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1 Identification of the master sex determining gene in 2 Northern pike (*Esox lucius*) reveals restricted sex 3 chromosome differentiation

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28 **Abstract**

29

30 Teleost fishes, thanks to their rapid evolution of sex determination mechanisms, provide
31 remarkable opportunities to study the formation of sex chromosomes and the mechanisms
32 driving the birth of new master sex determining (MSD) genes. However, the evolutionary
33 interplay between the sex chromosomes and the MSD genes they harbor is rather unexplored.
34 We characterized a male-specific duplicate of the anti-Müllerian hormone (*amh*) as the MSD
35 gene in Northern Pike (*Esox lucius*), using genomic and expression evidences as well as by
36 loss-of-function and gain-of-function experiments. Using RAD-Sequencing from a family
37 panel, we identified Linkage Group (LG) 24 as the sex chromosome and positioned the sex
38 locus in its sub-telomeric region. Furthermore, we demonstrated that this MSD originated from
39 an ancient duplication of the autosomal *amh* gene, which was subsequently translocated to
40 LG24. Using sex-specific pooled genome sequencing and a new male genome sequence
41 assembled using Nanopore long reads, we also characterized the differentiation of the X and Y
42 chromosomes, revealing a small male-specific insertion containing the MSD gene and a limited
43 region with reduced recombination. Our study depicts an unexpected level of limited
44 differentiation within a pair of sex chromosomes harboring an old MSD gene in a wild
45 population of teleost fish, highlights the pivotal role of genes from the *amh* pathway in sex
46 determination, as well as the importance of gene duplication as a mechanism driving the
47 turnover of sex chromosomes in this clade.

48

49 **Author Summary**

50

51 In stark contrast to mammals and birds, teleosts have predominantly homomorphic sex
52 chromosomes and display a high diversity of sex determining genes. Yet, population level
53 knowledge of both the sex chromosome and the master sex determining gene is only available
54 for the Japanese medaka, a model species. Here we identified and provided functional proofs
55 of an old duplicate of anti-Müllerian hormone (Amh), a member of the Tgf- β family, as the
56 male master sex determining gene in the Northern pike, *Esox lucius*. We found that this
57 duplicate, named *amhby* (Y-chromosome-specific anti-Müllerian hormone paralog b), was
58 translocated to the sub-telomeric region of the new sex chromosome, and now *amhby* shows
59 strong sequence divergence as well as substantial expression pattern differences from its
60 autosomal paralog, *amha*. We assembled a male genome sequence using Nanopore long reads
61 and identified a restricted region of differentiation within the sex chromosome pair in a wild
62 population. Our results provide insight on the conserved players in sex determination pathways,
63 the mechanisms of sex chromosome turnover, and the diversity of levels of differentiation
64 between homomorphic sex chromosomes in teleosts.

65

66 **Introduction**

67

68 The evolution of sex determination (SD) systems and sex chromosomes have sparked
69 the interest of evolutionary biologists for decades. While initial insights on sex chromosome
70 evolution came from detailed studies in *Drosophila* and in mammals [1–5], recent research on
71 other vertebrate groups, such as avian [6,7], non-avian reptiles [8,9], amphibians [10–13], and
72 teleost fishes [14–17], has provided new information that helps us understand the evolution of
73 SD systems and sex chromosomes.

74 Teleosts display the highest diversity of genetic sex determination systems in
75 vertebrates, including several types of monofactorial and polygenic systems [14,18]. In
76 addition, in some species, genetic factors can interact with environmental factors, most
77 commonly temperature, *i.e.* in *Odontesthes bonariensis* [19], generating intricate sex
78 determination mechanisms. Moreover, sex determination systems in fish can differ between
79 very closely related species, as illustrated in the group of Asian ricefish (genus *Oryzias*) [20–
80 25], and sometimes even among different populations of one species, like in the Southern
81 platyfish, *Xiphophorus maculatus* [26]. Beside this remarkable dynamic of sex determination
82 systems, the rapid turnover of sex chromosomes in teleosts provides many opportunities to
83 examine sex chromosome pairs at different stages of differentiation. Finally, recent studies on
84 fish sex determination have revealed a dozen new master sex determining (MSD) genes
85 [14,16,27], adding additional insights on the forces driving the turnover of SD systems and the
86 formation of sex chromosomes.

87 The birth of new MSD genes drives the formation of sex chromosomes and transitions
88 of SD systems. The origin of new MSDs falls into two categories: either gene duplication
89 followed by sub- or neo-functionalization, or allelic diversification [14]. To date, teleosts is the
90 only group where examples of both gene duplication and allelic diversification mechanisms

91 have been found [14–17]. Yet, sex chromosomes with a known MSD gene have only been
92 characterized extensively on the sequence level in two teleost species: the Japanese medaka
93 (*Oryzias latipes*), whose MSD gene originated from gene duplication followed by sub- or neo-
94 functionalization mechanism [28–31], and the Chinese tongue sole, whose MSD gene
95 originated from allelic diversification [32]. Therefore, to form a rich knowledgebase allowing
96 advances of theories of sex chromosomes evolution, additional empirical studies are urgently
97 needed, in particular studies on how the mechanism by which MSDs originate impacts the
98 formation and history of sex chromosomes.

99 Among identified teleost MSD genes, the *salmonid* MSD gene, named *sdY*, is the most
100 intriguing because it revealed a previously unexpected flexibility in SD pathways in teleosts.
101 While all other currently identified MSDs belong to one of three protein families (SOX, DMRT
102 and TGF- β and its signaling pathway) that were known to be implicated in the SD pathways,
103 the *salmonid* MSD gene *sdY* arose from duplication of an immune-related gene [33]. Despite
104 *sdY* being conserved in the majority of salmonid species [34], it was not found in *Esox lucius*,
105 the most studied member of the salmonid’s sister order the Esociformes [34]. The restriction of
106 *sdY* to the salmonids raised the question of what was the ancestral MSD before the emergence
107 of the “unusual” *sdY*. The first step to answer this question was then to identify the genetic
108 component responsible for sex determination in *E. lucius*.

109 *E. lucius*, commonly known as the Northern pike, is a large and long-lived keystone
110 predatory teleost species found in freshwater and brackish coastal water systems in Europe,
111 North America, and Asia [35]. It has emerged as an important model species for ecology and
112 conservation because of its pivotal role as a top predator that shapes the structure of local fish
113 communities, and also as a valuable food and sport fish [36]. Consequently, genomic resources
114 have been recently generated for *E. lucius*, including a whole genome assembly anchored on
115 chromosomes [37] and a tissue-specific transcriptome [38]. Yet, little is known about genetic

116 sex determination in *E. lucius* beyond the knowledge that males are the heterogametic sex [39],
117 and its sex locus and MSD gene remain elusive.

118 In this study, we identified a duplicate of anti-Müllerian hormone (*amh*) with a testis-
119 specific expression pattern as a candidate male MSD gene for *E. lucius*. Using pooled
120 sequencing (pool-seq) reads from a wild population and a new draft genome sequenced with
121 Nanopore long reads, we found limited differentiation between the homomorphic sex
122 chromosomes and that this male-specific duplicate of *amh*, which we call *amhby*, is located
123 within the Y-specific sequence. Using RAD-sequencing of a family panel, we identified
124 Linkage Group 24 as the sex chromosome and positioned the sex locus in its sub-telomeric
125 region. In addition, we showed that *amhby* has an expression profile characteristic of a male
126 MSD gene and is functionally both sufficient and necessary to trigger testis development,
127 providing robust support for *amhby* as the MSD gene in this species. Finally, through
128 phylogenetic and synteny analyses, we showed that this *amh* duplication took place around 40
129 million years ago and is lineage-specific and that *amhby* was translocated after its duplication,
130 which likely initiated the formation of the proto-Y chromosome.

131 Taking advantage of recent advances in functional genomics and sequencing
132 technologies, our study combines the location and characterization of the sex locus and the
133 identification of a master sex determining (MSD) gene with substantial functional validation in
134 a non-model species. Our results expand the knowledge of sex determination genes and provide
135 insight on the evolution of sex chromosomes in teleosts.

136

137 **Results**

138

139 **Identification of a male-specific duplicate of *amh* with testis-specific** 140 **expression in *E. lucius***

141 Two *amh* transcripts sharing 78.9% nucleotide identity were identified in the tissue-
142 specific transcriptomes of *E. lucius* ([38], phylofish.siggenae.org), one transcript predominantly
143 expressed in the adult testis with a low expression found also in adult ovary and adult muscle,
144 and the other transcript exclusively expressed in the adult testis (**Fig S1**). PCR amplification on
145 genomic DNA from 221 wild-caught individuals, whose phenotypic sex was determined by
146 gonadal inspection, showed that the genomic sequence of one *amh* copy was present in all
147 phenotypic males and females, while the genomic sequence of the testis-specific *amh* was
148 present in 98% of phenotypic males (157/161) and 0% of phenotypic females (0/60) (**Fig 1A**),
149 demonstrating a highly significant association between this testis-specific copy of *amh* and male
150 phenotype (Chi-squared test, $p < 2.2e-16$), and indicating that the genomic sequence of this
151 testis-specific copy of *amh* transcript is Y chromosome specific. This male-specific *amh* was
152 named *amhby* (Y-chromosome-specific anti-Müllerian hormone paralog b) and the other
153 autosomal gene was named *amha* (*amh* paralog a).

154 To compare the genomic regions containing *amha* and *amhby*, clones were isolated from
155 a phenotypic male genomic fosmid library and sequenced. The *amha*-containing fosmid
156 included the entire 5' intergenic region of *amha* up to the closest gene, *dot11*, and the *amhby*-
157 containing fosmid included a 22 kb region upstream of *amhby* which contained no coding
158 sequence for other proteins (blastx search against Teleostei, taxid:32443). Nucleotide identity
159 between *amha* and *amhby* exon sequences ranges from 74% to 95% (**Fig 1B and 1C**) and the
160 only gross structural difference between the two genes is a 396 bp specific insertion of a

161 repeated region in *E. lucius* genome in *amhby* intron 1. Little sequence similarity was found
162 between the proximal sequences of the two genes, except for a 1020 bp repetitive element
163 (transposase with conserved domain HTH_Tnp_Tc3_2) (**Fig 1D**). Predicted proteins contain
164 580 amino-acids (AA) for *amha* and 560 AA for *amhby*, sharing 68.7% identity and 78.4%
165 similarity. Both proteins have a complete 95 AA C-terminal TGF- β domain with seven
166 canonical cysteines (**Fig S2**), sharing 62.5% identity and 74.0% similarity.

167

168 **Assembly of an XY genome containing *amhby* using Nanopore long reads**

169

170 Blast results revealed that the sequence of the *amhby*-containing fosmid was absent from
171 the available genome assembly (GenBank assembly accession: GCA_000721915.3), which
172 suggests that the sequenced individual could have been a genetic female. Therefore, to
173 characterize the sex locus, we sequenced and assembled the genome of a genetic male with
174 Nanopore long reads. Results from BUSCO show that this new assembly has comparable
175 completeness to that of the reference assembly (**Table S1**). In this Nanopore assembly, the
176 entire sequence of the *amhby*-containing fosmid was included in a 99 kb long scaffold,
177 tig00003316, from 24,050 bp to 60,989 bp, with *amhby* located from 27,062 bp to 30,224 bp.

178

179 **The Y chromosome harbors a small sex locus containing *amhby***

180 To locate and characterize the sex locus of *E. lucius*, we first aligned sex-specific pool-
181 seq reads from 30 phenotypic males with *amhby* and 30 phenotypic females without *amhby* on
182 the available Northern pike reference assembly (GenBank assembly accession:
183 GCA_000721915.3), which we suspected to be an XX genome, and computed the number of
184 male-specific SNPs (MSS) in a 50 kb non-overlapping window across the genome (**Fig 2A**).
185 Genome average was 0.08 MSS per window, and two windows located on unplaced scaffold

186 1067 contained 136 and 89 MSS respectively, close to four times the number of MSS in the
187 next highest window (23 MSS). A further analysis with a higher resolution (i.e., 2.5 kb windows)
188 revealed that the majority (94%) of the MSS on scaffold 1067 is restricted to a 40 kb region
189 located between ~ 143 kb and ~ 184 kb on this scaffold. Together, these results indicate that
190 the sex locus is located on scaffold 1067, and Y-derived sequences show strong differentiation
191 from the XX genome only in a small 40 kb region on this scaffold (**Fig 2B, Fig S3**).

192 In a second step, to identify male-specific regions, we aligned the pool-seq reads on the
193 XY Nanopore assembly and identified 94 non-overlapping 1 kb regions covered only by reads
194 from the male pool (MR1k). These regions were located on only three Nanopore contigs
195 (tig00003316 = 53 MR1k, tig00003988 = 22 MR1k, tig00009868 = 19 MR1k). In contrast,
196 there were only four non-overlapping 1 kb regions covered only by reads from the female pool,
197 each located on a different contig, indicating a low false discovery rate for sex-specific regions
198 with our method. Moreover, we found no MR1k from the same analysis on the reference
199 assembly (GenBank assembly accession: GCA_000721915.3), further confirming that the
200 individual sequenced for this assembly has an XX genotype. Blasting results on the three
201 MR1k-containing Nanopore contigs showed that *amhby* is the only protein coding gene apart
202 from transposable elements (TEs) associated proteins in the sex locus of *E. lucius* (**Table S2**).

