

# Phylogenetic Distribution and Evolutionary Dynamics of the Sex Determination Genes *doublesex* and *transformer* in Insects

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## Key Words

*doublesex* · Evolution · Gene duplication · Insects · Sex determination · *transformer*

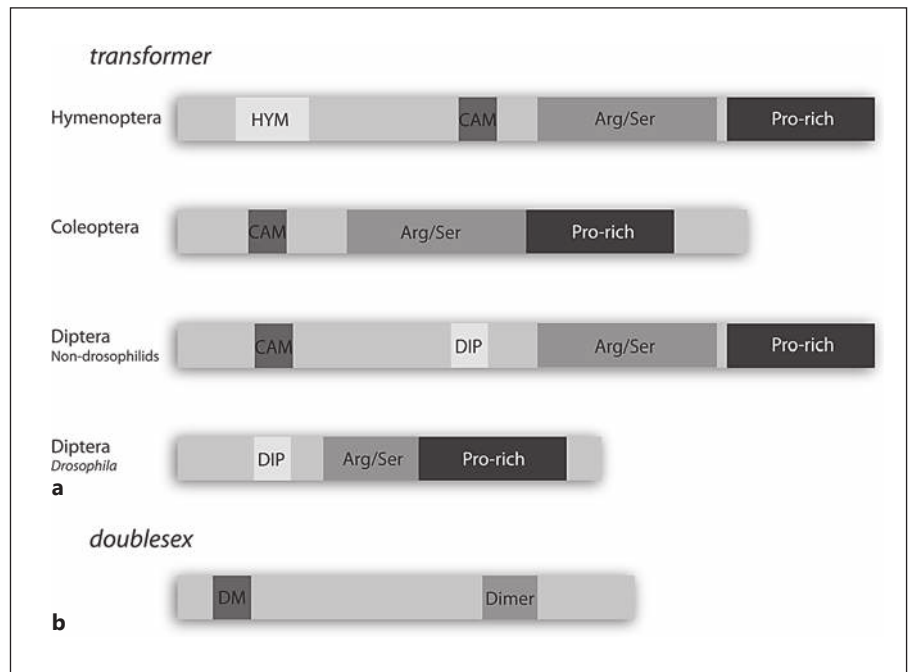
## Abstract

Sex determination in insects is characterized by a gene cascade that is conserved at the bottom but contains diverse primary signals at the top. The bottom master switch gene *doublesex* is found in all insects. Its upstream regulator *transformer* is present in the orders Hymenoptera, Coleoptera and Diptera, but has thus far not been found in Lepidoptera and in the basal lineages of Diptera. *transformer* is presumed to be ancestral to the holometabolous insects based on its shared domains and conserved features of autoregulation and sex-specific splicing. We interpret that its absence in basal lineages of Diptera and its order-specific conserved domains indicate multiple independent losses or recruitments into the sex determination cascade. Duplications of *transformer* are found in derived families within the Hymenoptera, characterized by their complementary sex determination mechanism. As duplications are not found in any other insect order, they appear linked to the haplodiploid reproduction of the Hymenoptera. Further phylogenetic analyses combined with functional studies are needed to understand the evolutionary history of the *transformer* gene among insects.

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The development into different sexes is a widespread phenomenon in eukaryote organisms. Despite its fundamentality, the mechanisms governing the determination and development of the sexes are widely diverged, even on small evolutionary time scales and between related organismal groups [Bull, 1983; Marshall Graves, 2008]. We are still far from grasping the full breadth of this diversity, and we have very little understanding of the evolutionary forces that drive it. Only a limited number of animal taxa have been investigated to a considerable extent. Sex determination in mammals and birds is rather invariable, but notably fish and insects are known for their rapid turnovers in the function and nature of sex determination genes [Devlin and Nagahama, 2002; Sanchez, 2008]. In this short review, we consider the genetic regulation of sex determination in insects with a focus on the phylogenetic distribution of 2 important genes, *doublesex* and *transformer*. Sex determination in insects is characterized by a gene cascade that is conserved at the bottom but contains diverse primary signals at the top. The bottom master switch gene is *doublesex* and is found in all insects studied to date. Its upstream regulator is *transformer* whose phylogenetic distribution shows some remarkable patterns, being present in some groups but not in others (see below).

**Fig. 1.** The domain structure of *transformer* (a) and *doublesex* (b) in the various holometabolous insect orders. **a** The *transformer* order-specific domains of Hymenoptera (HYM) and Diptera (DIP) are shown in light grey. The autoregulation domain (CAM) is found in all examined species except *Drosophila*. The arginine- and serine-rich region (Arg/Ser) and the proline-rich region (Pro-rich) are present in all orders. **b** The DM-domain and the dimerization domain (Dimer) of *doublesex* are shown.



