Y-linked *Dmrt1* 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 Dmrt1, 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.

Highlights

- The first heterogametic sex-linked *Dmrt* identified in an invertebrate: Sv-iDMY.
- Sv-iDMY embryonal expression predicts the male sex-determining period.
- Sv-iDMY dominantly suppresses its autosomal paralogue iDmrt1.
- iDMY suggested to be the master sex-determinant in *S. verreauxi*.
- Transactivation domain predicted to be fundamental to Dmrt functionality across phyla.

Graphical Abstract

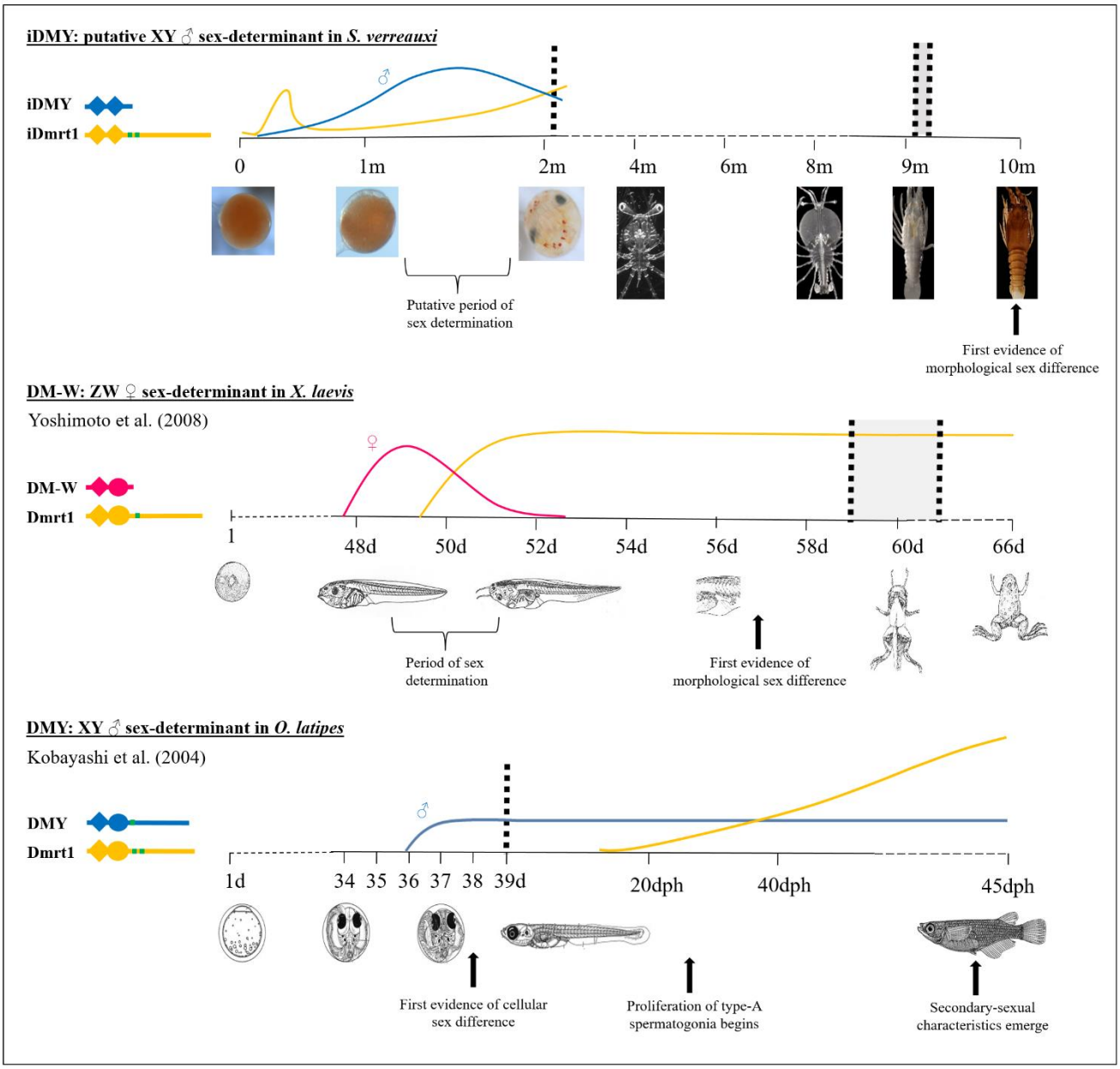

Figure caption: Illustration of the temporal expression profiles of the three heterogametic sex-linked Dmrts: the novel ♂ iDMY from lobster (*S. verreauxi*) alongside, ♀ *DM-W* from frog (*X. laevis*) and ♂ *DMY* from medaka (*O. latipes)*. Embryogenesis and early development is described in months (m) for the lobster and days (d) and days post hatch (dph) for frog and medaka, dashed lines represent hatching in the case of the lobster and medaka and dashed grey regions represent terminal metamorphosis in the case of lobster and frog. Domain architecture of each Dmrt is shown where diamonds represent DM domains, circles the specialised vertebrate Dmrt1 domains and green boxes the predicted TADs. Our analyses highlight that although each of the DM paralogue pairs exerts a unique mechanism of sex determination, there appear to be common features that dictate their regulation. Credit for developmental illustrations to Nieuwkoop and Faber (1994) for *X. laevis* and Iwamatsu (2004) for *O. latipes.*

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[*] 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 the 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[†], 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 medaka

[*]**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).

[†]**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.

(*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; Ventura et al., 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 transcriptional activation 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) (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 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.

Results

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.

The predicted Sv-iDMY protein sequence generates a truncated (179 amino acid) paralogue of the full Sv-iDmrt1 (504 amino acids), showing strong conservation with the N' DM-domains of Sv-iDmrt1 (Fig.1*B*), including the six intertwined cysteines and two histidines characteristic of the DM-domain (Fig. S2*A*). The C' of Sv-iDmrt1 is predicted to contain two TAD motifs. TADs were also predicted for the transcriptionally active: medaka- Dmrt1a; medaka- DMY (aka Dmrt1bY); and frog- Dmrt1a. TAD motifs were not predicted for Sv-iDMY (Fig. 2) or for the transcriptionally inactive frog- DW-W (Fig. S2*B*). We also applied TAD prediction to the splice variants of *Drosophila* Dsx and found that Dsx^{M} contains a predicted TAD motif while Dsx^{F} does not (Fig. S2*C*).

***Sv-DMY* shares similar patterns of emergence with *DM-W* and medaka *DMY*.** We quantified gene copy number of *Sv-iDmrt1* and *Sv-iDMY* in genomic DNA (gDNA). Using both an absolute and normalised measure (relative to *Sv-18S*), we showed a consistent 1:1 ratio of *Sv-iDmrt1* gene copy number in males and females (a 0.98 absolutely and 1.06 relatively), suggesting an autosomal positioning. The same analyses also showed a 1:1 ratio of *Sv-iDmrt1* to *Sv-iDMY* in males (a 1.01 absolutely and 0.87 relatively), with a 1:0 ratio obviously existing in females (Fig. S3). This indicates a gene duplication of *iDmrt1* in males to give rise to the male-specific *iDMY* paralogue, which these analyses suggest to be present in two(x) copies.

Spatiotemporal expression of *Sv-iDmrt1* and *Sv-iDMY*. A broad spatial expression profile for *Sv-iDmrt1* and *Sv-iDMY* was generated from sexually mature male and female tissues

through both digital gene expression and semi-quantitative PCR (Fig. 2). The primary tissue responsible for *iDmrt1* expression appears to be the antennal gland (females > males). *iDMY* is expressed in the male antennal gland, as well as the sex-related tissues of the testis, male-specific AG and fifth walking leg (the site of the AG and reproductive openings or gonopores in males); hypertrophy of the AG (induced by eyestalk ablation to remove neuroendocrine regulation) does not appear to induce any notable change in *iDMY* expression.

We then conducted qPCR analyses on the antennal glands and gonads of sexually immature (defined as sexually differentiated but not yet reproductively capable) males and females (Fig. 3*A*). *iDMY* showed expression similar to that described in mature tissues, with a significant difference (*$P < 0.01$) demonstrated (Fig. 3*A*, **AnG > TS), whilst *iDmrt1* showed an altered expression profile in immature tissues, lacking the sexually dimorphic expression seen at maturity. *iDmrt1* also showed significantly higher expression in the ovary than the testis (Fig. 3*A*, *OV >TS).

In situ hybridisation performed on testis and male antennal gland clearly identified the cell types responsible for *iDMY* expression (Fig. 3*B*). Although cellular differentiation within the testicular cords is in its infancy, a germinal layer of spermatogonia (Sp) are localised on one side of each cords' periphery (Fig. 3*B*, TS: H&E), identified as containing a large granular nucleus with sparse cytoplasm (Bell and Lightner, 1988); *Sv-iDMY* expression is highly specific to these Sp (Fig. 3*B*, TS: AS).The antennal gland is comprised of cuboidal cells forming a complex, channel-like structure, amongst which secretory-like cells appear to open up into a duct and channel network; *Sv-iDMY* is highly localised to these secretory-like cells.

