Comparative Genomics Studies on the dmrt Gene Family in Fish

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Doublesex and mab-3-related transcription factor (dmrt) genes are widely distributed across various biological groups and play critical roles in sex determination and neural development. Here, we applied bioinformatics methods to examine cross-species changes in the dmrt family members and evolutionary relationships of the dmrt genes based on genomes of 17 fish species. All the examined fish species have dmrt1–5 while only five species contained dmrt6. Most fish harbored two dmrt2 paralogs (dmrt2a and dmrt2b), with dmrt2b being unique to fish. In the phylogenetic tree, 147 DMRT are categorized into eight groups (DMRT1–DMRT8) and then clustered in three main groups. Selective evolutionary pressure analysis indicated purifying selections on dmrt1–3 genes and the dmrt1–3–2(2a) gene cluster. Similar genomic conservation patterns of the dmrt1–dmrt3–dmrt2(2a) gene cluster with 20-kb upstream/downstream regions in fish with various sex-determination systems were observed except for three regions with remarkable diversity. Synteny analysis revealed that dmrt1, dmrt2a, dmrt2b, and dmrt3–5 were relatively conserved in fish during the evolutionary process. While dmrt6 was lost in most species during evolution. The high conservation of the dmrt1–dmrt3–dmrt2(2a) gene cluster in various fish genomes suggests their crucial biological functions while various dmrt family members and sequences across fish species suggest different biological roles during evolution. This study provides a molecular basis for fish dmrt functional analysis and may serve as a reference for in-depth phylogenomics.

Keywords: fish, comparative genomics studies, dmrt genes, phylogenetic evolution, synteny analysis

INTRODUCTION

Doublesex and mab-3-related transcription factor (dmrt) genes are originally homologous to Doublesex (Dsx) in Drosophila melanogaster and Male abnormal 3 (Mab-3) in Caenorhabditis elegans, both of which play important roles in sex determination (Burtis and Baker, 1989; Zhu et al., 2000; Zarkower, 2001). In recent years, a large number of genes from the dmrt family have been identified from lower invertebrates to higher vertebrates, including corals, nematodes, fruit flies, frogs, fish, birds, and mammals, some of which have been confirmed to be related to sex differentiation (Hodgkin, 2002). Currently, in addition to Dsx and Mab, the dmrt family in...
vertebrates include nine dmrt genes (dmrt1–8 and dmrt2b) that share common characteristics with Dsx and Mab-3. Almost all of the encoded polypeptide chains contain a conserved DNA-binding motif, known as the Doublesex and Mab-3 (DM) domain, which is composed of six conserved cysteines and two histidines (locus 1 of CCHC and locus 2 of HCCC). Both loci form two highly intertwined zinc-finger-like DNA-binding regions can bind to the minor groove in DNA. Notably, this domain is highly conserved among organisms of different evolutionary types (Erdman and Burris, 1993; Zhu et al., 2000).

Fish dmrt genes were first discovered in Nile tilapia (Oreochromis niloticus) and rainbow trout (Oncorhynchus mykiss) (Guan et al., 2000; Marchand et al., 2000). These genes in the dmrt family have now been identified in more than 30 fish species. Seven dmrt genes have been found in fish, including dmrt1–6 and dmrt2b. DMRT1 plays an important role in sex determination and testicular development (Matson and Zarkower, 2012), except the DM-W gene, a DMRT1 W-linked paralog in Xenopus laevis, play the opposite roles in primary ovary development (Yoshimoto et al., 2010). DMRT1 is specifically expressed only in the embryonic genital ridge and adult testes of human males, and is related to the expression of sex-determining genes and differentiation of primordial germ cells (Raymond et al., 1998; Moniot et al., 2000; Matson et al., 2011). Alternatively, studies on more than 20 fish species have determined that fish dmrt1 expression is related to male development regardless of the various sex determination mechanisms (Kobayashi et al., 2004, 2008; Johnsen et al., 2010), indicating that dmrt1 plays a key role in male germ cells self-renewal and differentiation, testicular development and spermatogenesis of fish (Herpin and Scharl, 2011; Lin et al., 2017). Furthermore, in the medaka Orzias latipes, a Y-specific dmy gene, copy of autosome dmrt1, is the master sex-determining gene inducing male formation too.

The genomes of amphibians, reptiles, birds, and mammals contain only a single dmrt2 gene, whereas fish harbor two dmrt2 genes (dmrt2a and dmrt2b) (Liu et al., 2009; Su et al., 2015; Lyu et al., 2019). DMRT2 is widely distributed in the tissues of mammals and fish, and is expressed in both testes and ovaries (Kim et al., 2003; Winkler et al., 2004; El-Mogharbel et al., 2006). However, the function of DMRT2 has not been conserved during the evolution of species (Meng et al., 1999; Seo et al., 2006). For example, mouse DMRT2 is mainly involved in somite differentiation, in particular the patterning of the axial skeleton system (Lourenco et al., 2010). In contrast, both zebrafish dmrt2a and dmrt2b are involved in somite development, of which dmrt2a is necessary for symmetric somite formation and fast muscle differentiation (Saude et al., 2005; Lu et al., 2017), and dmrt2b regulates asymmetric organ positioning via the Hedgehog signaling pathway and therefore it is related to branchial arch and slow muscle development (Zhou et al., 2008; Li et al., 2018). This indicates that differences exist in the expression and functionality of dmrt2a and dmrt2b in fish.

Mammalian DMRT3 is highly expressed in the testis but not in the ovary; hence, it may be related to testicular differentiation and development (Hong et al., 2007). In mice, DMRT3 is also expressed in numerous non-gonadal tissues such as the embryonic forebrain and olfactory placode, in addition to spinal cord neurons, and thus it may be involved in neuronal specification (Smith et al., 2002; Kim et al., 2003; Andersson et al., 2012). Fish Dmrt3 is highly expressed in the testis and nervous system, and has accordingly been speculated to play a role in the developmental processes of the nerves and germ cells (Yamaguchi et al., 2006; Li et al., 2008; Dong et al., 2010).

