1   A compendium of novel genomics technologies provides a chromosome-scale

2   assembly and insights into the sex determining system of the Greenland Halibut

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2

35   ABSTRACT

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37   Despite the commercial importance of Greenland Halibut (*Reinhardtius hippoglossoides)*,

38   important gaps still persist in our knowledge of this species, including its reproductive biology

39   and sex determination mechanism. In this study, we combined single molecule sequencing of

40   long reads (Pacific Sciences) with Chromatin Conformation Capture sequencing (Hi-C) data to

41   provide the first chromosome-level genome reference for this species. The high-quality assembly

42   encompassed more than 598 Megabases (Mb) assigned to 1 594 scaffolds (scaffold N50 = 25

43   Mb) with 96 % of its total length distributed among 24 chromosomes. The investigation of its

44   syntenic relationships with other economically important flatfish species revealed a high

45   conservation of synteny blocks among members of this phylogenetic clade. Sex determination

46   analysis revealed that flatfishes do not escape the rule applied to other teleost fish and exhibit a

47   high level of plasticity and turnover in sex-determination mechanisms. A whole-genome

48   sequence analysis of 198 individuals allowed us to draw a full picture of the molecular sex

49   determination (SD) system for Greenland Halibut, revealing that this species possesses a very

50   nascent male heterogametic XY system, with a putative major effect of the sox2 gene, also

51   described as the main SD driver in two other flatfishes. Interestingly, our study also suggested for

52   the first time in flatfishes that a putative Y-autosomal fusion could be associated with a

53   reduction of recombination typical of early steps of sex chromosome evolution.

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57 INTRODUCTION

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60   The comprehension of sex-determining (SD) mechanisms is of high importance in biology,

61 as they affect developmental processes, the evolution of genomes, and are responsible for

62 determining sex ratio in a population, a key demographic parameter for its viability and stability

63 (Bull 1983, Uller et al 2007). In recent years, genetic analyses in non-model species have

64 revealed a greater degree of sex chromosome diversity and turnover than previously

65 appreciated (Abbott et al 2017). In fact, some lineages, including lizards, fish, amphibians,

66 insects, and plants show frequent changes in the location of SD genes and high rates of turnover

67 of sex chromosomes. This led Furman et al (2020) to propose a new conceptual framework, in

68 which sex chromosome evolution is more cyclical than linear and in which exceptions are the

69 rules in sex determination (McGrath 2020). In this cycle, a new master sex-determining locus

70 arises on an autosome, leading to sex chromosome formation generally characterized by

71 recombination suppression between the Y and X, or the Z and W. As under a classic sex

72 chromosome evolution model, this may progress towards increased divergence between male

73 and female chromosomes, up to chromosome heteromorphism characterized by genomic

74 degradation including rearrangements and loss of genetic material (Charlesworth et al 2005;

75 Bachtrog 2013). However, sex chromosomes and sex-determining regions can also undergo

76 turnover with either the evolution of a new sex-determining gene or the transposition of the sex-

77 determining gene to a new location in the genome, leading to another cycle (Bachtrog 2006).

78 The increasing availability of high-quality genome assemblies in non-model taxonomic groups is

79 now providing exciting opportunities to revisit the evolution of genetic sex-determination.

80

81   Fish form a particularly relevant group to investigate the evolutionary dynamics of sex

82 chromosomes, as they exhibit a remarkable gonad development plasticity and a high

83 evolutionary SD turnover (Kitano and Peichel 2012). Fish display highly diverse chromosome SD

84 systems, including models analogous to the XX/XY (male heterogametic) and ZZ/ZW (female

85 heterogametic) systems of mammals and birds respectively, but also others involving multiple

86 sex chromosomes (Capel 2017). In accordance with this rapid turnover, chromosome

87 heteromorphisms are rare in fish, which generally show limited sex chromosome differentiation

88 (Chen et al 2014), characteristic of early sex-determination mechanisms. Until recently, the DM-

89 domain gene on the Y chromosome (dmy) gene was the only known gene coding for a functional

90 protein in the Y-specific chromosome region (Nanda et al 2002, Kondo et al 2006) and was

91 described as the master SD gene in a wide range of invertebrates and vertebrates (including

92 fish). However, thanks to new genomics tools, the genomic features underlying SD have been

93 more intensively studied and several other strong candidates of master SD genes have been

94 found in fish (amhy, gsdf, amhr2, sdY, Guerrero-Estévez and Moreno-Mendoza 2010, Yano et al

95 2013). These novel master SD genes highlight the importance of non-transcriptional factors in

96 sex determination and differentiation. In fact, three of these genes (amhy, amhr2, and gsdf) are

97 involved in the transforming growth factor beta (TGF-β) signaling pathway, while the sdY, dmrt1,

98 and dmy genes code for transcription factors (Rastetter et al 2015). Insights into SD genomics

99 goes beyond master genes in fish, as recent studies have documented mixed determination

5

100   involving both genetic and environmental factors, polygenic SD systems, extensive within-species

101   variation in SD system, and complex rearrangements of sex chromosomes favouring species

102   diversification (Capel 2017, Martinez et al 2014). Yet, with such dynamism and variability in fish

103   sex chromosome evolution, we need improved genomic resources in non-model species,

104   including chromosome-scale reference genomes and whole-genome sequences from both sexes.

105   In exploited species, improved genomic resources and the understanding of the genetic

106   basis for SD is also relevant to manage fisheries in order to meet the need for seafood around

107   the world, and to secure the economies and livelihoods that depend on them (Bernatchez et al

108   2017). Genomics approaches allow for the investigation of genome wide diversity and to

109   accurately identify fish stock within commercial species (Ovenden et al 2013). Moreover,

110   accounting for sex-differentiation and sex-linked loci is necessary to avoid unexpected biases due

111   to sex-associated markers (Benestan et al 2017) as well as to improve the management of sex-

112   ratio. This is particularly true for species harbouring a size-related sexual dimorphism like flatfish

113   species, generally favouring females in their size dimorphism (e.g. European Plaice (*Pleuronectes*

114   *platessa*; Rijnsdorp and Ibelings 1989), Turbot (*Scotphtalmus maximus*; Imsland et al 1997),

115   Atlantic Halibut (*Hippoglossus hippoglossus*; Hagen et al 2006). In such species, sex control

116   and/or identification is of high importance either for flatfish production in aquaculture or

117   sustainable fisheries. The selection of large individuals in fisheries is a well-established concept in

118   fisheries management that increases yield per recruit. However, in the case of sex-related sexual

119   dimorphism, fisheries targeting large individuals may reduce the stock's reproductive potential,

120   produce bias in the assessment of the number of reproductive females, and feed erroneous data

121   to fisheries management decision makers (Keyl et al 2015). Overall, applied and fundamental

122   evolutionary biology thus benefits from moving beyond genetic markers towards contiguous

123   reference genomes annotated for functional elements and documented for sex-determination.

124   Greenland Halibut (*Reinhardtius hippoglossoides*) is one of the main demersal fish

125   exploited by fisheries in Eastern Canada (Gulf of St. Lawrence, Newfoundland and the Arctic),

126   representing around 4 000 tones of landings per year across the two last decades (DFO, 2018).

