The decapod researchers’ guide to the galaxy of sex determination

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Abstract
Sex determination systems in Animalia encompass a diverse array of genes, functioning in complex regulatory networks. This diversity is even pronounced within taxonomic ranks and the crustacean Order Decapoda is no exception. The commercial importance of the decapods and the ambition to develop their potential in aquaculture has resulted in the necessity to better understand the processes of sexual development. However, due to a lagging understanding of the regulation of sex determination, systems characterised in other model species often serve as the basis for these investigations. This work presents a collated summary of the current information of sex determination in Decapoda, including all determined chromosomal mechanisms and identified “sex-regulator” homologues, often focussing on genes characterised in the model arthropod Drosophila melanogaster (namely Sxl, Tra, Tra-2, Fru and Dsx), the nematode Caenorhabditis elegans (Fem-1 and Mab-3) and Mammalia (Sry, Sox9, Foxl2 and Dmrt1). Although homologue analyses such as these offer a good method to guide investigations in non-model species, the low conservation and variability of sex determination systems cautions against the assumption of conserved functionality. Thus, we propose a better suited approach to guide studies into sex determination in Decapoda, primarily relating to the functionally conserved sex-regulators, the Dmrt1.

Keywords: Sex determination; Sex chromosome; Malacostraca; Decapoda; Isopoda; Double-sex and mab-3 related transcription factor (Dmrt)
Sexual differentiation in Malacostraca

The integrated signalling cascades of sexual development make it difficult to clearly distinguish the processes of sex determination from sexual differentiation (Matson and Zarkower, 2012). However, in the decapods (Crustacea, Malacostraca), as members of the Malacostraca, the onset of male sexual differentiation is more clearly defined, due to the unique involvement of a male specific endocrine gland known as the androgenic gland (AG).

First characterised in the sister Order, Amphipoda (Crustacea, Malacostraca), the AG was determined to be the sole regulator of male primary and secondary sexual differentiation (Charniaux-Cotton, 1954). It was then in a second sister Order, Isopoda, that the specific regulatory hormone was first isolated, defined as an insulin-like peptide and so termed the insulin-like androgenic gland hormone (AGH) (Hasegawa et al., 1987; Martin et al., 1999; Okuno et al., 1999b; Suzuki, 1999).

As a sex-differentiating factor shared by all malacostracans, an understanding of the regulation of male sexual differentiation in Decapoda soon followed, now known to be regulated through a unique developmental axis known as the X-organ-sinus-gland neuroendocrine complex (XO-SG) – AG – Testis axis. In brief, the XO-SG (located in the eyestalk) secretes an array of neuropeptides including the gonad inhibiting hormone (GIH) (also known as vitellogenesis inhibiting hormone, in females) and molt inhibiting hormone (MIH) (Nagaraju, 2011; Rodríguez et al., 2007), both belonging to the crustacean hyperglycaemic hormone (CHH) family. In males, these neurohormones do not act directly on the gonad but instead modulate the proliferation of the AG; in the absence of their inhibitory signal, the AG develops bilaterally on each of the posterior sections of the sperm ducts (Charniaux-Cotton, 1954; Charniaux-Cotton, 1958; Charniaux-Cotton et al., 1966; Sagi et al., 1997). Once established, the male-specific AG is solely responsible for the synthesis and secretion of the insulin-like hormone, named IAG in the decapods, which in turn stimulates both testicular differentiation (Rodríguez et al., 2007) and the broad tissue effects of male sexual dimorphism through to full reproductive capacity (Manor et al., 2007; Martin et al., 1999; Okuno et al., 1999a; Rosen et al., 2010; Ventura et al., 2009; reviewed in Ventura et al. (2011a)).
The resulting sexual systems

Although the male sex-differentiating influence of IAG is a unifying feature of malacostracans, the hormone’s functional interaction with the broader networks of sexual development is not so conserved. While most decapods are gonochoristic, meaning that they develop into one distinct sex, multiple sexual systems have been described. First, there is a prevalence of complex gonochoristic species, where, although sex is genetically determined by a distinct genotype, simultaneous intersex (both male and female) phenotypes occur. This is particularly pronounced in certain Infraorders, such as the Astacidea (Grilo and Rosa, 2017); an example being the red-claw crayfish (Sagi et al., 1996), which can present as one of seven distinct intersex phenotypes, all of which develop from a ZW (female) genotype (Parnes et al., 2003).

