fishes

MDPI

Article

Development of an Accurate Polymerase Chain Reaction (PCR) Assay for Genetic Sex Identification in Lumpfish (*Cyclopterus lumpus*) Based on Male-Specific Anti-Mullerian Hormone (*amh*) Gene

Kasidis Chaiyasut [1], Paul Merviel [1], Peter Palma [1], Johanna Perschthaler [1], Eduardo Jimenez-Fernandez [2], Andrew Davie [3] and Alejandro P. Gutierrez [1,*,†]

[1] Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, UK; kasidis.chaiyasut@stir.ac.uk (K.C.)
[2] Otter Ferry Seafish Ltd., Tighnabruaich PA21 2DH, UK
[3] Aquascot Ltd., Fyrish Way, Alness IV17 0PJ, UK
* Correspondence: agutierrez@aquatechcenter.com
† Current address: Center for Aquaculture Technologies, San Diego, CA 92121, USA.

Abstract: The production of lumpfish (*Cyclopterus lumpus*) has become crucial in controlling sea lice levels in salmonid aquaculture. To improve their breeding, there is a need for early sex identification. The genomic region containing the *anti-Müllerian hormone* (*amh*) gene was suggested as the candidate master sex-determining gene in lumpfish. However, the genome of lumpfish contains three copies of *amh* with ambiguous sex specificity, designated *amh1*, *amh2*, and *amh3*. The study aims to analyse the male-specific region between these *amh* paralogues for its application as a sex marker. In this study, we utilised polymerase chain reaction (PCR)-based assays to identify the male-specific *amh* markers in lumpfish and estimate the length of the male-specific region in the lumpfish genome. Our results indicate that a specific genomic region of approximately 27 kilobases (kb), encompassing *amh1* and *amh2* genes, exhibits male specificity, whereas *amh3* is present in both sexes. The developed PCR-based genetic sex identification assays targeting *amh1* and *amh2* exhibited over 97% concordance with phenotypic records. Further experiments in other members of the Cyclopteridae: *Aptocyclus ventricosus*, *Eumicrotremus taranetzi*, and *E. asperrimus* revealed male-specific *amh* genome region only in *A. ventricosus*. Phylogenetic analyses using the available Cyclopteridae *amh* sequences suggest that male-specific *amh* arose early in the Cyclopteridae lineage. Our findings, along with the development of the PCR test, hold great promise for the field of lumpfish aquaculture and will also contribute significantly to future investigations aiming to enhance our understanding of the sex-determination system and the evolution of sex chromosomes in teleostean fish.

Keywords: *anti-Müllerian hormone*; *Cyclopterus lumpus*; male-specific marker; genetic sex identification

Key Contribution: This study developed molecular assays for sex identification in lumpfish based on the PCR amplification of the *amh* genes. These assays will allow early sex identification and, furthermore, facilitate the development of breeding programs for lumpfish.

Citation: Chaiyasut, K.; Merviel, P.; Palma, P.; Perschthaler, J.; Jimenez-Fernandez, E.; Davie, A.; Gutierrez, A.P. Development of an Accurate Polymerase Chain Reaction (PCR) Assay for Genetic Sex Identification in Lumpfish (*Cyclopterus lumpus*) Based on Male-Specific Anti-Mullerian Hormone (*amh*) Gene. *Fishes* **2023**, *8*, 327. https://doi.org/10.3390/fishes8060327

Academic Editor: Albert K. D. Imsland

Received: 16 May 2023
Revised: 14 June 2023
Accepted: 15 June 2023
Published: 19 June 2023

1. Introduction

One of the most damaging diseases to salmonid aquaculture is the infestation of the ectoparasitic copepods collectively called sea lice, which have been estimated to have cost the salmonid industry £700 million globally in 2015, and the impact is likely to keep increasing in the future [1]. The utilisation of lumpfish (*Cyclopterus lumpus*) as cleaner fish has become increasingly popular due to its effectiveness in providing long-term biological control and effectively managing lice abundance in sea cages [2]. This approach minimises the necessity for other delousing methods, such as chemotherapeutics, mechanical treatments,

and thermal treatments, which may compromise the welfare of the fish [3–5], have adverse effects on the ecosystem [6], and negatively influence public perception of the aquaculture industry [2]. Therefore, the salmonid aquaculture industry is utilising lumpfish as an environmentally friendly alternative for managing sea lice infestations. Apart from this, the roe of lumpfish is also highly valued as a delicacy in numerous countries, including France, Germany, and the United States of America [7].

While cleaner fish utilisation presents benefits, it is important to acknowledge that the utilisation of lumpfish in aquaculture, which currently heavily relies on capturing wild stocks [8], has the potential to negatively impact the species' wild populations. However, there is potential for a sustainable alternative to fishing as the lumpfish life cycle has been successfully closed, allowing for its captive breeding [9]. Unfortunately, the lack of pronounced sexual dimorphism in lumpfish before maturation [10] poses a significant challenge in managing the sex ratio of the breeding stock, which is crucial for the advancement of the species aquaculture. The ability to accurately identify the sex at an early stage would greatly facilitate the selection of broodstock and has the potential to minimise resource consumption for stock maintenance.

Sex determination techniques currently employed in other aquaculture species include ultrasound [11,12], plasma-based assay [11,13], gonad inspection [14,15], and PCR-based sex identification targeting conserved sex-specific molecular markers such as the female-specific region in various sturgeon species (Acipenseridae) [16,17] and the male-specific region in salmonids [18]. Nevertheless, the predominant method for sex identification in lumpfish remains gonad inspection [14]. PCR-based methods, on the other hand, offer an alternative approach to determining the sex at any life stage, unlike other methods that necessitate gonadal maturation [11,15], which typically occurs in lumpfish only after 3–5 years [19]. Given that lumpfish follow a XX/XY genetic sex-determination system [20], the utilisation of DNA markers located within the sex-determining chromosome regions possess the potential to accurately determine the sex of the organism at any life stage [21].

Recent genome-wide association studies (GWAS), which investigated markers related to phenotypical sex in lumpfish, revealed that chromosome 13 likely serves as the sex chromosome housing a male-specific region in the species [20,22]. Comparative sequence analysis of this region with master sex-determining (MSD) genes in other teleosts has suggested that the *anti-Müllerian hormone* (*amh*) could potentially serve as the putative MSD gene in lumpfish [20]. The available male lumpfish genome assemblies reveal the presence of three paralogues of *amh*, namely *amh1*, *amh2*, and *amh3* [20]. Among them, *amh1* and *amh2* exhibit a reversed orientation, being positioned adjacently to each other within the genome as inverted tandem repeats [20]. Studies in other species have already utilised male-specific *amh* as a sex-specific molecular marker for sex identification [23–25]. Thus, the region containing the male-specific *amh* in the lumpfish genome is expected to also allow their sex identification.

The objective of this study was to identify molecular markers located within the male-specific region of the lumpfish genome that can be used for the development of a polymerase chain reaction (PCR)-based test for accurate sex identification in the species. To accomplish this objective, our study aimed to (1) characterise the sex specificity of the three *amh* genes in the lumpfish genome, (2) determine the extent of the male-specific region within the species genome, and (3) investigate the presence of *amh* in related species within the Cyclopteridae family. Our study provides not only male-specific markers, which can assist sustainable lumpfish production, but also represents valuable insights into the mechanism and evolution of lumpfish's sex-determination system.

