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Functional relevance of the newly evolved sperm dynein intermediate chain multigene family in *Drosophila melanogaster* males

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Abbreviations: *AnnX*, *Annexin X*; EST, expressed sequence tag; mya, million years ago; RACE, rapid amplification of cDNA end; *sw*, *short wing*; *Sdic*, *sperm dynein intermediate chain*

In many animal species, traits associated with male fitness evolve rapidly. Intersexual conflict and male-male competition have been suggested to drive this rapid evolution. These fast evolutionary dynamics result in elevated rates of amino acid replacement and modification of gene expression attributes. Gene acquisition is another mechanism that might contribute to fitness differences among males. However, empirical evidence of fitness effects associated with newly evolved genes is scarce. The *Sdic* multigene family originated within the last 5.4 myr in the lineage that leads to *D. melanogaster* and encodes a sperm dynein intermediate chain presumably involved in sperm motility. The silencing of the *Sdic* multigene family, followed by the screening of relevant phenotypes, supports the role of the *Sdic* multigene family in sperm competition. The case of the *Sdic* multigene family illustrates the flexibility of genetic networks in incorporating lineage-specific gene novelties that can trigger an evolutionary arms race between males.

The gene *Sperm dynein intermediate chain* (*Sdic*) is located at the cytological location 19C1 of the *X* chromosome of *D. melanogaster* but is absent in its closest relatives (**Fig. 1A**).[1,2] This gene was discovered during the characterization of one of its adjacent genes, *short wing* (*sw*) [aka *Cdic*], which encodes a cytoplasmic dynein intermediate chain.[3] In fact, the structure of the gene *Sdic* includes exonic sequences from the gene *sw* and from its other flanking gene *Annexin X* (*AnnX*). Additionally, the gene *Sdic* possesses one newly evolved exon and regulatory sequences.[4] Its chimeric nature and its presence as a tandem array of multiple copies suggested an evolutionary scenario associated with several consecutive segmental duplications.[4-6] Early functional analysis of one of the *Sdic* copies, *Sdic1*, indicated that its expression is confined to testis with its encoded protein being present in seminal vesicles and maturing spermatocytes, especially along their tails.[4] Given the expression profile of *Sdic1* and its structural relationship with *sw* (only the exons of the parental gene *sw* are present in the transcript of *Sdic*), it was proposed that the SDIC protein is a sperm-specific axonemal dynein intermediate chain and therefore putatively relevant to the fertility of males. Genes associated with reproduction are potential targets of sexual selection, e.g., through male-male competition.[7] In the case of the *Sdic* multigene family, sexual selection may adopt the form of sperm competition, thus explaining the rapid evolution of *Sdic*. This notion would be consistent with the multiple rearrangements this region has undergone in a very short evolutionary period and with unusually low levels of nucleotide variation.[8,9] However, empirical evidence of the phenotypic effect associated with the *Sdic* multigene family, and therefore of its presumably adaptive role, was still lacking.

In a recent report, we knocked out the whole *Sdic* multigene family at 19C1.[10] Four copies of *Sdic* are annotated in the most recent release of the *D. melanogaster* genome. For at least two of these copies, *Sdic1* and *Sdic3*, there is unambiguous evidence of their expression in adult males.[4,10] We induced the deletion of the *Sdic* multigene family through an ectopic recombination event between two flanking FRT-bearing transposable elements. Since the deletion of the *Sdic* multigene family also spans the essential gene *sw*,[10-12] a *sw* transgene was introduced into the genome of the engineered stocks. The transgene recapitulates the endogenous expression of the gene *sw* in whole males[10] and in testis (**Fig. 2**). The absence of the *Sdic* multigene family did not result in obvious sperm malformations or significantly diminished fertility. However, the silencing of the *Sdic* multigene family did lead to an impairment of the competence of the sperm in experimental settings involving consecutive matings between one female and two individual males.[13] This phenotype is likely to be important for fitness because polyandry is well