203 To better delimitate the sex locus, we searched for Y-specific sequences, defined as
204 regions with no or little female reads mapped and male reads mapped at a depth close to half of
205 the genome average, on the three MR1k-containing contigs from the Nanopore assembly
206 (**Supplementary File 1, Fig S4**). In total, we identified ~ 180 kb of Y-specific sequences.

207 Taken together, these results indicate that the sex locus of *E. lucius*, is ~ 220 kb in size
208 including ~ 180 kb of Y-specific sequence and ~ 40 kb of X/Y differentiated region, and that
209 *amhby* is the only non-TE, protein-coding gene in this locus.

210

211 **The sex locus of *E. lucius* is located in the sub-telomeric region of LG24**

212 The pool-seq results located the sex locus on unplaced scaffold 1067. To identify the
213 sex chromosome in the genome of *E. lucius*, we generated RAD-Seq data from a single full-
214 sibling family of *E. lucius* with two parents, 37 phenotypic male offspring, and 41 phenotypic
215 female offspring. In total, 6,512 polymorphic markers were aligned to the 25 nearly
216 chromosome-length linkage groups and 741 polymorphic markers were aligned to unplaced
217 scaffolds of the reference assembly of *E. lucius* (GenBank assembly accession:
218 GCA_000721915.3). Genome-wide average F_{st} between males and females was 0.0033. Only
219 LG2 ($F_{st} = 0.006$) and LG24 ($F_{st} = 0.025$) had a higher average F_{st} between males and females
220 than the genome average, and only markers on LG24 showed genome-wide significant
221 association with sex phenotype (**Fig 3A**), indicating that LG24 is the sex chromosome of *E.*
222 *lucius*. The F_{st} of markers aligned to LG24 gradually increased along the length of the
223 chromosome and reached 90 times the genome average F_{st} (0.38) at the distal end of the
224 chromosome (**Fig 3B**), and the strongest association with sex was also identified for markers
225 aligned to this region (**Fig 3C**), pinpointing the location of the sex locus to the sub-telomeric
226 region of LG24. In addition, with our parameters, 32 non-polymorphic RAD markers were
227 found only in the father and all male offspring, 15 of which could be aligned to the reference
228 XX genome (**Table S3**): five (33%) to a region located distal to 22.1 Mb on LG24; one (7%) at
229 position 17.3 Mb on LG24; seven (22%) to unplaced scaffold 0213, and two (6%) to other LGs.
230 Moreover, two of the other 17 male-specific markers which could not be aligned to the reference
231 genome aligned perfectly to the sequence of *amhby*, showing that *amhby* has a strict father-to-
232 son inheritance pattern.

233 Collectively, these results locate the sex locus, containing *amhby*, in the sub-telomeric
234 region of LG24 of *E. lucius*. In addition, unplaced scaffold 0213, which is enriched in male-

235 specific RAD markers, and unplaced scaffold 1067, which is enriched with male-specific SNPs
236 identified with the pool-seq analysis, should also be placed in the sub-telomeric region of LG24.

237

238 ***amhby* is expressed prior to molecular gonadal differentiation in male *E.***

239 ***lucius***

240 To characterize the temporal and spatial expression of *amhby* in relation to the
241 molecular and morphological differentiation between male and female gonads, both
242 quantitative PCR (qPCR) and *in-situ* hybridization (ISH) were performed.

243 Expression of *amhby*, *amha*, three other genes (*drmt1*, *cyp19a1a*, and *gsdf*) known for
244 their role in gonadal sex differentiation, and *amhrII*, the putative receptor for the canonical *amh*,
245 was measured by qPCR at four time points from 54 days post-fertilization (dpf) to 125 dpf,
246 prior to the onset of gametogenesis. The entire trunks were used for the first three time points
247 when the gonads were too small to be isolated, and only gonads were used at 125 dpf for both
248 males and females. Expression of *amha* was detected in both males and females starting from
249 75 days post-fertilization (dpf), with a significantly higher expression in males than in females
250 at 100 dpf (Wilcoxon signed-rank test, $p=0.043$) (**Fig 4A**). In contrast, expression of *amhby*
251 was detected only in males starting from 54 dpf and increasing exponentially thereafter till 125
252 dpf ($R^2=0.79$) (**Fig 4B**). Expression of *drmt1*, *cyp19a1a*, and *gsdf* was only detected from 100
253 dpf onwards, *i.e.* much later than the first detected expression of *amhby* (**Fig S5**). Moreover,
254 among these three genes, only *cyp19a1a* showed significantly different expression between
255 sexes with a higher expression in females at 100 dpf (Wilcoxon signed-rank test, $p=0.014$).
256 Expression of *amhrII* was not detected until 100 dpf and did not differ significantly between
257 sexes at any stage (**Fig S5**).

258 Expression of *amha* and *amhby* was also characterized by *in-situ* hybridization (ISH)
259 performed on histological sections of the entire trunk of male and female *E. lucius* sampled at
260 80 dpf. Expression of *amha* was detected in the gonads of both female (**Fig 4C**) and male (**Fig**
261 **4D**) samples, but the signal was much stronger in male gonads. In contrast, expression of *amhby*
262 was strong in male gonads (**Fig 4F**) but not detected in female gonads (**Fig 4E**), confirming the
263 specificity of the probe for *amhby*. In addition, no morphological differences were observed
264 between male and female gonads at 80 dpf, even though expression of *amhby* was already
265 detected by qPCR and by ISH at this stage. Our ISH results show that the expression of both
266 *amha* and *amhby* is high in male gonads before the first signs of histological differentiation
267 between male and female gonads.

268 Collectively, these results show that *amhby* is expressed in the male gonads prior to both
269 molecular and morphological sexual-dimorphic differentiation of gonads in *E. lucius*.

270

271 **The *amhby* gene is both necessary and sufficient to trigger testicular** 272 **differentiation**

273 To further investigate the functional role of *amhby* in initiating testicular development,
274 we performed both loss-of-function and gain-of-function experiments.

275 We knocked out *amhby* using three pairs of TALENs targeting exon 1 and exon 2 of
276 *amhby* (**Fig S4A**). Only the T2 TALEN pair targeting exon 1 was effective in inducing deletions
277 in the *amhby* sequence. Overall, 12 of 36 (33.3%) surviving G0 males possessed a disrupted
278 *amhby* that resulted in truncated proteins (**Fig S4B**). G1 XY offspring obtained from three
279 *amhby* mosaic G0 males crossed with wild-type females were maintained until the beginning
280 of testicular gametogenesis at 153 dpf and then processed for histology. Gonads from 23 G1
281 *amhby* positive XY mutants were compared with those of wild-type control XY males (N=4)

282 and control XX females (N=4) of the same age. Control animals developed normal ovary and
283 testis (**Fig 5A and 5B**), but all 23 XY F1 *amhby* mutants failed to develop a normal testis.
284 Among these 23 G1 XY mutants, 20 (87%) showed complete gonadal sex reversal,
285 characterized by the formation of an ovarian cavity and the appearance of previtellogenic
286 oocytes (**Fig 5C**); the three (13%) remaining mutants developed potentially sterile gonads with
287 no clear ovarian nor testicular structure.

288 To investigate whether *amhby* alone is sufficient to trigger testicular development, we
289 overexpressed *amhby* in XX genetic females. Two G0 XY mosaic transgenic males possessing
290 the *amhby* fosmid were crossed to wild-type females, and 10 G1 XX offspring carrying the
291 *amhby* fosmid were maintained along with control wild-type siblings until the beginning of
292 testicular gametogenesis at 155 dpf. Upon histological analysis of the gonads, all ten (100%)
293 XX transgenics carrying *amhby* fosmid developed testis with testicular lumen and clusters of
294 spermatozooids (**Fig 5D**), while all 12 control genetic males developed testis and 19 of 24 (79%)
295 control genetic females developed ovaries. The other five control genetic females (21%)
296 developed testes. Such sex reversal was also observed in the natural population at a rate of 2%,
297 and this might have been exacerbated by culture conditions, a phenomenon previously
298 documented in other teleosts [40]. Despite this effect, the XX transgenics with *amhby* fosmid
299 had a significantly higher rate of sex reversal than their control female siblings raised in
300 identical condition (Chi-squared test, $p= 0.0001148$).

301 Taken together, these results show that *amhby* is both necessary and sufficient to trigger
302 testicular development in *E. lucius*, and further support the functional role of *amhby* as the MSD
303 gene in this species.

304

305 **A chromosomal translocation involved *amhby* after a lineage-specific**
306 **duplication of *amh***

307 To determine the origin of the two *E. lucius amh* paralogs, we generated a map of
308 conserved synteny for *amh* in several teleost species, including the spotted gar (*Lepisosteus*
309 *oculatus*) as outgroup (**Fig 6A**). Genes located upstream (*i.e.* *dot11*, *ell*, and *fkbp8*) and
310 downstream (*i.e.* *oazla*) of *amha* on *E. lucius* LG08 showed conserved synteny in all teleost
311 species included in the analysis, indicating that LG08 is the conserved location of the *E. lucius*
312 ancestral *amh*, now called *amha*, and that *amhby* evolved from a duplication of *amha* that was
313 later translocated to the sub-telomeric region of the future sex chromosome, LG24. We
314 estimated the duplication event to be ~ 38 and ~50 million years old (**Supplementary file 1**),
315 and found no homology between the ~ 180 Kb Y-specific sequence identified in the Nanopore
316 assembly and the sequence of LG08 from the reference assembly, besides the two *amh* genes,
317 suggesting that this translocation event is also likely to be ancient.

318 Prior to the discovery of *amhby* in *E. lucius*, male-specific duplications of *amh* have
319 been identified in Patagonian pejerrey (*Odontesthes hatcheri*) [41] and in Nile tilapia
320 (*Oreochromis niloticus*) [42]. To test whether these duplications have a shared origin, we
321 constructed a phylogeny of Amh from nine teleost species, including these three species with
322 male-specific *amh* duplications, and including spotted gar Amh as outgroup (**Fig 6B**). In this
323 protein phylogeny, each sex-specific Amh paralog clusters as a sister clade to its own species'
324 'canonical' Amh with significant bootstrap values, indicating that these three pairs of *amh*
325 paralogs were derived from three independent and lineage-specific duplication events.

326

327 **Discussion**

328

329 ***amhby*, the male-specific duplicate of *amh*, is the master sex determination**
330 **gene in *E. lucius***

331 Since the discovery of *dmrt1bY* in the Japanese rice fish [28,29], the first identified
332 teleost master sex determination gene, studies in teleosts have unveiled a dozen novel genes as
333 master regulators for sex determination [14–16,27]. Interestingly, many of these master sex
334 determining genes belong to the TGF- β superfamily. To date, this finding has been mostly
335 restricted to teleosts, highlighting the crucial role of TGF- β signaling in the sex determination
336 pathway in this vertebrate group. In the present study, we identified an old duplicate of *amh*, a
337 member of the TGF- β superfamily, as the male MSD gene in *E. lucius*. Results from genotyping
338 demonstrate a strong and significant association of *amhby* with male phenotype. RNA-seq,
339 qPCR and ISH showed that *amhby* is expressed in the male gonadal primordium before
340 histological testis differentiation, thus fulfilling another criterion for being an MSD.
341 Furthermore, knockout of *amhby* leads to complete gonadal sex reversal of XY mutants, while
342 overexpression of *amhby* in XX animals leads to the development of testis, demonstrating that
343 *amhby* is both necessary and sufficient for testicular differentiation. Together, these
344 independent lines of evidence provide strong confidence that *amhby* is the MSD in *E. lucius*.

345 This work provides a third functionally validated case of an *amh* duplicate evolving into
346 the MSD gene in a teleost species, along with the Patagonian pejerrey, *Odontesthes hatcheri*,
347 [41], and the Nile tilapia, *Oreochromis niloticus*, [42]. Besides these three examples,
348 association of *amh* duplicates with phenotypic sex was also found in other teleosts: in *O.*
349 *bonariensis*, the sister species of the Patagonian pejerrey, a male-specific *amhy* was found to
350 interact with temperature in determining sex [19], and in the ling cod, *Ophiodon elongatus*, a
351 male-specific duplicate of *amh* was also identified using molecular marker sequences [43].
352 More recently, a duplicated copy of *amh* was found in an *Atheriniformes* species, *Hypoatherina*

353 *tsurugae*, and is suspected to be involved in male sex determination [44]. Our phylogenetic
354 analysis on *Amh* sequences from several teleost species revealed that the three confirmed male-
355 specific *Amh* duplications are independent, lineage-specific events rather than the product of
356 shared ancestry. This finding supports the “limited option” hypothesis for master sex
357 determining genes [45] and makes *amh* duplicates the most frequently and independently
358 recruited master sex determining genes identified in any animal group so far.

