

Article

The Sex-Specific Splicing of Doublesex in Brine Shrimp *Artemia franciscana*

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Abstract: The understanding of sex determination and differentiation in animals has recently made remarkable strides through the use of advanced research tools. At the gene level, the Mab-3-related transcription factor (*Dmrt*) gene family, which encodes for the typical DNA-binding doublesex/Mab-3 (DM) domain in their protein, is known for its contribution to sex determination and differentiation in insects. In this study, DNA-binding DM domain screening has identified eight transcripts from *Artemia franciscana* transcriptomic that encode proteins containing one conserved DNA-binding DM domain. The genome mapping confirmed that these eight transcripts are transcribed from six different loci on the *A. franciscana* genome assembly. One of those loci, the *Af.dsx-4* locus, is closely related to *Doublesex*, a gene belonging to the *Dmrt* gene family. This locus could be transcribed into three alternative transcripts, namely *Af.dsx⁴*, *Af.dsx^F* and *Af.dsx^M*. While *Af.dsx⁴* and *Af.dsx^F* could putatively be translated to form an identical *Af.dsx^F* protein of 186 aa long, *Af.dsx^M* translates for an *Af.dsx^M* protein of 289 aa long but shares a DNA-binding DM domain. Interestingly, *Af.dsx^F* and *Af.dsx^M* are confirmed as sex-specific transcripts, *Af.dsx^F* is only present in females, and *Af.dsx^M* is only present in male individuals. The results suggest that the sex-specific splicing mechanism of the *doublesex* described in insects is also present in *A. franciscana*. *Af.dsx-4* locus can be used in further studies to clarify the sex determination pathways in *A. franciscana*.

Keywords: *Artemia franciscana*; *Doublesex*; sex-specific splicing



Citation: Viet, D.N.; Christiaens, O.; De Vos, S.; Smagghe, G.; Bossier, P. The Sex-Specific Splicing of Doublesex in Brine Shrimp *Artemia franciscana*. *Genes* **2022**, *13*, 1997.

<https://doi.org/10.3390/genes13111997>

Received: 25 August 2022

Accepted: 24 October 2022

Published: 1 November 2022

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1. Introduction

The *Artemia* genus contains species with sexual as well as parthenogenetic reproduction modes. *Artemia* has a short life cycle, and is cultured easily under laboratory conditions. This species is being used as an animal model to discover genes involved in biological processes. *Artemia* has a WZ-ZZ sex-determining system which means that males are homogametic (ZZ), while females are heterogametic (ZW) [1,2] such as other crustacean species including *Cherax quadricarinatus* [3], *Penaeus monodon* [4], *Macrobrachium rosenbergii* [5] and *Eriocheir sinensis* [6]. In an effort to explore the molecular mechanism of sex determination in *Artemia*, a few molecular biology techniques have been employed to understand this mechanism. The Z-chromosome of *Artemia. franciscana* has recently been characterized by detecting genomic regions that show lower genomic coverage in female than in male samples, and regions that harbor an excess of female-specific SNPs [7]. Eight sex-linked AFLP marker alleles have been reported in *A. franciscana* that are inherited from the female parent [1]. Some genes related to sexual differentiation in *A. franciscana* have been identified and shown as sex-biased gene expression [8]. Masculinizer gene (*Ar-Masc*) was identified in *A. franciscana* [9], the MASC protein encoded in the Z chromosome activates a sex-determining gene that regulates gene splicing of *Doublesex* (*dsx*) into the

male-specific isoform in the silkworm *Bombyx mori* [10]. Until now, the sex-determining genes in *A. franciscana* have not yet been identified and no genetic sex-specific marker is reported.