Identification of the putative sex-determining period. Considering the master sex determining function of DMY in medaka and DM-W in frog, we postulated Sv-iDMY may take a pivotal role in early development during male sex determination. First we defined embryogenesis in *S. verreauxi* (Fig. S4). With the exception of Stage 2, *Sv-iDmrt1* remains at relatively low levels throughout embryogenesis, increasing slightly in later development. *iDMY* is expressed after Stage 1, with a dramatic increase from Stage 3 (coinciding with the first evidence of cellular differentiation) to Stage 5b (Fig. 4). Stages 3 to 7, where *iDMY* > *iDmrt1* (boxed in grey), have therefore been identified as the putative male sex-determining period; *iDMY* > *iDmrt1* at Stages 5a and 5b (*$P < 0.01$).

Transcriptional activity: a clue to mechanism. The TAD predictions and the elevated expression of *Sv-iDMY* to *Sv-iDmrt1* suggest that iDMY may function as a dominant negative suppressor to iDmrt1. In this scenario, the transcriptionally-inactive iDMY would act as an antagonist to the transcriptionally-active iDmrt1, reducing its transcriptional response. We therefore employed a transactivation assay using GAL4 fusion proteins of the full iDmrt1/iDMY sequences and their C' (Fig. 5*A*) co-transfected with a 9xUAS reporter plasmid to assess the transcriptional activity of the Dmrt paralogues in COS-7 cells. Based on preliminary analyses (Fig. 5*B*) we then used the full iDmrt1/ iDMY fusion proteins to transfect cells at the expression ratios observed during embryogenesis (Fig. 4). The luciferase activity observed from the GAL4-iDmrt1 fusion protein was significantly reduced in the presence of iDMY from as little as 4:1 (Fig. 6), indicating that the transcriptional activity driven by iDmrt1 was supressed in the presence of the transcriptionally inactive iDMY (Fig. 5*B*, Fig. 6).

Discussion

This work is to our knowledge, the first example of a sex-linked *Dmrt* in an invertebrate species. Our analyses demonstrate that *Sv-iDMY* displays similar patterns of emergence to that of *DMY* in medaka and *DM-W* in the frog, *X .laevis.* Medaka *DMY* (Bewick et al., 2011; Matsuda et al., 2002; Nanda et al., 2002) and frog *DM-W* (Yoshimoto et al., 2008) have evolved a master sex-determining function in the heterogametic sex through the duplication and neofunctionalisation of the autosomal *Dmrt1*. *Sv-iDMY* is also a heterogametic (Y-linked) paralogue of an autosomal *iDmrt1* gene, which appears to be present in two(x) copies in the male genome equating to the copy number of the autosomal *iDmrt1*. In medaka and frog ~~all three cases~~ the emergence of the sex-linked Dmrt is ~~appears~~ ~~to be~~ highly species-restricted. Medaka *DMY* has only been identified in one other *Oryzias* species (Matsuda et al., 2003), lacking in other close relatives (Kondo et al., 2004; Myosho et al., 2012); and *DM-W* is not common amongst *Xenopus* species (Bewick et al., 2011). Although not conclusive, we performed analogous transcriptomic analyses assessing for gender-specific transcriptomic SNP patterning (which identified Sv-iDMY (Fig S1.)) in the closely related Southern rock lobster (*Jasus edwardsii*) and tropical rock lobster (*Panulirus ornatus*) and were unable to identify the presence of a similarly expressed sex-linked Dmrt gene. The full validation of the phylogenetic distribution of iDMY holds significant evolutionary perspectives and is an important area of future study. Together, these characteristics are indicative that *Sv-iDMY* may also have evolved as a master sex-determinant from an autosomal *Dmrt1* in the invertebrate *S. verreauxi*.

Although male specific- indicating Y-linkage similar to *DMY* in medaka- Sv-iDMY shows multiple similarities to the female specific W-linked DM-W in frog (Yoshimoto et al., 2008). This similarity is initially apparent in the striking sequence conservation that both Sv-iDMY and frog DM-W share with the N' DM-domains of their Dmrt1 paralogues, a conservation halted by a truncation resulting in loss of the C'. Work in *X. laevis* has demonstrated the importance of the Dmrt1 C', as it contains a TAD (Yoshimoto et al., 2006); a domain which is lacking in the DM-W paralogue (Yoshimoto et al., 2008), as is true of Sv-iDMY. Expression analyses of *DM-W* and *Dmrt1* during the sex determining period show that *DM-W* exhibits a peak prior to significant *Dmrt1* expression (Yoshimoto et al., 2008), a second similarity shared with *Sv-iDmrt1* and *Sv-iDMY*. The group went on to demonstrate that DM-W acts as an inactive competitive inhibitor to Dmrt1 at its DNA binding site, resulting in female sex determination (Yoshimoto et al., 2010). This mechanistic interaction is also demonstrated during the putative

male sex determining period of *S. verreauxi*, as iDMY exerts transcriptional suppression over the active iDmrt1. This work suggests that Sv-iDMY functions akin to DM-W, acting as a dominant negative regulator to its Dmrt1 paralogue and thus putatively responsible for male sex determination. If so, this provides the third evolutionary example of a viable sex determination mechanism achieved through a heterogametic sex-linked *Dmrt* paralogue. Interestingly, in all vertebrate cases described to date, Dmrt1 is the male promoting gene, whereas in *S. verreauxi* iDmrt1 (in the absence of iDMY) is predicted to promote female development. Although disparate to what has been characterised in vertebrates, it must be remembered that Sv-iDmrt1 is far removed from its vertebrate counterparts, containing a second repeat DM domain, rather than the specialised Dmrt1 domain limited to the vertebrates. As this is the first and thus far only, invertebrate example of a paralogous Dmrt pair, a similar male-promoting function of Sv-iDmrt1 should not necessarily be presumed. Thus, as the only master sex-determining gene common to multiple Classes (Bachtrog et al., 2014), the functional role of sex-linked *Dmrt* may be more diverse than first thought, a hypothesis that can only be tested by non-model species research such as that presented here.

Mechanistically, the transcriptional activity and resulting active (medaka DMY) or suppressive (DM-W) function of these master sex-determinants is determined by their C' TADs (Beukeboom and Perrin, 2014; Mapp and Ansari, 2007; Piskacek et al., 2007). It appears that a loss of the C' results in the suppressive mechanisms described for DM-W and proposed for Sv-iDMY. This contrasts drastically to that described in medaka, where DMY shows strong sequence conservation across the entire Dmrt1 peptide and thus retains the transcriptional activity of its paralogue (Kobayashi et al., 2004). In *S. verreauxi* we have demonstrated that the full length iDmrt1, but not the truncated iDMY, is capable of eliciting a transcriptional response when tested in a GAL4-UAS system (further targeted mutations of the predicted TADs would confirm their specific capacity to regulate transcriptional activity). This suppressive mechanism was also demonstrated in the frog (Yoshimoto et al., 2006), since validated using the DNA-specific binding motif (Yoshimoto et al., 2010). We note the oddity in this work that the C' of iDmrt1 was not capable of eliciting a transcriptional response and suggest it may reflect the use of the GAL4 system; the use of the DNA-specific sequence motif would allow for a more realistic depiction of the binding interaction.

Our bioinformatic predictions support these *in vitro* observations, identifying a TAD in all of the transcriptionally active Dmrts, namely: *S. verreauxi*- iDmrt1, frog- Dmrt1, medaka- Dmrt1

and medaka- DMY, but not in the suppressive Sv-iDMY or DM-W. It is worth noting that the other Dmrts identified in *S. verreauxi*, Sv-Dsx and Sv-Dmrt11E, were also predicted to contain TADs in their C' (Fig. S5). In the case of *Drosophila* Dsx, our TAD predictions highlighted that Dsx^M contains a TAD motif but Dsx^F does not. The lack of a TAD in Dsx^F has previously been suggested (Siegal and Baker, 2005) and is notable with regard to the differential transcriptional effects (Chatterjee et al., 2011) of the *Dsx* splice variants; for example the need for Dsx^F to recruit two coactivators (hermaphrodite (HER) and intersex (IX)) for transcriptional activity (Garrett-Engele et al., 2002; Pultz and Baker, 1995). Indeed, the male-specific sequence that we predict to be a TAD in Dsx^M, corresponds to the female-specific binding site for the transcriptional co-activator IX (Yang et al., 2008) in Dsx^F; and IX does contain a TAD (Garrett-Engele et al., 2002). Considered with the apparent inactivity of Sv-DMY and DM-W, the fact that the TAD-lacking Dsx^F is reliant on the TAD-containing IX to promote female differentiation (Garrett-Engele et al., 2002) is strong evidence for the vital importance of this small domain to Dmrt activity.

