Molecular evolution of vertebrate sex-determining genes

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Abstract Y-linked Dmy (also called dmrt1bY) in the teleost fish medaka, W-linked Dm-W in the African clawed frog (Xenopus laevis), and Z-linked Dmrt1 in the chicken are all sex chromosome-linked Dmrt1 homologues required for sex determination. Dmy and Dm-W both are Dmrt1 palalogues evolved through Dmrt1 duplication, while chicken Dmrt1 is a Z-linked orthologue. The eutherian sex-determining gene, Sry, evolved from an allelic gene, Sox3. Here we analyzed the exon–intron structures of the Dmrt1 homologues of several vertebrate species through information from databases and by determining the transcription initiation sites in medaka, chicken, Xenopus, and mouse. Interestingly, medaka Dmrt1 and Dmy and Xenopus Dm-W and Dmrt1 have a noncoding-type first exon, while mouse and chicken Dmrt1 do not. We next compared the 5′-flanking sequences of the Dmrt1 noncoding and coding exons 1 of several vertebrate species and found conservation of the presumptive binding sites for some transcription factors. Importantly, based on the phylogenetic trees for Dmrt1 and Sox3 homologues, it was implied that the sex-determining gene Dmy, Dm-W, and Sry have a higher substitution rate than their prototype genes. Finally, we discuss the evolutionary relationships between vertebrate sex chromosomes and the sex-determining genes Dmy/Dm-W and Sry, which evolved by neofunctionalization of Dmrt1 and Sox3, respectively, for sex determining function. We propose a coevolution model of sex determining gene and sex chromosome, in which undifferentiated sex chromosomes easily allow replacement of a sex-determining gene with another new one, while specialized sex chromosomes are restricted a particular sex-determining gene.

Keywords Sex determination · neofunctionalization · noncoding exon · Dmrt1 · Sry · promoter

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| DM           | Doublesex and mab-3 |
| Dmrt1        | Doublesex and mab-3 related transcription factor 1 |
| Dmy          | Doublesex and mab-3 related gene Y—the sex-determining gene on the medaka Y chromosome |
| Dm-W         | Doublesex and mab-3 related gene W—the sex-determining gene on the Xenopus W chromosome |
| FISH         | Fluorescence in situ hybridization |
| HMG          | High mobility group |
Introduction

Both genotypic and environmental sex-determining systems exist in vertebrates (Graves 2008). In the former, heterogametic sex chromosomes determine the fate of sex, male (XY) or female (ZW). In the XX/XY-type sex-determining system, the Y-linked Sry gene of most eutherian mammals and the Dmy (also known as dmrt1bY) gene of the teleost fish medaka (Oryzias latipes) function as sex-determining genes that trigger testis formation (Sinclair et al. 1990; Koopman et al. 1991; Matsuda et al. 2002, 2007; Nanda et al. 2002). In contrast, the molecular mechanisms of ZZ/ZW-type systems are poorly understood, although our recent studies have proved that a W-linked gene Dm-W is a female sex-determining gene in the African clawed frog (Xenopus laevis) (Yoshimoto et al. 2008, 2010; Okada et al. 2009; Yoshimoto and Ito 2011). Importantly, a Dmrt1 duplication caused the emergence of Xenopus Dm-W (Yoshimoto et al. 2008), as well as medaka Dmy (Matsuda et al. 2002; Nanda et al. 2002). Our findings indicated that DMRT1 and its paralogous protein DM-W could have mutually opposite roles in sex determination, supporting a novel ZZ/ZW-type system model in which DM-W dominantly orients female development by antagonizing DMRT1 (Yoshimoto et al. 2010). Smith et al. (2009) also have recently reported that the two copies of Z-linked Dmrt1 gene are necessary for male sex determination in the chicken (Gallus gallus domesticus). The avian Dmrt1 gene may have been located in the Z chromosome to induce male development by maintaining its two-fold gene dosage in ZZ males (Nanda et al. 2000; Smith et al. 2009). The Y-linked Dmy in medaka and the W-linked Dm-W in X. laevis emerged as a Medaka1 positive and dominant-negative type parologue during species divergence, respectively (Kondo et al. 2004; Bewick et al. 2011), suggesting that both the sex-determining genes of the XX/YY and ZZ/ZW systems were generated by neo-functionalization of Dmrt1 (Yoshimoto et al. 2010). These findings support the idea that a DMRT1-driven male-determining system is involved in non-mammalian vertebrate species (Yoshimoto et al. 2010; Yoshimoto and Ito 2011). In contrast, the male sex-determining gene Sry evolved from Sox3 during evolution of eutherian mammals by neo-functionalization and established an SRY/SOX9-driven male-determining system.