359 Among the three teleost species with confirmed *amh* duplicate MSD genes, *E. lucius*
360 has the highest degree of divergence in sequence between paralogs, with ~ 79.6% genomic
361 sequence identity on average. In the Nile tilapia, *amhy* is almost identical to the autosomal *amh*,
362 differing by only one SNP [42]. In the Patagonian pejerrey, the shared identity between paralogs
363 ranges from 89.1% to 100% depending on the exon [41]. Because of the low divergence
364 between the two paralogous sequences in the Patagonian pejerrey and the Nile tilapia, the new
365 MSD function of *amhy* was, in both cases, mainly attributed to its novel expression pattern.
366 Yet, low sequence divergence, as little as one amino acid-changing SNP in *amhrII* alleles in
367 *Takifugu*, was shown to be sufficient to impact the signal transduction function of a protein
368 [46]. In *E. lucius*, *amhby* also have an expression profile different from that of *amha*, likely due
369 to a completely different sequence in the putative promoter region. However, because of the
370 relatively high level of divergence between *amha* and *amhby* sequences in *E. lucius*, especially
371 in the C-terminal bioactive domain of the proteins [47], it is tempting to hypothesize that the
372 two proteins could have also diverged in their function, with for instance a different affinity for
373 their canonical AmhrII receptor or even the ability to bind to different receptors, leading to
374 divergences in their downstream signaling pathways. Because we estimated the duplication of
375 *amh* in *E. lucius* to be between ~ 38 and ~ 50 million-year-old, the long divergence time
376 between the two paralogous *amh* genes potentially provided opportunities for the accumulation
377 of these sequence differences. Further functional studies would be required to unravel the

378 downstream signaling pathways of *Amha* and *Amhby* in *E. lucius* to better understand the
379 mechanisms leading to the novel function of *amhby* as the MSD gene in this species.

380 Surprisingly, the sequence of *amhby* and the ~ 90 kb male specific sequence flanking
381 *amhby* are absent from the reference genome assembly sequenced from DNA of a
382 phenotypically male sample of *E. lucius* [37], suggesting that this sequenced individual had an
383 XX genotype. The absence of male specific regions when mapping pool-seq reads to this
384 assembly further confirms that the reference genome sequence likely comes from an XX genetic
385 individual. This apparent discordance of genotype and phenotype could be due the difficulty in
386 correctly identifying the phenotypic sex of an individual with immature gonads, as Northern
387 pikes do not display sexual dimorphic external traits. Alternatively, the sequenced individual
388 may have been a sex-reversed genetic female, as found in a low proportion (~ 2%) in a wild
389 population and at higher proportion (~ 20%) in captivity raised Northern pikes.

390

391 **The birth of the MSD and sex chromosomes in *E. lucius***

392 The analysis of the genomic neighborhood of both *amh* duplicates showed that *amha* is located
393 on LG08 in a cluster of genes regulating sexual development and cell cycling with conserved
394 synteny in teleosts [48,49], while *amhby* is located near the telomeric region of LG24 with no
395 other identified gene besides transposable elements within at least 99 kb in its close vicinity.
396 These results indicate that *amha* is likely to be the ancestral *amh* copy in *E. lucius*, and that
397 *amhby* was translocated near the telomere of the ancestor of LG24 after its duplication. This
398 scenario fits the description of a proposed mechanism of sex chromosome emergence and
399 turnover through gene duplication, translocation and neofunctionalization [31,50]. Following
400 this model, the translocation of a single copy of *amh* into another autosome triggered the
401 formation of proto sex chromosomes, possibly because the newly translocated genomic

402 segment containing the *amh* copy halted recombination with the X chromosome *ab initio* due
403 to a complete lack of homology. This mechanism came to light after the discovery of *dmrt1bY*
404 in the Japanese medaka, which acquired a pre-existing *cis*-regulatory element in its promoter
405 through a transposable element [51]. Besides *dmrt1bY*, the only other well-described case of
406 sex chromosome turnover via gene duplication, translocation and neofunctionalization is the
407 salmonid *sdY* gene, which maps to different linkage groups in different salmonid species. Our
408 study provides a third empirical example of gene duplication and translocation giving rise to
409 new MSD genes and bolster the importance of this mechanism in the birth and turnover of sex
410 chromosomes.

411 Theories of sex chromosomes evolution predict suppression of recombination around
412 the sex determining locus, eventually leading to degeneration of the locus because it lacks the
413 ability to purge deleterious mutations and repeated elements [52,53]. Here, we found that only
414 a small part (around 220 kb) of the sex chromosome shows differentiation between X and Y
415 chromosome, suggesting that this sex locus encompasses less than 1% of LG24 in *E. lucius*.
416 Sex chromosome differentiation has been characterized in a few other teleost species, including
417 Chinese tongue sole [32], Japanese medaka [30,54], stickleback [55], Trinidad guppy [56] and
418 a few cichlid species [57,58]. Compared to these examples, we found that the Northern pike
419 displays a very limited region of suppressed recombination between the sex chromosomes. A
420 usual explanation for a small sex locus is that the rapid transition of sex chromosomes
421 frequently observed in teleosts, facilitated by a duplication and translocation mechanism, can
422 readily produce neo-sex chromosomes showing little differentiation except for the recently
423 acquired MSD genes, as was demonstrated in the salmonids with the vagabond MSD gene *Sdy*
424 [34,59,60]. However, the ~ 40 million year of divergence time between *amhby* and *amha*, and
425 the lack of homology between the sequence from the sex locus and that of the vicinity of *amha*
426 on LG08, suggest that the sex locus of *E. lucius* is not nascent. Further comparative studies that

427 include sister species in the same clade will be needed to better estimate the age of the MSD
428 gene and the sex chromosome, but a nascent sex locus is likely not the explanation for the
429 restricted region suppression of recombination between the X and Y chromosome of *E. lucius*.
430 On the other hand, old yet homomorphic sex chromosomes have been observed, for instance in
431 ratite birds [61] and in pythons [62]. Furthermore, in *Takifugu*, a single SNP conserved for 30
432 million years determines sex and the rest of the sex chromosomes does not show evidence of
433 suppressed recombination, raising the possibility that decay is not the only possible fate for sex
434 chromosomes [46]. One mechanism for the maintenance of a small sex locus has been proposed
435 in the Japanese rice fish, where long repeats flanking the sex locus on the Y chromosome may
436 recombine with the same repeats on the X chromosomes, thus hindering the spread of
437 suppression of recombination around the MSD gene [50]. We found a slight enrichment of
438 repeated elements on the sequences from the sex locus of *E. lucius*, however a better assembly
439 of the sex locus would be needed to investigate whether a similar mechanism could have
440 contributed to the very limited differentiation between X and Y chromosomes in *E. lucius*.

441 To date, empirical support for non-decaying sex chromosome is still rare, possibly due
442 to the difficulties in identifying small differences between sex chromosomes, and because
443 environmental factors affecting the sex determination pathways could weaken the association
444 between genotype and phenotype, particularly in non-endothermic vertebrates. However, our
445 study, as well as other recent effort in characterizing sex loci in non-model species [63], shows
446 that using current population genomic approaches, we can now relatively easily identify and
447 characterize small sex loci. Future studies surveying sex chromosomes at various stages of
448 differentiation and understanding the factors influencing their level of differentiation will form
449 a new empirical basis to update the current models of sex chromosome evolution.

450 In conclusion, our study identified an old duplication of *amh* in *E. lucius* which
451 generated a Y-chromosome-specific copy that we named *amhby*. We showed that *amhby* is

452 functionally necessary and sufficient to trigger testicular development, and is expressed in the
453 male gonadal primordium, fulfilling key requirements for a classic MSD gene. Furthermore,
454 we located *amhby* on the sub-telomeric region on LG24, a region showing very limited
455 differentiation between the X and Y chromosome. The recurrent identification of *amh*
456 duplicates as MSD genes in teleosts highlights the pivotal role of Amh signaling pathway in
457 teleosts sex determination, and encourages further analysis on how *amh* MSDs genes initiate
458 testicular differentiation. Moreover, our results provide an intriguing empirical example of an
459 unexpected small sex locus with an old MSD gene and highlight the power of exciting new
460 sequencing technologies and population genomics approaches to identify and characterize sex
461 loci in non-model species.

462

463 **Material and Methods**

464

465 **Fish rearing conditions**

466 Research involving animal experimentation was conformed to the principles for the use
467 and care of laboratory animals, in compliance with French and European regulations on animal
468 welfare. The ethical agreement number for this project is 01676.02. Fertilized eggs from
469 maturing Northern pike females were obtained from the fish production unit of the fishing
470 federation of Ille-et-Vilaine (Pisciculture du Boulet, Feins, France). Fish were maintained
471 indoors under strictly controlled conditions in individual aquaria to avoid cannibalism, with
472 running dechlorinated local water (pH=8) filtered with a recirculating system. Rearing
473 temperature and photoperiod followed the trend of the ambient natural environment. Northern
474 pike fry were fed with live prey such as artemia larvae, daphnia, and adult artemia depending

475 on their size. After reaching a length of 4-5 cm, Northern pike juveniles were fed with rainbow
476 trout fry.

477

478 **Fosmid library screening, sub-cloning and assembling**

479 The Northern pike fosmid genomic DNA library was constructed by Bio S&T (Québec,
480 Canada) from high molecular weight DNA extracted from the liver of a male *E. lucius* from Ille
481 et Vilaine, France, using the CopyControl Fosmid Library Production Kit with pCC1FOS
482 vectors (Epicentre, USA) following the manufacturer's instructions. The resulting fosmid
483 library contained around 500,000 non-amplified clones that were arrayed in pools of 250
484 individual fosmids in ten 96-well plates.

485 Northern pike fosmid clones were screened by PCR (**Table S5**) to identify individual
486 fosmids containing *amhby* and *amha*. To sequence the two ~ 40 kb fosmids, purified fosmid
487 DNA was first fragmented into approximately 1.5 kb fragments using a Nebulizer kit supplied
488 in the TOPO shotgun Subcloning kit (Invitrogen, Carlsbad, CA), and then sub-cloned into
489 pCR4Blunt-TOPO vectors. Individual plasmid DNAs were sequenced from both ends with
490 Sanger sequencing using M13R and M13F primers. The resulting sequences were then
491 assembled using ChromasPro version 2.1.6 (Technelysium Pty Ltd, South Brisbane, Australia).

492

493 **Phylogenetic and synteny analyses**

494 The protein and cDNA sequences of *amhby* and *amha* were predicted from their
495 genomic sequences using the FGENESH+ suite [64], and the resulting cDNA sequences were
496 compared to the corresponding transcripts publicly available in the Phylofish database [38].
497 Shared identity between cDNA sequences was calculated after alignment using Clustal Omega
498 [65] implemented on EMBL-EBI [66,67] with default settings.

499 Global pairwise alignment of the two transcripts was performed using the mVISTA
500 LAGAN program with default settings [68]. Shared identity and similarity between protein
501 sequences were calculated using EMBOSS Water [69]. For each *amh* paralog, we compared
502 the 5 kb genomic sequence upstream of the start codon using PromoterWise [69] and mVISTA
503 LAGAN [68]. For *amha*, this 5 kb upstream region included the entire intergenic sequence up
504 to and including part of the *dot1l* gene, located 4.3 kb from the start codon of *amha*, so the
505 promoter for *amha* is likely located in this region.

506 Phylogenetic relationship reconstruction of Amh proteins was performed using the
507 maximum likelihood method implemented in PhyML 3.0 [70], and the proper substitution
508 model was determined with PhyML-SMS [71]. Amh protein sequences from eight teleost
509 species and from the spotted gar (*Lepisosteus oculatus*), which was used as outgroup, were
510 retrieved from the NCBI Protein database. The accession number for each sequence is
511 referenced in **Table S4**.

512 A synteny map of the conserved genes in blocks around *amh* was constructed with nine
513 teleost species and spotted gar as outgroup. For spotted gar, zebrafish (*Danio rerio*), Atlantic
514 cod (*Gadus morhua*), three-spined stickleback (*Gasterosteus aculeatus*), Japanese pufferfish
515 (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*), Nile tilapia (*Oreochromis niloticus*),
516 Southern platyfish (*Xiphophorus maculatus*) and Amazon molly (*Poecilia formosa*), the
517 synteny map was created with the Genomicus website (www.genomicus.biologie.ens.fr,
518 accessed in July 2017 [72,73]). For Northern pike, genes located upstream and downstream of
519 *amha* on LG08 were deduced based on the location of each gene in the genome assembly
520 (Eluc_V3, GenBank assembly accession: GCA_000721915.3).

521

522 **DNA extraction and genotyping**

523 Small fin clips were taken from caudal fins of Northern pikes after administrating fish
524 anesthetics (3 ml of 2-phenoxyethanol per L). When needed, fish were euthanized with a lethal
525 dose of anesthetics (10 ml of 2-phenoxyethanol per L). All fin clips were collected and stored
526 at 4°C in 75-100% ethanol until DNA extraction.

527 To obtain DNA for genotyping, fin clips were lysed with 5% Chelex and 25 mg of
528 Proteinase K at 55°C for 2 hours, followed by incubation at 99°C for 10 minutes. After brief
529 centrifugation, the supernatant containing genomic DNA was transferred to clean tubes without
530 the Chelex beads [74]. Primers for genotyping were designed using Perl-Primer (version 1.1.21,
531 [75]). Primer sequences and corresponding experiments can be found in **Table S5**.

