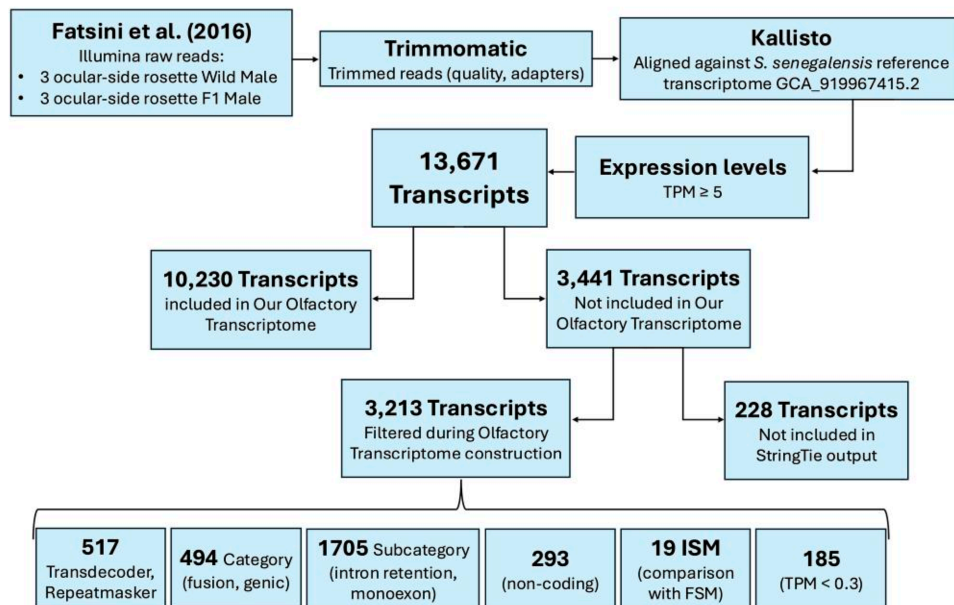

Fig. 4. Overview of the pipeline for the olfactory transcriptome comparison with previous short-read RNA-seq data by Fatsini et al.[60] ISM, incomplete splice match; FSM, full-splice match; TPM, transcripts per million of reads.

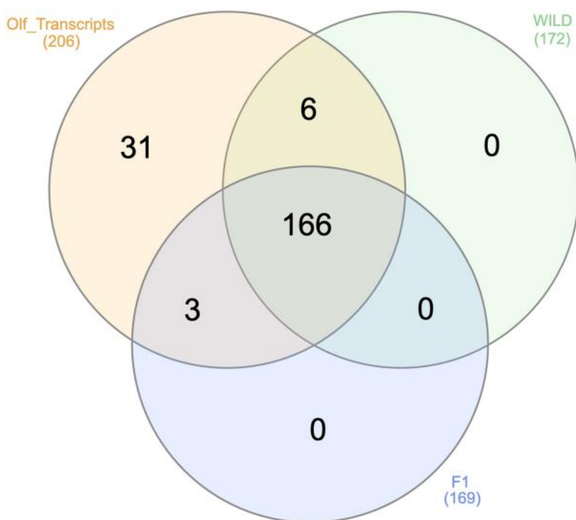

Fig. 5. Venn diagram comparing the datasets of olfactory receptor transcripts within our olfactory transcriptome (Olf_Transcripts; 206 transcripts), wild (Wild; 172 transcripts) and F1 (F1; 169 transcripts) male individuals from Fatsini et al.[60]

olfactory receptors in mediating sensory perception in fish, we retained these 16 olfactory receptor transcripts into the final olfactory transcriptome dataset. Notably, all these genes successfully passed our expression level TPM > 0.3 threshold. The reanalysis of previous data in our study highlights the importance of using all available resources for transcriptome reconstruction, which has made feasible incorporating some genes directly involved in sensory perception through olfaction.

Conversely, when the sequencing data[60] was aligned against our olfactory transcriptome, we observed that half of our transcriptome (10,440 genes) was not detected in the expressed list of the previous study.[60] This is not unexpected, given the broader biological conditions included in our study, encompassing both ocular- and blind-side olfactory rosettes, as well as sexually immature juveniles (both sexes) and adults from both wild and F1 origins, while that by Fatsini et al. study was limited to the ocular-side olfactory rosettes of adult wild and F1 males. Notably, RNA-seq Illumina data aligned to 423 novel transcripts (TPM > 5) encoded by genes at intergenic regions of the current genome version, and to 4,337 novel transcripts (TPM > 5) from our transcriptome characterization, thus validating a high proportion of the novel transcripts (>70%).

