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Y-linked *iDmrt1* paralogue (*iDMY*) in the Eastern spiny lobster, *Sagmariasus verreauxi*: The first invertebrate sex-linked *Dmrt*

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ABSTRACT

Sex determination pathways are extensively diverse across species, with the master sex-determinants being the most variable element. Despite this, there is a family of DM-domain transcription factors (*Dmrts*), which hold a highly conserved function in sexual development. This work is the first to describe a heterogametic sex-linked Dmrt in an invertebrate species, the Eastern spiny lobster, *Sagmariasus verreauxi*. We have termed the Y-linked, truncated paralogue of the autosomal iDmrt1, Sv-iDMY. Considering the master sex-determining function of both DMY in medaka and DM-W in frog, we hypothesised a similar function of Sv-iDMY. By conducting temporal expression analyses during embryogenesis we have identified a putative male sex-determining period during which *iDMY* > *iDmrt1*. Employing a GAL4-transactivation assay we then demonstrate the dominant negative suppression of iDMY over its autosomal iDmrt1 paralogue, suggesting the mechanism with which iDMY determines sex. Comparative analyses of Sv-iDMY, DM-W and medaka DMY, highlight the C′-mediated features of oligomerisation and transactivation as central to the mechanism that each exerts. Indeed, these features may underpin the plasticity facilitating the convergent emergence of these three sporadic sex-linked master-Dmrts.

1. Introduction

Since the discovery of the sex-determining region Y (*SRY*) gene in mammals in 1990 (Sinclair et al., 1990), only a handful of other master sex-determinants[1] have been identified, such as the Y-linked *amhY* in the Patagonian pejerrey (Hattori et al., 2012), the Y-linked *SdY* in rainbow trout (Yano et al., 2012) and the W-linked *Fem* in the silkworm (Kiuchi et al., 2014); see Bachtrog et al. (2014) for a full list. This is in part due to the rapidly diverging nature of metazoan sex determination mechanisms but is also reflective of the fact that these initial triggers tend to be the most variable element of the pathway (Beukeboom and Perrin, 2014; Wilkins, 1995). Downstream, the genetic cascade that ensues in response to the master sex-determining signal comprises the major effectors,[2] the genes that are responsible for integrating cues of sexual identity alongside other developmental and positional signals to mediate dimorphic sexual development (Beukeboom and Perrin, 2014). In contrast, the major effectors tend to be the most conserved element of the sex determination pathway and are commonly from a family of transcriptional regulators known as the Dsx- and mab-3-related transcription factors (Dmrt) (Bachtrog et al., 2014; Kopp, 2012; Matson and Zarkower, 2012). This family takes its name from the major effector genes characterised in the invertebrates *Drosophila* (*Doublesex, Dsx*) (Burtis and Baker, 1989) and *Caenorhabditis elegans (Male abnormal-3*, *Mab-3*) (Raymond et al., 1998; Shen and Hodgkin, 1988), orthologues of which have since been identified across *Metazoa* (Wexler et al., 2014), from mammals, notably the male sex-differentiating *Dmrt1*, to Cnidaria (Traylor-Knowles et al., 2015).

Indeed, the pivotal role that the *Dmrts* hold within metazoan sexual development is evident in the fact that they also feature in the few master sex-determinants that have been identified. These master sex-determining Dmrts include: 1) the male-specific, Y-linked DMY in

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[1] **Master sex-determinant**: the genetic trigger that occurs at the very top of the sex determination cascade, acting as the primary sex-determinant. Most variable element across sex determination mechanisms. Examples: Genetic, Y-linked *SRY* in mammals or X: A ratio in *Drosophila*; environmental, temperature; or social, population sex-ratio).

[2] **Major effector**: the downstream genetic factors that maintain the continuity of the initial trigger. Responsible for integrating cues of sexual identity with other spatial and developmental information to mediate the process of dimorphic sexual development. Most conserved element of sex determination mechanisms, most commonly from the Dsx- and mab-3-related transcription factors (Dmrt) family. Example: *Drosophila* Dsx.