Doublesex and Mab-3-related transcription factor (*Dmrt*) genes family are reported to be very highly conserved, as well as essential for sexual development in mammal, fish, and insects. In *Dmrt* genes family, *Dmrt1*, *Dmrt7*, and *Dmrt8* are only found in vertebrate; *dmrt2a/2b*, *dmrt3*, *dmrt4/5* and *dmrt93B* are commonly present in invertebrate bilateria; whereas *Dmrt1* and *dsx* have suggested independent evolution for sex determination and differentiation in the *Dmrt* gene family [11]. *Doublesex* bearing a doublesex/Mab-3 (DM) domain, regulates the sexual differentiation in *Drosophila melanogaster*, was the first *Dmrt* family member to be discovered [12]. *Dsx* and another two *Dmrt* genes including *Dmrt99B* and *Dmrt11E* could only be found in arthropod. *Dsx* gene is a critical transcription factor considered to be at the end of the sex determination cascade in many other insect species as a master gene in genotypic sex determination pathway. *Dmrt* genes family are involved in the formation of sex-linked traits including sexual behavior, gonadal development and sex-specific morphology in many insects [13]. In *D. melanogaster*, *dsx* gene is transcribed to produce a common primary transcript that is alternatively spliced and polyadenylated to yield male (*dsxM*) and female (*dsxF*) specific transcript [12]. *Dsx* and *Dmrt* have been characterized in a few crustacean species including *M. rosenbergii* [14–17], *M. nipponense* [18], *E. sinensis* [19], *F. chinensis* [20], *Litopenaeus. vannamei* [21], and four *Daphnia* species [22]. In *F. chinensis*, *Dsx* exhibited a sex-biased expression pattern in different tissues and its expression level increased along with developmental stages. Its binding site was identified on the promoter region of insulin-like androgenic gland gene [20]. Two *dsx* genes have been identified in *D. magna* (*DapmaDsx1* and *DapmaDsx2*), which are expressed in males but not in females. *DapmaDsx1* has been reported as a sex determination gene which is responsible for the male trait development [22]. Recently, *Sagmariasus verreauxi*, a heterogametic sex-linked *iDMY/iDmrt1* has been recently reported, in which the dominant negative suppression of *iDMY* over its autosomal *iDmrt1* paralogue might play an important key in the mechanism of determines sex this species [23]. These findings suggest the Doublesex and Mab-3-related transcription factor genes is good clues to look for the sex-determining genes in *Artemia*. In this study, the *Dsx/Dmrt* gene family is a target candidate for studying the sex determination mechanism in *A. franciscana*. We are looking for sex-specific splicing of *Dsx/Dmrt* gene family in the transcriptome database of *A. franciscana*. The expression profile of those genes was evaluated in both males and females using RT-PCR.

2. Materials and Methods

2.1. Animal Culture

A. franciscana cysts (Great Salt Lake, UT, USA) were hydrated in autoclaved seawater with strong aeration at 28 °C until hatching (approximately 20–24 h). The nauplii (larvae) were then transferred to a 5 L plastic tank with autoclaved seawater for rearing. The larvae were cultured at 28 °C in autoclaved seawater with aeration under fluorescent light and were fed with live marine microalgae *Tetraselmis suecica*. At 15–20 DAH (days after hatching), the juvenile females (before egg-sac formation) were identified by their brown-red color. At the juvenile stage, 20 individuals (10 males and 10 females) were analyzed separately to confirm sex-specific transcripts by RT-PCR. Adult males and females are morphologically completely different 20–25 DAH with the hooked grasper phenotype in males and the egg-sac in females. At the adult stage, pooled samples of the whole body of 10 adult females or 10 adult males were used to confirm sex-specific transcripts by RT-PCR.

2.2. Total RNA Extraction and cDNA Synthesis

The total RNA of the samples (the hydrated cysts, nauplii, juveniles and adults) was prepared using the RNeasy mini kit (Qiagen, Hilden, Germany). The total RNA was used to synthesize cDNA in a 20 µL reaction by using poly-T primer and RevertAid H Minus First Strand cDNA Synthesis Kit (Thermal Scientific, Waltham, MA, USA). The 20 µL of

reaction mixture contained 4 μL of 5X reaction buffer, 2 μL of 10 μM dNTPs mix, 20 units of ribonuclease inhibitor, 200 units of RevertAid H Minus M-MuLV Reverse Transcriptase, 1 μL of oligo-dT primer and 500 ng of total RNA. Subsequently, the reaction mixture was incubated for 70 min at 42 $^{\circ}\text{C}$. The reaction was terminated by heating at 70 $^{\circ}\text{C}$ for 5 min and then cooled to 4 $^{\circ}\text{C}$. Complementary deoxyribonucleic acids (cDNA) were then used as a template in PCR for further steps.