2. Materials and Methods

2.1. Samples

A total of 143 archived samples of lumpfish were utilised in this study, comprised of 67 males and 76 females. A total of 13 males and 34 females were sampled from the wild population of the British Isles. A total of 22 males and 17 females were sampled from

the wild population of Iceland. A total of 32 males and 25 females were sampled from Otter Ferry Seafish (OFS) Ltd., Tighnabruaich, UK, which was an offspring of four families derived from wild Norwegian parents. The phenotypic sex of the samples was determined through gonad inspection, and fin-clipping was carried out in 2017 to collect DNA samples for subsequent molecular analysis. The detailed records and the identification code of the samples are available in Table S1.

Tissue samples of two male and one female *Aptocyclus ventricosus*, all from the Sea of Japan, one male and female *Eumicrotremus taranetzi*, both from the Sea of Okhotsk, and one male and female *E. asperrimus*, from the Sea of Japan and the Sea of Okhotsk, respectively, were also utilised for interspecies phylogenetic analyses of *amh* gene sequence within the Cyclopteridae family.

2.2. DNA Extraction

Genomic DNA was extracted from the samples using the SSTNE extraction protocol [26]. The purity (260/280 and 260/230 ratios) and concentration of the extracted DNA were then measured using Nanodrop ND-1000 Spectrophotometer with ND-1000 version 3.8.1 software (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples with a 260/280 ratio of approximately 1.8 and the 260/230 ratio between 2.0 and 2.2 were then diluted to 50 ng μL^{-1} for downstream applications. The DNA samples of *A. ventricosus*, *E. taranetzi*, and *E. asperrimus* were diluted to 20 ng μL^{-1} instead due to low concentration.

2.3. Primer Design

The putative genomic DNA sequences of the three lumpfish *amh* genes available in the Ensembl genome database project [27], *amh1* (Ensembl accession no. ENSCLMG00005014163), *amh2* (Ensembl accession no. ENSCLMG00005014165), and *amh3* (Ensembl accession no. ENSCLMG00005016820), were used for designing pairs of *amh*-specific PCR markers. As a result, four primer sets were designed that bind to the three identified *amh* paralogues (Table 1). Primer set AMH1_E3I6 was designed to bind to exon 3 (forward) and intron 6 (reverse) of *amh1* to amplify 1410 bp amplicons. AMH2_I6E4 binds to intron 6 (forward) and exon 4 (reverse) of *amh2* to amplify 1804 bp amplicons. AMH3_E3I6 binds to exon 3 (forward) and intron 6 (reverse) of *amh3* to amplify 1136 bp amplicons. The AMH1+3_E6I6 binds to the shared regions of exon 6 (forward) and intron 6 (reverse) of *amh1* and *amh3*, which contain *amh1*-specific 91 bp deletion, to amplify 481 bp and 566 bp amplicons, respectively. Visual presentation of the primer binding sites is available in Figure S1.

Table 1. Sequence of the primer sets utilised to characterise the sex specificity of the three *anti-Müllerian hormone* (*amh*) paralogues in lumpfish (*Cyclopterus lumpus*).

Primer Set	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
AMH1_E3I6	TTTGACCTCCCACCGTTTAC	TATGCCCTCGTGTTGATTCC
AMH2_I6E4	GAGCCGGATTACTGACAGAC	GAAAAGCACAAGAGGGCAAC
AMH3_E3I6	TCGTGTTGACCTTTGACCTC	GGGATGTGGACTAAAGGAGC
AMH1+3_E6I6	GTCATACGGGAGGAGCAAGT	CTCGTTCCCACCACAGATCT

Moreover, additional primer sets were designed to estimate the length of the male-specific region (Table 2). For this purpose, a genomic sequence region encompassing 30 kilobases (kb) upstream and downstream of the *amh* paralogues was extracted from the publicly available lumpfish genome assembly (GCA_009769545.1). Primer sets 4K_Up, 6K_Up, and 10K_Up were designed to bind to approximately 4 kb, 6 kb, and 10 kb upstream of the male-specific *amh* region to amplify 683 bp, 540 bp, and 653 bp amplicons, respectively. Primer set 4K_Down, 14K_Down, and 16K_Down was designed to bind to approximately the 4 kb, 14 kb, and 16 kb downstream of the male-specific *amh* region to amplify 733 bp, 704 bp, and 670 bp amplicons, respectively.

Table 2. Sequence of the primer sets utilised for estimating the coverage of the male-specific region in lumpfish genome.

Primer Set	Forward Primer (5′→3′)	Reverse Primer (5′→3′)
4K_Up	ACCGGAAGAGTGAGCTTTGA	GTTAAGGGCCCCAAAACAGG
6K_Up	TTTAAGCGGCGGAAAGATCG	CTGAGTTTGGAAGGCTGCTC
10K_Up	ACCCTGAAGAAGCACCTCTC	CATCTGTATGGTTCGCGCAA
4K_Down	TGCGCCAGAAGTTTTCCAAA	GGGATCTCTGTCTACACATGC
14K_Down	CAACAGGTCGATGCAGATGG	GCAGCAGAAAACGTCAGGAA
16K_Down	AAACAACGACAGGTCAGTGC	CAGCAACACACTCCAAACGA

All the primers used in this study were designed using the online software Primer3 [28] and manufactured by Integrated DNA Technologies, Inc. (IDT, Coralville, IO, USA).

2.4. Determination of Sex Specificity of Markers

PCR amplifications were performed using four designed pairs of *amh*-specific markers (Table 1) on 12 randomly selected male and female lumpfish to characterise the sex specificity of each *amh* paralogue. PCR reactions were performed with the standard MyTaq mix protocol from the manufacturer but in a final volume of 12.5 µL mixture per reaction (one-fourth of the recommended reaction volume), consisting of 6.25 µL of 2×MyTaq™ HS Mix (Meridian Life Science, Memphis, TN, USA), 0.25 µL of the forward primer (10 µM), 0.25 µL of the reverse primer (10 µM), 4.75 µL of nuclease-free water, and 1 µL template DNA (50 ng μL^{-1}). In addition, blanks were also made with the same procedure, excluding the DNA, to serve as the negative control.

The PCR amplification condition was based on the PCR cycling conditions suggested by the manufacturer with annealing temperature and extension time specific to the primer sets. Accordingly, for the primer sets AMH1_E3I6, AMH2_I6E4, and AMH3_E3I6, the cycle was 1 min at 95 °C, followed by 28 cycles of 15 s at 95 °C, 15 s at 57 °C, and then 1 min at 72 °C, and a final extension of 5 min at 72 °C. The PCR amplification condition for the primer set AMH1+3_E6I6 was 1 min at 95 °C, followed by 28 cycles of 15 s at 95 °C, 15 s at 57 °C, and then 20 s at 72 °C, and a final extension of 5 min at 72 °C.

PCR products and GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were loaded into a 2.0% agarose gel (Agarose, Molecular Grade (Meridian Life Science, Memphis, Tennessee, USA); 0.5× Tris-acetate-EDTA (TAE) buffer; and 100 ng mL^{-1} ethidium bromide), submerged in 0.5× TAE buffer, and applied with 90 V for 40 min. The gel images were visualised by InGenius 3 Manual Gel Documentation System with the GeneSys software v.1.8.7 (Syngene, Cambridge, UK).

To further confirm the reliability of the developed markers, primer sets that provided male-specific amplification results were selected and used to test the remaining lumpfish samples. Chi-square tests were employed to determine the significant association between the phenotypic sex and the genotype displayed by each primer set. Chi-square tests were performed in R version 4.1.3 [29], using RStudio version 2022.02.1+461 [30].