There are also examples of protandrous hermaphroditic species, where individuals are born as males and later develop into females. This appears to be common in the Caridea, such as the Peppermint shrimp (Lysmata wurdemanni) (Bortolini and Bauer, 2016; Zhang et al., 2017) and is a process regulated through the sex differentiating effects of IAG (Zhang et al., 2017). Such hermaphroditism has also been documented in the Thalassinidae and Anomura (Subramoniam, 2017). To complicate things further, the shrimp species, L. wurdemanni, can also exist as a simultaneous hermaphrodite, where an individual can function as a male and a female at any one time (a protandrous simultaneous hermaphrodite) (Bauer and Holt, 1998).

More staggeringly, the Order also contains one documented parthenogenic species. The marbled crayfish (Procambarus fallax forma virginalis), only exists as female, of which all offspring is genetically identical to the mother and therefore, female (Scholtz et al., 2003; Vogt et al., 2015). Finally, to truly demonstrate the plasticity of the IAG-mediated system, one should consider the sister Order, Isopoda, where the influence of the AG is superseded by the feminisation effects of the bacterial endosymbiont, Wolbachia. This parasitism is thought to prevent the initial differentiation of the male-specific AG (Bouchon et al., 2008; Cordaux et al., 2011; Rigaud et al., 1997), allowing for female development. So dramatic are these AG-disruptive effects, not only do they result in total feminisation of ZZ males but ultimately, the loss and re-emergence of the W chromosome (Cordaux and Gilbert, 2017; Leclercq et al., 2016).
How is this diversity in sexual systems achieved?

Based on our understanding of conserved mode of sexual differentiation amongst these species, as well as the resulting diversity of sexual systems, the regulatory axis of the malacostracan AG, although ubiquitous, is certainly labile. This suggests that the preceding regulation of sex determination, which serves as the foundations of sexual development within each species, may be that responsible for the diversity of sexual systems observed. Sex determination mechanisms are known to be highly divergent across Animalia, even across closely related species, particularly amongst those genes acting at the very top of the genetic cascades, the master sex-determinants (Bachtrog et al., 2014). This variability is owing to the relaxed evolutionary restraint on sex determination genes (Meiklejohn et al., 2003; Parsch and Ellegren, 2013), readily apparent in the increased rates of several fundamental evolutionary phenomena: (1) Gene duplication and neofunctionalization: examples are found in the master sex-determinants of medaka (Oryzias latipes) (Matsuda et al., 2002; Nanda et al., 2002) and the African clawed frog (Xenopus laevis) (Yoshimoto et al., 2008), both of which have evolved through the sex-specific gene duplication of autosomal Dmrt1 genes. Neofunctionalization can result in the recruitment of entirely unrelated genes, such as the immune related gene duplicate SdY, acting as the master sex-determinant in rainbow trout (Yano et al., 2012) and more staggeringly still, the Wolbachia bacterial element gained through horizontal gene transfer to become the master female sex-determinant in the common pill bug (Armadillidium vulgare) (Leclercq et al., 2016). (2) Hierarchical rearrangement: resulting in loss of function between seemingly well conserved genes across species, such as the master sex-determinant Sxl in Drosophila melanogaster, a gene which has been expelled from the master regulatory role in the fly species Musca domestica and Ceratitis capitata (Meise et al., 1998), where it actually lacks any role in sex determination. (3) The increased occurrence of functional mutations: like the single nucleotide mutation in the male pufferfish (Takifugu rubripes) anti-Müllerian hormone receptor (Amhr2), a polymorphism which now acts as the master male sex-determining signal (Kamiya et al., 2012). (4) Altered translational and temporal gene expression patterns (Bachtrog et al., 2014; Beukeboom and Perrin, 2014) and (5) epigenetic effects (Piferrer, 2013). These factors culminate to make the characterisation of each sex determination systems highly cryptic.