Here, to clarify the molecular evolution of the sex-determining genes Dmy, Dm-W, and Sry, and their prototype genes Dmrt1 and Sox3 in vertebrates (Matsuda et al. 2002; Yoshimoto et al. 2008; Foster and Graves 1994), we compared the gene structures, presumptive promoter regions, and/or substitution rates among Dmrt1 and Sox3 family members in various species. Finally, we discuss the relationships between sex-determining genes and sex chromosomes during vertebrate evolution.

Materials and methods

Animal care and use

The Institutional Animal Care and Use Committee of Kitasato University approved all experimental procedures involving O. latipes, X. laevis, G. gallus, and Mus musculus.

RNA ligase-mediated rapid amplification of cDNA 5′ ends (RLM-5′RACE)

Total RNA was isolated using an RNeasy mini kit (Qiagen) from the gonads of M. musculus (C57BL/6), G. gallus, X. laevis, and O. latipes (Carbio). The Dmrt1 orthologue transcription initiation sites were determined using RNA obtained with the FirstChoice RLM-RACE kit (Ambion). Primers used for PCR were those supplied with the kit, and Dmrt1-specific primer sets were as follows: M. musculus Dmrt1, 5′-AAT CAGGCTGCACTTCTTGC-3′ and 5′-ACCTTTCTTCCAGAAACC-3′; G. gallus Dmrt1, 5′-CAGCCTTCTTGGACTGGGAGTC-3′ and 5′-ACATGAGAAGCGCTTGC-3′; X. laevis Dmrt1α, 5′-ATAAACCGGTGCTTCTCTG-3′ and 5′-GCCCTCTTCGAGAAGCGTG-3′; O. latipes Dmrt1, 5′-TCCATGATTTCTGGACATCGG-3′ and 5′-GGCCCTTTCGAGAAGCGTG-3′. The former primer set was used for first-round PCR, the latter for the second round. Products of the second round PCR were cloned and sequenced.
were inserted into pBluescript-KS (+) (Agilent Technologies) and sequenced.

Comparative analysis of genomic sequences

Comparisons of the 5'-flanking regions of Dmrt1 orthologues and paralogues were performed with mVISTA using the alignment program AVID (http://genomelx.lbl.gov/vista/index.shtml), which is suitable for globally aligning DNA sequences with its accuracy and ability to detect weak homologies. The Dmrt1 5'-flanking sequences of eight species (Homo sapiens, M. musculus, Canis lupus familiaris, Monodelphis domestica, Ornithorhynchus anatinus, G. gallus, Anolis carolinensis, Xenopus (Silurana) tropicalis) were obtained from the UCSC Genome Browser (http://genome.ucsc.edu). The 5'-flanking sequences of X. laevis Dmrt1α was isolated and sequenced, which was deposited in the GenBank/EBI Data Bank under accession number AB678700. We compared the 5'-flanking regions from the transcription start sites of M. musculus Dmrt1, G. gallus Dmrt1, X. tropicalis Dmrt1, O. latipes (Carbio) Dmrt1, or O. latipes (Carbio) Dmy, and Dmrt1 sequences of H. sapiens, C. familiaris, M. domestica, O. anatinus, or A. carolinensis to the 3' end of the next upstream gene, kank1. About 3 kbp sequences upstream of the transcription start sites of X. laevis Dmrt1α and Dm-W were also compared.