## Sex Determination Cascades

Sex determination in insects is organized as a hierarchical order of genes which is termed a genetic cascade. A primary signal is passed through a series of sex determination genes ending with a downstream master switch gene which regulates sexual differentiation genes. The downstream genes are more conserved than the upstream ones, which is explained by the cascade evolving from the bottom upwards [Wilkins, 1995]. The master switch at the bottom of the sex determination cascade is identified as *doublesex* in a range of insect species, including Diptera (flies and mosquitoes), Hymenoptera (ants, bees, wasps, and sawflies) and Lepidoptera (butterflies and moths) [Shukla and Nagaraju, 2010a]. *doublesex* belongs to a family of DM-domain genes which appear to play a role in sex determination or sexual differentiation in all Metazoa [Suzuki et al., 2001; Hodgkin, 2002; Kopp, 2012]. It is a transcription factor that regulates downstream sexual differentiation genes [Shukla and Nagaraju, 2010a]. *doublesex* is alternatively spliced by *transformer* into sex-specific variants, which leads to the production of male- and female-specific DSX proteins.

Genes upstream of *doublesex* are less conserved in the sex determination cascade. The gene directly upstream of *doublesex* is *transformer* which was considered a constant feature in insects, but this idea is now challenged (see be-

low). The *transformer* gene is not functionally conserved outside of insects. It is for example present in the water flea *Daphnia magna*, but not involved in the environmental sex determination of this crustacean [Kato et al., 2010]. *Transformer* belongs to a class of SR-type proteins that have sequences containing high frequencies of arginine (R) and serine (S) residues (fig. 1a). It contains several conserved domains, one of which is only shared amongst the Diptera ('the dipteran domain'), and another only present in the Hymenoptera ('the hymenopteran domain'). The function of these domains remains unknown. *transformer* is a very rapidly evolving gene and regulates *fruitless* splicing as well [Ryner et al., 1996; Gailey et al., 2006], in addition to *doublesex*. Sex-specific *transformer* mRNA is produced by alternative splicing, a regulatory mechanism that is sustained by an autoregulatory loop. When female *transformer* transcripts are blocked by e.g. RNA interference, it results in male development, as shown for several dipterans [Pane et al., 2002; Lagos et al., 2007] and the hymenopterans *Apis mellifera* [Hasselmann et al., 2008] and *Nasonia vitripennis* [Verhulst et al., 2010a].

The primary signals on top of the sex determination cascade vary strongly among insects. In *Drosophila melanogaster*, the X chromosome:autosome ratio functions as the primary signal. The concentration of X-linked signal elements activates the *Sex-lethal* early promoter [Erick-

son and Quintero, 2007], which in turn regulates *transformer* splicing. In other dipterans, *Sex-lethal* is not an intermediary between the primary signal and *transformer*, but *transformer* is directly regulated by the absence or presence of a male-determining M factor [Dübendorfer et al., 2002; Pane et al., 2002; Hediger et al., 2010]. The M factor has not been identified in any insect yet. In haplodiploid Hymenoptera, the primary signal is the ploidy level of the egg (diploid = female, haploid = male), but how this signal is transmitted towards *transformer* is still subject of active research [Verhulst et al., 2013; Verhulst and van de Zande, this issue]. In Lepidoptera, which have female heterogamety, a presumably dominant female determiner on the W chromosome regulates a splicing inhibitor (PSI) of *doublesex* [Suzuki et al., 2008]. The number of investigated insect species is still small, and given their enormous variety in genetic systems, it is to be expected that many more regulatory mechanisms of sex determination will be discovered in the future.

### Phylogenetic Distribution of *doublesex*

The downstream master switch gene *doublesex* was first identified in *D. melanogaster* [Baker and Wolfner, 1988]. The protein is characterized by 2 functional domains: a DNA-binding domain (DM or OD1) and a dimerization domain (OD2) (fig. 1b). The DM domain is found in multiple genes of the DM superfamily group consisting of *doublesex*, *mab3* and related transcription factors (*Dmrt*), while the dimerization domain is exclusively found in *doublesex*. Orthologs of *doublesex* were found in each insect species examined thus far (references in table 1), covering a range of Diptera, Lepidoptera, Coleoptera, and Hymenoptera. All of these belong to the derived group of holometabolous insects, while no information has been published from more basal insects. Other DM superfamily genes outside of the insects do not contain a dimerization domain and do not exhibit the sex-specific splicing typical of *doublesex*.

The increasing public availability of genomic and transcriptomic databases gave us the opportunity to screen more insect species for the presence of *doublesex*. We used the presence of the dimerization domain in combination with an upstream DM domain as evidence for the presence and functional conservation of *doublesex* (see Appendix). We found indications for putative orthologs of *doublesex* in a number of primitive insect groups (table 1; online supplementary table 1, see [www.karger.com/doi/10.1159/000357056](http://www.karger.com/doi/10.1159/000357056)). The genome of *Pe-*

*diculus humanus corporis* (human body louse) contains a hypothetical protein (XM\_002427625.1) which is a candidate for *doublesex*, consisting of both a DM domain and a putative dimerization domain. A homolog of the dimerization domain could, however, not be found in 2 hemipteran genomes, *Acyrtosiphon pisum* and *Rhodnius prolixus*, whereas both species do contain 2 DM superfamily genes. The closest relative to the insects examined thus far is the water flea *D. magna* which is claimed to have 2 *doublesex* genes, neither of which shows the sex-specific splicing characteristic of insects, but both do contain a putative dimerization domain [Kato et al., 2011]. It thus appears that the sex-specific splicing feature of *doublesex* was acquired early in the evolution of insects. More species from primitive groups such as Palaeoptera, Zygentoma and Archaeognatha need to be investigated to assert its precise origin. Further, identification of *doublesex* orthologs in genomic assemblies provides no evidence for sex-specific splicing and its coincidence with the origin of a conserved dimerization domain. Functional analysis outside the holometabolous insects is needed to ascertain its appearance in the sex determination cascade and the conservation of this role in various groups.

