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ORIGINAL ARTICLE

The comparative study of five sex-determining proteins across insects unveils high rates of evolution at basal components of the sex determination cascade

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Abstract In insects, the sex determination cascade is composed of genes that interact with each other in a strict hierarchical manner, constituting a coadapted gene complex built in reverse order from bottom to top. Accordingly, ancient elements at the bottom are expected to remain conserved ensuring the correct functionality of the cascade. In the present work, we have studied the levels of variation displayed by five key components of the sex determination cascade across 59 insect species, including *Sex-lethal*, *transformer*, *transformer-2*, *fruitless*, *doublesex*, and *sister-of-Sex-lethal* (a paralog of *Sxl* encompassing sex-independent functions). Surprisingly, our results reveal that basal components of the cascade (*doublesex*, *fruitless*) seem to evolve more rapidly than previously suspected. Indeed, in the case of *Drosophila*, these proteins evolve more rapidly than the master regulator *Sex-lethal*. These results agree with the notion suggesting that genes involved in early aspects of development will be more constrained due to the large deleterious pleiotropic effects of mutations, resulting in increased levels of purifying selection at top positions of the cascade. The analyses of the selective episodes involved in the recruitment of *Sxl* into sex-determining functions further support this idea, suggesting the presence of bursts of adaptive selection in the common ancestor of drosophilids, followed by the onset of purifying selection preserving the master regulatory role of this protein on top of the *Drosophila* sex determination cascade. Altogether, these results underscore the importance of the position of sex determining genes in the cascade, constituting a major constraint shaping the molecular evolution of the insect sex determination pathway.

Keywords Evolution · Development · Sex-specific genes · Insects · Evolutionary rates · Selection

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Introduction

In insects, the sex determination pathway constitutes a regulatory cascade that evolved in reverse order, from the final step in the hierarchy that creates the required product to the first step in the pathway that allows synthesis of the initial precursor (Bopp et al. 2014; Gempe and Beye 2011; Wilkins 1995). *Drosophila melanogaster* has been the paradigm for understanding the genetic and molecular basis underlying sex determination in this group (Bopp et al. 2014; Sánchez 2008). In this insect, the program committing the embryo to either the male or the female pathway is under the control of the gene *Sex lethal* (*Sxl*) (Cline 1978; Peñalva and Sánchez 2003). The study of the epistatic relationships between *Sxl* and the other genes involved in sex determination [i.e., *transformer* (*tra*), *transformer-2* (*tra-2*), *fruitless* (*fru*), and *doublesex* (*dsx*)] has revealed a hierarchical interaction among them during development (Baker and Ridge 1980), with the product of one gene controlling the sex-specific splicing of the primary transcript of the gene immediately downstream [reviewed in (Sánchez 2008)] (Fig. 1a). The search for genes homologous to the sex

determination genes of *D. melanogaster* has been undertaken in other insects [reviewed in (Gempe and Beye 2011; Sánchez 2008; Verhulst et al. 2010)]. It has been found a conserved relationship among *dsx*/*tra*/*tra-2* across dipterans so that this axis represents the ancestral state of the sex determination cascade, with the recruitment of *Sxl* as master regulator constituting an innovation acquired later on in *Drosophila*.

The evolution of the insect sex determination cascade has involved the sequential acquisition of genetic switches, each one reversing the action of the previous one, with the final step in the cascade (bottom) representing the oldest (Pomiankowski et al. 2004; Wilkins 1995). Under this model, trans-regulatory elements more recently recruited into sex determining pathways are expected to cause divergence toward the top because of recent regulatory change (e.g., the *Sxl* gene in *Drosophila*) while ancient elements at the bottom would remain conserved (e.g., the *dsx* gene in *Drosophila*) ensuring the correct functionality of the cascade (Verhulst et al. 2010) (Fig. 1b). However, an alternative interpretation of the evolution of the cascade (Artieri et al. 2009) suggests that genes involved in early aspects of development (which, as in the case of *Sxl*, are likely to regulate a large number of downstream effectors through hierarchical regulatory cascades) would be more constrained due to the large deleterious pleiotropic effects of mutations, resulting in increased levels of purifying selection at top positions of the cascade (Fig. 1c).