2.3. Identification of Putative of Dsx/Dmrt Transcripts

The primary amino acid sequences of *doublesex* from *Daphnia magna* were used for BLAST against the *A. franciscana* transcriptome database of the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Ghent, Belgium [24]. The identity of the selected hit contigs was subsequently verified using the BLASTX algorithm with default parameters on the nucleotide database of the National Center for Biotechnology Information (NCBI) [25]. Contigs matching Dsx/Dmrt gene family were selected for coding DNA sequence (CDS) analysis. The amino acid sequence of the selected contigs and open reading frame (ORF) was determined by the translation tool from the website Expasy (<http://web.expasy.org/translate> accessed on 26 January 2017). For contigs containing a full coding sequence (CDS), PCR primers were designed for isolation and sequencing. In contrast, for contigs containing only a partial CDS, their respective full-length cDNAs were identified by 3' and 5' rapid amplification of cDNA ends (RACE).

2.4. Isolation Full-Length of Dsx/Dmrt Transcript

Based on contigs containing only a partial CDS, gene-specific primers (Table 1) were designed to isolate the UTR (UnTranslated Region) using the SMARTer RACE Kit (Clontech, Palo Alto, CA, USA) with a modified protocol. The cDNA of each transcript from different lifecycle stages was used as a template for the PCR reaction to increase the chance of transcript amplification. Briefly, the 20 μL cDNA reaction for the isolation of the 5'UTR contained 4 μL 5X First-Strand Buffer, 0.5 μL DTT (100 mM), 1 μL dNTPs (20 mM) 5'-CDS Primer A, 10 μL total RNA, 1 μL of the SMARTer II A Oligonucleotide, 0.5 μL RNase Inhibitor (40 U/ μL) and 2 μL SMARTScribe Reverse Transcriptase (100 U). The mixture was incubated at 42 $^{\circ}\text{C}$ for 70 min. The cDNA reaction for the isolation of the 3'UTR followed the same protocol as used for the isolation of the 5'UTR, except that SMARTer II A Oligonucleotide was not added, and the 5'-CDS Primer was replaced by 3'-CDS Primer from the kit. Subsequently, a PCR reaction (50 μL) was carried out to amplify the UTR sequence: 1 μL cDNA (for 5' end UTR cDNA or 3' end UTR cDNA), 5 μL PCR buffer, 1 μL dNTPs (10 mM), 5 μL 10X UMP primer, 0.5 mM gene-specific primer (for 5'UTR or 3'UTR) and 0.25 μL DreamTaq DNA polymerase. The PCR thermal cycle was: denaturation for 3 min at 95 $^{\circ}\text{C}$, followed by 35 cycles of amplification; 30 s at 95 $^{\circ}\text{C}$; 30 s at 56 $^{\circ}\text{C}$; and 2 min at 72 $^{\circ}\text{C}$. The reaction was extended for 10 min at 72 $^{\circ}\text{C}$ and then cooled to 4 $^{\circ}\text{C}$. The UTR PCR products were purified from agarose gel using the Wizard[®] SV gel and PCR Clean-up System (Promega, Madison, WI, USA) for subsequent, direct sequencing. The full length of the UTR sequence was assembled by Vector NTI software (Invitrogen, Waltham, MA, USA). For mapping analysis, the full length of the isolated transcript was mapped to the genome of *A. franciscana* (Unit of Polar Genomics, Korea Polar Research Institute, version 1.0, http://antagen.kopri.re.kr/project/genome_info_iframe.php?Code=AF01 accessed on 26 June 2022) using BLASTN with default parameters.

Table 1. PCR primers used to confirm the Dsx/Dmrt transcripts of *A. franciscana*.

Genes	Gene-Specific Primers (5'-3')	Size in bp
Doublesex-1 (Dmrt93B)	For: ATCCTTTGGGACAATGGATGTTACACTC Rev: CTTTCATAGTGTGTCAAAAACGCGC	903
Doublesex-2 (Dmrt99B)	For: TGGGAGAGAGACAGGAATGTAAATACATAC Rev: GCAGCTCTGAGTGATACCAAAAATTAAG	1459

Table 1. Cont.