127   However, it suffers from a lack of genomic resources, since no karyotype, linkage map nor

128   genome assembly has been developed so far, thus hampering the use of genomics for improved

129   management. Since juvenile growth potential and size are sexually dimorphic in this species

130   (Ghinter et al 2019), it is valuable to better understand the sex-determining mechanisms to

131   control sex imbalance in catches. Moreover, the recent genome assemblies of flatfish species

132   (Figure 1, Table1, Chen et al 2014, Figueras et al 2016, Shao et al 2017, Einfeldt et al 2021)

133   together with studies on sex-determining loci in this group (Chen et al 2014, Liang et al 2018,

134   Drinan et al 2018, Einfeldt et al 2021) provides an opportunity to investigate the evolutionary

135   turnover of sex chromosomes and sex-determination systems. Flatfishes studied so far are

136   characterized by a variable heterogametic system and different sex-determining genes. For

137   instance, a similar female heterogametic system (ZZ/ZW) is used both by the Tongue Sole

138   (*Cynoglossus semlaevis*) and the Turbot, but the candidate SD drivers differ, being sox2 in the

139   latter (Martinez et al 2021, Table1), and dmrt1 in the former species (Chen et al 2014). Japanese

140   Flounder, *Paralichthys olivaceus* (also called the Olive flounder,) has an XX/XY system (Liang et al

141   2018) but no sex determination gene has been found (Shao et al 2017) and environment is

142   known to alter the gender (Ospina-Alvarez and Piferrer 2008). Within the righteye flounder

143   family (*Pleuronectidae*), the *Hippoglossinae* subfamiliy harbors variable genetic SD systems. The

7

144   Pacific Halibut (*Hippoglossus stenolepis*) has a ZZ/ZW system and several sex-linked loci aligned

145   in three different linkage groups (Drinan, Loher, & Hauser 2018) while its sister-species, the

146   Atlantic Halibut (*H. hippoglossus*), has a XX/XY system and a putative SD factor called gsdf on

147   chromosome 12 (Palaiokostas et al. 2013; Einfeldt et al 2021).

148   In this context, the first goal of this study was to provide high-quality genomic resources

149   and to investigate the genetic basis of sex determination in Greenland Halibut. We produced the

150   first chromosome-scale scaffolded genome assembly for this species, combining single molecule

151   sequencing of long reads (Pacific Sciences) with Chromatin Conformation Capture sequencing

152   (Hi-C) data. Furthermore, we also identified and characterized the molecular SD system by

153   mapping whole genome sequencing data of hundreds of individuals onto the assembly to screen

154   for divergent genetic markers between sexes. Finally, we analyzed our results in a comparative

155   genomic framework by assessing synteny (i.e. block of genes in the same relative positions in the

156   genome) with related species of flatfish and targeting candidate sex-determining genes in this

157   group.

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159

160   METHODS

161

162   **Reference genome assembly**

163

164   We built a reference genome assembly for the Greenland Halibut through two steps.

165   First, we created a draft assembly using PacBio sequencing approach (Pacific Biosciences, Menlo

166   Park, California) and then we used Dovetail™ Hi-C (Lieberman-Aiden et al 2009) – from Dovetail

167   Genomics to increase contiguity, break up mis-joins, and orient and join scaffolds into

168   chromosomes.

169

170   • *Sampling*

171

172   The individual used for the reference was a juvenile female caught in the St. Lawrence

173   River Estuary (48°39'11"N, 68°28'37"W) with a Comando-type trawl aboard the CCGS Leim

174   (Fisheries and Oceans Canada [DFO] survey) at the end of May 2016 and kept alive in common

175   garden environment at the Maurice Lamontagne Institute (Ghinter et al 2019). The female was

176   anaesthetized for 5 min in a solution of MS 222 (tricaine methane sulfonate 0.18 g L^{-1}; Sigma -

177   Aldrich Co, Missouri, USA). Blood was then sampled from the caudal artery using a 23-gauge

178   needle in a 1ml TB syringe (Becton, Dickinson & co., New Jersey, USA), both of which were

179   previously heparinized in a 100 U ml $^{-1}$ heparin solution. Blood was immediately flash frozen in

180   liquid nitrogen and then stored at $-80^{o}C$ until further manipulation, as well as multiple tissues

181   including liver, muscle, heart, brain, eye, gonads, gall bladder and kidney that were excised from

182   the fish.

183

184   • *DNA extraction, sequencing and assembly*

185

186   High Molecular Weight DNA was extracted using Qiagen MagAttract kit, (Valencia, CA).

187   Then a library was created using the SMRTbell Template Prep Kit 1.0 (Pacific Biosciences, Menlo

188   Park, California), which includes a DNA Damage Repair step after size selection. Size selection

189   was made for >10 kb using a Blue Pippin instrument (Sage Science, Beverly, Massachusetts)

190   according to the manufacturer recommended protocol for 20 kb template preparation. Five µg

191   of concentrated DNA was then used as input for the library preparation reaction. Library quality

192   and quantity were assessed using the Pippin Pulse field inversion gel electrophoresis system

193   (Sage Science), as well as with the dsDNA Broad Range Assay kit and Qubit Fluorometer (Thermo

194   Fisher). Single-Molecule Real Time sequencing was performed on the Pacific Biosciences RS II

195   instrument at McGill University using an on-plate concentration of 125 pM, P6-C4 sequencing

196   chemistry, with magnetic bead loading, and 360-minute movies. A total of 14 SMRT Cells were

197   run, generating a mean sequence coverage of ∼70-fold, with half of the 522 Megabases (Mb) of

198   data contained in reads longer than 10.6 kb. An initial assembly was carried out on the PacBio

199   long reads using Wtdbg v2.5 (-x sq gg 0.7g; Ruan and Li (2020), resulting in 2 037 contigs (N50 =

200   31.7 Mb), for a total assembly size of 598 MB. One Hi-C proximity ligation library was generated

201   from a flash frozen piece of muscle of the same individual using the Dovetail's ^{TM} Hi-C Kit v.1.0 to

202   aid with assembly scaffolding. This kit employs the restriction enzyme *Dpn*II which recognizes the

203   sequence 5' /GATC 3'. The long-range library was then sent to Dovetail Genomics to be

204   sequenced and run with the initial PacBio assembly through Dovetail's HiRiseTM scaffolding

205   pipeline. The completeness of the final assembly was assessed with BUSCO v 4.0.5 (Simão et al

206   2015), the eukaryota_odb10 database (created in October 2020) and the -m genome option.

207   Occurrence of repeat elements in our assembly as well as in Atlantic and Pacific halibut was

208   assessed using RepeatMasker v.4.0.6 (Smit, Hubley and Green, 2013).