The mouse dmrt4 gene is expressed in the testis and ovary, in addition to other various tissues (Kim et al., 2003). It can regulate the formation and development of ovarian follicles (Balciuniene et al., 2006). Alternatively, Xenopus DMRT4 is involved in the regulation of neurogenesis in the olfactory system (Huang et al., 2005b). In some fish species, the expression of dmrt4 in the ovary is significantly higher than that in the testis (Guan et al., 2000; Su et al., 2013; Wang, 2013); in other species, its expression is significantly higher in the testis than the ovary (Kondo et al., 2002; Dong and Chen, 2013; Sheng et al., 2014), whereas yet other species show high expression in both organs (Yamaguchi et al., 2006). In addition, dmrt4 is also expressed in the spleen (Yamaguchi et al., 2006; Sheng et al., 2014), kidney (Kondo et al., 2002; Sheng et al., 2014), gills (Kondo et al., 2002; Wang, 2013), and brain (Dong and Chen, 2013) in fish. Hence, it has been speculated to be related to immune and nervous system development.

Mouse DMRT5 is mainly expressed in brain tissue and is necessary for the early embryonic development of the cerebral cortex (Veith et al., 2006a; Konno et al., 2012). As a novel neurogenic factor, DMRT5, together with DMRT3, jointly controls hippocampal development and neocortical area map formation (Muralidharan et al., 2017; De Clercq et al., 2018). Fish dmrt5 is highly expressed primarily in the brain but can also be found in the gonads, eyes, and pituitary gland (Guo et al., 2004; Veith et al., 2006a; Yamaguchi et al., 2006; Gu et al., 2019). Furthermore, dmrt5 plays a key role in zebrafish neurogenesis in the telencephalon (Yoshizawa et al., 2011) and can regulate corticosterone and gonadotrope differentiation in the pituitary (Graf et al., 2015), in addition to spermatogenesis (Xu et al., 2013).

Mammalian DMRT6 is mainly expressed in gonadal intermediate cells and differentiating spermatogonia. It plays a crucial role in coordinating the transition of primordial germ cells from the mitotic to meiotic developmental programs during spermatogenesis (Zhang X.et al., 2014) and is also expressed in the embryonic brain of mice (Kim et al., 2003). Early studies have suggested that the dmrt6 gene is missing in fish (Veith et al., 2006b). However, recent studies have found that certain fish, such as coelacanth, tilapia, and Southern catfish also carry the dmrt6 gene, and that tilapia dmrt6 is involved in spermatogenesis (Forconi et al., 2013; Zhang X.et al., 2014). However, DMRT7 and DMRT8 are only present in mammals. The two genes are very similar, although DMRT8 does not have a complete DM domain. DMRT7 is specifically expressed in the male and female gonads and is related to mouse gonadal development and spermatogenesis (Kawamata and Nishimori, 2006; Hong et al., 2007). In comparison, DMRT8 is highly expressed in the male gonads and may have evolved from DMRT7 (Ottolenghi et al., 2002; Veith et al., 2006a).
Currently, reports are only available regarding the phylogenetic analysis of pan-arthropod and pan-metazoan DMRT family members (Volff et al., 2003; Wexler et al., 2014; Panara et al., 2019); to our knowledge, no studies have yet been published on the phylogeny of fish dmrt family. However, fish comprise a wide variety of species and previous reports have shown that members of the fish dmrt family own unique features such as two paralogs of dmrt2 genes (dmrt2a and dmrt2b), in addition to diverse tissue expression of the same gene family member in various fish (e.g., dmrt4), thus suggesting a remarkable difference in function. As the sequences of DMRT family members are highly variable with only the DM domain [~49 amino acids (aa)] exhibiting high sequence homology (Volff et al., 2003), it is difficult to accurately determine the evolutionary relationship among the family members based on such short sequences, which in turn has limited our understanding of the history of DMRT functional development.

Nevertheless, in recent years the whole-genome sequencing of many fish species has significantly facilitated the in-depth and systematic analysis on the evolutionary relationships among gene family members. In this study, we therefore employed the fine genomic map of largemouth bass recently obtained using third-generation sequencing by our team and collected the dmrt sequences of 16 fish species with different taxonomic positions from published whole-genome sequences, in order to analyze the sequence structure, phylogenetic relationship, sequence conservation, and synteny of members of the fish dmrt family. These findings will lay a solid foundation for a more systematic understanding of the structural characteristics of these members in fish dmrt family, and for further investigations into the different functions of fish dmrt family members in sex determination or differentiation along with their underlying mechanisms.

MATERIALS AND METHODS

Sequence Collection
In the present study, we employed two strategies to collect nucleotide or deduced amino acid sequences for dmrt family members in various vertebrates (Supplementary Table S1). For those with publicly available sequences, such as in human (Homo sapiens) and mouse (Mus musculus), we downloaded the sequences from NCBI or Ensembl (Supplementary Table S2). Other dmrt sequences were extracted from corresponding genome databases through BLAST (Altschul et al., 1990) and Genewise (Birney et al., 2004).

In brief, we used zebrafish (Danio rerio), Japanese medaka (Oryzias latipes), and mouse DMRT protein sequences from NCBI as the references, and mapped them onto the examined genomes using tBLASTn with an E-value <1e−5 and an alignment rate >0.6. Solar v0.9.6 was applied to connect high-identity segment pairs. Subsequently, we discarded those low-quality results with alignment rate <0.6 and mapping identity <0.5. Finally, each gene sequence was predicted on the target genomic region using Exonerate v2.2.0 (Slater and Birney, 2005), and extended 5 kb in the upstream and downstream directions to obtain the integrated gene model. A total of 147 dmrt sequences were derived from 23 representative vertebrate species, including 2 mammals (human and mouse), 2 birds (chicken Gallus gallus and zebra finch Taeniopygia guttata), 1 reptile (green Anole Anolis carolinensis), 1 amphibian (Western clawed frog Xenopus tropicalis), and 17 fish species belonging to two classes (Actinopterygii and Sarcopterygii), and ten superorders (Percomorpha: largemouth bass, Asian sea bass Lates calcarifer, European sea bass Dicentrarchus labrax, Japanese pufferfish Takifugu rubripes, Chinese tongue sole Cyprinus carpio semilaevis, and threespine stickleback Gasterosteus aculeatus; Atherinomorpha: Japanese medaka and southern platyfish Xiphophorus maculatus; Proteocephalopterygii: Atlantic cod Gadus morhua; Ostariophys: channel catfish Ictalurus punctatus and electric eel Electrophorus electricus; Clupeomorpha: Atlantic herring Clupea harengus; Elopomorpha: Japanese eel Anguilla japonica; Osteoglossomorpha: Asian arowana Scleropages formosus; Holostei: spotted gar Lepisosteus oculatus; Coelacanthiformes: African coelacanth Latimeria chalumnae).