Thus, considering the diversity of sexual systems amongst Decapoda and the apparent plasticity of IAG endocrinology, it is apparent that to fully understand the regulation of sexual
development, we must integrate the genetic networks that serve as the basis of each system. Furthermore (knowing the evolutionary characteristics and diversification of sex determination genes) to gain an accurate understanding of this highly complex regulatory process, this characterisation must be tackled on a species by species basis. In response, this review has collated the current research findings regarding the molecular basis of sex determination in Decapoda, in attempt to serve both as a reference for preliminary investigations and to provide a critical evaluation of the current data. To provide evolutionary context, we have included the sister Order Isopoda (Crustacea, Malacostraca), which shares the commonality of the AG and the model crustacean species Daphnia pulex and D. magna (Crustacea, Branchiopoda) from the sister Class Branchiopoda. It is thought that Insecta evolved from the freshwater branchiopod crustaceans around 410 MYA (Glenner et al., 2006). Hence the Daphnia sp. are included as a phylogenetic link between the decapods and the insects (Hexapoda, Insecta), from which a significant proportion of the model sex determination genes have been characterised.

The genetic (chromosomal) modes of sex determination characterised to date are presented, followed by a list of candidate genes implicated in sex determination in Decapoda. These genes were primarily identified through transcriptomic homologue screening and therefore the list is heavily biased by systems characterised in the model species D. melanogaster, Caenorhabditis elegans and Mammalia. Given the high evolutionary rates of sex determination systems, this review highlights the significance of functional genomics to ensure that genes are assigned with appropriate functions. To reiterate such, we have included a brief functional description of the genes most commonly targeted for homologue analyses. It is of note that there is only a single gene family common to all three model systems: namely Drosophila- Dsx, C. elegans-Mab-3 and Mammalia- Dmrt1 (denoted with an * in Table 2). These genes are from the DM domain transcription factor family, collectively known as the doublesex and male abnormal 3-related transcription factors (Dmrts).
Functional definitions of sex-regulator genes in model species

1. The fruit fly, *Drosophila melanogaster* (Arthropoda, Insecta)

**Sex lethal: pre-mRNA splicing protein**

Sex lethal (Sxl) is a splicing factor, defined by an RNA-binding domain. This gene is responsive to the higher X: Autosome chromosome ratio in females. The X chromosome gene products (e.g. Runt, Sisterless-A and Sisterless-B) bind to the Sxl promoter and induce its activation. Thus, Sxl is the master sex-determinant for female development. Its primary target is *Tra* mRNA (Beukeboom and Perrin, 2014; Hashiyama et al., 2011).

**Transformer: pre-mRNA splicing protein**

Transformer (Tra) is an mRNA splicing factor, defined by an RNA-recognition motif and an arginine/serine rich domain followed by a proline rich region. The action of Sxl results in the active splice variant of *Tra* in females. Tra then acts as a downstream splicing factor, regulating female-specific splicing of target RNAs (Kulathinal et al., 2003).

**Transformer-2: pre-mRNA splicing protein**

Transformer-2 (Tra-2) is an mRNA splicing factor, defined by an RNA-recognition motif and arginine/serine rich domain. Tra-2 is also a downstream splicing factor, which is constitutively produced (in both males and females) but only in females can it act in concert with Tra to regulate female-specific splicing of target RNAs. A primary target of Tra and Tra-2 is *Dsx* mRNA (Amrein et al., 1990).