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medaka (*O. latipes*) (Matsuda et al., 2002; Nanda et al., 2002); 2) the female-specific W-linked DM-W in the African Clawed Frog, (*Xenopus laevis*) (Yoshimoto et al., 2008); and 3) the Z-linked Dmrt1 in the domestic chicken (*Gallus gallus domesticus*) (Smith et al., 2009); a homologue of which was also recently identified in a forth species, the flatfish (*Cynoglossus semilaevis*) (Chen et al., 2014). Although all three genes have adopted the master sex-determining role in these vertebrate species, each functions through a very different mechanism. Medaka DMY (XY system) and *X. laevis* DM-W (ZW system) are the only examples of sex-specific genes, linked to the heterogametic sex chromosome, initiating sex determination through their sex-specificity. In contrast, the homogametic *Dmrt1* genes in chicken and flatfish function through a dose-dependent effect, determining male sexual development through their uncompensated expression in ZZ males. These limited examples provide real evolutionary evidence of the diversity of viable sex determination mechanisms that exist across species, elegantly illustrated in experimental work (Hodgkin, 2002), highlighting the plasticity of the system.

In vertebrates, it is broadly accepted that sexual fate is first determined in the genital ridge, which then differentiates into the gonad, from which the endocrine system is employed to convey the gonadal sex across somatic tissues (Beukeboom and Perrin, 2014). This can be described as a gonad-centric sex determination system (exceptions are noted (Cutting et al., 2013)). This contrasts quite drastically to that described in the invertebrates that have been studied. In these model species, sexual identity appears to be determined in a cell autonomous fashion, in other words cell by cell in a mosaic-like pattern (Beukeboom and Perrin, 2014; Robinett et al., 2010). This can result in the emergence of sex-specific characteristics before the establishment of a gonad. In decapod crustaceans a slightly different mechanism exists, encompassing the role of a sex-specific accessory gland in males, termed the androgenic gland (AG). In male decapods (of both XY and ZZ) the AG develops prior to the testis and is responsible for stimulating testicular development (as well as the broader differentiation of male sexual characteristics) through the secretion of an insulin-like peptide known as the insulin-like AG hormone (IAG) (Sagi et al., 1997; Ventura et al., 2009, 2011b).

The Dmrts feature in both gonad-centric and cell autonomous mechanisms of sex determination. Mammalian Dmrt1 is responsible for the initiation and maintenance of male regulatory signalling from the testis (Matson et al., 2011). While in the invertebrate *Drosophila*, the male-specific splice variant of *Dsx* (Dsx^M) mediates the cell specific development of sex combs on the first pair of legs (Beukeboom and Perrin, 2014; Robinett et al., 2010). Similarly, localised expression of *Dsx1* stimulates male-specific dimorphism in the crustacean *Daphnia magna* (Kato et al., 2011). Classified by their DNA-binding (DM) domain, the Dmrt family comprises a group of non-classical zinc fingers. The highly conserved DM-domain is characterised by its ability to stabilise two zinc ions, each residing within a hydrophobic core coordinated by three cysteines and a histidine (Zhang et al., 2006; Zhu et al., 2000). Outside of the DM-domain, there is minimal sequence conservation across the family. As a consequence, while the N terminus (N′) DM-domain defines the Dmrts, the non-conserved C terminus (C′) is evidence of the adaptation that is central to the versatility of this gene family within metazoan sex determination.

Work in both human Dmrt1 and *Drosophila* Dsx, has demonstrated the critical importance of the C′ in coordinating binding stability and successful activation. While the DM-domain binds the DNA's minor groove, it is the helical C′ tail that inserts into the major grove, stabilising the DNA-protein interface, facilitating the assembly of the Dmrt binding complex (Murphy et al., 2015). In the case of Dsx, dimerization occurs *in vivo* (Zhang et al., 2006) although tri and higher oligomers readily form *in vitro* (An et al., 1996). Human Dmrt1 forms di/tri/tetramer forms *in vivo* (Murphy et al., 2015). The correct assembly of such binding complexes is a fundamental feature of successful activation (Zhang et al., 2006; Zhu et al., 2000).