Within the Holometabola, *doublesex* appears to be ubiquitously present, with possible identifications from this study in genomes of Coleoptera (*Dendroctonus ponderosae*) and Diptera (*Mayetiola destructor* and *Phlebotomus papatasi*) (table 1; online suppl. table 1). We could also find it with both of its conserved functional domains in the order of Strepsiptera (*Mengenilla moldrzyki*).

### Phylogenetic Distribution of *transformer*

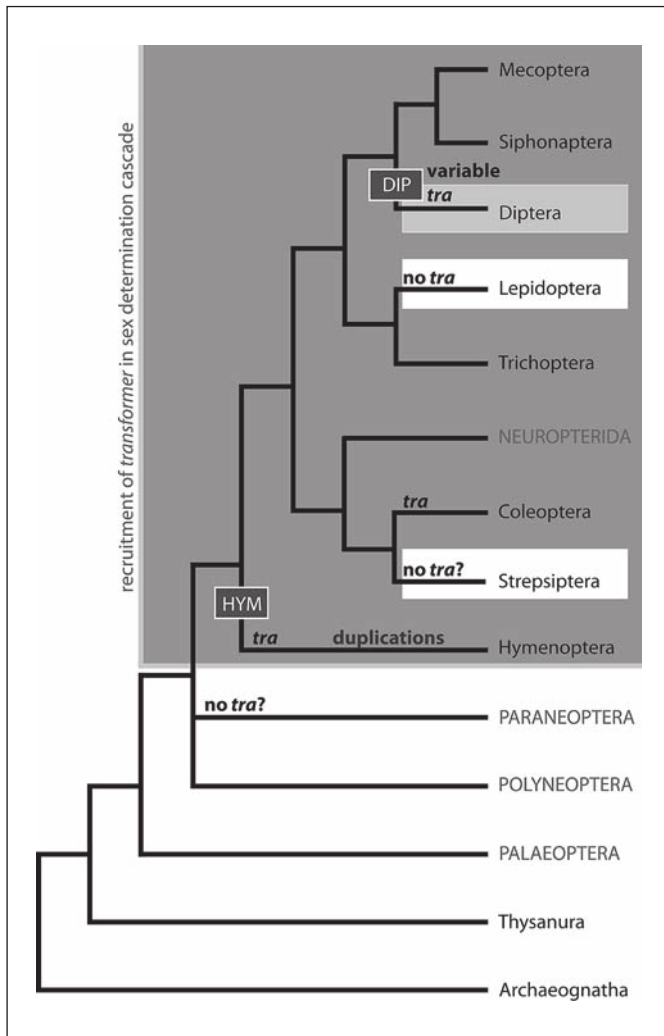
*transformer*, the upstream splicing controller of *doublesex*, shows a distinctly more patchy distribution among insects (fig. 2). It is also more diverged in sequence than *doublesex*, which complicates its identification in genome sequences. The most conserved part of *transformer* is the autoregulation domain CAM (from *Ceratitis capitata*-*Apis mellifera*-*Musca domestica*; nomenclature based on Hediger et al. [2010]). This domain is present in all *transformer* genes, except in that of *D. melanogaster*. In *Drosophila*, *Sex-lethal* has been added upstream of *transformer*, and it has taken over autoregulation, making the CAM domain of *transformer* obsolete [Bopp, 2010]. Other features of *transformer* include order-specific domains of unknown function. One of these is shared among all Hymenoptera, the other is present in all Diptera (fig. 1a). In addition, 2 regions are present in all *transformers*: an ar-

**Table 1.** Presence of *doublesex*, *transformer*, *transformer* paralogs and the *transformer* autoregulation domain (CAM) in insect species. Species with a genomic database from which *transformer* was characterized were not assessed for presence of *doublesex* if the family belonged to a well-studied order (Diptera and Hymenoptera)

