

1 **The decapod researchers' guide to the galaxy of sex determination**

2
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12
13 **Abstract**

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

30
31 **Keywords:** Sex determination; Sex chromosome; *Malacostraca*; *Decapoda*; *Isopoda*; Double-
32 sex and mab-3 related transcription factor (*Dmrt*)

34 **Sexual differentiation in *Malacostraca***

35 The integrated signalling cascades of sexual development make it difficult to clearly
36 distinguish the processes of sex determination from sexual differentiation (Matson and
37 Zarkower, 2012). However, in the decapods (*Crustacea, Malacostraca*), as members of the
38 *Malacostraca*, the onset of male sexual differentiation is more clearly defined, due to the
39 unique involvement of a male specific endocrine gland known as the androgenic gland (AG).
40 First characterised in the sister Order, *Amphipoda (Crustacea, Malacostraca)*, the AG was
41 determined to be the sole regulator of male primary and secondary sexual differentiation
42 (Charniaux-Cotton, 1954). It was then in a second sister Order, *Isopoda*, that the specific
43 regulatory hormone was first isolated, defined as an insulin-like peptide and so termed the
44 insulin-like androgenic gland hormone (AGH) (Hasegawa et al., 1987; Martin et al., 1999;
45 Okuno et al., 1999b; Suzuki, 1999).

46

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

63

64 **The resulting sexual systems**

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

74

75 There are also examples of protandrous hermaphroditic species, where individuals are born as
76 males and later develop into females. This appears to be common in the *Caridea*, such as the
77 Peppermint shrimp (*Lysmata wurdemanni*) (Bortolini and Bauer, 2016; Zhang et al., 2017) and
78 is a process regulated through the sex differentiating effects of IAG (Zhang et al., 2017). Such
79 hermaphroditism has also been documented in the *Thalassinidae* and *Anomura* (Subramoniam,
80 2017). To complicate things further, the shrimp species, *L. wurdemanni*, can also exist as a
81 simultaneous hermaphrodite, where an individual can function as a male and a female at any
82 one time (a protandrous simultaneous hermaphrodite) (Bauer and Holt, 1998).

83

84 More staggeringly, the Order also contains one documented parthenogenic species. The
85 marbled crayfish (*Procambarus fallax* forma *virginalis*), only exists as female, of which all
86 offspring is genetically identical to the mother and therefore, female (Scholtz et al., 2003; Vogt
87 et al., 2015). Finally, to truly demonstrate the plasticity of the IAG-mediated system, one
88 should consider the sister Order, *Isopoda*, where the influence of the AG is superseded by the
89 feminisation effects of the bacterial endosymbiont, *Wolbachia*. This parasitism is thought to
90 prevent the initial differentiation of the male-specific AG (Bouchon et al., 2008; Cordaux et
91 al., 2011; Rigaud et al., 1997), allowing for female development. So dramatic are these AG-
92 disruptive effects, not only do they result in total feminisation of ZZ males but ultimately, the
93 loss and re-emergence of the W chromosome (Cordaux and Gilbert, 2017; Leclercq et al.,
94 2016).

95 **How is this diversity in sexual systems achieved?**

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

125

126 Thus, considering the diversity of sexual systems amongst *Decapoda* and the apparent
127 plasticity of IAG endocrinology, it is apparent that to fully understand the regulation of sexual

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

142

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

155 **Functional definitions of sex-regulator genes in model species**

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

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

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

163

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

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

169

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

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

176

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

178 Doublesex (Dsx) is a transcription factor defined by a zinc finger domain, termed the DM
179 domain. In the presence of the Tra – Tra-2 complex, the female splice-variant of *Dsx* is
180 produced, generating the Dsx^F protein. Dsx^F works in concert with an array of regulatory genes
181 as the major effector of sexual development ((Beukeboom and Perrin, 2014; Hoshijima et al.,
182 1991).

183

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

185 Fruitless (Fru) is a transcription factor defined by its zinc fingers. *Fru* is a male promoting
186 gene, regulating development of the male central nervous system and male sexual behaviour.

