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The roles of *Dmrt* (Double sex/Male-abnormal-3 Related Transcription factor) genes in sex determination and differentiation mechanisms: Ubiquity and diversity across the animal kingdom

Rôle des gènes Dmrt *(*Double sex/Male-abnormal-3 Related Transcription factor*) dans le déterminisme sexuel ou la différenciation sexuelle : ubiquité et diversité dans le règne animal*

Marion Anne-Lise Picard, Céline Cosseau, Gabriel Mouahid, David Duval, Christoph Grunau, Ève Toulza, Jean-François Allienne, Jérôme Boissier *

Université Perpignan Via Domitia, IHPE UMR 5244, CNRS, IFREMER, Université de Montpellier, 58, avenue Paul-Alduy, bâtiment R, 66860 Perpignan, France

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ABSTRACT

The *Dmrt* (Double sex/Male-abnormal-3 Related Transcription factor) genes have been intensively studied because they represent major transcription factors in the pathways governing sex determination and differentiation. These genes have been identified in animal groups ranging from cnidarians to mammals, and some of the genes functionally studied. Here, we propose to analyze (i) the presence/absence of various *Dmrt* gene groups in the different taxa across the animal kingdom; (ii) the relative expression levels of the *Dmrt* genes in each sex; (iii) the specific spatial (by organ) and temporal (by developmental stage) variations in gene expression. This review considers non-mammalian animals at all levels of study (i.e. no particular importance is given to animal models), and using all types of sexual strategy (hermaphroditic or gonochoric) and means of sex determination (i.e. genetic or environmental). To conclude this global comparison, we offer an analysis of the DM domains conserved among the different DMRT proteins, and propose a general sex-specific pattern for each member of the *Dmrt* gene family.

Mots clés :
Gènes *Dmrt*
Profil d'expression génique
Diversité fonctionnelle

RÉSUMÉ

Les gènes *Dmrt* (*Double sex/Male-abnormal-3 Related Transcription factor*) ont été particulièrement étudiés, car ils représentent des facteurs de transcription majeurs dans le déterminisme sexuel ou dans la différenciation sexuelle. Ces gènes ont été identifiés, et parfois fonctionnellement étudiés, des cnidaires aux mammifères. Cet article propose d'analyser (i) la présence/absence des différentes familles de gènes *Dmrt* dans différents

* Corresponding author.
E-mail address: boissier@univ-perp.fr (J. Boissier).

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taxa du règne animal ; (ii) de relever leur expression relative male *vs* femelle ; (iii) de souligner leur expression spatiale (c'est-à-dire dans quel organe les gènes s'expriment) et temporelle (c'est-à-dire à quelle phase du développement les gènes s'expriment). Cette synthèse considère tous les animaux, qu'ils soient bien étudiés ou pas (c'est-à-dire qu'il n'y a pas d'importance particulière donnée aux organismes dits « modèles »), qu'ils soient hermaphrodites ou gonochoriques et que leur déterminisme sexuel soit génétique ou environnemental. Seuls les mammifères sont exclus de cette synthèse. En guise de conclusion, nous proposons une analyse permettant de discriminer les différentes familles de protéines *Dmrt* et une vision globale de l'expression sexe-spécifique des gènes *Dmrt*.

1. Introduction

The existence of *Dmrt* (Double sex/Male-abnormal-3 Related Transcription factor) genes was first formally proposed in the fruit fly, *Drosophila* [1], where the spliceforms of DSX are sex-specific: the female-specific spliceform activates genes, while the male-specific spliceform represses its targets [2]. The transcription factor family is characterized by an unusual zinc finger motif called DM domain [3,4]. The domain consists of six conserved intertwined cysteines and two histidines (site 1, CCHC and site 2, HCCC) that form a groove for the binding of DNA [4]. The presence of *Dmrt* genes in non-bilaterian animals (e.g., corals and placozoa) suggests that this gene family originated before eumetazoans [5]. In contrast, *Dmrt* genes appear to be lacking in the genomes of sponges, choanoflagellates and fungi. The members of the *Dmrt* family include nine *Dmrt* genes (*Dmrt*1 to *Dmrt*9) plus DSX from insects and *Mab* from *Caenorhabditis* [5,6]. This classification reflects monophyletic gene groups having at least one DM domain. Some species (e.g., Planaria) possess two paralogs [7]. Members of the *Dmrt* gene family are broadly represented in animal kingdom but they lack clear linkages with species clades, with some exceptions. For example, DSX only present in arthropoda, *Dmrt1* is only present in vertebrates, and *Dmrt6-9* are only present in mammals.

Dmrt genes have been intensively studied because they represent major transcription factors of the sex determination or differentiation pathways. It is generally agreed that a gene may be considered to be a sex determination gene when modification of its expression induces sex reversal. However, the identification of a gene as a sex determination gene may also consider the temporal window for sex determination, the sex chromosome localization and the genetic strict necessity of the gene. In this context, *Dmrt* genes have been proposed as sex determination genes for the medaka fish, *Oryzias latipes* [8], the African clawed frog, *Xenopus laevis* [9], the chicken, *Gallus gallus* [10], the daphnia, *Daphnia magna* [11] and the planarian, *Schmidtea mediterranea* [7]. In several other species, *Dmrt* genes have been implicated later in sexual development (i.e. downstream of the sex differentiation cascade), as changes in their expression levels induce sexual abnormalities rather than sex reversal. For example, the loss of *Dmrt1* causes undifferentiated mouse spermatogonia to precociously exit the normal program of proliferation/differentiation to spermatids [12,13]. For the majority of species in which *Dmrt* genes have been identified, however, the role of such genes in sex determination or differentiation remains unknown.

