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Generation of a chromosome-level genome assembly for Pacific halibut (*Hippoglossus stenolepis*) and characterization of its sex-determining genomic region

Andrew J. Jasonowicz\(^1\) | Anna Simeon\(^1,2\) | Margot Zahm\(^3\) | Cédric Cabau\(^4\) | Christophe Klopp\(^3\) | Céline Roques\(^5\) | Carole lampietro\(^5\) | Jérôme Lluch\(^5\) | Cécile Donnadieu\(^5\) | Hugues Parrinello\(^6\) | Daniel P. Drinan\(^2\) | Lorenz Hauser\(^2\) | Yann Guiguen\(^7\) | Josep V. Planas\(^1\)

\(^1\)International Pacific Halibut Commission, Seattle, Washington, USA
\(^2\)School of Aquatic and Fishery Science, University of Washington, Seattle, Washington, USA
\(^3\)SIGENAE, Bioinfo Genotoul, UMIAT, INRAE, Castanet-Tolosan, France
\(^4\)SIGENAE, GenPhySE, Université de Toulouse, INRAE, ENVT, Castanet-Tolosan, France
\(^5\)INRAE, GeT-PlaGe, Castanet-Tolosan, France
\(^6\)MGX-Montpellier GenomiX, University of Montpellier, CNRS, INSERM, Montpellier, France
\(^7\)INRAE, LPGP, Rennes, France

Correspondence
Josep V. Planas, International Pacific Halibut Commission, Seattle, Washington, WA 98199, USA.
Email: josep.planas@iphc.int

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Abstract
The Pacific halibut (*Hippoglossus stenolepis*) is a key species in the North Pacific Ocean and Bering Sea ecosystems, where it also supports important fisheries. However, the lack of genomic resources limits our understanding of evolutionary, environmental and anthropogenic forces affecting key life history characteristics of Pacific halibut and prevents the application of genomic tools in fisheries management and conservation efforts. In the present study, we report on the first generation of a high-quality chromosome-level assembly of the Pacific halibut genome, with an estimated size of 602 Mb, 24 chromosome-length scaffolds that contain 99.8% of the assembly and a \textit{N}50 scaffold length of 27.3 Mb. In the first application of this important resource, we conducted genome-wide analyses of sex-specific genetic variation by pool sequencing and characterized a potential sex-determining region in chromosome 9 with a high density of female-specific SNPs. Within this region, we identified the \textit{bmpr1ba} gene as a potential candidate for master sex-determining (MSD) gene. \textit{bmpr1ba} is a member of the TGF-\(\beta\) family that in teleosts has provided the largest number of MSD genes, including a paralogue of this gene in Atlantic herring. The genome assembly constitutes an essential resource for future studies on Pacific halibut population structure and dynamics, evolutionary history and responses to environmental and anthropogenic influences. Furthermore, the genomic location of the sex-determining region in Pacific halibut has been identified and a putative candidate MSD gene has been proposed, providing further support for the rapid evolution of sex-determining mechanisms in teleost fish.

KEYWORDS
candidate master sex-determining gene, genome assembly, Pacific halibut, pool-sequencing, sex-associated genomic region, whole genome sequencing

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1 | INTRODUCTION

While the phenotypic outcome of sex determination is highly conserved, a diverse array of mechanisms underlie sex determination throughout the tree of life with a large portion of this diversity being represented in vertebrate species (Bachtrog et al., 2014; Pennell et al., 2018). In some vertebrate clades, such as mammals and birds, sex determination systems are highly conserved (Pennell et al., 2018). Both rely on genetic sex determination mechanisms (GSD) where, in mammals, males are the heterogametic sex (XX/XY system) and in birds, females are the heterogametic sex (ZZ/ZW system) (Bachtrog et al., 2014; Capel, 2017). Teleost fishes on the other hand, exhibit a remarkable array of sex determination mechanisms. This includes, but is not limited to, temperature-dependent sex determination (TSD), behavioural sex determination (BSD), and GSD mechanisms (Bachtrog et al., 2014; Godwin & Roberts, 2018).

In some cases, taxonomically closely related species display alternate mechanisms of sex determination. Both XX/XY and ZZ/ZW systems have been documented in medaka (Oryzias spp.) (Takehana et al., 2008), mosquitofish (Gambusia spp.) (Kottler et al., 2020), Esociformes (Pan, Feron, et al., 2021), and the Pleuronectiformes or flatfishes.

The diversity of sex determination mechanisms exhibited among flatfishes makes them a particularly interesting group to study from an evolutionary perspective. Not only are both XX/XY and ZZ/ZW systems represented but there is some evidence that environmental factors may play a role in sex determination for some flatfish species (Luckenbach et al., 2009). It is estimated that flatfishes diverged from a common ancestor with threadfins (Polynemidae) between 57.3–72.6 million years ago (Ma) (Harrington et al., 2016). Among the Pleuronectidae family, the genus Hippoglossus consists of two species, the Pacific (Hippoglossus stenolepis) and Atlantic halibut (H. hippoglossus). Levels of genetic divergence suggest that a relatively recent common ancestor was shared between the sister species as it is estimated that these species diverged from one another 0.9–4.5 Ma and that a common ancestor dispersed from the Pacific to the Atlantic ocean sometime after the Bering Strait opened or that some degree of interocean migration was maintained until relatively recently in an evolutionary time scale (Grant et al., 1984).

Interestingly, alternate genetic sex determination systems have been documented for these sister species. Females are the heterogametic sex in Pacific halibut (ZZ/ZW) (Drinan et al., 2018; Galindo et al., 2011) while males are the heterogametic sex (XX/XY) in Atlantic halibut (Einfeldt et al., 2021; Palaiokostas et al., 2013) and in Greenland halibut (Ferchaud et al., 2022). Moreover, both XX/XY and ZZ/ZW sex determination systems are documented in more distantly related flatfish species. The turbot (Scophthalmus maximus) (Martinez et al., 2021) and half-smooth tongue sole (Cynoglossus semilaevis) (Chen et al., 2014), utilize a ZZ/ZW system while an XX/XY system has been proposed for the Senegalese sole (Solea senegalensis) (Guerrero-Czar et al., 2021; Portela-Bens et al., 2017). While sex determination systems in other vertebrate lineages are much older (therian mammals ~166 Ma (Wallis et al., 2008), avian ~102 Ma (Zhou et al., 2014)), flatfish offer a unique opportunity to study the biological mechanisms behind sex determination.