532 To obtain DNA for Sanger and Illumina short read sequencing, DNA was extracted
533 from fin clips using NucleoSpin Kits for Tissue (Macherey-Nagel, Duren, Germany) following
534 the producer's protocol. To obtain high molecular weight DNA for long read sequencing, DNA
535 was extracted from blood samples lysed in TNES-Urea buffer (TNES-Urea: 4 M urea; 10 mM
536 Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% SDS) [76] followed by phenol-chloroform
537 extraction [77]. Afterwards, the DNA pellet was washed three times with 80% ethanol before
538 being stored at 4°C in 80% ethanol.

539 When preparing DNA for sequencing, DNA concentration was first quantified with a
540 NanoDrop ND2000 spectrophotometer (Thermo scientific, Wilmington, DE) to estimate the
541 range of concentration, and was then measured again with Qubit3 fluorometer (Invitrogen,
542 Carlsbad, CA) to determine the final concentration.

543

544 **RNA extraction, cDNA synthesis and qPCR**

545 Ten Northern pikes from a local French population were sampled at 54, 75, 100, and
546 125 days post fertilization (dpf). For the first three time points, the whole trunk, defined as the
547 entire body without head and tail, was collected for RNA extraction because gonads were too
548 small to be dissected routinely at these stages. At 125 dpf, gonads were large enough to be
549 isolated and were thus collected for RNA extraction. The genotypic sex of each animal was
550 determined based on *amhby* amplification and results are listed in **Table S6**.

551 Samples (trunks or gonads) were immediately frozen in liquid nitrogen and stored at -
552 70°C until RNA extraction. RNA was extracted using Tri-Reagent (Molecular Research Center,
553 Cincinnati, OH) following the manufacturer's protocol and RNA concentration was quantified
554 with a NanoDrop ND2000 spectrophotometer (Thermo scientific, Wilmington, DE). Reverse
555 transcription was performed by denaturing a mix of 1µg of RNA and 5 µL of 10 mM dNTP at
556 70°C for 6 minutes, followed by 10 minutes on ice. Random hexamers and M-MLV reverse
557 transcriptase (Promega, Madison, WI) were then added, and the mixture was incubated at 37°C
558 for 75 minutes followed by 15 minutes of enzyme inactivation at 70°C, and then chilled at 4°C.
559 During these last two steps, a negative control without reverse transcriptase was prepared for
560 each sample. The resulting cDNA was diluted 25-fold before qPCR.

561 Primers for qPCR were designed using Perl-Primer (version 1.1.21, [75]) on intron-exon
562 junctions to avoid genomic DNA amplification (primers are listed in **Table S5**). Primer pairs
563 were diluted to 6 µg/µL for qPCR, which was performed with SYBER GREEN fluorophore
564 kits (Applied Biosystems, Foster City, CA) on an Applied Biosystems StepOne Plus instrument.
565 For each reaction, 4 µL of diluted cDNA and 1 µL of diluted primers were added to 5 µL of
566 SYBER Green master mix. The following qPCR temperature cycling regime was used: initial
567 denaturation at 50°C for 2 minutes and then at 95°C for 2 minutes, followed by 40 PCR cycles
568 at 95°C for 15s, 60°C for 30s and 72°C for 30s, and a final dissociation step at 95°C for 3s,

569 60°C for 30s and 95°C for 15s. Primer pairs were checked for nonspecific amplification and
570 primer-dimers using the dissociation curve implemented in the StepOne software. Relative
571 abundance of each target cDNA was calculated from a standard curve of serially diluted pooled
572 cDNA from all samples, and then normalized with the geometric mean of the expression of six
573 housekeeping genes (*β-actin*, *ef1a*, *gapdh*, *eftud2*, *ubr2*, and *ccdc6b*) following classical qPCR
574 normalization procedures [78].

575

576 **Whole mount *in situ* hybridizations**

577 *In situ* hybridization RNA probes for *amhby* and *amha* were synthesized from cDNA
578 PCR products amplified from 125 dpf testis samples using primers including T7 sequences in
579 the reverse primer (**Table S5**). These PCRs were performed with the Advantage2 Taq
580 Polymerase (Clontech, Mountain View, CA) for high fidelity. Gel electrophoresis was
581 performed on the PCR products, and products of the expected size were cut out and purified
582 using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany). 10 ng of
583 purified PCR product was then used as template for RNA probe synthesis. RNA probes were
584 synthesized using T7 RNA polymerase (Promega, Harbonnieres, France) following the
585 manufacturer's protocol, with digoxigenin-11-UTP for *amha* and Fluorescein-12-UTP for
586 *amhby*. Afterwards, probes were purified using NucleoSpin Gel and PCR Clean-up Kit
587 (Macherey-Nagel, Duren, Germany), re-suspended in 50 µL of DEPC water, and stored at -70
588 °C.

589 Samples used for *in situ* hybridization were entire trunks of males and females collected
590 at 85 dpf and fixed overnight at 4°C in 4% paraformaldehyde solution, then wash and stored at
591 -20°C in 100% methanol. Hybridization was performed using an *in situ* Pro intavis AG robotic
592 station according to the following procedure: male and female samples were rehydrated,

593 permeabilized with proteinase K (25 mg/ml) for 30 minutes at room temperature, and incubated
594 in post-fix solution (4% paraformaldehyde and 0.2% glutaraldehyde) for 20 minutes. Then,
595 samples were incubated for 1 h at 65 °C in a hybridization buffer containing 50% formamide,
596 5% SCC, 0.1% Tween 20, 0.01% tRNA (0.1 mg/ml), and 0.005% heparin. RNA probes were
597 added and samples were left to hybridize at 65 °C for 16 h. Afterwards, samples were washed
598 three times with decreasing percentages of hybridization buffer, incubated in blocking buffer
599 (Triton .1%, Tween 20 0.2%, 2% serum/PBS) for 2 h, and incubated for 12 h with addition of
600 alkaline phosphatase coupled with anti-digoxigenin antibody for *amha* and anti-Fluorescein
601 antibody for *amhby* (1:2000, Roche Diagnostics Corp, Indianapolis, IN). Samples were then
602 washed with PBS solution, and color reactions were performed with NBT/BCIP (Roche
603 Diagnostics Corp, Indianapolis, IN). After visual inspection of coloration, samples were
604 dehydrated and embedded in plastic molds containing paraffin with a HistoEmbedder (TBS88,
605 Medite, Burgdorf, Germany) instrument. Embedded samples were sectioned into 5 µm slices
606 using a MICRO HM355 (Thermo Fisher Scientific, Walldorf, Germany) instrument. Imaging
607 of the slides was performed with an automated microscope (Eclipse 90i, Nikon, Tokyo, Japan).
608

609 **TALENS knock-out**

610 Three pairs of TALENs [79,80], called T1, T2 and T3, were designed to target exon 1
611 and exon 2 of *amhby* (**Fig S4A**). TALENs were assembled following a method derived from
612 Huang *et al.* [81]. For each subunit, the target-specific TALE DNA binding domain consisted
613 of 16 RVD repeats obtained from single RVD repeat plasmids kindly provided by Bo Zhang
614 (Peking University, China). Assembled TALE repeats were subcloned into a pCS2 vector
615 containing appropriate $\Delta 152$ Nter TALE, +63 Cter TALE, and FokI cDNA sequences with the
616 appropriate half-TALE repeat (derived from the original pCS2 vector [81]). TALEN expression
617 vectors were linearized by *NotI* digestion. Capped RNAs were synthesized using the

618 mMMESSAGE mMACHINE SP6 Kit (Life Technologies, Carlsbad, CA) and purified using the
619 NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany).

620 Groups of embryos at the one-cell stage were microinjected with one pair of TALENs
621 at the concentration of either 50 ng/L or 100 ng/L. Among surviving embryos, 66 were injected
622 with T1 (51 embryos at 50 ng/L, 15 embryos at 100 ng/L); 101 were injected with T2 (73
623 embryos at 50 ng/L, 28 embryos at 100 ng/L); and 45 were injected with T3 (all at 50 ng/L). At
624 two months post fertilization, fin clips were collected from 36 surviving animals for genotyping
625 with primers flanking the TALENs targets. Amplification of primers flanking the TALENs
626 targets on *amhby* was also used for sex genotyping. Only genetic males with a disrupted *amhby*
627 sequence were raised until the following reproductive season. The sperm of three one-year old
628 G0 mosaic phenotypic males with a disrupted *amhby* sequence was collected and then used in
629 *in vitro* fertilization with wild-type eggs. G1 individuals were genotyped for *amhby* TALEN
630 targeting sites using primers flanking the TALENs targets, and XY mutants were kept until 153
631 dpf. G1 *amhby* mutants were then euthanized and dissected, and their gonads were subjected to
632 histological analysis to inspect the phenotypes resulting from *amhby* knockout.

633

634 **Additive transgenesis with *amhby* fosmid**

635 Northern pike embryos from wild-type parents were microinjected with the *amhby*
636 fosmid at a concentration of 100 ng/L at the one-cell stage. Two months post fertilization, fin
637 clips were collected and used for genotyping with primer pairs in which one primer was located
638 on the fosmid vector sequence, which does not come from the Northern pike genome, and the
639 other primer was located in the insert sequence from Northern pike genomic DNA to ensure
640 specificity (**Table S5**). Only G0 males possessing the *amhby* fosmid were kept until the
641 following reproductive season when spawning was induced with Ovaprim (Syndel, Ferndale,
642 Washington) following the protocol from [82]. Sperm from two G0 males possessing the *amhby*

643 fosmid was collected and used for *in vitro* fertilization with wild-type eggs. F1 XX individuals
644 were identified due to the absence of amplification of Y-specific sequences outside of the
645 sequence contained in the *amhby* fosmid. The XX transgenics with *amhby* fosmid were kept
646 until 155 dpf together with wild-type sibling control genetic males and females and then
647 dissected. Gonads from mutant and control fish were subjected to histological analysis.

648

649 **Histology**

650 Gonads to be processed for histology were fixed immediately after dissection in Bouin's
651 fixative solution for 24 hours. Samples were dehydrated with a Spin Tissue Processor Microm
652 (STP 120, Thermo Fisher Scientific, Walldorf, Germany) and embedded in plastic molds in
653 paraffin with a HistoEmbedder (TBS88; Medite, Burgdorf, Germany). Embedded samples were
654 cut serially into slices of 7 μm using a MICRO HM355 (Thermo Fisher Scientific, Walldorf,
655 Germany) and stained with Hematoxylin. Imaging was performed with an automated
656 microscope (Eclipse 90i, Nikon, Tokyo, Japan).

657

658 **Statistical analyses**

659 All statistical analyses, including Wilcoxon signed rank test and Chi-squared test, were
660 performed with R (version 3.5.1 [83]).

661

662 **Population analysis for male and female *E. lucius* RAD-Seq markers**

663 A Restriction Associated DNA Sequencing (RAD-Seq) library was constructed from
664 genomic DNA extracted from the fin clips of two parents, 37 male offspring and 41 female
665 offspring according to standard protocols [84]. The phenotypic sex of the offspring was

666 determined by histological analysis of the gonads at 8-month post fertilization. The library was
667 sequenced in one lane of Illumina HiSeq 2500. Raw reads were analyzed with the Stacks
668 (Catchen et al., 2011) program version 1.44. Quality control and demultiplexing of the
669 169,717,410 reads were performed using the *process_radtags.pl* script with all settings set to
670 default. In total, 128,342,481 (76%) reads were retained after this filtering step, including ~ 1.6
671 M. retained sequences from the father, ~ 0.9 M. retained sequences from the mother, and
672 between 1.0 M. and 2.2 M. retained sequences from each offspring.

673 Demultiplexed reads were mapped to the genome assembly of *E. lucius* (Elu_V3,
674 GenBank assembly accession: GCA_000721915.3) using BWA (version 0.7.15-r1140, [86])
675 with default settings. The resulting BAM files were run through the *ref_map.pl* pipeline with
676 default settings except a minimum stack depth of 10 (m=10). Results of *ref_map.pl* were
677 analyzed with *populations* using the --fstats setting to obtain population genetic statistics
678 between sexes. Fisher's exact test was performed on all polymorphic sites using Plink (version
679 1.90b4.6 64-bit, [87]) to estimate association between variants and phenotypic sex. A Manhattan
680 plot was constructed in R with homemade scripts showing $-\log_{10}$ (Fisher's test *p*-value) for all
681 25 LGs of the *E. lucius* genome.

682

683 **Genome sequencing and assembly**

684 The genome of one local phenotypic male Northern pike (*Esox lucius*) was assembled
685 using Oxford Nanopore long reads and polished with Illumina reads.

686 Illumina short reads libraries were built using the Truseq nano kit (Illumina, ref. FC-
687 121-4001) following the manufacturer's instructions. First, 200 ng of gDNA was briefly
688 sonicated using a Bioruptor sonication device (Diagenode, Liege, Belgium), end-repaired and
689 size-selected on beads to retain fragments of size around 550 bp, and these fragments were A-

690 tailed and ligated to Illumina's adapter. Ligated DNA was then subjected to eight PCR cycles.
691 Libraries were checked with a Fragment Analyzer (AATI) and quantified by qPCR using the
692 Kapa Library quantification kit. Libraries were sequenced on one lane of a HiSeq2500 using
693 the paired end 2x250 nt v2 rapid mode according to the manufacturer's instruction. Image
694 analysis was performed with the HiSeq Control Software and base calling with the RTA
695 software provided by Illumina.