Overall, the current body of knowledge hints the presence of diverse specific constraints operating at different levels of the cascade, probably imposed by the epistatic interactions of its constituting components with upstream regulators and downstream target genes (Sánchez 2008), as well as by pleiotropic effects [e.g., additional functions unrelated to sex (Kunte et al. 2014)]. However, the nature and origin of the constraints shaping the evolution of the insect cascade still remain uncertain, mainly because of the lack of comparative studies across different levels of this pathway in diverse insect species. To fill this gap, the present work investigates the levels of variation displayed by five sex-determining proteins across 59 insect species, finding high rates of evolution at basal components of the cascade. In addition, our results provide clues for understanding the modifications in the evolutionary constraints resulting from the recruitment of *Sxl* into sex determination functions at the top of the *Drosophila* sex determination pathway.

Materials and methods

Evolutionary rates of sex determination proteins

Extensive data mining experiments were performed to build up the dataset of sex determination proteins used in the present work, consisting of 166 sequences (40 SXL, 27 TRA, 30 TRA-2, 25 FRU, 22 DSX-Male, and 22 DSX-Female). In addition, 12 SSX protein sequences from *Drosophila* representatives (sex-independent functions) were also included for further comparisons. Altogether, the taxonomic range covered by these sequences spans six insect orders (Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Phthiraptera) encompassing 59 different insect species: *Acromyrmex echinatior*, *Acyrthosiphon pisum*, *Aedes aegypti*, *Anastrepha amita*, *Anastrepha bistrigata*, *Anastrepha fraterculus*, *Anastrepha grandis*, *Anastrepha ludens*, *Anastrepha obliqua*, *Anastrepha serpentina*, *Anastrepha sororcula*, *Anastrepha striata*, *Anastrepha suspensa*, *Anopheles darlingi*, *Anopheles gambiae*, *Antheraea assama*, *Apis cerana*, *Apis dorsata*, *Apis florea*, *Apis mellifera*, *Bactrocera oleae*, *Bombus impatiens*, *Bombus terrestris*, *Bombyx mori*, *Bradysia coprophila*, *Camponotus floridanus*, *Ceratitis capitata*, *Chrysomya rufifacies*, *Culex quinquefasciatus*,

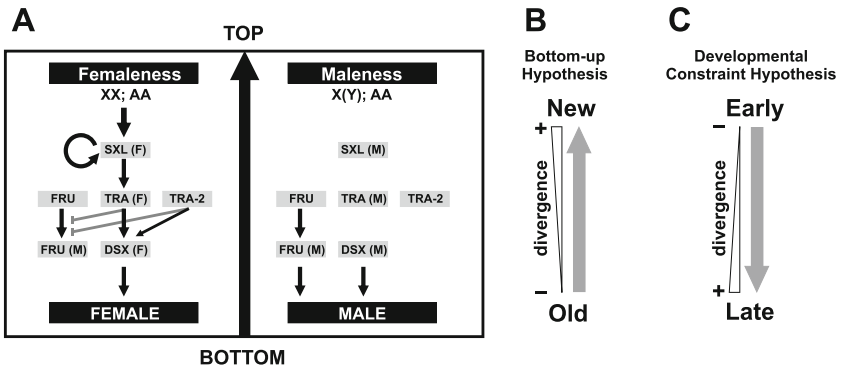

Fig. 1 Schematic representation of the hierarchical epistatic interactions constituting the sex determination cascade in *Drosophila* [adapted from (Sánchez 2008)] evolving from *bottom* to *top* (*DSX doublesex*, *FRU fruitless*, *TRA-2 transformer-2*, *TRA transformer*, *SXL Sex-lethal*). **a** In the absence of X/A signal in males, truncated SXL and TRA proteins will be produced leading to the synthesis of male-specific FRU and DSX that will eventually result in maleness. The major components of the cascade analyzed in the present work are indicated in *gray background*. **b** Under the bottom-up hypothesis, genes more recently recruited into sex determining pathways are expected to cause divergence toward the top of the cascade. **c** According to the developmental constraint hypothesis, genes involved in early aspects of development would be more constrained due to the large deleterious pleiotropic effects of mutations