The Pacific halibut is a migratory demersal flatfish species of the order Pleuronectiformes that occupies a top trophic role in the North Pacific Ocean and Bering Sea ecosystems (Best, 1981). It is one of the largest flatfish species worldwide and exhibits adult sexual dimorphic growth, females growing larger than males to sizes reaching 230 kg in weight and 2.4 m in length (Figure 1a). This species also supports important commercial, recreational, and subsistence fisheries. Recent research to identify genetic markers diagnostic of sex in Pacific halibut for their application in fisheries management, as the commercial fishery is currently operating under minimum size limits (Stewart et al., 2021), resulted in the identification of a number of sex-linked loci that, in the absence of a Pacific halibut genome, mapped to the Z chromosome of the half-smooth tongue sole and largely to single linkage groups in Atlantic halibut and turbot, suggesting that genetic sex in Pacific halibut is likely under the control of loci present on a single chromosome (Drinan et al., 2018). Through the use of high-resolution genetic marker assays derived from the identified sex-linked loci (Drinan et al., 2018) it is now known that the Pacific halibut commercial catches in waters off Canada and the United States (including Alaska) are comprised of over 80% females (Stewart & Webster, 2021), providing direct evidence for sex-specific fishing pressure. Therefore, while promoting improved understanding of the mechanisms responsible for sex determination is a relevant aspect to the basic biology of the species, studies on sex determination in Pacific halibut have profound implications for fisheries management and conservation. In this study, we present the first chromosome-level reference genome assembly for Pacific halibut and utilize this genomic resource to identify genomic regions associated with sex in Pacific halibut using a pool sequencing approach. We identified a potential sex-determining region in chromosome 9 of Pacific halibut and, within this region, a potential candidate master sex-determining (MSD) gene. The genomic resource developed here will be essential to future genomic studies aimed at resolving population structure and dynamics and at understanding the evolutionary history of Pacific halibut populations.

2 | MATERIALS AND METHODS

2.1 | Sampling and genomic DNA extraction

Freshly sampled blood (500μl) from an adult Pacific halibut female captured by angling from the mouth of Resurrection Bay near Seward, Alaska (59.80°N, 149.23°W) was mixed with 25 ml of TNE-Urea lysis buffer (4 M urea; 10 mM Tris–HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% SDS; (Asahida et al., 1996). From this blood sample, high-molecular weight genomic DNA (gDNA) was purified by phenol-chloroform extraction. A separate blood sample from the same Pacific halibut female (1.5 ml) was gently mixed with 270μl of dimethyl sulfoxide (DMSO) in a 2 ml cryotube (15% DMSO) and
slowly cryopreserved in an isopropanol-containing Nalgene Mr Frosty Freezing container (Sigma) at −80°C for generating a chromosome contact map (Hi-C) (see Section 2.2.3).

Fin clip samples from 30 adult female and 30 adult male Pacific halibut captured by a chartered commercial longline vessel in the Portlock Bank region of the Gulf of Alaska (56°59’N-58°55’N, 148°41’W-152°44’W) were collected for whole-genome sequencing of pools of individuals (Pool-seq). Genomic DNA from fin clips was extracted using the DNeasy Blood & Tissue Kit (Qiagen) and the concentration was standardized across all samples and pooled in equimolar ratios by individual and sex, resulting in two gDNA pools, one for females and another for males. Ovarian and testicular samples were collected from the same fish, preserved in RNAlater (Invitrogen) and stored at −80°C until processed for RNA extraction. Ovarian and testicular samples were also collected, fixed in 10% buffered formalin and processed for histological analyses to determine the reproductive stage, as described previously (Fish et al., 2020). Collected females and males for Pool-sequence were at the early vitellogenic and early spermatogenic stages, respectively (data not shown).

2.2 DNA library construction and sequencing

2.2.1 Illumina sequencing

DNA library construction was performed according to the manufacturer’s instruction using the Truseq DNA nano library prep kit (Illumina). Briefly, gDNA was quantified using the HS dsDNA Assay kit on the Qubit (Invitrogen). 200 ng of gDNA were sonicated on a Bioruptor (Diagenode). Sonicated gDNA was end repaired and size selected on magnetic beads aiming for fragments of an average size of 550 bp. Selected fragments were adenylated on their 3’ ends before ligation of Illumina’s indexed adapters. The library was amplified using an eight cycle PCR and verified on a fragment analyser using the HS NGS fragment kit (Agilent). The library was quantified by qPCR using the KAPA Library quantification kit (Roche, ref. KK4824) and sequenced on half a lane of Hiseq2500 in paired-end 2x250 nt using the clustering and SBS rapid kit following the manufacturer’s instructions. This sequencing generated 57,277,046 raw clusters, leaving 56,057,418 clusters after Illumina’s purity filtering.

2.2.2 Nanopore sequencing

The quality and purity of gDNA were assessed using spectrophotometry, fluorometry and capillary electrophoresis. Additional purification steps were performed using AMPure XP beads (Beckman Coulter). All library preparations and sequencing were performed using Oxford Nanopore Ligation Sequencing Kit SQK-LSK109 according to the manufacturer’s instructions “1D gDNA selecting for long reads.” Genomic DNA (10 μg) was purified and then sheared to 20 kb using the megaruptor system (Diagenode). A size selection step using a 10 kb cutoff was performed on the BluePippin Size Selection system (Sage Science) with the 0.75% agarose cassettes, Marker 51 high Pass 6–10 kb. For each library, a one-step-DNA-damage repair + ENDrepair + dA-tail-of-double-stranded-DNA-fragments procedure was performed on 2 μg of DNA. Adapters were then ligated to DNAs in the library. Libraries were loaded onto R9.4.1 flowcells and sequenced on either a GridION or PromethION instrument at a concentration of 0.019 pmol for 48 h and 0.013 pmol for 64 h, respectively. The GridION flowcell produced 8.6 Gb of data and the PromethION flowcell produced 46.3 Gb of data.
2.2.3 | Hi-C sequencing