PCR amplifications were also performed for the *A. ventricosus*, *E. taranetzi*, and *E. asperrimus* samples using the primer set AMH1+3_E6I6. The PCR reaction mixtures were prepared in a total volume of 12.50 µL per reaction, following the modified manufacturer's instruction to include dimethyl sulfoxide (DMSO) to improve amplification, comprised of 6.250 µL of Q5® Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA, USA), 0.375 µL of DMSO, 0.625 µL of the forward primer (10 µM), 0.625 µL of the reverse primer (10 µM), 3.625 µL of nuclease-free water, and 1 µL template DNA (20 ng μL^{-1}). The PCR amplification condition was 30 s at 98 °C, followed by 32 cycles of 10 s at 98 °C, 20 s at 55 °C, and then 15 s at 72 °C, and a final extension of 2 min at 72 °C. PCR products were run on agarose gels in similar conditions as described above but utilised Tris-borate-EDTA (TBE) buffer instead of TAE and were applied with 120 V for 150 min instead.

2.5. DNA Sequencing

Amplicons obtained from randomly chosen single male and female lumpfish amplified with the primer sets from Table 1 were sequenced.

Amplicons of primer set AMH1_E3I6, AMH2_I6E4, and AMH3_E3I6 were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). For the primer set AMH1+3_E6I6, gel band excision was performed to extract each amplicon for sequencing. The excised bands were purified with QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany).

The samples were submitted to LightRun Tube Sanger sequencing (Eurofins genomics, Ebersberg, Germany) for Sanger sequencing. The obtained consensus sequences were then aligned with the lumpfish *amh* sequences obtained from the Ensembl database using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) alignment algorithm [31] to calculate the percentage of identical sites to identify the most similar sequences.

Amplicons of the primer set AMH1+3_E6I6 from the *A. ventricosus*, *E. taranetzi*, and *E. asperrimus* samples were also purified and sequenced with similar protocols.

2.6. Estimation of the Male-Specific Region Coverage

PCRs were performed on six randomly selected male and female lumpfish individuals using primer sets designed to estimate the length of the male-specific region in the species (Table 2). The PCR reactions were similar to the reaction utilising 2× MyTaq™ HS Mix detailed in Section 2.4. PCR amplification conditions for these primer sets were 1 min at 95 °C, followed by 28 cycles of 15 s at 95 °C, 15 s at 57 °C, and then 20 s at 72 °C, and a final extension of 5 min at 72 °C. Amplicons underwent the previous gel electrophoresis protocol (as detailed in Section 2.4) but ran at 80 V for 30 min.

2.7. Phylogenetic Analysis

The autosomal amplicon sequences from primer set AMH1+3_E6I6 of male *A. ventricosus*, *E. taranetzi*, and *E. asperrimus*, along with the sequences of the three *amh* paralogues from lumpfish obtained in this study, autosomal *amh* and male-specific *amhy* sequences from lingcod (*Ophiodon elongatus*), and an autosomal *amh* sequence from zebrafish (*Danio rerio*) as an outgroup (GenBank accession nos. KP686074.1 and KP686073.1; Ensembl accession no. ENSDARG00000014357), were utilised for phylogeny reconstruction.

Sequence alignments using the MUSCLE algorithm and the best substitution model determination were performed with MEGA11, Molecular Evolutionary Genetics Analysis ver 11.0 [32]. Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) methods. ML tree was reconstructed using Tamura 3-parameter model [33] with 1000 bootstrap replicates by MEGA11. BI tree was reconstructed using the same model (lset nst = 2 rates = equal) by MrBayes ver. 3.2.7a [34]. Bayesian posterior probabilities were estimated using the Metropolis-coupled Markov Chain Monte Carlo method with four chains (three heated and one cold), with the temperature set to 0.2. Chains were run for 1,000,000 generations and sampled every 1000 trees, with 25% of the trees discarded as burn-in. The resulting phylogenies were processed by Figtree ver. 1.4.4 [35].

3. Results

3.1. Male-Specificity of the Markers

On the 12 males and 12 females initially tested, only primer set AMH1_E3I6 and AMH2_I6E4 amplified the anticipated 1410 bp and 1804 bp amplicons, respectively, in the males exclusively, while primer set AMH3_E3I6 amplified an 1136 bp band in both sexes (Figure 1A–C). The primer set AMH1+3_E6I6 amplified two bands of the anticipated sizes only in males, designated as the upper (566 bp) and the lower (481 bp) band, but amplified one band with a similar size to the upper band in females (Figure 1D).

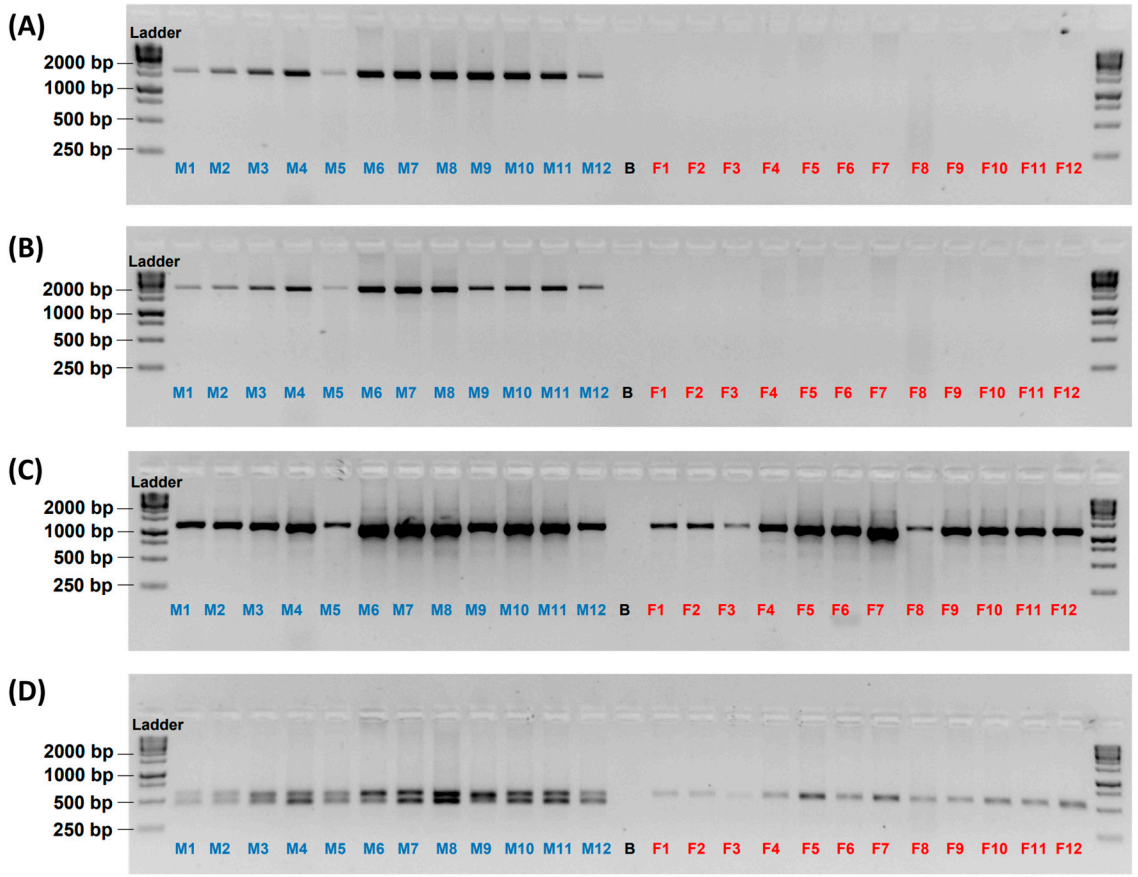

Figure 1. PCR amplification results of the primer set utilised to characterise the sex specificity of the three *amh* paralogues in lumpfish. Lanes with blue labels represent the amplification results of the phenotypic males (M1–12); lanes with red labels represent the amplification results of the phenotypic females (F1–12); and lanes with the "B" labels represent blanks as the negative control. (**A**) Results of the primer set AMH1_E3I6. (**B**) Results of the primer set AMH2_I6E4. (**C**) Results of primer set AMH3_E3I6. (**D**) Results of the primer set AMH1+3_E6I6. The ladder used is GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA). The samples were run on ethidium bromide-stained 2.0% agarose gel at 90 V for 40 min.