Several previous reviews on *Dmrt* have focused on a particular animal group, such as fishes [14] or mammals [15,16], while more generalist reviews have mainly focused on knowledge gained from model organisms, such as the mouse, *Caenorhabditis* or *Drosophila* [17,18]. Except in the case of model organisms such as the hermaphroditic *C. elegans*, the existing reviews have largely focused on gonochoric species with chromosomal sex-determining systems (ZW or XY systems). Here, we systematically review all non-mammalian animals regardless of their level of study, their sex determination strategy (hermaphroditic or gonochoric) and whether their sex is genetically or environmentally controlled. We exclude mammals because two very recent reviews exist on their *Dmrt* genes (i.e. *Dmrt*6 to -*9*) [15,16]. We do not focus on the detailed downstream mechanisms of the sex determination or differentiation pathways, but instead look at different aspects of the diversity found within the *Dmrt* gene family. For each animal, we first examine the presence/absence of the family members [6] in various animal species and taxa. Second, we assess sex-specific differences in the expression levels of each known gene. Third, when possible, we discuss any known spatial (organ-level) and temporal (developmental stage-level) variations in *Dmrt* gene expression. Finally, we introduce a database and its use in a discriminant analysis of the DM domains conserved among the different proteins. A comprehensive phylogeny of the *Dmrt* gene family was very recently described by Wexler et al. [5].

2. Cnidaria

Dmrt genes have been identified in two stony corals (*Lopheliapertusa* and *Acroporamillepora*), one hydra (*Hydra magnipapillata*) and one sea anemone (*Nematostellavectensis*). The temporal expression of the DM domain-containing gene, *AmDM1*, has been studied in *Acropora millepora* [19]. In this species, it is difficult to assess sex-specific spatial expression because the individuals are hermaphroditic, with male and female cells adjacent to each other. Furthermore, the presence of a hard calcium carbonate matrix makes it difficult to access the gonad for *in situ* hybridization. With respect to temporal expression, however, Northern blot analysis revealed that the expression of *AmDM1* was correlated with the annual cycle of sexual differentiation. No expression was detected in the egg and embryonic stages, whereas expression was detected in all adult stages, with maximum expression

observed during October in the branch tip compared to the base of the branch) [19]. This expression pattern is consistent with the sex differentiation of this species.

3. Platyhelminthes

Dmrt genes have been identified in a planarian (*Schmidtea mediterranea*, free living and hermaphroditic), the Chinese liver fluke (*Clonorchis sinensis*, parasitic and hermaphroditic) and a schistosome (*Schistosoma mansoni*, parasitic and gonochoric). Four DM domain-containing genes have been identified in each genome. A complete functional study was recently performed on *Smed-dmd-1* from *Schmidtea mediterranea* [7]. In terms of spatial expression, this *Dmrt* gene is expressed in both the nervous system (in neurons) and the reproductive system, where it is expressed in somatic cells of the testes and in the accessory male sexual organs (seminal vesicles, sperm duct and penis papilla) [7]. Temporally, *Smed-dmd-1* is expressed: (i) during the early stage of development, when RNAi experiments have shown that the gene is required for the development of both male and female reproductive systems; (ii) in sexually adult individuals, when RNAi was found to affect only males;(iii) during the post-amputation regeneration of males but not females. Considering this expression pattern, the authors suggest that *Smed-dmd-1* functions in male development and in the subsequent development of the female consistent with the protandrous nature of the planarian. Finally, the authors showed that *Schistosoma mansoni* expresses a homolog of *Smed-dmd-1* (called *Sm-dmd-1*) that is more highly expressed in adult males than in adult females [7].

4. Mollusca

Dmrt genes are known in six mollusk species and have been studied in three bivalves and one gastropod species of economic importance: the pacific oyster (*Crassostrea gigas*), the Akoya pearl oyster (*Pinctada martensis*), the Japanese scallop (*Chlamys farreri*) and the tropical abalone (*Haliotis asinina*). The oysters are protandrous hermaphrodites, while the scallop and abalone are both gonochoric. A number of environmental factors have been reported to play important roles in the sex determination and differentiation of mollusks, including nutrition, water temperature, and the proportion of males and females [20]. Table 1 summarizes the spatial expression of the *Dmrt* genes in the different mollusk species, showing how the pattern varies greatly by species and tissue. In gonads, *Dmrt* expression is specific to the testes of *Pinctada martensis*, *Chlamys farreri* and *Haliotis asinina*, and more highly expressed in males than females in *Crassostrea gigas*. In the context of temporal expression, maximum gene expression is detected at the adult stage in all tested species. For *Pinctada martensis*, the *Dmrt* gene expression pattern follows the reversal of sex from male to female [21]: it is absent from juvenile gonads, increases as the testes develop, and decreases once more as the animal transitions from male to female, until no expression is detected in the mature female gonad [21].

5. Nematoda

5.1. Caenorhabditis elegans

The Nematoda include *Caenorhabditis elegans*, which is one of the most widely studied animal models. *C. elegans* uses an $XX_{hermaphrodites}/XO_{male}$ system, and the molecular aspects of sex determination and differentiation have been extensively studied in this organism (see [22,23] for review). Among the 11 DM genes predicted from genomic sequences, three genes, *mab-3*, *mab-23* (*mab* stands for "male abnormal") and *dmd-3*, play central roles in male differentiation and behavior.

Among them, *mab-3* was the first identified, in a 1988 paper [24] reporting that: (i) a mutation in *mab-3* causes the synthesis of yolk protein in male worms but has no effect on hermaphrodite worms; and (ii) *mab-3* is required for the development of the male-specific sense organs (called V rays) required for mating. Later studies showed that *mab-3*, which represses the transcription of genes related to the production of yolk proteins [25], is required for transcription of male-specific genes in sensory neurons of the head and tail [26–28] and is necessary for male-specific muscle differentiation [29]. Behavioral assays showed that male *mab-3* mutants present deficits in attraction to and interaction with hermaphrodites [27]. It was recently shown that *mab-3* suppresses the expression of several target genes whose transcription has been implicated in the oxidative stress, perhaps explaining why male worms are much more sensitive to oxidative stress than their hermaphroditic counterparts [30].