187 *Fru* is spliced in the absence of the female Tra – Tra-2 complex, carried out by non sex-specific
188 splicing machinery (Billeter et al., 2006).

189
190 2. The nematode, *Caenorhabditis elegans* (Nematoda, Chromadorea)

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

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

197
198 **Male Abnormal 3: DM domain, zinc finger protein (transcription factor)***

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

204
205 3. Mammals (*Chordata, Mammalia*)

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

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

211
212 **Sex-determining region Y-box 9: HMG-box DNA-binding protein (transcription factor)**

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

218
219 **Doublesex and male abnormal-3 related transcription factor 1: DM domain, zinc finger
220 protein (transcription factor)***

221 Doublesex and male abnormal-3 related transcription factor 1 (*Dmrt1*) is a transcription factor
222 defined by a novel zinc finger domain, termed the DM domain. It is upregulated in response to
223 *Sox9* via *Fgf9*. *Dmrt1* works in concert with an array of regulatory genes as the major effector
224 of male sexual differentiation, specifically testicular development and maintenance. It also acts
225 to inhibit the female developmental pathway (e.g. by suppressing *Foxl2*) (Matson et al., 2011).
226

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

228 Forkhead box L2 (*Foxl2*) is a transcription factor that is defined by a unique DNA-binding
229 domain. In the absence of the Sry driven expression of *Sox9*, *Foxl2* expression ensues and acts
230 to inhibit the male pathway, whilst promoting the female pathway through the action of *Rspo1*,
231 *Wnt4* and *B-catenin* (Matson et al., 2011).
232

233 **Table 1) Modes of sex determination in *Brachiopoda*, *Isopoda* and *Decapoda*.** Unless
234 otherwise indicated modes are genetic.

235

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

241 **Acting at the top of the cascade: the sex chromosomes**

242 The summary presented in Table 1 provides clear demonstration of the diversity of sex
243 determination modes present in *Crustacea*. Both *D. pulex* (Chen et al., 2014; Crease et al.,
244 1989) and *D. magna* (Kato et al., 2011; Kleiven et al., 1992) are subject to environmental sex
245 determination, through cyclic parthenogenesis. This contrasts to the decapods where, thus far,
246 genetic sex determination is common across the Order, although both XY/XX (heterogametic
247 male) and ZW/ZZ (homogametic male) mechanisms exist sporadically across families
248 (Chandler et al., 2016b). In *Isopoda*, male homogamety (ZZ) seems to be more common than
249 male heterogamety (XY) (Becking et al., 2017). There is also strong evidence for frequent sex
250 chromosome turnover, with an estimated three to thirteen heterogametic transitions occurring,
251 accounting for the dramatic XY and ZW diversity observed within *Genera* (Becking et al.,
252 2017).

253

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

263

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

274 perhaps only differing in the one master sex-determining gene responsible for flipping the
275 male/female switch.

276

277 **The mechanism: evaluating the role of sex-regulating genes**

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

284

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

291

292 There is some level of conservation within *Insecta*, as a functional *Tra* orthologue (termed
293 *Feminiser*, *Fem*) also acts as the female master sex-determinant in the honey bee (*Apis*
294 *mellifera*) (Hasselmann et al., 2008). *Fem* lacks complete conservation of the sequence motifs
295 described in *Drosophila* but does contain the same arginine/serine and proline-rich domain
296 organisation and one conserved sequence motif, also described in *C. capitata* (Pane et al.,
297 2002). Although diverged in both sequence and function across these three species (telling of
298 significant independent evolution over the ~300 million years that separates the Orders) the
299 conserved role of the three orthologues is indicative of a *Tra*-based ancestral pathway of sex
300 determination in *Insecta* (Hasselmann et al., 2008). Furthermore, the discovery of *Fem* suggests
301 that the *complementary sex determiner* (*csd*) gene may not be the universal master sex-
302 determinant across hymenopteran insects (bees, ants and wasps), as was previously thought
303 (Hasselmann et al., 2008; Heimpel and Boer, 2008).