209

210   • *Synteny with other flatfish species*

211

212   Synteny blocks between the genomes of Greenland Halibut and other flatfish species for

213   which a reference genome is available (Tongue Sole, Turbot and Japanese Flounder as well as

214   Atlantic and Pacific Halibut) were computed using SyMAP v4.2 (Soderlund, Bomhoff and Nelson

215   2011). Genomic sequences were first aligned using promer/MUMmer (Kurtz et al 2004). Raw

216   anchors resulting from MUMmer were clustered into (putative) gene anchors, filtered using a

217   reciprocal top-2 filter and used as input to the synteny algorithm (Soderlund et al 2006). The

218   algorithm constructs maximal-scoring anchor chains based on a given gap penalty. It also

219   searches a range of gap penalties to generate the longest chains subject to several quality

220   criteria, which are based on the Pearson correlation coefficient applied to the anchors in the

221   chain as well as the anchors in its bounding box. The chains are not required to be entirely

222   colinear and may incorporate local inversions relative to the overall chain orientation. Synteny

223   analysis results were visualized using the Chromosome Explorer and the Circle view options in

224   SyMAP v4.2. Finally, we named scaffolds by mapping our assembly to the Pacific Halibut

225   assembly (IPHC_HiSten_1.0, Genbank accession: GCA_013339905.1).

226

227   • *Transcriptome assembly and annotation of the assembly*

228

11

229   In order to annotate the reference assembly, total RNA from 8 different tissues (liver,

230   muscle, heart, brain, eye, gonads, gall bladder and kidney) from the same individual was

231   extracted using the RNeasy Mini kit from Quiagen then treated using DNase I, Amplification

232   Grade (1 unit per μl; Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA

233   concentration of each tissue was quantified with QuantiT Picogreen dsDNA Assay Kit 669 (Invitrogen)

234   and fragment size distribution was estimated with an Agilent BioAnalyzer before being shipped to the

235   Genome Québec Centre d'Expertise et de Services (Montréal, Canada) to prepare libraries and

236   perform sequencing. One RNA-seq library was constructed for each tissue using the

237   NEBNext® Ultra™ II Directional RNA Library Prep Ki (Illumina, San Diego, Calif., USA) and sequenced

238   with a 2×100bp (paired-end) read module using the NovaSeq 6000, generating 50M reads.

239

240   For each of the 8 paired-end tissue-specific RNAseq datasets, raw reads were trimmed

241   with trimmomatic (v0.36, ILLUMINACLIP:univec.fasta:2:20:7, LEADING:20, TRAILING:20,

242   SLIDINGWINDOW:30:30, MINLEN:80). The tissue-specific paired-end datasets were then each

243   assembled separately using Trinity (v2.1.1, --seqType fq, --min_contig_length 200) to produce 8

244   assembled transcriptomes that contained between 68 481 and 276 168, with a mean of 131 038,

245   assembled expressed sequences. The 1 048 307 assembled expressed sequences were used to

246   annotate   the   assembled   genome   using   the   GAWN   pipeline   v0.3.4

247   (https://github.com/enormandeau/gawn). The annotations were then simplified by using the

248   included simplify_genome_annotation_table.py script, which removes consecutive almost-

249   identical annotations from the genome annotation table.

250

251

252   *Genomic sex determination*

253

254   • *Sampling of individuals, DNA extraction, libraries and sequencing*

255

256   A total of 432 Greenland Halibut were collected aboard the R/V Paamiut, from October to

257   November 2016, as part of the annual multispecies survey conducted by the Department of

258   Fisheries and Oceans in Northwest Atlantic Fisheries Organization Subarea 0 in Davis Strait,

259   Canada. Each fish collected has been measured and weighed as well as phenotypically sex

260   identified. From those individuals, 99 females and 99 males have been selected for sex

261   determination analysis (see sample selection details in Supplementary Material).

262   Genomic DNA was extracted using a salt-extraction protocol (Aljanabi and Martinez 1997)

263   with a RNase A treatment 645 (Quiagen). DNA quality of each extract was evaluated with

264   nanodrop and on a 1% agarose gel electrophoresis. Following Therkildsen and Palumbi 2017, we

265   removed DNA fragments shorter than 1kb by treating each extract with Axygen magnetic beads

266   in a 0.4:1 ratio, and eluted the DNA in 10mM Tris-Cl, pH 8.5. We measured DNA concentrations

267   with Accuclear ultra high sensitivity dsDNA quantification kit (Biotium) and normalized all

268   samples at a concentration of 5ng/ μL. Then, sample DNA extracts were randomized, distributed

269   in plates (96 -well see details about randomization and number of plates used in Supplementary

270   Material) and re-normalized at 2ng/μL.

271   Whole-genome high-quality libraries were prepared for each sample according to the

272   protocol described in (Baym et al. 2015; Therkildsen and Palumbi 2016). Briefly, a tagmentation

13

273   reaction using enzyme from the Nextera kit, which simultaneously fragments the DNA and

274   incorporates partial adapters, was carried out in a 2.5 µl volume with approximately 2 ng of input

275   DNA. Then, we used a two-step PCR procedure with a total of 12 cycles (8+4) to add the

276   remaining Illumina adapter sequence with dual index barcodes and to amplify the libraries. The

277   PCR was conducted with the KAPA Library Amplification Kit and custom primers derived from

278   Nextera XT set of barcodes A, B, C and D (total 384 combinations). Amplification products were

279   purified from primers and size-selected with a two-steps Axygen magnetic beads cleaning

280   protocol, first with a ratio 0.5:1, keeping the supernatant (medium and short DNA fragments),

281   second with a ratio 0.75:1, keeping the beads (medium fragments). Final concentrations of the

282   libraries were quantified with Accuclear ultra high sensitivity dsDNA quantification kit (Biotium)

283   and fragment size distribution was estimated with an Agilent BioAnalyzer for a subset of 10 to 20

284   samples per plate. Finally, libraries were pooled (see details in Supplementary Material) for

285   sequence lanes of paired-end 150 bp reads on an Illumina HiSeq 4000 at the Norwegian

286   Sequencing Center at the University of Oslo. Given our multiplexing (up to 96 individuals per

287   lane) and genome size (~ 600 Mb), we targeted to a low coverage sequencing (~ 1.26 X).

288

289   • *Sequencing filtering and processing*

290

291   Raw reads were trimmed, filtered for quality, mapped to the reference genome (obtained

292   above), cleaned for mapping quality and duplicates reads and then re-aligned using the pipeline

293   available   at   https://github.com/enormandeau/wgs_sample_preparation,   inspired   by

294   (Therkildsen and Palumbi 2016) and fully described in Mérot et al (2021). Given the high

14

295   percentage of the assembly comprised into the anchored 24 chromosomes (> 96 %, see results

296   section), raw reads were mapped against a reduced version of the genome including only the 24

297   chromosomes and not the unplaced scaffolds.

298

299   For low coverage whole genome sequencing (lcWGS) data, the recommended practice is to

300   avoid basing downstream analysis on raw counts of sequenced bases or called genotypes

301   (Nielsen et al 2011), but instead use a probabilistic approach based on the use of Genotype

302   Likelihoods. Several models for computing genotype-likelihood-based on read data have been

303   implemented in the program ANGSD (Korneliussen, Albrechtsen and Nielsen et al 2014), which is

304   currently the most widely used and versatile software package for the analysis of lcWGS (Lou et

305   al 2021). Therefore, the program ANGSD v0.931 was used for most of our subsequent analyses,

306   according   to   the   pipeline   documentation   available   at

307   https://github.com/clairemerot/angsd_pipeline. For all analyses, input reads were filtered to

308   remove reads with a samtools flag above 255 (not primary, failure and duplicate reads, tag -

309   remove_bads = 1), with mapping quality below 30 (-minMapQ 30) and to remove bases with

310   quality below 20 (-minQ 20). We also filtered in order to keep only SNPs covered by at least one

311   read in at least 30 % of individuals (-minInd 60) and remove SNPs in putative repeated regions

312   allowing a maximum depth of 3 times the number of individuals (-setMaxDepth 594). Finally, for

313   most of the subsequent analysis (unless mentioned otherwise) we kept SNPs with minor allele

314   frequency above 5%.