Construction of molecular phylogenetic trees and substitution rate calculations

Phylogenetic analyses were performed using integrated tool MEGA 5 (Tamura et al. 2011). The nucleotide sequences used for the analyses were obtained from the GenBank/EBI Data Bank as follows. O. latipes (HNI) Dmrt1, AY1577712; O. latipes (HNI) Dmy, AY12924; O. latipes (Carbio) Dmrt1, AF319994; O. latipes (Carbio) Dmy, AY129240; O. latipes (YZ) Dmrt1, AY442916 and AY524417; O. latipes (YZ) Dmy, AY448017; Oryzias marmoratus Dmrt1, AY521023; X. laevis Dmrt1β, AB252635; X. laevis Dm-W, NM00114842; Xenopus andrei Dmrt1β1, HQ220773; X. andrei Dmrt1β2, HQ220774; X. andrei Dm-W, HQ220853; Xenopus itombwensis Dmrt1β1, HQ220748; X. itombwensis Dmrt1β2, HQ220749; X. itombwensis Dm-W, HQ220850; Bufo marinus Dmrt1, FJ697175; H. sapiens SOX3, NM005634; H. sapiens SRY, NM003140; Pan troglodytes Sox3, AC149044; P. troglodytes Sry, NM000108988; Nomascus leucogenys Sox3, XM003272598; N. leucogenys Sry, HM759741; Macaca mulatta Sox3, NM001193752; M. mulatta Sry, NM001032836; Ornithorhynchus anatinus Sox3, XP001511549. DNA sequences were aligned using the MUSCLE (Edgar 2004) and gaps (insertions/deletions) were removed. Phylogenetic trees for the DNA binding (DM or HMG) domain regions, the non-DNA binding (non-DM or non-HMG) domain regions, and their combined regions were constructed using a maximum likelihood method after selecting the substitution model that best fitted the data using MODELTEST (Bayesian information criterion). Nucleotide numbers used: medaka DM domain, 111 bp; medaka non-DM domain, 672 bp; Xenopus DM domain, 105 bp; Xenopus non-DM region, 35 bp; Sox3 or Sry HMG domain, 216 bp; Sox3 non-HMG region, 1124 bp; and Sry non-HMG region, 225 bp. The bootstrap consensus tree inferred from 500 replicates was generated. Branches corresponding to partitions reproduced in less than 60% bootstrap replicates were collapsed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (indicated next to the branches). The substitution rates of sex-determining genes and their paralogues from each divergence were calculated using the phylogenetic tree branch lengths. Tajima’s nonparametric relative-rate test (Tajima 1993) was performed for testing the molecular evolutionary clock hypothesis.

Results

Xenopus and medaka Dmrt1 homologues possess a noncoding first exon

We previously showed that both the Xenopus tropicalis (also called Silurana tropicalis) Dmrt1 and X. laevis Dm-W genes have a noncoding exon 1 (Yoshimoto et al. 2006, 2008), as does medaka Dmy (Matsuda et al. 2002). To confirm the existence of the noncoding exon 1 and analyze the 5′-flanking sequences containing the promoter regions in the X. laevis Dmrt1α and medaka Dmrt1 genes, we determined their transcription initiation sites by performing rapid amplification
of the 5′ end using X. laevis and medaka testis RNAs and by cloning the products into a plasmid (see “Materials and methods”). X. laevis is an allotetraploid species (Hughes and Hughes 1993) and has two Dmrt1 genes, Dmrt1α and β, while Xenopus (Silurana) tropicalis is a diploid (Evans 2008). Because we were not able to obtain the genomic sequence corresponding to the Dmrt1β gene, we did not confirm the existence of the noncoding exon 1 of the β gene. The comparison between the obtained sequences and the genomic sequence revealed that both X. laevis Dmrt1α and medaka Dmrt1 had a noncoding exon 1, consisting of 140 and 115 bases, respectively (Fig. 1). These findings suggest that authentic Dmrt1 homologues in teleost fish and amphibians have a noncoding exon 1.

The Dmrt1 noncoding exon 1 degenerated during vertebrate evolution

We next examined whether or not the exon–intron structures of Dmrt1 orthologue and paralogue were conserved during vertebrate evolution, using the genomic and/or EST databases of teleost fish medaka, amphibian Xenopus, chicken, and mammalian species (human and mouse). We also predicted the Dmrt1 structures of monotreme platypus and marsupial opossum by comparative analysis between their genomic databases. The exon–intron structures, including the splicing sites, are well conserved among various vertebrate species except for the noncoding exons (Fig. 1). Interestingly, the comparative analysis did not detect a Dmrt1 noncoding exon 1 in mouse, human, or chicken. To confirm the absence of the noncoding exon 1, we then determined the Dmrt1 transcription initiation sites using mouse and chicken testis RNAs. The transcription initiation sites of the mouse and chicken Dmrt1 genes were located at 236 and 54 bases, respectively, upstream of the ATG translation initiation codon, indicating that each Dmrt1 gene has a coding first exon. These findings suggest that a Dmrt1 noncoding exon 1 has degenerated during amniote or homoiothermal evolution.