Danaus plexippus, *Drosophila ananassae*, *Drosophila erecta*, *Drosophila grimshawi*, *Drosophila hydei*, *Drosophila mauritiana*, *Drosophila melanogaster*, *Drosophila mojavensis*, *Drosophila persimilis*, *Drosophila pseudoobscura*, *Drosophila sechellia*, *Drosophila simulans*, *Drosophila subobscura*, *Drosophila virilis*, *Drosophila willistoni*, *Drosophila yakuba*, *Glossina morsitans*, *Harpegnathos saltator*, *Lucilia cuprina*, *Megachile rotundata*, *Megaselia scalaris*, *Musca domestica*, *Nasonia vitripennis*, *Pediculus humanus corporis*, *Rhynchosciara americana*, *Sciara ocellaris*, *Stomoxys calcitrans*, *Tribolium castaneum*, *Trichomegalosphys pubescens* (see Supplementary Table 1 for details). Multiple alignments of protein and nucleotide sequences were implemented using the BIOEDIT program (Hall 1999) and visually inspected for errors. Estimations of protein divergence among insects for each component of the sex determination cascade were carried out using *p* distances with partial deletion (95 %), as this approach is known to give better results for distantly related taxa owing to its smaller variance. Estimations were performed using MEGA version 6 (Tamura et al. 2013). Estimations of divergence times between all pairs of taxa studied were manually retrieved from the TimeTree database (Hedges et al. 2006). Divergence times between taxa are listed together with the corresponding pairwise protein divergences in Supplementary Table 2. Regression analyses describing the relationships between protein divergence estimates and divergence time estimates were implemented for each sex-determining protein as well as for SSX using the program STATGRAPHICS Plus version 5.1 (Warrenton, VA). The rates of evolution for the studied proteins (amino acid substitutions/site per million years) were subsequently inferred based on the calculated regression coefficients.

Molecular evolutionary analyses and episodic diversifying selection in *Sxl*

Most part of the molecular evolutionary analyses was carried out using the program MEGA version 6 (Tamura et al. 2013) except where noted. The global molecular clock hypothesis was tested in each sex-determining protein by using likelihood ratio tests based on the models of evolution defined (see Table 1 for details). Additional tests for the presence of local molecular clocks were carried out in the case of SXL (insects) by using the program HyPhy (Pond et al. 2005). The SXL phylogeny was reconstructed using the maximum-likelihood approach based on the model of evolution that best fit the sets of sequences analyzed, rooting the tree with the cladoceran *Daphnia pulex*, diverging from the order Diptera approximately 443.2 MYA (Hedges et al. 2006). The reliability of the reconstructed topology was contrasted by nonparametric bootstrap (1000 replicates) and further examined by Bayesian analysis using the program BEAST version 1.7 (Drummond et al. 2012). Three independent Markov chain Monte Carlo (MCMC) runs of 10,000,000 generations each were performed to generate posterior probabilities, sampling tree topologies every 1000 generations to ensure the independence of successive trees and discarding the first 1000 trees of each run as burn-in.

Table 1 Best-fit models of evolution and global molecular clock tests in insect sex determination proteins

Protein	Model	lnL	lnL (clock)	*p* value
SXL (*Drosophila*)	JTT+G	−1225.3	−1234.3	0.0677
SXL (insects)[a]	WAG+G	−3764.2	−3874.0	4.06×10^{-14}*
TRA	JTT+G+F	−3615.2	−3637.3	0.7721
TRA-2	JTT+G	−2146.0	−2184.6	0.0763
FRU	JTT+G	−999.1	−1008.1	0.5633
DSX(c)	JTT+G+I	−3582.5	−3607.5	0.1829
DSX(f)	JTT+G	−2881.0	−2909.5	0.8055
DSX(m)	JTT+G+I	−3017.0	−3042.2	0.1255

Whelan and Goldman (WAG) and Jones and Taylor and Thornton (JTT) models of protein evolution (Goldman and Whelan 2001; Jones et al. 1992), including gamma distributed variation across sites (G) and invariant sites (I)

*Global molecular clock hypothesis rejected ($p<0.001$)

[a] The molecular clock is rejected in SXL from insects (excluding *Drosophila*) where this protein does not have sex determining roles