Sequence Alignment and Phylogenetic Analysis
We performed phylogenetic analysis on these collected dmrt sequences. MAFFT v7.273 (Katoh et al., 2002) was employed to align these sequences. Gblocks was used to find conserved fragments with the following parameter settings: minimum number of sequences for a conserved/flank position (75/75), maximum number of contiguous non-conserved positions (50), minimum length of a block (50), allowed gap positions (all). ProtTest v3.4.2 was operated to determine the best-fit models of amino acid replacement (Darriba et al., 2011). Based on the Akaike Information Criterion (AIC) algorithm, we set the best-fit model as “JTT+I+G+F.” Finally, we utilized PhyML 3.0, MrBayes v3.24 7, and MEGA v7.0 8 to analyze these sequences.

Identification of Conserved Synteny for the dmrt1–dmrt3–dmrt2(2a) Gene Cluster (Synteny Analysis)
To evaluate the conservation of the dmrt1–dmrt3–dmrt2(2a) gene cluster, we explored conserved genes in the upstream and downstream regions (20 kb) within the genomes of 19 examined species, using zebrafish genomic sequence as the reference, since the zebrafish genome is currently the best fish genome assembly with the highest quality and the complete genome annotation. These examined genome assemblies were explored using tBLASTn (Altschul et al., 1990), and the best-fit results were selected using a Perl script and Adobe Illustrator.

Substitution Rate Estimation and Comparison (Ka/Ks Analysis)
We calculated the average non-synonymous substitutions (Ka), synonymous substitutions (Ks), and Ka/Ks among dmrt1, dmrt2(2a), dmrt3, and the dmrt1–dmrt3–dmrt2(2a)
gene cluster to test the selective pressure at the codon-based sequence level among various species. First, we aligned dmrt gene sequences from each species to spotted gar (L. oculatus; as the reference sequence) by using Prank v100802 with the “-codon” model (Loytynoja and Goldman, 2005). Subsequently, we calculated the Ka, Ks, and Ka/Ks values of each pair using Ka/Ks Calculator v2.0 with four different algorithms, including gMYN (Wang D.P.et al., 2009), gYN (Wang D.et al., 2009), MYN (Zhang et al., 2006), and YN (Yang and Nielsen, 2000).

Analysis of Regulatory Regions and Cross-Species Comparisons of the dmrt1–dmrt3–dmrt2(2a) Gene Cluster

Complete genomic sequences with 20 kb-upstream/downstream regions of the dmrt1–dmrt3–dmrt2(2a) gene cluster were extracted from various species. We applied mVISTA (Frazer et al., 2004) to align these relevant genomic sequences. This tool can align and compare long sequences based on the window-based comparisons of sequence conservation. Repetitive elements were annotated using RepeatMasker v4.06 software (Chen, 2004), and the zebrafish genomic sequence was used as the reference. Pair-wise sequence comparisons were determined with a threshold of 70% identity in each 50-bp window. In addition, five typical regulatory elements, including BRE, CAAT box, E box, GC box, and TATA box, were predicted in each sequence using a Perl script (the motif function in Primer 5.0 and Genomatix MatInspector). Finally, Adobe Illustrator and R were applied to produce graphs for the information obtained.

RESULTS

Cross-Species Changes in dmrt Family Members and Copy Numbers

A total of 147 dmrt sequences were derived from 23 representative vertebrate species (Table 1 and Supplementary Tables S1, S2). Among them, 128 dmrt sequences for 17 species were downloaded from the

| Class               | Superorder | Species      | Common name | dmrt1 | dmrt2 | dmrt3 | dmrt4 | dmrt5 | dmrt6 | dmrt7 | dmrt8 |
|---------------------|------------|--------------|-------------|------|------|------|------|------|------|------|------|
|                     |            |              |             |      |      |      |      |      |      |      |      |
| Mammalian           | –          | H. sapiens   | Human       | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
|                     | –          | M. musculus  | Mouse       | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
| Birds               | –          | G. gallus    | Chicken     | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
|                     | –          | T. guttata   | Zebra finch | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | -    |
| Reptilia            | –          | A. carolinensis | Anole lizard | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
| Amphibia            | –          | X. tropicalis | Clawed frog | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
| Fish (Actinopterygii)| Percomorpha | M. salmoides | Largemouth bass | 1   | 2    | 1    | 1    | 1    | 1    | -    | -    |
|                     |            | L. calcarifer | Asian seabass | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | 1*   | -    |
|                     |            | D. labrax     | European seabass | 1   | 2    | 1    | 1    | 1    | 1    | -    | -    |
|                     |            | T. rubripes   | Japanese pufferfish | 1* | 2*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
|                     |            | C. semilaevis | Tongue sole | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
|                     |            | G. aculeatus  | Stickleback | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
|                     | Atherinomorpha | O. latipes | Japanese medaka | 1* | 2*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
|                     |            | X. maculatus  | Southern platyfish | 1* | 2*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
| Protacanthopterygii |            | S. salar     | Atlantic salmon | 1* | 4*   | 2*   | 1*   | 2*   | -    | -    | -    |
| Paracanthopterygii |            | G. morhua    | Atlantic cod | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
| Ostariophysi        |            | D. rerio     | Zebrafish    | 1*   | 2*   | 1*   | -    | 1*   | -    | -    | -    |
|                     |            | I. punctatus | Channel catfish | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
|                     |            | E. electricus | Electronic eel | 1   | 2    | 1    | 1    | 1    | 1    | -    | -    |
| Clupeomorphi        |            | C. harengus  | Atlantic herring | 1* | 2*   | 1*   | 1*   | 1*   | 1*   | -    | -    |
| Elpomorphi          |            | A. japonica  | Japanese eel | 1*   | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | 1*   |
| Osteoglossomorphi   |            | S. formosus  | Asian arowana | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |
| Holostei            |            | L. oculatus  | Spotted gar | 1*   | 2*   | 1*   | 1*   | 1*   | 1*   | 1*   | 1*   |

