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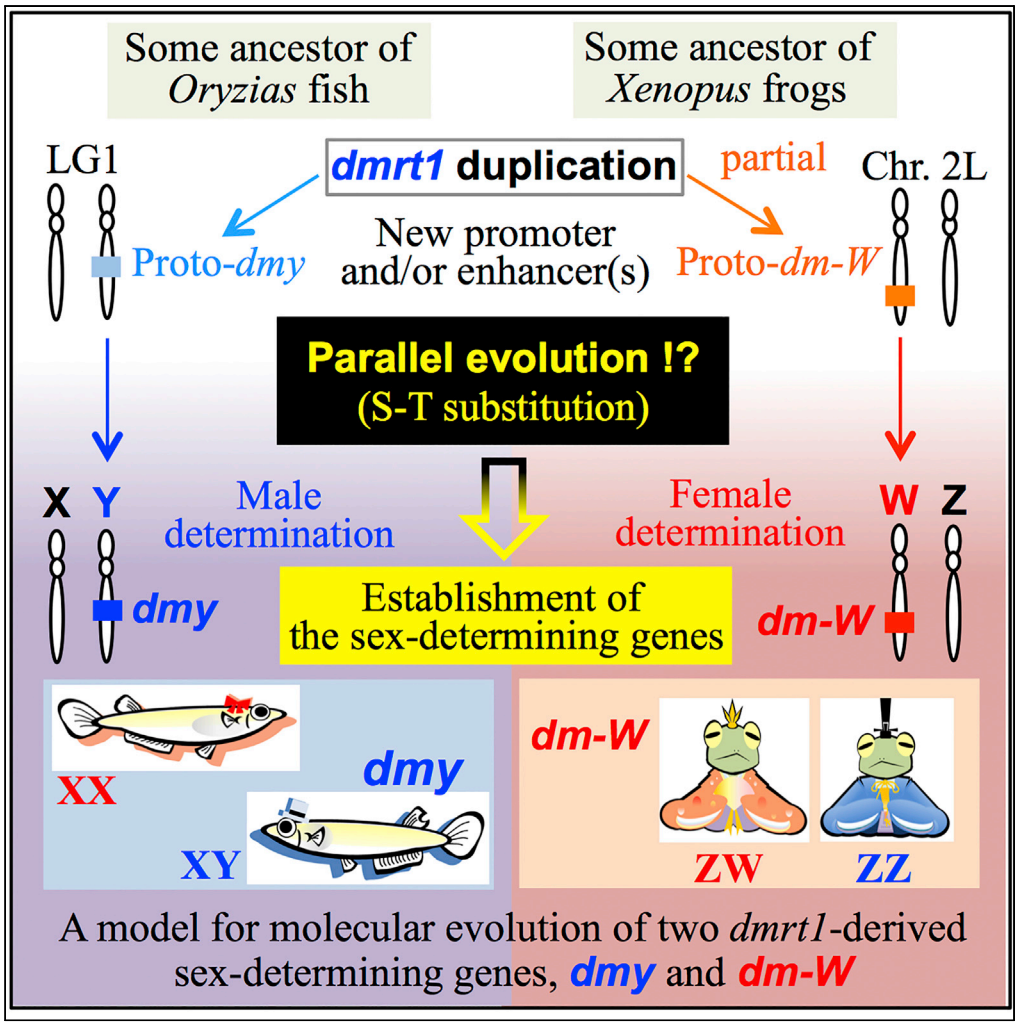

Yusaku Ogita, Shuuji Mawaribuchi, Kei Nakasako, ..., Shigetaka Yoneda, Nobuhiko Takamatsu, Michihiko Ito

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HIGHLIGHTS

We detected parallel amino acid substitutions in two sex-determining genes, *dmy* and *dm-W*

Both the substitutions from *dmrt1* duplication are under positive selection

These substitutions enhanced their DNA-binding activity as transcription factors

These substitutions might have contributed to the establishment of *dmy* and *dm-W*

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Parallel Evolution of Two *dmrt1*-Derived Genes, *dmy* and *dm-W*, for Vertebrate Sex Determination

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SUMMARY

Animal sex-determining genes, which bifurcate for female and male development, are diversified even among closely related species. Most of these genes emerged independently from various sex-related genes during species diversity as neofunctionalization-type genes. However, the common mechanisms of this divergent evolution remain poorly understood. Here, we compared the molecular evolution of two sex-determining genes, the medaka *dmy* and the clawed frog *dm-W*, which independently evolved from the duplication of the transcription factor-encoding masculinization gene *dmrt1*. Interestingly, we detected parallel amino acid substitutions, from serine (S) to threonine (T), on the DNA-binding domains of both ancestral DMY and DM-W, resulting from positive selection. Two types of DNA-protein binding experiments and a luciferase reporter assay demonstrated that these S-T substitutions could strengthen the DNA-binding abilities and enhance the transcriptional regulation function. These findings suggest that the parallel S-T substitutions may have contributed to the establishment of *dmy* and *dm-W* as sex-determining genes.

INTRODUCTION

Sexual reproduction in multi-cellular organisms results in the mixture of two types of genomes. Most bilaterian animals have two types of gonads, ovaries and testes. Gonadal sex determination can be defined as a decision whether bipotential gonads develop into ovaries or testes. In vertebrates, there are various sex-determining systems, which can be divided into two categories: environmental sex determination or genetic sex determination. In the latter system, sex chromosome(s) have sex-determining gene(s), which primarily induce a female or male gonad. Previously, we proposed a coevolution model for sex-determining genes and sex chromosomes, in which homomorphic (undifferentiated) sex chromosomes easily allowed the turnover of a sex-determining gene with another one, whereas heteromorphic (differentiated) sex chromosomes tended to be restricted to a specific sex-determining gene (Mawaribuchi et al., 2012). Most vertebrate species, except mammals and birds, have homomorphic sex chromosomes. Therefore, many sex-determining genes should be present in ectothermic vertebrates; however, only about 10 sex-determining genes have been identified so far (Ito and Mawaribuchi, 2013). The turnover of sex chromosomes and sex-determining genes has been evaluated in certain vertebrate species (Kitano and Peichel, 2012; Matsuda and Sakaizumi, 2016; Miura, 2017; Jeffries et al., 2018).

dmrt1 (*doublesex- and mab-3-related transcription factor 1*) belongs to the DM domain gene family, which encodes transcription factors characterized by a DNA-binding region called the DM domain. *dmrt1* is a key gene in testis formation and/or gonadal somatic-cell masculinization in various vertebrate species, including the teleost fish *Oryzias latipes*, anuran amphibian *Xenopus laevis*, slider turtle *Trachemys scripta elegans*, and house mouse *Mus musculus* (Yoshimoto et al., 2010; Masuyama et al., 2012; Matson and Zarkower, 2012; Zhao et al., 2015; Ge et al., 2018). In the chicken *Gallus*, a Z-linked *dmrt1* is required as a sex-determining gene for testicular formation (Smith et al., 2009). In addition, *dmrt1* can also be involved in the germ-cell development from jawless vertebrates to mammals (Matson et al., 2010; Zarkower, 2013; Mawaribuchi et al., 2017a). In our recent study of the promoters and conserved noncoding sequences of *dmrt1* orthologs during vertebrate evolution, we found that *dmrt1* regulated germ-cell development in the vertebrate ancestor, acquiring another promoter to regulate somatic-cell masculinization during gnathostome evolution (Mawaribuchi et al., 2017a).

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In 2002, two groups independently reported the Y-linked gene, *dmy/dmrt1bY*, as a sex (male)-determining gene in *O. latipes*; this gene evolved for male determination from the whole duplication of *dmrt1* and led to apparition of an XX/XY-type sex-determining system in the ancestor of this species (Matsuda et al., 2002; Nanda et al., 2002). In 2008, we identified the W-linked *dm-W*, as a sex (female)-determining gene in *X. laevis*; this gene evolved for female (anti-male) determination from the partial duplication of *dmrt1*, including the DM domain sequences, thus generating a ZZ/ZW-type sex-determining system in the ancestor of *X. laevis* (Yoshimoto et al., 2008, 2010; Yoshimoto and Ito, 2011). Notably, the two genes, *dmy* and *dm-W*, independently emerged for sex determination through neofunctionalization after the duplication of *dmrt1*, during species diversity in the *Oryzias* and *Xenopus* genera, respectively (Yoshimoto and Ito, 2011; Mawaribuchi et al., 2012). We also reported that *dm-W* established the ZZ/ZW-type system after allotetraploidization through hybridization between two closely related diploid *Xenopus* species around 17–18 million years ago (Session et al., 2016; Mawaribuchi et al., 2017b). Collectively, these findings indicate the convergent gene evolution from *dmrt1* to *dmy* and *dm-W* for sex determination.

Almost all the sex-determining genes in vertebrates emerged independently during species diversification through neofunctionalization. These genes include *dmy*, *dm-W*, and a Y-linked gene, *Sry*, in therian mammals, which encode transcription factors (Mawaribuchi et al., 2012). Our previous evolutionary analyses showed that all the DNA-binding domain-coding regions in these three genes show higher substitution rates than their prototype genes, *dmrt1* and *Sox3* (Mawaribuchi et al., 2012). However, whether the emergence of such neofunctionalization-type sex-determining genes shared common evolutionary mechanisms remains to be elucidated.

In this study, we focused on the molecular evolution of *dmy* and *dm-W*, as both derived from *dmrt1*. In order to determine how *dmy* and *dm-W* evolved from *dmrt1* for sex determination, we searched for shared common mechanisms in the convergent evolution of the *dmrt1*-derived sex-determining genes *dmy* and *dm-W*. In 2004, Zhang (2004) reported that the DM domain in DMY is under positive selection. In this study, we found a common amino acid substitution from serine (S) to threonine (T) at position 15 on the DM domains of both ancestral DMY and DM-W. Importantly, the parallel S15T substitutions in both DMY and DM-W are under positive selection. In addition, both the parallel substitutions were associated with increased DNA-binding and transregulation activities, suggesting a common mechanism for the molecular evolution of *dmy* and *dm-W* in sex determination.

RESULTS

Amino Acid Substitutions Accumulate in the DM Domain of Sex-Determining Gene Products DMY and DM-W

Because *dm-W* and *dmy* exhibit higher substitution rates than their ancestral gene *dmrt1* (Mawaribuchi et al., 2012), we phylogenetically analyzed the amino acid substitutions on the DM domain consisting of 56 or 41 amino acid residues for *Oryzias* DMY and DMRT1 or *Xenopus* DM-W and DMRT1, respectively. For *Xenopus* DM-W and DMRT1, only 41 amino acid sequences corresponding to the N-terminal region of the DM domains were obtained from several *Xenopus* species, except for *X. laevis*, in the database. Figure 1A shows the amino acid alignment of these sequences. Using the maximum likelihood method in MEGA6, we constructed two phylogenetic trees (Figures S1A and S1B, and Table S1) and inferred the ancestral amino acid sequence on each branchpoint (Figures 1B and 1C, and Table S2).