315   First, we ran ANGSD to estimate genotype likelihoods (GL) with the GATK model (-doGlf 2 -GL

316   2 -doCounts 1), the spectrum of allele frequency (-doSaf 1) and minor allele frequency (-doMaf

317   1). The major allele was based on the genotype likelihood and was the most frequent allele (-

318   doMajorMinor 1). From this first analysis, we generated (i) a beagle file with GL estimates and (ii)

319   a list of variants passing those filters and their respective major and minor alleles that were used

320   for most subsequent analysis. The R program (R Core Team 2020) was employed for graphic

321   output in subsequent analyses, *via* the package ggplot (Wickham 2016).

322

323   • Genome-wide coverage difference between sexes

324

325   Total depth along the entire genome was calculated using the function -doDepth 1 in ANGSD,

326   excluding high coverage (-setMaxDepth 594 and -maxDepth 1000) and low coverage regions (-

327   minInd 60), along with the following arguments for depth: -dumpCounts 2 -doCounts 1 and then

328   averaged across sliding-windows of 25Kb with a step of 5 Kb using the winScan() function of the

329   R package windowscanr (Tavares 2021). The sliding approach allowed us to visualize individual

330   coverage along the genome, while a ratio of total depth of females above total depth of males

331   was also plotted using ggplot(). This ratio is expected to deviate from 1 in genomic regions in

332   which a sex-bias in coverage exists.

333

334   • *Genome wide variation between sexes*

335

336   Genome-wide variation across samples was explored using PCAangsd (Meisner and

337   Albrechtsen 2018) on the genotype likelihoods. This program extracts a covariance matrix that is

338   then decomposed into principal component analysis (PCA) with R using a scaling 2

16

339   transformation adding an eigenvalues correction, to obtain the individuals PC scores (Legendre

340   and Legendre 1998).

341

342   • *Identify genomic regions implied in sex determination*

343

344   In order to identify the genomic regions involved in the sex differentiation, two analyses

345   were performed. First, we ran PCAangsd on genotype likelihoods in non-overlapping windows of

346   1 000 SNPs in each chromosome to extract local covariance matrices and obtained local PCAs of

347   genomic variation (as detailed above). For each local PCA, we analyzed the correlation between

348   individuals PC1 scores and PC scores from the global PCA performed on the entire genome. The

349   resulting correlation indices were then plotted across their relative position in each

350   chromosome. Second, genome-wide Fst was estimated between males and females. To do this,

351   allele frequency spectrum (-doSaf 1) and minor allele frequencies were calculated for each sex

352   with the previous list of variant positions (-sites) and their polarisation as major or minor alleles

353   (-doMajorMinor 3). Genome-wide Fst was estimated using the realSFS function in ANGSD

354   between males and females and then summarized across sliding-windows of 25 Kb with a step of

355   5 Kb.

356   Observed heterozygosity was computed for each sex and each SNP using the function -

357   doHWE in ANGSD and then averaged across sliding-windows of 25Kb with a step of 5 Kb using

358   the winScan() function of the R package windowscanr.

359

360

17

361   • *LD estimation*

362   For the two chromosomes associated with sex determination (see results below), intra-

363   chromosomal as well as inter-chromosomal linkage disequilibrium was calculated within each sex

364   for a reduced number of SNPs, filtered with more stringent criteria (MAF > 10%, at least one

365   read in 75 % of the samples). Pairwise R^2 were calculated using ngsLD (Fox et al 2019) based on

366   genotype likelihood reported in the beagle file obtained previously. The 10th percentile of

367   pairwise R^2 values was averaged across windows of 250 Kb and plotted for each sex using

368   custom scripts available at https://github.com/clairemerot/angsd_pipeline. Then changes in LD

369   along the sex identified chromosomes were analyzed by calculating mean R^2 for males and

370   females separately.

371

372   • *Mapping known sex locus*

373

374   To localize homolog genes of known sex-associated genes in our assembly, we aligned all sex-

375   associated markers from Drinan et al (2018) as well as others previously described to have a sex-

376   determination effect in flatfish or teleost species, to our reference assembly using LAST v.1179

377   (Kielbasa et al 2011; see Table S1 for details about those genes and their accession number).

378

379   DATA AVAILABILITY:

380   Genome assembly is available upon the GCA_006182925.2 accession. Raw data for the 8 RNA-

381   seq lanes are available on NCBI upon following accession numbers: SAMN18221283 to

18

382   SMAN18221290 and 198 individual whole genome sequences are submitted under the

383   BioProject ID PRJNA737354 on NCBI SRA and will be available upon publication.

384

385   RESULTS AND DISCUSSION

386

387   •   A high-quality reference genome serving flatfish management and research

388

389   We generated a final genome assembly of 598.5 Mb, with overall high contiguity

390   (N50=25MB, N90=20MB), of which 96.35% was comprised into 24 chromosome-length scaffolds

391   (GenBank accession: GCA_006182925.3). These 24 scaffolds are consistent with the number of

392   diploid chromosomes (2n=48) for Atlantic Halibut (Einfeldt et al 2021) and Pacific Halibut

393   (GCA_013339905.1). The Hi-C data supported a high degree of accuracy in the overall assembly

394   into these 24 scaffolds, as indicated by the strong concentration of data points along the

395   diagonal rather than elsewhere in the contact maps (Figure S1). Altogether, this suggests that

396   these 24 scaffolds can be considered as chromosomes. The completeness of the assembly based

397   on single-copy orthologs across eukaryotes was 98.04%, with 96.47% complete and single copy

398   orthologs and 1.57% duplication.

399   There were 60 313 transcripts detected from multi-tissue transcriptomics data, with 97.7%

400   of them (58 908 transcripts) within the 24 anchored chromosomes. A total of 19 252 genes and

401   pseudogenes were annotated, of which 19 053 (99%) were located within the 24 chromosomes.

402   Repeated elements accounted for 8.20% of chromosome length (49.1 Mb). These measures of

403   completeness and assessments of repeat elements in Greenland Halibut were highly concordant

19

404   with the statistics obtained from Atlantic and Pacific Halibut as well as the three other flatfish

405   species with reference genome and important for fisheries and/or aquaculture production (Table

406   1). SyMap revealed shared syntenic blocks with other flatfish species, allowing the identification

407   of homologous chromosomes (Table 2 and Figure 1). Most of the chromosomes harbor a single

408   syntenic block compared with the most closely-related *Hippoglossinae*, the Pacific Halibut (16

409   out of 24 chromosomes) and the Atlantic Halibut (15/24). In contrast, mapping with the three

410   other flatfish species displayed several syntenic blocks for all chromosomes (Table 2). As a result,

411   syntenic blocks are larger with Pacific and Atlantic Halibut than with the three other flatfishes

412   (Table 2, and Figure 1). Figure 1 displays this negative relationship between the size of syntenic

413   blocks and phylogenetic distance.