All in all, these results highlight the value of integrating diverse sampling data and advanced sequencing technologies to achieve a more complete and functionally relevant transcriptome. While the examination of the publicly available data supports the robustness of our olfactory transcriptome characterization, the differences observed underscore the impact of methodological choices, including sample dissection and processing, sample conditions selection, sequencing technology, and data analysis.

3.4. Olfactory receptor genes

S. senegalensis reference genome included 190 genes annotated as olfactory receptors, (69 OlfC, 51 OR, 3 ORA, and 67 TAAR). These receptors constitute the olfactory repertoire and likely play a crucial role in chemical communication.[4,12] A large variation in the number of olfactory receptor genes has been reported for 185 different Actinopterygii species,[27] including Pleuronectiformes (range: 76 to 234 olfactory receptor genes). Therefore, *S. senegalensis* is a flatfish in the upper range of the number of genes currently annotated as olfactory receptors. However, since the *S. senegalensis* genome is still under revision in the Ensembl database, an increase in the number of olfactory receptor genes is expected in a better-annotated version of the genome, potentially adding novel transcripts, new genes or previously unannotated genes to the list.

Among the genes of the *S. senegalensis* olfactory genome repertoire, 143 were included in our olfactory transcriptome

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Fig. 6. Main features of the olfactory receptor transcripts within *S. senegalensis* olfactory transcriptome. a) Boxplot of the expression of each olfactory receptor gene family. b) Distribution of structural categories: FSM, ISM, NIC, and NNC. c) Transcript length distribution; d) Exon number distribution.

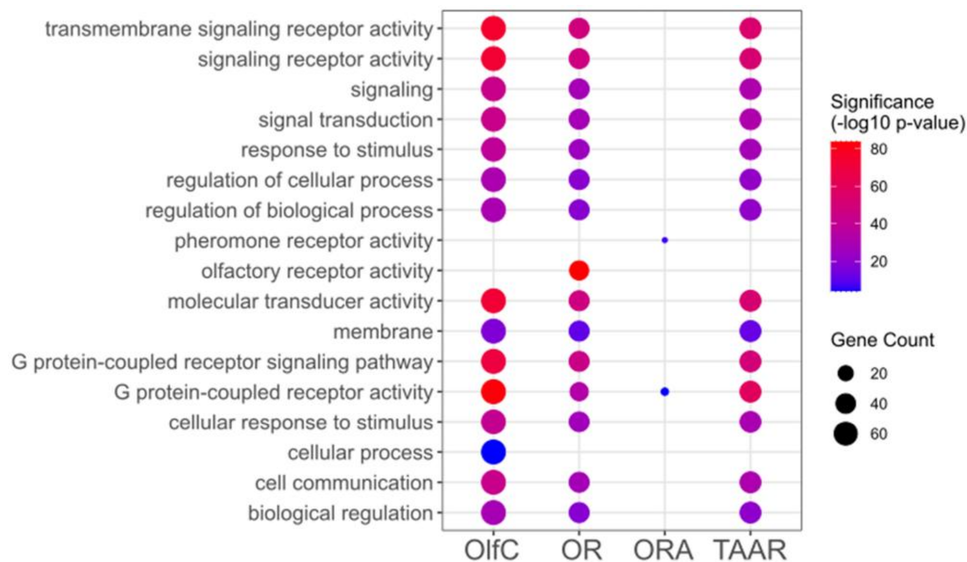

Fig. 7. Dotplot representing the GO enrichment analysis of the four olfactory receptor gene families (OlfC, OR, ORA, and TAAR). Circle size corresponds to the number of genes contributing to each GO term, while colour intensity represents the significance level (−log10 *P*-value).

(190 transcripts), and therefore, expressed in the olfactory rosettes of the individuals included in our study. Furthermore, after adding the 15 olfactory receptor genes retrieved from Fatsini et al.[60] (16 transcripts), the final list would include a total of 206 transcripts: 104 OlfC, 46 OR, 3 ORA, and 53 TAAR (Table S2), encoded by 158 genes actively expressed (TPM > 0.3) in the olfactory rosette. When comparing the data from Fatsini et al.[60] with our transcriptome, 172 and 169 olfactory receptor transcripts were detected in the wild and F1 individuals, respectively (Fig. 5), six of them

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corresponding to six genes expressed only in wild individuals (5 OlfC and 1 TAAR) while three to as many genes of F1 (2 OlfC and 1 TAAR). Future studies may help to confirm this preliminary observation for understanding the mechanisms underlying the divergent reproductive performance of wild and F1 individuals on farms.