Human Dmrt1, *Drosophila* Dsx and *C. elegans* MAB-3 all bind their target DNA as oligomeric complexes (Murphy et al., 2015; Zhang et al., 2006). It is therefore highly likely that the formation of oligomers is a common feature underlying the transcriptional activity of Dmrts. In the context of our sex-determining paralogue-pairs, the significance of this oligomeric binding may extend further. During the period of female sex determination in the frog, it has been suggested that as the expression of the transcriptionally active Dmrt1 begins, its activity is initially sequestered by the formation of inactive DM-W/Dmrt1 hetero-mers, prolonging the period of DM-W's inhibition (Yoshimoto et al., 2010). Unlike the frog, Sv-iDmrt1 and Sv-iDMY show overlapping temporal expression during embryogenesis. Although we cannot ascertain which sex accounts for this iDmrt1 expression, if it is present in males, we hypothesise that the dominant negative suppression achieved by iDMY is maximised through the formation of inactive iDMY/iDmrt1 hetero-mers to minimise any potential activation of iDmrt1. Such a mechanism would dramatically increase the suppressive efficiency of iDMY in *S. verreauxi*, which is supported by the dramatic reduction of iDmrt1 activity seen with even the smallest amount of iDMY. Although acting through a very different mechanism, these oligomeric binding characteristics may also function in the regulation of sex determination in medaka. As Dmrt1 becomes functionally critical in spermatogonial differentiation (Kobayashi et al., 2004), DMY exerts an auto-feedback mechanism to reduce its own expression. As the expression of Dmrt1 increases, it is also capable of binding this regulatory element in DMY

(Herpin et al., 2010). Considering the repeated evidence of Dmrts' oligomeric binding (29, 30, 41), it seems highly probable that as the relative amounts of DMY and Dmrt1 shift, both homo and hetero-meric complexes are employed to efficiently coordinate DMY's plateauing expression in concert with increasing *Dmrt1*.

One of the main differences of *Sv-iDmrt1* and *Sv-iDMY* compared with the other master sex-determinants discussed, is their non-gonadal expression. Frog *Dmrt1* (Yoshimoto et al., 2006) and *DM-W* (Yoshimoto et al., 2008), medaka *Dmrt1* and *DMY* (Kobayashi et al., 2004) as well as most other vertebrate *Dmrt1* orthologues that have been analysed (Beukeboom and Perrin, 2014; Hong et al., 2007) show gonad-localised expression. This is probably reflective of the gonad-centric mode of sex determination that occurs in these vertebrates, compared to the cell autonomous mode described in the invertebrates (Beukeboom and Perrin, 2014; Robinett et al., 2010). As an invertebrate, it follows that male sexual development in *S. verreauxi* is a cell autonomous process, explaining the non-gonadal expression of *iDMY* and *iDmrt1*. The notable expression of *Sv-iDmrt1*in the antennal glands of both sexes (Fig. 3) illustrates this point. Work has already highlighted the role of the antennal gland in the sexual development of *S. verreauxi*, specifically the gland's significant expression of *Sv-TKIR* (a receptor involved in mediating male sexual differentiation) (Aizen et al., 2016) and the sexually dimorphic expression patterns that emerge by sexual maturity (Chandler et al., 2016b), including *Sv-iDmrt1* itself (Fig. 2). Furthermore, it must be considered that there are up to eight known *Dmrt* genes in the vertebrates, specialised to govern a range of developmental functions (Hong et al., 2007), a contrast to the three *Dmrt* genes identified in *S. verreauxi* (Chandler et al., 2016a). Thus it may be that Sv-iDmrt1 has a broader transcriptional role in this species, encompassing processes other than sexual development.

The first emergence of sexual dimorphism in *S. verreauxi* occurs in the early juvenile phase, with the development of sex-specific gonopores at the base of the fifth walking-legs in males and third walking-legs in females. Interestingly it appears that this developmental phase coincides with a dramatic and stage-specific increase of *iDmrt1* in females and of *iDMY* and *iDmrt1* in males (Fig. S6); supporting a cell autonomous mode of sexual differentiation mediated by iDMY/iDmrt1. The emergence of these sex-specific gonopores, long before establishment of the gonad in either sex (personal observation) and prior to any significant expression of the male sex differentiating hormone IAG in males (Ventura et al., 2014) is in an oddity we have previously noted (Chandler et al., 2016b). Thus perhaps iDMY/iDmrt1 are

providing the sexual identity necessary to mediate a cell autonomous mode of primary sexual differentiation. Further support is offered by the observation that all of the male sexually dimorphic tissues are those that show maintained, elevated *iDMY*: *iDmrt1* expression into adulthood (AG, the gonopore region of the fifth walking leg, and the testis). The above are consistent with iDMY being not only a master sex-determinant but also an ongoing major effector of male-cell sexual-identity.

Finally, in medaka and frog, the establishment of these sex-specific *Dmrts* coincides with a rearrangement in the hierarchy of sex determination (Herpin et al., 2010) and is even postulated to play a role in speciation, creating an isolation event through an altered sex determination mechanism (Bewick et al., 2011; Matsuda, 2005). This work adds to the evidence for these hypotheses. *S. verreauxi* was previously known as *Jasus verreauxi* as part of the *Jasus* genus, but has since been reclassified to form its own subgenera (*Sagmariasus*), based on significant morphological and genetic divergence from other *Jasus* species (Booth et al., 2002; Ovenden et al., 1998; Tsang et al., 2009). Although only speculative, perhaps the species divergences described in *S. verreauxi* are evidence of the genetic isolation initiated through the emergence of *iDMY*.

Conclusions

The discovery of *Sv-iDMY* marks the first evidence of a sex-linked *Dmrt* in an invertebrate species. Although the *Dmrts* have been extensively identified across Metazoa due to their early emergence within the kingdom (Wexler et al., 2014), there are only two other known examples of heterogametic, sex-specific master sex-determinant *Dmrts*. Our work in *S. verreauxi* sheds new light on the prevalence of Dmrts as master sex-determinants, now described in three distinct Classes, encompassing two Phyla. With further screening across other non-model species, the rarity of the master sex-determining Dmrt may well diminish. Furthermore, this suggests the stabilisation of a third, distinct regulatory mechanism through which a Dmrt1 paralogue has evolved to govern a mechanism of sex determination, reiterating the dramatic plasticity and consequential potential that exists within the regulation of sex determination.

However, within this plasticity, there appear to be certain features which explain the prevalence of the Dmrt family in sex determination, most critically the C' tail. These comparative analyses highlight that this region is ultimately responsible for the transcriptional activity and in turn, the mechanistic role that the Dmrt adopts. Structurally, the C' allows the stable assembly of oligomeric binding complexes, a common feature of all those Dmrts studied. Indeed, we suggest that the Dmrts' conservation across metazoan sex determination systems and the successful stabilisation of the *S. verreauxi*, frog and medaka Dmrt1 paralogues, may be in part driven by these versatile binding characteristics.

The second regulatory feature of the Dmrt C' relates to the presence of the TAD motif. The truncation of Sv-iDMY and lacking TAD motif, suggests it functions akin to DM-W and is incapable of transcriptional activation, dictating its role as a negative suppressor of iDmrt1. In contrast, the sex determination mechanism of medaka relies on the retained transcriptional activity of DMY, fitting with the TAD motif identified in this work. Furthermore, we highlight that the sex-specific regions of Dsx^{M} and Dsx^{F} correspond to the sexually dimorphic presence and absence (respectively) of a TAD motif in *Drosophila*. By bringing attention to this more conspicuous, but fundamentally critical domain, we hope to stimulate future work that considers the implications of this regulatory element in complex regulatory processes such as sexual development.

Materials and Methods

Transcript identification and validation. For description of animal husbandry, transcriptome generation and identification of *Sv-iDmrt1*, *Sv-Dsx* and *Sv-Dmrt11E* see (Chandler et al., 2016a). FASTQ libraries of brain, eyestalk, gonad and antennal gland were mapped to *Dmrt* transcripts (using CLC (v7.5.1)) and screened for sex-related SNP pattern. For *Sv-iDmrt1*, primers were designed to specifically amplify the male-specific SNP region as well as for the regions common to both males and females. Primers were tested for their sex-specificity on male and female genomic DNA (gDNA) as described in (Ventura et al., 2011a); note these primers have been further validated on > 200 individuals. The complete *Sv-iDmrt1* (male and female) and *Sv-iDMY* (male) mRNA sequences were obtained by 3' and 5' rapid amplification of cDNA ends (RACE) as described in (Ventura et al., 2014) using the Clontech SMARTer™ RACE kit (BD Biosciences, NJ, USA). The resulting *Sv-iDmrt1* (both male and female) and *Sv-iDMY* sequences were validated by Sanger sequencing (AGRF).

Sequence analysis. Transcripts were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and domain architecture defined with NCBI BLAST (http://www.ncbi.nlm.nih.gov/) and previous structural descriptions (Murphy et al., 2015; Zhang et al., 2006). To assess for transactivation domains (TAD) we used the Nine Amino Acids Transactivation Domain (9aaTAD) Prediction Tool (Piskacek et al., 2007), examining *S. verreauxi*- iDmrt1 and iDMY, alongside: *O. latipes*-Dmrt1a (AAL02165.1); *O. latipes*-DMY/Dmrt1bY (Q8JIR6.1); *X. laevis*-Dmrt1a (Q3LH63.1)*; X. laevis* DW-W (AB259777.1); Dsx^{M}: isoforms A, D, E (AAF54169.1); and Dsx^{F}: isoforms B C, F ((AAN13385.1). As non-yeast, non-mammalian transcription factors, we applied the "less stringent pattern" but enforced a "100% match" criteria.