Comparative analysis of the 5′-flanking sequences among vertebrate Dmrt1 homologues

To understand the conservation and molecular evolution of the 5′-flanking sequences of Dmrt1 orthologues during vertebrate evolution and of the neofunctionalization-type sex-determining genes Dmy and Dm-W after Dmrt1 duplication, we conducted a comparative analysis of the 5′-flanking sequences of the Dmrt1 homologues in several vertebrate species using a comparative genomics tool, mVISTA (see “Materials and methods”). The analysis detected no significant homologous regions among mouse, chicken, clawed frog, and medaka fish over approximately 5 kb of the Dmrt1 5′-flanking sequences. However, in the more closely related species, such as human/mouse Dmrt1 (Fig. 2a), and X. tropicalis/X. laevis Dmrt1 (Fig. 2b), some homologous regions were found within 500 bp of the transcription start site. Since about 2.5 kb repeated sequence is inserted into the medaka Dmy promoter region (Herpin et al. 2010), we also searched the upstream region of the repeated sequence. As expected, two homologous regions were identified in the region −500 to −75 of medaka Dmrt1 and −3,120 to −2,386 of medaka Dmy (Fig. 2c). Curiously, we could not detect any sequence homology between X. laevis Dm-W and X. tropicalis or X. laevis Dmrt1. This may be because Dm-W emerged earlier than Dmy and much more base substitutions were accumulated—Dm-W and Dmy diverged from Dmrt1 13–64 million years ago (Bewick et al. 2011) and about 10 million years ago (Kondo et al. 2004), respectively. In any case, it will be necessary to obtain the gene structure and sequence information of X. laevis Dmrt1β, which is inferred to be an ancestral paralogue of Dm-W (Bewick et al. 2011).

To understand basic mechanisms for transcription of Dmrt1 orthologues, we searched for DNA-binding motifs of transcription factors in the 5′-flanking 500 bases upstream of the transcription start sites of the Dmrt1 homologues, using the program TFSEARCH 1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html). We could find a few motifs common to the human and mouse Dmrt1 promoter regions upstream of the coding exon 1, except for the binding elements for AML-1a, C/EBP, and GATA2 within the homologous regions detected by mVISTA (Supplementary Fig. 1A). In the promoter regions upstream of the noncoding exon 1, we could not find any common motifs arranged in order among the Xenopus and medaka Dmrt1 homologues. However, there was a conserved array of DNA-binding motifs, that is, Sox5/Nkx-2.5/AML-1a/Sox5/HNF-1/GATA-1/CdxA/YY1, in the homologous regions corresponding to the region −500 to −250 of medaka Dmrt1 and the −3,120 to −2,862 of medaka Dmy (Supplementary Fig. 1B).
The medaka and *Xenopus* *Dmrt1* genes have a non-coding exon 1. Therefore, we predicted there would be a homologous region between the upstream regions of the noncoding exons 1 of *Xenopus*/*medaka* *Dmrt1* and the far upstream regions of the coding exons 1 of mouse/human and chicken *Dmrt1*. We searched the regions from the transcription start sites of *Xenopus* and *medaka* *Dmrt1* to the 3' end of the next upstream gene, *kank1* but did not find any sequence homology. Besides, a Blat search (http://genome.ucsc.edu/cgi-bin/hgBlat) using the wider region between the *Dmrt1* and *kank1* genes revealed a significantly homologous region, consisting of about 600 bases, among the various species of mammals, chicken, and lizard (Supplementary Fig. 2). The region on the human genome is located at about 40 kb upstream of *Dmrt1* (Supplementary Table 1), which corresponds to about 60 kb downstream of *kank1*. Figure 2d shows the results of mVISTA analysis on this region. There was a high sequence conservation among the regions of eutherian human, mouse and dog, marsupial opossum, and monotreme platypus. Moreover, the human region showed a significant homology with those of chicken and lizard, but not of frog. From mammals to lizards, the region included common DNA-binding motifs, such as Cdxα, GATA-X, Pbx-1, AP-1, and Nkx-2.5 (Supplementary Fig. 2), which might regulate the transcription of *Dmrt1* and/or *kank1*. Lei and Heckert (2002, 2004) reported that transcription factors Sp1 and Egr1/Gata4 regulate transcription of the rat *Dmrt1* gene in the testes by binding to about 100 b and 3 kb upstream of the gene, respectively. Our search in the upstream regions of vertebrate *Dmrt1* genes identified no consensus sequences for Sp1 and Egr1/Gata4 binding except for rats/mice and rats, respectively.