| Species                           | <i>doublesex</i> |  | <i>transformer</i> |            |             |  |
|-----------------------------------|------------------|--|--------------------|------------|-------------|--|
|                                   | presence         | reference                                      | presence           | CAM-domain | duplication | reference  |
| <b>Order Phthiraptera</b>         |                  |  |                    |            |             |  |
| <i>Pediculus humanus corporis</i> | yes              | this study                                     | no                 | no         | no          | this study   |
| <b>Order Hemiptera</b>            |                  |  |                    |            |             |  |
| <i>Acyrtosiphon pisum</i>         | DM only          | this study                                     | no                 | no         | no          | this study   |
| <i>Rhodnius prolixus</i>          | DM only          | this study                                     | no                 | no         | no          | this study   |
| <b>Order Hymenoptera</b>          |                  |  |                    |            |             |  |
| <i>Acromyrmex echinatior</i>      |                  |  | yes                | yes        | no          | Nygaard et al., 2011;<br>Schmieder et al., 2012;<br>Privman et al., 2013 |
| <i>Apis cerana</i>                |                  |  | yes                | yes        | yes         | Hasselmann et al., 2008b   |
| <i>Apis dorsata</i>               |                  |  | yes                | yes        | yes         | Hasselmann et al., 2008b   |
| <i>Apis florea</i>                |                  |  | yes                | yes        | yes         | Schmieder et al., 2012   |
| <i>Apis mellifera</i>             | yes              | Cho et al., 2007                               | yes                | yes        | yes         | Beye et al., 2003  |
| <i>Atta cephalotes</i>            |                  |  | yes                | yes        | yes         | Schmieder et al., 2012;<br>Privman et al., 2013                          |
| <i>Bombus impatiens</i>           | yes              | GenBank prediction                             | yes                | yes        | yes         | Schmieder et al., 2012   |
| <i>Bombus terrestris</i>          | yes              | GenBank prediction                             | yes                | yes        | yes         | Hasselmann et al., 2010;<br>Schmieder et al., 2012                       |
| <i>Camponotus floridanus</i>      |                  |  | yes                | yes        | yes         | Schmieder et al., 2012;<br>Privman et al., 2013                          |
| <i>Euglossa hemichlora</i>        |                  |  | yes                | yes        |             | Hasselmann et al., 2010  |
| <i>Harpegnathos saltator</i>      |                  |  | yes                | yes        | yes         | Schmieder et al., 2012;<br>Privman et al., 2013                          |
| <i>Linepithema humile</i>         |                  |  | yes                | yes        | yes         | Schmieder et al., 2012;<br>Privman et al., 2013                          |
| <i>Megachile rotundata</i>        | yes              | this study                                     | yes                | yes        | no          | this study   |
| <i>Melipona compressipes</i>      |                  |  | yes                | yes        |             | Hasselmann et al., 2008a   |
| <i>Nasonia giraulti</i>           | yes              | Oliveira et al., 2009                          |                    |            |             |  |
| <i>Nasonia longicornis</i>        | yes              | Oliveira et al., 2009                          |                    |            |             |  |
| <i>Nasonia vitripennis</i>        | yes              | Oliveira et al., 2009                          | yes                | yes        | no          | Hasselmann et al., 2008a;<br>Werren et al., 2010                         |
| <i>Pogonomyrmex barbatus</i>      |                  |  | yes                | yes        | yes         | Schmieder et al., 2012;<br>Privman et al., 2013                          |
| <i>Solenopsis invicta</i>         |                  |  | yes                | yes        | yes         | Wurm et al., 2011  |
| <b>Order Lepidoptera</b>          |                  |  |                    |            |             |  |
| <i>Antheraea assama</i>           | yes              | Shukla and Nagaraju, 2010b                     |                    |            |             |  |
| <i>Antheraea mylitta</i>          | yes              | Shukla and Nagaraju, 2010b                     |                    |            |             |  |
| <i>Bombyx mori</i>                | yes              | Ohbayashi et al., 2001;<br>Suzuki et al., 2001 | no                 | no         |             | Mita et al., 2004  |
| <i>Danaus plexippus</i>           | yes              | this study                                     | no                 | no         | no          | this study   |
| <i>Heliconius melpomene</i>       | yes              | this study                                     | no                 | no         | no          | this study   |
| <i>Ostrinia scapulalis</i>        | yes              | Sugimoto et al., 2010                          |                    |            |             |  |