In situ Hi-C sequencing was performed as previously described (Foissac et al., 2019). Cryopreserved blood cells were thawed, washed twice with PBS and counted. Five million cells were then cross-linked with 1% formaldehyde in PBS, quenched with 0.125 mM glycine and washed twice with PBS. Membranes were disrupted using a Dounce pestle, and nuclei were permeabilized using 0.5% SDS and then digested with HindIII endonuclease. Five-prime overhangs at HindIII-cut restriction sites were filled-in, in the presence of biotin-dCTP with the Klenow large fragment, and then religated at an NheI restriction site. Nuclei were lysed and DNA was precipitated and then purified using Agencourt AMPure XP beads (Beckman Coulter) and quantified using the Qubit fluorometric quantification system (Thermo). T4 DNA polymerase was used to remove unligated biotinylated ends. Then, the Hi-C library was prepared according to Illumina’s protocols using the Illumina TruSeq Nano DNA HT Library Prep Kit with a few modifications: DNA (1.4 μg) was fragmented to 550bp by sonication. Sheared DNA was then sized (200–600bp) using Agencourt AMPure XP beads, and biotinylated ligation junctions were captured using M280 Streptavidin Dynabeads (Thermo) and then purified using reagents from the Nextera Mate Pair sample preparation kit (Illumina). Using the TruSeq nano DNA kit (Illumina), the 3’ ends of blunt fragments were adenyalted. Next, adapters and indexes were ligated and the library was amplified for 10 cycles. Library quality was assessed by quantifying the proportion of DNA cut by endonuclease NheI using a fragment analyser (Advanced Analytical Technologies). Finally, the library was quantified by qPCR using the Kapa Library Quantification Kit (Roche). Sequencing was performed on an Illumina HiSeq3000 apparatus (Illumina) using paired-end 2 × 150nt reads. This produced 51 million read pairs (1.5 Gb of raw nucleotides).

2.2.4 | Pool genome sequencing (Pool-seq) and analysis

Male and female pooled DNA libraries were constructed using the TruSeq Nano DNA HT Library Prep Kit (Illumina) following the manufacturer’s protocol. Briefly, DNA from each male and female pool (200ng/pool) was sonicated using a M220 focused-ultrasonicator (COVARIS) and size-selected using SPB beads (kit beads) to retain fragments of ~550bp. Blunt DNA fragments were monoadenylated, ligated to indexes and Illumina’s adapter and amplified using Illumina-specific primers. Libraries were checked for quality using a fragment analyser (Advanced Analytical Technologies Inc.) and quantified by qPCR using the Kapa Library Quantification Kit (Roche Diagnostics Corp.). Sequencing of male and female Pool-seq libraries was performed on a NovaSeq S4 lane (Illumina) using a paired-end read length of 2 × 150nt mode Illumina NovaSeq reagent kits following the manufacturer’s instructions. The run produced 105 million read pairs for the male pool library and 96 million read pairs for the female pool library. Reads from the male and female pools were analysed as previously described (Adolfi et al., 2021; Imarazene et al., 2021; Pan, Feron, et al., 2021), using the pooled sequencing analyses for sex signal (PSASS; https://github.com/SexGenomicsToolkit/PSASS), a software that compares pooled sequencing data sets from two sexes, and our Pacific halibut assembly as a reference assembly. PSASS was run with default parameters to compute three metrics in 50kb nonoverlapping windows over the Pacific halibut genome: (1) \( F_{ST} \) between males and females over the window, (2) density of sex-specific SNPs, defined as SNPs heterozygous in one sex and homozygous in the other sex, and (3) read depth for each sex.

2.2.5 | Estimation of linkage disequilibrium on chromosome 9

In order to investigate patterns of linkage disequilibrium (LD) throughout the Pacific halibut genome, the restriction-site associated DNA (RAD) sequence data generated by Drinan et al. (2018) was reanalysed using our Pacific halibut assembly as a reference. Complete details regarding RAD sequencing and raw data processing can be found in Drinan et al. (2018). Trimmed sequence reads were aligned to the Pacific halibut reference using minimap2 (v2.23) (Li, 2018) and invoking the short read preset option (−x sr). Samtools (v1.14) (Li et al., 2009) was used to sort and filter unmapped reads and alignments with mapping qualities (MAPQ) less than 20. The reference aligned data were processed using stacks (v2.60). First, the gstacks module was used to create RAD loci, identify SNPs, and genotype individuals while removing PCR duplicates. Then, the populations module was used to filter loci and export a vcf file of variants. Loci were retained for further analysis if they had a minor allele frequency (MAF) of at least 0.01 and genotypes for at least 20% of the individuals, these filters were applied using the −filter-haplotype-wise option. Patterns of linkage disequilibrium were analysed separately for males and females. First, vcftools (v0.1.16) (Danecek et al., 2011) was used to apply more stringent SNP filters to each data set separately, requiring a MAF of at least 0.05 and genotypes for at least 80% of the individuals within each data set. Plink (v1.9) (Chang et al., 2015; www.cog-genomics.org/plink/1.9/) was used to calculate pairwise \( r^2 \) for all SNPs located on Chr 09. Pairwise \( r^2 \) values were summarized in nonoverlapping 100kb bins by taking the median within each bin and plotting.

2.3 | Genome assembly and analysis

GridION and PromethION data were trimmed using porechop version 0.2.1 (Wick, 2017/2019) and assembled using Flye version 2.9 (Kolmogorov et al., 2019) with default parameters. The assembly base pair quality was improved by two polishing steps, including one round of long read alignment to the draft genome with minimap2 version 2.7 (Li, 2018) followed by samtools sort and index version 1.10 then by racon version 1.3.1 (Vaser et al., 2017), as well as one round of short read alignments using bwa mem version 0.7.17
followed by samtools sort and index and Hapo-G version 1.2 (Aury & Iстасе, 2021). Default parameters were used for all these software packages. The polished genome assembly was then scaffolded using Hi-C as a source of linking information. Reads obtained by in situ Hi-C sequencing (see Section 2.2.3) were aligned to the draft genome using juicer (Durand et al., 2016) with default parameters. A candidate assembly was then generated with the 3D de novo assembly (3D-DNA) pipeline (Dudchenko et al., 2017) with the -r 0 parameter. Finally, the candidate assembly was manually reviewed using juicebox assembly tools (Durand et al., 2016). Genome completeness was estimated using Benchmarking universal single-copy orthologues (BUSCO) version 4.0 (Simão et al., 2015) based on 3640 BUSCO orthologues derived from the Actinopterygii lineage.