**Doublesex: DM domain, zinc finger protein (transcription factor)**

Doublesex (Dsx) is a transcription factor defined by a zinc finger domain, termed the DM domain. In the presence of the Tra – Tra-2 complex, the female splice-variant of *Dsx* is produced, generating the Dsx<sup>F</sup> protein. Dsx<sup>F</sup> works in concert with an array of regulatory genes as the major effector of sexual development ((Beukeboom and Perrin, 2014; Hoshijima et al., 1991).

**Fruitless: Zinc finger protein (transcription factor)**

Fruitless (Fru) is a transcription factor defined by its zinc fingers. *Fru* is a male promoting gene, regulating development of the male central nervous system and male sexual behaviour.
Fru is spliced in the absence of the female Tra–Tra-2 complex, carried out by non sex-specific splicing machinery (Billeter et al., 2006).

2. The nematode, Caenorhabditis elegans (Nematoda, Chromadorea)

**Feminization of XX and XO animals-1: Ankyrin repeat containing protein**

Feminization of XX and XO animals-1 (Fem-1) is a second messenger protein, defined by ankyrin-repeats. It acts in male sex determination, as a signal-transducing regulator between the membrane receptor Tra-2 (not to be confused with the mRNA splicing factor, Tra-2 in Drosophila) and the transcription factor Tra-1. It works in concert with Fem-2 and Fem-3 (Haag, 2005; Yi et al., 2000).

**Male Abnormal 3: DM domain, zinc finger protein (transcription factor)**

Male Abnormal 3 (Mab-3) is a transcription factor defined by a novel zinc finger domain, termed the DM domain. In males, Her-1 binds the Tra-2 receptor, releasing Fem-1,2,3 which then sequester Tra-1. This in turn activates the major effector Mab-3. Mab-3 is vital for the male sexual differentiation of the peripheral nervous system and the intestine, causing the repression of vitellogenesis (Haag, 2005; Yi et al., 2000).

3. Mammals (Chordata, Mammalia)

**Sex-determining region Y: HMG-box DNA-binding protein (transcription factor)**

Sex-determining region Y (Sry) is the master sex-determinant in males. Sry, along with SF-1, binds and activates Sox9. Its function is transient, after its activation of Sox9, other major effector genes are responsible for the continuity of its signal (Matson et al., 2011; Sinclair et al., 1990).

**Sex-determining region Y-box 9: HMG-box DNA-binding protein (transcription factor)**

Sex-determining region Y-box 9 (Sox9) is a transcription factor defined by its Sry-related HMG-box. Once activated by Sry it is responsible for upregulating the expression of the male promoting Dmrt1 via Fgf9. These genes then function in an auto-regulating loop, maintaining their own expression, stimulating (and maintaining) the male-specific programme of development (Jakob and Lovell-Badge, 2011; Matson et al., 2011).

**Doublesex and male abnormal-3 related transcription factor 1: DM domain, zinc finger protein (transcription factor)**
Doublesex and male abnormal-3 related transcription factor 1 (Dmrt1) is a transcription factor defined by a novel zinc finger domain, termed the DM domain. It is upregulated in response to Sox9 via Fgf9. Dmrt1 works in concert with an array of regulatory genes as the major effector of male sexual differentiation, specifically testicular development and maintenance. It also acts to inhibit the female developmental pathway (e.g. by suppressing Foxl2) (Matson et al., 2011).

**Forkhead box L2: Forkhead box DNA-binding protein (transcription factor)**

Forkhead box L2 (Foxl2) is a transcription factor that is defined by a unique DNA-binding domain. In the absence of the Sry driven expression of Sox9, Foxl2 expression ensues and acts to inhibit the male pathway, whilst promoting the female pathway through the action of Rspo1, Wnt4 and B-catenin (Matson et al., 2011).
Table 1) Modes of sex determination in *Brachiopoda, Isopoda and Decapoda*. Unless otherwise indicated modes are genetic.