414   By generating this chromosome-level genome assembly for Greenland Halibut, among the

415   most contiguous of fish assemblies to date, we provide a genomic resource for this unique

416   species of the genus *Reinhardtius.* This reference genome will now become a fundamental tool

417   to understand the genomic variability of Greenland Halibut in the context of comparative

418   genomics and fisheries applications.

419

420

421   • *Nascent XY sex determination system*

422

423   After mapping the reads of 99 male and 99 female Greenland Halibut samples to the

424   reference genome, cleaning for quality and processing for SNP identification using GL approach,

425   we identified 7 301 470 Single Nucleotide Polymorphisms (SNPs) with a minor allelic frequency

426   (MAF) above 5%. These SNPs were then used for further differentiation analyses.

427   Genome-wide variation analyzed by a global PCA displayed two clusters corresponding to

428   males and females on PC1, which explained 5.7 % of the genetic variance (Figure S2). Local PCAs

429   on windows of 1 000 SNPs along the genome revealed that this sex differentiation was mainly

430   explained by genetic variation on two chromosomes (Chr10 and Chr21 Figure 2A). The

431   correlation between the 1st PC of the global PCA and each local PCA was very low at the very

432   beginning of each of those two chromosomes but it increased rapidly along both chromosomes.

433   A large portion of each of these two chromosomes explains the total genetic variation observed

434   between the two sexes in PC1. Particularly, the last 66.5 % of Chr10 (16.5 Mb out of 24.8 Mb)

435   and 52.2 % of Chr21 (10.5 Mb out of 20.1 Mb) explained more than 90 % the sex differentiation

436   observed on global PC1 (Figure 2B). Genome-wide Fst estimations between males and females

437   corroborate this result revealing high differentiation on Chr10 and Chr21 (Figure 2C). While the

438   mean genome-wide Fst between the sexes is as low as 0.01, Chr10 and Chr21 harbored a large

439   region with higher Fst values with a mean Fst of 0.21 and 0.15, respectively (Figure 2D). Few

440   localized peaks of sex differentiation were also observed on Chr9, 11, 12, 13 and 15 (Figure 2B).

441   The observed proportion of heterozygotes followed the same pattern, with similar values

442   between males and females across most of the genome (Figure 2E), but with a much higher

443   fraction of heterozygotes in males than females in the two major regions previously described.

444   This difference between males and females was more pronounced in Chr10 than in Chr21. Both

445   Chr10 and Chr21 were also characterized by strong linkage disequilibrium, which was markedly

21

446   higher in males than in females (Figure 3 and Figure S4), suggesting that recombination events

447   are less frequent in males than females for these genomic regions.

448   In contrast with differentiation and heterozygosity, coverage did not differ between males

449   and females, neither genome-wide nor in regions of sex differentiation (Figure S3), suggesting

450   that Greenland Halibut is not harboring sex chromosome heteromorphism and that the putative

451   sex chromosomes Chr10 and Chr21 have not undergone degradation, which is typical of early SD

452   systems.

453   Altogether, these results point towards a major role of Chr10 and Chr21 in genetic sex

454   determination. The higher heterozygosity and LD in males suggests that Greenland Halibut has a

455   XX/XY sex determination mechanism. The putative male Y chromosome exhibits two

456   characteristics of sex chromosome evolution; it is divergent from the X chromosome and exhibits

457   a reduction of recombination. However, it does not appear degraded, suggesting very nascent

458   sex chromosomes at an early evolutionary stage. This is corroborated by the syntenic

459   relationships with closely related flatfish species which indicate that homologues of Chr10 and

460   Chr21 are autosome chromosomes. Chr10 and Chr21 neither correspond to locations where sex-

461   determining regions have been defined in other species (Figure 1, Table1). Overall, the resolved

462   sex-determining system in Greenland Halibut is highly consistent with the rapid turnover of sex

463   chromosomes in fish, particularly in flatfish, given that the Atlantic Halibut has also undergone a

464   recent evolution of an XY sex-determination mechanism (Einfeldt et al 2021).

465   Observing high sex differentiation on two different chromosomes was nevertheless

466   unexpected and puzzling. The Hi-C contact map very strongly supported that Chr10 and Chr21

467   are distinct chromosomes in the specimen used for the assembly, a juvenile female (Figure S1).

468   Inter-chromosomal linkage disequilibrium was also low in females, confirming the independent

469   segregation of Chr10 and Chr21. Conversely, LD between Chr10 and Chr21 was very high in

470   males (Figure 3), suggesting that the male haplotypes of those two chromosomes segregate

471   together. One possible mechanism explaining such pattern would be a male-restricted fusion

472   between the Y chromosome and one autosome. Chromosome fission and fusion events are

473   frequent in the evolution of vertebrates, particularly in fish, in which they could explain the

474   enormous species-richness and rapid evolution of karyotypes (Cheng et al 2020; Sutherland et al

475   2017). Fusions may also be polymorphic in fish and play an adaptive role, as reported in Atlantic

476   salmon (*Salmo salar*), in which fused and unfused karyotypes have been shown to coexist

477   (Lehnert et al 2019; Wellband et al 2019). Moreover, fusions between a Y chromosome and an

478   autosome are known to occur more frequently than autosomal fusions or W-autosome fusions,

479   particularly in fishes Pennell et al (2015). The most probable explanation for the preponderance

480   of Y-autosome fusions involved both selection and sex biases, as the smallest effective size (Ne)

481   of the Y makes it more likely to fix rearrangements including those with partially deleterious

482   effects. This process may be even more prevalent in species with polygynous mating system like

483   fish, which increases the variance of male reproductive success, and in species with skewed sex-

484   ratio, as observed in the halibut. Because of its significant impact on recombination,

485   chromosomal fusion may be one of the key factors underlying the evolution of differentiated sex

486   chromosomes. Fusions are a peculiar class of rearrangements that not only link chromosomes

487   that previously segregated independently but also reduce recombination rates within each arm

488   and prevent recombination between fused and unfused homologous chromosomes in

489   polymorphic populations (Dumas and Britton-Davidian 2002). This typically leads to gradual

490   differentiation from the fusion point as observed here for Greenland Halibut or the young sex

491   chromosomes found in three-spined stickleback (*Gasterosteus aculeatus*) (Kitano et al 2009).

492   This represents a typical step in the birth and establishment of new sex chromosomes

493   (McAllyster 2003, Kirkpatrick 2010), which is often mediated *via* large chromosomal

494   rearrangements such as inversions or fusions. One of the main determinants of this process is

495   selection against recombination, because linkage between sex-determination loci and alleles

496   beneficial to one sex has the potential to resolve sexual conflict (Charlesworth et al 2005). As

497   discussed below, we identified three sex-associated loci spread on two chromosomes and one

498   may speculate whether epistatic interactions may occur between them. Further work would be

499   needed to better understand gene interactions in this region and more formally confirm the

500   putative male-limited fusion (Guerrero and Kirkpatrick 2014; Wright et al 2016).