In the genus *Oryzias*, two amino acid substitutions, F13L and S15T, at positions 13 and 15 on the DM domain (see Figure 1A), were detected on the branch of a common ancestral molecule between DMY and DMRT1 in the three species, *O. latipes*, *O. sakaizumii*, and *O. curvinotus* (Figure 1B). There were also 5, 6, or 9 amino acid substitutions from the ancestral DMY of the three species, *O. sakaizumii*, *O. curvinotus*, or *O. latipes*, respectively. In contrast, there may only be three or four amino acid substitutions from the common ancestral molecule between DMY and DMRT1 in *O. sakaizumii* or *O. latipes*, respectively (Figure 1B). In *Xenopus*, we detected four amino acid substitutions, S15T, M25I, K32N, and I37T, at positions 15, 25, 32, and 37 in the DM domain, on the branch of a common ancestor of DMRT1 and DM-W to a common ancestral DM-W among *X. laevis*, *X. itombwensis*, and *X. clivi* (Figure 1C). In addition, none, one, and two amino acid substitutions were found on the branches from ancestral DM-W to *X. clivii*, *X. itombwensis*, and *X. laevis* DM-W, respectively. In contrast, there may be none or only one amino acid substitution from the common ancestral molecule between DM-W and DMRT1 to *X. laevis* DMRT1.S or

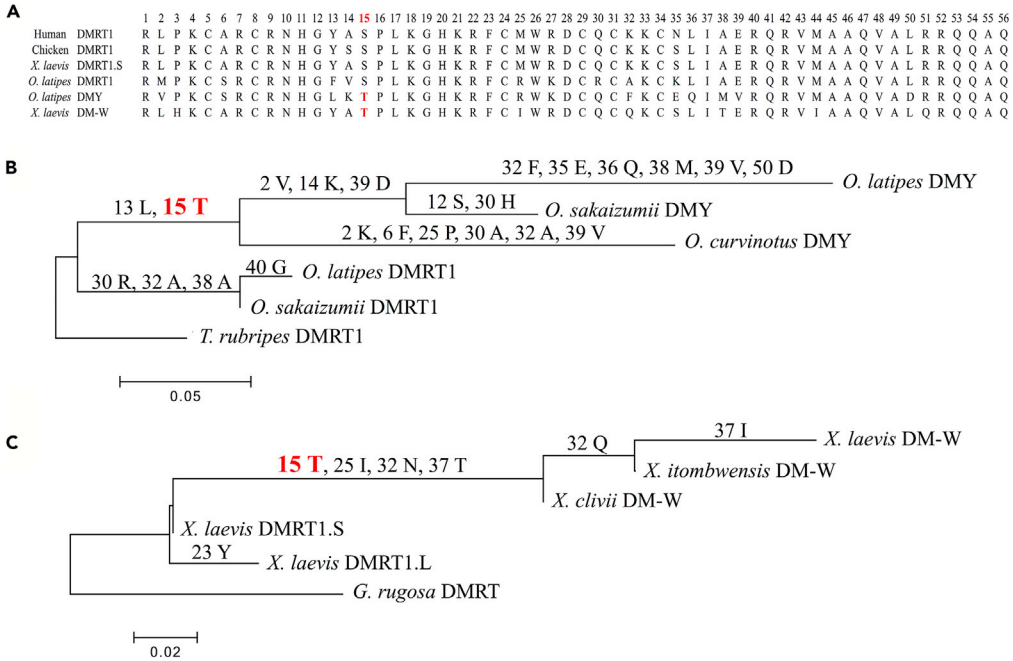

Figure 1. Phylogenetic Trees of the DM Domains of DMRT1 Subfamily Including DMY and DM-W in the Genus *Oryzias* and *Xenopus* and Estimated Amino Acid (aa) Substitutions on Their Ancestral Sequences

The DM domain sequences consisting of 56 aa residues from human, chicken, *Xenopus laevis*, *Oryzias latipes* DMRT1, *O. latipes* DMY, and *X. laevis* DM-W were aligned (A). Two maximum likelihood trees including DMY (B) and DM-W (C) were constructed from the alignments of the DM domains including 56 aa and 41 aa sequences, respectively, in DMRT1 subfamily. Each number and letter corresponds to the position from the 5′-terminal of the DM domain. One letter code denotes substituted amino acids. The only conserved substitution form S in DMRT1 to T in DMY and DM-W in the 15th position was displayed in red. Scale bar = 0.02 estimated amino acid substitutions per site.

DMRT1.L, respectively (Figure 1C). These results suggest a higher nucleotide substitution rate for *dmy* and *dm-W* compared with *dmrt1* (Mawaribuchi et al., 2012).

Remarkably, the common serine (S) to threonine (T) substitutions at position 15 on the DM domain were shared during the molecular evolution from the DMRT1 ancestors to the ancestor of DMY and DM-W (Figure 1). Importantly, S or T at position 15 represents an exclusive conservation in *the dmrt1* gene of all jawed vertebrates or the *dm-W* gene in *Xenopus* and the *dmy* gene in *Oryzias*, respectively (Figure 1A). The S15T substitutions on DMY and DM-W must have occurred independently as *dmy* and *dm-W* emerged independently from the duplication of *dmrt1*.

Parallel Amino Acid Substitutions under Positive Selection in the Early Lineages of the Two Sex-Determining Genes, *dmy* and *dm-W*

Because more amino acid substitutions were found in the molecular evolution of *dmy* and *dm-W* (Figure 1), we examined whether *dm-W* or *dmy* evolved under positive selection during species diversification in *Xenopus* or *Oryzias*, respectively, through the dN/dS ratio test with codeml in PAML4.8 (see Transparent Methods). Then, we reconstructed two phylogenetic trees of the *dmrt1* subfamily, containing *dmy* and *dm-W*, and denoted the branches of *dmy* and *dm-W* for each estimation as the foreground ("Figures S1C and S1D, and Table 1), which revealed positive selection in both lineages of *dmy* and *dm-W* ($p < 0.05$) (Table 1). Bayes empirical Bays (BEB) analysis demonstrated that positions 6 and 1 on the DM domain of the *dmy* and *dm-W* lineages, respectively, were under positive selection (see Figures S2 and S3). It is worth noting that these positive selection sites included the common S15T substitutions between DMY and DM-W. In other words, the parallel S15T substitutions under positive evolution may take place in the early lineages of *dmy* and *dm-W* during the convergent evolution of *dmrt1*-derived sex-determining genes.

Gene	Fix Omega	Parameter Estimated					lnL	2Δ lnL (p value[a])	BEB Analysis		
		Site Class	0	1	2a	2b			Positively Selected Sites (DM Domain Position[b])	Amino Acid	Probablity ω > 1
dmy	Estimated	Proportion	0.654	0.204	0.107	0.033	−2646	5.627	8 (–)	R	0.991
		Background ω	0.052	1	0.051	1			9 (–)	P	0.952
		Foreground ω	0.052	1	999	999			25 (14)	K	0.979
									26 (15)	T	0.961
	1	Proportion	0.546	0.173	0.212	0.067	−2649	(p < 0.01)	47 (35)	Q	0.952
		Background ω	0.05	1	0.05	1			49 (37)	M	0.988
		Foreground ω	0.05	1	1	1			50 (38)	V	0.994
									61 (49)	D	1
dm-W	Estimated	Proportion	0.714	0.147	0.114	0.024	−2969	19.7	38 (15)	T	0.951
		Background ω	0.043	1	0.043	1			86 (–)	Y	0.999
		Foreground ω	0.043	1	9.418	9.419			107 (–)	Q	1
	1	Proportion	0.331	0.069	0.496	0.103	−2979	(p < 0.01)			
		Background ω	0.043	1	0.042	1					
		Foreground ω	0.043	1	1	1					

Table 1. Likelihood Ratio Test of Branch-Site Model for *dmy* and *dm-W*

[a]p-value was calculated by the 50:50 mixture of point mass 0 and χ_1^2-test.

[b]The selected sites are numbered from the N-terminal residues in the whole proteins and the DM domains. The number in parentheses shows that from the DM domain (see Figure S2).

Parallel S15T Substitutions in Both DM-W and DMY May Enhance Their DNA-Binding Activity

Next, we investigated the advantage of parallel S15T substitutions in the molecular evolution of both sex-determining genes *dmy* and *dm-W*. We investigated whether the S15T or T15S substitutions could affect the DNA-binding properties of DMRT1 or DMY and DM-W, respectively. As described in the Introduction section, *X. laevis* has allotetraploid genome consisting of L and S subgenomes (Session et al., 2016). It is considered that *dm-W* emerged from the S-subgenome-derived *dmrt1.S* after allotetraploidization (Bewick et al., 2011; Mawaribuchi et al., 2017b). Therefore, we used DMRT1.S in this experiment. We first constructed expression plasmids for four FLAG-tagged DMRT1 subfamily proteins (*O. latipes* DMRT1, *O. latipes* DMY, *X. laevis* DMRT1.S, and *X. laevis* DM-W) and their mutant proteins (*O. latipes* DMRT1(S15T), *O. latipes* DMY(T15S), *X. laevis* DMRT1.S(S15T), and *X. laevis* DM-W(T15S)), which contain one amino acid substitution. Each protein was produced and validated by *in vitro* transcription/translation, confirmed by immunoblotting with the anti-FLAG antibody (Figure S4), and then the DNA-binding affinity for the DMRT1-binding consensus DNA sequence (Murphy et al., 2007) was examined using an electrophoretic mobility shift assay (EMSA). Intriguingly, the shifted bands corresponding to the DNA-protein complexes from *O. latipes* or *X. laevis* mutant protein DMRT1(S15T), which had the DMY/DM-W-specific threonine at position 15 (15T), were significantly thicker than those corresponding to wild-type DMRT1 (Figure 2A). Conversely, the mutant proteins, DMY(T15S) or DM-W(T15S), which had a DMRT1-specific serine at position 15 (15S), exhibited weaker DNA-binding affinity than each corresponding wild-type protein. We detected similar amounts of proteins between the wild-types and the mutants by western blot analysis using the anti-FLAG antibody (Figure 2B) and confirmed that "15T" is involved in stronger DNA-binding activity than "15S" by quantification of the DNA-binding activity to protein content (Figure 2C). These reciprocal results indicate that the S15T substitutions in the DM domains of the two sex-determining gene's products, DMY and DM-W, may be responsible for the enhancement of both of their DNA-binding activities.