Mab-23 was first characterized in 2002 [29]. Male *mab-23* mutants have morphological abnormalities in their tails, gonads and nervous system, and are unable to sire progeny. In males, *mab-23* has been implicated in the patterning of the nervous system [28,29], the development of male-specific muscles [28,29] and the development of

Table 1
Comparative spatial expression of different *Dmrt* genes in mollusks.

	Organ	Gills		Mantle		Adductor Muscle		Digestive gland		Gonad		Reference
Species	Gene	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Crassostrea gigas	*Cg-DMl*	+	+	+	+	+	+	+	+	++	+	[96]
Pinctada martensis	*pmDmrt2*	+	+	–	–	–	–	–	–	+	–	[21]
Chlamys farreri	*Cf–dmrt4–like*	+	–	+	+	+	+	NP	NP	+	–	[97]
Haliotis asinine	*Ha–Dmrt1*	NP	NP	NP	NP	NP	NP	NP	NP	+	–	[98]

"+" and "++": low and high level of expression respectively; "–": no expression detected; NP: not performed.

the proctodeum [28]. *Mab-23 mutants* are defective in the stereotyped sequence of copulatory behavior and show impairments in the transfer of sperm during copulation [28,29]. In hermaphrodites, *mab-23* is expressed in both sex- and non-sex-specific tissues; however, hermaphrodite *mab-23* mutants do not show any detectable phenotype, suggesting that this gene is unnecessary or functionally redundant in hermaphrodites [29].

Dmd-3 was first characterized in 2008 [31]. In *C. elegans*, the tail tip is highly dimorphic between males and hermaphrodites; male *dmd-3* mutant exhibit abnormal tail tips, and *dmd-3/mab-3* double mutant show more severe tail-tip abnormalities. The expression of *dmd-3* is the same in the tail tips of males and hermaphrodites, suggesting that while it is essential for the development of the former, it may play a redundant role in the latter [31].

Considering their apparent position in the pathways of sexual development, *mab-3, mab-23* and *dmd-3* seem to be implicated in sexual differentiation rather than sex determination. The roles of the other *Dmrt* genes found in the genome of *C. elegans* (*dmd-4* through *dmd-11*) are unknown.

5.2. Other nematodes

Dmrt gene sequences have been identified in free-living *Caenorhabditis briggsae*, *C. remanei* and *C. brenneri*, and in the parasitic species, *Ascaris suum*, *Trichinella spiralis*, *Brugia malayi*, *Loa loa* and *Trichostrongylus vitrinus*. Characterization (a partial one) has only been performed to date for the parasite, *Trichostrongylus vitrinus* [32]. This worm possesses four undifferentiated larval stages before the gonochoric adult stage. Larval stage 3 (L3) is the infective stage. The *Dmrt* gene, *Tv-mab-23* (similar to *mab-23* of *C. elegans*), was identified and the gene product was quantified in L3 larvae, L4 larvae, and male and female adults. Gene expression was detected at all stages. The levels were similar in the L3 and adult stages, while the maximum expression level was obtained from the morphologically undifferentiated L4 stage. In males, this stage corresponds to the sperm-producing stage of free-living and parasitic nematodes [33], suggesting that *Tv-mab-23* plays a specific role in males. Similar to *C. elegans*, in which *mab-23* is expressed in both the male and hermaphroditic forms, the expression level of *Tv-mab-23* is the same in male and female adults of *T. vitrinus*.

6. Hexapoda

In Hexapoda (insects), the primary signal for sex determination varies greatly by order, but the bottom of the cascade is conserved (see [34–36] for details). *Dmrt* genes, which are known as doublesex (DSX) genes in insects play a pivotal role in insect sex determination. As sex determination systems, insects can use XX/XY, ZZ/ZW, XX/XO or homomorphic chromosomes with a male-determining factor [36]. There is no known link between the sex determination system and the *DSX* gene, but sex-specific alternative splicing of the *DSX* gene can produce DSX^M or DSX^F transcripts for males and females, respectively.

Detailed studies have been performed on *Drosophila* as a model organism (see [35,37,38] for review). A recent cell-level study revealed that the *DSX* gene shows dynamic and precise spatial and temporal expression patterns in *Drosophila* [39]. In the embryo, the *DSX* gene is only expressed in the gonad; in the larval stage, the expression extends to other tissues; and in the adult stage, the expression is localized to tissues in which DSX is known to play developmental roles, such as the peripheral and central nervous systems (see [39] for a detailed description). Notably, not all cells in a given tissue show expression of the *DSX* gene; instead, both males and females are mosaics of sexually differentiated and undifferentiated cells. Functional studies have shown that the sex-specific transcripts of DSX are implicated in several morphological and behavioral traits that show sexual dimorphism, as well as being intimately associated with the sex-specific transcripts of *fru* [37,38,40].

DSX genes have been identified in all insects studied to date, including 21 species of Diptera, four species of Hymenoptera, four species of Lepidoptera, one Coleopteran, one Phthirapetran and one Hemipteran.