**Table 1** (continued)

| Species                        | <i>doublesex</i> |   | <i>transformer</i> |            |             |                             |
|--------------------------------|------------------|---|--------------------|------------|-------------|-----------------------------|
|                                | presence         | reference                                   | presence           | CAM-domain | duplication | reference                   |
| <b>Order Coleoptera</b>        |                  |   |                    |            |             |                             |
| <i>Dendroctonus ponderosae</i> | yes              | this study                                  | no                 | no         | no          | this study                  |
| <i>Onthophagus sagittarius</i> | yes              | Kijimoto et al., 2012                       |                    |            |             |                             |
| <i>Onthophagus taurus</i>      | yes              | Kijimoto et al., 2012                       |                    |            |             |                             |
| <i>Tribolium castaneum</i>     | yes              | Shukla and Palli, 2012b                     | yes                | yes        |             | Shukla and Palli, 2012a     |
| <b>Order Strepsiptera</b>      |                  |   |                    |            |             |                             |
| <i>Mengenilla moldrzyki</i>    | yes              | this study                                  | no                 | no         | no          | this study                  |
| <b>Order Diptera</b>           |                  |   |                    |            |             |                             |
| <i>Aedes aegypti</i>           | yes              | Salvemini et al., 2011                      | no                 |            |             | Salvemini et al., 2013      |
| <i>Anopheles gambiae</i>       | yes              | Scali et al., 2005                          | no                 |            |             | Gailey et al., 2006         |
| <i>Anastrepha</i> (12 species) | yes              | Ruiz et al., 2005, 2007a                    | yes                | yes        |             | Ruiz et al., 2007b          |
| <i>Bactrocera correcta</i>     | yes              | Permpoon et al., 2011                       |                    |            |             |                             |
| <i>Bactrocera dorsalis</i>     | yes              | Chen et al., 2008;<br>Permpoon et al., 2011 |                    |            |             |                             |
| <i>Bactrocera oleae</i>        | yes              | Lagos et al., 2005                          |                    |            |             |                             |
| <i>Bactrocera tryoni</i>       | yes              | Shearman and Frommer, 1998                  | yes                | yes        |             | Lagos et al., 2007          |
| <i>Ceratitis capitata</i>      | yes              | Saccone et al., 1996                        | yes                | yes        |             | Pane et al., 2002           |
| <i>Cochliomyia hominivorax</i> |                  |   | yes                | yes        |             | Li et al., 2013             |
| <i>Cochliomyia macellaria</i>  |                  |   | yes                | yes        |             | Li et al., 2013             |
| <i>Culex quinquefasciatus</i>  | yes              | this study                                  | no                 | no         | no          | this study                  |
| <i>Drosophila americana</i>    |                  |   | yes                | no         |             | McAllister and McVean, 2000 |
| <i>Drosophila erecta</i>       |                  |   | yes                | no         |             | O'Neil and Belote, 1992     |
| <i>Drosophila hydei</i>        |                  |   | yes                | no         |             | O'Neil and Belote, 1992     |
| <i>Drosophila mauritiana</i>   |                  |   | yes                | no         |             | Kulathinal et al., 2003     |
| <i>Drosophila melanogaster</i> | yes              | Burtis and Baker, 1989                      | yes                | no         |             | Boggs et al., 1987          |
| <i>Drosophila sechellia</i>    |                  |   | yes                | no         |             | Kulathinal et al., 2003     |
| <i>Drosophila simulans</i>     |                  |   | yes                | no         |             | O'Neil and Belote, 1992     |
| <i>Drosophila virilis</i>      | yes              | Hertel et al., 1996                         | yes                | no         |             | O'Neil and Belote, 1992     |
| <i>Glossina morsitans</i>      |                  |   | yes                | yes        |             | Hediger et al., 2010        |
| <i>Lucilia cuprina</i>         | yes              | Concha et al., 2010                         | yes                | yes        |             | Concha and Scott, 2009      |
| <i>Lucilia sericata</i>        |                  |   | yes                | yes        |             | Li et al., 2013             |
| <i>Lutzomyia longipalpis</i>   | yes              | this study                                  | no                 | no         | no          | this study                  |
| <i>Mayetiola destructor</i>    | yes              | this study                                  | no                 | no         | no          | this study                  |
| <i>Megaselia scalaris</i>      | yes              | Sievert et al., 1997;<br>Kuhn et al., 2000  | no?                | no?        | no          | this study                  |
| <i>Musca domestica</i>         | yes              | Hediger et al., 2004                        | yes                | yes        |             | Hediger et al., 2010        |
| <i>Phlebotomus papatasi</i>    | yes              | this study                                  | yes                | no         | no          | this study                  |



**Fig. 2.** Phylogeny of insects showing the presence of *transformer* (*tra*) across orders. Orders are noted on the right and superorders are marked in capital letters. Proposed gain of *transformer* into the sex determination cascade is indicated by the grey box, with inserted white boxes indicating secondary losses. Question marks refer to uncertainties as data are based only on this study and require validation. The Diptera are in a light grey box to mark the variable presence of *transformer* in this order, which is characterized by an absence in basal lineages and a presence in derived lineages, with the exception of *Drosophila*. The addition of the Hymenoptera and Diptera domains in *transformer* is marked by boxes labeled HYM and DIP. The phylogeny is based on Trautwein et al. [2012].

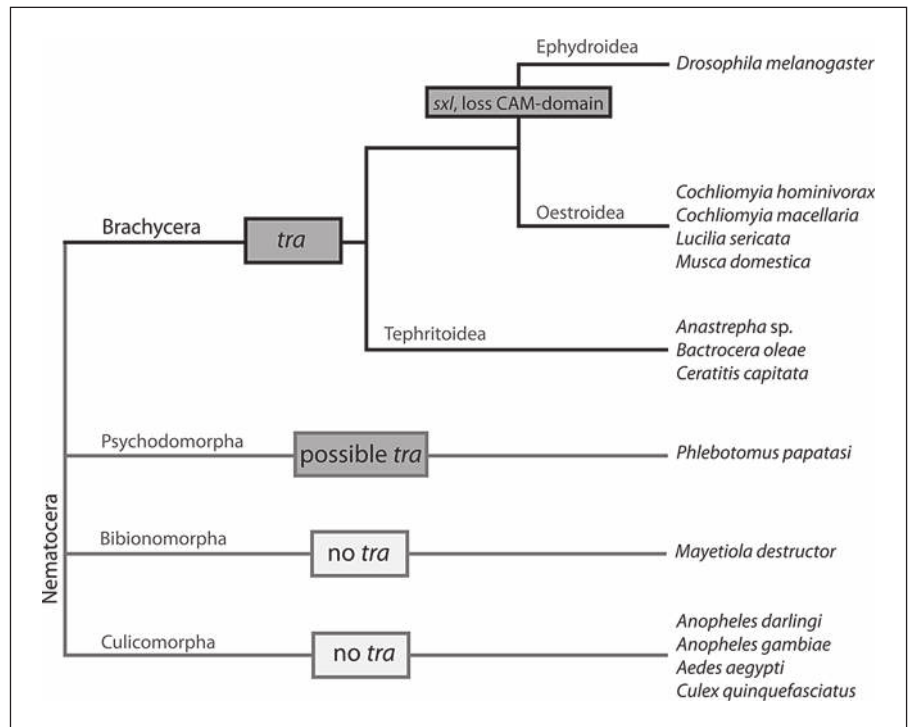
arginine/serine-rich region and a proline-rich region. The location of these regions towards the C-terminal is conserved across all *transformer*-containing insect species studied to date, while the location of the CAM domain and the order-specific domains varies. In Hymenoptera,

the order-specific domain is located upstream of the CAM domain, whereas in Diptera, its order-specific domain is close to the arginine/serine region. In non-drosophilid Diptera, the CAM domain is positioned towards the N-terminal of the gene, a feature that it shares with the short *transformer* gene in the beetle *Tribolium castaneum*.