The evolution of *Sxl* was examined for lineages displaying evidence of diversifying selection episodes ($\omega>1$) by using the branch-site random effects likelihood (REL) model (Pond and Frost 2005). Codon positions were examined using the phylogeny of insects as a reference (Wheeler et al. 2001; Wiegmann et al. 2011), without making any prior assumptions about which lineages have been subject to diversifying selection. The proportion of sites inferred to be evolving under diversifying selection at each branch was estimated using likelihood ratio tests (LRTs), resulting in a *p* value for episodic selection corrected for multiple testing using the Holm-Bonferroni method. The strength of selection was partitioned into three categories ($\omega>5$, $\omega=1$, $\omega=0$) for descriptive purposes, using three different corrected significance levels ($p<0.001$, $p<0.01$, and $p<0.05$) to assess the obtained results. Selection analyses were further expanded to single codon positions in *Sxl* sequences by using a mixed effects model of evolution (MEME), modeling variable ω across lineages at an individual site (Murrell et al. 2012). The numbers of synonymous and nonsynonymous substitutions at these codon positions were estimated and subsequently located in the corresponding functional regions of the SXL protein (N- and C-terminal domains, RNA binding domain). *Sxl* codons subject to diversifying selection were also analyzed in a phylogenetic context, providing information on internal branches accumulating higher numbers of nonsynonymous mutations. All analyses

in this section were carried out using the Datamonkey webserver (Delport et al. 2010; Poon et al. 2009).

Results and discussion

Rates of evolution in sex-determining proteins from insects

The study of the rates of molecular evolution in key components of the sex determination pathway from insects yielded three interesting results: first, the sex-determining proteins studied in the present work evolve at constant rates, as suggested by global molecular clock tests (Table 1). Second, sex-determining proteins located at bottom positions of the cascade (i.e., *DSX* and *FRU*) display relatively high rates of evolution in insects (Fig. 2a). This is specially evident in the case of *Drosophila* (Fig. 2b), where basal proteins display higher evolutionary rates compared with proteins located at top positions (i.e., *SXL*). The high rates of evolution found in DSX could be due, at least in part, to sexual selection operating on this gene in order to keep up with modifications in downstream components at the bottom of the cascade (e.g., sexual cytodifferentiation genes). Similarly, sexual selection and/or conflict have been also suggested as the main driver of FRU diversification (Sobrinho and de Brito 2010). Third, TRA represents the fastest evolving protein in the sex determination cascade, encompassing a rate of evolution of approximately 2.57×10^{-3} and 1.25×10^{-2} substitutions/site per MY in insects and in *Drosophila*, respectively (see Table 2 for detailed evolutionary rates). Although unusually high rates of neutral functional evolution have been previously reported for this gene in *Drosophila* (Kulathinal et al. 2003; McAllister and McVean 2000), the present results constitute the first evidence showing rapid evolution of TRA in other insect species. We believe that this observation bears relevance, as *transformer* plays a master regulatory role on top of the sex determination cascade in some non-drosophilid insects.

Table 2 Rates of protein evolution (amino acid substitutions/site per million years) in the components of the sex determination cascade from insects (excluding *Drosophila*) and from *Drosophila*

Protein	Insects	*Drosophila*
SXL	$0.95\times10^{-3}\pm1.23\times10^{-5}$[a]	$2.80\times10^{-3}\pm3.89\times10^{-4}$
SSX	N/A	$4.32\times10^{-3}\pm1.65\times10^{-4}$[a]
TRA	$2.57\times10^{-3}\pm1.41\times10^{-4}$	$1.25\times10^{-2}\pm3.12\times10^{-4}$
TRA-2	$1.00\times10^{-3}\pm2.42\times10^{-5}$	$4.56\times10^{-3}\pm2.16\times10^{-4}$
FRU	$2.34\times10^{-3}\pm5.02\times10^{-5}$	$2.90\times10^{-3}\pm1.47\times10^{-4}$
DSX(c)	$1.61\times10^{-3}\pm4.06\times10^{-5}$	$5.59\times10^{-3}\pm1.47\times10^{-4}$
DSX(f)	$1.73\times10^{-3}\pm3.94\times10^{-5}$	$5.59\times10^{-3}\pm1.47\times10^{-4}$
DSX(m)	$1.54\times10^{-3}\pm4.22\times10^{-5}$	$5.83\times10^{-3}\pm1.58\times10^{-4}$

N/A, not applicable

[a] Although SXL do not play sex-determining roles in insects other than *Drosophila*, and SSX lacks sex determining fuctions, evolutionary rate estimations were performed in both cases for comparison purposes