### 2.4 | Genome annotation

The first annotation step was to identify repetitive content using RepeatMasker v4.0.7 (Tarailo-Gravoac & Chen, 2009), DustMasker of NCBI toolkit v18 (described in Morgulis et al., 2006), and TandemRepeatFinder version 4.09 (Benson, 1999). A species-specific de novo repeat library was built with RepeatModeler version 1.0.11 (http://www.repeatmasker.org/RepeatModeler/) and repeated regions were located using RepeatMasker with the de novo and Danio rerio libraries. Bedtools version 2.26.0 (Quinlan & Hall, 2010) was used to aggregate repeated regions identified with the three tools and to soft mask the genome. 23.3% of the assembly was soft masked.

The second annotation step aimed to generate a transcriptome annotation using Maker3 genome annotation pipeline version 3.01.02-beta (Holt & Yandell, 2011). It combines annotations and evidence from three approaches: (1) similarity with fish proteins, assembled transcripts (see below), and de novo gene predictions. Protein sequences from 11 fish species (Astyanax mexicanus, D. rerio, Gadus morhua, Gasterosteus aculeatus, Lepisosteus oculatus, Oreochromis niloticus, Oryzias latipes, Poecilia formosa, Takifugu rubripes, Tetraodon nigroviridis, Xiphophorus maculatus) found in Ensembl were aligned to the masked genome using exonerate version 2.2.0 (Slater & Birney, 2005) with the alignment model protein2genome that allows translated alignments with modelling of introns. (2) RNA-seq reads from 12 tissues (white muscle, liver, ovary, testis, red muscle, heart, pituitary, brain, spleen, gill, retinas and head kidney) (Jasonowicz et al., 2022) were used for genome annotation and aligned to the chromosomal assembly using HISAT2 version 2.2.1 (Kim et al., 2019). Bam files were merged using samtools merge and signal wiggle files were generated using STAR version 2.5.1b (Dobin et al., 2013) in run mode inputAlignmentsFromBAM with outWigType and outWigStrand options. Cufflinks version 2.2.1 (Trapnell et al., 2010) was used to assemble the transcripts, which were used as RNA-seq evidence. (3) Braker version 2.0.4 (Hoff et al., 2016) provided de novo gene models with wiggle files provided by STAR as hint files for GeneMark and Augustus training. The best supported transcript for each gene was chosen using the quality metric called annotation edit distance (AED) (Eilbeck et al., 2009). Genome annotation gene completeness was assessed by BUSCO using the Actinopterygii group. From the 52 scaffolds of the genome assembly, 40 were annotated. These annotated scaffolds were kept for further analyses.

### 2.5 | Real-time quantitative PCR

The mRNA expression levels of bmpr1ba in testis and ovary from adult Pacific halibut were measured by real-time quantitative PCR (rt qPCR) and normalized to the mRNA expression levels of two housekeeping genes: elongation factor 1-alpha 1 (ef1a1) and ubiquitin (ubq). The housekeeping genes ef1a1 and ubq were identified by geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) as the most suitable in both sets of samples. Primers to bmpr1ba, ef1a1 and ubq were designed in Primer3 using Geneious (https://www.geneious.com) (Table S1). RNA was extracted from testis (N = 7) and ovary (N = 10) samples from fish used in Pool-Seq using the PureLink RNA Mini Kit (Invitrogen) with β-mercaptoethanol (Thermofisher Scientific) added to the lysis buffer at a final concentration of 4%. One microgram of total RNA from each sample was treated with DNase I amplification grade (Life Technologies) to remove contaminating gDNA. Resulting RNA concentrations were measured on a NanoDrop 8000 spectrophotometer (Thermo Scientific) and 300ng of each RNA sample were reverse transcribed using the SuperScript IV VILO Master Mix cDNA kit with ezDNase (Invitrogen) using the protocol specified by the manufacturer. All PCR reactions were run in a QuantStudio 6 real-time PCR system (Applied Biosystems) and followed the requirements of the MIQE guidelines. Each sample was run in triplicate 10 μl reactions with 2 μl of cDNA (diluted 1:20), 400nM of each primer, and PowerUp SYBR Green Mastermix (Applied Biosystems) using the manufacturer’s standard PCR and melt curve protocols. The specificity of the reaction and absence of primer dimers were confirmed by the melt curve profile, and products of new primer pairs were run on a 1.5% agarose gel to confirm expected amplicon size. Primer efficiency was calculated by analysing serial dilutions of cDNA samples. The expression level of bmpr1ba was normalized to the geometric mean of the two reference genes (M<0.5) and calculated using the 2−ΔΔC_T method (Livak & Schmittgen, 2001).

### 2.6 | Chromosome synteny among flatfish genomes

D-genies (Cabanettes & Klopp, 2018) was used to compare the Pacific halibut genome assembly (RefSeq: GCF_022539355.2) to representative genome assemblies that are publicly available for other flatfish species: Atlantic halibut (H. hippoglossus) (RefSeq: GCF_009819705.1), Greenland halibut (Reinhardtius hippoglossoides) (GenBank: GCA_000618295.3), smooth-tongue sole (C. semilaevis) (RefSeq: GCF_000523025.1), Senegalese sole (S. senegalensis)
2.7 Bone morphogenetic protein receptor type-1B sequence comparison

Multiple sequence alignments of the Bmpr1ba protein sequences where done using the MUSCLE aligner (v3.8.1551) (Edgar, 2004). Sequences from the flatfish Bmpr1ba proteins were also compared to sequences from zebrafish (D. rerio), and medaka (O. latipes). A phylogenetic tree was generated using the web version of PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/) (Guindon et al., 2010). The substitution model was selected using the Smart Model Selection (SMS) (Lefort et al., 2017) implementation in PhyML using Akaike information criterion (AIC) as a model selection criterion. Branch support values were estimated using approximate likelihood-ratio test for branches (aLRT) (Anisimova & Gascuel, 2006).