Hymenoptera are the most basal order of the holometabolous insects (fig. 2). *transformer* has been identified in more than a dozen hymenopteran species, all belonging to the more derived lineages within the order. On the other hand, in Lepidoptera no *transformer* homologs have been found thus far. Most information is available for the well-studied species *Bombyx mori* [Suzuki, 2010], but genomic data of *Danaus plexippus* and *Heliconius melpomene* indicate lack of a *transformer* homolog as well (table 1; Appendix). With only 1 published Strepsiptera genome, that of *Mengenilla moldrzyki* [Niehuis et al., 2012], information about this group is scarce, but this species appears to lack a *transformer* homolog. This is remarkable because as a sister order of the Coleoptera, it was presumed to carry *transformer*, based on its presence in *T. castaneum* [Shukla and Palli, 2012a]. However, in the genome of the coleopteran *D. ponderosae*, which was published recently [Keeling et al., 2013], we could also not find a homolog of *transformer*. This leaves the question of whether *transformer* is conserved in Coleoptera open until more species are examined.

An interesting pattern of *transformer* distribution emerges in the Diptera, where recent studies have suggested it to be absent in mosquitoes, which form a basal dipteran lineage [Salvemini et al., 2013]. *transformer* is found in all more derived Brachycera species (references in table 1) including *D. melanogaster*, where it has lost its CAM domain (fig. 3). In addition to its apparent absence in several mosquito species, we could not find *transformer* in *M. destructor* (table 1; Appendix), which belongs to the basal lineage of the Bibionomorpha, in *Lutzomyia longipalpis* (Psychodomorpha) and, peculiarly, in *Megaselia scalaris* (a derived brachyceran species). On the other hand, the recently published genomic assembly of *P. papatasi*, a member of the basal Psychodomorpha, contains a putative *transformer* homolog, but misses the CAM domain. A cautionary note is needed here, as this absence of its most reliable recognition feature complicates its identification (see Appendix). The putative *P. papatasi* homolog does contain a Diptera domain and structurally resembles the CAM domain-lacking *D. melanogaster transformer*. Outside of the Hymenoptera, no paralogs of *transformer* have been identified, thus indi-

**Fig. 3.** Phylogeny of dipteran insects showing the distribution and structure of *transformer* (*tra*). The figure is based on phylogenetic trees by Bertone et al. [2008], Lambkin et al. [2013] and Wiegmann et al. [2011]. There is no consensus about the resolution of the basal lineages.



cating that this putative homolog in *P. papatasi* has a likely orthologous origin. Interestingly, the CAM domains of the more derived Diptera show strong similarity with the more than a dozen known sequences of the Hymenoptera and the coleopteran *Tribolium*. The *D. magna transformer* also contains a highly conserved CAM domain, but it is located downstream of the arginine/serine-rich region and is not involved in sex determination [Kato et al., 2010]. This suggests that other selective pressures have acted to preserve its presence; an explanation for this could be that the domain serves additional functions in development.

The presence of *transformer* in *Daphnia* indicates that it arose before the branching of Crustacea and Hexapoda. Under the assumption of a unique *transformer* recruitment into the sex determination cascade at the basis of the holometabolous insects, secondary loss of *transformer* must have occurred on at least 2 occasions based on current studies: in the Lepidoptera [Mita et al., 2004] and the basal Diptera [Salvemini et al., 2013]. We found evidence for 2 additional possible losses, i.e. in a strepsipteran and a coleopteran species. Secondary loss of the CAM domain was only known from *D. melanogaster*, but our study suggests a second dipteran case, i.e. the sand fly *P. papatasi*. The absence of *transformer* in groups other than holome-

tabolous insects and crustaceans suggests that the gene can disappear again. This is at odds with the presumed conserved role in sex determination, but may reflect that *transformer* was only introduced in the sex determination cascade at the rise of the holometabolous insects.

The current knowledge on the distribution and structural features of *transformer* is based on a small collection of insect species. Studies have only been performed on 4 orders (Coleoptera, Diptera, Hymenoptera, and Lepidoptera) out of the 12 that make up the holometabolous insects. This review includes a first indication of a lack of *transformer* in a fifth order (the Strepsiptera), but no attention has been given to the remaining 7 orders, due in part to lack of genomic/transcriptomic data. Non-holometabolous insects comprise another 17 orders, amongst which no species have been studied regarding their sex determination (genes).