304

305 It is interesting to note that the brachiopods *D. pulex* (Chen et al., 2014) and *D. magna* (Kato
306 et al., 2010) do have *Tra* homologues, however both show even greater divergence in domain

307 organisation from that of *Drosophila* Tra. Furthermore, it is only in *D. magna* that a function
308 in sexual development has been suggested, not in the female sex determination pathway (as
309 with the insect sp.), but in the maintenance of male phenotype (Chen et al., 2014). This
310 evolutionary emergence and divergence of *Tra* is in support of the proposed decapod–
311 branchiopod – hexapod trajectory of the *Pancrustacea* (Glenner et al., 2006). A similar story
312 is true of the highly conserved *Sxl* splice variants, which although seemingly well conserved in
313 sequence throughout *Insecta* and *Decapoda* (Table 2), have not retained conserved
314 functionality. These evolutionary patterns exemplify why the functional conservation of the
315 genes summarised in this work need to be appropriately evaluated; a conserved function to that
316 described in each model species should not be assumed *a priori*.

317

318 **Dmrts: the functionally conserved sex-regulators**

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

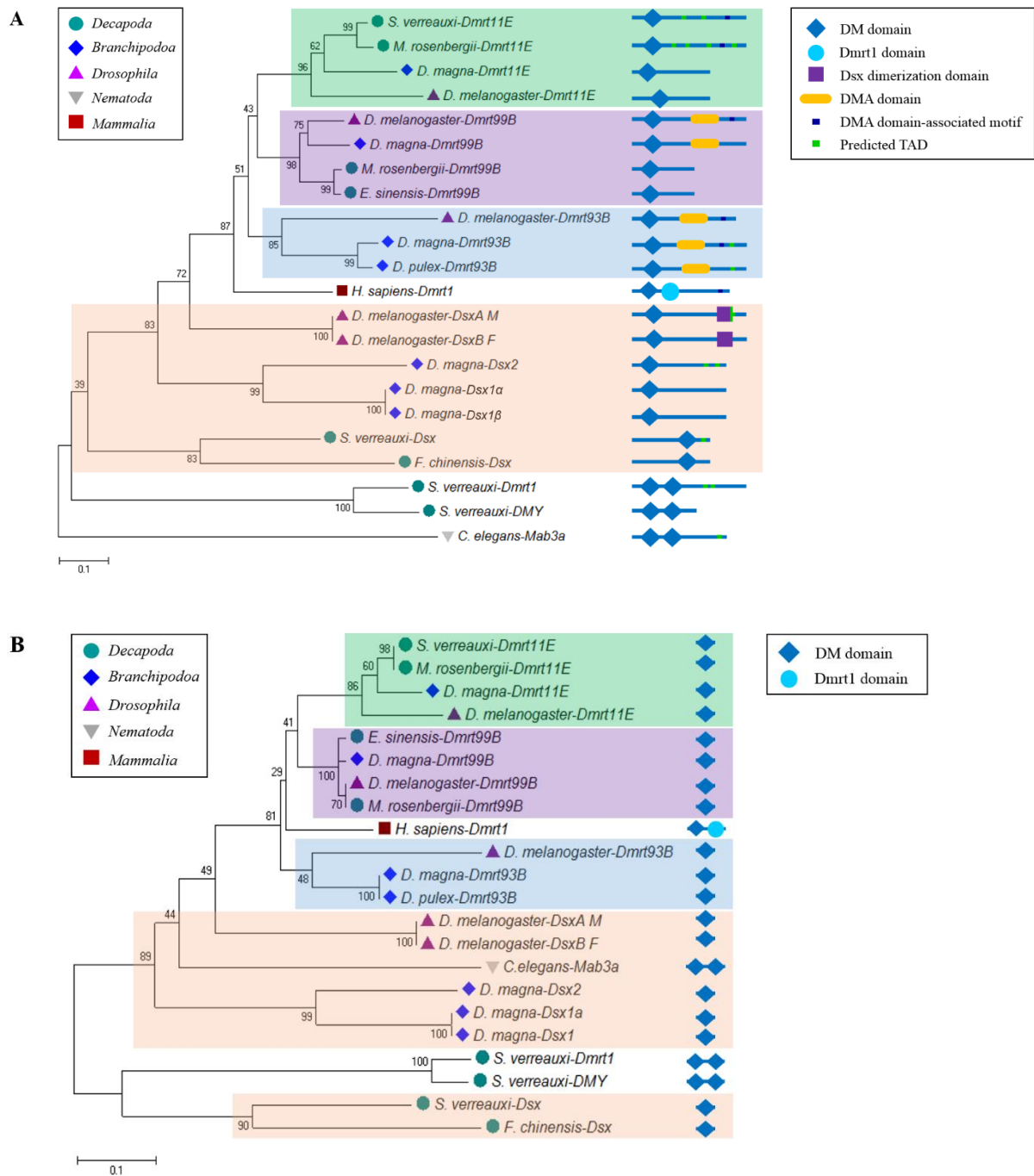
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332 The functional conservation of the model genes characterised in *D. melanogaster* (*Dsx*),
333 *C. elegans* (*Mab-3*) and *Mammalia* (*Dmrt1*) is readily illustrated in *D. magna*. In this
334 branchiopod, a Dmrt homologue (*Dsx1*) was determined to be the master male sex-determinant
335 (Kato et al., 2011). It is of note however, that four additional *Dmrts* were also identified,
336 *Dmrt11E*, *Dmrt93B*, *Dmrt99B* (Kato et al., 2008) and *Dsx2* (Kato et al., 2011) and determined
337 to have no clear function in sexual development. This highlights the critical importance of the
338 functional analyses to reliably determine the explicit regulatory role of *Dapma-Dsx1*. The
339 functional conservation of the *Dmrts* continues in the decapods, as recently a Y-chromosome-