7. Crustacea

7.1. Brachiopoda

The water flea, *Daphnia magna*, is known to switch between sexual and asexual reproduction depending on the quality of the environment [41]. This crustacean parthenogenetically produces males when the photoperiod is shortened, food is lacking or the population density increases. In the sexual part of the water flea life cycle, the expression levels of three DM domain-containing genes (*DMRT11E*, *DMRT93B* and *DMRT99B*) have been compared between the sexes [41]. *DMRT11E* and *DMRT99B* are much more highly expressed in the ovary than in the testis, while *DMRT93B* is only expressed in the testis [41]. A recent study showed that these genes are not differentially expressed during embryonic development, suggesting that they are not involved in sex determination, but rather in sex differentiation [11]. Two additional DM domain genes were recently identified; they were designated *DapmaDsx1* and *DapmaDsx2* based on their similarity to the *DSX* genes of *Drosophila* [11]. *DapmaDsx1* encodes two mRNAs (*DapmaDsx1alpha* and *DapmaDsx1beta*) that differ in their 5′UTRs and show male-specific expression both temporally and spatially [11]. The expression levels of these two genes increase exclusively in male embryos during the development of sexually dimorphic organs (72 hours post-ovulation). Spatially, the *DapmaDsx1alpha* mRNA is only expressed in testes, while the *DapmaDsx1beta* and *DapmaDsx2* mRNAs are more highly expressed in testis than in ovary. *DapmaDsx1* and *DapmaDsx2* show even higher expression levels in the sexually dimorphic organs of the male daphnia (i.e. the first antennae and first thoracic segment). Finally, using gain (transient transgenesis) and loss (dsRNA-based gene knockdown)-of-function analyses, the same authors showed that *DapmaDsx1*, but not *DapmaDsx2*, plays a primordial role in male determination [11].

7.2. Decapoda

Eriocheir sinensis is the only Decapoda in which *Dmrt* genes have been studied. This mitten crab is a gonochoric animal, but the genetic bases of its sex determination remains unknown. Only one *Dmrt* gene has been isolated in this species [42]: the *EsDMRT-like* gene, which shows 37% homology with DMRT99B isolated from *Drosophila*. This gene is present in both males and females, but is only expressed in the testis (not in ovary, heart, hepatopancreas, muscle, thoracic ganglion or gill) [42]. Interestingly, the transcript level is higher in immature testis compared to mature testis. *In situ* hybridization showed that *EsDMRT-like* transcripts in the testis can be detected in both Sertoli and germ cells. Together, these findings suggest that this gene plays a role in male differentiation. Its possible role in male determination cannot be assessed, however, as the kinetic expression pattern has not yet been analyzed.

8. Amphibia

In general, amphibian sex determination is genetically controlled by XY/XX (i.e. *Ranalivida*) or ZW/ZZ (i.e. *Xenopus*) systems [43]. However, some species possess a certain amount of plasticity. For instance, different populations of the frog, *Ranarugosa*, possess different determination systems [44], and the sex of the salamander, *Hynobiusretardatus*, can be modified by environmental cues (genetic males can convert to phenotypic females at a high temperature) [45].

8.1. Anura

The important role of *Dmrt* genes in the sex determination process has been clearly demonstrated in the African clawed frog, *Xenopus laevis*. In this species, two DM domain-containing genes have been identified: *Dmrt1* [46] and its paralog, *Dm-W* [9]. *Dmrt1* is found on chromosomes 2 and 3, as *Xenopus* experienced genomic duplication during evolution [9]. Both genes are expressed early in embryonic development and are candidates to be sex determination genes. Spatially, *Dmrt1* is exclusively expressed in male and female primordial gonads, while *Dm-W* is only expressed in female primordial gonads [9]; both genes co-localize in the somatic cells surrounding primordial cells [45]. Temporally, *Dmrt1* is expressed during all gonadal differentiation steps in testis and ovary [9,47], while *Dm-W* is only expressed at the time of sex differentiation [9]. *Dm-W* was shown to bind the same DNA sequence as *Dmrt1*, thereby antagonizing its transcriptional activity [9]. *Dm-W* plays a primordial role in female sex determination, as shown using gain- and loss-of-function analyses. These studies yielded the following three main results: First, constitutive expression of the female *Dm-W* gene in ZZ male *Xenopus* was shown to induce the development of ovotestis but not normal ovaries, suggesting that another W- or Z-specific gene might contribute to the latter. Second, knockdown of *Dm-W* expression in ZW female *Xenopus* induced male development. Third, constitutive expression of male *Dmrt1* in ZW female *Xenopus* induced male development. Based on the above-described results, the following scenario was proposed for sex determination in *Xenopus*: in female tadpoles, *Dm-W* binds the *Dmrt1* target sequence to block the development of testes, while in male tadpoles, *Dmrt1* acts freely as a testis-forming factor [48]. To date, the *Dm-W* gene is the only known female-determining factor. It was recently shown that *Dm-W* is not present in all *Xenopus* species [46]; instead, some closely related species differ in possessing or not possessing the *Dm-W* gene (i.e. *X. laevis* versus *X. borealis*), suggesting that the mechanism of sex determination might differ even within the same order.

Dmrt genes have been identified in various other frog species, including *Rana plancyi*, *R. nigro maculata*, *R. livida*, *R. rugosa*, *Microhyla ornata*, *Engystomops pustulosus* and *Odorrana schmackeri*. *R. rugosa* is an interesting animal model for studying sex differentiation processes, because the sex of an XX female tadpole can be modified by injection of testosterone [49]. Four *Dmrt* genes have been identified in *R. rugosa* and their spatial expression is summarized in Table 2. All of these genes are expressed in testis but only *Dmrt5* is expressed in ovary. *Dmrt1* is expressed by interstitial cells, Sertoli cells and germinal cells of the testes [50]. Except for ovary, the organ-level gene expression pattern has only been tested in male adult frogs; thus, we lack detailed information on the expression of *Dmrt1* in males versus females. The expression patterns vary according to the gene and organs considered [50–52]. However, as these studies used RT-PCR, not quantitative-RT-PCR, the relative expression levels should be interpreted with caution.

In terms of temporal expression, the patterns exhibited by all four genes are similar for males and females in the developing gonads of embryos and tadpoles, and for each of the seven developmental stages tested [51]. In sex-reversal experiments performed using the XX female tadpole, researchers observed that *Dmrt1* is not expressed in the ovary, but rather appears after testosterone injection [50]. Because all four *Dmrt* genes are expressed in both male and female embryos, they would appear to be implicated in sex differentiation rather than sex determination. Consistent

Table 2
Comparative spatial expressions of different *Dmrt* genes in *Rana rugosa*.