The high rate of evolution observed in TRA proteins can be reconciled with its top position in the cascade of many insects based on the molecular mechanism of TRA function (Black 2003). Accordingly, TRA participates in splicing regulation through its interaction (through their SR domains) with other proteins carrying RNA-binding domains (e.g., TRA-2). Although SR dipeptide content can vary among TRA proteins, it appears that TRA functionality depends on the presence of a minimum number of SR dipeptides located at very conserved positions (Ruiz et al. 2007). Therefore, while SR regions must remain conserved to assure TRA function, this protein can still accept high levels of neutral variation on regions not involved

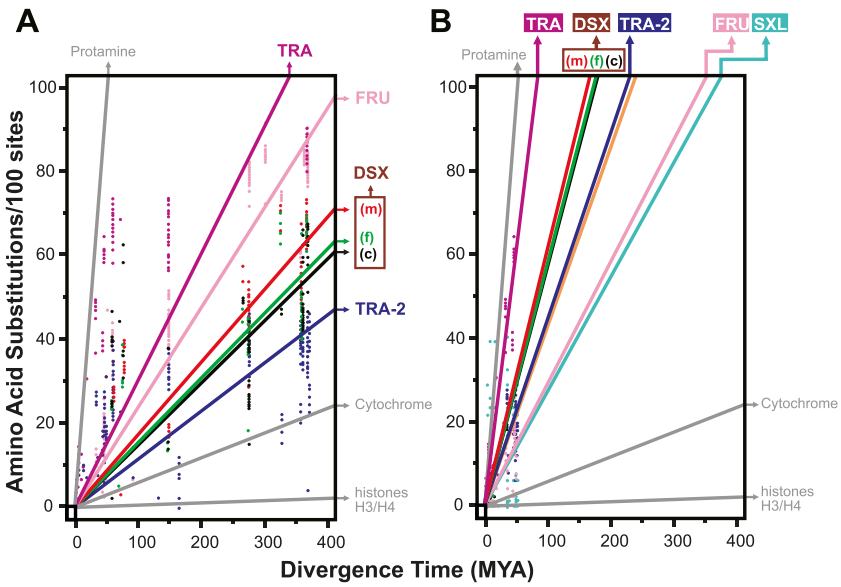

Fig. 2 Rates of evolution in sex-determining proteins from insects including *Drosophila* (**a**) and rates of evolution in sex-determining proteins only from *Drosophila* (**b**). Evolutionary rate estimations for DSX have been divided into the male/female common region (*c*), the female-specific DSX protein (*f*), and the male-specific DSX protein (*m*). Evolutionary rates for fast-evolving protamines and slow-evolving cytochrome and histones H2A/H2B are included as references

in protein-protein interactions (Kulathinal et al. 2003; McAllister and McVean 2000). Similarly to TRA, TRA-2 also participates in protein-protein interactions. However, this protein constitutes a general splicing factor that also interacts with RNAs, requiring a higher degree of conservation to preserve its functionality, especially at RNA recognition motifs (Sarno et al. 2010). That is mirrored by the low evolutionary rate displayed by this protein in insects (Fig. 2).

SXL (the top component of the *Drosophila* sex determination cascade) constitutes the slowest evolving sex determining protein in drosophilids (Fig. 2b) as well as a slow evolving protein in other insect species (see Table 2 for details). However, there is still the possibility that such a high degree of conservation is a result of the lack of sex-specific functions in insects other than *Drosophila* (Cline et al. 2010; Sánchez 2008). Two approaches were followed in order to explore this scenario: first, the analysis of SXL in non-drosophilid insects revealed an evolutionary rate of 0.95×10^{-3} substitutions/site per MY (Table 2), constituting a much lower rate than the one estimated for *Drosophila* (2.80×10^{-3} substitutions/site per MY). Indeed, it seems that all sex-determining proteins from *Drosophila* evolve significantly faster than their orthologs in other insects (Fig. 2b, Table 2). These results agree with the rapid evolution of the sex determination cascade in *Drosophila*, with *Sxl* occupying a top position, after medfly and fruitfly diverged (Civetta and Singh 1998; Cline et al. 2010). Second, the analysis of SSX [a paralog of SXL which took on roles unrelated to sex through a process of neofunctionalized after duplication (Cline et al. 2010)] revealed that this protein evolves almost twice as fast as SXL in drosophilids (4.32×10^{-3} substitutions/site per MY, Table 2), in agreement with previous reports describing a signature of rampant positive selection and relaxation of purifying selection in this gene (Mullon et al. 2012). Altogether, these results suggest a reinforcement in the selective constraints operating on SXL, most likely resulting from its recruitment into sex-related roles at the top of the *Drosophila* cascade (Cline et al. 2010; Mullon et al. 2012), as well from its role in controlling dosage compensation [reviewed in (Peñalva and Sánchez 2003)].