 Asterisks indicate the dmrt genes were downloaded from the NCBI/Ensembl databases.
NCBI/Ensembl databases (asterisk in Table 1 and Accession number in Supplementary Table S2). The remaining 19 dmrt sequences for three species were extracted from genomes through the method described in section “Similarities and Variances of the dmrt Gene Family Members in Various Fish Species.” These nucleotide sequences and corresponding deduced protein sequences were used for our further data analysis.

In mammals, eight dmrt genes (dmrt1–dmrt8) were identified in their genomes. However, in other species, dmrt7 and dmrt8 were lost. In addition, dmrt4 was also lost in birds. In the fish dmrt gene family, dmrt1–dmrt5 showed relatively high conservation. Among these, dmrt2 usually consisted of two paralogs (dmrt2a and dmrt2b) in most fish species, with only three species (Atlantic cod, Japanese eel, and coelacanth) carrying a single paralog. dmrt6 was only found in five fish species, i.e., largemouth bass, Asian sea bass, channel catfish, spotted gar, and African coelacanth. In addition, some of the dmrt genes were duplicated in Atlantic salmon (dmrt2, 3, 5) and Japanese eel (dmrt3; see Table 1).

### Structural Characterization and Evolutionary Analysis of the dmrt Family Genes

The gene structure of dmrt1 is composed of five exons in all examined species except for Atlantic salmon (Table 2 and Figure 1A), and a highly conserved DM domain (with a total of 49 aa) is located in the DMRT1 protein. In comparison, dmrt2 contains three exons and dmrt3–dmrt4 contain two exons in most examined species. Dmrt5 consists of 2 to 4 exons in higher vertebrates but only two in all examined fish species except for Stickelback (Table 2). Dmrt6 contains four exons in higher vertebrates, whereas the number of exons in fish varies greatly (from 2 to 4). dmrt7 and dmrt8 can only be identified in mammals, and both contain a large number of exons (8 for dmrt7, 6–7 for dmrt8). Except for DMRT8, all DMRT proteins (DMRT1–7) contain a conserved DM domain, often locating in the first exon of each gene (Figure 1A).

Using DMRTA protein sequence of the sea anemone (Nematostella vectensis) as the out-group, we constructed a
protein-based phylogenetic tree (Figure 1B), in which the DMRT family is distinctly categorized into eight groups (DMRT1–DMRT8). All DMRT proteins are distributed in the following three main groups: Group 1 includes five subfamilies, i.e., DMRT1, 2, 6, 7, and 8. DMRT2 was placed as the sister of DMRT7/8 and DMRT1 as the sister of DMRT6, suggesting a closer evolutionary relationship among these subfamilies. The subfamilies DMRT2, 7, and 8 were together placed as a sister group to the DMRT1 and 6 subfamilies. Group 2 includes DMRT4 and DMRT5. Group 3 contains only one subfamily DMRT3 (see more details in Figure 1B).

The dmrt1-dmrt3-dmrt2(2a) Cluster in Fish Genomes

Syntenic relationships of the dmrt1-dmrt3-dmrt2(2a) gene cluster were analyzed in 17 fish species, and using zebrafish (Cypriniformes, Ostariophysi) as the base reference. The dmrt1–dmrt3–dmrt2(2a) gene cluster is relatively conserved in various fish species; consistent with this, fish within the same superorder were clustered together (Figure 2A). A total of 11 genes (gas1a, dapk1, ctsla, fbpl2, fbpl1a, kank1a, smarca2, adams3, npff2a, gc, and slc4a4a) neighbor the zebrafish dmrt1–dmrt3–dmrt2(2a) gene cluster (Supplementary Table S3). All these genes were also found to neighbor the dmrt1–dmrt3–dmrt2(2a) gene cluster in channel catfish and Atlantic herring, whereas some genes were lost around this gene cluster in the remainder fish species (with frequent loss of the gc).

Mammals have lost larger numbers of genes next to this cluster, which also happens in two fish species (Atlantic salmon and coelacanth). Furthermore, in some fish species, such as largemouth bass, the dapk1 gene experienced a polyploidization event to generate four tandem duplicated copies. This phenomenon was also observed in the southern platyfish, which harbors two copies of the ctsla gene in its genome. Moreover, in both largemouth bass and southern platyfish, the gas1a gene experienced a translocation and inversion event as well (see more details in Figure 2B).

Substitution Rates (Ka/Ks) of the dmrt1-dmrt3-dmrt2(2a) Cluster in Fish Genomes

Ka/Ks represents the ratio of non-synonymous substitutions (Ka) to synonymous substitutions (Ks). This ratio can be used to determine whether there is selective pressure on a given protein-coding gene. It is generally believed that synonymous mutations are not subjected to natural selection, whereas non-synonymous mutations are. Ka/Ks > 1 implies the existence of positive selection; Ka/Ks = 1 suggests neutral selection; and Ka/Ks < 1 indicates purifying selection.