696 Nanopore long reads libraries were prepared and sequenced according to the
697 manufacturer's instruction (SQK-LSK108). DNA was quantified at each step using the Qubit
698 dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA). DNA purity was checked using a NanoDrop
699 ND2000 spectrophotometer (Thermo scientific, Wilmington, DE) and size distribution and
700 degradation were assessed using a Fragment analyzer (AATI) High Sensitivity DNA Fragment
701 Analysis Kit. Purification steps were performed using AMPure XP beads (Beckman Coulter).
702 In total, five flowcells were sequenced. For each flowcell, approximately 7 µg of DNA was
703 sheared at 20 kb using the megaruptor system (Diagenode, Liege, Belgium). A DNA damage
704 repair step was performed on 5 µg of DNA, followed by an END-repair and dA tail of double
705 stranded DNA fragments, and adapters were then ligated to the library. Libraries were loaded
706 on R9.4.1 flowcells and sequenced on a GridION DNA sequencer (Oxford Nanopore, Oxford,
707 UK) at 0.1 pmol for 48 h.

708 In total, 1,590,787 Nanopore reads corresponding to 17,576,820,346 nucleotides were
709 used in the assembly. Adapters were removed using Porechop (version 0.2.1,
710 <https://github.com/rrwick/Porechop>). The assembly was performed with Canu (version 1.7 [88])
711 using standard parameters, with genomeSize set to 1.1g to match theoretical expectations [89]
712 and maxMemory set to 240. Two rounds of polishing were performed with racon version 1.3.1
713 using standard parameters. For this step, Nanopore long reads were mapped to the assembly
714 with Minimap2 (version 2.5 [90]) using the map-ont parameter preset. Afterwards, four

715 additional rounds of polishing were performed with Pilon [91] using default parameters and the
716 Illumina short reads mapped to the assembly with BWA mem (version 0.7.17 [86]). Metrics for
717 the resulting assembly were calculated with the `assemblathon_stats.pl` script [92]. The
718 assembly's completeness was assessed with BUSCO [93] using the *Actinopterygii* gene set
719 (4,584 genes) and the default gene model for Augustus. The same analysis was performed on
720 the Elu_V3 reference genome (GenBank assembly accession: GCA_000721915.3) for
721 comparison.

722

723 **Sequencing of male and female pools**

724 DNA from 30 males and 30 females from the fish production unit of the fishing
725 federation of Ille-et-Vilaine (Pisciculture du Boulet, Feins, France) was extracted with a
726 NucleoSpin Kit for Tissue (Macherey-Nagel, Duren, Germany) following the manufacturer's
727 instructions. DNA concentration was quantified using a Qubit dsDNA HS Assay Kits
728 (Invitrogen, Carlsbad, CA) and a Qubit3 fluorometer (Invitrogen, Carlsbad, CA). DNA from
729 different samples was normalized to the same quantity before pooling for male and female
730 libraries separately. Libraries were constructed using a Truseq nano kit (Illumina, ref. FC-121-
731 4001) following the manufacturer's instructions. DNaseq shorts reads sequencing was
732 performed at the GeT-PlaGe core facility of INRA Toulouse, France
733 (<http://www.get.genotoul.fr>). Two DNA poolseq libraries were prepared using the Illumina
734 TruSeq Nano DNA HT Library Prep Kit (Illumina, San Diego, CA) following the
735 manufacturer's protocol. First, 200ng of DNA from each sample (male pool and female pool)
736 was briefly sonicated using a Bioruptor sonication device (Diagenode, Liege, Belgium), and
737 then end-repaired and size-selected on beads to retain fragments of size around 550 bp, and
738 these fragments were A-tailed and ligated to indexes and Illumina's adapter. Libraries were
739 checked with a Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, IA) and

740 quantified by qPCR using the Kapa Library Quantification Kit (Roche Diagnostics Corp,
741 Indianapolis, IN). Sequencing was performed on a NovaSeq S4 lane (Illumina, San Diego, CA)
742 using paired-end 2x150 nt mode with Illumina NovaSeq Reagent Kits following the
743 manufacturer's instruction. The run produced 129 millions of read pairs for the male pool
744 library and 136 millions of read pairs for the female pool library.

745

746 **Identification of sex-differentiated regions in the reference genome**

747 Reads from the male and female pools were aligned separately to the reference genome
748 (GenBank assembly accession: GCA_000721915.3) using BWA mem version 0.7.17 [86] with
749 default parameters. Each resulting BAM file was sorted and PCR duplicates were removed
750 using Picard tools version 2.18.2 (<http://broadinstitute.github.io/picard>) with default
751 parameters. Then, a pileup file combining both BAM files was created using samtools mpileup
752 version 1.8 [94] with per-base alignment quality disabled (-B). A sync file containing the
753 nucleotide composition in each pool for each position in the reference was generated from the
754 pileup file using popoolation mpileup2sync version 1.201 [95] with a min quality of 20 (--min-
755 qual 20).

756 An in-house C++ software was developed to identify sex-specific SNPs, defined as
757 positions heterozygous in one sex while homozygous in the other sex, compute per base
758 between-sex F_{ST} , and coverage for each sex from the output of popoolation mpileup2sync
759 (PSASS v1.0.0, doi: 10.5281/zenodo.2538594). PSASS outputs 1) all positions with sex-
760 specific SNPs or high F_{ST} , 2) the number of such positions in a sliding window over the genome,
761 3) average absolute and relative coverage for each sex in a sliding window over the genome,
762 and 4) number of sex-specific SNPs as well as coverage for each sex for all genes and CDS in
763 a user-supplied GFF file.

764 We used PSASS to identify non-overlapping 50 kb windows enriched in sex-specific
765 SNPs in *E. lucius*, using the following parameters: minimum depth 10 (--min-depth 10), allele
766 frequency for a heterozygous locus 0.5 ± 0.1 (--freq-het 0.5, --range-het 0.1), allele frequency
767 for a homozygous locus 1 (--freq-het 1, --range-het 0), --window-size 50000, and --output-
768 resolution 50000. For scaffold1067, the number of sex-specific SNPs and coverage for each sex
769 were similarly computed in 2,500 bp windows, only changing the parameters --window-size to
770 2500 and --output-resolution to 2500.

771

772 **Identification of Y specific sequences in the Nanopore assembly**

773 A sync file containing the nucleotide composition in each pool for each position in the
774 reference was generated as described in the previous section, using the Nanopore assembly
775 (NCBI accession number SDAW000000000) to align the reads. This time, because we were
776 comparing reads coverage levels, the BAM files were filtered with samtools version 1.8 [94] to
777 only retain reads with a properly mapped pair and a mapping quality higher than 30 to reduce
778 the impact of false positive mapped reads. The resulting sync file was used as input for PSASS
779 to compute coverage for each sex in 1 kb non-overlapping windows along the genome using
780 the following parameters: --min-depth 10, --window-size 1000, and --output-resolution 1000.
781 Results from this analysis were filtered in R (version 3.5.1 [83]) to identify 1 kb regions with
782 mean relative coverage between 0.3 and 0.7 in males, and mean absolute coverage lower than
783 1 in females.

784 To identify protein coding sequences, we performed alignments between the Y-specific
785 sequence and the teleostei (taxid:32443) non-redundant protein database using blastx
786 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, version 2.8.1 [96]) with the parameters “Max target

787 sequences” set to 50 and “Max matches in a query range” set to 1. Regions matching potential
788 homologs with an e-value < 1E-50 were considered as protein coding sequences.

789 To determine repeat content of the Y-specific sequence, RepeatMasker (version open
790 4.0.3 [97]) was run on these Y-specific sequences with NCBI/RMBLAST (version 2.2.27+)
791 against the Master RepeatMasker Database (Complete Database: 20130422). The same analysis
792 was also performed on the entire Nanopore assembly.

793

794

795 **Supporting information**

796

797 Supplementary file 1, Figure and Text: Figure S1 to Figure S6 and additional results.

798 Supplementary file 2, Tables: Table S1 to Table S8

799

800 **Data availability**

801 Sequencing data and assembly for the *Esox lucius* genome can be found under NCBI Bioproject
802 PRJNA514887. Sequencing data for identifying and charactering the sex locus, including RAD-
803 seq, pool-seq reads, can be found under the NCBI Bioproject PRJNA514888.

804

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806

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819

820 **Author contribution**

821

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822

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1091

1092 **Figure captions**

1093

1094 **Figure 1: Sequence identity between *amha* and *amhby* and amplification in males and**

1095 **females. A) PCR amplification of *amha* (500 bp band) and *amhby* (1500 bp band) in male (n=4)**

1096 and female (n=4) genomic DNA samples from *E. lucius*. Each lane corresponds to one
1097 individual. The number of tested animals of each sex positive for *amha* and *amhby* is indicated
1098 in the table on the right. B) Sequence identify of global pairwise alignment between *amha* and
1099 *amhby* genomic sequences with *amhby* sequence as the reference. C) Schematic representation
1100 of *amha* and *amhby* gene structure in *E. lucius* from the start to the stop codon. Exon1-Exon7
1101 are represented by green boxes with shared percentage identity indicated and introns are
1102 represented by white segment. The red segment in intron 1 represents the *amhby* specific
1103 insertion. TGF- β domains are indicated with diagonal lines. D) Global pairwise alignment
1104 between 5 kb upstream region of *amha* and *amhby* with *amhby* sequence as the reference. The
1105 start codon of each gene is positioned at 0 bp and the number on the y-axis indicates distance
1106 from the start codon upstream to the coding sequence.

1107

1108 **Figure 2: Regions enriched with male-specific SNPs (MMS) in the genome of *E. lucius***
1109 **based on pooled sequencing analysis. A:** Number of MSS in a 50 kb non-overlapping window
1110 is plotted for each linkage group (LG) and all unplaced scaffolds in the reference genome
1111 (GenBank assembly accession: GCA_000721915.3). The two highest peaks of MMS density
1112 are located on unplaced scaffold1067. **B:** Number of male and female-specific SNPs in 50 kb
1113 non-overlapping windows is plotted along scaffold1067. The male data are represented in blue
1114 and female in red. The ~ 40 kb region located between ~ 143 kb to ~ 184 kb (11% of the
1115 scaffold) contained 211 MMS, showing the strongest differentiation between males and
1116 females.

1117

1118 **Figure 3: A: Rad-Seq marker association with sex across the 25 linkage groups of *E. lucius***
1119 **genome.** The \log_{10} of p-value from the association test between each marker and sex phenotype
1120 is mapped against the linkage group (LG) they belong. Linkage group 24 showed a

1121 concentration of markers significantly associated with sex phenotype. The dotted horizontal
1122 lines are indications for genome-wide significance level from the association test, with the
1123 lower dotted line indicating p-value of 1×10^{-5} and higher dotted line indicating p-value of 1×10^{-8} . **B**: Differentiation of polymorphic markers demonstrated by F_{ST} between males and females
1125 and their association with sex phenotypes on LG24. Between male and female F_{ST} value is
1126 plotted against the mapped position of each marker on the upper panel. Average F_{ST} value of
1127 markers on LG24 is presented as the dotted line (higher panel). **C**: The \log_{10} of p-value from
1128 the association test of each marker with sex phenotype is mapped against the mapped position
1129 on the lower panel. Significant threshold of p-value for this test is presented as the dotted line
1130 (lower panel). Both between-sex F_{ST} and marker association with sex indicate that the sex locus
1131 is near the end of LG24.

1132

1133 **Figure 4: Temporal and spatial expression of *amha* and *amhby* mRNA in male and female**
1134 **developing gonads. A-B**: Boxplots showing the first quantile, median, and the third quantile
1135 of the temporal expression of *amha* (A) and *amhby* (B) during early development of *E. lucius*
1136 measured by qPCR. Outliers are displayed as a dot. The mRNA expression of *amha* and *amhby*
1137 were measured at 54, 75, 100, and 125 days post fertilization (dpf) in male and female samples
1138 of *E. lucius* and the \log_{10} of their relative expression is presented on the graph. Significant P-
1139 values (<0.05) for Wilcoxon signed rank test between male and female expression at each time
1140 point are indicated by * and 'ns' indicates 'non-significant P-values. Statistical tests were not
1141 performed on *amhby* expression between sexes because of the complete absence of *amhby*
1142 expression in females. **C-F**: *In situ* hybridization on histological sections revealed the
1143 localization of *amha* in both 80 dpf female (C) and male (D) gonads, with a stronger expression
1144 in male gonads. A high *amhby* mRNA expression is detected in the male gonad (F), with no

1145 signal detected in female gonad (E). The red scale bars denote 20 μm and the dashed lines
1146 outlines the gonadal sections.