	Brain	Heart	Pancreas	Kidney	Lung	Liver	Muscle	Spleen	Testis	Ovary	Reference
Dmrt1	–	–	–	–	–	–	–	–	++	–	[49]
Dmrt2	+	–	–	++	NP	NP	NP	NP	++	–	[51]
Dmrt3	++	–	–	–	NP	NP	NP	NP	+	–	[51]
Dmrt5	++	++	++	++	NP	NP	NP	NP	++	++	[51]

"+" and "++": low and high level of expression respectively; "–": no expression detected; NP: not performed.

with this, *Dmrt1* is localized on an autosomal chromosome [50].

In Bufonidae, males possess a special feature: the Bidder's organ, which is an undeveloped ovary located anterior to the testis in male toads. Following removal of the testes, the Bidder's organ develops and produces viable oocytes [53]. Thereafter, the female accessory organs develop and the male becomes a functional female. Four partial *Dmrt* gene sequences have been identified in the Asiatic toad (*Bufo gargarizans*) and one *Dmrt* gene has been identified and studied in the cane toad (*Bufo marinus*) [54]. The latter gene is highly similar to the *Dmrt1* gene from various species, such as frog, *Xenopus*, chicken and mouse [54]. Spatial analysis showed that this gene is expressed in testis, ovary and the Bidder's organ, but not in brain, heart, lung, liver, kidney or muscle. Temporal analysis showed that: (i) newly metamorphosed individuals do not express the gene; (ii) in the early stages of sex differentiation, males and females show similar patterns of *Dmrt1* expression; (iii) later, when the testis and ovary are visible, this expression increases in males and decreases to a basal level in females. These elements suggest that in the cane toad, *Dmrt1* may be implicated in sex differentiation but not in sex determination.

8.2. Caudata

One *Dmrt* gene, analogous to *Dmrt1*, was found in the salamander, *Hynobius retardatus* [55]. The sex of this species is temperature-sensitive, with genetic males becoming phenotypic females when reared at 28 °C. The spatial and temporal expression patterns of this *Dmrt* gene have been analyzed during the sexual differentiation period (i.e. 25 to 35 days after hatching) at different rearing temperatures [55]. Spatially, at normal temperature (20 °C), the gene was only expressed in testes, while at the female-producing temperature (28 °C), it was completely suppressed [55]. Temporally, a kinetic analysis performed using end-point PCR revealed that the transcript could be detected at all time-points at the normal temperature (20 °C), but was absent at the higher temperature (28 °C).

9. Testudines

Testudines (turtles) use a variety of sex determination mechanisms, including TSD (Temperature Sex Determination) and GSD (Genetic Sex Determination). Morphologically, the gonads of turtles and other reptiles develop as part of a heterogeneous tissue complex called the AKG (Adrenal, Kidney and Gonad) complex. This special feature makes it difficult to perform a precise analysis of spatial expression. In turtles, TSD has been proposed to be the ancestral trait [56]. The red-eared slider turtle, *Trachemys scripta*, may be considered the model organism for studying sex determination mechanisms in reptiles with TSD systems [57–61]. An analog of the chicken *Dmrt1* gene was isolated in *Trachemys scripta*, and its expression was followed in the genital ridge during the critical period of sex determination [60]. Spatially, *Dmrt1* is exclusively expressed in the gonads, and is not found in other tissues of the AKG complex [57]. Temporally, the gene is expressed prior to sexual differentiation (in stage 17 embryos). At a male-promoting temperature (26 °C), the expression increases over time, showing a strong peak at stage 19 [59]. *Dmrt1* expression was found to be inhibited by injection of estradiol, which is known to produce females at a male-promoting temperature [59]. Similarly, a shift from a female-promoting temperature to a male-promoting temperature during the embryonic stage immediately increases the expression level of the gene [58]. At a female-promoting temperature (32 °C) the expression remains low before and after sex determination. Similar results have been obtained in the sea turtles, *Lepidochelys olivacea* [62] and *Chelydra serpentine* [63]. In these two species, there is no observable difference in *Dmrt1* expression at male- and female-promoting temperature in the early stages of development, but as development progresses, the expression of *Dmrt1* increases at a male-promoting temperature (26 °C) and decreases to an undetectable level at a female-promoting temperature (33 °C) [62]. In the Reeves turtle (*Mauremys reevesii*), the expression of *Dmrt1* was studied only in male individuals, where it was found to be expressed in testes but not in liver, spleen, brain or kidney [64]. Finally, DM genes have been isolated from other turtle species, but none has yet been subjected to a functional analysis [65].

10. Lepidosauria

Lizards generally have either GSD or TSD systems, and the two can co-occur within the same family. However, the Indian garden lizard, *Calotes versicolor*, is unusual because it does not have any distinguishable sex chromosomes [66–68]. One *Dmrt1* gene was identified in this species [69]. Temporally, it is expressed during all embryonic stages, from day 3 to the hatchling stage. Spatially, expression during the early stages is limited to the testicular pathway, while expression in the later stages can be detected in both testis and ovary [69]. *Dmrt* genes have been identified but not studied in four additional lizard species: *Podarcis sicula*, *Anolis carolinensis*, *Eremias brenchleyi* and *Aspidoscelis inornata*.

11. Bony fishes

The *Dmrt1* genes and their functions in fishes were recently reviewed [14]. Here, we summarize the functional studies of these DM genes and mention those that have not yet been functionally studied.