Next, we examined the kinetics of association and dissociation between the DMRT1-binding consensus DNA sequence and the wild-type or mutant proteins by bio-layer interferometry (Table 2). Due to the large

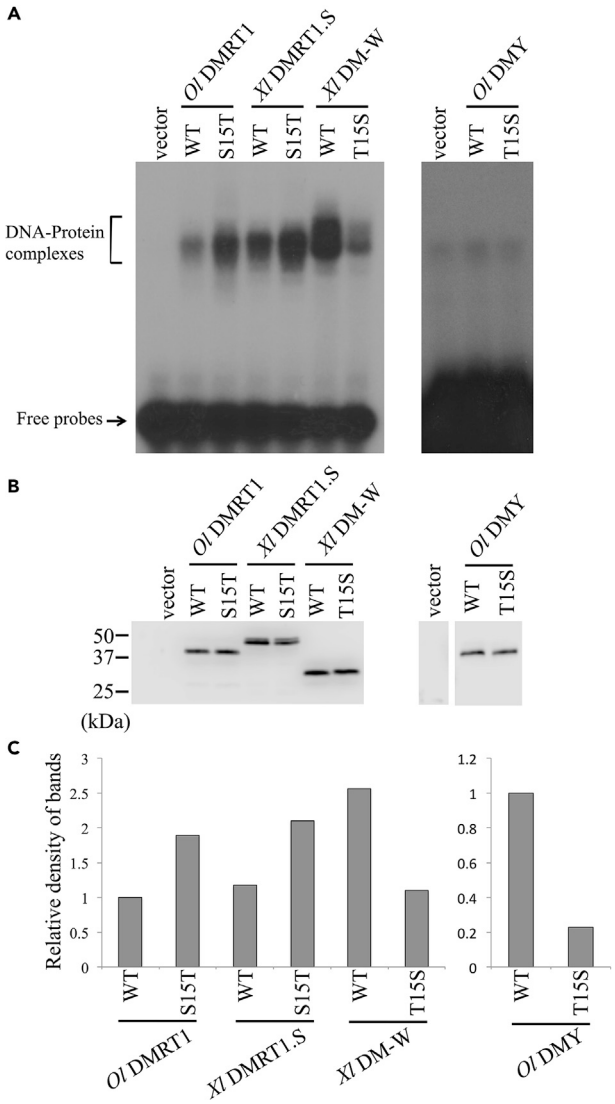

Figure 2. DNA-Binding of *X. laevis* (*Xl*) DMRT1.S, *Xl*. DM-W, *O. latipes* (*Ol*) DMRT1, *Ol* DMY, and Their S15T or T15S Mutant proteins to a Consensus DMRT1-Binding Sequence by EMSA

Each FLAG-tagged protein produced by *in vitro* transcription/translation was used for EMSA (A), and its amount was analyzed by western blotting (WB) using an anti-FLAG antibody (B). The densities of both the shifted bands (A) and the reacted bands with the antibody (B) were quantified using ImageJ, and the relative values were calculated (C). Vector, pcDNA3-flag empty vector; WT, wild-type.

amounts of proteins needed to measure the kinetics, DMY, DM-W, and DMRT1 and their T15S or S15T mutant proteins were overexpressed in bacteria (Figure S5 and Table S3). Each purified protein was mixed with biotinylated dsDNA containing the DMRT1-binding consensus sequence to measure their association, followed by the immersion of the biosensor to measure their dissociation. The association and dissociation rate constants, K_a or K_d, respectively, were measured for the dsDNA and each protein. As expected from the EMSA results (Figure 2), *X. laevis* DMRT1.S or *O. latipes* DMRT1 had a lower K_a and a higher K_d than each S15T mutant protein (Table 2), whereas DMY and DM-W had a higher K_a and a lower K_d than each T15S mutant. These results indicate that 15T favors faster DNA association and slower DNA dissociation of DMRT1 subfamily proteins compared with 15S. Moreover, the values of the dissociation constant (K_D), calculated as $K_D = K_d/K_a$, were lower in the 15T proteins, *O. latipes* DMRT1(S15T), *X. laevis* DMRT1.S(S15T), DMY, and DM-W, than their corresponding 15S proteins, *O. latipes* DMRT1, *X. laevis* DMRT1.S, DMY(T15S), and DM-W(T15S), respectively.

Species	Protein	KD (M)	Ka (Ms^{-1})	Kd (s^{-1})
	Trx-S-His	1.57×10^{-3}	5.82×10^{-1}	9.13×10^{-4}
Xenopus laevis	DMRT1.S	7.16×10^{-7}	2.01×10^{3}	1.44×10^{-3}
	DMRT1.S (S15T)	3.11×10^{-7}	4.13×10^{3}	1.29×10^{-3}
	DM-W	2.88×10^{-7}	9.18×10^{3}	2.65×10^{-3}
	DM-W (T15S)	2.95×10^{-6}	2.43×10^{3}	7.17×10^{-3}
Oryzias latipes	DMRT1	1.21×10^{-7}	1.50×10^{4}	1.81×10^{-3}
	DMRT1 (S15T)	4.97×10^{-8}	2.21×10^{4}	1.10×10^{-3}
	DMY	4.56×10^{-6}	2.37×10^{3}	1.08×10^{-2}
	DMY (T15S)	1.45×10^{-5}	1.64×10^{3}	2.38×10^{-2}

Table 2. Kinetics of Association and Dissociation between a DMRT1-Binding Consensus DNA Sequence and DMRT1 Subfamily Proteins or Their Mutant Proteins with One Amino Acid Substitution by Bio-layer Interferometry

K_a, association rate constant; K_d, dissociation rate constant; K_D, dissociation constant (K_d/K_a).

Parallel S15T Substitutions in Both DM-W and DMY May Contribute to Their Higher Transcriptional Regulation Abilities

The T15S substitutions decreased the DNA-binding abilities of DMY and DM-W (Figure 2 and Table 2). Consequently, we then verified whether the decrease in DNA-binding activity resulting from the substitutions in DMY and DM-W could influence their transcriptional regulation activity using a luciferase reporter assay. A DMRT1-driven luciferase reporter plasmid containing four tandem repeats of the consensus DMRT1-binding sequence (Yoshimoto et al., 2010) and expression plasmids for DMRT1, DMY, DM-W, and/or its mutant proteins were co-transfected in HEK293T cells. As expected, higher levels of luciferase activity were observed after the introduction of 10 ng of an expression plasmid for DMY than the same amount of an expression plasmid for its T15S substitution mutant, DMY(T15S) (Figure 3A). DM-W did not have a region corresponding to the transregulation domain of DMRT1; however, it did show transrepression functions against the transactivation induced by DMRT1 (Yoshimoto et al., 2008, 2010; Yoshimoto and Ito, 2011). We then examined the transrepression activity of DM-W against transactivation by DMRT1. The luciferase activity induced by the introduction of 10 ng of an expression plasmid for *X. laevis* DMRT1.S was downregulated by 44% or 61% compared with the same amount of an expression plasmid for DM-W or DM-W(T15S), respectively. There was a significant difference ($p < 0.01$) in the downregulation between the two proteins, indicating that DM-W(T15S) has a lower transrepression activity against DMRT1 transactivation than the wild-type DM-W (Figure 3B). Collectively, these findings demonstrate that parallel S15T substitutions in the early lineage of *dmy* and *dm-W* may play a role in their DNA-binding activity, resulting in an increase in their transregulation activity.

DISCUSSION

Transcription-factor-encoding sex-determining genes in vertebrates include Y-linked *Sry* in therian mammals, Z-linked *dmrt1* in chickens, W-linked *dm-W* in *X. laevis*, Y-linked *dmy* in *O. latipes*, and Y-linked *sox3y* in *O. dancena* (Ito and Mawaribuchi, 2013; Takehana et al., 2014). Except for *dmrt1* in chickens, these genes are considered to have emerged as a result of gene duplication or allelic mutations and neofunctionalization for sex determination. The molecular evolution of promoters and enhancers must have been essential for the establishment of the four genes, *Sry*, *sox3y*, *dm-W*, and *dmy*, as sex-determining genes from their prototype genes, *sox3* and *dmrt1* (Mawaribuchi et al., 2012; Takehana et al., 2014). Moreover, the coding regions of *Sry*, *dm-W*, and *dmy* have a higher substitution rate than those of their prototype genes, resulting in several amino acid substitutions (Mawaribuchi et al., 2012). Here, we detected two and four amino acid substitutions in the DM domains of the early lineage of DM-W and DMY, respectively (Figure 1). Among them, common S15T substitutions were found and were estimated to result from positive selection (Table 1). Most importantly, the parallel S15T substitutions in the early lineage of DMY and DM-W may enhance their DNA-binding activity (Figure 2 and Table 2), which may contribute to the establishment of *dmy* and *dm-W* as sex-determining genes. Compared with S15T, we found that the other substitutions 13L or 25I, 32N, and 37T in the DM domains of the early lineage of DM-W or DMY, respectively (Figure 1),

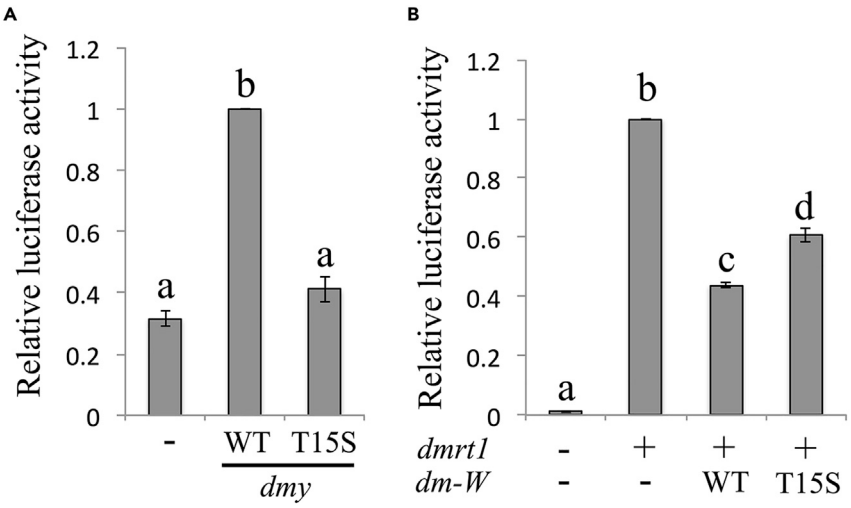

Figure 3. Effects of One Amino Acid Substitution T15S in DMY or DM-W on DMRT1-Driven Transactivation by Luciferase Reporter Assay in HEK293T Cells

Here, 100 ng of DMRT1-driven firefly luciferase reporter plasmid; 5 ng of the internal control *Renilla* luciferase plasmid pRL-TK; and 10 ng of each expression plasmid for *O. latipes* DMY, DMY(T15S) (A), or *X. laevis* DMRT1.S (B) were co-transfected into HEK293T cells as indicated. In the case of (B), 10 ng of an additional expression plasmid for *X. laevis* DM-W or DM-W(T15S) was also co-transfected, as indicated. As a negative control, 20 ng of pcDNA3-FLAG empty vector was used (left lanes in A and B). Twenty-four h post-transfection, luciferase activities were measured. The data are shown relative to the luciferase activity in the case of *O. latipes* DMY (the second left lane in A) or *X. laevis* DMRT1.S (the second left lane in B). The results are expressed as the mean ± SEM for three independent experiments, performed in technical triplicate. One-way ANOVA and Tukey's HSD test were used for statistical analysis. Different letters indicate significant differences ($p < 0.01$). WT, wild-type.

are not under positive selection (Table 1). However, we could not deny the possibility that these substitutions contributed to the establishment of *dmy* or *dm-W* as sex-determining gene. In contrast, we detected seven and two amino acid sites in DMY and DM-W, respectively, different than S15T, which are under positive selection (Table 1). It is possible that the substitutions, which were likely to occur after the early lineage of DMY and DM-W, contributed lineage-specific neofunctionalization of DMY or DM-W.