1147

1148 **Figure 5: Gonadal phenotypes of *E. lucius* in *amhby* knockout (KO) and additive**
1149 **transgenesis experiments.** Gonadal histology of a representative control XX female (A),
1150 control XY male (B), an *amhby* KO XY male (C), and an *amhby* transgenic XX mutant female
1151 (D). The *amhby* KO XY male (C) developed ovaries with oocytes and an ovary cavity,
1152 indistinguishable from the ovary of the control females (A). The *amhby* transgenic XX mutant
1153 female (D) developed testis with clusters of spermatozooids and testicular lobules identical to
1154 that of the control males (B). **PVO**: previtellogenic oocytes; **SP**: spermatozooids; **L**: testicular
1155 lobules; **T**: testis; and **O**: ovary.

1156

1157 **Figure 6: Evolution of Amh in teleosts. A:** Synteny map of genomic regions around *amh*
1158 genes (highlighted by the red box) in teleosts. Orthologs of each gene are shown in the same
1159 color and the direction of the arrow indicates the gene orientation. Ortholog names are listed
1160 below and the genomic location of the orthologs are listed on the right side. For *Esox lucius*,
1161 *amha*, which is located on LG08, is used in this analysis. **B:** Phylogenetic reconstruction of
1162 teleost Amh protein orthologs. The phylogenetic tree was constructed with the maximum
1163 likelihood method (bootstrap=1000). Numbers at tree nodes are bootstrap values. Spotted gar
1164 (*Lepisosteus oculatus*) Amh was used as an outgroup. Branches with Amh duplication are
1165 indicated by the red pinwheels.

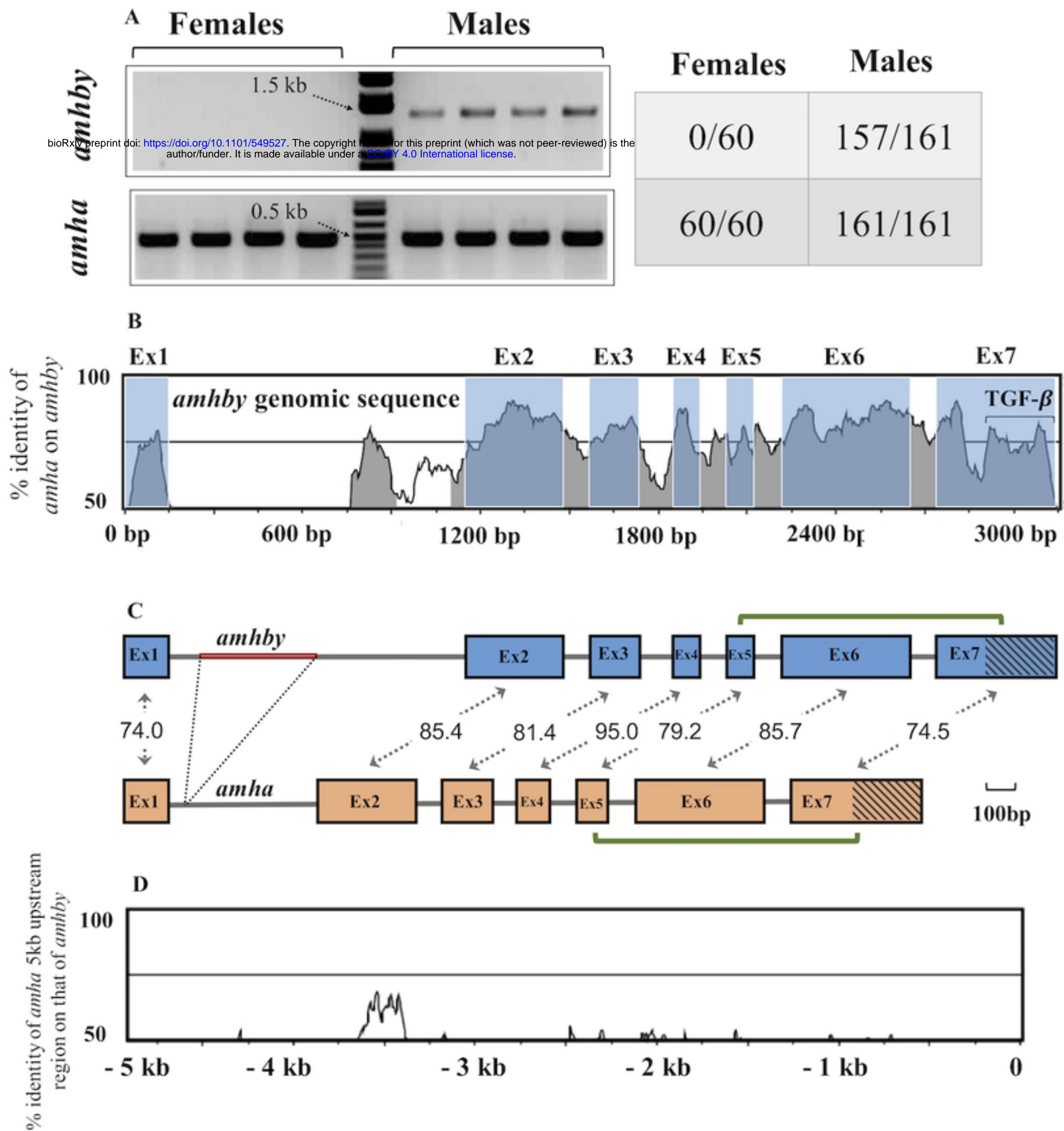


Figure 1

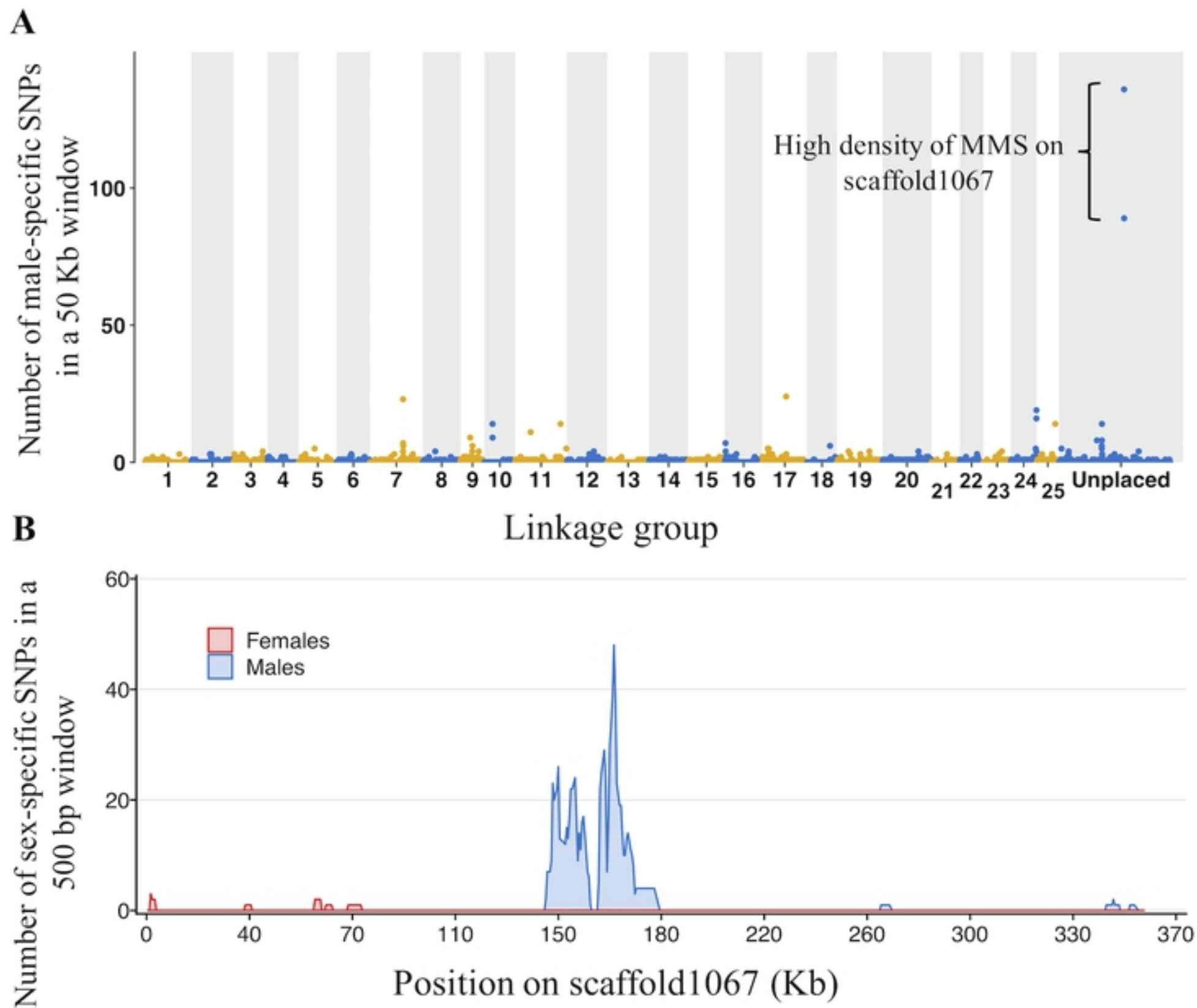


Figure 2

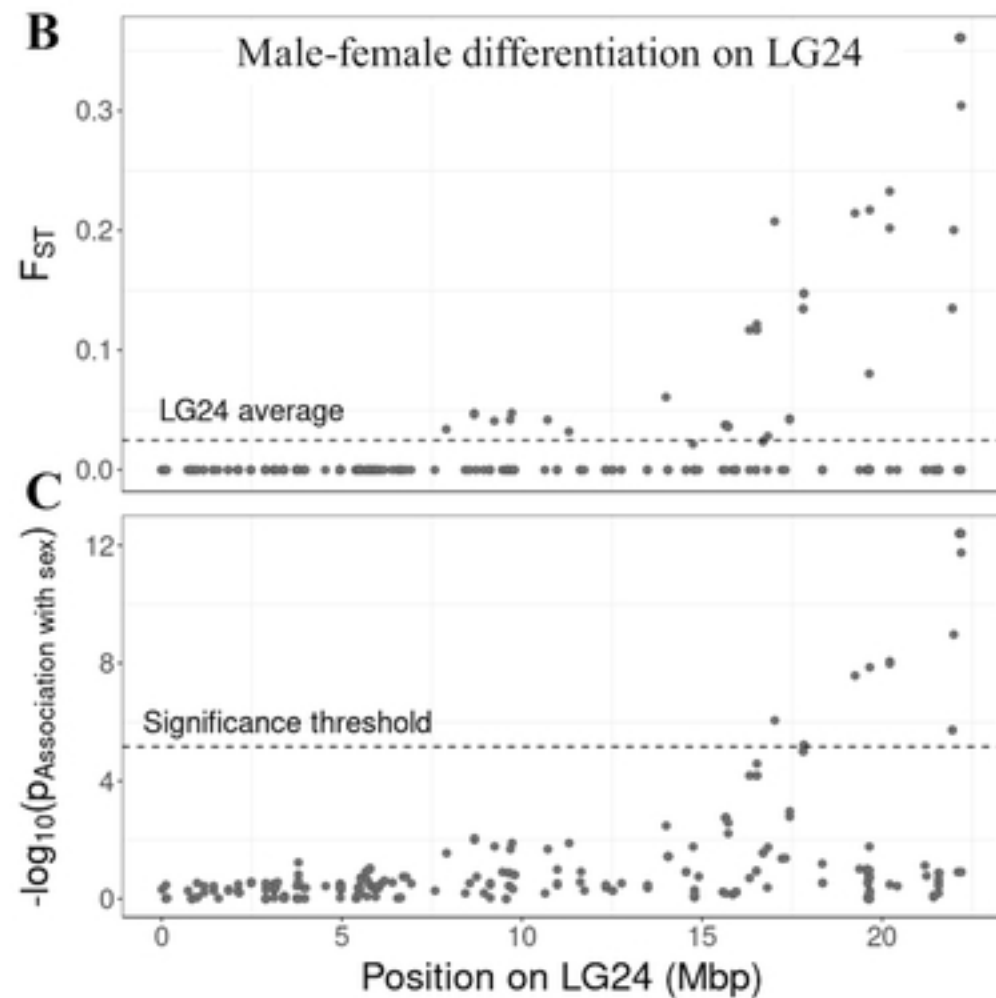
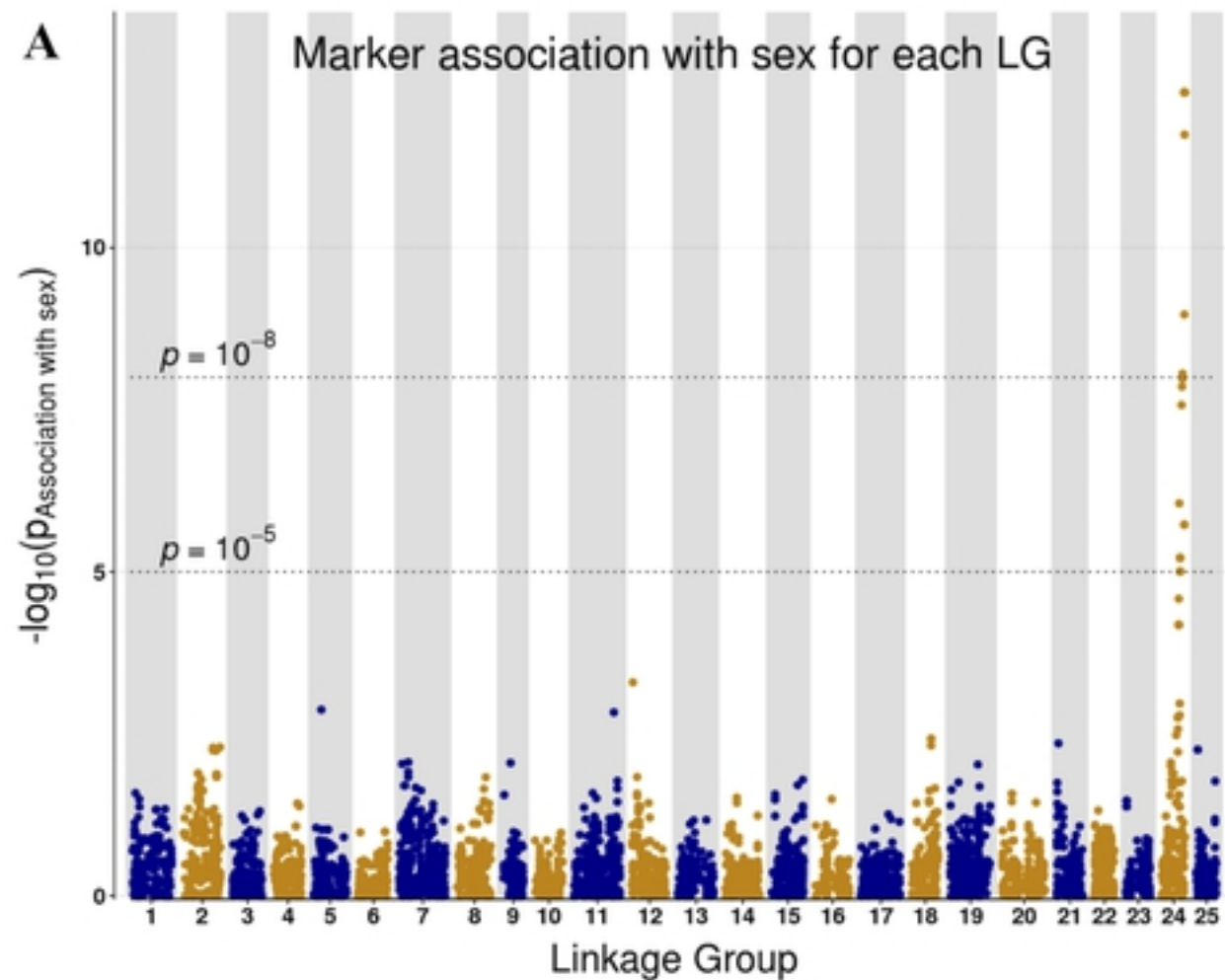