Five *Dmrt* genes have been identified and studied in fishes. The *Dmrt1* gene has been the most widely studied, as it was shown to be the sex determination factor in the Medaka fish, *Oryzias latipes* (see [70] for review). Because it was found on the Y chromosome, it is called *Dmy* or *Dmrt1bY* [71]. This is the only functional gene in the male-specific region of the fish's Y chromosome, making it a good (and possibly the only) candidate to be the sex determination factor [72]. The injection of a *Dmy*-encoding genomic fragment into XX females is sufficient to reverse the sex of 20% of fishes [8], and *Dmy* knockdown in XY male embryos initiates the female pathway [73]. Functional

analyses have been performed on 18 other fish species, where *Dmrt1* expression appears to be correlated with male development regardless of the sex determination system [14]. In gonochoric species, the gene is either exclusively expressed in males or more highly expressed in testes than ovary. In seasonal gonochoric species, the expression pattern is synchronous with the reproductive season. In hermaphroditic species, the gene expression increases or decreases in protogynous or protandrous species, respectively, during the sex-reversal period. Finally, in fish that use temperature-dependent sex determination the *Dmrt1* gene is more highly expressed at the male-producing temperature than at the female-producing temperature. It is important to note that the *Dmrt1* gene is not the universal sex-determining gene in fishes [74]; for example, it is absent from the genomes of some species related to *Oryzias latipes*, including *Oryzias celebensis* and *Oryzias mekongensis* [75,76].

Four other *Dmrt* genes (*Dmrt2-5*) have been identified and partially characterized in bony fishes (see Table 3). Temporally, all of these genes are active during both embryogenesis and various adult physiological processes [74,77–85]. *Dmrt2* (also named *Terra* gene) implicated in neurogenesis [78] and left-right patterning [79] during embryonic development, while it is mainly expressed in male and female gonads or gills in adult fish. Exclusively, female expression has been identified in the gonad of the Atlantic cod [77]. In contrast, *Dmrt3* has presented clearly male-gonad-biased expression in all studied species (Table 3). Such bias was also detected in other organs of the Japanese pufferfish, prompting the authors to propose that *Dmrt3* functions cooperatively with *Dmrt1* after gonadal differentiation in both fishes and mammals [82]. The gonadal sex-specific expression of *Dmrt4* varies by species: in the Tilapia (*Oreochromis aureus*), *Dmrt4* is only expressed in the ovary [83,85]; in the olive flounder (*Paralichthys olivaceus*) and the Medaka fish, it is only expressed in the testis [80,84]; and in the Atlantic cod and the Japanese pufferfish, it is expressed in the ovary and testis [77,82].

12. Archosauria

12.1. Birds

Three DM genes have been identified in birds (*Dmrt1-3*) [15,86]. *Dmrt1*, which is carried by the Z chromosome and absent from the W chromosome of all birds, including the ratites, has been demonstrated to be a key sex-determining factor in chicken [10,87–89]. The higher-dose expression of *Dmrt1* in ZZ male chicken embryos induces male testicular development, while the lower dose arising in ZW female embryos produces the ovary [86,89,90]. In a recent functional validation study [10], the authors successfully knocked down *Dmrt1* gene expression in the male embryo using RNA interference, creating a male loss-of-function model. However, experimental obstacles prevented the over-expression of *Dmrt1* necessary to obtain a gain-of-function (i.e. masculinized) female embryo, which would be needed to fully validate the function of *Dmrt1* and exclude (or not) a role for W-linked genes in female development. Compared to the chicken model, very few studies have been performed in other birds; however, *Dmrt1* sequences are available for 16 Neognathes and 2 Ratites.

Relatively few *Dmrt2* and *Dmrt3* sequences (three and two, respectively) from birds are available in the relevant databases. However, these genes have been implicated in the embryonic development of the chicken: *Dmrt2* has been implicated in left-right synchronization during chicken development [91], while *Dmrt3* is expressed in several non-gonadal embryonic tissues (e.g., the nasal placode, telencephalon, spinal cord, somite, branchial arches and Mullerian ducts) [92].

Table 3
Comparative spatial expression of different *Dmrt* genes in fishes.

	Gene	Brain		Eye		Gills		Liver		Gonad		Muscle		Reference
Species		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
Xiphophorus maculates	*Dmrt2*	–	–	–	–	+	+	–	–	–	–	–	–	[99,100]
Oryziaslatipes	*Dmrt2*	–	–	–	–	+	+	–	–	+	+	–	–	[80,101]
Takifugurubripes	*Dmrt2*	–	–	+	+	+	+	+	+	+	+	+	+	[82]
Gadusmorhua	*Dmrt2*	–	–	–	–	+	+	–	–	–	+	–	–	[77]
Takifugurubripes	*Dmrt3*	+	+	++	+	+	+	++	+	++	+	++	+	[82]
Oryziaslatipes	*Dmrt3*	–	–	–	–	–	–	–	–	++	+	–	–	[80,101]
Daniorerio	*Dmrt3*	–	–	NP	NP	NP	NP	–	–	++	+	–	–	[81]
Gadusmorhua	*Dmrt3*	–	–	–	–	+	+	–	–	++	+	–	–	[77]
												–	–	
Xiphophorus maculates	*Dmrt4*	–	–	–	–	+	+	–	–	–	–	–	–	[99,100]
Oryziaslatipes	*Dmrt4*	+	+	+	+	+	+	–	–	+	–	–	–	[100]
Takifugurubripes	*Dmrt4*	–	–	–	–	+	+	–	–	+	+	–	–	[82]
Oreochromisaureus	*Dmrt4*	–	–	–	–	–	–	–	–	–	+	–	–	[83]
Paralichthys olivaceus	*Dmrt4*	+	+	–	–	+	+	NP	NP	+	–	NP	NP	[102]
Gadusmorhua	*Dmrt4*	+	+	+	–	+	+	–	–	+	+	–	–	[77]
Xiphophorus maculates	*Dmrt5*	+	+	–	+	–	–	–	–	–	–	–	–	[99,100]
Daniorerio	*Dmrt5*	+	+	+	+	–	–	–	–	+	+	–	–	[103]
Gadusmorhua	*Dmrt5*	–	–	–	–	+	+	–	–	+	+	–	–	[77]
Takifugurubripes	*Dmrt5*	+	+	+	+	–	–	–	–	–	–	–	–	[82]

"+" and "++": low and high level of expression respectively; "–": no expression detected; NP: not performed.