Selective episodes involved in the recruitment of *Sxl* into sex-specific functions

Modifications in the specific components of any network are expected to impact their hierarchical organization and their interactions, especially in those cases where components at top regulatory positions have been modified very recently (Bopp et al. 2014; Gempe and Beye 2011). Since that is precisely the case of *Drosophila* (*Sxl* has been recruited into sex-specific functions at the top of the cascade), this group provides us with a very powerful model to investigate the evolutionary consequences of such modification. To do so, this work addresses the following question: what was the nature of the selective episodes responsible for the recruitment of *Sxl* into sex-specific functions, and when (during the evolution of insects) and where (in the SXL protein) did they take place? To answer the first part of the question, we screened the phylogeny of insects for lineages at which *Sxl* experienced episodic adaptive selection ($\omega>1$), finding 12 significant

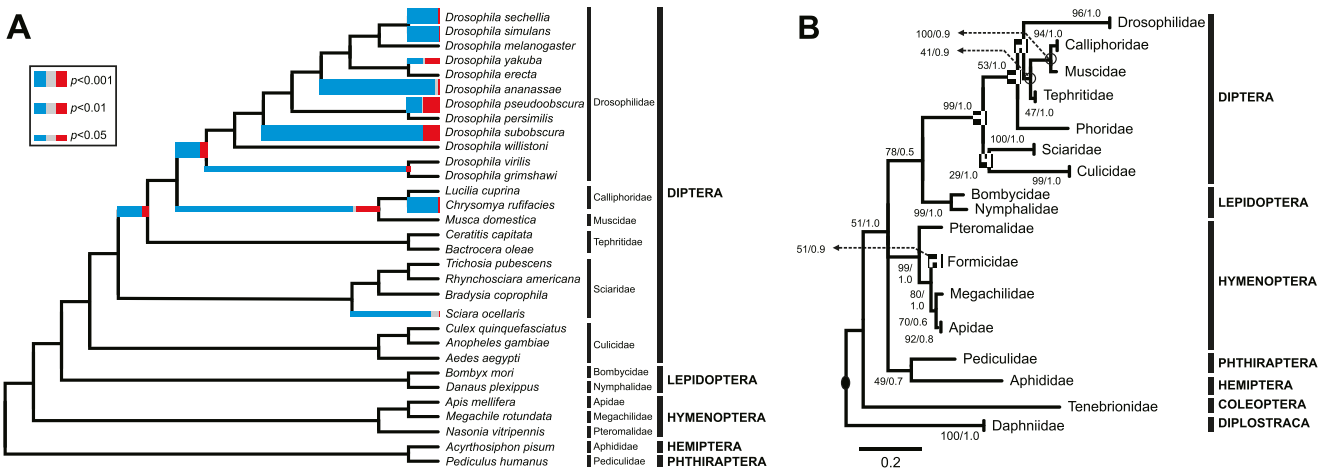

Fig. 3 Molecular evolution and diversifying selection in *Sxl* across insects. The taxonomic classification of the insect species studied (family/order) is indicated in the right margin of the trees. **a** Episodes of diversifying selection acting on *Sxl* throughout the phylogeny of insects [according to (Wheeler et al. 2001; Wiegmann et al. 2011)]. The strength of selection at significant branches is represented in *red* ($\omega>5$), *gray* ($\omega=1$), and *blue* ($\omega=0$), with the proportion of sites within each class represented by the color width. Thicker branches have been classified as undergoing episodic diversifying selection at corrected $p<0.001$ (thickest branches), $p<0.01$ (medium thickness), and $p<0.05$ (thinnest branches). **b** Protein phylogeny showing local SXL lineages deviating from a clock-like mode of evolution in insects. *Black boxes* at internal nodes indicate subtrees at which the molecular clock hypothesis was rejected ($p<0.001$). The numbers for interior branches represent bootstrap probabilities (only shown when ≥50 %) followed by the corresponding Bayesian posterior probabilities (only shown when ≥0.5). Topologies were rooted using the cladoceran *Daphnia* as an outgroup