Genes	Gene-Specific Primers (5'-3')	Size in bp
Doublesex-3	For: ATATGAAGTGAGCAATGGTGATACGC Rev: CGAATTCGTTAATCACACATTAAGGCCAC	1169
Af.dsx-4	Af.dsx ^F -For: TCTTCAACCATGAAAACAAACAGGCATCTACC Af.dsx ^F -Rev-A: ATGTTTTCTGGTGTCTTCTCTGTACAAC	920
	Af.dsx ⁴ -For: TCTTCAACCATGAAAACAAACAGGCATCTACC Af.dsx ⁴ -Rev-B: AGAAAGAGATGGTGGAAATTGC	1010
	Af.dsx ^M -For: AGTTCGGTTTGTGGTTCCTCACGG Af.dsx ^M -Rev-C: CAGGCCAATTTTTGAATATTATCTAGAAGC	1124
Doublesex-5	For: CTAATCTTACACCTAAGTGCCTGAG Rev: GCAACGATTGAAGAGAAATGGGAAAGGATC	918
Doublesex-6 (Dmrt11E)	For: GATTAACGAACTAAGGCCAGAGAGA Rev: CACAAAGAACGAAAATAAGAACTAACACAC	966
Tubulin	For: GCAGTGGTCTACAAGGTTTC Rev: TGCATTGACGTCTTTTGGTACGACATCTC	605

2.5. Phylogenetic Analysis

The phylogenetic tree was generated using website NGPhylogeny (<https://ngphylogeny.fr>, accessed on 17 September 2022), a phylogeny software based on the maximum-likelihood [26], wherein the usual bootstrapping procedure is replaced by a fast approximate likelihood ratio test (aLRT), which is proven to be a good alternative to the (time-consuming) bootstrap analysis. The putative polypeptide sequence from the isolated transcripts and known polypeptide sequences of homologs from NCBI were introduced under FASTA format.

2.6. Transcription Proof of Dsx/Dmrt Transcript

Gene-specific PCR primers was designed for each *Dsx/Dmrt* gene to amplify the complete transcript (Table 1). The applied PCR conditions were the same for all genes in this study. In addition, the PCR reaction contained 150 ng cDNA, 5 µL reaction buffer, 1 µL dNTPs (10 mM), 0.5 mM gene-specific primers, (Table 1) and 0.25 µL DreamTaq DNA polymerase (Thermal Scientific, Waltham, MA, USA). The PCR reaction was carried out in a thermal cycler (2720 Thermal Cycler (Thermal Scientific, Waltham, MA, USA) programmed for 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 5 min; followed by 72 °C for 10 min. The amplicons were then separated on agarose gel and cleaned using Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). These cleaned amplicons were used directly for sequencing by the Sanger sequencing method using the same PCR primers.

3. Results

3.1. Identification of Dsx/Dmrt Transcripts

Six contigs encoding for polypeptides with a Doublesex/Mab-3 domain [27] were found in the *A. franciscana* transcriptome. These contigs were named doublesex-1, doublesex-2, doublesex-3, doublesex-4, doublesex-5 and doublesex-6. Based on the nucleotide sequence, only doublesex-3, doublesex-5 and doublesex-6 contained a full CDS encoding for the full putative polypeptide of the protein, while the others contained only a partial CDS. Therefore, the full doublesex-1, doublesex-2, and doublesex-4 cDNA sequences were then identified by the RACE method. Interestingly, RACE results revealed three different isoform from the doublesex-4 contig, namely doublesex-4 α isoform A of 920 bp, doublesex-4 α isoform B of 1010 bp; and doublesex-4 β isoform C of 1156 bp. Their nucleotide sequences were submitted to NCBI GenBank as doublesex-1 (*dsx-1*) (MF287957), doublesex-2 (*dsx-2*) (MF287958), doublesex-3 (*dsx-3*) (MF287959), doublesex-4 α isoform A (*dsx-4 α A*) (MF287960), doublesex-4 α isoform B (*dsx-4 α B*) (MF993793), doublesex-4 β isoform C (*dsx-4 β C*) (MF287961), doublesex-5 (*dsx-5*) (MF287962) and doublesex-6 (*dsx-6*) (MF287963).

The presence of the isolated Dsx/Dmrt transcripts were confirmed in adult *A. franciscana* animals by PCR method. The sex specific PCR analysis was conducted on cDNA from whole body RNA extraction of pooled male or female adults showed the presence

of *Dsx-1*, *Dsx-2*, *Dsx-3*, *Dsx-5* and *Dsx-6* transcripts in both sexes (Figure 1A). However, *dsx-4βC* transcript could only be seen in the pooled males, while *dsx-4αA* transcript could only be found in the pooled females. Interestingly, *dsx-4αB* transcript was present in both sexes (Figure 1B).