Every generated consensus sequence from the amplicons has the highest pairwise percentage identity with their expected *amh* gene paralogue, with the amplicon of AMH1_E3I6 matching 99.6% with *amh1*, AMH2_I6E4 matching 99.5% with *amh2*, AMH3_E3I6 matching 99.2% with male and 99.4% with female *amh3*, AMH1+3_E6I6 matching 94.1% with the male upper band for *amh3*, 99.0% with the male lower band for *amh1*, and 85.8% with the female fragment for *amh3*. The DNA sequences of the PCR amplicons sequenced in this study can be found in File S1.

3.2. Accuracy of the Male-Specific Markers

Based on the preliminary tests, the primer sets that amplified male-specific amplicons (AMH1_E3I6, AMH2_I6E4, and AMH1+3_E6I6) were further assessed with the DNA samples of 67 male and 76 female lumpfish from different sources. The three chosen primer sets all correctly identified 65 out of 67 males (97.0%) and 76 out of 76 females (100%). Note that the two samples which were identified as male but exhibit the genotypes of a female are identical throughout the three tests. A summary of the results is available in Table S1.

The genotype exhibited by the three primer sets and phenotypic sex are significantly associated, based on the chi-square tests ($p < 2.2 \times 10^{-16}$).

3.3. Determination of the Male-Specific Region Length

Additional primers for estimating the size of the male-specific region were based on the flanking regions upstream of *amh1* and downstream of *amh2* (Table 2).

On the six males and the six females utilised for this purpose, only the primer set 4K_Up exhibited male-specific amplification among the upstream primer sets, suggesting the extension of the male-specific region upstream is at least 4 kb from *amh1*. Similarly, only the primer sets 4K_Down and 14K_Down exhibited male-specific amplifications among the downstream primer sets, suggesting the extension of the male-specific region downstream is at least 14 kb from *amh2*. The gel images can be found in Figures S2 and S3.

Based on these results and the reference genome, the male-specific region of lumpfish was estimated to cover at least 27 kb but no more than 30 kb.

3.4. Sequence Homogeneity and Evolutionary Relationship of the amh Gene in Cyclopterida

By utilising the primer set AMH1+3_E6I6, only *A. ventricosus* showed a male-specific pattern among the available Cyclopteridae samples other than lumpfish, exhibiting two male-specific bands above the autosomal bands at approximately 450 bp and 600 bp (Figure 2). However, the size and the number of male-specific fragments are different between lumpfish and *A. ventricosus*. The DNA sequences of the amplicons from the non-lumpfish Cyclopteridae, which were successfully sequenced, are available in File S1.

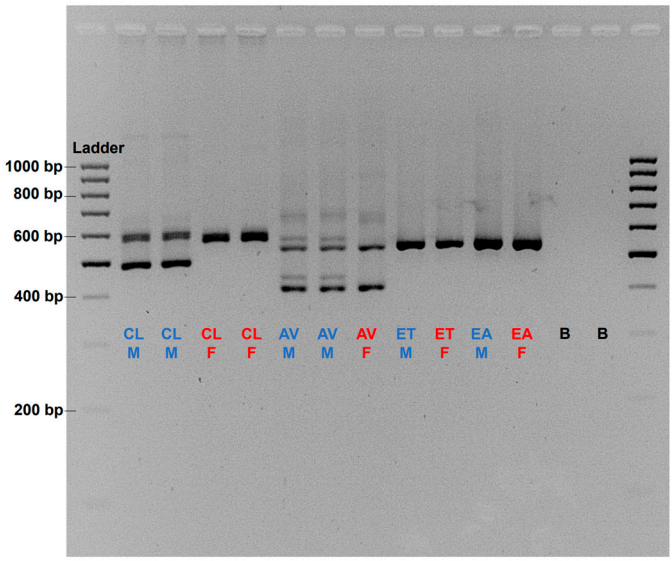

Figure 2. PCR amplification results of the primer set AMH1+3_E6I6 in lumpfish (CL), *Aptocyclus ventricosus* (AV), *Eumicrotremus taranetzi* (ET), and *Eumicrotremus asperrimus* (EA). Lanes with blue labels represent the amplification results of the phenotypic males (M); lanes with red labels represent the amplification results of the phenotypic females (F); and lanes with the "B" labels represent blanks as the negative control. The ladder used is GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA). The samples were run on ethidium bromide-stained 2.0% agarose gel at 120 V for 150 min.

Using the available DNA sequences from Cyclopteridae samples, we conducted additional phylogenetic reconstructions to infer the evolutionary relationship of the *amh* gene within this family. After gap deletion, a final alignment of 200 sites with 100 variable sites (50%) and 27 parsimony-informative sites (13.5%) was generated from ten *amh* sequences of Scorpaeniform fishes and zebrafish (File S2). The ML tree and BI tree reconstructed from this alignment share a similar topology, with the Cyclopteridae autosomal *amh* and male-specific *amh* segregated into two clades (bootstrap support = 80% and posterior probability = 0.69), so the topology of the BI consensus tree with the bootstrap values from the ML analysis was chosen to represent both analyses (Figure 3). These topologies suggest that the male-specific *amh* of lumpfish emerged after the split of Cyclopteroidea

and Hexagrammoidea and before the split of *Aptocyclus*, *Cyclopterus*, and *Eumicrotremus* lineages rather than independently arising in the lineage of lumpfish.