The sex-determining genes *Dmy/Dm-W* and *Sry* show a higher substitution rate than their prototype genes, *Dmrt1* and *Sox3*, respectively.

To elucidate the molecular evolution of the sex-determining genes, we first examined the substitution rates of the sex-determining gene *Dmy* and its prototype gene *Dmrt1* from three groups of medaka (*O. latipes* HNI, Carbio, and YZ), and *Dm-W* and *Dmrt1β* from three species of *Xenopus* (*X. laevis*, *X. andrei*, and *X. itombwensis*). The substitution rates of *Dmy* and *Dm-W* were higher than those of *Dmrt1* and *Dmrt1β*, respectively (Fig. 3). Particularly, the DNA-binding DM domains of *Dmy* and *Dm-W* showed a
higher substitution rate, about 46 and 7 times, respectively (Fig. 3). To examine whether substitution rates vary significantly between the sex-determining genes and their prototype genes, Tajima's relative rate test was performed on the DNA sequences corresponding to Fig. 3. The Tajima's test showed that the molecular clock hypothesis was rejected ($p<0.05$) between each region of medaka Dmrt1 and Dmy (Supplementary Table 3) and between the DM domain region or combined region of Xenopus Dmrt1 and Dm-W except for $X$. itombwensis (Supplementary Table 4). These results indicated that the sex-determining genes Dmy and
**Dm-W** significantly have a higher substitution rate than their prototype genes. The substitution rates of the DM domains of medaka and *Xenopus Dmrt1* were lower than those of their non-DM domain regions (Fig. 3). This was expected because the DNA-binding domain is known to be functionally conservative. Interestingly, the substitution rate of the DM domain, on the contrary, was higher than that of the non-DM domain region in *Dmy* and was equal to in *Dm-W*.

We next examined substitution rates of the mammalian sex-determining gene *Sry* and its prototype gene *Sox3* from four species of primates—*H. sapiens*, *P. troglodytes*, *N. leucogenys*, and *M. mulatta* (Fig. 4a). Because there is little homology in the non-HMG
domains between Sox3 and Sry, we calculated the substitution rates of only the HMG domain to compare the two genes (Fig. 4a). As expected, the rate of the DNA-binding HMG domain of Sry was about seven times higher than that of Sox3. Tajima's test was also performed, indicating that the molecular clock hypothesis was rejected between the HMG regions of Sox3 and Sry (Fig. 4a) in three species of primates and Ornithorhynchus anatinus (Oa) as an outgroup, Sox3 (b) or Sry (c) in three species of primates and Macaca mulatta (Mm) as an outgroup, using the maximum likelihood method based on the Kimura 2-parameter model (a), Hasegawa-Kishino-Yano model (b), or the Kimura 2-parameter model (c). The three trees in (b) and (c) were derived from the HMG domain region (upper), the non-HMG domain region (middle), and their combined region (lower). Calculations of the substitution rates were performed as described in Fig. 3. Sry diverged from the prototype genes 148–166 million years ago (Marques-Bonet et al. 2009). A common ancestor of Homo sapiens, Pan troglodytes, and Nomascus leucogenys diverged from M. mulatta 18 million years ago (Marques-Bonet et al. 2009). Hs, H. sapiens; Pt, Pan troglodytes; Nl, Nomascus leucogenys