Table 2) Sex-regulator homologues identified in *Decapoda*, based on model systems characterised in *Drosophila melanogaster, Caenorhabditis elegans* and *Mammalia*; (p) denotes partial sequences and (?) indicates lack of conclusive data. In cases where the authors have arbitrarily named *Dmrt* genes but we have shown they fall into a given clade, the clade name is given after in brackets. Authors’ conclusions are highlighted in bold.
Acting at the top of the cascade: the sex chromosomes

The summary presented in Table 1 provides clear demonstration of the diversity of sex determination modes present in *Crustacea*. Both *D. pulex* (Chen et al., 2014; Crease et al., 1989) and *D. magna* (Kato et al., 2011; Kleiven et al., 1992) are subject to environmental sex determination, through cyclic parthenogenesis. This contrasts to the decapods where, thus far, genetic sex determination is common across the Order, although both XY/XX (heterogametic male) and ZW/ZZ (homogametic male) mechanisms exist sporadically across families (Chandler et al., 2016b). In *Isopoda*, male homogamety (ZZ) seems to be more common than male heterogamety (XY) (Becking et al., 2017). There is also strong evidence for frequent sex chromosome turnover, with an estimated three to thirteen heterogametic transitions occurring, accounting for the dramatic XY and ZW diversity observed within *Genera* (Becking et al., 2017).

This suggests that the disruptive effects of *Wolbachia* have had significant consequence on the evolution of sex determination mechanisms in isopods. Furthermore, the dramatic influence of *Wolbachia* has given rise to a third, distinct mode of sex determination: cytoplasmic. Genetic and environmental (including social) modes of sex determination are both extensively characterised across species (Beukeboom and Perrin, 2014), but cytoplasmic sex determination appears to be specific to the *Crustacea*, resulting from the feminising effect of parasites (Rigaud et al., 1997). Hence, this mode of inheritance described in the isopods, accounts for a third mode of sex determination in the already diverse regulation of sexual development in *Crustacea*.

One unifying factor shared by the malacostracans, is the potential for sex to be manipulated, whether by parasitic infection, acting to prevent the differentiation of the AG in males (*Isopoda*) (Bouchon et al., 2008; Cordaux et al., 2011; Rigaud et al., 1997), or the RNAi-induced prevention of IAG expression (*Decapoda*) (Ventura et al., 2012); both causing fully functional feminisation. Taken together, this suggests that both sexes, irrelevant of their genetic background, have the genetic potential to develop as male or female, suggesting that the sex chromosomes must be predominantly homologous. This is further supported by the viability of WW females and YY males observed in both isopods (Becking et al., 2017; Rigaud et al., 1997) and decapods (Shpak et al., 2016), therefore suggesting that the sex chromosomes of these Orders are in the primary stages of differentiation (Charlesworth, 1991; Rigaud et al., 1997).
perhaps only differing in the one master sex-determining gene responsible for flipping the male/female switch.

The mechanism: evaluating the role of sex-regulating genes

The data presented in Table 2 clearly illustrates how transcriptomics (and genomics) have greatly advanced the identification of new gene families in the decapods. From an evolutionary perspective, this has enhanced our understanding of the divergence of these genes both within the Order and across Animalia. However, with regards to the functional context of sexual development, some of these data must be interpreted with a critical understanding of the evolutionary rates that define sex-determining genes.

The Tra homologues provide an apt example: the Tra genes are known to be highly divergent in sequence even amongst Drosophila sp. (Kulathinal et al., 2003); in other non-Drosophilid fly species such as the housefly (M. domestica) (Hediger et al., 2010) and medfly (C. capitata) (Pane et al., 2002) the Tra orthologues show functional divergence, having evolved up the hierarchy (expelling Sxl) to adopt the master sex-determining role; and in the distantly related decapods, thus far Tra homologues appear to be lacking.