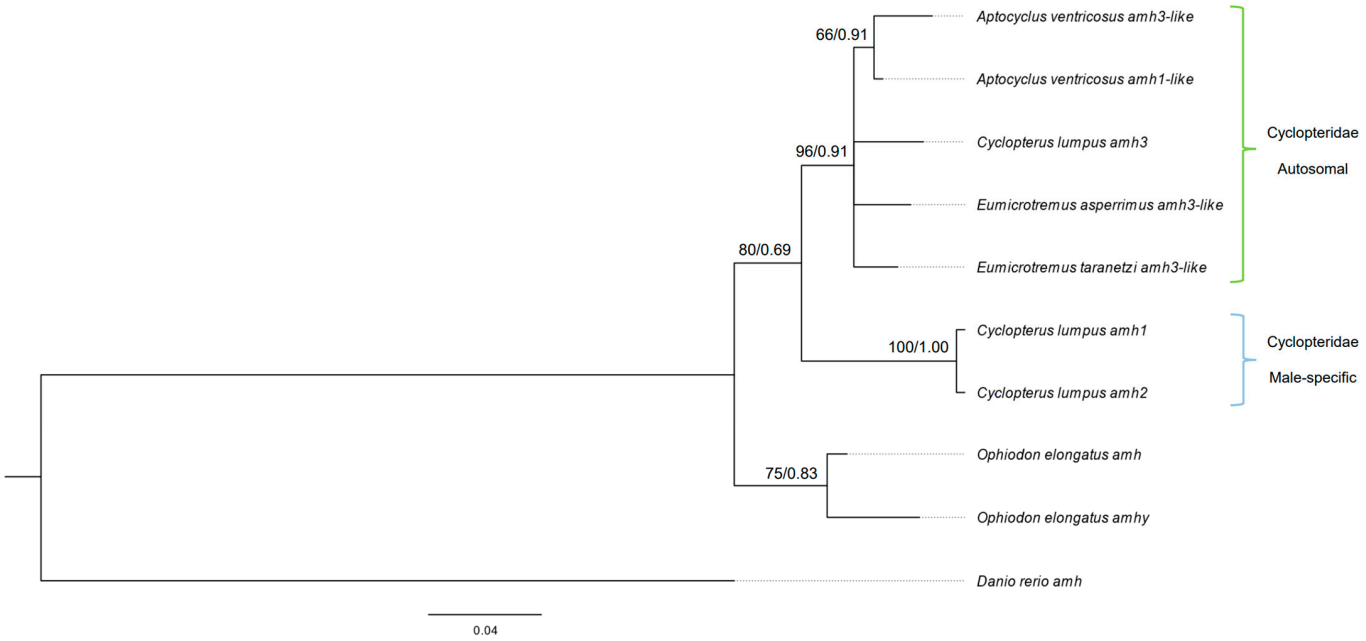

Figure 3. Bayesian inference consensus tree constructed using sequences of *amh* homologues from male lumpfish and the males of their closely related species. The green highlight denotes the clade of Cyclopteridae autosomal *amh*. The blue highlight denotes the clade of Cyclopteridae male-specific *amh*. The *amh* sequences obtained from the non-lumpfish Cyclopteridae samples were designated as *amh1*-like (lower band) and *amh3*-like (upper band), depending on their similarity in size to the fragment of *amh1* and *amh3* amplified by the primer set AMH1+3_E6I6 (Figure 2). Numbers at the nodes denote the branch support values from maximum likelihood (bootstrap value) and Bayesian inference (posterior probability), respectively. Zebrafish (*Danio rerio*) *amh* was chosen as the outgroup. The alignment for phylogenetic analysis and the sequence's source is available in File S2.

4. Discussion

Our findings suggest that *amh1* and *amh2* are found only in male lumpfish, while *amh3* seems to be of an autosomal nature. We also found evidence suggesting the presence of a male-specific *amh* in another closely related species, *A. ventricosus*, albeit seemingly different from lumpfish. Our results agree with previous research on lumpfish sex GWAS, where a male-specific region in chromosome 13 was described [20,22]. Based on these results and previous studies, we can confirm that *amh1* and *amh2* can function as male-specific molecular markers for sex identification in lumpfish.

4.1. Application in Aquaculture

Prior to maturity, lumpfish show minimal sexual dimorphism, but upon reaching maturity, they can be visually identified by pronounced sexual dichromatism, especially during the spawning season [10]. Nevertheless, the prolonged maturation period in lumpfish [19,36], along with the associated resources required, renders the visual sex identification method unsuitable for efficient aquaculture production. Sex identification is essential for managing the sex ratio of broodstock to develop successful breeding schemes for offspring production; therefore, the ability to identify the sex of lumpfish at early stages will facilitate more efficient production.

Genetic sex identification, like the PCR-based techniques applied in this study, is a powerful alternative to morphological sex identification, and can ultimately improve the management of the sex ratio within the broodstock [37]. In addition, PCR-based sex identification has been developed in many important aquaculture species, such as

salmonids [18], Sturgeon species [16,17], Nile tilapia (*Oreochromis niloticus*) [38–40], and the large yellow croaker (*Larimichthys crocea*) [41], mainly for the screening of mono-sex production and early sex identification of potential broodstock. Although a previous study did not find any significant difference in grazing efficacy between sexes in juvenile lumpfish [14], the production of mono-sex female lumpfish could potentially boost the profitability of lumpfish aquaculture by the addition of the roe as a by-product.

In our study, the accuracy rate of the PCR-based sex identification technique utilising male-specific *amh* duplicates was comparable to that of developed tests for other teleost fish species, with an accuracy rate ranging from 97.5% to 100% [23–25]. The consistent occurrence of male-specific *amh* in numerous species could be attributed to its role as a male MSD gene [24,25,42]. The model of sex-determination cascade in Nile tilapia suggested that male-specific *amh* is the key trigger for testicular differentiation through inhibition of the transcription of *cytochrome P450*, *family 19*, *subfamily A*, *polypeptide 1a* (*cyp19a1a*), the gene encoding teleost ovarian aromatase for oestrogen synthesis, during the sex-determination period via binding to anti-Mullerian hormone receptor type 2 (Amhr2) to activate the suppressor of mothers against the decapentaplegic (Smad) transcription factor cascade that alters the gene expression [43]. Therefore, our molecular tools, which utilise male-specific *amh* paralogues of lumpfish, should be suitable for genetic sex identification to improve the efficiency of the lumpfish breeding program.

4.2. Implication in the Sex-Determination System of Lumpfish

The concordance between genotypic and phenotypic sex in our PCR tests supports the hypothesis that lumpfish possess a genetic sex-determination system, and furthermore, it provides evidence that a major influence from environmental factors is unlikely. A male-specific region in the lumpfish genome suggests a XX/XY genetic sex-determination system, as supported by the previous GWAS analysis [20,22] and observation from a feminisation experiment [44]. Our study also identified *amh1* and *amh2* as male-specific *amh* paralogues, which may function as the MSD gene in lumpfish.

Presently, male-specific *amh* duplicates have been found in various teleosts, including the *Odontesthes* clade of the order Atheriniformes [45], notably Patagonian silverside (*O. hatcheri*) [24] and Argentinian silverside (*O. bonariensis*) [46], Nile tilapia [39,40,43], lingcod [23], cobaltcap silverside (*Hypoatherina tsurugae*) [47], northern pike (*Esox lucius*) [25], Korean rockfish (*Sebastes schlegelii*) [42], and the *Gasterosteus* clade of the family Gasterosteidae [48,49]. Moreover, some of these homologues have also been recognised as the male MSD gene, highlighting the important role that this gene plays in sexual development. Our phylogenetic analyses suggest that the male-specific *amh* in lumpfish is more likely to have emerged independently in the Cyclopteridae lineage rather than being a shared ancestral trait in the Scorpaeniform lineage. Furthermore, our phylogenetic analyses exhibit a comparable topology to another neighbour-joining phylogeny constructed using *amh* sequences from lumpfish and their closely related species [20], providing additional support for this hypothesis.

Phylogenetic analyses of *amh* sequences from other teleosts also indicated that most sex-determining *amh* genes emerged independently in clades of closely related species as a form of convergent evolution rather than due to shared ancestry of teleost [25,42,46]. One of the theories for these independent recruitments is that *amh* encodes a protein belonging to the Tgf-β family, which is composed of versatile and flexible signalling networks that interact with the gonad development cascade in vertebrates [50]. Due to this connectivity, the male-specific *amh* paralogues are prime candidates for the MSD gene in lumpfish.

Most male-specific *amh* paralogues of teleost species were theorised to arise as an MSD gene through neofunctionalisation via the gene duplication (GD) model [24,25,42]. This model is well described in the MSD gene of Japanese medaka, *dmrt1bY*, which was proposed to emerge via the events of gene duplication from its ancestral gene, translocation to a new chromosome, and neo-functionalisation by acquiring a pre-existing *cis*-regulatory element necessary for rewiring its expression for the sex determination through a transposable

element [51]. Following this model [51], the male-specific *amh* duplicates in lumpfish could be derived from the autosomal *amh3*, translocated to their current sex chromosome, and neo-functionalised into an MSD gene by acquiring a novel expression pattern, mutation, or a combination of both that can induce gonad development.