Sample collection. Samples from sexually immature (~1kg) individuals of each sex were collected in April 2015, N=8; animals supplied by IMAS aquaculture facility in Hobart, Tasmania, reared as described in (Fitzgibbon et al., 2017). Although sexually immature, all individuals had undergone primary sexual differentiation characterised by the presence of sex-specific gonopores but were not yet reproductively capable (Chandler et al., 2016b). Tissues were sampled, rinsed in PBS and either placed in liquid nitrogen and stored at -80°C or processed for histology as described in (Ventura et al., 2009). To analyse the expression of *iDmrt1* and *iDMY* during the putative period of sex determination, we first had to define embryogenesis in *S. verreauxi*. Embryo samples were collected from five unrelated females

from Day 0 (defined as egg extrusion and fertilisation) to Day 65 (hatching). Each major morphological event was defined as a stage, representative images were taken and a sample of embryos were removed from the mother and processed as previously described.

Digital and molecular gene expression. Spatial-temporal expression was evaluated through digital gene expression, as reads per kilobase per million reads (RPKM). RPKM values were calculated for the re-assembled *iDMY* transcript. Semi-quantitative PCR analyses were conducted as described in (Aizen et al., 2016) using the same *Sv-iDmrt1* and *Sv-iDMY* primers designed for gDNA sexing; *Sv-16S* was used as the positive control. *Sv-iDmrt1* and *Sv-iDMY* gene copy number were assessed through qPCR using gDNA of previously sexed animals, N=12. qPCR analyses were also run on cDNA from immature male / female gonad and antennal gland, N=8, and embryo samples, N=5, as described in (Ventura et al., 2015). Statistical analysis was performed using ANOVA, followed by Mann-Whitney U-test with $*P < 0.01$ considered as statistically significant. For *in-situ* hybridisation, *Sv-iDMY* sense (S) and antisense probes (AS) corresponding to nucleotides 5 to 400 of the 3' UTR of *iDMY* were generated and tissue sections of 5µm were processed as described in (Chandler et al., 2015). Hybridisation was carried out as described in (Ventura et al., 2014). To ensure specificity of the *iDMY* probe ovary and female antennal gland controls were included.

Gal4-UAS luciferase transactivation assay. Transient transfection in COS-7 cells was conducted as previously described in (Aizen et al., 2016). Co-transfection of 10ng of the UAS reporter vector pGL4.35[luc2P/9XGAL4UAS/Hygro] (Promega) and 20ng of the GAL4-fusion plasmids pFN26A (BIND) hRluc-neo Flexi® Vector (Promega) containing the GAL4 binding domain fused to: *iDmrt1* open reading frame (iDmrt1); *iDmrt1 C*' corresponding to amino acids (aa) 157-504 (iDmrt C'); *iDMY* open reading frame (iDMY); *iDMY C'*, corresponding to aa 143- 179 (iDMY C'); and empty vector (plasmid constructs generated by Genscript). All following ratio transfections were conducted using the iDmrt1/iDMY plasmids, using both 20ng total DNA and 20ng constant of iDmrt1 and corresponding ratio amounts of iDMY; total DNA was kept constant with pcDNA3.1. Both methods gave highly similar results, with the 20ng total data presented. Transfection was carried out in a 24 well plates using TransIT-LT1 Transfection Reagent (Mirus), according to manufacturer's instructions. After 24 hours the luciferase activities were measured, normalised against *Renilla* to control for

transfection efficiency using the Dual Luciferase Assay system (Promega). Statistical analyses performed as previously described.

Western blot. Total cell lysates from the previously described transfections were collected through incubation with 0.6ml RIPA buffer at 4°C on a rocking platform for 15 minutes. The cell monolayer was removed through cell scraping, transferred to a microcentrifuge tube and incubated for one hour on ice and centrifuged at 10,000g for 10 minutes at 4°C. Lysates (30ug/lane) were loaded and run on 10% Tris-Glycine gels (Biorad), transferred to nitrocellulose membrane and Western blot analyses conducted as described in (Aizen et al., 2016). The membranes were probed with a GAL4 Antibody (DBD) (#sc-577 Santa Cruz) and visualised on Odessy CLx.

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Competing interests

No competing interests declared

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Figures and legends

Fig. 1.

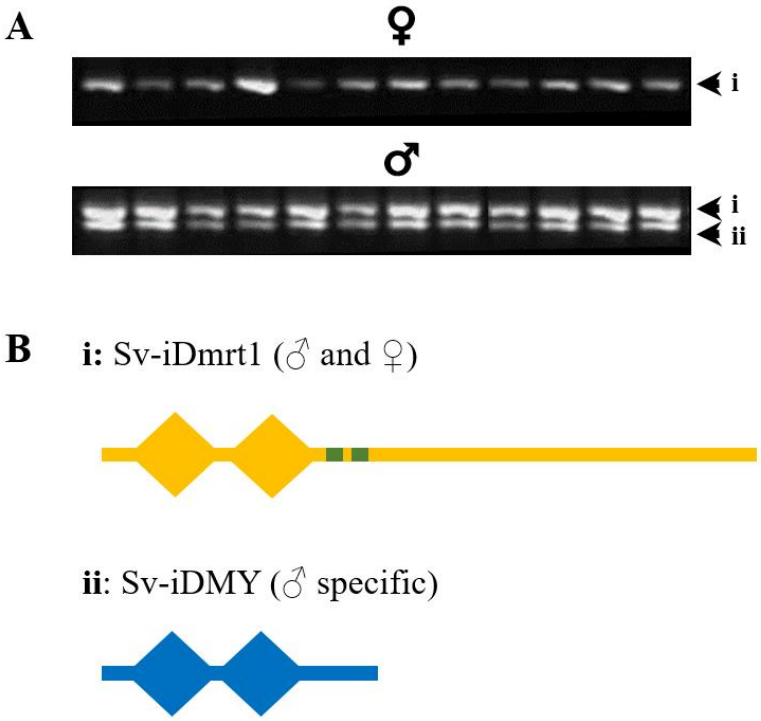

Fig. 1. *S. verreauxi iDmrt1* and *iDMY* structure and sex specificity. (*A*) Genomic sex-specificity of (i) *Sv-iDmrt1* and (ii) *Sv-iDMY*. (*B*) Scaled illustration of domain architecture of (i) Sv-iDmrt1 and (ii) Sv-iDMY, DM-domains represented by diamonds and the predicted transactivation domains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

Fig. 2.

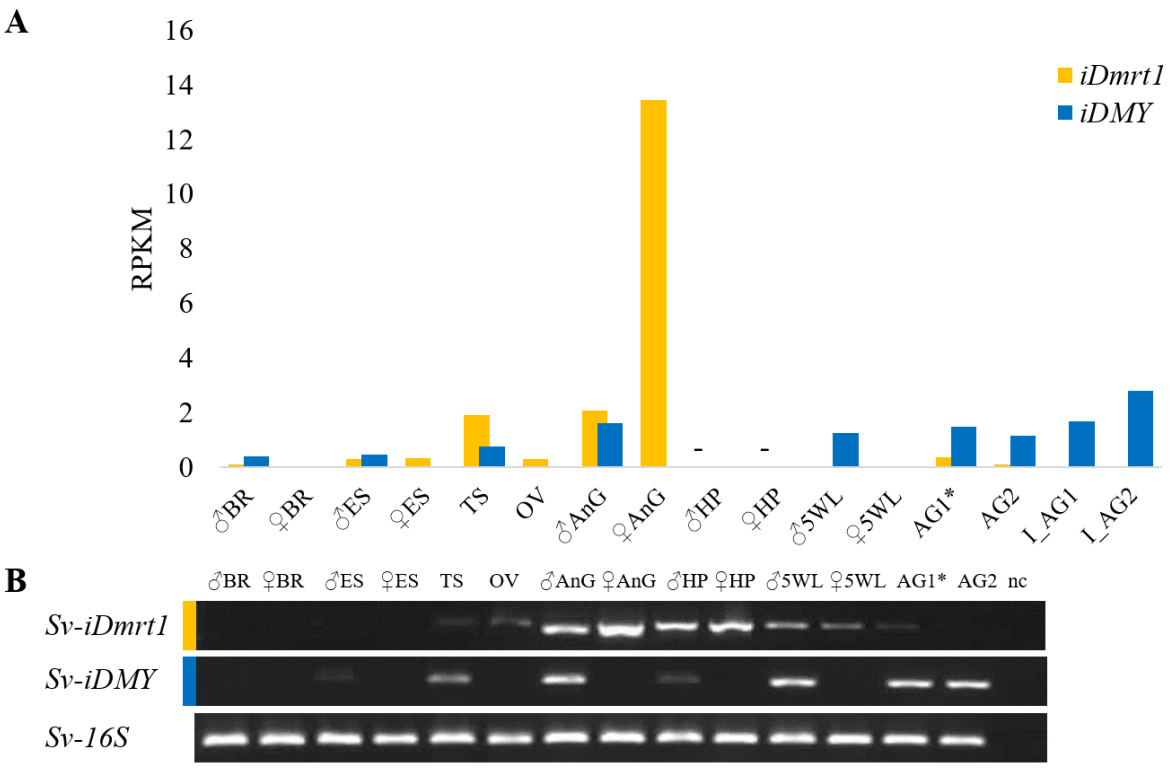

Fig. 2. Spatial expression of *Sv-iDmrt1* and *Sv-iDMY* in sexually mature tissues. (*A*) Transcriptomic spatial expression of *Sv-iDmrt1* and *Sv-iDMY* as RPKM for male and female brain (BR), eyestalk (ES), gonads (TS and OV), antennal gland (AnG) and fifth walking leg (5WL), androgenic glands (AG1* and AG2, where * indicates a hypertrophied gland) and immature androgenic glands (I_AG1 and I_AG2). - indicates a lack of transcriptomic data. (*B*) The RT-PCR expression profile of *Sv-iDmrt1* and *Sv-iDMY* including all the tissues used for transcriptomic analyses, lacking immature AGs, with the addition of hepatopancreas (HP). Negative control (nc) in the fifteenth lane, *16S* as positive control.