2.8 Regulatory elements of the bone morphogenetic protein receptor type-1ba gene

Putative transcription factor (TF) binding sites were identified by scanning the genomic region containing the bmpr1ba gene and 5 kb of upstream sequences using the R package TFBSTools (v1.26) (Tan & Lenhard, 2016). Transcription factor binding profiles were obtained as position weight matrices from the JASPAR 2020 database (Fornes et al., 2019). Two scans were carried out. The JASPAR POLI collection was used to identify binding sites linked to RNA polymerase II core promoters. Additionally, a scan using vertebrate binding profiles from the JASPAR CORE collection was carried out to identify binding sites for other TFs that may play a role in the expression of the bmpr1ba gene. To evaluate the potential for functional significance of the sex-specific SNPs, their location was compared to the structure of the bmpr1ba gene (introns, exons, etc.) and the potential transcription factor binding sites identified. First, the bmpr1ba gene annotation was manually curated as (1) 5'UTR, defined as the transcript upstream of start codon in the annotation, (2) 3'UTR, defined as the transcript downstream of the stop codon in the annotation, (3) Intron-exon boundaries, defined using the annotation, and (4) 5' flanking region, defined as the 5 kb region upstream of 5'UTR (as indicated above).

Sex-specific SNPs within the bmpr1ba gene and 5 kb of upstream sequences were compared to the manual curation of the bmpr1ba gene to identify the gene regions containing SNPs and then this list was joined to the list of putative TF binding sites using bedtools (v2.29.2) (Quinlan & Hall, 2010). With this, the sex-specific SNP positions were compared to putative TF binding sites within the bmpr1ba gene to assess potential changes in the transcriptional regulation of this gene due to the presence of SNPs in regulatory regions.

2.9 Functional annotation of Pacific halibut genes and gene ontology analysis

To infer the potential function of genes identified in the Pacific halibut genome annotation, InterProScan (v5.52-86.0) (Jones et al., 2014) was used to screen all protein products identified in the genome annotation against the InterPro consortium member databases (Blum et al., 2021). Gene ontology (GO) terms for the InterPro scan matches were assigned to genes in Pacific halibut genome annotation. The R (v4.0.5) (R Core Team, 2019) packages VISEAGO (v1.4.0) (Brionne et al., 2019) and topGO (v2.44.0) (Alexa & Rahnenfuhrer, 2020) were used to identify enriched GO terms associated with genes in the putative sex determining region of chromosome 9 using GO terms associated with the entire genome as a background. GO terms were scored using the classic algorithm and fisher test implemented in topGO for all three GO domains (cellular component, biological process, and molecular function).

2.10 Mapping of RAD-tags associated with sex to the Pacific halibut genome assembly

Drinan et al. (2018) used restriction-site associated DNA (RAD) sequencing to identify 56 RAD-tags associated with sex in Pacific halibut. The consensus sequences for these RAD-tags were aligned to Pacific halibut genome assembly using minimap2 (v2.17) using the short read preset (–x sr).
3 | RESULTS AND DISCUSSION

3.1 | Generation of a chromosome-level assembly of the Pacific halibut genome

We have generated the first high-continuity, chromosome-length de novo assembly of the Pacific halibut genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_022539355.2) by combining Oxford Nanopore Technologies (ONT) long reads, Illumina short linked reads, and a chromosome contact map (Hi-C). The complete genome assembly after the Hi-C integration step yielded 52 scaffolds with a total genome size of 602.15 Mb, an N\textsubscript{50} scaffold length of 27.29 Mb and an L\textsubscript{50} scaffold number of 11 (Table 1). The complete genome assembly includes 24 chromosome-length scaffolds that represent 99.78% of the complete genome sequence. Based on the discrete blocks identified in the Hi-C contact map (Figure 1b) and the reported 24 chromosomes in Atlantic halibut, as determined by karyotype (Brown et al., 1997) and linkage analyses (Reid et al., 2007) and genome sequencing (Edvardsen et al., 2022; Einfeldt et al., 2021), we propose that the 24 chromosome-length scaffolds identified here correspond to the 24 chromosomes in Pacific halibut. Pacific halibut chromosome sizes range from 11.27 to 32.85 Mb (Table S2). Genome assembly completeness, as estimated by BUSCO in genome mode with the Actinopterygii lineage as reference, is demonstrated by the high percentage (98.4%) of complete BUSCOs. By using protein, transcript and de novo gene prediction analyses we annotated 26,772 protein-coding genes in the Pacific halibut genome, whereas the annotation conducted by the NCBI Eukaryotic Genome Annotations Pipeline (NCBI Hi Annotation Release 100 [https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Hippoglossus_stenolepis/101/]) yielded 27,944 annotated genes.

Table 1 Pacific halibut (H. stenolepis) genome assembly statistics and assembly completeness

| Assembly metrics | Complete assembly | Chromosomes only |
|------------------|------------------|------------------|
| Number of scaffolds | 52 | 24 |
| Total size of scaffolds | 602,147,347 | 600,901,890 |
| Longest scaffold | 32,845,235 | 32,845,235 |
| Shortest scaffold | 582 | 11,267,222 |
| Mean scaffold size | 11,579,757 | 25,037,579 |
| Median scaffold size | 184,878 | 25,468,064 |
| N\textsubscript{50} scaffold length | 27,288,303 | 27,288,303 |
| L\textsubscript{50} scaffold count | 11 | 11 |
| % of assembly in chromosomes | — | 99.78% |
| % of assembly in unanchored scaffolds | — | 0.22% |

TABLE 1 Pacific halibut (H. stenolepis) genome assembly statistics and assembly completeness