Figure 3

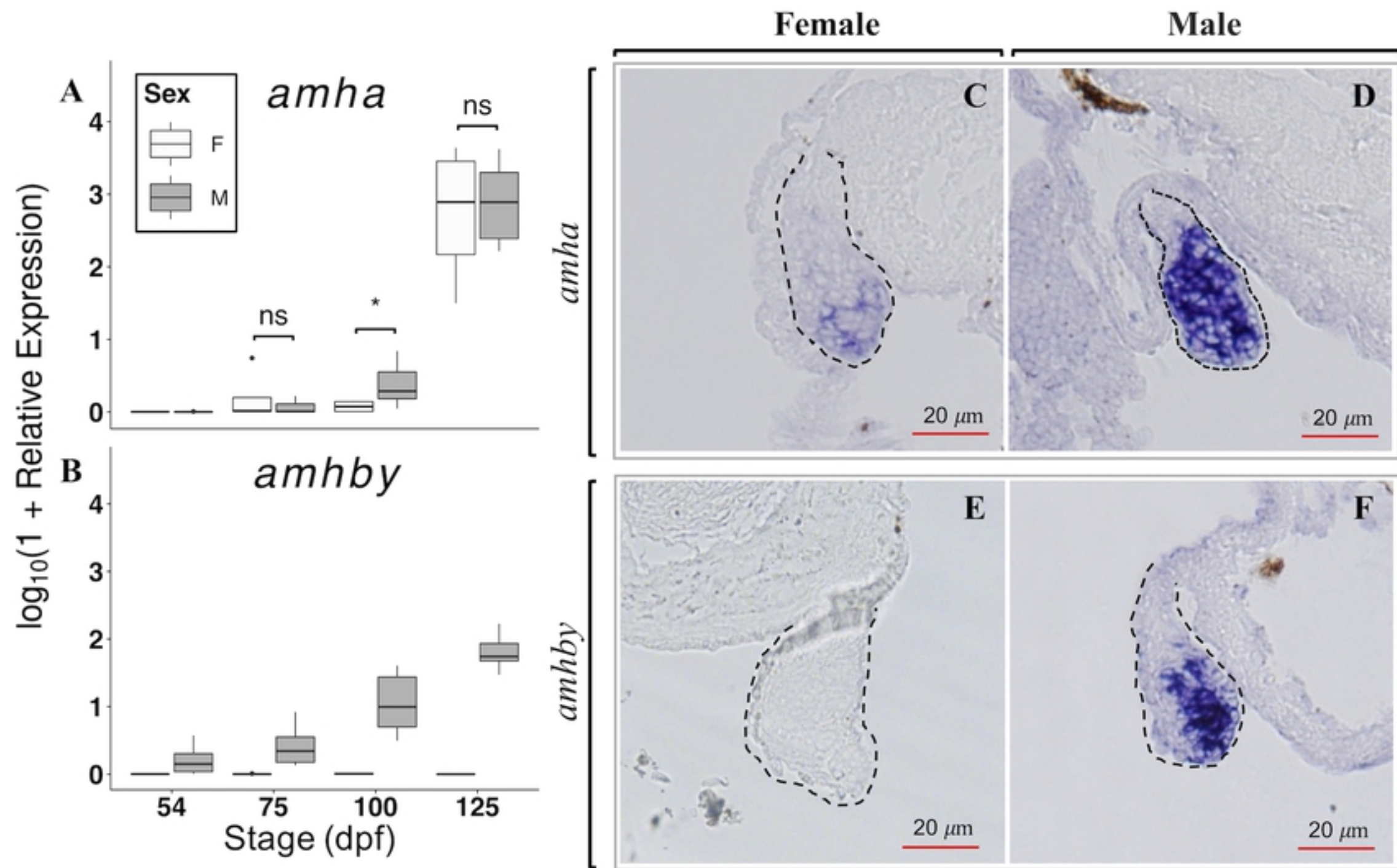
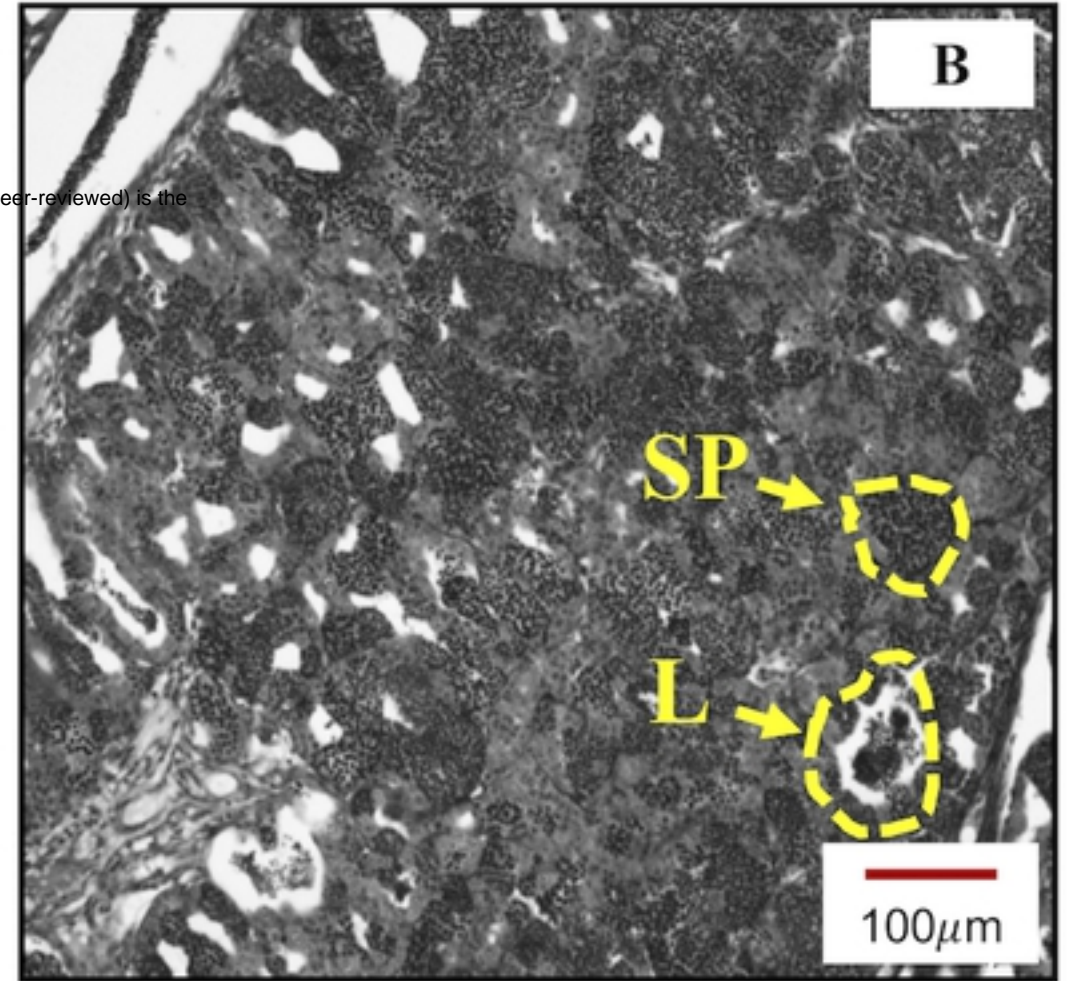
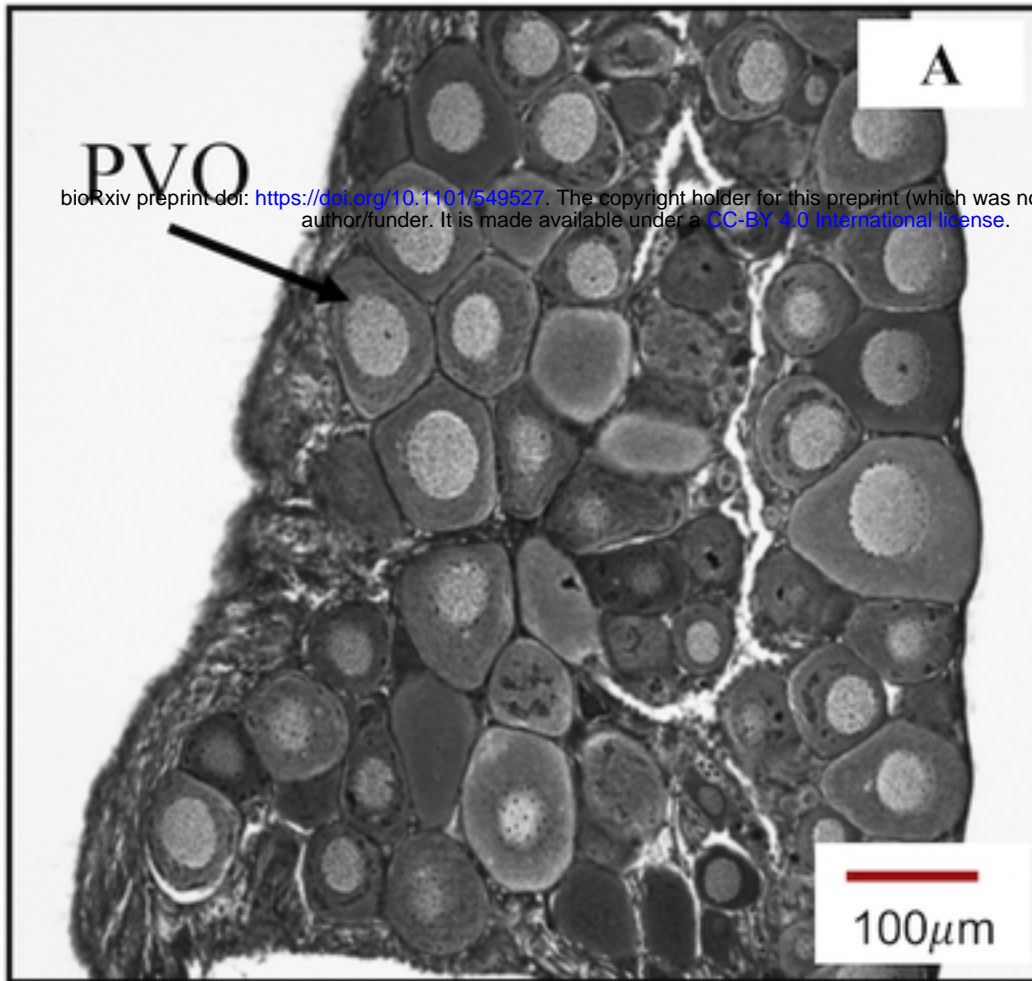