Comparing the Ka and Ka/Ks values using four different methods (gMYN, gYN, MYN, and YN), we found conserved substitution rates in dmrt1, dmrt2(2a), dmrt3, and the dmrt1–dmrt3–dmrt2(2a) gene cluster. In detail, all the mean Ka/Ks values were less than 1 (Figure 3), indicating a purifying selection on these genes. However, dmrt1 showed a higher average Ka/Ks value (0.0900) than dmrt2 (0.0576), dmrt3 (0.0645), and the dmrt1–dmrt3–dmrt2(2a) gene cluster (0.0606), suggesting that
Conserved Sequences and Regulatory Elements in the Fish
dmrt1–dmrt3–dmrt2(2a) Gene Clusters

To visualize the genomic conservation, the mVISTA tool was employed to generate a VISTA plot (Figure 4A). We used Chinese tongue sole (ZZ/ZW) (Chen et al., 2014), Japanese medaka (XX/XY) (Kasahara et al., 2007), southern platyfish (males are XY or YY, females are WX, WY, or XX) (Volff and Schartl, 2001), European sea bass (polygenic sex determination system, PSD system) (Vandeputte et al., 2007; Mei and Gui, 2015), Asian sea bass (protandrous hermaphrodite) (Wang et al., 2018), and largemouth bass which was previously reported as WZ/ZZ) (Glennon et al., 2012), whereas our recent analysis combining genomic map, ddRAD-Seq and sex-reversal experiments suggests a XX/XY sex determination system (data unpublished) to evaluate regulatory elements and genomic sequence changes in the dmrt1–dmrt3–dmrt2(2a) gene cluster with 20-kb upstream/downstream regions in various sex-determination systems.

Overall, a similar conservation pattern in both coding and non-coding sequences was observed. Comparisons of these six fish species along with zebrafish showed considerable homology within and between these dmrt genes. We also identified three regions with remarkable diversity among these fish (lower panels in Figure 4A). Region 1 covers 207 bp located at the 11-kb upstream region of the dmrt1 gene and contains nine TATA boxes (63 bp), which only exists in Chinese tongue sole. Region 2 is located in the third exon of dmrt1 with 18-bp missing in Chinese tongue sole. Comparing the protein sequences of tongue sole and other fish species, we determined that six amino acids (-P/S-A/S/T/P-YY-S/G/N-N-) were missing (Figure 4B). Region 3, located in the second exon of dmrt3, shows a 21-nucleotide (nt) deletion in Japanese medaka and a 15-nt deletion in southern platyfish (Figure 4B).
Synteny of Other dmrt Genes [Excluding dmrt1, dmrt2(2a), and dmrt3] in Fish Genomes

Based on the whole-genome sequence of largemouth bass and other eight representative vertebrate species (including O. niloticus, T. rubripes, O. latipes, L. oculatus, A. carolinensis, and H. sapiens) obtained from NCBI, we performed a synteny analysis of four dmrt genes, including dmrt2b, dmrt4, dmrt5, and dmrt6. The results (Figure 5) indicated that among these fish species, the KN motif and ankyrin repeat domain-containing protein 4 (kank4) and low-density lipoprotein receptor-related protein 8 (lrp8) genes in the upstream of dmrt2b were conserved. The ELAV-like protein 2 (elavl2) and caspase activity and apoptosis inhibitor 1 (caap1) genes in the downstream of dmrt4, and the elavl4 and FAS-associated factor 1 (faf1) genes in the downstream of dmrt5 were also conserved, which is consistent with the findings in reptiles and humans. This suggests that dmrt2b, dmrt4, and dmrt5 were relatively conserved during the evolutionary process.

Although dmrt6 was lost in most fish species including T. rubripes and O. latipes, in L. oculatus and I. punctatus, lrp8 was present in the upstream of dmrt6, which is consistent with higher vertebrates; whereas in M. salmoides and O. niloticus, dmrt6 was located between the conserved plectin (plec) and epilakin-F-box/LRR-repeat protein 6 (eppk1-fbxl6) genes (see more details in Figure 5).
FIGURE 4 | Species variances of the regulatory regions in the dmrt1-dmrt3-dmrt2(2a) gene cluster. (A) A VISTA plot of the dmrt1-dmrt3-dmrt2(2a) gene clusters among six examined fish species. Peaks of similarity in pair-wise sequence alignments between zebrafish (D. rerio) are compared with Chinese tongue sole (C. semilaevis), Japanese medaka (O. latipes), southern platyfish (X. maculatus), Asian sea bass (L. calcarifer), European sea bass (D. labrax), and largemouth bass (M. salmoides). Blue peaks represent coding exons and pink peaks denote non-coding sequences. The horizontal axis shows relative positions in the zebrafish genomic sequence, whereas the vertical axis indicates percentage of identity (50–100%). Three distinct regions, named as Region 1, Region 2, and Region 3, were identified. (B) Protein sequence alignments of the Regions 2 and 3 in 17 examined fish species. (C) Numbers of different regulatory elements in six representative fish species. Additional details regarding the sequences of each regulatory element are provided in Supplementary Table S4.

DISCUSSION

Fish are the oldest and most diverse group among vertebrates, containing about 32,000 species and accounting for more than half of the vertebrate species. Fish have undergone a long history of emergence, development, and evolution. The increasing amount of fish genomic information provides an important resource for studying the evolution, structure, and function of key genes through comparative genomics analysis. Seven dmrt genes have been identified in fish to date, including dmrt1–6 and dmrt2b. dmrt genes have also been reported in more than 30 fish species and a number of functional studies have been performed to reveal that regardless of the sex determination mechanism, the majority of fish dmrt genes (Table 3) are related to sexual development (Li et al., 2008, 2018; Liu et al., 2009; Herpin and Schartl, 2011; Yoshizawa et al., 2011; Xu et al., 2013). However, the phylogenetics of the dmrt gene family in fish have not yet been reported.

To obtain a better understanding of the functional diversification of this gene family, we therefore examined dmrt gene complements from the whole genome sequences of 17 representative fish species representing 10 various superorders and several non-fish outgroups. The evolutionary relationships of the dmrt genes in fish were subsequently examined using both phylogenetic and synteny analyses.