There is some level of conservation within Insecta, as a functional Tra orthologue (termed Feminiser, Fem) also acts as the female master sex-determinant in the honey bee (Apis mellifera) (Hasselmann et al., 2008). Fem lacks complete conservation of the sequence motifs described in Drosophila but does contain the same arginine/serine and proline-rich domain organisation and one conserved sequence motif, also described in C. capitata (Pane et al., 2002). Although diverged in both sequence and function across these three species (telling of significant independent evolution over the ~300 million years that separates the Orders) the conserved role of the three orthologues is indicative of a Tra-based ancestral pathway of sex determination in Insecta (Hasselmann et al., 2008). Furthermore, the discovery of Fem suggests that the complementary sex determiner (csd) gene may not be the universal master sex-determinant across hymenopteran insects (bees, ants and wasps), as was previously thought (Hasselmann et al., 2008; Heimpel and Boer, 2008).

It is interesting to note that the brachiopods D. pulex (Chen et al., 2014) and D. magna (Kato et al., 2010) do have Tra homologues, however both show even greater divergence in domain
organisation from that of *Drosophila* *Tra*. Furthermore, it is only in *D. magna* that a function in sexual development has been suggested, not in the female sex determination pathway (as with the insect sp.), but in the maintenance of male phenotype (Chen et al., 2014). This evolutionary emergence and divergence of *Tra* is in support of the proposed decapod–branchiopod trajectory of the *Pancrustacea* (Glenner et al., 2006). A similar story is true of the highly conserved *Sxl* splice variants, which although seemingly well conserved in sequence throughout *Insecta* and *Decapoda* (Table 2), have not retained conserved functionality. These evolutionary patterns exemplify why the functional conservation of the genes summarised in this work need to be appropriately evaluated; a conserved function to that described in each model species should not be assumed *a priori*.

### DmRTs: the functionally conserved sex-regulators

There is however, a gene family that does display the functional conservation that appears to be somewhat assumed in other cases: the DmRTs. Indeed, the DmRTs are the only gene family with a conserved function in sex determination across *Animalia* (Beukeboom and Perrin, 2014; Kopp, 2012), being identified in all investigated species to date, with the only exception being the sponge *Amphimedon queenslandica* (Wexler et al., 2014) (which may well reflect its lack of tissue differentiation). There has been good progress in the identification of DmRTs in the decapods, with homologues identified in seven species comprising the *Penaeidae* (prawns), *Palinuridae* (lobsters), *Palaemonidae* (shrimp) and the *Portunidae* (crab). Although from our data collation, they appear yet to be identified in the isopods. Indeed, this work may suggest that the characterisation of the DmRTs in Isopoda is a promising avenue of further study regarding the mechanism by which the *f* element disrupts the native pathway of sexual development.

The functional conservation of the model genes characterised in *D. melanogaster* (*Dsx*), *C. elegans* (*Mab-3*) and *Mammalia* (*Dmrt1*) is readily illustrated in *D. magna*. In this branchiopod, a Dmrt homologue (*Dsx1*) was determined to be the master male sex-determinant (Kato et al., 2011). It is of note however, that four additional DmRTs were also identified, *Dmrt11E, Dmrt93B, Dmrt99B* (Kato et al., 2008) and *Dsx2* (Kato et al., 2011) and determined to have no clear function in sexual development. This highlights the critical importance of the functional analyses to reliably determine the explicit regulatory role of Dapma-Dsx1. The functional conservation of the DmRTs continues in the decapods, as recently a Y-chromosome-
linked *Dmrt* was identified in the Eastern spiny lobster (*S. verreauxi*) (Chandler et al., 2017). This gene, termed *Sv-iDMY*, offers the third example of a sex-linked *Dmrt* across *Animalia* (joining medaka DMY and frog DM-W) and is the first example in an invertebrate. As is the case in both medaka and frog, our functional analyses suggest that *Sv-iDMY* has evolved as the master sex-determinant in this decapod species (Chandler et al., 2017).