The four olfactory receptor gene families showed different levels of expression (TPM) (Kruskal–Wallis test; $P = 0.0003$). Differences were detected between OlfC and OR families (Dunn's test $P\text{adj} = 0.0001$), and between OlfC and TAAR ($P\text{adj} = 0.0244$) (Fig. 6a). This pattern might reflect the specialization of the OlfC family for detecting specific chemical cues in *S. senegalensis*, as has been described in zebrafish.[29,96] These findings align with the functional specialization observed in other teleost species, where different olfactory receptor families can detect distinct semiochemicals, including adaptations at the species-specific level.[26,27] We identified 60 novel isoforms, substantially increasing the known olfactory receptor transcript repertoire in *S. senegalensis* (Fig. 6b), thus expanding the functional specialization of olfactory receptors.[96,121] Improving our understanding on the physiological basis of chemoreception, including pheromone candidates, will be very helpful for aquaculture production.[30]

Olfactory receptor transcripts ranged from 660 to 10,170 bp, with a median similar to the total olfactory transcriptome (2,620 *vs* 2,604 bp) and a mean of 2,631 bp (Fig. 6c). However, olfactory receptor transcripts contained fewer exons on average (5.3) than the olfactory transcriptome (10.7), including a higher proportion of mono-exonic (12%; Fig. 6d).

GO term enrichment revealed that each olfactory receptor gene family was associated with functions directly related to stimulus perception and transmembrane signalling (Fig. 7), playing a crucial role in *S. senegalensis* chemosensory perception (Table S3). Notably, ORA genes showed specific enrichment in pheromone receptor activity, supporting their role as sexual pheromone detectors in this species. Studies in zebrafish proved that an ORA receptor directly mediates reproductive behaviour through detection of sexual pheromones.[14]

Furthermore, the expression in our olfactory transcriptome (TPM > 0.3) of hormone receptors of prostaglandin, oestrogen, and androgen (Table S1), highlights the link between olfaction and reproduction in this species.[28] Their expression in the olfactory epithelium has been reported in fish[13,28,122] and mammals,[85] where they might modulate the sensitivity to olfactory stimuli.[13,123] Additionally, stress-related hormone genes, such as corticotropin and vomeronasal receptor genes involved in pheromone detection, were identified (Table S1), showing the complex modulation of reproduction and stress responses through olfaction.[85,124] Hence, our olfactory transcriptome resource lays the foundation for future functional studies aimed at identifying potential chemical cues, including sex pheromones, that drive reproduction, ultimately optimize reproductive strategies in aquaculture.[30]

Our olfactory transcriptome significantly enriched the genomic information in *S. senegalensis*. Our findings establish a foundation for investigating the role of olfaction in social communication and reproduction in this species, where the full-length mRNA sequencing proved its potential to uncover an unprecedent number of novel transcripts emerging from alternative transcription initiation or splicing, consistent with findings reported in analogous studies.[99] Further exploration of additional developmental stages and environmental conditions will help enrich this resource. Our study presents a confident and comprehensive *S. senegalensis* olfactory transcriptome at the isoform level, an essential sensory organ for demersal species. The diversity of olfactory receptor gene families in *S. senegalensis*, including expression pattern and functional enrichment, underscores their specialized roles in chemical communication. Future research will benefit from integrating our transcriptomic information with functional studies that might help to elucidate mechanisms underlying the reproductive dysfunction in the species, ultimately contributing to improving fish aquaculture production.

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Author contributions

D.T.S.: Conceptualization, Investigation, Data curation, Formal analysis, Writing—original draft; A.B.H.: Software, Formal analysis; P.R.V.: Conceptualization, Investigation, Formal analysis, review and editing; I.M. and I.R.: Resources, review and editing; C.B.: review and editing; D.R.: Conceptualization, Funding acquisition, review and editing; and P.M.: Conceptualization, Funding acquisition, Project administration, Supervision, review and editing, Methodology, Writing—original draft, review and editing

Supplementary material

Supplementary data are available at *DNARES* online.

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Conflict of interest

The authors declare that they have no competing interests.

Data availability

The sequencing datasets generated during the current study can be accessed through the NCBI SRA repository, BioProject accession PRJNA1315123. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GLIU00000000. The version described in this paper is the first version, GLIU01000000. The dataset will be made available upon acceptance of the manuscript. To facilitate the reviewing

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Ethics approval

All animal experiments were conducted in accordance with the guidelines of the University of Santiago de Compostela, the Instituto Español de Oceanografía de Santander and Stolt Sea Farm, in accordance with EU guidelines (86/609/EU).

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