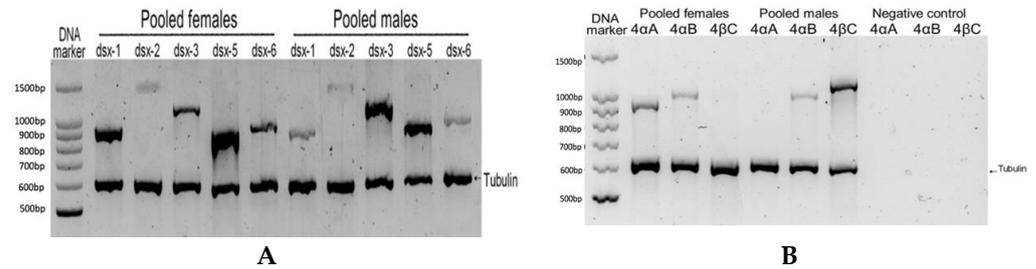


Figure 1. The evidence of transcription of *Dsx/Dmrt* genes from the cDNA samples of the pooled adult females and pooled adult males in *A. franciscana*. (A): The presence of *dsx-1*, *dsx-2*, *dsx-3*, *dsx-5* and *dsx-6* transcripts by semi-quantitative PCR (tubulin was used as the internal control). (B): The alternative transcript of *A. franciscana Af.dsx-4*, including *dsx-4αA*, *dsx-4αB* and *dsx-4βC*, confirmed the presence by PCR from the cDNA of the pooled adult animals.

3.2. Genome Mapping and Putative Protein Translation

The nucleotide sequence BLAST results of isolated *Dsx/Dmrt* transcripts against the *A. franciscana* genome assembly (NCBI Bioproject PRJNA589114) confirmed these transcripts are transcribed from different loci on six different scaffolds. These loci encoded putatively different DSX proteins bearing one DM domain [28,29]. Among those, the DSX protein that is translated by *dsx-2* transcript beared an additional ubiquitin—associated domain (UBA domain) at its C-terminal, which is a common domain found in DSX proteins in insect species [28]. Genome mapping of the full-length of three transcripts from putative *dsx-4* contig to the genome of *A. franciscana* revealed a DNA scaffold (scaffold901_size138921) bearing an intact locus, namely *Af.dsx-4* locus, which contained six different exons and its arrangement on this scaffold. The organization of six exons on *Af.dsx-4* locus and the sequence of *dsx-4αB*, *dsx-4αA*, and *dsx-4βC* transcripts suggest that *Af.dsx-4* locus could produces three alternative transcripts including *dsx-4αB*, *dsx-4αA*, and *dsx-4βC* as namely *Af.dsx4*, *Af.dsx^F* and *Af.dsx^M*, respectively. Therefore, these three transcripts of *Af.dsx-4* locus are a probable consequence of a splicing event (Figure 2).

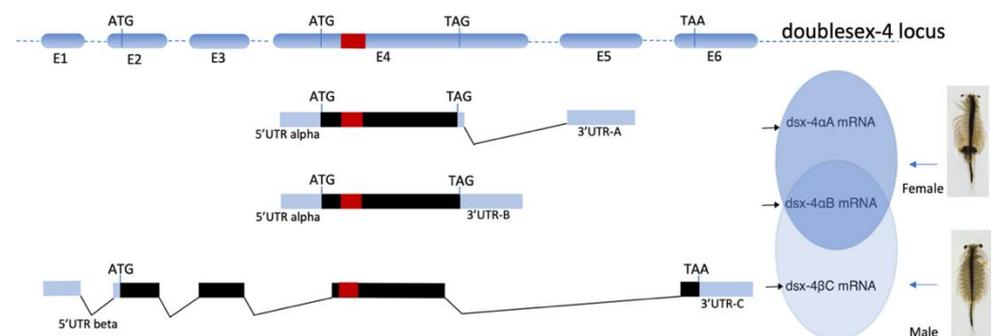


Figure 2. The alternative splicing model of the *Af.dsx-4* locus in *A. franciscana*. E1 to E6 stand for six exons on the DNA scaffold containing the *Af.dsx-4* locus. The red bar indicates the location of the DM domain. The black bar is the open reading frame of *Af.dsx-4*, starting at ATG and stopping at TAG or TAA. The *dsx-4αA* (*Af.dsx^F*) is female-specific splicing, while the *dsx-4βC* (*Af.dsx^M*) is male-specific splicing. The *dsx-4αB* (*Af.dsx⁴*) is present in males and females.