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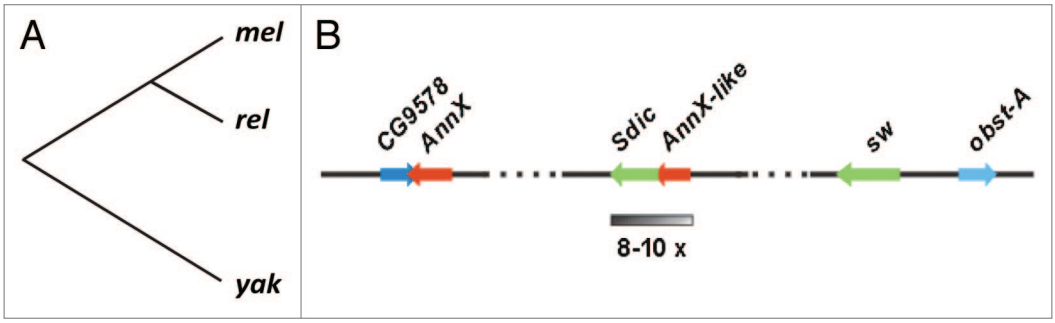


Figure 1. (A) Phylogeny of *D. melanogaster* (*mel*), its closest relatives (*rel*: *D. simulans*, *D. sechellia*, and *D. mauritiana*), and the outgroup species *D. yakuba* (*yak*). The *Sperm dynein intermediate chain* (*Sdic*) multigene family is only present in *D. melanogaster* and therefore it must have been originated after its lineage branched off from that leading to its closest relatives 5.4 mya.[36] (B) Details of the molecular organization of the *Sdic* multigene family at 19C1 of the *X* chromosome. Although four copies are annotated in FlyBase,[25] our computational analyses revealed the existence of additional haplotypes that denote the existence of further copies, in good agreement with earlier estimates (see main text). The presence of additional copies of the gene *Sdic* could conflict with models previously proposed for the generation of the multigene family.[6]

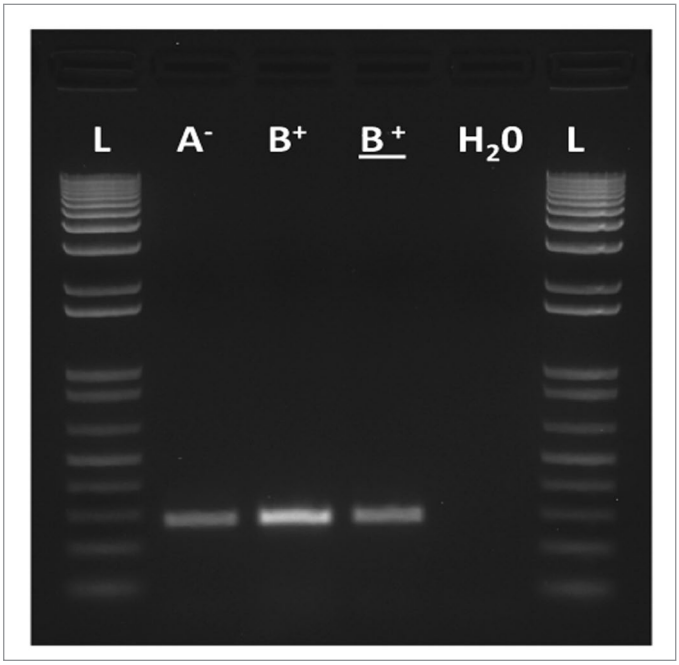
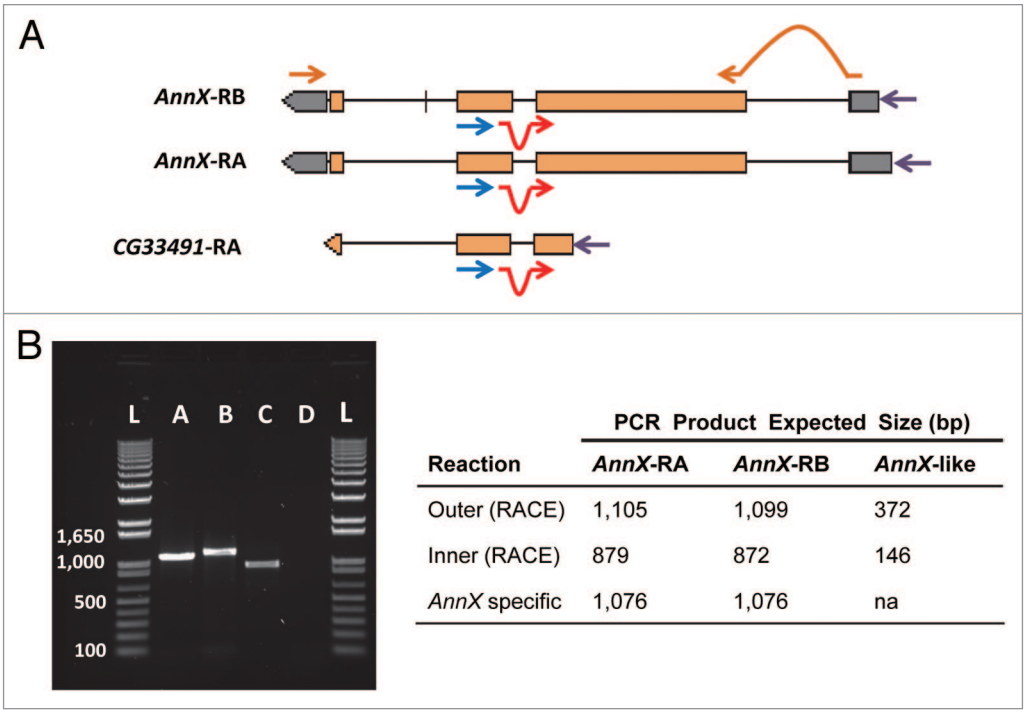