Discussion

Degeneration of a Dmrt1 noncoding exon 1 during vertebrate evolution

Here, we performed a comparative analysis of Dmrt1 homologue genomic and cDNA sequences in medaka,
Xenopus, chicken, and mouse, with determination of the transcription initiation sites (Fig.1) and showed that a Dmrt1 noncoding exon 1 exists in the fish and frog, but not in chicken or mouse. This suggests that non-coding exon 1 degenerated during vertebrate evolution. Why did it degenerate? It may have been due to the need for a promoter change to modify the transcription machinery for the Dmrt1 mRNA expression. In mice, DMRT1 is expressed in primordial germ cells (PGCs) and somatic cells of XX- and XY-indifferent gonads (Lei et al. 2007). In chicken and Xenopus, Dmrt1 is expressed in somatic cells of ZZ- and ZW-indifferent gonads (Oréal et al. 2002; Yoshimoto et al. 2010). In medaka, Dmy and Dmr1 are expressed in PGC- and spermatogonium-supporting cells, respectively, of XY gonads (Kobayashi et al. 2004). Dmrt1 is not essential for fetal testis and ovary development in mice (Raymond et al. 2000) but is necessary for male and female germ cell development (Matson et al. 2010; Krentz et al. 2011) and for postnatal testicular development (Matson et al. 2011). In contrast, Dmr1 is required for male determination in chicken (Smith et al. 2009) and has the potential to induce primary male development in Xenopus (Yoshimoto et al. 2010). Promoter reporter analyses of a transgenic mouse carrying about 9-kb upstream of the rat Dmr1 coding exon 1 and of transgenic Xenopus carrying about 3-kb upstream of the X. tropicalis Dmr1 noncoding exon 1 revealed that this region is necessary for the transgene expression in Sertoli cells and male germ cells (Lei et al. 2009) and in indifferent gonads (Yoshimoto et al. 2006), respectively. Totally, these findings do not show any differences in the promoter activity and regulation of Dmr1 between the animals with and without non-coding exon 1.

We propose one hypothesis that some important regulatory sequences upstream of the Dmr1 noncoding exon 1 required for sex determination and differentiation in poikilothermic vertebrate species were abolished during homeotherm evolution (Fig. 2; Supplemental Fig. 1).
It is possible that these regulatory sequences are involved in temperature sensitivity because \textit{Dmrt1} expression is related to the temperature-dependent sex determination and sex reversal in some poikilothermic reptile, amphibian, and teleost species (Kettlewell et al. 2000; Murdock and Wibbels 2003; Sakata et al. 2006; Hattori et al. 2007; Anand et al. 2008; Graves 2008). It is important to clarify whether or not reptiles have a noncoding exon 1 within the \textit{Dmrt1} gene.

\textit{Dmy}, \textit{Dm-W}, and \textit{Sry} as neofunctionalization-type sex-determining genes

In general, all of the new genes might arise from redundant copies of the preexisting genes (Ohno 1970). In the neofunctionalization model of gene duplication, one copy retains the original function, and the other evolves a new function (Lynch et al. 2001). The vertebrate sex-determining genes \textit{Sry} and \textit{Dmy}/\textit{Dm-W} may have evolved from \textit{Sox3} and \textit{Dmrt1}, respectively, as a neofunctionalization-type gene for sex determination. Neofunctionalization-type genes have higher substitution rates than those of their prototype genes (Fig. 3; Fig. 4a; Supplemental Tables 3–5). This coincides with the results of amino acid sequence comparisons among vertebrate DMRT1 family proteins (Supplementary Table 2). In this context, the chicken Z-linked \textit{Dmrt1} does not appear to be a neofunctionalization-type gene (data not shown; Supplementary Table 2), although avian Z-linked \textit{Dmrt1} is a strong candidate for the male-determining gene.