Similarities and Variances of the dmrt Gene Family Members in Various Fish Species

Zhou et al. (2008) showed that unlike mammals and other groups that only harbored one dmrt2, zebrafish carries a second paralog of dmrt2(2a), dmrt2b, which was subsequently identified in many other fish species (Zhou et al., 2008; Liu et al., 2009; Su et al., 2015; Lyu et al., 2019). The 17 representative fish species analyzed in the present study belong to Actinopterygii, with the exception of coelacanth L. chalumnae that belongs to Sarcopterygii. Among the 16 actinopterygians, 14 harbored the two paralogs of dmrt2 (dmrt2a and dmrt2b), however, dmrt6, which is commonly found...
FIGURE 5 | Synteny of dmrt genes in various fish species. The synteny analyses were performed in eight vertebrate species (M. salmoides, O. niloticus, T. rubripes, O. latipes, I. punctatus, L. oculatus, A. carolinensis, and H. sapiens). Chr., chromosome; LG, linkage group. The most conserved surrounding genes of dmrt genes were shown in green and bold fonts. The most conserved were shown in red fonts.
in mammals and other groups, was only identified in four actinopterygians including *M. salmoides* and the sarcopterygian *L. chalumnae*.

Among the 17 fish species, only *A. japonica* and *G. morhua* carried *dmrt2a* alone and lacked *dmrt6*. *L. chalumnae* only had one *dmrt2* (2a) and one *dmrt6*, similar to higher vertebrates. A search through the database revealed that two other sarcopterygians (*Protopterus annectens* and *Sarcopteryx salamandra*) also only carried one *dmrt2a* and one *dmrt6* (similar to higher vertebrates). A search through the database revealed that two other sarcopterygians (*Protopterus annectens* and *Sarcopteryx salamandra*) also only carried one *dmrt2a* and one *dmrt6* (similar to higher vertebrates).

**Table 3**  
The relevant references for *dmrt* family genes in fish.

| Genes   | Number of species | References |
|---------|------------------|------------|
| *dmrt1* | 23               | *Acipenser gueldenstaedtii* (Fajkowska et al., 2016); *A. japonica* (Jeng et al., 2019); *Acanthopagrus schlegelii* (Wu and Chang, 2018); *Anoplopoma fimbria* (Smith et al., 2013); *C. semilaevis* (Cui et al., 2017); *D. rerio* (Lin et al., 2017; Webster et al., 2017); *Epinephalus cooides* (Lyu et al., 2019); *G. morhua* (Johnsen and Andersen, 2012); *Gobio gobio* (Cao et al., 2012); *Halichoeres poecilopterus* (Miyake et al., 2012); *L. chalumnae* (Forconi et al., 2013); *M. amblycephala* (Su et al., 2015); *M. salmoides* (Yan et al., 2019); *O. latipes* and *X. maculatus* (Kondo et al., 2002); *O. niloticus* (Wei et al., 2019); *Odontesthes bonarvicensis* (Fernando et al., 2008); *O. mykiss* (Marchand et al., 2008); *Plecoglossus altivelis* (Wang et al., 2014); *Sebastes schlegelii* (Ma et al., 2014); *Solea senegalensis* (Portela-Bens et al., 2017); *T. rubripes* (Yamaguchi et al., 2006); *X. maculatus* (Veith et al., 2006b). |
| *dmrt2* (2a) | 15              | *A. japonica* (Zhu et al., 2019); *Carassius auratus* (Liu and Gu, 2017); *D. rerio* (Zhou et al., 2008; Lu et al., 2017); *G. morhua* (Johnsen and Andersen, 2012); *E. coioidea* (Lyu et al., 2019); *Labeo rohita* (Sahoo et al., 2019); *M. aibus* (Sheng et al., 2014); *M. amblycephala* (Su et al., 2015); *O. latipes* and *X. maculatus* (Kondo et al., 2002); *S. senegalensis* (Portela-Bens et al., 2017); *Scophthalmus maximus* (Robledo et al., 2015); *T. rubripes* (Xu et al., 2013); *X. maculatus* (Veith et al., 2006b). |
| *dmrt2b* | 5               | *A. japonica* (Zhu et al., 2019); *Carassius auratus* (Liu and Gu, 2017); *D. rerio* (Li et al., 2018); *E. coioidea* (Lyu et al., 2019); *G. morhua* (Johnsen and Andersen, 2012); *M. aibus* (Sheng et al., 2014); *M. amblycephala* (Su et al., 2015); *T. rubripes* (Yamaguchi et al., 2006). |
| *dmrt3* | 10              | *D. rerio* (Li et al., 2008); *E. coioidea* (Lyu et al., 2019); *G. morhua* (Johnsen and Andersen, 2012); *M. aibus* (Sheng et al., 2014); *M. amblycephala* (Su et al., 2015); *O. latipes* and *X. maculatus* (Kondo et al., 2002); *S. senegalensis* (Portela-Bens et al., 2017); *T. rubripes* (Yamaguchi et al., 2006). |
| *dmrt4* (2a) | 11              | *C. semilaevis* (Dong and Chen, 2013); *G. morhua* (Johnsen and Andersen, 2012); *M. aibus* (Sheng et al., 2014); *M. amblycephala* (Su et al., 2015); *O. aureus* (Cao et al., 2007); *O. latipes* and *X. maculatus* (Kondo et al., 2002); *Paralichthys olivaceus* (Wen et al., 2009); *S. senegalensis* (Portela-Bens et al., 2017); *T. rubripes* (Yamaguchi et al., 2006); *X. maculatus* (Veith et al., 2006b). |
| *dmrt5* (2a) | 7               | *D. rerio* (Guo et al., 2004); *G. morhua* (Johnsen and Andersen, 2012); *M. aibus* (Sheng et al., 2014); *O. latipes* (Shirak et al., 2006); *P. altivelis* (Wang et al., 2014); *Scaphophagus argus* (Gu et al., 2019); *T. rubripes* (Yamaguchi et al., 2006); *X. maculatus* (Veith et al., 2006b). |
| *dmrt6* (2b) | 2               | *L. chalumnae* (Forconi et al., 2013); *O. niloticus* (Zhang et al., 2014). |
the gonads. Specifically, dmrt1, 3, and 6 are highly expressed in the testis (dmrt1: (Guan et al., 2000; Marchand et al., 2000; He et al., 2003; Guo et al., 2005; Huang et al., 2005a; Veith et al., 2006b; Yamaguchi et al., 2006; Johnsen et al., 2010; Su et al., 2015); dmrt3(Yamaguchi et al., 2006; Dong et al., 2010; Sheng et al., 2014; Su et al., 2015); dmrt6: (Forconi et al., 2013; Zhang X.et al., 2014), whereas dmrt2a, 2b, and dmrt4 are expressed in both male and female gonads, with different fish species showing different expression profiles [dmrt2a/b(Yamaguchi et al., 2006; Zhou et al., 2008; Liu and Gui, 2011; Sheng et al., 2014; Su et al., 2015); dmrt4: (Kondo et al., 2002; Veith et al., 2006b; Yamaguchi et al., 2006; Cao et al., 2007; Wen et al., 2009; Dong and Chen, 2013; Sheng et al., 2014; Jiang et al., 2019)]. Current studies have shown that fish dmrt family members may mainly be involved in embryonic sex differentiation, gonadal development, and gametogenesis (Herpin and Scharlt, 2011; Xu et al., 2013; Zhang X.et al., 2014; Graf et al., 2015), in addition to other functions such as neural development (Li et al., 2008; Lourenco et al., 2010; Yoshizawa et al., 2011).