Serine at position 15 of the DM domain of DMRT1s may play an important role in determining DNA-binding properties, as serine is completely conserved in all vertebrate species except for Agnatha fish (Mawaribuchi et al., 2017a) (Figure 1A). Surprisingly, except for DMY and DM-W, there are no DM domains with T at position 15 in the DMRT family, including invertebrate-sex-determination-related proteins DSX and MAB-3. Murphy et al. (2015) found that S15 on the human DMRT1 does not come into contact with the specific-binding DNA sequence or other DM domains in its homodimer or trimer form in crystallography and nuclear magnetic resonance. These findings suggest that T15 has unique and important roles in both DMY and DM-W for sex determination, despite S and T sharing the most characteristics among the 20 amino acid residues. Based on the three-dimensional structural information of human DMRT1 (Murphy et al., 2015), we compared the stability of complexes between the specific DNA and human DMRT1 or its S15T substitution mutant protein by root-mean-square deviation and found that the substitution DMRT1(S15T) protein had a slightly higher stability with DNA than its wild-type version (data not shown). In addition, the *in vitro* binding analysis and transactivation assay in cultured cells, as shown in Figures 2 and 3 and Table 2, suggest that the S15T substitution of DMRT1 could modulate the DM domain structure, leading to alternation in its DNA-binding activity.

Convergent evolution is the independent evolution of similar features in two or more lineages. Convergence also occurs at the protein level, which can be separated into two types: convergent and parallel amino acid substitutions. The latter have a higher probability to occur by chance than the former (Zhang and Kumar, 1997). Zhang (22) reported that the molecular evolution of *dmy* from *dmrt1* duplication is under positive selection. The author predicted that a single amino acid substitution from DMRT1 to DMY may be responsible for the establishment of *dmy* as a sex-determination gene in medaka fish (Zhang, 2004). This

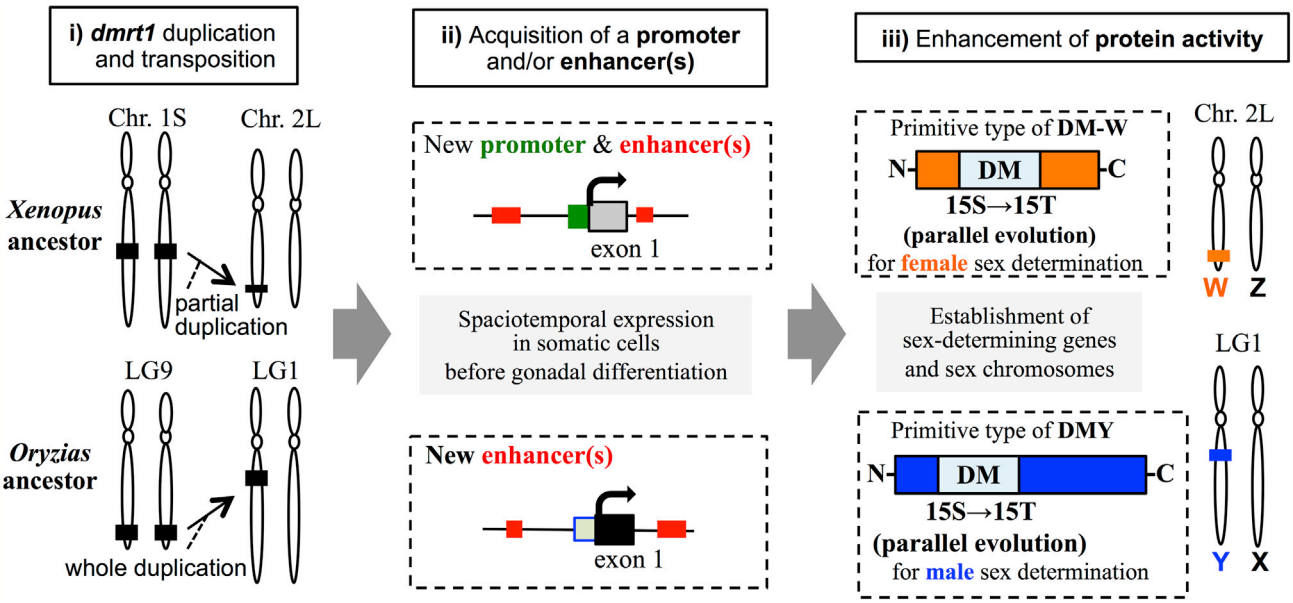

Figure 4. A Proposed Model for the Establishment of *dmrt1*-Derived Sex-Determining Genes, *dm-W* and *dmy*

study could support the validity of the prediction; we found the positive selection of S15T in medaka DMY, which might be involved in the sex-determining activity (Figures 2 and 3, Tables 1 and 2). In addition, we detected positive selection at the same position, S15T of the DM domains, in the early lineage of *Xenopus dm-W* after *dmrt1* duplication (Figure 1 and Table 1). Such parallel amino acid substitutions have been reported in several species, including three voltage-gated sodium channel genes in garter snakes for tetrodotoxin resistance (McGlothlin et al., 2014) and two testis-specific duplicates of the broadly expressed aldehyde dehydrogenase gene in different *Drosophila* lineages (Chakraborty and Fry, 2015). To our knowledge, this is a first example of parallel amino acid substitutions for sex determination.

Were the parallel S15T amino acid substitutions involved in homologous recombination suppression? It is believed that *dmy* and *dm-W* have evolved under recombination suppression. We previously reported that recombination suppression might lead to accumulation of DNA replication errors during population and species diversification (Mawaribuchi et al., 2016). Therefore, it is possible that amino acid substitutions might occur with increased frequency under the suppression of recombination.

Based on these findings, we propose a model for the molecular evolution of *dmy* and *dm-W* (Figure 4). In some ancestor of *Oryzias* or *Xenopus*, the whole or partial duplicate of *dmrt1* was inserted into another chromosome via transposable element, resulting in the emergence of the prototype *dmy* and *dm-W* genes (Matsuda et al., 2002; Nanda et al., 2002; Yoshimoto et al., 2008; Herpin et al., 2010; Bewick et al., 2011; Yoshimoto and Ito, 2011; Matsuda and Sakaizumi, 2016; Mawaribuchi et al., 2017b). Each prototype gene happened to obtain a (additional) promoter and enhancer(s) for expression in gonadal somatic cells before gonadal differentiation, leading to the appearance of the ancestor *dmy* or *dm-W* genes for sex determination (Herpin et al., 2010; Mawaribuchi et al., 2012). After obtaining this new promoter/enhancer system, the *dmy* or *dm-W* genes mutated, causing the T15S substitution, which may enhance DNA-binding ability (Figures 1 and 2, and Table 2). As a result, these reinforced *dmy* or *dm-W* genes were established as sex-determining genes in *Oryzias* or *Xenopus*.

Limitations of the Study

This work indicated that the parallel amino acid substitutions from S to T on ancestral DMY and DM-W could enhance their DNA-binding activity and transcriptional regulation function, which might have independently supported the establishment of the *dmrt1*-type sex-determining genes, *dmy* and *dm-W*. However, it remains unknown whether each S15T substitution was involved in the enhancement of the sex-determining function in the ancestor of the fish *O. latipes* or frog *X. laevis*. It would be helpful for

the question to investigate an effect of the S15T substitution in DMRT1 or T15S substitution in DMY/DM-W on sex determination in knock-in transgenic *O. latipes* and *X. laevis* individuals.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.100757.

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AUTHOR CONTRIBUTIONS

Y. O., S. M., and M. I. designed the study; Y. O., S. M., and K. N. performed the experiments; Y. O., K. T., M. M., T. K., H. O., G. W., S. Y., and N. T. analyzed the data; and Y. O. and M. I. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

Bewick, A.J., Anderson, D.W., and Evans, B.J. (2011). Evolution of the closely related, sex-related genes DM-W and DMRT1 in African clawed frogs (Xenopus). Evolution *65*, 698–712.

Chakraborty, M., and Fry, J.D. (2015). Parallel functional changes in independent testis-specific duplicates of Aldehyde dehydrogenase in Drosophila. Mol. Biol. Evol. *32*, 1029–1038.

Ge, C., Ye, J., Weber, C., Sun, W., Zhang, H., Zhou, Y., Cai, C., Qian, G., and Capel, B. (2018). The histone demethylase KDM6B regulates temperature-dependent sex determination in a turtle species. Science *360*, 645–648.

Herpin, A., Braasch, I., Kraeussling, M., Schmidt, C., Thoma, E.C., Nakamura, S., Tanaka, M., and Schartl, M. (2010). Transcriptional rewiring of the sex determining dmrt1 gene duplicate by transposable elements. PLoS Genet. *6*, e1000844.

Ito, M., and Mawaribuchi, S. (2013). Molecular Evolution of Genes Involved in Vertebrate Sex Determination (eLS. John Wiley and Sons, Ltd), p. a0024948, https://doi.org/10.1002/9780470015902.

Jeffries, D.L., Lavanchy, G., Sermier, R., Sredl, M.J., Miura, I., Borzée, A., Barrow, L.N., Canestrelli, D., Crochet, P.A., Dufresnes, C., et al. (2018). A rapid rate of sex-chromosome turnover and non-random transitions in true frogs. Nat. Commun. *9*, 4088.

Kitano, J., and Peichel, C.L. (2012). Turnover of sex chromosomes and speciation in fishes. Environ. Biol. Fishes *94*, 549–558.

Masuyama, H., Yamada, M., Kamei, Y., Fujiwara-Ishikawa, T., Todo, T., Nagahama, Y., and Matsuda, M. (2012). Dmrt1 mutation causes a male-to-female sex reversal after the sex determination by Dmy in the medaka. Chromosome Res. *20*, 163–176.

Matson, C.K., and Zarkower, D. (2012). Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. Nat. Rev. Genet. *13*, 163–174.