branches ($p \leq 0.05$) located exclusively within dipterans (Fig. 3a). Interestingly, eight of these branches fall within the drosophilid subtree, including a highly significant branch at the root of this lineage ($p \leq 0.001$). Combined with local molecular clock analyses (Fig. 3b), these results indicate that episodic adaptive selection was probably responsible for the nonclock-like behavior of *Sxl* during its recruitment into sex-specific functions in drosophilids (Mullon et al. 2012).

To answer the second part of the question, we studied the specific protein positions targeted by selection in SXL. Significant evidence of adaptive selection was found at 15 codons ($p \leq 0.05$) predominantly located at N- and C-terminal regions (Fig. 4a). These results are consistent with functional studies showing that the sex-specific properties of extant *Drosophila* SXL depend on its global structure, and that modifications at N- and C-terminal domains of SXL in the drosophilid lineage represented coevolutionary changes determining the appropriate folding of SXL to carry out its sex-specific function (Ruiz et al. 2013). The analysis of the episodes of adaptive selection in *Sxl* revealed significantly higher proportions of nonsynonymous substitutions ($p < 0.05$) (Fig. 4b). More specifically, higher numbers of nonsynonymous substitutions were found at 33.3 % of the codons subject to episodic adaptive selection in the common ancestor of *Sxl* in Diptera (5 out of 15 codons); 13.3 % in the common ancestor of Drosophilidae, Calliphoridae, Muscidae, Tephritidae, and Sciaridae (2 out of 15 codons); 53.3 % in the common ancestor of Drosophilidae, Calliphoridae, Muscidae, and Tephritidae (8 out of 15 codons, highlighted with red boxes in Fig. 4b); 6.7 % in the common ancestor of Drosophilidae, Calliphoridae, and Muscidae (1 out of 15 codons); and 60 % in the common ancestor of drosophilids (9 out of 15 codons, highlighted with red circles in Fig. 4b). Two major conclusions can be drawn from these results: first, the diversification of *Sxl* in dipterans seems to have been driven by episodes of adaptive selection involving amino acid replacements at