The open reading frame prediction of *Af.dsx^F* and *Af.dsx⁴* transcripts showed that they only contained different 3'UTR sequences which are namely 3'UTR-A and 3'UTR-B, respectively. Therefore, they are predicted to encode for an identical putative polypeptide of 186 aa long, namely *Af.DSX-F*. In contrast, the *Af.dsx^M* transcript is predicted to encode

a polypeptide of 289 aa long, namely Af.DSX-M. The sequence at the 5' end (consisting of a 5' untranslated region (5'UTR) and a partial CDS encoding for the N-terminal) of Af.dsx^M and Af.dsx^F transcripts are completely different sequences. Additionally, the Af.dsx^M transcript had a different 3'UTR sequence, namely 3'UTR-C, in comparison to the 3'UTR-A and 3'UTR-B sequence of Af.dsx^F and Af.dsx⁴ transcripts, respectively. The Af.dsx⁴, Af.dsx^F and Af.dsx^M transcripts shared a sequence of 477 nucleotides encoding a polypeptide, including the DM domain. The polypeptide alignment suggested that Af.DSX-M is probably an isoform of Af.DSX-F but bears an extended polypeptide at its N-terminal (Figure 3).

Af .DSX-M	MVIQTLKHRADRERMEEIQQTQAIIVTSSTEKTTNESNSNSAAPDSVTEYAGSGHVQOMP	60
Af .DSX-F	-----	0
Af .DSX-M	SASHTYTDASYSPEPRDLSRKRRESDENEDDIQIKIGNMSNEKDDESLSDM--GDMSD	118
Af .DSX-F	-----MNRRIFEKYPDFPIIPGFI	19
	*.: *.: *.:	
Af .DSX-M	AGSQGDKMGKMRMPTCARCRNHGQVVKLRGHKRYCSFRHCLCDRCALTSEKQRVMAAQVA	178
Af .DSX-F	SGSQGDKMGKMRMPTCARCRNHGQVVKLRGHKRYCSFRHCLCDRCALTSEKQRVMAAQVA	79
	:*****	
Af .DSX-M	LRRAQKQDEENGIVRPVPIPKRPEIYPPPRIPSPPSAPLPLQLPIPPQPLNYIDSYLK	238
Af .DSX-F	LRRAQKQDEENGIVRPVPIPKRPEIYPPPRIPSPPSAPLPLQLPIPPQPLNYIDSYLK	139

Af .DSX-M	FSLLSMKDELKLGHEHNPLLYLAYSFVGWDRHRLIKELHVARNSLVKLGLL	289
Af .DSX-F	FSLLSMKDELKLGHEHNPLLYLAYSFVGWDRHRLIKELHVGKLGSSS----	186
	*****.: .	

Figure 3. The alignment of primary polypeptide *A. franciscana* Af.DSX-F and Af.DSX-M. The box indicates the DM domain sequence; the asterisks indicate the conserved amino acids.

3.3. Phylogenetic Tree

Eight transcripts were identified encoding seven putative DSX proteins in *A. franciscana*. The polypeptide phylogenetic tree showed that *A. franciscana* DSX-1, DSX-2 and DSX-6 are related to DMRT93B, DMRT99B and DMRT11E protein from crustaceans *D. magna*, *E. sinensis*, *M. rosenbergii* and *S. verreauxi*. In contrast, putative *A. franciscana* DSX-3, Af.DSX-F, Af.DSX-F and DSX-5 polypeptides are closely related to the sex-specific DSX proteins cluster (Figure 4).