Figure 2. Molecular verification by RT-PCR of the proper expression of the sw transgene in testis of males with (A^-) and without *Sdic* (B^+). The lane of the underlined B^+ corresponds to males carrying the endogenous copy of *sw* but not the transgene. H_2O, negative control (no cDNA was added); L, ladder. Primers and methods are as described.[10]

documented in natural populations of *D. melanogaster*.[14-18] The type of assay performed compares the ability of the sperm from males with and without the *Sdic* multigene family (the experimental males) to displace or inactivate the sperm of a reference wild-type male, which results in differences in the fraction of the progeny fathered by each male. The experimental males can be first or second to mate relative to the reference male depending on the assay. In the assay in which the experimental males were second to mate, we detected that the fraction of progeny fathered by the knockout experimental male was significantly reduced compared with that of the experimental male carrying the *Sdic* multigene family. The molecular mechanisms whereby this diminished competence appears remain unknown. It is possible that mobility of the sperm, which has been proposed to affect fertilization efficiency,[19] had been subtly altered. This altered mobility can also affect sperm utilization upon contact with particular accessory gland proteins, some of which have been shown to influence the outcome of sperm competition due to their involvement in sperm storage and displacement.[20] Likewise, there could be an altered interaction with the proteins secreted by the spermathecal secretory cells in females[21] or a combination of any of these scenarios. The phenotype detected supports that the *Sdic* multigene family indeed has a measurable impact on male fertility in spite of its recent origin.

Several important aspects of our findings warrant further clarification. First, the phenotype observed as a consequence of the silencing of the *Sdic* multigene family does not seem to diminish sperm motility qualitatively in non-competitive conditions. One explanation is that at least one other gene is performing this function. An alternative explanation is the presence of at least one additional functional copy of the gene *Sdic* outside 19C1 on the *X* chromosome, which would have not been knocked out by our silencing approach. Computational analysis using raw sequences absent in the main assembly of the reference strain of *D. melanogaster* y^1; *cn* bw^1 sp^1 [22] supported the existence of additional haplotypes that must belong to other copies of the gene *Sdic* (**Fig. 1B**). This result would be in good agreement with previous estimates based on Southern blot experiments,[4] and the restriction profile of BACs (Berkeley Drosophila Genome Project; unpublished results) and P1 phages (J.M. Ranz, unpublished results). In situ hybridization experiments on mitotic chromosomes rejected this scenario, eliminating the possibility that extra copies outside the cluster of 19C1 on the *X* chromosome exist, e.g., in poorly annotated regions of the *D. melanogaster* genome like the *Y* chromosome. Another possibility is that the gene *sw*, which is also known to be involved in sperm differentiation,[23] may encode protein isoforms functionally equivalent to the SDIC protein. Although multiple SW protein isoforms exist, they all differ significantly from the SDIC