In addition, as a family of transcriptional activators, the C′ of the Dmrts serves a second function. While the DM-domain is responsible for DNA specificity, it is the C′ that ultimately dictates transcriptional activation through the transactivation domain (TAD) (Beukeboom and Perrin, 2014; Mapp and Ansari, 2007). Unlike the readily defined DM-domain, TADs have proven far harder to characterise, as they lack a conserved motif or folding pattern (Mapp and Ansari, 2007). A combination of studies have suggested that hydrophobicity is particularly important for transcriptional potency and that acidic residues are also therefore necessary for solubility, but as these features can be achieved with a range of amino acids, TADs are highly conspicuous (Mapp and Ansari, 2007; Piskacek et al., 2007). Therefore Piskacek et al. (2007) focussed on creating a TAD prediction tool which can accurately predict TAD motifs based on hydrophobic and hydrophilic signatures.

In this work, we have identified the first invertebrate sex-linked (Y-linked) *Dmrt* gene, *Sv-iDMY*. We have considered this novel discovery in the context of the two previously characterised sex-linked master sex-determinant Dmrts, medaka DMY and frog DM-W, where notable shared characteristics suggest that Sv-iDMY may also act as the master sex-determinant in the Eastern spiny lobster, *Sagmariasus verreauxi*. Through these comparative analyses, although it is clear that each Dmrt exerts a unique mechanism of sex determination, we have also recognised key features, other than the DM-domain itself, that are central to Dmrt functionality.

2. Results

2.1. Identification of a male Y-linked iDmrt1 paralogue, Sv-iDMY

Three *Dmrt* genes, *Sv-iDmrt1* (KY427006), *Sv-Dsx* (KY427007) and *Sv-Dmrt11E* (KY427008) were previously identified in the decapod crustacean, *S. verreauxi* (Chandler et al., 2016a). In the case of *Sv-iDmrt1,* male-specific SNPs were identified (Fig. S1). Using SNP specific primers we were able to confirm the presence of a second, male specific *Sv-iDmrt1* gene (Fig. 1*A*). The complete transcript was obtained using rapid amplification of cDNA ends (RACE) and validated with Sanger sequencing (as was *Sv-iDmrt1* which was identical in both sexes); we have named the transcript *Sv-iDMY* (KY427009), similar to the male-specific, Y-linked *DMY* from the vertebrate medaka (*O. latipes*) (Matsuda et al., 2002; Nanda et al., 2002). *Sv-iDMY* is the first identification of a sex-specific (heterologous sex-linked) *Dmrt* gene in an invertebrate species.

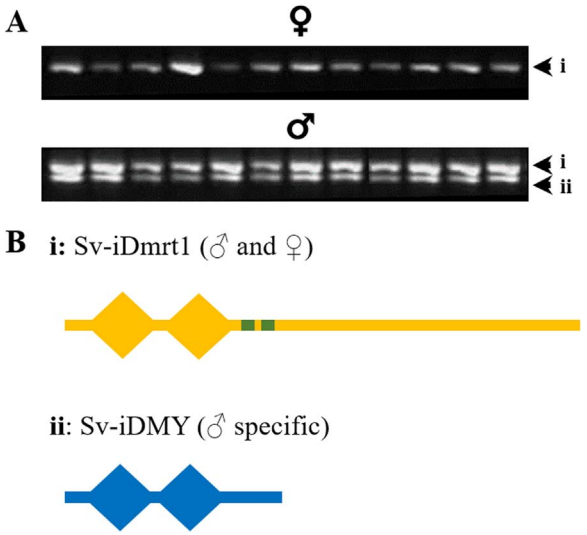

Fig. 1. *S. verreauxi iDmrt1* and *iDMY* structure and sex specificity. (*A*) Genomic sex-specificity of (i) *Sv-iDmrt1* and (ii) *Sv-iDMY*. (*B*) Scaled illustration of domain architecture of (i) Sv-iDmrt1 and (ii) Sv-iDMY, DM-domains represented by diamonds and the predicted transactivation domains of iDmrt1 shown in green.