### Duplications of *transformer*

For a long time, *D. melanogaster* was the only species known to have an additional regulator upstream of *transformer* in the sex determination cascade, i.e. *Sex-lethal*. A few years ago, Beye et al. [2003] and Hassel-

mann et al. [2008a] found that the honey bee (*A. mellifera*) had another gene inserted at the top of its cascade. This turned out to be a duplication of *transformer* (=feminizer in *A. mellifera*). The duplication initiates the female-specific splicing of *feminizer* transcripts. The zygosity of this *complementary sex determiner (csd)* locus determines the switching on of the autoregulation loop of *transformer/feminizer* [Beye et al., 2003; Hasselmann et al., 2008a]. *Feminizer* genes coupled with paralogous *csd* genes were also found in 2 sister species, *Apis cerana* and *Apis dorsata* [Hasselmann et al., 2008b]. The 3 species showed signs of convergent evolution based on sharing of the same nonsynonymous substitutions in different alleles across species. The *Apis* genus belongs to the Aculeata, consisting of bees, vespoid wasps and ants, which is considered a derived lineage within the Hymenoptera. Two recent reports [Schmieder et al., 2012; Privman et al., 2013] found a similar duplication of *transformer* in 2 bumblebees and 6 out of 7 sequenced ant species (listed in table 1), all belonging to the Aculeata. The presence of *transformer* paralogs in these aculeate species is explained by a single duplication event after which the genes evolved through concerted evolution [Schmieder et al., 2012; Privman et al., 2013]. This places the duplication of *transformer/feminizer* leading to the *csd* gene before the appearance of the Aculeata lineages, around 120 million years ago [Schmieder et al., 2012]. In 3 examined cases, the *transformer/feminizer* genes show sex-specific splicing, while the duplicated *transformer* genes are expressed but without a sex-specific splicing pattern [Schmieder et al., 2012]. No functional analysis has been performed on these paralogs of *transformer*, thus leaving open the question whether they function similar to the *csd* gene and are part of the sex determination cascade.

Duplications of *transformer* have thus far only been identified in Hymenoptera, being haplodiploid, and exclusively in species presumed to have a complementary sex-determining mechanism (CSD). Under CSD, individuals homozygous at the *csd* locus develop into males, while heterozygosity at the *csd* locus starts female development [Whiting, 1943; Beye et al., 2003]. To counteract the costs of inbreeding, where decreasing variation would result in diploid homozygous males, the *csd* gene shows high allelic variation [Hasselmann et al., 2008b]. This effect of inbreeding, which results in diploid males, often sterile or inviable, allows simple testing for presence of CSD, at least in species that can be cultured in the laboratory. CSD has been inferred in over 60 species [van Wilgenburg et al., 2006] and is presumed to be ancestral to

the Hymenoptera [Cook, 1993; Heimpel and de Boer, 2008; Asplen et al., 2009]. Most studies indicated CSD based on a single locus (sl-CSD), but studies in the *Cotesia* genus have found evidence for multiple loci (multi-locus, or ml-CSD) [de Boer et al., 2008, 2012].

Based on the widespread occurrence of both *transformer* paralogs and CSD within the Aculeata, it is tempting to propose that the CSD locus consists of the *transformer* paralog in this group, as documented for the honey bee [Beye et al., 2003; Hasselmann et al., 2008a]. The paralog would then need to contain a hypervariable region as in the *A. mellifera csd* gene, or at least a high variety of alleles. However, within the Aculeata, 2 of the thus far examined species appear to lack a duplication of *transformer*: the leaf-cutting ant *Acromyrmex echinatior* [Nygaard et al., 2011; Schmieder et al., 2012; Privman et al., 2013], which is known to produce high levels of diploid males, and the solitary bee *Megachile rotundata* (table 1). No duplications have yet been found outside the Aculeata, but the availability of databases in Hymenoptera is strongly skewed towards eusocial derived species. Unfortunately, of the many other species that have CSD [van Wilgenburg et al., 2006], no genomic or transcriptomic information is available. Data on lineages of sawflies, which are basal to the Hymenoptera that are in turn one of the basal orders of the holometabolous insects, is crucial for determining whether *transformer* is involved in the presumed CSD ancestry. In the chalcidoid *N. vitripennis*, *csd* is absent, and there are no duplications of *transformer*. Its sex determination is based on a maternal effect genomic imprinting model [Verhulst et al., 2010a, 2013; Verhulst and van de Zande, this issue]. *N. vitripennis* is the first species outside the Aculeata that has no CSD and for which genomic data is available. More studies on both non-CSD and non-Aculeata species are needed to assess the commonality of the *csd/transformer* mechanism and the abundance of maternal imprinting sex determination among Hymenoptera.

## Conclusions and Outlook

In this article, we considered the phylogenetic distribution and evolutionary dynamics of 2 important sex determination genes, *doublesex* and *transformer*, among the insects. Although members of only a limited number of orders have been investigated, it is safe to conclude that *doublesex* serves as the master switch at the bottom of the sex determination cascade in all insects. The gene and its characteristic domains, as well as its sex-specific splicing



feature, are found across holometabolous insects. Branches outside of the Holometabola, i.e. *P. humanus corporis* in the Phthiraptera, contain homologous genes which show less conservation in the insect-specific dimerization domain. This latter feature cannot be identified when moving further into the basal lineages.