Matson, C.K., Murphy, M.W., Griswold, M.D., Yoshida, S., Bardwell, V.J., and Zarkower, D. (2010). The mammalian doublesex homolog DMRT1 is a transcriptional gatekeeper that controls the mitosis versus meiosis decision in male germ cells. Dev. Cell *19*, 612–624.

Matsuda, M., and Sakaizumi, M. (2016). Evolution of the sex-determining gene in the teleostean genus Oryzias. Gen. Comp. Endocrinol. *239*, 80–88.

Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C.E., Shibata, N., Asakawa, S., Shimizu, N., et al. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. Nature *417*, 559–563.

Mawaribuchi, S., Yoshimoto, S., Ohashi, S., Takamatsu, N., and Ito, M. (2012). Molecular evolution of vertebrate sex-determining genes. Chromosome Res. *20*, 139–151.

Mawaribuchi, S., Ito, M., Ogata, M., Oota, H., Katsumura, T., Takamatsu, N., and Miura, I. (2016). Meiotic recombination counteracts male-biased mutation (male-driven evolution). Proc. Biol. Sci. *283*, 1823.

Mawaribuchi, S., Musashijima, M., Wada, M., Izutsu, Y., Kurakata, E., Park, M.K., Takamatsu, N., and Ito, M. (2017a). Molecular evolution of two distinct dmrt1 promoters for germ and somatic cells in vertebrate gonads. Mol. Biol. Evol. *34*, 724–733.

Mawaribuchi, S., Takahashi, S., Wada, M., Uno, Y., Matsuda, Y., Kondo, M., Fukui, A., Takamatsu, N., Taira, M., and Ito, M. (2017b). Sex chromosome differentiation and the W- and Z-specific loci in Xenopus laevis. Dev. Biol. *426*, 393–400.

McGlothlin, J.W., Chuckalovcak, J.P., Janes, D.E., Edwards, S.V., Feldman, C.R., Brodie, E.D., and Pfrender, M.E. (2014). Parallel evolution of tetrodotoxin resistance in three voltage-gated sodium channel genes in the garter snake Thamnophis sirtalis. Mol. Biol. Evol. *31*, 2836–2846.

Miura, I. (2017). Sex determination and sex chromosomes in amphibia. Sex. Dev. *11*, 298–306.

Murphy, M.W., Zarkower, D., and Bardwell, V.J. (2007). Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. BMC Mol. Biol. *8*, 58.

Murphy, M.W., Lee, J.K., Rojo, S., Gearhart, M.D., Kurahashi, K., Banerjee, S., Loeuille, G.A., Bashamboo, A., McElreavey, K., Zarkower, D., et al. (2015). An ancient protein-DNA interaction

underlying metazoan sex determination. Nat. Struct. Mol. Biol. *22*, 442–451.

Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., et al. (2002). A duplicated copy of DMRT1 in the sex-determining region of the Y chromosome of the medaka, Oryzias latipes. Proc. Natl. Acad. Sci. U S A *99*, 11778–11783.

Session, A.M., Uno, Y., Kwon, T., Chapman, J.A., Toyoda, A., Takahashi, S., Fukui, A., Hikosaka, A., Suzuki, A., Kondo, M., et al. (2016). Genome evolution in the allotetraploid frog Xenopus laevis. Nature *538*, 336–343.

Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., and Sinclair, A.H. (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. Nature *461*, 267–271.

Takehana, Y., Matsuda, M., Myosho, T., Suster, M.L., Kawakami, K., Shin-I, T., Kohara, Y., Kuroki, Y., Toyoda, A., et al. (2014). Co-option of Sox3 as the male-determining factor on the Y chromosome in the fish Oryzias dancena. Nat. Commun. *5*, 4157.

Yoshimoto, S., and Ito, M. (2011). A ZZ/ZW-type sex determination in Xenopus laevis. FEBS J. *278*, 1020–1026.

Yoshimoto, S., Okada, E., Umemoto, H., Tamura, K., Uno, Y., Nishida-Umehara, C., Matsuda, Y., Takamatsu, N., Shiba, T., and Ito, M. (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in Xenopus laevis. Proc. Natl. Acad. Sci. U S A *105*, 2469–2474.

Yoshimoto, S., Ikeda, N., Izutsu, Y., Shiba, T., Takamatsu, N., and Ito, M. (2010). Opposite roles of DMRT1 and its W-linked paralogue, DM-W, in sexual dimorphism of Xenopus laevis: implications of a ZZ/ZW-type sex-determining system. Development *137*, 2519–2526.

Zarkower, D. (2013). DMRT genes in vertebrate gametogenesis. Curr. Top. Dev. Biol. *102*, 327–356.

Zhang, J. (2004). Evolution of DMY, a newly emergent male sex-determination gene of medaka fish. Genetics *166*, 1887–1895.

Zhang, J., and Kumar, S. (1997). Detection of convergent and parallel evolution at the amino acid sequence level. Mol. Biol. Evol. *14*, 527–536.

Zhao, L., Svingen, T., Ng, E.T., and Koopman, P. (2015). Female-to-male sex reversal in mice caused by transgenic overexpression of Dmrt1. Development *142*, 1083–1088.

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Supplemental Information

Parallel Evolution of Two *dmrt1*-Derived Genes, *dmy* and *dm-W*, for Vertebrate Sex Determination

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Supplementary Information
Supplementary Figures

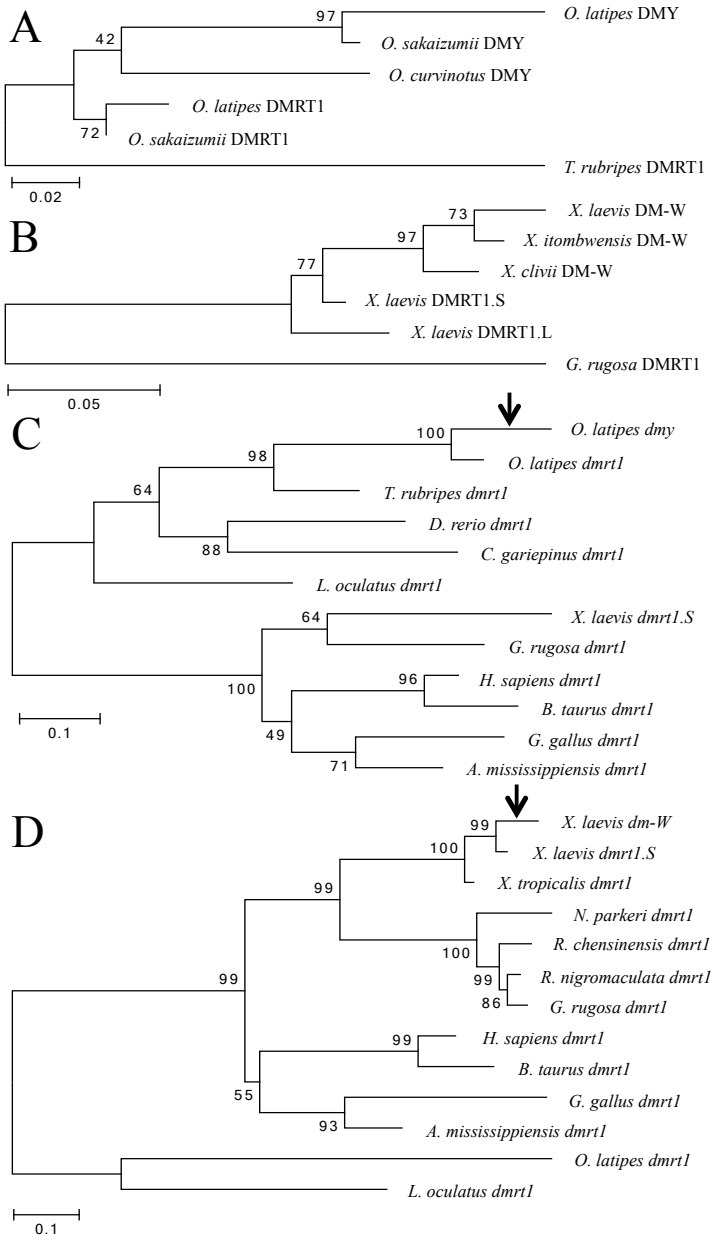

Figure S1. [Phylogenetic trees of *dmrt1* subfamily], Related to Figure 1 and Table 1. The trees were constructed using the nucleotide alignments of the DM domain-coding 168 sequences (**A and B**), or 339- and 345-coding sequences (**C and D,** respectively) within the two exons including the DM domain-coding region, by MUSCLE in MEGA6. The Jukes-Cantor model with gamma distribution (**A**), Kimura 2-parameter model (**B**), and Tamura 3-parameter model with gamma distribution (**C**) or gamma distribution and invariant site categories (**D**) were used. **A and B** or **C and D** were used to infer the ancestral sequences in Fig. 1 and the dN/dS ratio test in Table 1, respectively. Arrows in (**C**) and (**D**) show the foreground branches used for the dN/dS ratio test by codeml. A 1,000 bootstrap value is shown at each branch point. Scale bar = estimated nucleotide substitutions per site.