To better illustrate the evolutionary and mechanistic features of the Dmrt family, Figure 1 presents the phylogeny of all the available decapod Dmrt sequences, as well as those from *D. pulex* and *D. magna* and the model Dsx, Mab-3 and Dmrt1. Figure 1A shows the full protein sequence, highlighting the clear clustering of Dmrt subclasses, but more significantly, the pronounced variation in domain organisation outside of the DM domain itself. In contrast, Figure 1B shows the phylogeny of the DM domains only, emphasising the extreme conservation of the DNA-binding domain that defines the family. Taken together, Figure 1 demonstrates how the mechanistic diversity of this family is achieved: routed in the sequence and domain variation outside of the DM domain itself. The only exception being the specialised repeat DM domain (defined as the Dmrt1 domain) of *H. sapiens* Dmrt1, which defines all vertebrate Dmrt1 homologues and is not present in any invertebrates thus far (Wexler et al., 2014).
Figure 1) Neighbour-joining phylogram and domain illustration of Dmrt in Decapoda, alongside model comparative species. Bootstrap values are shown at each node and were performed with 1000 replicates to ensure reliability. Scale bar indicates number of amino acid substitutions per site. Fig 1A) Displays phylogeny of full length Dmrt peptides, TAD predictions were conducted using the Nine Amino Acids Transactivation Domain (9aaTAD) Prediction Tool (Piskacek et al., 2007) as described in (Chandler et al., 2017) B) shows phylogeny of isolated DM domains. Throughout, the Dmrt11E cluster is highlighted in green, 99B cluster in purple, 93B cluster in blue and Dsx cluster in orange. Domain illustration and taxonomy keys are provided in figure. All decapod GenBank Accession Numbers are given in Table 2, with the addition of H. sapiens Dmrt1 (Q9Y5R6.2); D. melanogaster Dmrt11E (AAF48261.2), Dmrt99B (AAF56919.1), Dmrt93B (AAF55843.1), DsxM (AAF54169.1), DsxF (AAN13385.1); and C. elegans Mab3 (O18214.1).
The mechanistic diversity of the Dmrts

A pronounced example of the domain variation illustrated in Figure 1A is the DMA domain. This domain has been identified in multiple animal taxa, including the decapods, cnidarians and the ctenophores and tends to be associated with the Dmrt93B and 99B clades, lacking from Dsx homologues (Wexler et al., 2014). However, a function for this domain is yet to be determined. The transactivation domain (TAD) however, has an explicit functional significance, responsible for the transcriptional activation of the bound Dmrt-DNA complex (Beukeboom and Perrin, 2014; Mapp and Ansari, 2007; Piskacek et al., 2007). Hence its occurrence has dramatic potential to shape the mechanistic action of each Dmrt. In cases where the TAD is absent, such as Drosophila Dsx^F (Figure 1A), additional coactivators (hermaphrodite (HER) and intersex (IX)) are required to achieve transcriptional activity (Garrett-Engele et al., 2002; Pultz and Baker, 1995). Or indeed, in the absence of such coactivators, there is potential for the Dmrt to exert suppressive mechanisms, such as the antagonistic mechanisms described for DM-W (Yoshimoto et al., 2006; Yoshimoto et al., 2008) and iDMY (Chandler et al., 2017). When one considers the functional significance of each additional domain, it becomes apparent how the seemingly well conserved Dmrts adopt the diversity of mechanisms observed across species. Moreover, this domain variation points towards the varied necessity to recruit additional genes to support functionality (explaining the diversity of described sex determination networks), from which the integration of co-regulatory genes can be better elucidated.