340 linked *Dmrt* was identified in the Eastern spiny lobster (*S. verreauxi*) (Chandler et al., 2017).
341 This gene, termed *Sv-iDMY*, offers the third example of a sex-linked *Dmrt* across *Animalia*
342 (joining medaka DMY and frog DM-W) and is the first example in an invertebrate. As is the
343 case in both medaka and frog, our functional analyses suggest that *Sv-iDMY* has evolved as the
344 master sex-determinant in this decapod species (Chandler et al., 2017).

345

346 To better illustrate the evolutionary and mechanistic features of the *Dmrt* family, Figure 1
347 presents the phylogeny of all the available decapod *Dmrt* sequences, as well as those from
348 *D. pulex* and *D. magna* and the model *Dsx*, *Mab-3* and *Dmrt1*. Figure 1A shows the full protein
349 sequence, highlighting the clear clustering of *Dmrt* subclasses, but more significantly, the
350 pronounced variation in domain organisation outside of the DM domain itself. In contrast,
351 Figure 1B shows the phylogeny of the DM domains only, emphasising the extreme
352 conservation of the DNA-binding domain that defines the family. Taken together, Figure 1
353 demonstrates how the mechanistic diversity of this family is achieved: routed in the sequence
354 and domain variation outside of the DM domain itself. The only exception being the specialised
355 repeat DM domain (defined as the *Dmrt1* domain) of *H. sapiens* *Dmrt1*, which defines all
356 vertebrate *Dmrt1* homologues and is not present in any invertebrates thus far (Wexler et al.,
357 2014).



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Figure 1) Neighbour-joining phylogram and domain illustration of Dmrts 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), Dsx^M (AAF54169.1), Dsx^F (AAN13385.1); and *C. elegans* Mab3 (O18214.1).