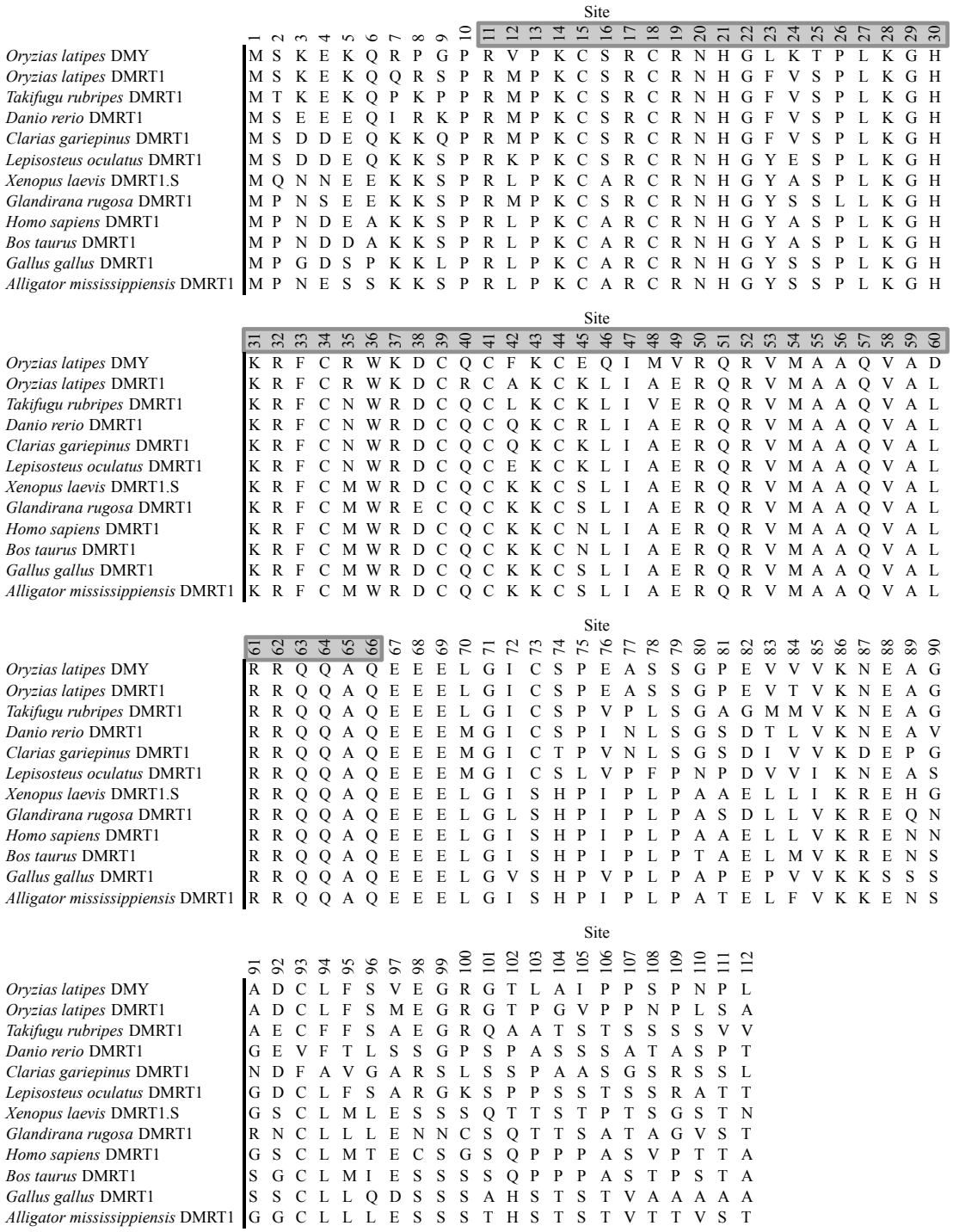

Figure S2. [The amino acid sequence alignments of various vertebrate DMRT1 and *Oryzias latipes* DMY for the BEB analysis in codeml], Related to Table 1. The DMRT1 subfamily sequences containing DMY were aligned by MUSCLE in MEGA6. The numbers on the DM domain are shown by gray squares.

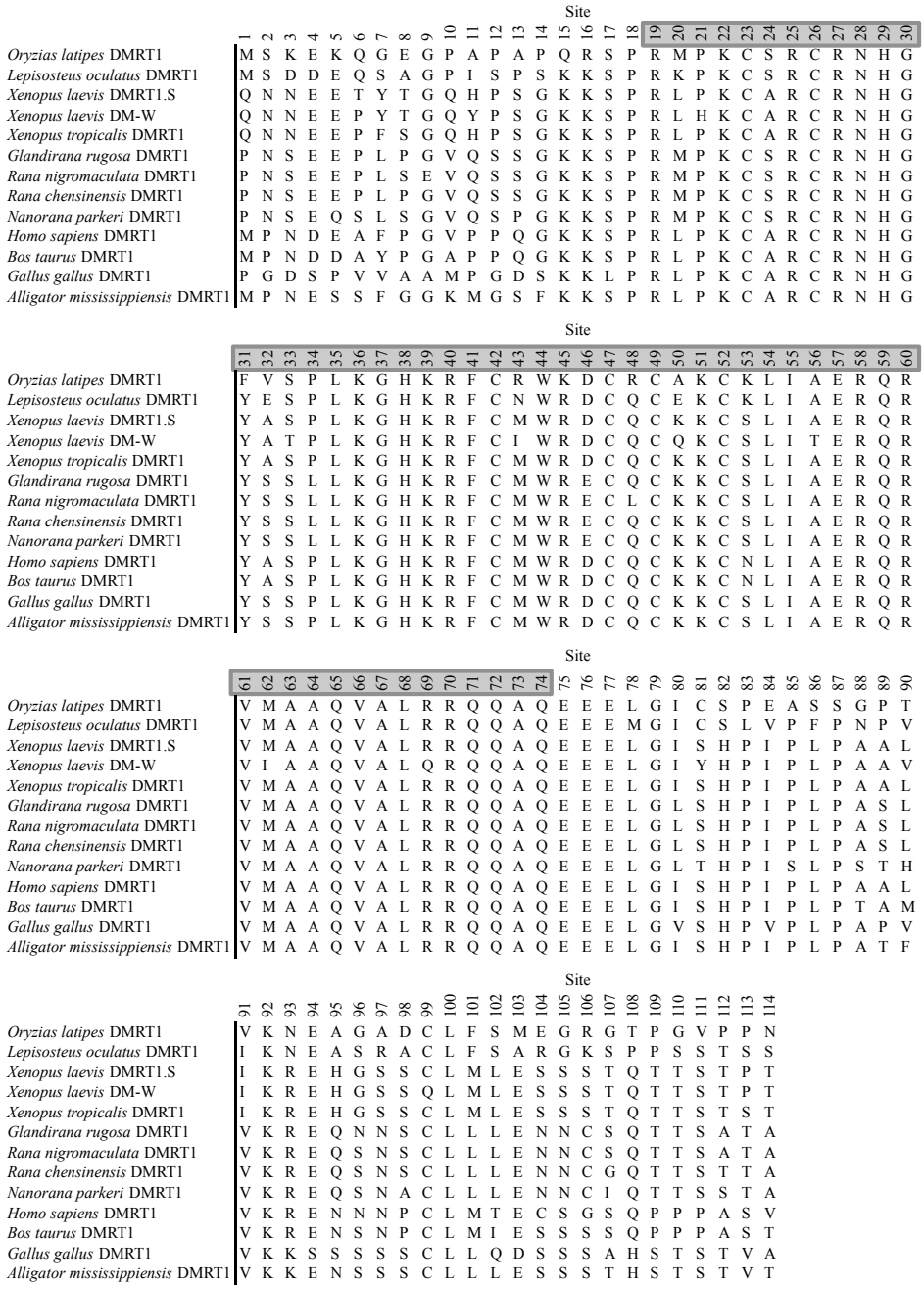

Figure S3. [**The amino acid sequence alignments of various vertebrate DMRT1 and *Xenopus laevis* DM-W for the BEB analysis in codeml], Releted to Table 1.** The DMRT1 subfamily sequences containing DM-W were aligned by MUSCLE in MEGA6. The numbers on the DM domain are shown by gray squares.

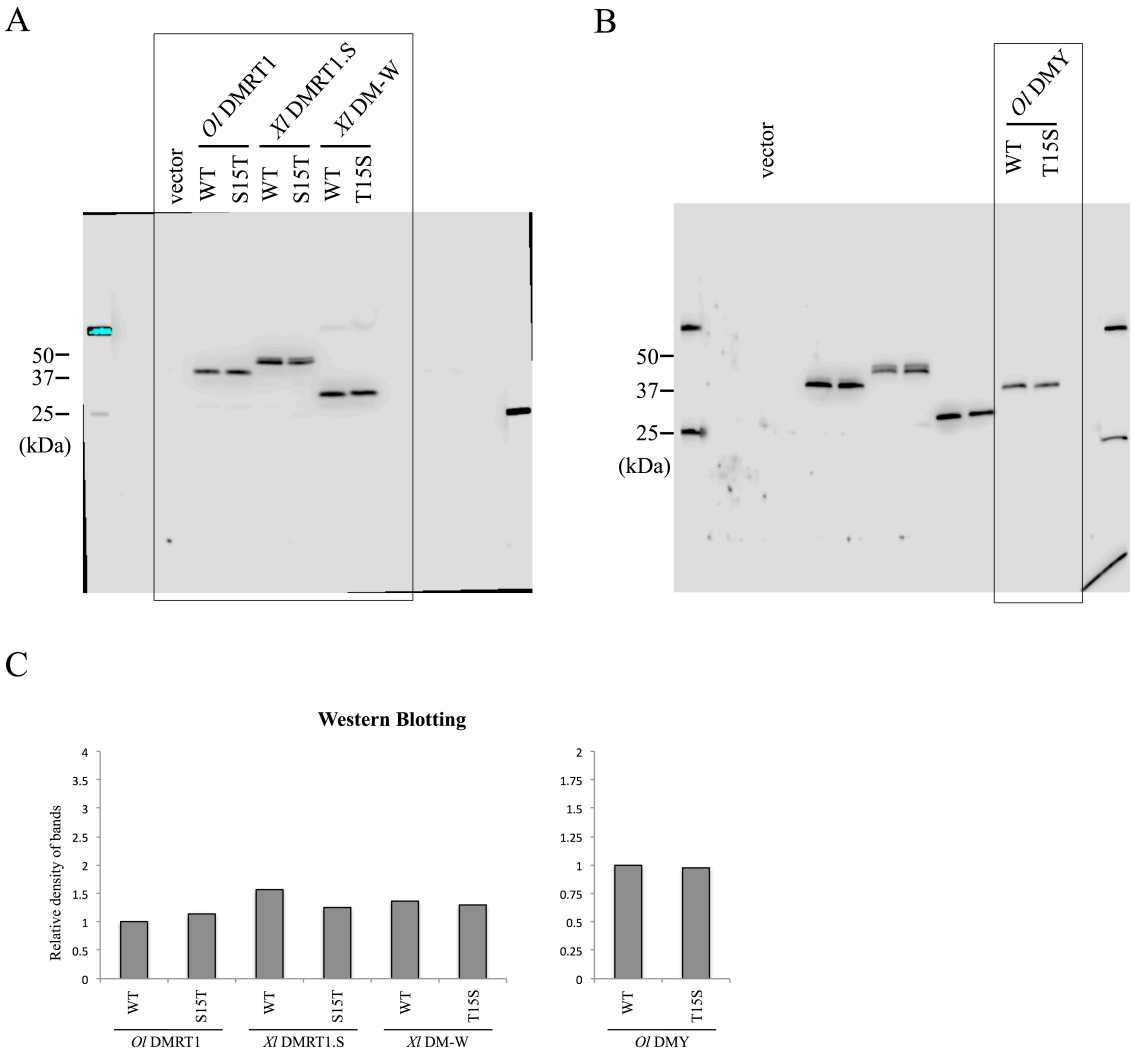

Figure S4. [**Immunoblotting analysis of FLAG-tagged DMRT1, DM-W, and DMY produced by *in vitro* transcription/translation with an anti-FLAG antibody], Related to Figure 2.** (**A**) Enlarged version of Fig. 2B. Dashed square indicated the lanes used in Fig. 2B. (**B**) Relative density of the immunoreactive bands, compared to *O. latipes* wild-type (WT) DMRT1 (left panel) or *O. latipes* WT DMY (right panel) in **A**. The band density was quantified using Image J.