**Similarities and Variances of the dmrt1–dmrt3–dmrt2(2a) Gene Cluster in Various Fish Genomes**

In vertebrate genomes, the dmrt1, dmrt2(2a), and dmrt3 genes are in tandem in the order of dmrt1–dmrt3–dmrt2(2a) (Johnsen and Andersen, 2012). Our phylogenetic analysis based on this dmrt1–dmrt3–dmrt2(2a) cluster confirmed the clustering in fish within the same superorder, thus indicating that the dmrt1–dmrt3–dmrt2(2a) gene cluster is highly conserved in various fish species (Figure 2). Further analysis of the conserved genes flanking this cluster revealed that D. rerio carried 11 neighboring genes, as did I. punctatus and C. harengus. However, other fish species showed partial loss (such as the gc gene), duplication (dapl1 in M. salmoides and ctsla in X. maculatus), and transversion (gas1a in M. salmoides and X. maculatus). This may have been caused by genomic polyploidization events during the evolutionary process of fish (Braasch and Postlethwait, 2012). Despite the large variations in the flanking genes among different fish species, the number and location of the dmrt1–dmrt3–dmrt2(2a) genes have been stable. Thus, the high conservation of the dmrt1–dmrt3–dmrt2(2a) gene cluster in various fish genomes suggests their crucial biological functions in fish.

Among the fish genomes analyzed in this study, the Ka/Ks ratios of the dmrt1–dmrt3–dmrt2(2a) gene cluster and the three dmrt genes were less than 0.2, implying that after the examined actinopterygians diverged from L. oculatus, the dmrt1–dmrt3–dmrt2(2a) gene cluster was subjected to relatively strong purification selection in its evolutionary process, whereas its positive selection may have occurred prior to the divergence from L. oculatus. These low Ka/Ks ratios across various fish species indicate that the dmrt1–dmrt3–dmrt2(2a) genes are highly conserved during evolution. Occurrence of a non-synonymous substitution would alter the conformation and function of the corresponding protein, thereby affecting any individual’s sex differentiation, which in turn would affect the inheritance of the mutation site by its offspring (Wang D.et al., 2009). Therefore, the high conservation of the dmrt1/2/3 genes across fish suggests that its key role in sex differentiation.

Analysis of the conserved sequences and regulatory elements was performed on the dmrt1–dmrt3–dmrt2(2a) gene cluster of three representative fish genomes with different sex determination systems [i.e., C. semilaevius (ZW) (Chen et al., 2012), O. latipes (XY) (Otake et al., 2006), X. maculatus (WXY) (Schultheis et al., 2009)]. Three distinct regions 1–3 were identified (Figure 4A). C. semilaevius showed 207-bp only exists in Region 1 and 18-bp deletions in Regions 2, respectively, and Region 1 contained nine TATA boxes. O. latipes and X. maculatus showed 21- and 15-bp deletions, respectively, in Region 3. Analysis of the regulatory elements for this gene cluster indicated that the number of TATA boxes in C. semilaevius was higher than that in other fish species (twice of O. latipes), whereas O. latipes had significantly more E box, GC box, and BRE elements than other fish species. Fish with various sex-determination systems showed significant differences in their conserved sequences and regulatory elements, suggesting that the dmrt1–dmrt3–dmrt2(2a) gene cluster may be related to the sex-determination systems in fish. In our recent study, it reveals that M. salmoides is a XY/XX system species (Sun et al., 2020). In conserved sequences analysis of fish dmrt1–dmrt3–dmrt2(2a) gene clusters, it had much difference in Regions 1–3 between M. salmoides and C. semilaevius (ZW/ZZ). The Region 2 of M. salmoides was more similar to O. latipes (XX/XY). The Region 3 of M. salmoides was similar to D. labrax (PSD) and L. calcarifer (hermaphrodite) (Wang et al., 2018). Therefore, the sex-determination systems of M. salmoides might be preferred to XY/XX system species.

**Conserved Synteny of the dmrt Genes in Fish Genomes**

The synteny analysis performed in this study showed that apart from dmrt6, all other six dmrt genes in the fish dmrt gene family (dmrt1, dmrt2a, dmrt2b, and dmrt3–5) were relatively conserved. Fish dmrt1–dmrt3–dmrt2(2a) clusters are located in tandem in genomes, which is consistent with higher vertebrates. Fish dmrt4 is usually located on a different chromosome from the cluster, and the downstream elav2 gene is conserved. In contrast, dmrt4 is located on the same chromosome as this dmrt1–dmrt3–dmrt2(2a) cluster in higher vertebrates, but the downstream elav2 gene is also conserved. Fish dmrt5 gene is the same as that in higher vertebrates, in which the upstream and downstream elav4 and fis1 genes are also conserved. dmrt2b gene can be found in most fish species, and the upstream kank4 and lrp8 genes are conserved. The dmrt6 gene is lost in most fish genomes. However, in L. oculatus and I. punctatus, dmrt6 is conserved with downstream lrp8, which is consistent with higher vertebrates, however, in M. salmoides and O. niloticus, the dmrt6 gene is located between the conserved plec and eppk1–foxi6 genes (see Figure 5).