501

502   • *Sox2 gene as the major driver of Greenland Halibut SD?*

503

504   Alignment of DNA or mRNA sequences of previously described sex-associated markers

505   revealed that those genes were widely distributed throughout Greenland Halibut assembly (on

506   seven chromosomes, Table S1). Most of those genes are located in regions of low differentiation

507   between males and females: amhr2, cyp19a1A on chr05; cyp11b2 and rspo1 on chr08; dmrt1 on

508   chr09, foxl2 on chr10 and gsdf on chr18, making them unlikely to underlie sex differentiation.

509   However, three candidate genes mapped to the putative sex chromosomes: gdf6 and *sox2* on

24

510   Chr10, and *sox9a2* on Chr21 (Figure 2, Table S1), and are thus possible drivers of sex-

511   determination in Greenland Halibut.

512

513   Growth differentiation factor 6 (*Gdf6*) is a member of the TGF-β family involved in

514   vertebral segmentation and cell differentiation. While no gonadal function was reported

515   previously, *gdf6Y* emerged recently as a putative SD gene in the Turquoise Killifish

516   (*Nothobranchius furzeri*) (Reichwald et al 2015) because sex-linked allelic variations affect the

517   Gdf6 protein function. The Y chromosome allele, *gdf6Y*, also exhibits sex-linked expression

518   during testicular differentiation in the Turquoise Killifish, supporting its SD role but functional

519   evidence of its role as a SD gene is yet not available.

520

521   Sox2 is a member of the SOX (SRY-related HMG-box) family with a high-mobility-group

522   (HMG) DNA-binding domain. It is an important transcription factor, which is involved in

523   developmental processes of vertebrates, such as embryogenesis, maintenance of stem cells,

524   neurogenesis. In the Rohu Carp (*Labeo rohita)*, sox2 mRNA is expressed in various organs, as well

525   as in the culture proliferating spermatogonial stem cells (Patra et al (2015). Sox2 is also a

526   candidate SD gene in two aquaculture species, the mollusc Zhikong scallop (*Chlamys varia*) (Liang

527   et al 2019) and the crustacean black Tiger Shrimp (*Penaeus monodon)* (Guo et al 2019). In

528   flatfish, sox2 is a strong candidate for SD, in Japanese Flounder, sox2 transcripts is expressed in

529   gonadal tissues and the transcript abundance in ovaries is higher than in testis (Gao et al 2014),

530   and, in Turbot, sox2 is defined as a consistent candidate gene putatively driving SD (Martinez et

531   al (2021).

532   Sox9 gene has been related to sexual differentiation in several teleost species: Rainbow

533   Trout, (*Oncorhynchus mykiss,* Takamatsu et al. 1997; Vizziano et al. 2007), Tilapia (*Oreochromis*

534   *niloticus,* Ijiri et al 2008), Zebrafish (*Danio rerio* Chiang et al 2001; von Hofsten and Olsson 2005),

535   medaka, *Oryzias latipes* (Yokoi et al 2002), Guppy (*Poecilia reticul*, Shen et al 2007), the Common

536   Carp (*Cyprinus carpio*, Du et al. 2007) and the Asian Swamp Eel (*Monopterus albus,* Zhou et al

537   2003). In the Rainbow Trout, two sox9 genes have been identified: sox9a1 and sox9a2. Sox9a1

538   shows higher expression levels in males than in females before sexual differentiation (Vizziano et

539   al 2007). In Tilapia, sox9 expression is similar in XX and XY gonads, but, after sexual

540   differentiation, it is positively regulated in XY gonads (Ijiri et al 2008). In Medaka, testicular sox9

541   (sox9a2) expression in somatic cells is similar in both sexes during early gonadal differentiation

542   and becomes positively regulated in males during the testicular lobe formation stage (Nakamoto

543   et al. 2005; Nakamura et al. 2008). Overall, these observations support the hypothesis of a

544   significant role for sox9 in either testicular differentiation or testicular development. This gene

545   may also be at play in flatfish since, in Atlantic Halibut, sox9a2 is included in a cascade of gene

546   activation starting with the dmrt1 gene which is activated by the putative SD factor, the gsdf

547   gene (Norris and Carr 2013, Einfeldt et al 2021).

548   In Greenland Halibut, sox2, gdf6, and sox9a2 represent three possible candidate genes

549   for sex determination. One can think that these three genes may be interacting in shaping the

550   determination of sex and/or the subsequent development of sex organs. However, several lines

551   of evidence suggest that sox2 might have a major role in Greenland Halibut SD. First, sox2 is

552   located in a region that is more highly differentiated between sexes than gdf6 and sox9a2 genes.

553   Furthermore, no functional evidence in sexual determination is available yet, neither for gdf6 nor

26

554   sox9a2 genes, in other fish. Conversely, sox2 has been reported as the main SD driver for several

555   species including another flatfish (Turbot, Martinez et al 2021).

556   In contrast with sox2 identified in Turbot, SD genes identified in the two most-closely

557   related flatfish species, Atlantic and Pacific Halibut (Table1), do not appear to differentiate males

558   and females in the Greenland Halibut. This is somewhat unsurprising given that even within the

559   same genus (*Hyppoglossus*), not only different species have different genes driving SD, but they

560   harbor a different heterogametic sex (XY for Atlantic Halibut and ZW for Pacific Halibut, Drinan,

561   Loher, & Hauser 2018, Einfeldt et al 2021).

562   The nascent SD system with low differentiation between sex chromosomes revealed in

563   Greenland Halibut might reflect the observed complex and still enigmatic sex maturation. For

564   example, in some Greenland Halibut individuals, the maturation cycle can be interrupted by a

565   sudden degeneration of the oocytes (Fedorov, 1971). More recently, it has been suggested that

566   this species utilizes a very unusual oocyte development pattern containing two simultaneously

567   developing cohorts of vitellogenic oocytes, not seen in any related species (Rideout et al 2012;

568   Dominguez-Petit et al 2017). Also, unusual and still unexplained, Greenland Halibut present high

569   densities and regular occurrence of rodlet cells in male and female gonads (Rideout et al 2015).

570   Furthermore, other layers may be added on the top of such an SD mechanism; environmental

571   factors such as temperature, pH, population density, and social interactions have been found to

572   influence the sex-ratio in fish (Crews 1996; Nakamura et al. 1998; Baroiller et al. 1999; Baroiller

573   and D'Cotta 2001). More research would be needed to better disentangle the relative

574   contribution of environmental factors and genetic factors in sex-determination in Greenland

575   Halibut.

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577

578 Conclusion:

579   Despite the commercial importance of Greenland Halibut, important gaps still persist in

580 our knowledge of this species, including gaps about its reproduction cycle and sex determination

581 mechanisms. In this study, we used a compendium of novel technologies (single molecule

582 sequencing of long reads (Pacific Sciences) with Chromatin Conformation Capture sequencing

583 (Hi-C) data and whole-genome sequencing of hundreds of individuals) to provide the very first

584 chromosome-level genome reference and elucidate the sex determination mechanism of this

585 demersal fish. The chromosome-level assembly and the investigation of its syntenic relationships

586 with other economically important flatfish species highlight the high conservation of synteny

587 blocks within the flatfish phylogenetic clade. Our sex determination analysis revealed that

588 flatfishes do not escape this rule and exhibit a high plasticity and turnover in sex-determination

589 mechanisms. In fish, the early evolutionary stage of most sex chromosomes and the subsequent

590 small differences between those chromosomes make it extremely difficult to distinguish

591 diagnostic differences between them. A whole-genome sequencing approach of hundreds of

592 individuals allowed us to draw a full picture of the molecular SD system in Greenland Halibut.