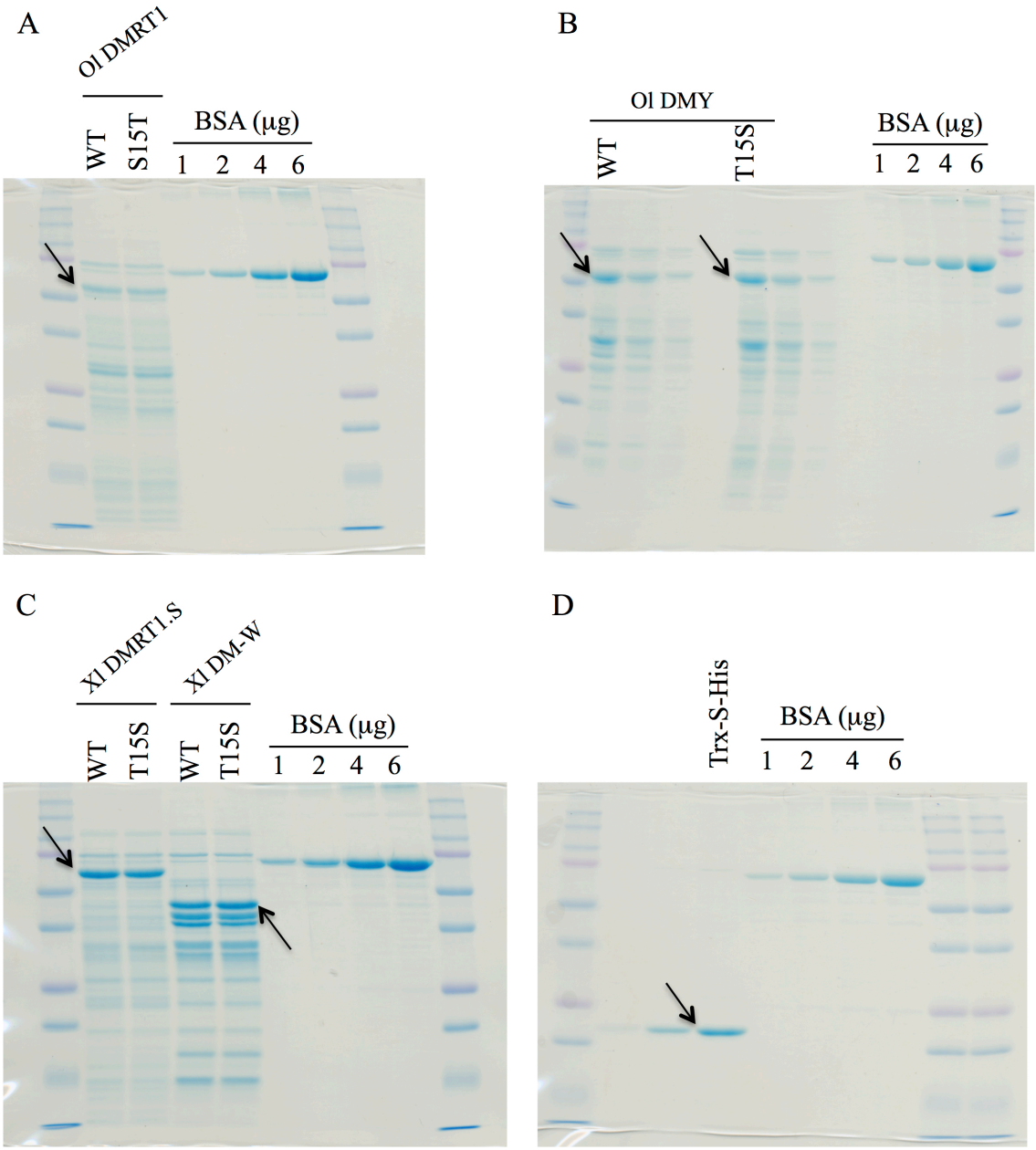

Figure S5. [Coomassie brilliant blue (CBB) staining of the DMRT1 subfamily and its mutant proteins used for bio-layer interferometry], Related to Table 2. The purified proteins and bovine serum albumin (BSA) were separated by SDS-PAGE and stained with CBB. The arrows indicate the bands of the DMRT1 subfamily protein.

Supplementary Tables

Table S1. [Species names and gene ID of *dmrt1* subfamily members, used for reconstruction of ancestral sequences and dN/dS ratio test], Related to Figure 1 and Table 1

Species	Gene	Gene ID
Oryzias latipes	*dmrt1*	NM_001104680.2
Oryzias latipes	*dmy*	AY129240.1
Oryzias sakaizumii	*dmrt1*	AY157712.1
Oryzias sakaizumii	*dmy*	NM_001104680.1
Oryzias curvinotus	*dmrt1*	AB091696.1
Oryzias curvinotus	*dmy*	AB091695.1
Takifugu rubripes	*dmrt1*	NM_001037949.1
Danio rerio	*dmrt1*	NM_205628.2
Clarias gariepinus	*dmrt1*	AF439561.1
Lepisosteus oculatus	*dmrt1*	XM_006627122.1
Xenopus laevis	*dmrt1.L*	NM_001096500.1
Xenopus laevis	*dmrt1.S*	NM_001085483.1
Xenopus laevis	*dm-W*	NM_001114842.1
Xenopus clivii	*dm-W*	HQ220852.1
Xenopus itombwensis	*dm-W*	HQ220850.1
Xenopus tropicalis	*dmrt1*	XM_018089347.1
Glandirana rugosa	*dmrt1*	AB272609.1
Rana nigromaculata	*dmrt1*	EF524050.1
Rana chensinensis	*dmrt1*	KC439687.2
Nanorana parkeri	*dmrt1*	XM_018559473.1
Homo sapiens	*dmrt1*	NM_021951.3
Bos taurus	*dmrt1*	NM_001078060.1
Gallus gallus	*dmrt1*	GU396269.1
Alligator mississippiensis	*dmrt1*	XM_006261265.3
Lethenteron reissneri	*dmrt1*	*LC035083.1*

Table S2. [**The ancestral amino acid sequences of the DM domains in DMRT1 subfamily], Related to Figure 1.** They were estimated by the maximum likelihood method of the Jones-Taylor-Thornton model with gamma distribution (two panels in the upper part) and the Dayhoff model (two panels in the lower part) in MEGA6.

	Site																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1. *Oryzias latipes* DMY	R	V	P	K	C	S	R	C	R	N	H	G	L	K	T	P	L	K	G	H	K	R	F	C	R	W	K	D	C	Q
2. *Oryzias sakaizumii* DMY	R	V	P	K	C	S	R	C	R	N	H	S	L	K	T	P	L	K	G	H	K	R	F	C	R	W	K	D	C	H
3. *Oryzias curvinotus* DMY	R	K	P	K	C	F	R	C	R	N	H	G	L	V	T	P	L	K	G	H	K	R	F	C	P	W	K	D	C	A
4. *Oryzias latipes* DMRT1	R	M	P	K	C	S	R	C	R	N	H	G	F	V	S	P	L	K	G	H	K	R	F	C	R	W	K	D	C	R
5. *Oryzias sakaizumii* DMRT1	R	M	P	K	C	S	R	C	R	N	H	G	F	V	S	P	L	K	G	H	K	R	F	C	R	W	K	D	C	R
6. *Takifugu rubripes* DMRT1	R	M	P	K	C	S	R	C	R	N	H	G	F	V	S	P	L	K	G	H	K	R	F	C	N	W	R	D	C	Q
7. (1 . 2)	R	V*	P	K	C	S	R	C	R	N	H	G	L	K	T	P	L	K	G	H	K	R	F	C	R	W	K	D	C	Q*
8. (7 . 3)	R	M*	P	K	C	S	R	C	R	N	H	G	L*	V	T	P	L	K	G	H	K	R	F	C	R	W	K	D	C	Q*
9. (4 . 5)	R	M	P	K	C	S	R	C	R	N	H	G	F	V	S	P	L	K	G	H	K	R	F	C	R	W	K	D	C	R
10. (8 . 9)	R	M	P	K	C	S	R	C	R	N	H	G	F	V	S	P	L	K	G	H	K	R	F	C	R	W	K	D	C	Q*

	Site																								
	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
1. *Oryzias latipes* DMY	C	F	K	C	E	Q	I	M	V	R	Q	R	V	M	A	A	Q	V	A	D	R	R	Q	Q	A	Q
2. *Oryzias sakaizumii* DMY	C	L	K	C	K	L	I	V	D	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
3. *Oryzias curvinotus* DMY	C	A	K	C	K	L	I	V	V	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
4. *Oryzias latipes* DMRT1	C	A	K	C	K	L	I	A	E	G	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
5. *Oryzias sakaizumii* DMRT1	C	A	K	C	K	L	I	A	E	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
6. *Takifugu rubripes* DMRT1	C	L	K	C	K	L	I	V	E	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
7. (1 . 2)	C	L*	K	C	K	L	I	V	D*	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
8. (7 . 3)	C	L*	K	C	K	L	I	V	E*	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
9. (4 . 5)	C	A	K	C	K	L	I	A	E	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q
10. (8 . 9)	C	L*	K	C	K	L	I	V	E	R	Q	R	V	M	A	A	Q	V	A	L	R	R	Q	Q	A	Q

	Site																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1. *Glandirana rugosa* DMRT1	K	S	P	R	C	S	R	C	R	N	H	G	Y	S	S	L	L	K	G	H	K	R	F	C	M	W	R	E	C	Q
2. *Xenopus laevis* DMRT1.L	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	S	P	L	K	G	H	K	R	Y	C	M	W	R	D	C	Q
3. *Xenopus laevis* DMRT1.S	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	S	P	L	K	G	H	K	R	F	C	M	W	R	D	C	Q
4. *Xenopus laevis* DM-W	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	T	P	L	K	G	H	K	R	F	C	I	W	R	D	C	Q
5. *Xenopus clivii* DM-W	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	T	P	L	K	G	H	K	R	F	C	I	W	R	D	C	Q
6. *Xenopus itombwensis* DM-W	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	T	P	L	K	G	H	K	R	F	C	I	W	R	D	C	Q
7. (4 . 6)	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	T	P	L	K	G	H	K	R	F	C	I	W	R	D	C	Q
8. (7 . 5)	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	T	P	L	K	G	H	K	R	F	C	I	W	R	D	C	Q
9. (8 . 3)	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	S	P	L	K	G	H	K	R	F	C	M	W	R	D	C	Q
10. (9 . 2)	K	S	P	R	C	A	R	C	R	N	H	G	Y	A	S	P	L	K	G	H	K	R	F	C	M	W	R	D	C	Q

	Site														
	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1. *Glandirana rugosa* DMRT1	R	E	C	Q	C	K	K	C	S	L	I	A	E	R	Q
2. *Xenopus laevis* DMRT1.L	R	D	C	Q	C	K	K	C	S	L	I	A	E	R	Q
3. *Xenopus laevis* DMRT1.S	R	D	C	Q	C	K	K	C	S	L	I	A	E	R	Q
4. *Xenopus laevis* DM-W	R	D	C	Q	C	Q	K	C	S	L	I	T	E	R	Q
5. *Xenopus clivii* DM-W	R	D	C	Q	C	N	K	C	S	L	T	A	E	R	Q
6. *Xenopus itombwensis* DM-W	R	D	C	Q	C	Q	K	C	S	L	T	A	E	R	Q
7. (4 . 6)	R	D	C	Q	C	Q	K	C	S	L	T	A	E	R	Q
8. (7 . 5)	R	D	C	Q	C	N	K	C	S	L	T	A	E	R	Q
9. (8 . 3)	R	D	C	Q	C	K	K	C	S	L	I	A	E	R	Q
10. (9 . 2)	R	D	C	Q	C	K	K	C	S	L	I	A	E	R	Q

***** Sites with maximum probability < 0.9 and ≥ 0.5 for any state

Table S3. [Quantification of DMRT1/DM-W and their mutant proteins by CBB staining in Figure S5], Related to Table 2.