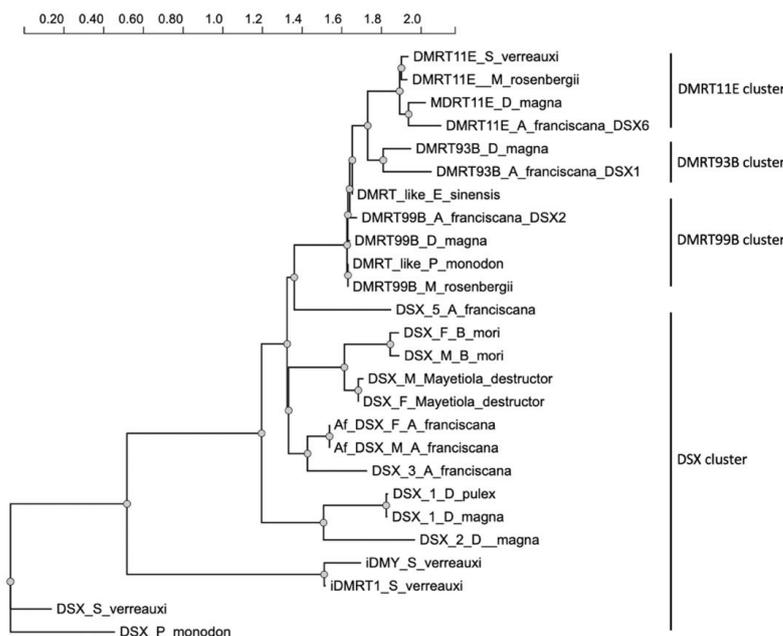


Figure 4. The DSX/DMRT protein phylogenetic tree of *Artemia franciscana* and other arthropods. The phylogeny tree was generated using NGPhylogeny (a free web service) [26].

3.4. Sex-Specific Splicing of Doublesex Gene

The presence of sex-specific transcripts originating from *Af.dsx-4* locus was searched on *A. franciscana* individuals, which were reared separately from the juvenile until the adult stage (full maturity). The sex-specific PCR analysis of *Af.dsx^F* and *Af.dsx^M* transcripts at the juvenile stage was performed on twenty juvenile individuals separately. It again confirmed that *Af.dsx^F* and *Af.dsx^M* were female and male-specific transcripts, respectively (Figure 5).

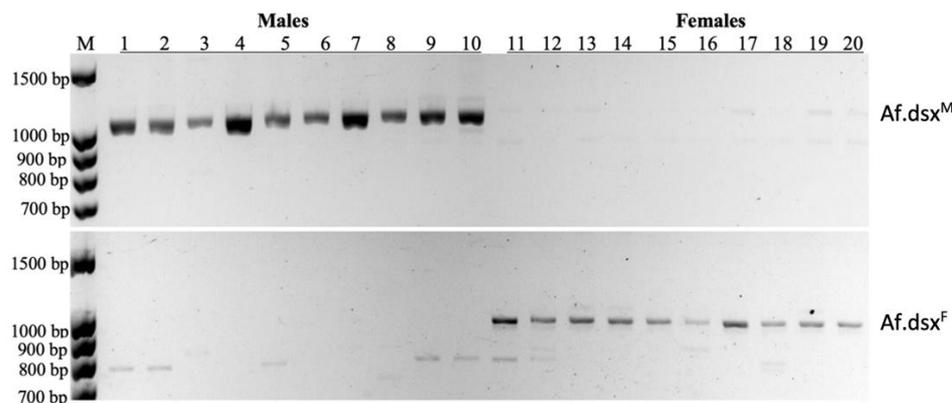


Figure 5. The presence of sex-specific transcript encoded by *Af.dsx-4* locus in *A. franciscana*. The sex-specific transcripts of *Af.dsx^F* and *Af.dsx^M* as determined by PCR from the cDNA samples of 20 juvenile animals individually. The amplification PCR products of *Af.dsx^M* transcript could only be seen in the male individuals (lanes 1 to 10), while *Af.dsx^F* transcript could only be found in female individuals (lanes 11 to 20). M: DNA marker.

4. Discussion

Extensive studies of the sex determination mechanism in invertebrate animal models such as *D. melanogaster* have shown that sex is determined in the embryonic stage by the involvement of so-called sex-determining genes. Particularly, *dsx* genes have been identified in many insects to be linked to the sex determination pathway [13]. In *A. franciscana*, the available RNAseq databases suggest that this species has many genes that are similar to sex-determining genes in fruit fly [30]. In *D. magna*, the transcription of *dmrt11E* and *dmrt99B* is higher in ovaries than in the testes [31]. In *M. rosenbergii*, the transcription of both *dmrt11E* and *dmrt99B* is found in spermatogonia and spermatozoa during spermatogenesis [15]. In *D. magna*, the *dmrt93B* is detected only in the testes, and not in the ovaries [31]. Based on polypeptide phylogenetic analysis, *A. franciscana* DSX-1, DSX-2 and DSX-6 are very close to DMRT93B, DMRT99B and DMRT11E, respectively, thus they are hypothesized to play a similar role in development of gonadal tissue in *Artemia*.