*transformer*, the upstream regulator of *doublesex*, has a less ubiquitous distribution. It is found outside holometabolous insects, but there it apparently has no function in sex determination, e.g. in *D. magna* [Kato et al., 2010]. It is apparently (partly) absent in 4 insect orders, notably the Lepidoptera, basal Diptera, Strepsiptera, and Coleoptera. *transformer* could have been recruited multiple times into the sex determination cascade, but its domain structure (CAM domain followed by an arginine/serine domain and a proline-rich domain) is conserved across all groups. An origin of a sex determination function at the basis of the holometabolous insects appears most parsimonious, but requires multiple independent losses. If *transformer*, however, originated before the branching-off of the crustaceans, it must have been lost in more groups outside of insects as well.

Many additional questions remain to be answered about *transformer* before we can make further inferences about its evolution in insects. First of all, a broader taxonomic screen is needed to determine how widespread *transformer* is present among insects. Particularly informative groups are the Paraneoptera (lice, thrips and bugs), a sister group of the Holometabola, and some of the other basal taxa, such as the Odonata (damselflies and dragonflies) and Ephemeroptera (mayflies). These could provide answers to the precise timing of *transformer* recruitment into the insect sex determination pathway. No information is available outside of the Holometabola, e.g. the Polyneoptera that span more than 10 orders of insects.

The evolutionary significance of *transformer* duplication in the Hymenoptera remains an enigma. Study of the basal lineages, such as the solitary Symphyta (sawflies), will be informative, for example to determine whether a link exists between *transformer* duplications and CSD. Only in the honey bee, the duplicated copy has been shown to have a functional role in sex determination. No functional studies have been performed on any other species with a *transformer* paralog. We also need more information about possible additional roles of *transformer* in development, as this may be revealing about the function of order-specific domains and may also yield insights into why some regions, such as the CAM domain, are conserved over a broad range of arthropod taxa. Our analysis

found further support for Wilkins [1995] hypothesis that the genetic pathway of sex determination started with the most downstream signal and evolved by adding genes upstream. We also adhere to the argument of Verhulst et al. [2010b] that *transformer* plays a central role in the evolving cascade in some insect groups. However, its absence in some groups also shows that *transformer* can be circumvented, and alternative regulators of *doublesex* can evolve. The frequent gains and losses of genes and gene domains are another signifying feature of the insect sex determination cascade.

Next generation sequencing is exponentially increasing the amount of data across an increasing number of arthropod groups. Large sequencing projects on hundreds of arthropod species, in particular insects, are currently underway. The i5K project (<http://arthropodgenomes.org/wiki/i5K>), for example, plans to sequence 5,000 insect and other arthropod genomes in the next couple of years. The 1KITE project (<http://www.1kite.org/>) focuses exclusively on insects and will yield 1,000 insect transcriptomes spanning all orders. These efforts, combined with the increasing amount of separately published genomic and transcriptomic datasets, provide a great wealth of information for gene identification studies. Transcriptomic datasets can offer immediate tests whether the putative sex determination genes are expressed in the sequenced tissue and may even provide a first glimpse at sex-specific splice variants. Genomic databases have the advantage of structure detection and, if the scaffolds are large enough, synteny of the sex determination regions. In combination, they make the future for insect sex determination researchers a bright one.

## Appendix

### *Methodology for Identification of doublesex and transformer (Duplication) Homologs with tBLASTn Searches on Publicly Available Genomic Databases*

The strong divergence of sex determination genes makes identification of homologs, particularly in thus far unexamined orders, not a straightforward task. Published studies which examined newly identified sex determination genes in genomic or transcriptomic databases relied on BLAST searches. Profile hidden Markov models could potentially be a more powerful method to detect distant homologs of these fast evolving genes.

The conserved domains provide some anchor points for the presence of a sex determination gene, though, on its own, this information cannot provide any evidence for an actual role in sex determination. Absence of a gene is more difficult to prove: is the gene truly lost (or never gained) in the species, or is it overlooked due to strong divergence and possible loss of conserved domains?

To increase detection of sex determination genes in unstudied insect orders and other relative outgroups, we searched for the presence of *doublesex* and *transformer* in a range of publicly available databases. We had no opportunity for any verification tests regarding the structure, expression or function of these genes. Moreover, some of the investigated species are distantly related to any studied species with an identified *transformer* or *doublesex* homolog, which added further difficulty to the scans.

Identification of *doublesex* and *transformer* homologs was performed in genomic databases of *Pediculus humanus corporis*, *Acyrtosiphon pisum*, *Rhodnius prolixus*, *Megachile rotundata*, *Danaus plexippus*, *Heliconius melpomene*, *Dendroctonus ponderosae*, *Menigenilla moldrzyki*, *Lutzomyia longipalpis*, *Mayetiola destructor*, *Megaselia scalaris*, and *Phlebotomus papatasi* with translated BLAST [Altschul et al., 1997]. As queries, the full *Doublesex* and *Transformer* protein sequences of *Apis mellifera* (ABW99105, NP\_001128300), *Nasonia vitripennis* (ACJ65507, NP\_001128299),

*Tribolium castaneum* (AFQ62106, AFQ62109), *Ceratitis capitata* (AAN63597, AAM88673), *Drosophila melanogaster* (NP\_731197, NP\_524114) and *Daphnia magna* (BAJ78307/BAJ78309, BAI66432) were used. In addition, the dimerization domain of *doublesex* and the CAM, Hymenoptera and Diptera domains of *transformer* were used, as these conserved regions allow more stringent detection where the full sequences may provide false positives.

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