Reaction	PCR Product Expected Size (bp)		
	AnnX-RA	*AnnX*-RB	*AnnX*-like
Outer (RACE)	1,105	1,099	372
Inner (RACE)	879	872	146
AnnX specific	1,076	1,076	na

Figure 3. Functional test of the expression of *AnnX*-like genes. (a) Outline of the strategy followed. 5' RACE experiments were performed to detect the presence of the putative transcripts corresponding to the *AnnX*-like genes in whole body and testis of 5-d-old males. The experiments were done following the instructions of the kit from Epicenter ExactSTART Eukaryotic mRNA 5'-&3'-RACE. Primers used: outer primer (blue; 5'-GGA TCC AAG TAG GGC TGT CA-3'), located on the third exon of *AnnX* and the second exon of *AnnX*-like (only *CG33491* is shown); inner primer (red; 5'-GAC TGG ACG CAC TCA ACT ATG G-3'), located at the junction of the second and third exons; and a 5' primer from the kit used (purple). The sequences of the outer and inner primers are complementary to those of the two *AnnX* transcripts and to that putatively transcribed from the *AnnX*-like genes according to FlyBase.[25] *AnnX* transcript specific primers (brown) were used for RT-PCR experiments with the only purpose to test for the quality of the cDNA and the presence of genomic DNA. (B) Results for the expression profiling experiments in testis. Left, 5' RACE products amplified from cDNAs of total RNA from males carrying the wild-type configuration for the *Sdic* multigene family (B^+). Lanes: 1) PCR product using *AnnX* specific primers; 2) PCR product using the outer primer and the 5' primer; 3) PCR product using the inner primer and the 5' primer; 4) H_2O, negative control (cDNA but no primers added); L) ladder. Right, summary table with the expected size of the PCR products for the two transcripts of *AnnX* and for the putative transcript of *AnnX*-like genes. Evidence for the presence of the two *AnnX* transcripts was detected but not for the putative transcript of *AnnX*-like genes. Experiments using total RNA from whole bodies as starting material shown identical results (not shown).

protein at both termini.[4,5] Recently, the first axonemal dynein intermediate chain gene has been characterized in *D. melanogaster*.[24] The gene *Dic61B* exists as a single copy, is expressed in testis, and shows homology to axonemal dynein intermediate chain encoding genes from other organisms, including that of the gene *DNAI1* in humans. Importantly, when the gene *Dic61B* is knockout, sperm individualization fails. Since the gene *Dic61B* is found in *D. simulans*, we hypothesize that it is the truly indispensable axonemal intermediate chain encoding gene, while *Sdic* may be still evolving its function affecting the competence of the sperm of *D. melanogaster* in a more subtle manner.

A second important aspect is the phenotype detected in the absence of the *Sdic* multigene family. In recent releases of the *D. melanogaster* genome assembly, the residual stretches of the parental gene *AnnX* still part of the *Sdic* gene structure have been annotated as independent transcriptional units (*CG33491*, *CG33496*, *CG33487*, and *CG33498*; collectively *AnnX*-like hereafter).[25] Evidence of the expression of these genes stemmed from an RNA-seq experiment,[26] which indicated that these *AnnX*-like genes reach their peak of expression in 5-d-old males. These transcriptional units are part of the deleted chromosomal stretch. If these *AnnX*-like genes can somehow affect male fertility, it is unclear whether the reduced sperm competence observed results partially or entirely from their deletion as opposed to the deletion of the gene copies of *Sdic*. 5' RACE experiments using 5-d-old males did not find evidence of transcription of the *AnnX*-like gene upstream *Sdic* (**Fig. 3**), in good agreement with the absence of supporting ESTs for these genes.[27] Collectively, these results point to a potential artifact in the current functional annotation of the *AnnX*-like genes.

The acquisition of new genes with the potential to affect male fitness is widespread across taxa.[28-30] Recently originated genes can become stable components of the genome if they contribute to differences in reproductive success.[7] Empirical evidence of the impact of newly evolved genes on male fertility has only been documented in very few cases.[31-35] Our functional characterization of the *Sdic* multigene family precisely illustrates the genomic consequences of the arms race established among males, which continuously, and in a very short evolutionary time, reshapes the genetic network that underlies male reproductive traits.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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