593 Specifically, our results suggested that Greenland Halibut has a very nascent male heterogametic

594 XY system, with a putative major effect of the sox2 gene, also described as the main SD driver in

595 the Turbot (*S. maximus*) and to a lesser extent in the Japanese Flounder. Interestingly, our study

596 also suggested for the first time in flatfish a putative Y-autosomal fusion that could be associated

597 with a reduction of recombination typical of early steps of sex chromosome evolution.

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870   FIGURES:

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872   **Figure 1: Phylogenetic relationships and syntenic distances between important flatfish species in**

873   **fisheries and aquaculture.** Phylogenetic trees were retraced according to previous studies,

874   Betancour et al (2014) for the top one and Vinnikov et al (2018) for the zoom into the sub-family

875   *Hippoglossinae* framed in red. Only the names of relevant species compared in our studies are

876   reported as well as the SD system, either "XY" for a male heterogametic system (XX/XY) or "ZW"

877   for a female heterogametic system (ZZ/ZW). The XY system for Greenland Halibut has been

878   assessed in the present study. Circos plots showing synteny between the Greenland Halibut

879   (displayed in low half circle) and the Tongue Sole, Turbot, Japenese Flounder, Atlantic and Pacific

880   Halibut. Black arrows in each circos plot refers to the sex chrosomosomes or the location of sex-

881   related markers. A positive relationship between the mean block size and phylogenetic distance

882   is also reported. Colors used for reporting the standard deviation and the mean of block size are

883   indicated next to each species name respectively. Note that the phylogenetic distance reported

884   on the x-axis is qualitative only and that species have been positioned from the most closely

885   related species to the most distant species. The same color code as the one near the circus plots

886   is used.

887   **Figure 2: Two genomic regions implied in sex differentiation. (A)** Correlation between PC1 scores

888   of the local PCAs performed on windows of 1000 SNPs and PC1 scores of the global PCA along

889   their genomic position in each chromosome. **(C)** Fst differentiation estimated between males

890   and females in sliding-windows of 25 Kb within each chromosome. **(E)** Mean observed

891   heterozygosity in each sex smoothed for visualization. **(B)**, **(D)**, and **(F)** are zooms on the two

42

892   differentiated chromosomes (chr 10 and 21) of the previous values, respectively (A), (C), and (E).

893   Vertical dashed grey lines indicate the positions of detected sex-related candidate genes.

894   **Figure 3: Intra- and Inter- chromosomal Linkage Disequilibrium (LD) among males and females,**

895   **revealing a putative Y-autosomal fusion**. Male values are displayed above the diagonal and female

896   values are displayed below the diagonal. The color scale corresponds to the 10th highest

897   percentile of the R2 value of each SNP pairwise summarized by windows of 250 Kb. Positions are

898   represented in megabases.

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900   Table 1: Statistics of genome assemblies of the important flatfish species in fisheries and aquaculture. n.a. is used for data not available

901   [1] This study, [2] Drinan, Loher, & Hauser, 2018, [3] Einfeldt et al 2021, [4] Liang et al 2018, [5] Martinez et al 2021, [6]Cheng et al

902   2014

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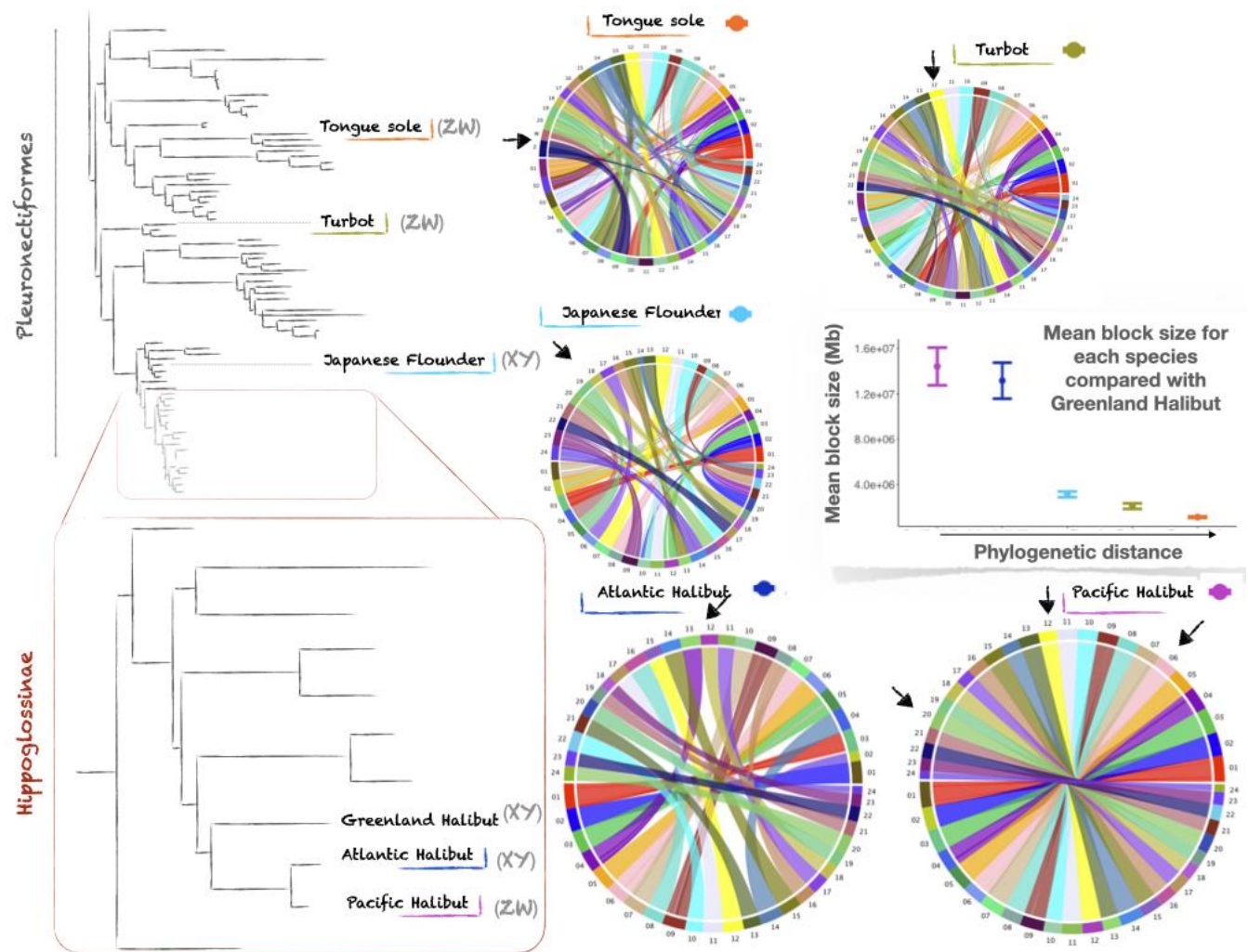|	Greenland Halibut	Pacific Halibut	Atlantic Halibut	Japenese flounder	Turbot	Tongue sole
Species latin name	*Reinhardtius hippoglossoides*	*Hippoglossus stenolepis*	*Hippoglossus hippoglossus*	*Paralichthys olivaceus*	*Scophthalmus maximus*	*Cynoglossus semilaevis*
total sequence length	598,525,902	594,145,200	596,792,615	545,775,252	524,979,463	470,199,494
number of scaffolds	1,594	120	57	9,525	22	31,181
max scaffolds lengh	31,982,681	32,413,155	30,753,830	27,810,759	31,901,587	34,528,841
scaffold N50	25,015,410	24,985,257	26,312,018	3,817,360	24,811,384	509,861
scaffold L50	11	11	11	40	10	289
total number of chromosomes	24	24	24	24	22	21
Genbank accession number	GCA_006182925.3	GCA_013339905.1	GCA_009819705.1	GCA_001904815.2	GCA_003186165.1	GCA_000523025.1
Reference	This study	International	Einfeldt et al 2021	Shao et al 2017	Martinez et al	Chen et al 2014