12.2. Crocodiles

All crocodiles possess a TSD system. *Dmrt1* expression in the crocodile was first reported in 1999 [93], in one of the first papers to show that the male-biased expression of DM genes is conserved in embryos of three distinct vertebrates: mouse, chicken and alligator (*Alligator mississippienis*). More recently, a detailed analysis was performed on another crocodile species, the Indian mugger (*Crocodylus palustris*) [66]. Eight *Dmrt1* isoforms were sequenced, and expression pattern analyses revealed that they were expressed in the AKG complex and brain (at a much lower level), but not in the heart or liver [66]. Moreover, their expression levels were found to be higher at a male-promoting temperature (32.5 °C) than at a female-promoting temperature (30 °C).

13. Conclusions

13.1. Analysis of the conserved DM domain among the DMRT proteins

A comprehensive phylogeny of the *Dmrt* gene family was very recently published by Wexler et al. [5]. In order to complete this phylogeny, we herein provide an amino-acid alignment of the DM domains from various metazoans, highlighting the differences and similarities among the *Dmrt* family members (Fig. 1). All of the sequences used for this analysis are listed in a supplementary file (Table S1). As expected, the structurally important cysteine and histidine residues of the Zn finger-like DNA-binding motif are highly conserved. A module consisting of the four amino acids beginning at position 9 of the DM domain can be used to distinguish among some. The VLSW and VVSC signatures appear to be specific to the *Dmrt3* and *Dmrt2* groups, respectively, whereas the Y/FVSP motif is shared exclusively by members of the *Dmrt1* group. This block is mainly composed of three hydrophobic residues and one polar amino acid. The DSX and DX (unclassified sequence) groups are highly variable in this motif, suggesting that it may play a non-essential role in DM. Consistent with this, mutagenesis of these residues in the *Drosophila* DSX factor are tolerated and do not affect the DNA-binding affinity [94]. The nineteenth amino acid of the DM sequence is also characteristically associated with certain groups: a tyrosine is mainly detected in the *Dmrt3*, *Dmrt5* and DSX groups, while a phenylalanine or histidine is present in the *Dmrt1*, *Dmrt2* and DX groups, respectively. Substitution of this position (regardless of the original residue) does not appear to disrupt the structure of the DM domain [94]. Two other modules located at positions 21–24 and 28–35 of the DM sequence: WRD/XXIAE has been found in *Dmrt1* group, WRD/LXVVE in *Dmrt2*, FKD/IXIIE in *Dmrt3*, WRD/TXIAE in *Dmrt4*, and WKD/TXIAE in *Dmrt5*. The XXTAD has been detected only in the DSX group, while over half of the DX sequences have a proline residue at position 21 of the sequence. All of the amino acids in these modules are functionally non-essential, regardless of their solvent accessibility and physicochemical properties [94]. Position 47 a conserved basic residue: a lysine or arginine for the DX group, and an arginine for the other groups. Its substitution induces a loss function, due to a lack of DNA binding [94]. However, this functional conservation has been detected in only 75% of the DX sequences.

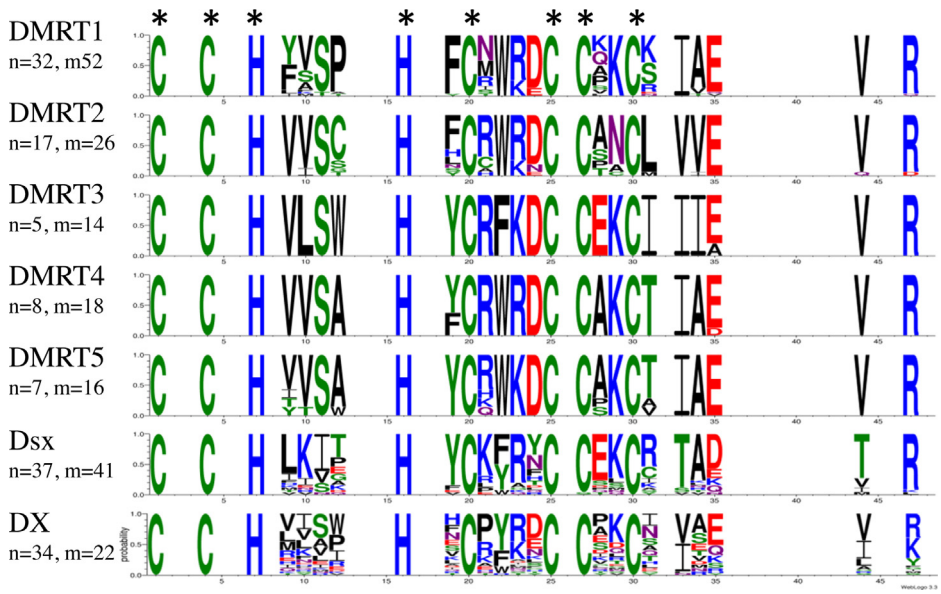

Fig. 1. (Color online.) *Analysis of the conserved DM domain among the different proteins.* Multiple amino acid sequence alignment of the DM domain from selected DMRT proteins (see supplementary Table S1) was performed using ClustalW software. Consensus sequences proposed for each DMRT protein groups and illustrated with WebLogo are indicated by the amino acid frequency at a given position. Undiscriminating amino acids are not shown. The six conserved cysteines and two histidines are marked by an asterisk, the others mentioned amino acids are represented by their physicochemical property: acidic residues (red), basic residues (blue), polar residues (green), hydrophobic residues (black) and neutral residues (purple). *n*: number of unique sequence considered. m: number of distinct species.

13.2. Sex-specific DMRT gene expression

Table 4 summarizes the sex-specific expression by taxon for each DM domain-containing gene group. The *Dmrt1* group is the most widely represented and thoroughly studied, and its expression pattern clearly favors the male developmental pathway. The single exception is DM-W, which is the female-determining factor on the W chromosome of *Xenopus laevis*. This exception is not explained by the fact that *Xenopus* possesses a female heterogametic sex determining system (ZZ/ZW), as the chicken possesses a similar system but uses a male-determining factor on the Z chromosome. In addition, while the other gene groups seem to show a male preference, it is difficult to infer a global rule given that not all of the genes have been functionally studied at this point.