The substitution rates of the transcription factor genes \textit{Dmrt1} and \textit{Sox3} indicated that their DM and HMG domains are relatively conserved, compared to other regions, during species divergence (Fig. 3; Fig. 4b); this indicates the functional importance of these sequence-specific DNA-binding domains. In contrast, the \textit{Dmrt1} and \textit{Sox3}-derived sex-determining genes were different in that point; the rate of the DNA-binding domain was significantly higher than that of the non-DNA binding region in \textit{Dmy}, was equal to in \textit{Dm-W}, and lower in \textit{Sry} (Figs. 3, 4c). The difference in substitution rates between \textit{Dmy} and \textit{Dm-W} could be due to the restricted small numbers of \textit{Dm-W} sequences used for the analysis. Zhang (2004) reported that the \textit{Dmy} DM domain is likely to be under positive Darwinian selection. Full sequencing of the \textit{Dm-W} orthologues in several \textit{Xenopus} species would answer whether or not there is a common evolutionary mechanism of DM domain in the \textit{Dmrt1}-derived sex-determining genes. On the other hand, the contrasting results between \textit{Dmy} and \textit{Sry} might have been caused by differences in the functional importance of the non-DNA binding regions. The results in the present study indicate that the HMG-domain sequence of \textit{Sry} is much more conserved than the other region during species divergence. In fact, it is proved that \textit{Sry} could be replaced with \textit{Sox3} for the male determination in the transgenic studies (Sutton et al. 2011), indicating that the target element of \textit{Sry} is still conserved after divergence from \textit{Sox3}. In contrast, the higher substitution rate of \textit{Dmy} DM-domain than the other region suggests that the target elements are modified after duplication. Further transgenic experiments with a replacement of \textit{Dmy} with \textit{Dmrt1} would give an answer to the intriguing question. In the future, it will be important to analyze the molecular evolution of the \textit{Dmrt1}- and \textit{Sox3}-derived sex-determining genes, from the view of the differences in the neofunctionalization-type emergence process; \textit{Dmy}/\textit{Dm-W} and \textit{Sry} evolved through individualized autosomal \textit{Dmrt1} duplication and allelic \textit{Sox3} mutation, respectively (Fig. 5). This molecular process may be closely related to the coevolution of the sex-determining genes and sex chromosomes.

A hypothesis—undifferentiated sex chromosome state allows a sex-determining gene to change

Closely related species to medaka fish (\textit{O. latipes}), which has \textit{Dmy} as a sex-determining gene on the Y chromosome, are \textit{O. curvifinis}, \textit{O. luzonensis}, and \textit{O. mekongensis}. These four species (including medaka) all have 48 chromosomes with a genetic XX/XY-type sex-determining system. \textit{O. curvifinis} has \textit{Dmy}, which is located on the orthologous Y chromosome (chromosome 1 of \textit{O. latipes}, called \textit{latipes} linkage group 1 (LG1)). Interestingly, the \textit{Dmy} gene degenerates into a pseudogene in \textit{O. luzonensis} (Kondo et al. 2004), and, there is no \textit{Dmy}-orthologous gene in \textit{O. mekongensis}, suggesting that the sex-determining gene \textit{Dmy} arose from a duplicated copy of the autosomal \textit{Dmrt1} gene after divergence of the three species from \textit{O. mekongensis}, approximately 10 million years ago (Kondo et al. 2004). Importantly, the Y chromosomes in \textit{O. luzonensis} and \textit{O. mekongensis} are not LG1, but LG12 and LG2, respectively (Tanaka et al. 2007; unpublished data in Takehana et al. 2008), suggesting that a new sex-determining gene arose in each of the two species. In addition, no sex-chromosomal heteromorphism was
observed in the genus *Oryzias* species, as is seen in *O. latipes* and *O. luzonensis* (Takehana et al. 2007; Tanaka et al. 2007). The sex-reversed XX males and XY females in these species are completely fertile, suggesting that there is no functional differentiation between the X and Y chromosomes except for the male determining role of *Dmy* in the Y chromosoome (Takehana et al. 2007; Tanaka et al. 2007). This idea is supported by the fertility of the transgenic medaka sex-reversed XX males carrying the *Dmy* expression vector and the XY females with spontaneous *Dmy* gene mutations (Matsuda et al. 2002, 2007). Consequently, it is plausible that a sex-determining gene is not stabilized during species diversification under the genomic condition in which sex chromosomes are undifferentiated (Fig. 4).