		Pacific Halibut Commission			2021	
Name of the assembly	UL_REHI_2.0	IPHC_HiSten_1.0	fHipHip1	ParOli_1.1	ASM318616v1	Cse_v1.0
Complete BUSCO	98.04%	na	96.90%	na	na	na
Repeat elements	8.20%	7.28%	7.14%	4.92%	6.35%	6.08%
Genetic sex-determinism	XX//XY	ZZ/ZW	XX/XY	XX/XY	ZZ/ZW	ZZ/ZW
SD genes or putative SD genes	sox2, sox9a2, gdf6 (1)	no gene has been specifically identified (2)	gsdf (3)	Cyp11a (4)	sox2 (5)	dmrt1 (6)
Homolog of sex chromosome in Greenland Halibut	-	GH06, GH12, G20	GH18	GH19	GH14	GH09

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Table 2: Syntenic relations between Greenland Halibut and related commercial flatfish species.
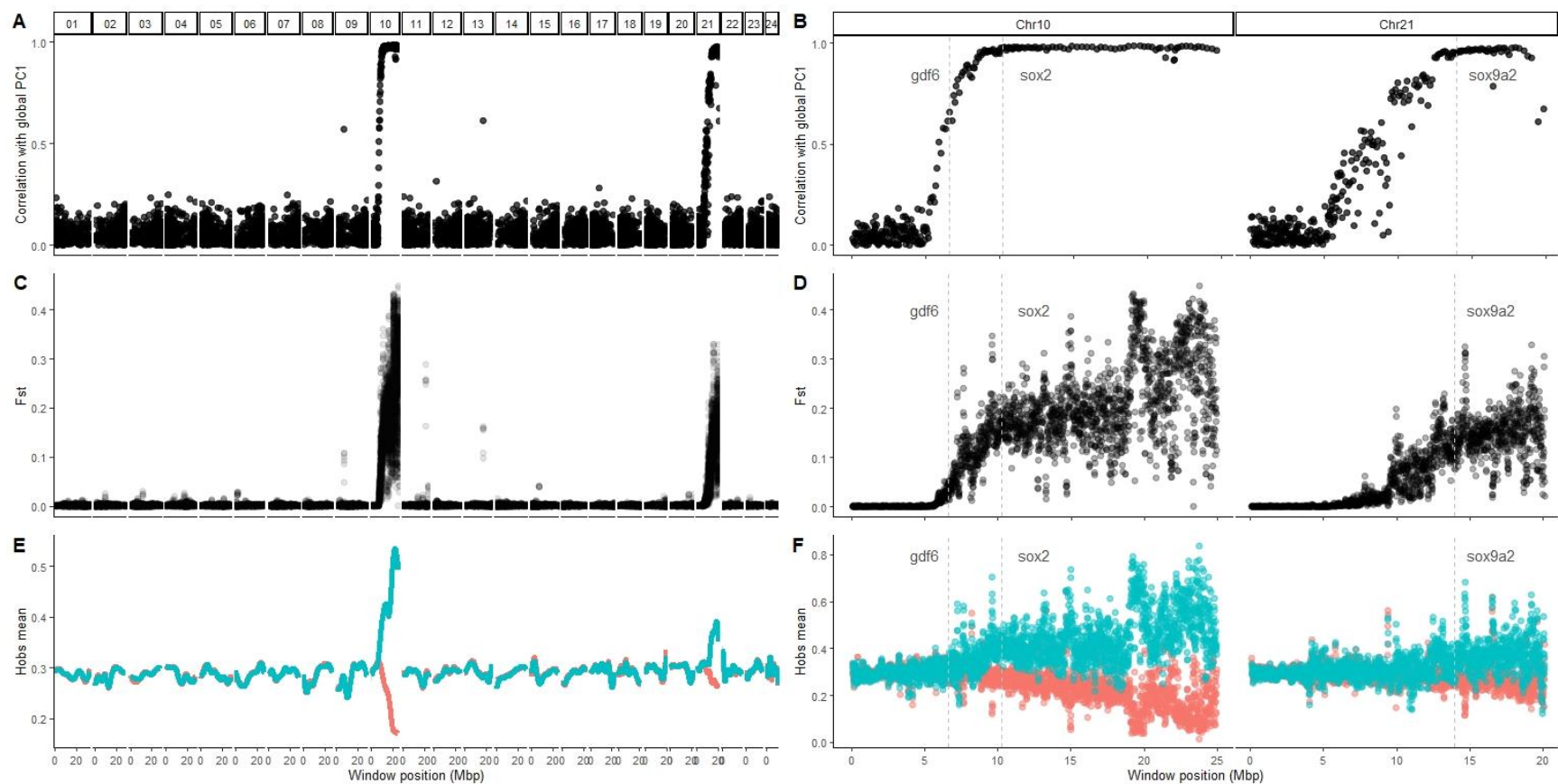
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	Greenland Halibut / Pacific Halibut	Greenland Halibut / Atlantic Halibut	Greenland Halibut / Japenese Flounder	Greenland Halibut / Turbot	Greenland Halibut / Tongue sole
number of blocks	40	45	191	333	745
number of chr with single block	16	15	0	0	0
min size	560,659	537,880	39,610	23,190	25,486
max size	28,459,961	30,361,793	21,363,777	24,314,634	16,925,444
median size	24,561,162	11,813,728	1,877,480	507,912	482,179
nb of block > 10Mb	24	24	11	23	6
percent of blocks > 10Mb (%)	60	53	6	7	1

910   Figure 1

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Tongue sole

Turbot

Japanese Flounder

Mean block size for each species compared with Greenland Halibut

Atlantic Halibut

Pacific Halibut

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914   Figure 2

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923   Figure 3

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