Contrary to our deduction, previous research proposed *amh3* as the potential MSD gene, as it is the only *amh* transcript detected in the testis tissue of mature male lumpfish [20]. However, their observation could be explained by differences in expression patterns between the matures and juveniles, as observed in male Japanese medaka, which exhibited a decline in their MSD gene expression as they matured [52]. Further expression and functional analyses are recommended to determine the level of expression of both *amh1* and *amh2* during the different development stages of both males and females. If one of the *amh* functions as an MSD gene in lumpfish, it is expected to exhibit male-specific expression before the sexual differentiation of the bipotential gonadal primordium that is requisite and adequate for testis development [24,25,42]. Further understanding of the sex-determination mechanism in lumpfish is expected to improve the management of the stock's sex ratio and fill in the knowledge gap in the sex-determination system of teleost, which is still limited to a few species.

4.3. Implication in the Sex Chromosome Evolution of Lumpfish

We estimated that the male-specific region of lumpfish covers approximately 27 kb, which is considerably minuscule compared to the length of the putative sex chromosome. Nevertheless, a study on Korean rockfish has estimated the male-specific region in this species to be approximately 5 kb [42], providing evidence of another small genomic region controlling such an important developmental process. Based on the assumption that the male-specific *amh* functions as the MSD gene in lumpfish, the compact size of the male-specific region and the observed homomorphic state of their chromosomes [53] indicate that the sex chromosome in lumpfish is in its early stages of development. It is worthwhile to mention that we designed and tested multiple primers upstream and downstream from the *amh* region apart from those presented in our methods, but PCR amplification of most primers was not possible for an unknown reason. We suspected potential assembly errors within this region in the currently available genome are the cause; therefore, the real male-specific region could be larger or shorter than we estimated according to the currently available coordinates. Future studies should attempt to characterise the male-specific region using other sequencing methods, such as Long-read Oxford Nanopore sequencing [54], or new assembly algorithms that could provide a more accurate sequence and will ultimately allow a more comprehensive analysis between male and female lumpfish genome.

The nascent sex chromosome could be explained by the "high turnover" theory, in which a new MSD gene frequently emerges on an autosome, replacing the ancestral sex chromosome and preventing it from further degradation [55]. This phenomenon can be observed in the genus *Oryzias,* which contains various MSD genes within the clade [56]. However, our phylogenetic analyses disagree with this theory, as the consensus phylogeny implies that the male-specific *amh* of lumpfish might have emerged early in the Cyclopteridae lineage and were retained in the lumpfish lineage. We may require additional samples from other members of the family, informative sites, and genomic analysis to better elucidate the evolution of *amh* in Cyclopteridae. Nevertheless, the species utilised in our study have been identified as the closest ones to lumpfish within the family; therefore, our phylogenetic analyses should reflect the actual evolutionary pathway of *amh* in the Cyclopteridae family.

Another possible theory is the "jumping sex locus" theory, in which the existing MSD gene is translocated to a new autosome by transposable elements, allowing a replacement of the ancestral sex chromosome without overhauling the current sex-determination mechanism, as observed in *sdY* of various salmonids [18,57]. Alternatively, it is possible that the MSD gene has remained on the same sex chromosome since the most recent turnover event without undergoing chromosomal degradation up to the present time. Similar to

the Northern pike, where the male-specific *amh* has been conserved as the MSD gene on the same chromosome for an estimated 40 million years while maintaining homomorphic sex chromosomes, this conservation might be facilitated by the presence of direct repeats flanking the sex-determining locus [25].

It would be informative to analyse the male-specific region and its flanking region to identify possible transposable elements that may facilitate translocation or potential novel mechanisms which prevent chromosomal degradation. With such a relatively small male-specific region, further studies on the sex chromosome of lumpfish may provide more insight into the sex chromosome evolution mechanism in teleosts.

5. Conclusions

Our PCR amplification results suggest that *amh1* and *amh2* paralogues are male-specific, while *amh3* is autosomal. PCR amplification in 143 samples showed significant associations between the male phenotype to the presence of both *amh1* and *amh2*, with more than 97% of the males amplifying fragments from both genes while every female did not. Further analysis of the flanking regions adjacent to *amh1* and *amh2* revealed that the male-specific region spans approximately 27 kb. These results support that *amh1* and *amh2* can function as male-specific molecular markers for genetic sex identification in lumpfish. The development of a precise PCR test holds significant potential for advancing lumpfish breeding programs, thereby reducing dependence on wild populations and promoting sustainable aquaculture practices. Additionally, these findings may also benefit future studies regarding the lumpfish's sex-determination system and sex chromosome evolution.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fishes8060327/s1, Table S1: Detailed record of the lumpfish (*Cyclopterus lumpus*) samples in this study, Figure S1: Binding sites of the primers utilised in this study to amplify *anti-Müllerian hormone* (*amh*) genes of lumpfish, Figure S2: PCR amplification results of the primer set utilised to estimate the upstream coverage of the male-specific region in lumpfish, Figure S3: PCR amplification results of the primer set utilised to estimate the downstream coverage of the male-specific region in lumpfish, File S1: DNA sequences of the amplicons sequenced in this study, File S2: DNA sequences alignment utilised for phylogenetic analyses.

Author Contributions: Conceptualisation: A.P.G. and K.C.; Data curation: K.C., A.P.G. and A.D.; Formal analysis: K.C. and A.P.G.; Funding acquisition: A.P.G. and A.D.; Investigation: K.C., P.M., E.J.-F., A.P.G. and J.P.; Methodology: K.C., P.P. and A.P.G.; Writing—original draft: K.C. and A.P.G.; Writing—review and editing: A.P.G., K.C., E.J.-F. and P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) as part of the Aqualeap project (BB/S004416/1).

Institutional Review Board Statement: The study was conducted following the UK Animals (Scientific Procedures) Act 1986 (revised 2013) and approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 2022 7735 636).

Data Availability Statement: The data presented in this study are available in File S1 and File S2 as part of the supplementary materials.

Acknowledgments: We would like to express our gratitude to Kai Yoshiaki of the University of Kyoto, who graciously shared tissue samples of *Aptocyclus ventricosus*, *Eumicrotremus taranetzi*, and *E. asperrimus* for this research.

Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Brooker, A.J.; Skern-Mauritzen, R.; Bron, J.E. Production, mortality, and infectivity of planktonic larval sea lice, *Lepeophtheirus salmonis* (Krøyer, 1837)*: Current* knowledge and implications for epidemiological modelling. *ICES J. Mar. Sci.* **2018**, *75*, 1214–1234. [CrossRef]
2. Bolton-Warberg, M. An overview of cleaner fish use in Ireland. *J. Fish Dis.* **2018**, *41*, 935–939. [CrossRef]
3. Østevik, L.; Stormoen, M.; Evensen, Ø.; Xu, C.; Lie, K.; Nødtvedt, A.; Rodger, H.; Skagøy, A.; Manji, F.; Alarcón, M. Effects of thermal and mechanical delousing on gill health of farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture* **2022**, *552*, 738019. [CrossRef]
4. Overton, K.; Dempster, T.; Oppedal, F.; Kristiansen, T.S.; Gismervik, K.; Stien, L.H. Salmon lice treatments and salmon mortality in Norwegian aquaculture: A review. *Rev. Aquac.* **2019**, *11*, 1398–1417. [CrossRef]
5. Barisic, J.; Cannon, S.; Quinn, B. Cumulative impact of anti-sea lice treatment (azamethiphos) on health status of Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) in aquaculture. *Sci. Rep.* **2019**, *9*, 16217. [CrossRef] [PubMed]
6. Parsons, A.E.; Escobar-Lux, R.; Sævik, P.N.; Samuelsen, O.B.; Agnalt, A. The impact of anti-sea lice pesticides, azamethiphos and deltamethrin, on European lobster (*Homarus gammarus*) larvae in the Norwegian marine environment. *Environ. Pollut.* **2020**, *264*, 114725. [CrossRef] [PubMed]
7. Johannesson, J. *Lumpfish Caviar: From Vessel to Consumer*; Food & Agriculture Organization of the United Nations: Rome, Italy, 2006; Available online: https://www.fao.org/3/a0685e/a0685e.pdf (accessed on 25 July 2022).
8. Brooker, A.J.; Papadopoulou, A.; Gutierrez, C.; Rey, S.; Davie, A.; Migaud, H. Sustainable production and use of cleaner fish for the biological control of sea lice: Recent advances and current challenges. *Vet. Rec.* **2018**, *183*, 383. [CrossRef]
9. Powell, A.; Treasurer, J.W.; Pooley, C.L.; Keay, A.J.; Lloyd, R.; Imsland, A.K.; Garcia de Leaniz, C. Use of lumpfish for sea-lice control in salmon farming: Challenges and opportunities. *Rev. Aquac.* **2018**, *10*, 683–702. [CrossRef]
10. Davenport, J.; Bradshaw, C. Observations on skin colour changes in juvenile lumpsuckers. *J. Fish Biol.* **1995**, *47*, 143–154. [CrossRef]
11. Kohn, Y.Y.; Lokman, P.M.; Kilimnik, A.; Symonds, J.E. Sex identification in captive hapuku (*Polyprion oxygeneios*) using ultrasound imagery and plasma levels of vitellogenin and sex steroids. *Aquaculture* **2013**, *384–387*, 87–93. [CrossRef]
12. Ledoré, Y.; Bestin, A.; Haffray, P.; Morvezen, R.; Alix, M.; Schaerlinger, B.; Fontaine, P.; Chardard, D. Sex identification in immature Eurasian perch (*Perca fluviatilis*) using ultrasonography. *Aquac. Res.* **2021**, *52*, 6046–6051. [CrossRef]
13. Chu-Koo, F.; Dugué, R.; Alván Aguilar, M.; Casanova Daza, A.; Alcántara Bocanegra, F.; Chávez Veintemilla, C.; Duponchelle, F.; Renno, J.-; Tello, S.; Nuñez, J. Gender determination in the Paiche or Pirarucu (*Arapaima gigas*) using plasma vitellogenin, 17β-estradiol, and 11-ketotestosterone levels. *Fish Physiol. Biochem.* **2009**, *35*, 125–136. [CrossRef] [PubMed]
14. Imsland, A.K.D.; Reynolds, P.; Hangstad, T.A.; Kapari, L.; Maduna, S.N.; Hagen, S.B.; Jónsdóttir, Ó.D.B.; Spetland, F.; Lindberg, K.S. Quantification of grazing efficacy, growth and health score of different lumpfish (*Cyclopterus lumpus* L.) families: Possible size and gender effects. *Aquaculture* **2021**, *530*, 735925. [CrossRef]
15. Hurvitz, A.; Jackson, K.; Degani, G.; Levavi-Sivan, B. Use of endoscopy for gender and ovarian stage determinations in Russian sturgeon (*Acipenser gueldenstaedtii*) grown in aquaculture. *Aquaculture* **2007**, *270*, 158–166. [CrossRef]
16. Kuhl, H.; Guiguen, Y.; Christin, H.; Kreuz, E.; Du, K.; Klopp, C.; Céline, L.; Elena, S.Y.; Ciorpac, M.; Jörn, G.; et al. A 180 My-old female-specific genome region in sturgeon reveals the oldest known vertebrate sex determining system with undifferentiated sex chromosomes. *bioRxiv* **2020**. [CrossRef]
17. Scribner, K.T.; Kanefsky, J. Molecular sexing of lake sturgeon. *J. Great Lakes Res.* **2021**, *47*, 934–936. [CrossRef]
18. Yano, A.; Nicol, B.; Jouanno, E.; Quillet, E.; Fostier, A.; Guyomard, R.; Guiguen, Y. The sexually dimorphic on the Y-chromosome gene (*sdY*) is a conserved male-specific Y-chromosome sequence in many salmonids. *Evol. Appl.* **2013**, *6*, 486–496. [CrossRef]
19. Whittaker, B.A.; Consuegra, S.; Garcia de Leaniz, C. Genetic and phenotypic differentiation of lumpfish (*Cyclopterus lumpus*) across the North Atlantic: Implications for conservation and aquaculture. *PeerJ* **2018**, *6*, e5974. [CrossRef]
20. Holborn, M.K.; Einfeldt, A.L.; Kess, T.; Duffy, S.J.; Messmer, A.M.; Langille, B.L.; Brachmann, M.K.; Gauthier, J.; Bentzen, P.; Knutsen, T.M.; et al. Reference genome of lumpfish *Cyclopterus lumpus* Linnaeus provides evidence of male heterogametic sex determination through the AMH pathway. *Mol. Ecol. Resour.* **2022**, *22*, 1427–1439. [CrossRef]
21. Patil, J.G.; Hinze, S.J. Simplex PCR assay for positive identification of genetic sex in the Japanese medaka, *Oryzias latipes*. *Mar. Biotechnol.* **2008**, *10*, 641–644. [CrossRef]
22. Alejandro, P.G.; Sarah-Louise, C.S.; John, B.T.; Kokkinias, P.; Thomas, C.; Eduardo, J.F.; Migaud, H.; Lein, I.; Davie, A.; Bekaert, M. Development of genomic markers associated to production traits in lumpfish (*Cyclopterus lumpus*). *bioRxiv* **2023**. [CrossRef]
23. Rondeau, E.B.; Laurie, C.V.; Johnson, S.C.; Koop, B.F. A PCR assay detects a male-specific duplicated copy of Anti-Müllerian hormone (*amh*) in the lingcod (*Ophiodon elongatus*). *BMC Res. Notes* **2016**, *9*, 230. [CrossRef] [PubMed]
24. Hattori, R.S.; Murai, Y.; Oura, M.; Masuda, S.; Majhi, S.K.; Sakamoto, T.; Fernandino, J.I.; Somoza, G.M.; Yokota, M.; Strüssmann, C.A. A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 2955–2959. [CrossRef] [PubMed]
25. Pan, Q.; Feron, R.; Yano, A.; Guyomard, R.; Jouanno, E.; Vigouroux, E.; Wen, M.; Busnel, J.; Bobe, J.; Concordet, J.; et al. Identification of the master sex determining gene in Northern pike (*Esox lucius*) reveals restricted sex chromosome differentiation. *PLoS Genet.* **2019**, *15*, e1008013. [CrossRef]