Figure 4

XX WT

XY WT



XY *amhby* KO

XX *amhby* transgenic

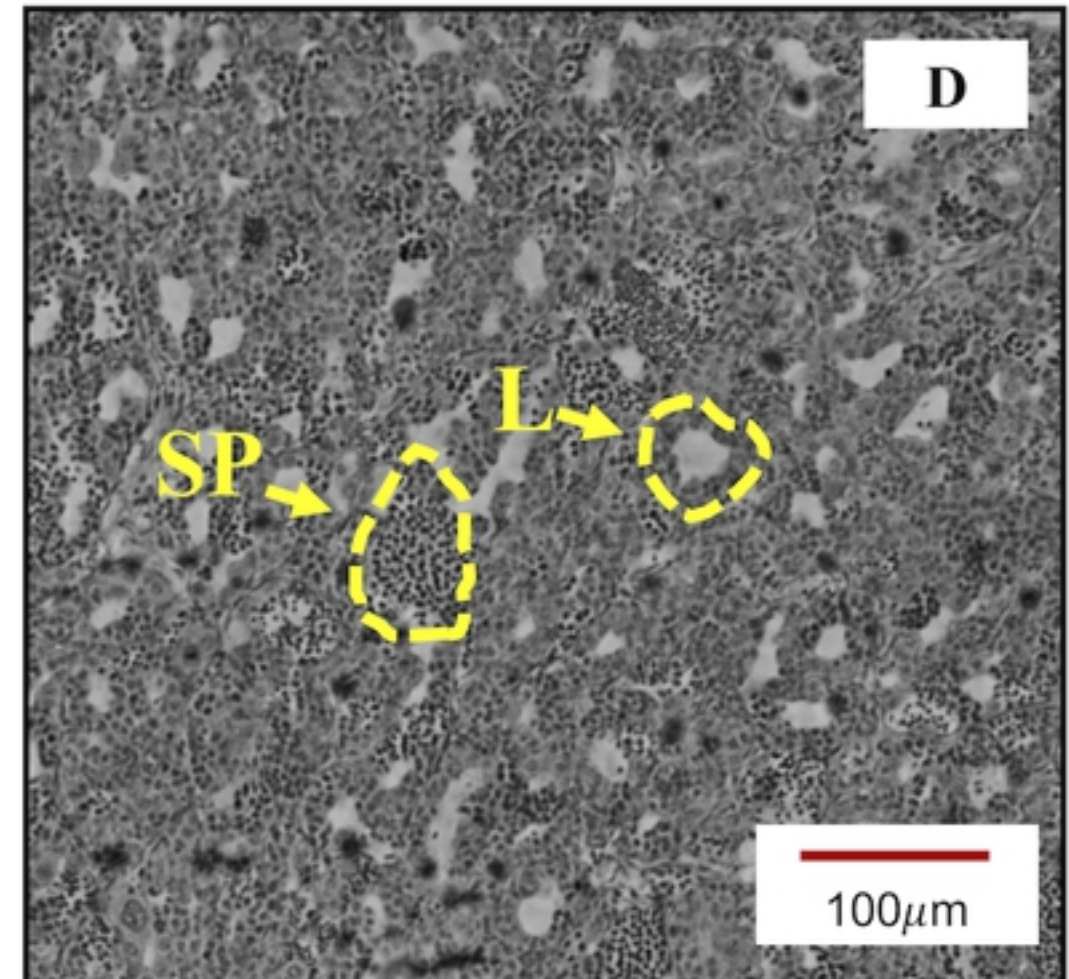
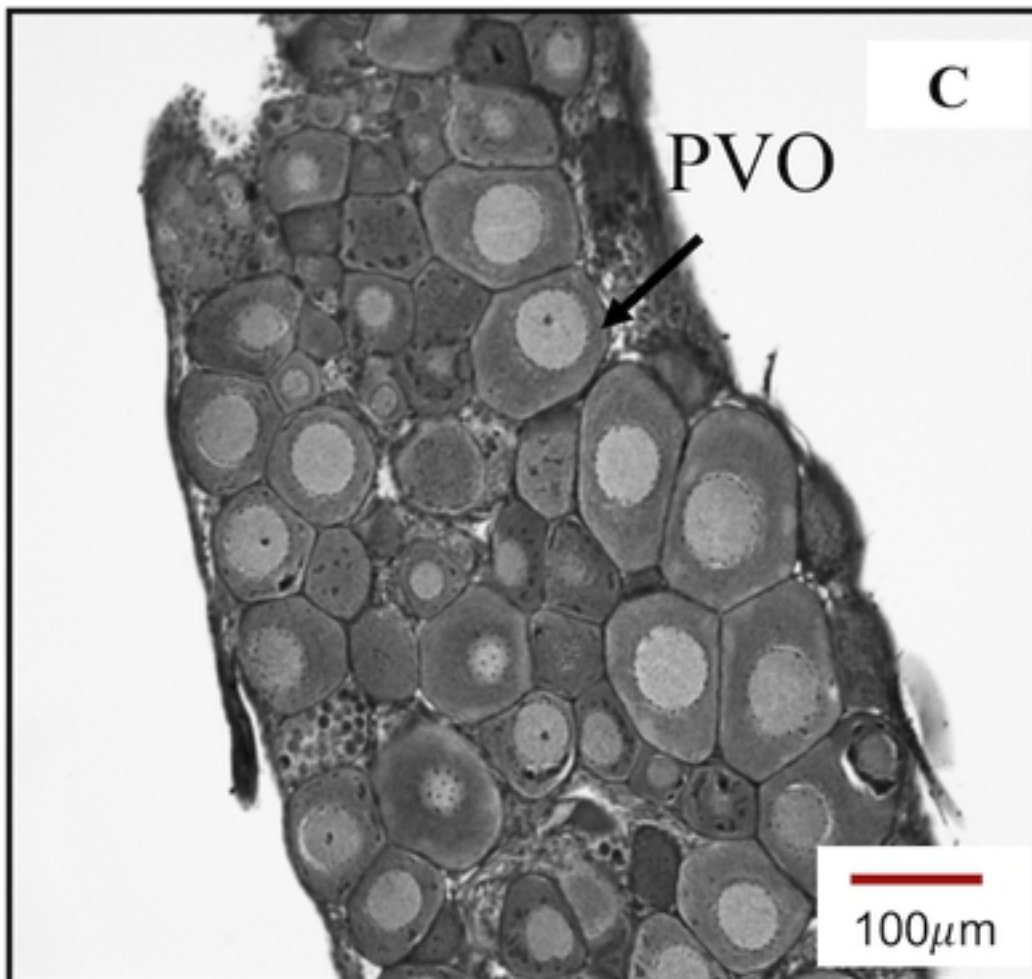


Figure 5

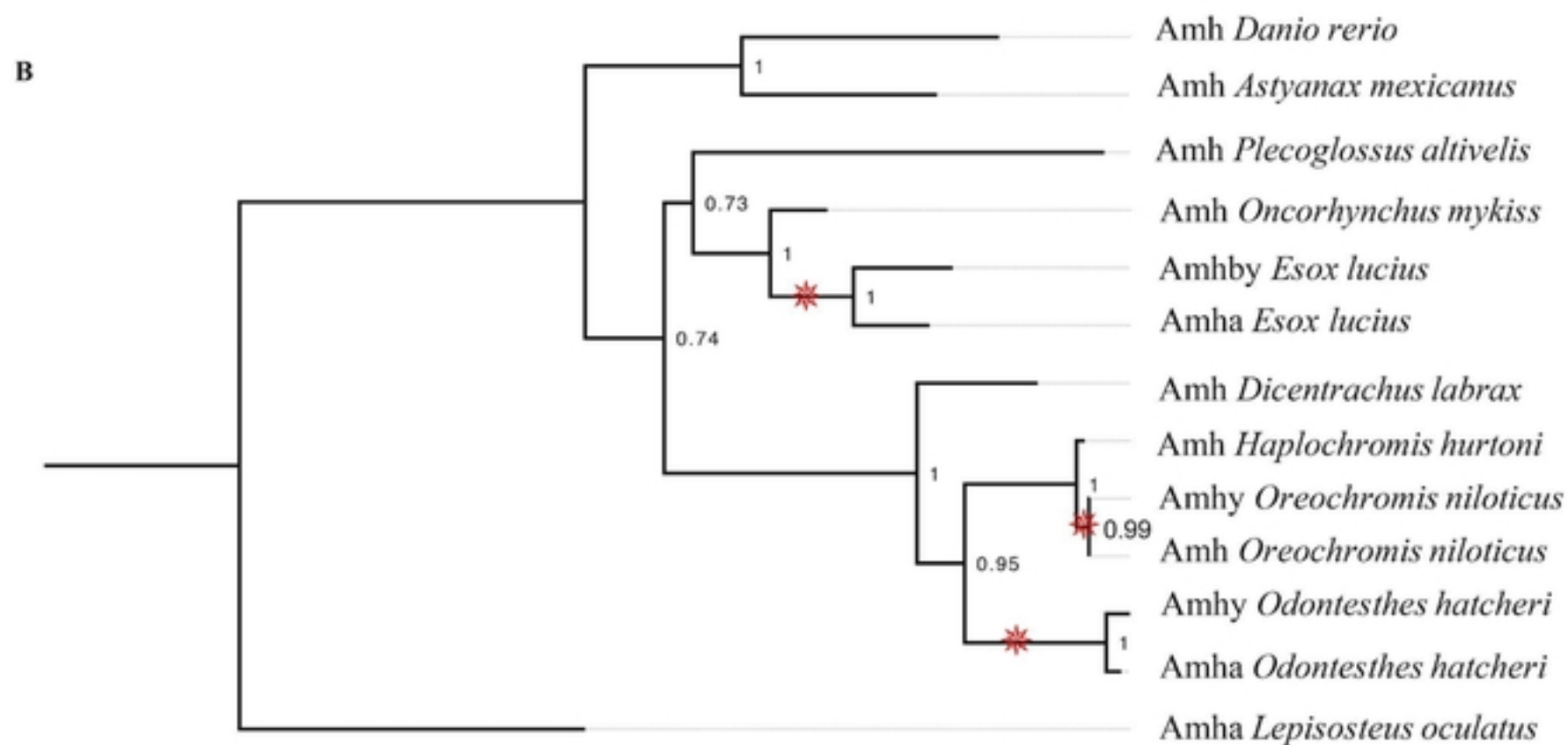
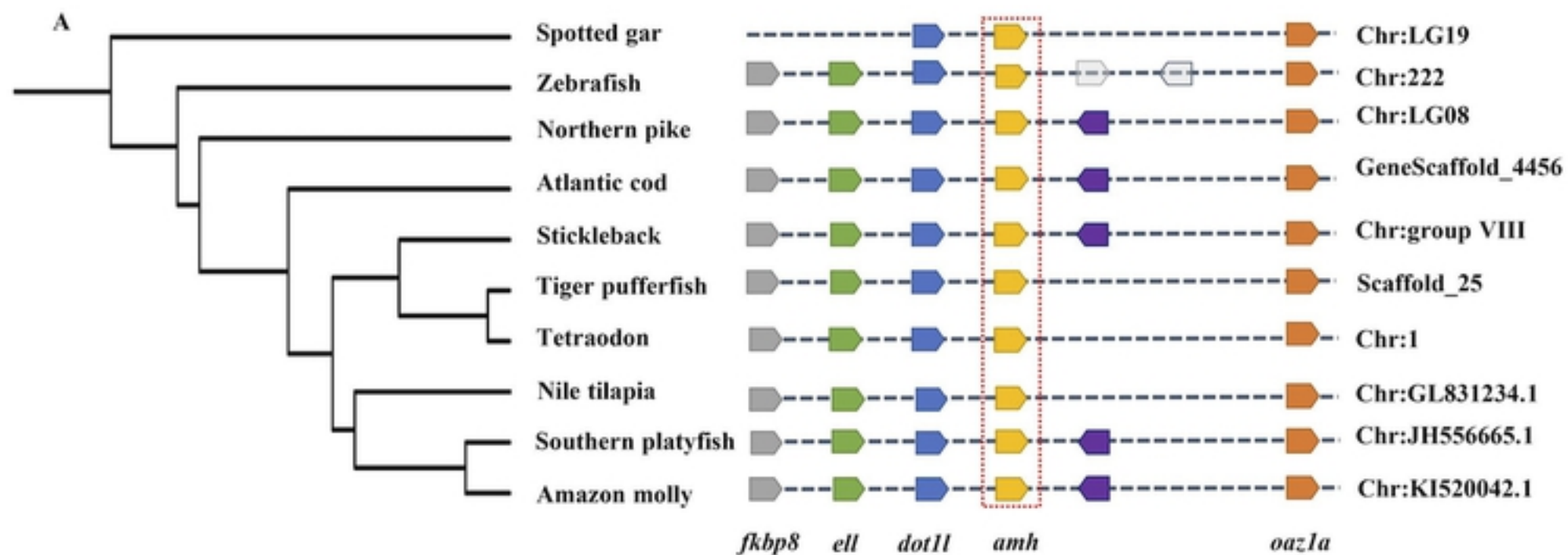


Figure 6