Fig. 3.

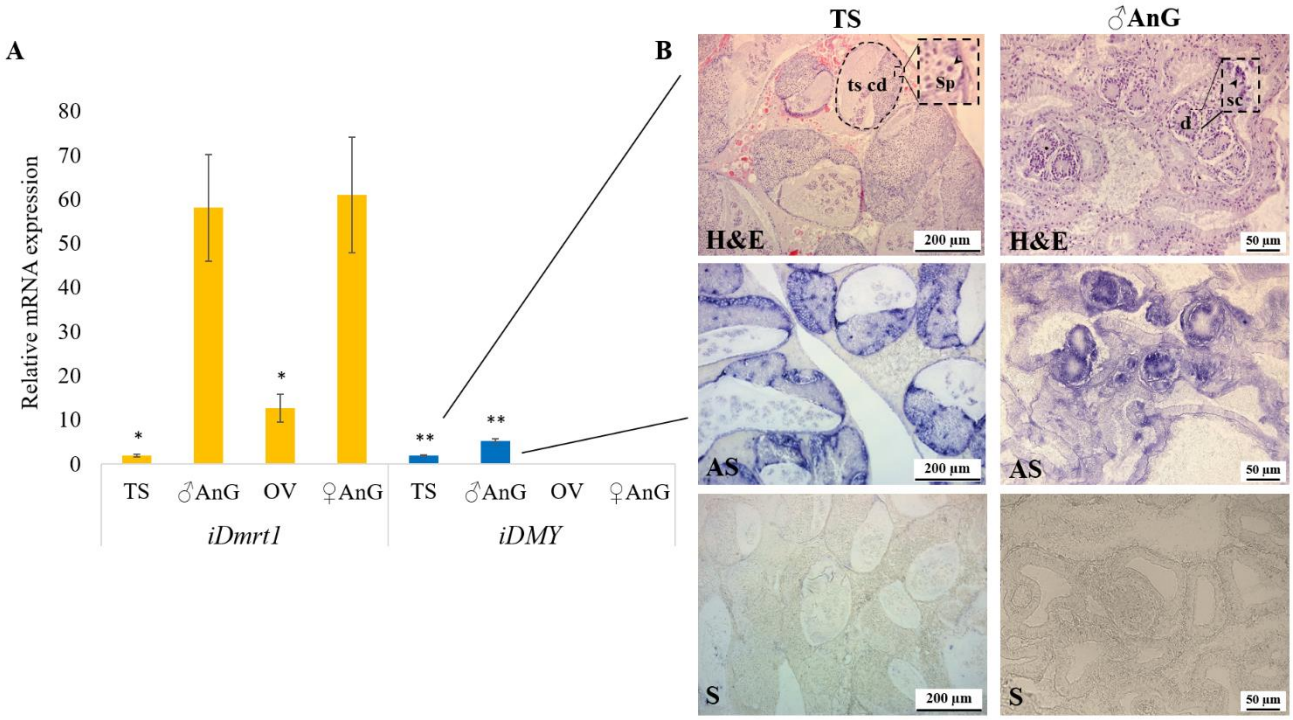

Fig. 3. Focal expression analyses of *S. verreauxi iDmrt1* and *iDMY* in sexually immature tissues. (*A*) qPCR quantified from male and female gonads (TS and OV) and antennal glands (AnG); data represent the mean ± SEM, N= 8. Significant differences ($P < 0.01$) are indicated between: *iDmrt1* OV >TS (*); *iDMY* ♂AnG > TS (**). (*B*) *In situ* hybridisation of *iDMY* on testis (TS) and male antennal gland (♂AnG). H&E shows hematoxylin and eosin staining, specific features are indicated: testicular cords (ts cd) and spermatogonia (Sp) in TS and secretory-like cells (sc) encapsulating central ducts (d) in AnG; AS corresponds to the anti-sense probe; S to the sense probe. Scale bars indicated.

Fig. 4.

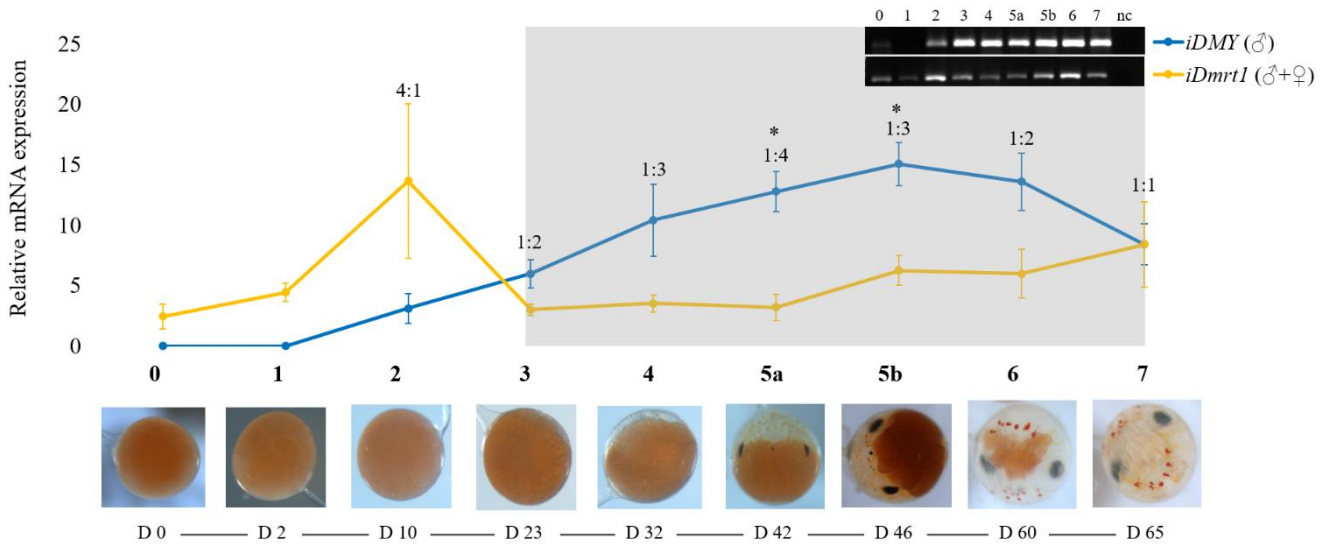

Fig. 4. *S. verreauxi iDmrt1* and *iDMY* during embryogenesis indicating the putative sex determining period. qPCR quantified from embryo samples; data represent the mean ± SEM, N= 5; supporting PCR gel image in the top right. Time course from fertilisation on Day 0 (D0) to hatching on Day 65 (D65) as defined in Fig. S3. The ratio of *iDmrt1*: *iDMY* expression is indicated at each stage and the putative male sex determining period boxed in grey; stages at which *iDMY* > *iDmrt1* (*$P < 0.01$) are indicated.

Fig. 5.