370 **The mechanistic diversity of the Dmrts**

371 A pronounced example of the domain variation illustrated in Figure 1A is the DMA domain.
372 This domain has been identified in multiple animal taxa, including the decapods, cnidarians
373 and the ctenophores and tends to be associated with the Dmrt93B and 99B clades, lacking from
374 Dsx homologues (Wexler et al., 2014). However, a function for this domain is yet to be
375 determined. The transactivation domain (TAD) however, has an explicit functional
376 significance, responsible for the transcriptional activation of the bound Dmrt-DNA complex
377 (Beukeboom and Perrin, 2014; Mapp and Ansari, 2007; Piskacek et al., 2007). Hence its
378 occurrence has dramatic potential to shape the mechanistic action of each Dmrt. In cases where
379 the TAD is absent, such as *Drosophila* Dsx^F (Figure 1A), additional coactivators
380 (hermaphrodite (HER) and intersex (IX)) are required to achieve transcriptional activity
381 (Garrett-Engle et al., 2002; Pultz and Baker, 1995). Or indeed, in the absence of such
382 coactivators, there is potential for the Dmrt to exert suppressive mechanisms, such as the
383 antagonistic mechanisms described for DM-W (Yoshimoto et al., 2006; Yoshimoto et al.,
384 2008) and iDMY (Chandler et al., 2017). When one considers the functional significance of
385 each additional domain, it becomes apparent how the seemingly well conserved Dmrts adopt
386 the diversity of mechanisms observed across species. Moreover, this domain variation points
387 towards the varied necessity to recruit additional genes to support functionality (explaining the
388 diversity of described sex determination networks), from which the integration of co-regulatory
389 genes can be better elucidated.

390

391 **The DM domain that defines the Dmrts**

392 In contrast, that depicted in Figure 1B, reiterates the dramatic sequence conservation of the
393 DNA-binding domain that defines the Dmrt family; the DM domain is that responsible for
394 binding the target DNA. This commonality across species therefore suggests that DNA-binding
395 motifs, which constitute the response elements targeted by each Dmrt, must also be relatively
396 well conserved. Indeed, such conservation of the Dmrt binding motif has already been
397 demonstrated amongst *Drosophila* sp. (Luo et al., 2011). This conservation therefore offers a
398 perfect method by which to bridge the gap, identifying the regulatory genes that act
399 downstream of these key node regulators, through the identification of the conserved DM
400 domain binding motif. This has already been done in *Mammalia* (Murphy et al., 2010),
401 *X. laevis* (Herpin et al., 2010) and *Drosophila* (Luo et al., 2011), informing of the auto and
402 cross-regulatory effects of the Dmrt genes (Murphy et al., 2010), the mechanisms by which

403 they coordinate their own expression (Herpin et al., 2010), as well as the extent of divergence
404 of the DNA-binding motif itself (Luo et al., 2011). Similar promoter analysis in the decapods
405 would facilitate the identification of genomic binding motifs, from which candidate response
406 genes can be elucidated and fully investigated through the more telling expression patterns
407 gained through transcriptomics. Similar analyses have been conducted on a gene-by-gene basis,
408 such as that conducted for the genomic regions of IAG in *S. paramamosain* (Zhang et al., 2014)
409 and *M. nipponense* (Ma et al., 2016b), identifying a range of transcription factor binding sites
410 in the 5' promotor region (Table 2). Although informative, when one considers the
411 interconnected nature of genetic networks, a genome-wide analysis would prove a far more
412 powerful tool to accurately assemble the regulatory map governing sex determination.