The DM domain that defines the Dmrts

In contrast, that depicted in Figure 1B, reiterates the dramatic sequence conservation of the DNA-binding domain that defines the Dmrt family; the DM domain is that responsible for binding the target DNA. This commonality across species therefore suggests that DNA-binding motifs, which constitute the response elements targeted by each Dmrt, must also be relatively well conserved. Indeed, such conservation of the Dmrt binding motif has already been demonstrated amongst Drosophila sp. (Luo et al., 2011). This conservation therefore offers a perfect method by which to bridge the gap, identifying the regulatory genes that act downstream of these key node regulators, through the identification of the conserved DM domain binding motif. This has already been done in Mammalia (Murphy et al., 2010), X. laevis (Herpin et al., 2010) and Drosophila (Luo et al., 2011), informing of the auto and cross-regulatory effects of the Dmrt genes (Murphy et al., 2010), the mechanisms by which
they coordinate their own expression (Herpin et al., 2010), as well as the extent of divergence of the DNA-binding motif itself (Luo et al., 2011). Similar promoter analysis in the decapods would facilitate the identification of genomic binding motifs, from which candidate response genes can be elucidated and fully investigated through the more telling expression patterns gained through transcriptomics. Similar analyses have been conducted on a gene-by-gene basis, such as that conducted for the genomic regions of IAG in *S. paramamosain* (Zhang et al., 2014) and *M. nipponense* (Ma et al., 2016b), identifying a range of transcription factor binding sites in the 5’ promoter region (Table 2). Although informative, when one considers the interconnected nature of genetic networks, a genome-wide analysis would prove a far more powerful tool to accurately assemble the regulatory map governing sex determination.

**Future directions**

In summary, this review serves as a detailed reference for those interested in sex determination in Decapoda, offering a list of putative candidates that (in most cases) can act to guide further functional investigations. However, we also intend for the critical assessment presented throughout this summary, to highlight the risks associated with the arbitrary identification of target genes without an appropriate consideration of functional conservation. Thus, we urge, that when evaluating each homologous candidate, one considers the divergence that defines the rapidly evolving genes of sex determination.

The spatial expression analyses that constitute the majority of the molecular studies presented, are a sound starting point for functional investigations; they are however indicative and not conclusive. This, considered with the fact that *Decapoda* is a non-model Order, advises that the best approach to advance current understanding should build on what is known in these species, rather than relying on that characterised in others. As described above, the well characterised (and conserved) function of IAG in sexual differentiation, offers an ideal functional basis with which to integrate putative candidates.

Following this, we advocate for a greater emphasis to be placed on the Dmrt genes, which of all the candidate homologues, are known to have the most significant functional conservation. Work should aim to thoroughly investigate the spatial and temporal expression of the Dmrts in *Decapoda*. The use of genome-guided promoter analyses would inform of Dmrt interconnectivity, as well as facilitate the identification of, as yet, unknown response genes. In
conjunction with (or, as is often the case, in the absence of) genomic resources, the use of RNAi knock-down is a well-suited tool to begin to understand the transcriptional integration of each Dmrt with each other, with IAG and with the regulatory elements of IAG, such as the CHH hormones (e.g. GIH) and the TKIR receptor (Aizen et al., 2016; Sharabi et al., 2016). Yu et al. (2014), present a sound example of such, using RNAi approaches to determine that of two Dmrts (both of which showed pronounced testicular-biased expression), Dmrt11E but not Dmrt99B, was functionally involved in the regulation of IAG expression. We therefore conclude with Figure 2, presenting our revised suggestion of the regulatory axis of sexual development in Decapoda, emphasising the network-like qualities of the system, rather than the linear ones described at present. In following these suggested avenues of future research, we can begin to identify and integrate associative genes, assembling a functionally valid understanding of sexual development in the decapods.

**Figure 2A** Illustration of the evolutionary relationships of the focal taxa discussed in this work, emphasising the extreme evolutionary distance over which the Dmrts are conserved, highlighted by the blue bar (note that the Dmrts remain yet to be identified in isopods). The shared role of IAG in the malacostracan Orders is highlighted in orange. **B) Revised depiction of the XO-SG – AG – TS axis of the Malacostraca (Decapoda)**, with inclusion of the Dmrts. Those interactions that are proven are displayed in black line (Yu et al., 2014), those that have some preliminary support in dark grey (Chandler et al., 2017) and those that remain to be investigated in light grey. The red-ended line indicates the inhibitory signals of GIH/MIH, which when removed allow for proliferation of the AG; colouration as described in the key.
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