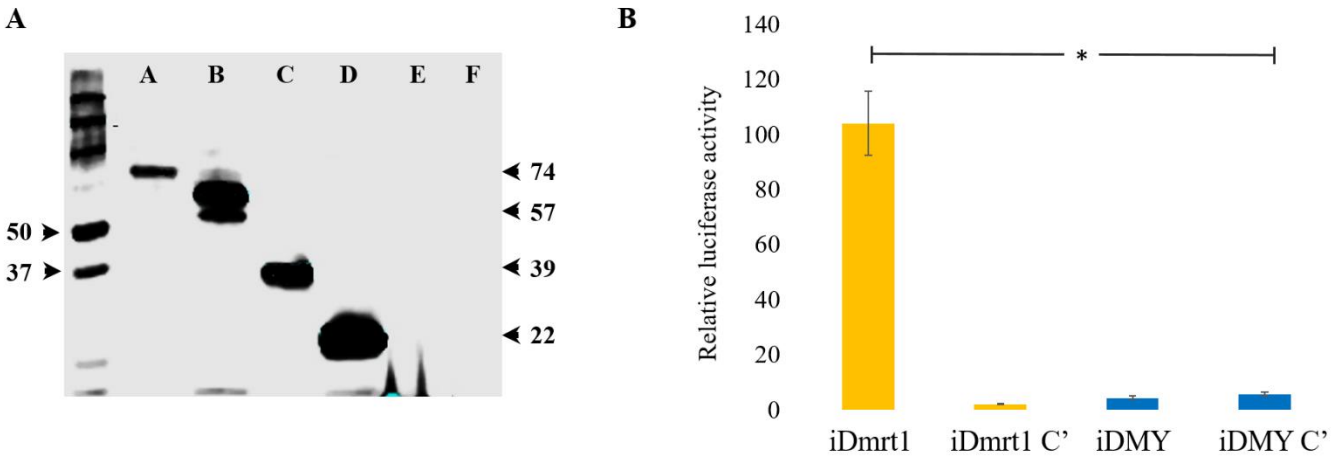

Fig. 5. Proof of concept GAL4 transactivation assay: (*A*) Cell lysate from transfected COS-7 cells demonstrating *in vitro* production of GAL4-fusion proteins, indicated by arrow heads, lanes as follows: A) iDmrt1, expected size of 74KDa; B) iDmrt1 C', 57KDa; C) iDMY, 39KDa; D) iDMY C', 22KDa; E) reporter and empty vector; and F) untransfected cells. (*B*) Relative luciferase activity of transfected COS-7 cells with 20ng of each GAL4-fusion protein. Activities shown as fold increase normalised against *Renilla*. The data represent the mean ± SEM from three independent experiments. (*$P < 0.01$).

Fig. 6.

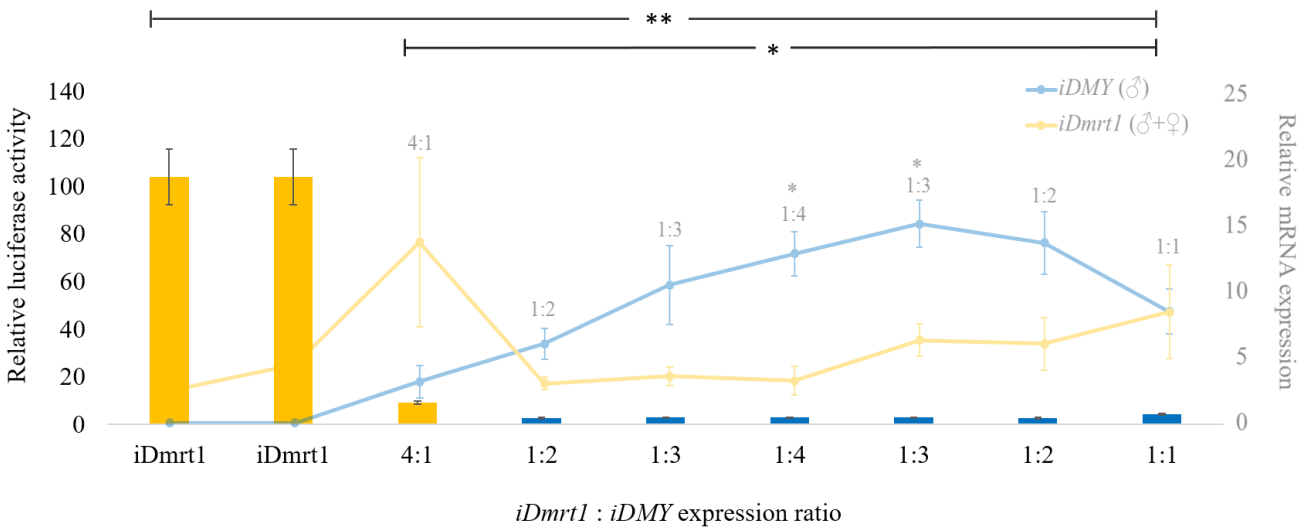

Fig. 6. GAL4 transactivation assay guided by embryonic expression of *iDmrt1: iDMY*. Relative luciferase activities of transfected COS-7 cells with 20ng total *iDmrt1: iDMY*, ratios guided by the expression quantified through qPCR analyses. Activities shown as fold increase normalised against *Renilla*. The data represent the mean ± SEM from three separate experiments, significant differences ($P < 0.01$) are indicated between: *iDmrt1* > all (*) and 4:1 > 1:1, 1:2, 1:3, 1:4 (**).

Supplementary figures and legends

Fig. S1.

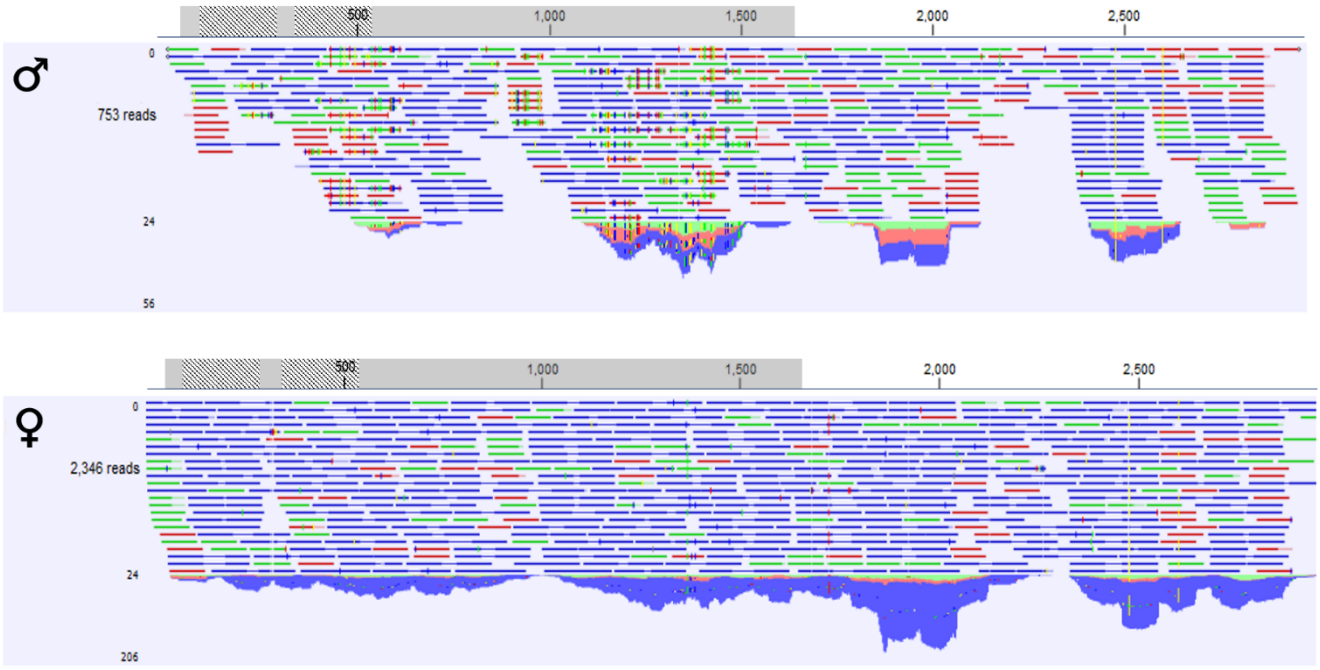

Fig. S1. Evidence of male sex-specific SNP pattern in *Sv-iDmrt1* transcript demonstrated by mapping sex-specific FASTQ-libraries. Sex-specific libraries were comprised of pooled male (♂) and female (♀) brain, eyestalk, gonad and antennal gland and were mapped against the *iDmrt1* transcript. The mapping profile shows distinct regions of nucleotide variance amongst male reads, each nucleotide that differs from the consensus is depicted as a vertical dash across the horizontal read, primarily occurring between 480-590nt and 900-1500nt. These variants tend to occur in ~50% of reads, suggesting that two distinct read-sets are mapping to the *iDmrt1* transcript in males; a lower depth of coverage amongst male libraries is also apparent. Taken together this provides strong indication of a second male-specific *iDmrt1* variant. Grey boxes indicate the open reading frame, corresponding to 55-1657nt and the hashed regions indicate the positioning of the DM-domains occurring at 100-256nt and 361-523nt.