26. Blanquer, A. Phylogéographie Intraspécifique d'un Poisson Marin, le Flet *Platichthys flesus* L. (Heterosomata): Polymorphisme des Marqueurs Nucléaires et Mitochondriaux. Ph.D. Thesis, University of Montpellier, Montpellier, France, 1990.
27. Cunningham, F.; Allen, J.E.; Allen, J.; Alvarez-Jarreta, J.; Amode, M.R.; Armean, I.M.; Austine-Orimoloye, O.; Azov, A.G.; Barnes, I.; Bennett, R.; et al. Ensembl 2022. *Nucleic Acids Res.* **2022**, *50*, D988–D995. [CrossRef] [PubMed]
28. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* **2012**, *40*, e115. [CrossRef]
29. R Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2022; Available online: https://www.R-project.org/ (accessed on 6 September 2022).
30. RStudio Team. *RStudio: Integrated Development for R*; RStudio, Inc.: Boston, MA, USA, 2022; Available online: http://www.rstudio.com/ (accessed on 6 September 2022).
31. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [CrossRef]
32. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef]
33. Tamura, K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol. Biol. Evol.* **1992**, *9*, 678–687. [CrossRef]
34. Ronquist, F.; Teslenko, M.; van der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Syst. Biol.* **2012**, *61*, 539–542. [CrossRef]
35. Rambaut, A. *FigTree*, version 1.4.4; Institute of Evolutionary Biology, University of Edinburgh: Edinburgh, UK, 2018. Available online: http://tree.bio.ed.ac.uk/software/figtree/(accessed on 6 February 2023).
36. Davenport, J. *Synopsis of Biological Data on the Lumpsucker, Cyclopterus lumpus (Linnaeus, 1758)*; Food and Agriculture Organization of the United Nations: Rome, Italy, 1985; Available online: https://www.fao.org/3/ap950e/ap950e.pdf (accessed on 6 May 2022).
37. Martínez, P.; Viñas, A.M.; Sánchez, L.; Díaz, N.; Ribas, L.; Piferrer, F. Genetic architecture of sex determination in fish: Applications to sex ratio control in aquaculture. *Front. Genet.* **2014**, *5*, 340. [CrossRef] [PubMed]
38. Taslima, K.; Wehner, S.; Taggart, J.B.; de Verdal, H.; Benzie, J.A.H.; Bekaert, M.; McAndrew, B.J.; Penman, D.J. Sex determination in the GIFT strain of tilapia is controlled by a locus in linkage group 23. *BMC Genet.* **2020**, *21*, 49. [CrossRef] [PubMed]
39. Li, M.; Sun, Y.; Zhao, J.; Shi, H.; Zeng, S.; Ye, K.; Jiang, D.; Zhou, L.; Sun, L.; Tao, W.; et al. A tandem duplicate of anti-Müllerian hormone with a missense SNP on the Y Chromosome is essential for male sex determination in Nile tilapia, *Oreochromis niloticus*. *PLoS Genet.* **2015**, *11*, e1005678. [CrossRef] [PubMed]
40. Eshel, O.; Shirak, A.; Dor, L.; Band, M.; Zak, T.; Markovich-Gordon, M.; Chalifa-Caspi, V.; Feldmesser, E.; Weller, J.I.; Seroussi, E.; et al. Identification of male-specific *amh* duplication, sexually differentially expressed genes and microRNAs at early embryonic development of Nile tilapia (*Oreochromis niloticus*). *BMC Genom.* **2014**, *15*, 774. [CrossRef]
41. Lin, A.; Xiao, S.; Xu, S.; Ye, K.; Lin, X.; Sun, S.; Wang, Z. Identification of a male-specific DNA marker in the large yellow croaker (*Larimichthys crocea*). *Aquaculture* **2017**, *480*, 116–122. [CrossRef]
42. Song, W.; Xie, Y.; Sun, M.; Li, X.; Fitzpatrick, C.K.; Vaux, F.; O'Malley, K.G.; Zhang, Q.; Qi, J.; He, Y. A duplicated *amh* is the master sex-determining gene for *Sebastes* rockfish in the Northwest Pacific. *Open Biol.* **2021**, *11*, 210063. [CrossRef]
43. Liu, X.; Dai, S.; Wu, J.; Wei, X.; Zhou, X.; Chen, M.; Tan, D.; Pu, D.; Li, M.; Wang, D. Roles of anti-Müllerian hormone and its duplicates in sex determination and germ cell proliferation of Nile tilapia. *Genetics* **2022**, *220*, iyab237. [CrossRef]
44. Martin-Robichaud, D.; Peterson, R.H.; Benfey, T.J.; Crim, L.W. Direct feminization of lumpfish (*Cyclopterus lumpus* L.) using 17β-oestradiol-enriched Artemia as food. *Aquaculture* **1994**, *123*, 137–151. [CrossRef]
45. Hattori, R.S.; Somoza, G.M.; Fernandino, J.I.; Colautti, D.C.; Miyoshi, K.; Gong, Z.; Yamamoto, Y.; Strüssmann, C.A. The duplicated Y-specific *amhy* gene is conserved and linked to maleness in Silversides of the genus *Odontesthes*. *Genes* **2019**, *10*, 679. [CrossRef]
46. Yamamoto, Y.; Zhang, Y.; Sarida, M.; Hattori, R.S.; Strüssmann, C.A. Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLoS ONE* **2014**, *9*, e102574. [CrossRef]
47. Bej, D.K.; Miyoshi, K.; Hattori, R.S.; Strüssmann, C.A.; Yamamoto, Y. A duplicated, truncated *amh* gene is involved in male sex determination in an Old World silverside. *G3 Genes | Genomes | Genet.* **2017**, *7*, 2489–2495. [CrossRef] [PubMed]
48. Sardell, J.M.; Josephson, M.P.; Dalziel, A.C.; Peichel, C.L.; Kirkpatrick, M. Heterogeneous histories of recombination suppression on stickleback sex chromosomes. *Mol. Biol. Evol.* **2021**, *38*, 4403–4418. [CrossRef] [PubMed]
49. Peichel, C.L.; McCann, S.R.; Ross, J.A.; Naftaly, A.F.S.; Urton, J.R.; Cech, J.N.; Grimwood, J.; Schmutz, J.; Myers, R.M.; Kingsley, D.M.; et al. Assembly of the threespine stickleback Y chromosome reveals convergent signatures of sex chromosome evolution. *Genome Biol.* **2020**, *21*, 177. [CrossRef]
50. Pan, Q.; Kay, T.; Depincé, A.; Adolfi, M.; Schartl, M.; Guiguen, Y.; Herpin, A. Evolution of master sex determiners: TGF-β signalling pathways at regulatory crossroads. *Philos. Trans. R. Soc. B Biol. Sci.* **2021**, *376*, 20200091. [CrossRef]
51. Herpin, A.; Braasch, I.; Kraeussling, M.; Schmidt, C.; Thoma, E.C.; Nakamura, S.; Tanaka, M.; Schartl, M. Transcriptional rewiring of the sex determining *dmrt1* gene duplicate by transposable elements. *PLoS Genet.* **2010**, *6*, e1000844. [CrossRef]
52. Hornung, U.; Herpin, A.; Schartl, M. Expression of the male determining gene *dmrt1bY* and its autosomal coorthologue *dmrt1a* in medaka. *Sex. Dev.* **2007**, *1*, 197–206. [CrossRef]

53. Li, M.F.; Clyburne, S. New cell line from the marine lumpfish, *Cyclopterus lumpus*. *J. Fish. Res. Board Can.* **1977**, *34*, 134–139. [CrossRef]
54. Feron, R.; Zahm, M.; Cabau, C.; Klopp, C.; Roques, C.; Bouchez, O.; Eché, C.; Valière, S.; Donnadieu, C.; Haffray, P.; et al. Characterization of a Y-specific duplication/insertion of the anti-Mullerian hormone type II receptor gene based on a chromosome-scale genome assembly of yellow perch, *Perca flavescens*. *Mol. Ecol. Resour.* **2020**, *20*, 531–543. [CrossRef]
55. Meisel, R.P. Evolution of sex determination and sex chromosomes: A novel alternative paradigm. *BioEssays* **2020**, *42*, 1900212. [CrossRef]
56. Myosho, T.; Takehana, Y.; Hamaguchi, S.; Sakaizumi, M. Turnover of sex chromosomes in *Celebensis* group medaka fishes. *G3 Genes Genomes Genet.* **2015**, *5*, 2685–2691. [CrossRef]
57. Woram, R.; Gharbi, K.; Sakamoto, T.; Høyheim, B.; Holm, L.; Naish, K.; Mcgowan, C.; Ferguson, M.; Phillips, R.; Stein, J.; et al. Comparative genome analysis of the primary sex-determining locus in salmonid fishes. *Genome Res.* **2003**, *13*, 272–280. [CrossRef] [PubMed]