Proteins		Concentration (μg/μl)
Trx-S-His		0.509
Ol DMRT1	WT	0.190
	S15T	0.165
Ol DMY	WT	0.901
	T15S	0.846
Xl DMRT1.S	WT	0.375
	S15T	0.335
Xl DM-W	WT	0.349
	T15S	0.388

Transparent Methods

Evolutionary analysis

For evolutionary analysis, the protein-coding sequences of the *dmrt1* subfamily genes were collected from the NCBI database (see Table S1).

The two alignments of nucleotide sequences encoding the DM domains (see Fig. 1A) of the *dmrt1* subfamily including *dmy* and *dm-W* were performed by MUSCLE in MEGA6, based on their deduced aa sequences. From each resultant alignment, its corresponding phylogenetic tree including *dmy* or *dm-W* was constructed using the maximum likelihood method of the Jukes-Cantor model with gamma distribution or the Kimura 2-parameter model, respectively. The ancestral aa sequences of DMRT1 subfamily proteins were inferred using each alignment and tree including *dmy* or *dm-W* with the maximum likelihood method of the Jones-Taylor-Thornton model with gamma distribution or the Dayhoff model, respectively, in MEGA6 (32) (see Table S2).

For analysis of positive selection, two alignments including the deduced aa sequences of DMY and DM-W were performed using the 339 and 345 coding sequences within the two exons, respectively, which include the DM domain-coding region, by MUSCLE in MEGA6. From the resultant alignments, the two phylogenetic trees including DMY and DM-W were constructed in MEGA6 with the maximum likelihood method of the Tamura 3-parameter model with gamma distribution or gamma distribution and invariant site categories, respectively. Then the alignments and phylogenetic trees were used for the branch-site test (33) using the maximum likelihood method in the codeml program of PAML4.8 as described (34).

For the construction of all phylogenetic trees, the nucleotide and amino acid substitution model was evaluated using Model Selection in MEGA6, according to the bayesian information criterion.

Plasmid construction

A full-length ORF, except without ATG (corresponding to an initiation codon), from *O. latipes dmrt1*, *O. latipes dmy*, *X. laevis dmrt1.S*, or *X. laevis dm-W* was cloned either into an *Eco*RV-digested pcDNA3-FLAG vector (35) with a FLAG-tag sequence for protein production by *in vitro* transcription/translation, or into an *Eco*RI/*Xho*I-digested pET-32b(+) vector (Novagen) with an N-terminal S/His-tag sequence for protein expression in bacteria. Each mutated plasmid for *O. latipes* DMRT1(S15T), *X. laevis* DMRT1.S(S15T), *O. latipes* DMY(T15S), or *X. laevis* DM-W(T15S), which has an S15T

or T15S substitution, was constructed by site-directed mutagenesis using the plasmid constructed above.

Electrophoretic mobility shift assay

FLAG-tagged proteins were synthesized using the TnT® Quick Coupled Transcription/Translation System (Promega, Wisconsin, USA). The reaction buffer included 20 μM $ZnSO_4$ for zinc-finger protein synthesis. For probe preparation, two types of oligonucleotides, 5'-GGGGAGATTTGATACATTGTTGCTCGATGG-3' and 5'-CCATCGAGCAACAATGTATCAAATCTC-3', were incubated for 10 min at 70°C in 100 mM NaCl, and then annealed by cooling down to room temperature. The resulting dsDNA (0.625 μM) was labeled with [^{32}P]-dCTP using Klenow DNA polymerase. Unincorporated nucleotides were removed using the MicroSpin G-25 Columns (GE Healthcare, Tokyo, Japan). A DNA-protein binding reaction was performed at room temperature for 30 minutes in a total volume of 10 μl of binding reaction mixture consisting of 10 mM Tris-HCl pH 8.0, 100 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM spermidine, 0.075% Triton-X 100, 1 mM dithiothreitol, 1 μg bovine serum albumin, 0.1 μg dI-dC, 0.1 mM $ZnSO_4$, 0.05 μM radio-labeled duplex probe, and 2 μl of the *in vitro* transcription/translation solution. The reaction solutions were resolved on a 5% native acrylamide gel in Tris/glycine buffer (50 mM Tris, 400 mM glycine). Gels were pre-run at 100 V at the same voltage for 1 hour at room temperature, and then dried and subjected to autoradiography.

Western blotting

Protein samples were electrophoresed on 12% SDS-PAGE gel and then transferred to a FluoroTrans® W Membrane (PALL) by semi-dry transfer. The membrane was treated with 5% skim milk, and incubated with a 1:50000 dilution of an M5 anti-FLAG monoclonal antibody (Sigma-Aldrich, Missouri, USA) overnight at 4°C. The membrane was washed with PBS containing 0.1% Tween-20 (PBST) for 5 min three times, then incubated with a 1:50000 dilution of an horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (Cell signaling technology, MA, USA) at room temperature, and washed with PBST for 15 min three times. The HRP was reacted by ImmnoStar® LD (Wako, Osaka, Japan), and then detected by C-DiGit® (LI-COR®, Nebraska, USA).

Protein expression in bacteria and purification

Escherichia coli BL21 (DE3) carrying the pET32b(+) bacterial expression plasmid

was grown to OD_{600} = 0.5, induced with a final concentration of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside for 2 h at 25℃, and then harvested by centrifugation. Pellets were resuspended in buffer A (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Triton X-100) containing 10 mM imidazole and a protease inhibitor cocktail (Sigma-Aldrich, P2714). The cells were lysed using an ultrasonic homogenizer (Sonifire 250, BRNSON), followed by centrifugation at 10,000 × g at 4°C for 10 min. The supernatant was loaded onto a nickel affinity column pre-equilibrated with buffer B (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.01% Triton X-100, and 10 mM imidazole), buffer C (50 mM Na-phosphate pH 7.8, 300 mM NaCl, 0.01% Triton X-100, 10 mM, and 10 mM imidazole). The column was washed with buffer B, followed by buffer D (50 mM Na-phosphate pH 6.0, 300 mM NaCl, and 0.01% Triton X-100) containing 50 mM imidazole. The His-tagged proteins were eluted using buffer D with 500 mM imidazole for 30 min at 4°C. The eluates were dialyzed with buffer E (10 mM Tris-HCl pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.01% Triton X-100, 0.1 mM $ZnSO_4$, and 1 mM DTT) in a cellulose tube. The proteins were concentrated using a Nanosep 30K Omega centrifuge (Pall) at 12,000 × g for 15 min at 4°C. The purified proteins and several amounts of bovine serum albumin (BSA) were separated by SDS-PAGE, then stained using Coomassie brilliant blue. The quantity of purified proteins was calculated from the standard curves produced by BSA. The density of the stained bands was quantified using Image J (see Table S3).

Bio-layer interferometry

A biotinylated oligonucleotide, 5'-GAGATTTGATACATTGTTGCTCGATGG-3', was annealed with its complementary oligonucleotide in 100 mM NaCl by cooling down to room temperature after heat treatment at 70℃ for 10 min. Then, 1 μM of the resultant dsDNA, which was diluted in a binding buffer (10 mM Tris-HCl pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.01% Triton X-100, 0.1 mM $ZnSO_4$, and 1 mM DTT), was immobilized on a streptavidin biosensor for 2 min. The binding kinetics of the biotinylated DNA to S/His-tag DM domain-containing proteins was estimated by bio-layer interferometry using BLitz (ForteBio, Menlo Park, CA, USA). Each of the S/His-tag proteins was incubated with the immobilized DNA in biding buffer for 2 min to determine the association. The biosensor was then immersed in binding buffer for another 2 min to measure the dissociation. KD was calculated using BLitz (version 1.2.1.5).

Cell culture, transfection, and luciferase assays

HEK293T cells were grown in Dulbecco's modified Eagle's medium containing 10%

FCS. Cells were plated 2×10^5 cells/well in 24-well dishes 24 h before transfection. The cells were transfected with 100 ng of luciferase reporter plasmid p4xDMRT1-luc (7), 10-20 ng of various combinations of effector plasmids, and 20 ng of TK-Luc *Renilla* luciferase expression control plasmid pRL-TK-Luc (Promega, Madison, WI) using 4 μg of Polyethylenimine-Max (Polysciences, Inc., Warrington, PA) as the transfection reagent. The amount of expression plasmids was adjusted to 0.25 μg by adding the pcDNA3-FLAG empty vector. Luciferase activity was measured 24 h after transfection using a luciferase assay system (Promega, Madison, WI) and normalized to *Renilla* luciferase activity.

Statistical analysis

All statistical analyses were conducted using R (version 3.3.3) and Excel. The results are expressed as the mean ± standard error of the mean (SEM). The one-tailed χ^2-test was carried out to compare the mean values of the two-nested hypothesis. Null models were rejected when the p-value was lower than 0.05. One-way ANOVA was used to compare three or four groups. Tukey's honestly significant difference (HSD) test was used to perform multiple tests using the ANOVA results. P-values lower than 0.01 were considered statistically significant.

References in Methods

Ito, M., Yoshioka, K., Akechi, M., Yamashita, S., Takamatsu, N., Sugiyama, K., Hibi, M., Nakabeppu, Y., Shiba, T., and Yamamoto, K. I. (1999) JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. Mol Cell Biol *19*, 7539-7548.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol *30*, 2725-2729.

Yang, Z. (2007) PAML 4: phylogenetic analysis by maximum likelihood. Mol Biol Evol *24*, 1586-1591.

Zhang, J., Nielsen, R., and Yang, Z. (2005) Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol Biol Evol *22*, 2472-2479.