Fig. S2*A*

A

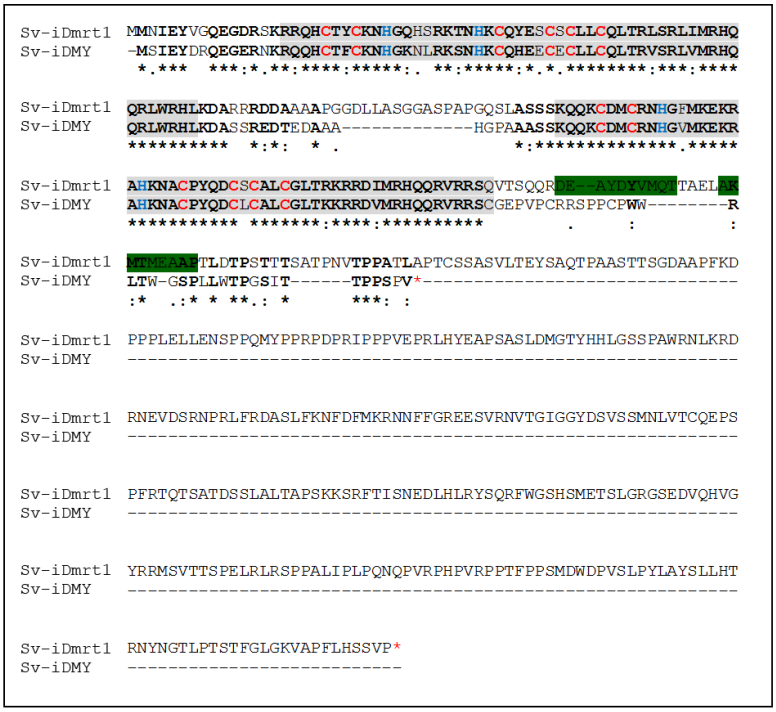

Fig. S2*B*

B

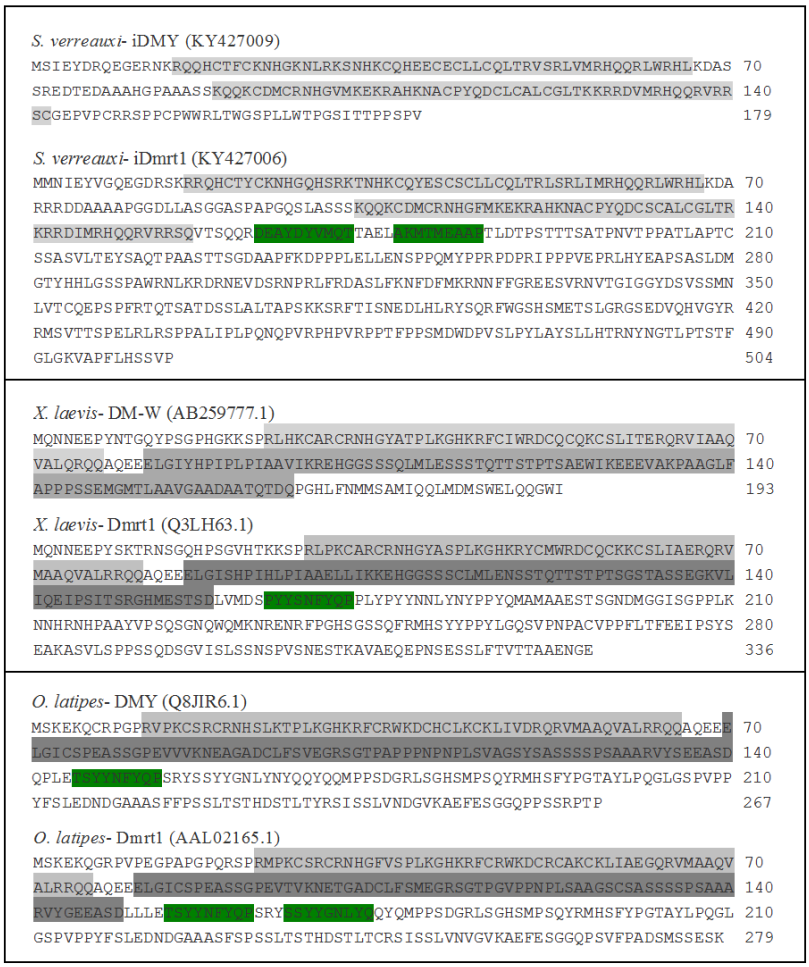

Fig. S2*C*

Fig. S2. (*A*) Sequence alignment of Sv-iDmrt1 and Sv-iDMY. DM-domains boxed in grey, highlighting the six conserved cysteines (in red) and two histidines (in blue). The predicted transactivation domains of iDmrt1 are boxed in green. An asterisk (*) indicates positions which have a fully conserved residue; a semi-colon (:) indicates conservation between amino acids with strongly similar properties (both of which are highlighted in bold); and a full stop (.) indicates conservation between groups with weakly similar properties. The stop codon is indicated by a red asterisk *. (*B*) Sequence summary of the three sex-linked Dmrts and their autosomal paralogues, highlighting the presence or absence of a C' TAD. DM-domains are boxed in light grey, the specialised vertebrate Dmrt1-domains in dark grey and the predicted TADs in green. (*C*) Same for the male and female splice variants of Dsx in *Drosophila*, with the addition of the dimerization domains underlined in red and in the case of Dsx^F the residues described (47) to be critical for the female-specific binding with the coactiavtor IX, boxed in yellow; note TAD predictions were the same across all Dsx^M and Dsx^F isoforms.

Fig. S3.

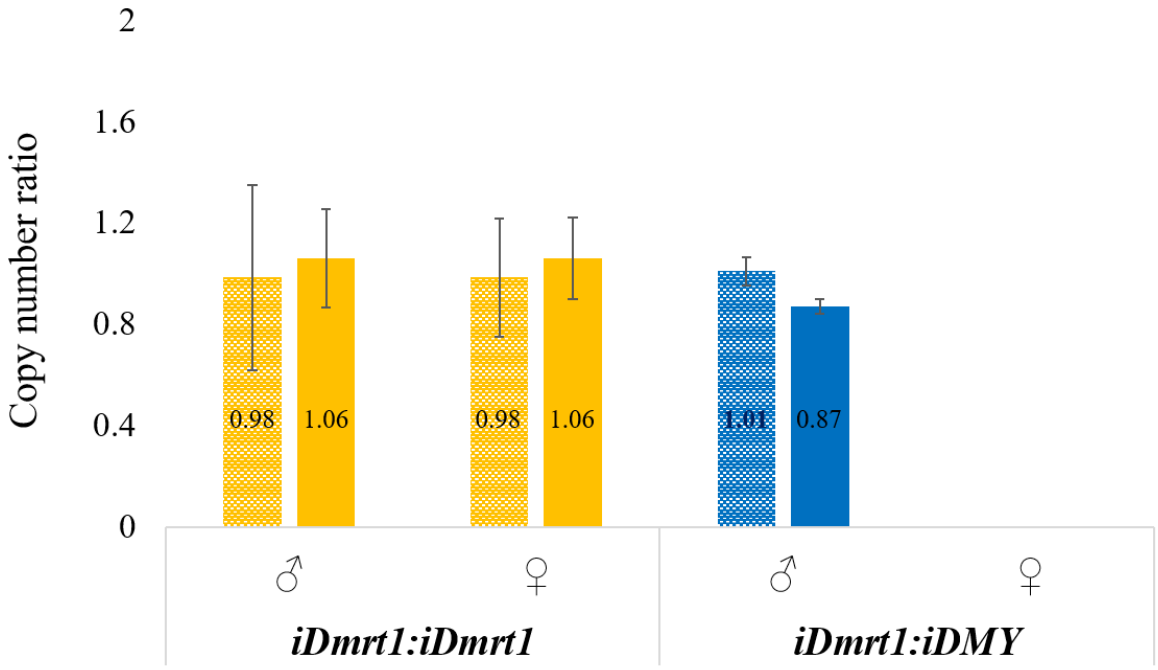

Fig. S3. Gene copy-number ratio of *iDmrt1* and *iDMY*. The comparison of *iDmrt1* gene copy number in ♂ and ♀ gDNA, ratios quantified using RT-qPCR. The data represent the mean ±SEM, N= 12, patterned bars represent absolute quantification and solid bars relative quantification.

Fig S4.