3.2 | Identification of the sex-determining region in the Pacific halibut genome

In order to identify the sex-determining (SD) region and putative MSD gene in Pacific halibut we set out to detect whole genome sex differences by Pool-seq analysis (Schlotterer et al., 2014). We mapped the Pool-seq reads from male and female pools to the Pacific halibut genome assembly to characterize genomic regions containing sex-specific single nucleotide polymorphism (SNP) differences. By conducting whole genome analysis of SNP distribution, we identified a compared to other related flatfish species with sequenced genomes, the size of the Pacific halibut genome assembly is most similar to that of Atlantic halibut (596.8 Mb) (Einfeldt et al., 2021) and Greenland halibut (598.5 Mb) (Ferchaud et al., 2022), followed by turbot (568 Mb) (Figueras et al., 2016), Japanese flounder (546 Mb) (Shao et al., 2017) and half-smooth tongue sole (477 Mb) (Chen et al., 2014). The quality of the Pacific halibut genome assembly was also comparable to that of some closely related species that have been sequenced recently, like Atlantic and Greenland halibut (Table 2). The genomic coverage of the Atlantic and Greenland halibut genomes by the Pacific halibut assembly was very high (99.31 and 99.60%, respectively (Figure S1). Furthermore, a high degree of nucleotide level synteny was observed between Pacific halibut and the other flatfish genomes, with the highest levels of synteny observed between its closest relatives in the subfamily Hippoglossinae (H. hippoglossus and R. hippoglossoides) (Atta et al., 2022) (Figure S2). Inspection of the dotplots identified orthologous chromosomes for all Pacific halibut chromosomes in both the Atlantic halibut and Greenland halibut genome assemblies (Figure S2) that is likely a reflection of their recent common evolutionary history (Grant, 1987; Grant et al., 1984).
clear sex-linked region in Pacific halibut females on chromosome 9 (Chr9) alone (Figure 2). This large region of approximately 12 Mb in size (Chr9: 14,000,000-26,000,000bp) shows differentiation between the two sexes, contains a high-density of female-specific SNPs that peak in regions of high differentiation (Figure 3) and is very likely the SD region in Pacific halibut. From a total of 20,048 sex-specific SNPs identified in this region, 18,452 SNPs were female-specific (strictly heterozygotes in females and homozygotes in males) and 1596 SNPs were male-specific. In addition, increased LD was observed in this region (Figure 4). Therefore, we propose that the female-specific region in Chr9 likely corresponds to the nonrecombining region on the sex chromosome in Pacific halibut. Read coverage between males and females was similar throughout Chr9 including the SD region, suggesting low levels of sex chromosome degeneration despite the accumulation of female-specific SNPs. However, close examination of the SD region revealed a small 18kb region at Chr9:17,625,400-17,643,850 with an approximately four-fold excess of female over male reads (Figure 3d). This region is probably a nonresolved W-specific copy number variation (CNV) region in the assembly that contains one complete gene annotated as kineticochore protein ndc80 (ndc80) and a 3’ end truncated gene annotated as nipped-B-like protein A (nipbla) (Figure S3).

Mapping of previously identified Pacific halibut RAD-tags associated with sex (Drinan et al., 2018) to the Pacific halibut genome assembly resulted in the alignment of 55 of the 56 RAD-tags, all of which mapped to the putative SD region (14,601,685-25,693,202bp) on Chr9 (NC_061491.1) (Figure 3a; Figure S4). The large number of sex-associated RAD-tags mapping to this region of Chr9 provides additional support that this region of the genome may play a key role in the sexual determination system of Pacific halibut.

Whole-genome macrosyntenic relationships between Pacific halibut and other flatfish species show that Chr9 of Pacific halibut is orthologous to Chr9 of Atlantic halibut, Greenland halibut and turbot, linkage group (LG) 5 of Senegalese sole, and to both Z and W chromosomes of half-smooth tongue sole (Figure 5). In contrast to the regions of Pacific halibut Chr9 that map to the half-smooth tongue sole sex chromosomes, highlighting the importance of this genomic region in the sex determination system of Pacific halibut, the reported SD regions in the genomes of Atlantic halibut, Greenland halibut, turbot and Senegalese sole are present in chromosomes or LGs that are not orthologous to Chr9 of Pacific halibut: Chr12 in Atlantic halibut (Einfeldt et al., 2021), Chr10 and 21 in Greenland halibut (Ferchaud et al., 2022), Chr12 in turbot (Martinez et al., 2021) and LG18 in Senegalese sole (Guerrero-Czar et al., 2021). These differences in SD chromosomes among phylogenetically related flatfish species are consistent with the known rapid turnover of sex chromosomes in teleosts, attributed to allelic diversification or gene duplication and translocation events associated with the emergence of new MSD genes (Kikuchi & Hamaguchi, 2013; Pan et al., 2018). Closer inspection of the alignment of Chr9 in Pacific halibut to orthologous chromosomes in the other Hipoglossinae species revealed the presence of structural differences between these species in this region of their genome (Figure 6). Both species contain inversions on Chr9 but only the inversion in Atlantic halibut overlaps with the SD region of Pacific halibut (Figure 6). It has been discussed that inversions offer a potential mechanism for the suppression of recombination which is often implicated in sex chromosome evolution (Furman et al., 2020). This is supported by our analysis of LD, which identified a large block of SNPs showing higher levels of LD on Chr9 in females than in males (Figure 4), suggesting a reduced recombination rate for females in this region of the Pacific halibut genome. Given the relatively recent divergence between Pacific and Atlantic halibut (Grant, 1987; Grant et al., 1984), we hypothesize that the increased levels of LD on Chr9 in Pacific halibut, coupled with the structural differences that exist between the SD region of Pacific halibut and the orthologous region of Chr9 of Atlantic halibut, could be associated with the early stages of sex chromosome degradation; however, further studies are needed to shed light onto this issue.

### 3.3 Search for a putative sex-determining gene in Pacific halibut

We next searched the putative SD region in Chr9 for potential candidate MSD genes in Pacific halibut. We compared the genome annotation to the locations of sex-specific SNPs in the putative SD region and identified 482 annotated genes that contained a total of 13,949 SNPs, with 12,915 female-specific and 1034 male-specific SNPs (Table S3).

Enrichment analysis of GO terms associated with genes in the SD region of Chr9 yielded several significantly enriched GO terms (biological process [BP]: 26; cellular component: 4; molecular function [MF]: 19), including “regulation of translational fidelity” as the top BP term and “hormone receptor activity” and “growth factor receptor binding” among the top MF terms (Table S4). Although several annotated genes containing a large number of sex-specific SNPs were identified in the SD region of Chr9, most notably netrin receptor UNC5C (unc5c), glutamate receptor ionotropic, delta-2 (grid2) and transmembrane protein 132C, with 702, 665 and 493 SNPs, respectively, none of these genes corresponded to known MSD genes in vertebrates.