In insects, sex-specific splicing generates sex-specific transcripts from the sex-specific *dsx* gene controlling sexually-dimorphic traits. The disruption of *dsx* gene function produces either male-specific sexually-dimorphic defects or intersexual phenotypes [10]. Sex-specific *dsx* splicing in *B. mori* depends on the presence of MASC protein in the embryo to promote the production of the male-specific splicing variants [32]. Recently, a *masc* gene has been characterized in *A. franciscana*. *Masc* RNAi during embryonic development of sexual *A. franciscana* could slightly change the ratio of females/males [9]. This suggests that *A. franciscana* probably also has a similar sex determining mechanism in which the splicing of *dsx* plays an important role in sex determination. In this study, the RACE method has not been applied on *A. franciscana dsx-3* and *dsx-5*, thus potential variant transcripts of these two genes remain unknown. However, three different transcripts of *Af.dsx-4* locus were isolated in *A. franciscana* suggesting that a specific splicing mechanism of *dsx* may exist in *A. franciscana*. In crustaceans, up to now, evidence for alternative splicing of the *dsx* gene could only be found in *D. magna*, involved in environmental sex determination. Doublesex-1 of *D. magna* is identified to directly determine the gender via the splicing at 5' end of mRNA to produce doublesex-1 α or doublesex-1 β transcript. Therefore, they

have different 5'UTR sequences in their structural transcript, which leads to the control of the translation of key regulator protein of the male phenotype [22,33]. In *A. franciscana*, the *Af.dsx^M* isoform has a different 5'UTR sequence in comparison to the *Af.dsx^F* and *Af.dsx⁴* isoforms, thus this may influence the efficient translation to corresponding protein. In comparison to the *AF.DSX-F* primary polypeptide sequence, *AF.DSX-M* has a longer N-terminal sequence, this difference could affect the formation of the dimer structure that is needed for its function [34].

Moreover, the transcription of *Af.dsx^M* could only be found in male animals, and *Af.dsx^F* was only present in female animals (Figure 5). This reinforces the hypothesis that sex-specific transcripts of *Af.dsx-4* are related to sex determination in *A. franciscana*. Interestingly, *Af.dsx⁴* was found present in both sexes (Figure 1B) and since *Af.dsx^F* and *Af.dsx⁴* probably encode for the same *Af.DSX-F* protein, females are predicted to have only *Af.DSX-F* homodimer. In contrast, the male has both *Af.dsx^M* and *Af.dsx⁴*, therefore their *Af.DSX-F* and *Af.DSX-M* proteins probably are present together, resulting in three different types of protein dimer (*Af.DSX-F* homodimer, *Af.DSX-M* homodimer and *Af.DSX-F/Af.DSX-M* heterodimer). In *B. mori*, the complication of cis- and trans-spliced transcripts of *dsx* has also been reported to generate seventeen alternatively spliced forms and eleven putative trans-spliced variants. However, all transcripts encode for only four female-specific, two male-specific *DSX* proteins and one *DSX* protein common to males and females [35,36]. Therefore, the alternative splicing of *Af.dsx-4* locus may need to be investigated further to unravel its precise role in sex determination.

5. Conclusions

Six *Dsx/Dmrt* gene family loci were identified on different scaffolds, and their transcripts were sequenced from a cDNA sample of *A. franciscana* in this study. The expression profiles showed that these genes are all expressed in both sexes except the *Af.dsx-4* locus. We determined that the *Af.dsx-4* locus potentially transcribes three different isoforms, including *Af.dsx^M*, *Af.dsx^F* and *Af.dsx⁴*, probably due to a splicing event. *Af.dsx^F* and *Af.dsx⁴* transcripts are found only in female animals, whereas *Af.dsx^M* and *Af.dsx⁴* transcripts are detected in male animals. These three transcripts of *Af.dsx-4* locus putatively encode *Af.DSX-F* proteins and *Af.DSX-M* protein, which is characterized by large differences in amino acid sequence at the N-terminal and minor changes at the C-terminal. Herein, *Af.dsx-4* is a suitable candidate for further investigations into sex determination in *A. franciscana*.

Author Contributions: D.N.V.: Methodology, Investigation, Validation, Writing—Original Draft; O.C.: Supervision, Review & Editing; S.D.V.: Resources, Review & Editing; G.S. and P.B.: Review & Editing, Project administration. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Vietnam International Education Development.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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