13.3. Future directions

The existing detailed analyses of the role of *Dmrt* genes in sex determination/differentiation have largely focused on model organisms, and spatial expression patterns have been more extensively studied than temporal expression patterns. In the future, analyses that use both dimensions will provide better insights into the roles of these genes in sexual development.

Due to technical difficulties, functional characterizations of the *Dmrt* genes (such as through gain- or loss-of-function experiments) have been performed only in animal models. Transgenic techniques are likely to remain an option only for such models, but other methods for testing loss of function, such as the use of morpholino antisense oligonucleotides, should continue to progress, potentially facilitating new advances in our understanding of how the *Dmrt* genes functions in sexual development.

This review focuses on genes that have been subjected to either partial or extensive functional characterization. The analysis of *Dmrt* gene expression lacks in groups were sequences are known to be present Echinodermata, Hemichordata, Chelicerata, Rotifera, Annelida, Placozoa, Tunicata, Cephalochordata, snakes and cartilaginous fish. Moreover, the sequence and diversity analyses of the *Dmrt* family members are biased by the fact that these genes have not been studied in some taxa. The development of next-generation sequencing programs should contribute to addressing this problem. This discrepancy between species studies is also notable in the context of gene expression. This is seen in the present review, where Table 1 summarizes information on the mollusk phylum, which has one gene per species, Table 2 addresses a single species of Anura in which several members of the *Dmrt* gene family have been analyzed, and Table 3 features a number of *Dmrt* genes in various species of fish.

Some animal species are of particular interest when considering the roles of *Dmrt* genes in sexual development. First, it could be interesting to analyze the role of these genes in an evolutionary young sex chromosome system, such as that of the *Schistosoma* Platyhelminth [95]. Second, studies on successive hermaphroditic organisms are also of great interest that has been shown in fishes or salamanders. Third, it could be useful to study *Dmrt* gene expression in both sexes of some animals that

Table 4
Dmrt gene families according to different phyla (DX: non classified *Dmrt* gene).

Phylum	DM domain gene families						
	Dmrt1	*Dmrt2*	*Dmrt3*	*Dmrt4*	*Dmrt5*	*Dsx*	*DX*
CNIDARIA		P (1)					P (3)
PLATYHELMINTHES		♂ (3)			♂ (1)		♂ (8)
NEMATODA					P (2)		♂ (15)
ARTHROPODA							
Hexapoda		P (2)		P (3)		P (58)	P (3)
Crustacea		P (1)		P (2)		P (4)	P (2)
Chelicerata						P (1)	
ROTIFERA				P (1)			
ECHINODERMATA				P (1)			P (1)
ANNELIDA				P (1)			P (1)
MOLLUSCA		♂ (1)		♂(2)	♂ (1)		♂ (1)
HEMICHORDATA		P (1)		P (1)			P (1)
CHORDATA							
Tunicate		P (1)			P (1)		
Cartilaginous fishes		P (1)			P (1)		
Bony fishes	♂ (38)	♂ = ♀ (6)	♂ (4)	♂ = ♀ (4)	♂ = ♀ (5)		
Amphibia (Anura)	♂/♀ (14)	♂ (7)	♂ (10)	P (2)	♂ = ♀ (4)		
Amphibia (Caudata)	♂ (1)	P (1)					
Archosauria (Crocodiles)	♂ (1)						
Archosauria (Birds)	♂ (1)	NS (2)	NS (1)				
Testudines (Turtles)	♂ (2)						
Lepidosauria (lizards)	♂ (1)		P (2)	P (1)	P (1)		
Lepidosauria (snakes)	P (2)						

♂: higher expression in male than in female; ♂ = ♀: similar expression in male and in female; NS: No expression linked to sexual development; P: presence of DM sequences in the phylum but sex specific expression not tested.

possess unusual sex-determining systems, such *Rana rugosa*, which possesses either XX/XY or ZZ/ZW sex chromosomes.

The analysis of *Dmrt* genes in the chicken has been important because in this model organism, sexual development is governed not only by the absolute gene expression, but also the relative dose between males and females at a given developmental stage. The balance between male and female expression needs to be studied in greater detail in future studies.

To date, DM-W from *Xenopus laevis* is the only known female-determining gene among the *Dmrt* genes. Indeed, the roles of *Dmrt* genes appear to be highly biased toward male development (see Table 4). However, additional studies are warranted to assess the spatial and temporal expression patterns of the *Dmrt* genes in species with female heterogametic systems.

Although we herein focused on the roles of *Dmrt* genes in sexual development, this gene family is also known to play a pivotal in neurogenesis. Our conclusion is, even if *Dmrt* genes play an important role in sex determining/differentiation mechanisms, no clear picture emerged. Relatively fewer studies (in fewer species) have examined the role of *Dmrt* genes in neurogenesis, but a review similar to this one could be warranted in the context of neurogenesis.

Finally, DM domain-genes are known to be the sex-determining genes in five very distinct species: the planarian, *Schmidtea mediterranea*; the crustacean, *Daphnia magna*; the fish, *Oryzias latipes*; the African clawed frog, *Xenopus laevis*; and the bird, *Gallus gallus*. Considering that DM domain-containing genes play such a fundamental role in individual development and species-level maintenance, it seems odd that this factor is not found in species that are very closely related to those listed above. For example, DM-Y (a paralog of *Dmrt1*) is absent from the genomes of some species related to *Oryzias latipes* (e.g., *Oryzias celebensis* and *Oryzias mekongensis*), while DM-W is absent from the genomes of some species related to *Xenopus laevis* (e.g., *Xenopus borealis*).

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2015.04.010.

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