However, by specifically searching among all the annotated genes present in this region for members of the few gene families known to have produced MSD genes in teleost species (e.g., SOX, DMRT, TGF-β (Pan et al., 2018), we identified the bone morphogenic protein receptor-type IB (bmpr1ba) gene in the SD region at position 17,407,676-17,433,892bp on Chr9, located ~2.2 kb downstream of the largest FST peak (Figure 3). The bmpr1ba gene is a member of the TGF-β superfamily of polypeptide growth factors and, therefore, is part of a complex TGF-β signalling pathway whose components have been recruited to play important roles in sex determination during teleost evolution (Pan, Kay, et al., 2021). The bmpr1ba gene codes for a type I transmembrane serine/threonine kinase receptor that upon ligand binding is activated by type II receptors (e.g., Amhr2) triggering a downstream signal, as part of the characteristic hexameric signalling complex through which TGF-β family ligands, such as anti-Müllerian hormone (Amh) or bone morphogenetic protein (Bmp), exert their function (Miyazono et al., 2005). Examples of teleost MSD genes belonging to the TGF-β family include genes coding for ligands and receptors that are derived either by gene duplication or...
allelic diversification from *amh* (*amhY* and *amhbY*), from amh receptor II or *amhr2* (*amhr2Y* and *amhr2bY*), from gonadal soma-derived growth factor or *gsdf* (*gsdfY*), from growth differentiation factor 6 or *gdf6* (*gdf6Y* and *gdf6bY*) and from bmp receptor 1bb or *bmpr1bb* (*bmpr1bbY*). Interestingly, *bmpr1ba* is a paralogue of *bmpr1bbY*, a gene encoding a truncated form of a BMP type I receptor with constitutive activity that originated by gene duplication and that is the candidate MSD gene in Atlantic herring (*Clupea harengus*) (Rafati et al., 2020). Therefore, we propose that *bmpr1ba* is a potential candidate MSD gene in Pacific halibut that, if confirmed, would represent the first potential MSD gene coding for a receptor to TGF-β family ligands identified in a flatfish species. Other candidate MSD genes identified in flatfish species include *dmrt1* in half-smooth tongue sole (Chen et al., 2014), *gsdf* in Atlantic halibut (Einfeldt et al., 2021) and *sox2* in turbot (Martinez et al., 2021) as well as in Greenland halibut (Ferchaud et al., 2022). Like species of the genus *Oryzias* and *Gasterosteus*, Pacific halibut and Atlantic halibut, with female and male heterogametic systems, respectively, provide another example of closely related teleost species that have recruited different sex-linked chromosomes and different MSDs, further establishing the lack of conservation of sex determining mechanisms in teleost fish (Pan et al., 2016). It is conceivable that the relatively recent evolutionary divergence of Atlantic halibut from Pacific halibut was accompanied by a shift from female to male heterogamety driven possibly by the emergence of a new MSD. A shift between male and female heterogamety driven by the rise of a new dominant female determinant was recently proposed for certain species of the Esocidae family (Pan, Feron, et al., 2021).

We found a total of 115 female-specific SNPs in the *bmpr1ba* gene (Table S5), with only four SNPs located within exons that are synonymous; therefore, no changes in the sequence of the translated protein nor its function are expected between males and females. In absence of obvious sequence characteristics of the product of the *bmpr1ba* gene that could support a potential role for this gene

| Assembly metrics                          | Pacific halibut | Atlantic halibut | Greenland halibut |
|-------------------------------------------|-----------------|------------------|-------------------|
| Genome size (Mb)                          | 602.1           | 596.8            | 598.5             |
| Chromosome-level scaffolds mapped to total assembly (%) | 99.8            | 99.8             | 96.3              |
| N50 (Mb)                                  | 27.3            | 26.3             | 25.0              |
| Complete BUSCO (%)                        | 98.5            | 96.9             | 96.5              |
in driving sex determination, we next investigated whether \textit{bmpr1ba} gene expression differed between males and females by measuring the mRNA expression levels of \textit{bmpr1ba} in male and female gonads from adult Pacific halibut by qPCR. Our results show similar levels of \textit{bmpr1ba} mRNA expression between ovaries and testes from adult fish (Figure S5). These results are not surprising given that sex-related differences in expression levels of candidate MSD genes are most likely to occur during early gonad development at the sex determination
Unfortunately, the lack of Pacific halibut captive broodstock to produce early embryos prevents us from testing this hypothesis. However, we searched for female-specific SNPs in non-coding regions of the \textit{bmpr1ba} gene for indications of potential sex-specific gene transcriptional differences. We identified 12 SNPs in the 5 kb region immediately upstream of the \textit{bmpr1ba} gene that overlap with 57 putative TF binding sites (Table S5). Despite the limited information on the mechanisms involved in the transcriptional regulation of the \textit{bmpr1ba} gene, the presence in the promoter region of SNPs in binding sites for TFs that interact with BMP signalling, such as MEIS1 (Erickson et al., 2010), GSX1 (Ma et al., 2020), Nkx2-5 (Smith et al., 2000; Theodosiou & Tabin, 2005) and RUNX-2 (Ito & Miyazono, 2003), is suggestive of possible sex-specific differences in the transcription of the \textit{bmpr1ba} gene during the sex determination stage. As with functional alterations of Amhr2 that promote a female phenotype in medaka (Morinaga et al., 2007), GSX1 (Ma et al., 2020), Nkx2-5 (Smith et al., 2000; Theodosiou & Tabin, 2005) and RUNX-2 (Ito & Miyazono, 2003), is suggestive of possible sex-specific differences in the transcription of the \textit{bmpr1ba} gene during the sex determination stage. As with functional alterations of Amhr2 that promote a female phenotype in medaka (Morinaga et al., 2007), GSX1 (Ma et al., 2020), Nkx2-5 (Smith et al., 2000; Theodosiou & Tabin, 2005) and RUNX-2 (Ito & Miyazono, 2003), is suggestive of possible sex-specific differences in the transcription of the \textit{bmpr1ba} gene during the sex determination stage. As with functional alterations of Amhr2 that promote a female phenotype in medaka (Morinaga et al., 2007), GSX1 (Ma et al., 2020), Nkx2-5 (Smith et al., 2000; Theodosiou & Tabin, 2005) and RUNX-2 (Ito & Miyazono, 2003), is suggestive of possible sex-specific differences in the transcription of the \textit{bmpr1ba} gene during the sex determination stage.
to speculate that changes in the expression levels of \textit{bmpr1ba} could potentially result in alterations of Amh signalling in Pacific halibut. Clearly, further studies are needed to provide evidence for a possible role of \textit{bmpr1ba} in sex determination in Pacific halibut.