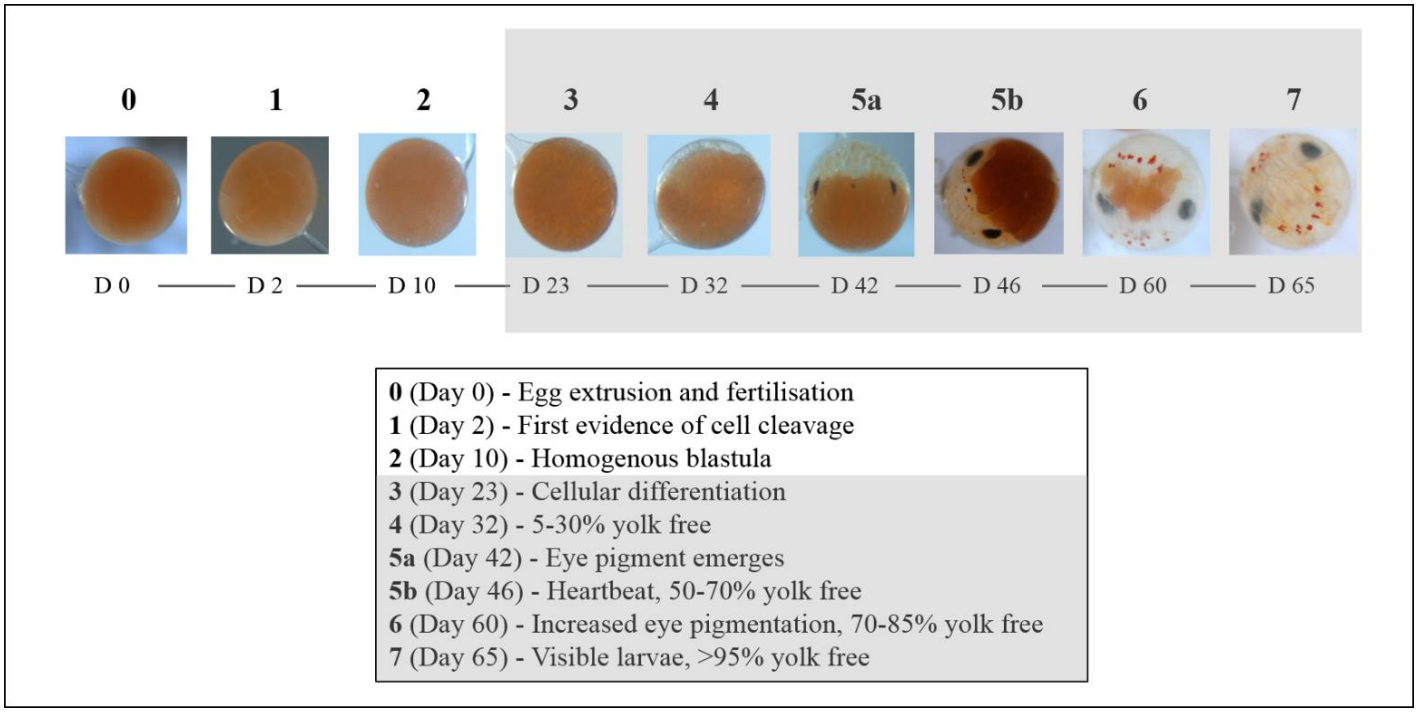

Fig. S4. Classification of embryogenesis in *S. verreauxi* (related to Fig. 4). Representative images of embryogenesis in *S. verreauxi*, which we have shown occurs over 65 days in culture, starting from egg extrusion and external fertilisation on Day 0 (Stage 0). By Day 2 cell cleavage is apparent (Stage 1) and within 10 days a homogeneous blastula exists (Stage 2). The first evidence of cellular differentiation can be seen by Day 23 (Stage 3) and as cellular differentiation continues, the yolk-sac reduces, so by Day 32 the embryo is 5-30% yolk-free (Stage 4). By Day 42 eye pigmentation is visibly apparent (signifying the differentiation and establishment of the neuroendocrine system) (Stage 5b) shortly followed by the emergence of the heartbeat by Day 46 (Stage 5b). By Day 60 the majority of the embryo is lacking yolk (>70%) and the development of the eyestalk is more pronounced (Stage 6). At Day 65 the yolk is near diminished and the visible larvae is ready to hatch (Stage 7). The period of putative male sex determination is boxed in grey.

Fig. S5.

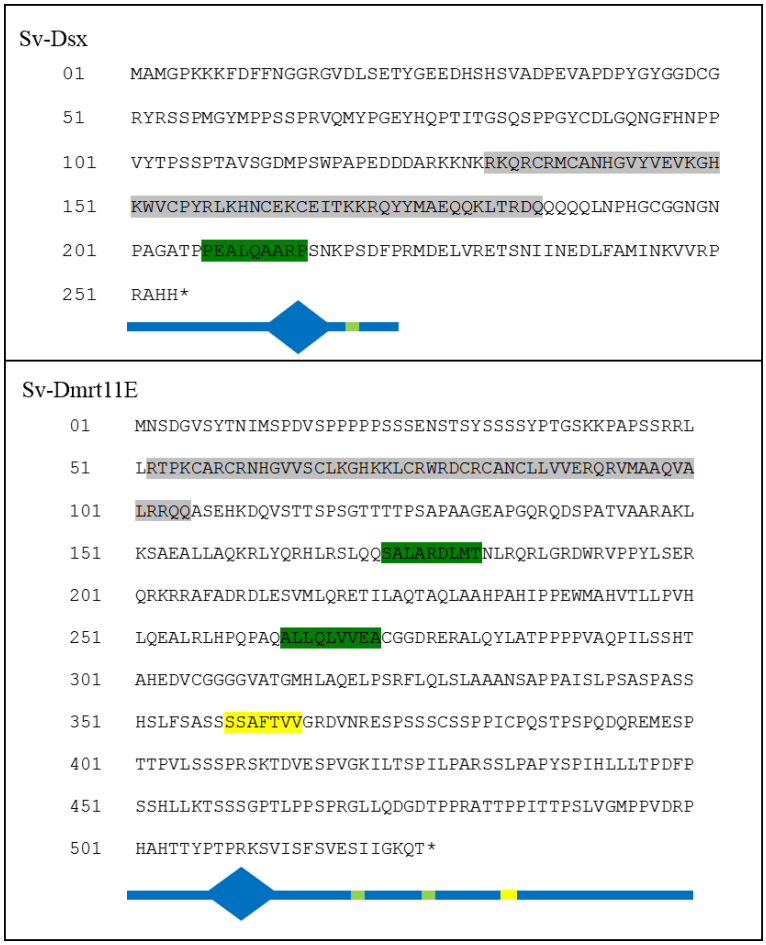

Fig. S5. Amino acid sequence of additional Dmrts identified in *S. verreauxi* (*A*) Sv-Dsx and (*B*) Sv-Dmrt11E. In both cases the DM-domains are boxed in grey in text and represented by the diamonds in the scaled illustration. Transactivation domains are highlighted in green in both text and illustration. Sv-Dmrt11E is the only sequence to contain the DMA-linked "SSAFTVV" motif (13) which is highlighted in yellow (although no DMA domain is present).

Fig. S6.

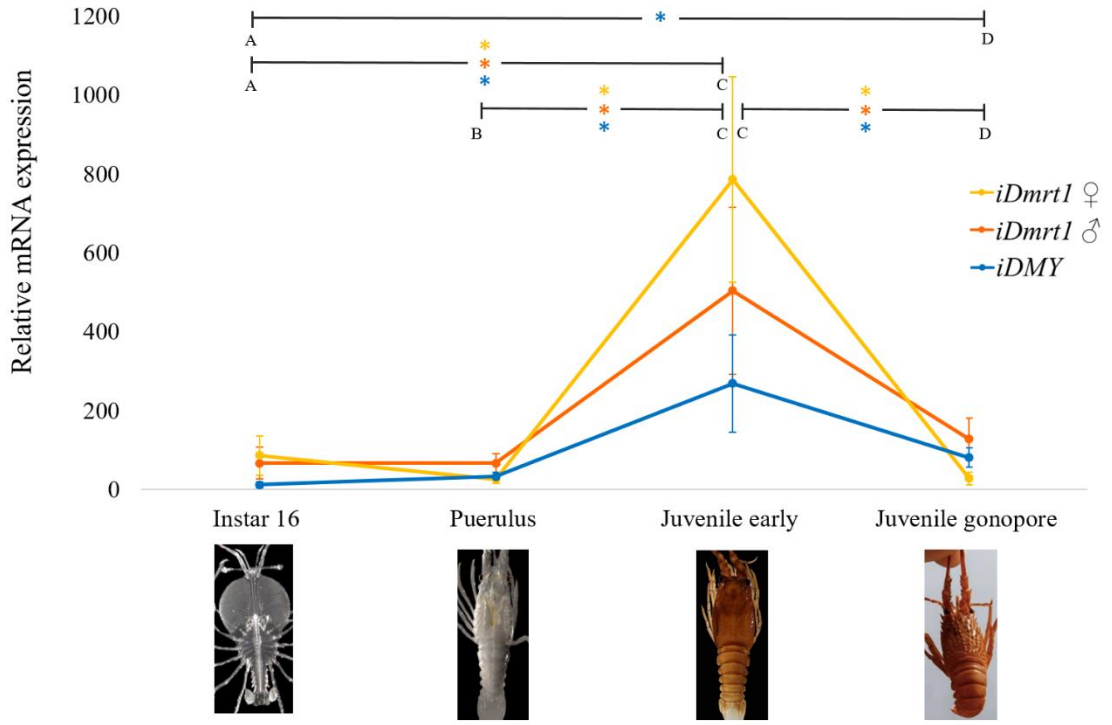

Fig. S6. qPCR quantified from sexed male and females from defined developmental stages preceding the first evidence of sexual differentiation, seen in the emergence of the gonopores at the so named "Juvenile gonopore" stage; representative images shown. The data represent the mean ±SEM, N=5. Statistical differences (*$P < 0.05$) of ♀ *iDmrt1*, ♂ *iDmrt1* and *iDMY* at each stage are noted: namely expression in juvenile early (C) > instar 16 (A), puerulus (B) and juvenile gonopore (D) with all ♀ *iDmrt1*, ♂ *iDmrt1* and *iDMY* and also juvenile gonopore (D) > instar 16 (A) in the case of *iDMY*. Thus a significant peak in ♀ *iDmrt1* expression and ♂ *iDmrt1* and *iDMY* expression is evident, specific to the developmental stage just prior to the first phonotypic evidence of primary sexual differentiation.