413

414 **Future directions**

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

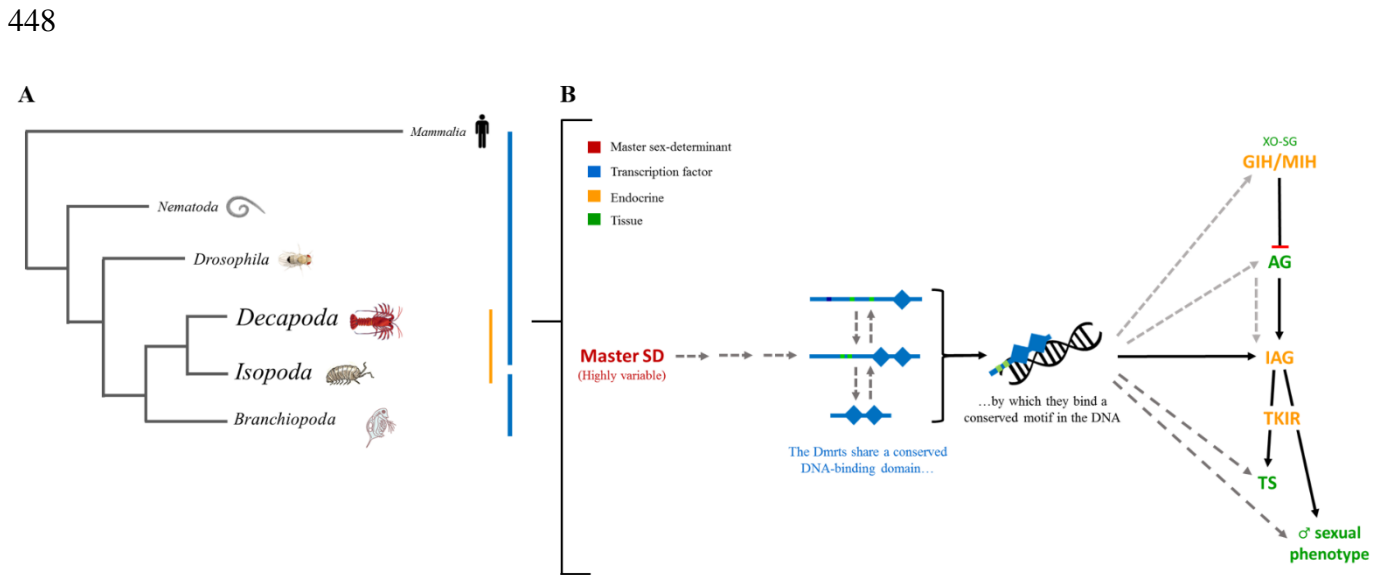
422

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

430

431 Following this, we advocate for a greater emphasis to be placed on the Dmrt genes, which of
432 all the candidate homologues, are known to have the most significant functional conservation.
433 Work should aim to thoroughly investigate the spatial and temporal expression of the Dmrts in
434 *Decapoda*. The use of genome-guided promoter analyses would inform of Dmrt
435 interconnectivity, as well as facilitate the identification of, as yet, unknown response genes. In

436 conjunction with (or, as is often the case, in the absence of) genomic resources, the use of *RNAi*
 437 knock-down is a well-suited tool to begin to understand the transcriptional integration of each
 438 *Dmrt* with each other, with *IAG* and with the regulatory elements of *IAG*, such as the CHH
 439 hormones (e.g. *GIH*) and the *TKIR* receptor (Aizen et al., 2016; Sharabi et al., 2016). Yu et al.
 440 (2014), present a sound example of such, using *RNAi* approaches to determine that of two
 441 *Dmrt*s (both of which showed pronounced testicular-biased expression), *Dmrt11E* but not
 442 *Dmrt99B*, was functionally involved in the regulation of *IAG* expression. We therefore
 443 conclude with Figure 2, presenting our revised suggestion of the regulatory axis of sexual
 444 development in *Decapoda*, emphasising the network-like qualities of the system, rather than
 445 the linear ones described at present. In following these suggested avenues of future research,
 446 we can begin to identify and integrate associative genes, assembling a functionally valid
 447 understanding of sexual development in the decapods.



449
 450 **Figure 2A) Illustration of the evolutionary relationships of the focal taxa** discussed in this
 451 work, emphasising the extreme evolutionary distance over which the *Dmrt*s are conserved,
 452 highlighted by the blue bar (note that the *Dmrt*s remain yet to be identified in isopods). The
 453 shared role of *IAG* in the malacostracan Orders is highlighted in orange. **B) Revised depiction**
 454 **of the XO-SG – AG – TS axis of the Malacostraca (Decapoda)**, with inclusion of the *Dmrt*s.
 455 Those interactions that are proven are displayed in black line (Yu et al., 2014), those that have
 456 some preliminary support in dark grey (Chandler et al., 2017) and those that remain to be
 457 investigated in light grey. The red-ended line indicates the inhibitory signals of *GIH/MIH*,
 458 which when removed allow for proliferation of the *AG*; colouration as described in the key.
 459

460 **Acknowledgements**

461 This work was supported by the Australian Research Council *via* a Discovery Early Career
462 Research Award [DE130101089 to TV], a Discovery [DP160103320 to TV], a University of
463 the Sunshine Coast (USC) Collaborative Research Networks (CRN) grant program and a USC
464 International Research Scholarship to JC.

465
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