We recently performed a FISH analysis of *Dm-W* in *X. laevis* and showed that the *Dm-W*-harboring chromosome 3 is the W sex chromosome, and its homologous partner is the Z sex chromosome (Yoshimoto et al. 2008), although conventional chromosomal staining did not identify any morphological differences between the Z and W chromosomes. In addition, the sex chromosomes are indistinguishable in the females and males of several other species examined in the genus *Xenopus*, including diploid and polyploid (tetraploid, octaploid, and tetraoctaploid) species (Tymowska and Fischberg 1973). It is interesting that most *Xenopus* species may not have developed differentiated sex chromosomes during and after the species divergence mediated through polyploidization. More recently, Bewick et al. (2011) have identified *Dm-W* orthologues in at least seven species of the genus *Xenopus*—four tetraploid species including *X. laevis* and *X. largeni*, and three octaploid species including *X. itombwensis*—but that many other *Xenopus* species lack a *Dm-W* orthologue. They also suggested that *Dm-W* might be degenerated in closely related *Xenopus* species to the ones bearing *Dm-W*, as in the case of the teleost fish *O. luzonensis*. They concluded that *Dm-W* arose from a partial *Dmrt1β* duplication in the *Xenopus* genus after diverged from its sister genus *Silurana*, which has a diploid genome, but before the divergence of *X. laevis* and *X. clivii*, that is, 13–64 million years ago. Therefore, there should be other kinds of sex-determining genes in *Xenopus* species lacking *Dm-W* gene. Importantly, *X. laevis* ZW transgenic individuals carrying the *Dm-W* knockdown vector develop testes (Yoshimoto et al. 2010); among them, one ZW individual formed sperm. In addition, *X. laevis* ZZ animals that had undergone male-to-female sex reversal were fertile (Hayes et al. 2010). Taken together, these findings suggest that there might be no functional differentiation between the Z and W chromosomes except for the sex-determining gene *Dm-W*. This is similar to the relationship between the X and Y chromosomes in the four closely related medaka species of the genus *Oryzias* described above. Thus, the heterogametic sex chromosome may be only a vehicle for the sex-determining gene in the two independent genera *Oryzias* and *Xenopus*.

In summary, there is likely some relationship between the undifferentiated state of sex chromosome and the change of sex-determining gene in the cases of both the *Oryzias* fish and the *Xenopus* frog. Therefore, we hypothesize that an undifferentiated state of sex chromosomes allows change of a sex-determining gene and provokes evolution of a neofunctionalization-type sex-determining gene, regardless of the heterogametic sex (Fig. 5).

Is sex chromosome specialization involved in sex-determining gene stabilization?

Compared to poikilotherm vertebrate evolution, male heterogametic (XX/XY) and female heterogametic (ZZ/ZW) sex-determining systems are almost perfectly conserved during mammalian and avian evolution, respectively, with some exceptions, such as the Ryukyu spiny rats and mole voles (Kobayashi et al. 2007; Matthey 1933; Fregda 1983). A similar phenomenon of the loss of a Y chromosome appeared to occur independently during species diversity of the spiny rats and mole vole (Just et al. 2007). In poikilotherm animals, flexibility of gene expression for sex determination and sex-reversal might be required in response to the environmental change, and the sex chromosomes are morphologically homomorphic in both sexes in many fishes, amphibians, and reptiles. On the other hand, it is possible that a homeothermic condition, as in birds and mammals, makes it possible to control the gene expression superior to the outdoor environment and requires no flexibility of the genetic systems, and thus might allow sex chromosomes to become highly differentiated between sexes. Once the sex chromosomes were highly differentiated, some functional differentiation might be accelerated between the female and male sex chromosomes except for the role of sex determination. As a result, the sex-determining gene becomes stabilized on the sex chromosome (Fig. 5). This could be one reason why *Sry* is highly conserved as a sex-
determining gene in most species of eutherian mammals. Based on this scenario, one same sex-determining gene might be common to most avian species—the avian Z-linked Dmrt1 gene is plausible to be a male-determining gene. Likewise, as for female determination, it is possible that a particular W-linked female determining gene is common to many avian species.

In the future, it will be interesting to clarify molecular mechanisms of coevolution between sex-determining genes and sex chromosomes in various vertebrate species including the spiny rats and mole voles mentioned above, the Japanese wrinkled frog *Rana rugosa*, which underwent change of heterogametic sex from XY male and ZW female, and some species of reptiles and fishes, which have temperature sex determination (TSD) and/or genetic sex determination (GSD). The identification of sex-determining genes in these species would lead to molecular understanding of the coevolution and supply information to discuss on our model proposed here (Fig. 5) with reference to TSD, GSD-TSD transitions (Quinn et al. 2007; Grossen et al. 2011), and ZW-XY transitions (Miura 2007; Quinn et al. 2011).

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Vertebrate sex-determining genes

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