Kondo et al. (2002) was the first report of conserved synteny analysis on the dmrt1–4 genes between fish and human. This study demonstrated that the dmrt1, 2, and 3 genes formed clusters in fish and constituted a part of a large number of genes in this cluster that exhibit conserved synteny between human
and fish. Johnsen and Andersen (2012) performed chromosomal synteny analysis on \textit{dmrt2a} and \textit{dmrt2b}, and proposed that these genes originated from the second round (2R) of whole genome duplication of the ancestral \textit{dmrt2} (Johnsen and Andersen, 2012). In turn, Mawaribuchi et al. (2019) performed phylogenetic cluster analysis of lower bilaterian and higher animal \textit{dmrt} genes, based on which they speculated that the \textit{dmrt3} gene emerged by genome duplication (1R), and \textit{dmrt1} and \textit{dmrt6} emerged after the 2R genome duplication; they also proposed an evolutionary history for the \textit{dmrt} family genes in bilateria (Mawaribuchi et al., 2019). Therefore, according to our data coupled with these relevant literatures, we hypothesized evolutionary history of the \textit{dmrt} genes in fish (Figure 6).

Firstly, we propose that \textit{dmrt} genes exist in various fish species through the fish-specific (3R) genome duplications (Johnsen and Andersen, 2012). Secondly, we propose that \textit{dmrt3} might originate from \textit{dmrt93B} and emerged by genome duplication (1R). According to our phylogenetic tree of the \textit{dmrt} family in vertebrates (Figure 1B), in addition to the conserved genes flanking \textit{dmrt1} and \textit{dmrt6} (\textit{kank1/4}) (Figure 5), we propose that \textit{dmrt1} and \textit{dmrt6} might originate from the same ancestral \textit{dmrt} (labeled with \textit{dmrt1/6?} in Figure 6) and they emerged after genome duplication (2R). Thirdly, (1) we propose that the common ancestor of chordates might have four ancestral \textit{dmrt} genes (\textit{dmrt4/5}, \textit{dmrt2a/2b}, \textit{dmrt93B}, and \textit{dmrt1/6}), and two ancestral surrounding genes (\textit{kank1/4} and \textit{elval2/4}). (2) A common ancestor of vertebrata might have possessed four \textit{dmrt} family genes, including \textit{dmrt1/6}, \textit{dmrt2a/2b}, \textit{dmrt3}, and \textit{dmrt4/5}. The four \textit{dmrt} family genes and their conserved surrounding genes are distributed in tandem on two pairs of chromosomes, specifically \textit{kank1/4–dmrt1/6–dmrt3–dmrt2a/2b–dmrt4/5–elavl2/4} and \textit{kank1/4–dmrt1/6–dmrt2a/2b–dmrt4/5–elavl2/4}. (3) After three rounds (3R) of whole genome duplication, the syntenies of \textit{kank1–dmrt1–dmrt3–dmrt2a}, \textit{dmrt4–elavl2–caap1}, \textit{dmrt4–elavl2–caap1}, and \textit{dmrt5–elavl4} are conserved in fish. (4) \textit{dmrt6} is lost in most fish species, although some retained it with downstream \textit{kank4–lrp8}, or it was recombined to other chromosomes with a location between \textit{plec} and \textit{eppk1}.

We should note that our present study has several limitations. First, although fish are the most numerous vertebrates on earth, whole genome sequences are currently available for only a small fraction of fish. In this study, 17 representative fish species from 10 superorders were selected for analysis; however, the number and coverage of species was still insufficient and may have limited the generalizability of our results. Second, this study was based on fish species with known genome sequences, which may have affected the accuracy of data analysis because genome assembly techniques and quality vary significantly among species. For example, since coelacanth genomes are not assembled to the chromosomal level, our syntenic analysis of their genes is affected. Third, it is expected that as sequencing coverage and quality
increase for fish genomes, future studies will be able to confirm and expand findings and generalizability from the present study.

In this study, we applied bioinformatics methods to perform phylogenetic and synteny analyses on dmrt genes in 17 fish species. (1) All the examined fish species have dmrt1–5 and most fish species harbored two dmrt2 paralogs (dmrt2a and dmrt2b). Phyletic evolution and structure of dmrt1–5 and dmrt2b genes were relatively conserved in most of fish. The dmrt6 gene is lost in most fish genomes and less conservative. (2) Purifying selections on the dmrt1, dmrt2(2a), dmrt3, and the dmrt1–dmrt3–dmrt2(2a) gene cluster were observed. (3) Fish with various sex-determination systems have the similar genomic conservation patterns of the dmrt1–dmrt3–dmrt2(2a) gene cluster. dmrt2b, dmrt4, and dmrt5 were also relatively conserved during the evolutionary process. The high conservation of the dmrt1–dmrt3–dmrt2(2a) gene cluster in various fish genomes suggests their crucial biological functions while various dmrt family members and sequences across fish species suggest different biological roles during evolution.

Furthermore, we hypothesized the evolutionary history of the dmrt genes in fish after fish-specific genome duplication(s). Moreover, here raised a series of new questions during the course of our data analysis. For example, in terms of evolutionary analysis, whether dmrt2b is homologous and functionally similar to a specific dmrt in higher animals, or does fish dmrt6 have similar functions to mammalian counterpart. We anticipate that these gene trees will help to place current dmrt research in a proper phylogenomic context. Our present study will provide a solid molecular basis for functional research on the fish dmrt family and may in particular serve as genetic reference for in-depth phylogenomics studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

XY and QS conceived and designed the project and revised the manuscript. JD and JL performed the genomic investigations and wrote the manuscript. JH, CPS, YT, NY, CXS, XS, and SY participated in discussion and figure preparation. All authors read and approve the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.563947/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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