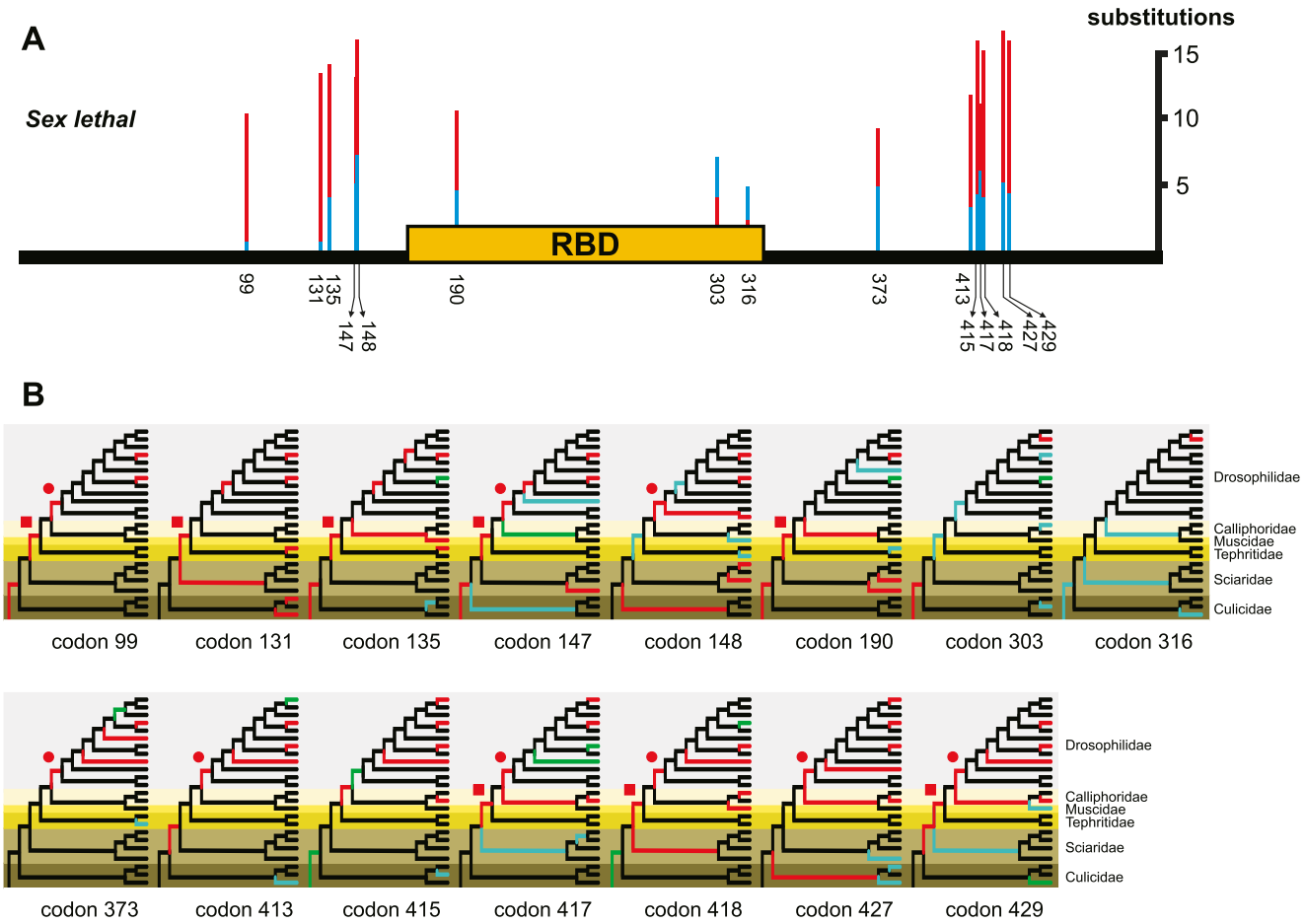

Fig. 4 Physical position and phylogenetic location of adaptive selection episodes involved in the recruitment of *Sxl* into sex-specific functions. **a** Numbers of synonymous (*blue bars*) and nonsynonymous (*red bars*) substitutions at codon positions subject to significant episodes of diversifying selection in dipterans ($p < 0.05$). **b** Phylogenetic location of the mutations involved in such episodes. *Branches in red* account for higher numbers of nonsynonymous mutations, *branches in blue* indicate higher numbers of synonymous mutations, and *branches in green* represent cases with same numbers of nonsynonymous and synonymous mutations. *Red squares* indicate codons displaying prevalence of nonsynonymous substitutions in the common ancestor of Drosophilidae, Calliphoridae, Muscidae, and Tephritidae. *Red circles* indicate the same but only in the common ancestor of Drosophilidae

specific codons in terminal protein domains. Second, the recruitment of *Sxl* into sex-specific roles required bursts of adaptive selection during the evolution of dipterans and most importantly in the common ancestor of drosophilids, probably taking advantage of its preexisting role as a general splicing factor (Ruiz et al. 2003; Serna et al. 2004).

Conclusions

The rates of evolution observed in sex-determining proteins suggest that the position of the different genes in the sex determination cascade has played a very important role shaping the molecular evolution of this pathway in insects. Accordingly, genes involved in early aspects of development (i.e., *Sxl*) are likely to remain more constrained than genes expressed later on (i.e., *dsx*, *fru*) due to the large deleterious pleiotropic effects of mutations at top positions of the cascade. Consequently, increased levels of purifying selection will observed at top positions of the cascade, while higher levels of variation will be observed at basal components interacting with diverse downstream factors (e.g., due to sexual selection). This is nicely illustrated by the recruitment of *Sxl* on top of the *Drosophila* cascade, involving bursts of adaptive selection in the common ancestor of drosophilids followed by the onset of purifying selection preserving the master regulatory role of this protein. In addition to providing us with a privileged insight into the mechanisms guiding the evolution of sex determination, the present work constitutes a powerful model for future studies on other functionally relevant coadapted gene complexes.

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