Related to other Pleuronectiform species, the \textit{bmpr1ba} gene is similarly located in Chr9 in both Atlantic halibut and turbot (Table S6). Interestingly, the \textit{bmpr1ba} gene is located in sex chromosome Z in half-smooth tongue sole, whereas in Senegalese sole it is located in LG05 and in Japanese flounder in an unplaced scaffold (NW_017859680.1). The syntenic arrangement of the \textit{bmpr1ba} gene is well conserved among Pacific halibut, Atlantic halibut, Japanese flounder and turbot and partially conserved when compared to Senegalese sole and half-smooth tongue sole (Figure 7). The conserved synteny of the \textit{bmpr1ba} gene is coupled with high

\textbf{FIGURE 6} Syntenic relationship of Pacific halibut (\textit{H. stenolepis}) chromosome 9 (Chr9) and orthologues in Atlantic halibut (\textit{H. hippoglossus}) (a) and Greenland halibut (\textit{R. hippoglossoides}) (b). The sex associated region of Chr9 in Pacific halibut is shaded in light blue, the genomic location of the \textit{bmpr1ba} gene is indicated in red.

\textbf{FIGURE 7} Syntenic comparison of the genes located around the \textit{bmpr1ba} gene in Pacific halibut (\textit{H. stenolepis}), Atlantic halibut (\textit{H. hippoglossus}), Japanese flounder (\textit{P. olivaceus}), turbot (\textit{S. maximus}), Senegalese sole (\textit{S. senegalensis}) and half-smooth tongue sole (\textit{C. semilaevis}). Orthologous genes are identified by colour, a blue line between genes represents a gap in the order of genes (i.e., nonorthologous genes are present in this region of the genome).
homology among the flatfish Bmpr1ba protein sequences, particularly between the Atlantic and Pacific halibut sequences that differ only in two amino-acid residues (Figure S6), as supported by their close phylogenetic relationship (Figure 8). Unfortunately, these observations do not provide indications on how the bmpr1ba gene may have emerged as a potential candidate MSD gene in Pacific halibut but we attribute this to the relatively recent evolutionary history of species within the Pleuronectidae family.

In summary, we have generated the first chromosome-level assembly of the Pacific halibut genome and used this resource to identify the sex-determining genomic region, leading to the identification of a putative candidate MSD gene for Pacific halibut. The generated genomic resource will be instrumental for future research on population genomics informing management and conservation strategies for Pacific halibut.

AUTHOR CONTRIBUTIONS
Yann Guiguen and Josep V. Planas designed the study. Anna Simeon, Céline Roques, Carole Iampietro, Jérôme Lluch, Cécile Donnadieu and Hugues Parrinello performed research. Andrew J. Jasonowicz, Margot Zahm, Cédric Cabau, Christophe Klopp, Hugues Parrinello, Daniel P. Drinan, Lorenz Hauser, Yann Guigen analysed the data. Andrew J. Jasonowicz and Josep V. Planas wrote the manuscript and Yann Guigen, Daniel P. Drinan and Lorenz Hauser edited the manuscript. All the authors read and approved the final manuscript.

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CONFLICT OF INTERESTS
The authors have no conflict of interest to declare.

DATA AVAILABILITY AND BENEFIT-SHARING STATEMENT
Data availability statement:
Genetic data:
The Pacific halibut whole genome sequencing data that support the findings of this study are openly available in NCBI at [https://www.ncbi.nlm.nih.gov/bioproject/622249](https://www.ncbi.nlm.nih.gov/bioproject/622249), under BioProject PRJNA622249. The Pacific halibut genome assembly is openly available in NCBI at [https://www.ncbi.nlm.nih.gov/assembly/GCA_022539355.2/](https://www.ncbi.nlm.nih.gov/assembly/GCA_022539355.2/) with GenBank accession number GCA_022539355.2. The RNA-seq data that support the findings of this study are openly available in NCBI at [https://www.ncbi.nlm.nih.gov/bioproject?LinkName=bioproject_biosample&LinkRead&leName=BioSample&ordinalpos=1&IdsFromResult=622249](https://www.ncbi.nlm.nih.gov/bioproject?LinkName=bioproject_biosample&LinkRead&leName=BioSample&ordinalpos=1&IdsFromResult=622249), under BioProject PRJNA634339.
Sample metadata:
The master record for the whole genome shotgun sequencing project has been deposited at DDBJ/ENA/GenBank under the accession JAKRZP000000000. The version described in this paper is version JAKRZP010000000 and is openly available in NCBI at [https://www.ncbi.nlm.nih.gov/nuccore/JAKRZP000000000](https://www.ncbi.nlm.nih.gov/nuccore/JAKRZP000000000). Sample metadata is openly available in NCBI at [https://www.ncbi.nlm.nih.gov/biosample?Db=biosample&DbFrom=bior project&Cmd=Link&LinkName=bioproject_biosample&LinkRead&leName=BioSample&ordinalpos=1&IdsFromResult=622249](https://www.ncbi.nlm.nih.gov/biosample?Db=biosample&DbFrom=bior project&Cmd=Link&LinkName=bioproject_biosample&LinkRead&leName=BioSample&ordinalpos=1&IdsFromResult=622249), under BioSamples SAMN14503176, SAMN25516224, SAMN25600010 and SAMN25600011.
Benefit-sharing statement:
Benefits from this research accrue from the sharing of our data and results on public databases as described above.

ORCID
Margot Zahm [https://orcid.org/0000-0001-8632-0109](https://orcid.org/0000-0001-8632-0109)
Cédric Cabau [https://orcid.org/0000-0001-8560-4003](https://orcid.org/0000-0001-8560-4003)
Christophe Klopp [https://orcid.org/0000-0001-7126-5477](https://orcid.org/0000-0001-7126-5477)
Carole Iampietro [https://orcid.org/0000-0002-8148-4785](https://orcid.org/0000-0002-8148-4785)

**FIGURE 8** Maximum likelihood phylogenetic reconstruction of flatfish Bmpr1ba protein sequences, including Pacific halibut (H. stenolepis), Atlantic halibut (H. hippoglossus), Japanese flounder (P. olivaceus), turbot (S. maximus), Senegalese sole (S. senegalensis) and half-smooth tongue sole (C. semilaevis), compared to zebrafish (D. rerio) and medaka (O. latipes). The values indicate branch support (estimated by aLRT).
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