The role of testis-specific gene expression in sex chromosome evolution of *Anopheles gambiae*.

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**ABSTRACT (50 word max.)**

Gene expression in *Anopheles gambiae* shows a deficiency of testis expressed genes on the X chromosome associated with an excessive movement of retrogene duplication. We suggest the degeneration of sex chromosomes in this monandrous species is related to the biological and evolutionary forces of X-inactivation, dosage compensation and sexual antagonism.

**NOTE**

In a heterogametic sex determination system, the sex chromosomes (X or Z) spend a disproportionate amount of evolutionary time in one gender over the other (VICOSO and CHARLESWORTH, 2006). For example, relative to autosome evolution, the X chromosome occurs twice as frequently in females compared to males, thus new sex-linked mutations will be exposed to selection two thirds of the time in females and only one third in males. One expected consequence of this relationship is that the sex-related content of autosomes and sex chromosomes should be unequally distributed through the genome as a result of antagonism between the sexes (RICE, 1984). It is predicted that dominant or partially dominant mutations beneficial to females (and detrimental to males) spread more easily if they are X-linked, whereas recessive mutations beneficial to males (and detrimental to females) should accumulate on the X chromosome, because sexual antagonism will be masked in heterozygous females.

Even though the outcome of sexual antagonism is expected to be highly dependent on the dominance of mutations in question, several genomic studies have found significant differences in the gene content of autosomes and the X chromosome. First, sex-biased gene expression in somatic and germline tissue is often non-randomly distributed among chromosomes (PARISI et al. 2003; RANZ et al. 2003; KHIL et al. 2004). Second, several studies have reported an excess of duplicated genes moving from the X chromosome to the autosomes (BETRAN et al. 2002; EMERSON et al. 2004; VIBRANOVSKI et al., 2009; MEISEL et al., 2009). While sexual antagonism no doubt contributes to the observed deficiency of sex-biased genes in somatic tissues, a further consideration within the male germline is X inactivation. Evidence from *Drosophila* indicates that gene duplications escaping the X chromosome have a higher likelihood of testis expression and are retained on the autosomes because they are required during...
spermatogenesis (BETRAN et al. 2002; VIBRANOVSKI et al., 2009; MEISEL et al., 2009). Thus, X inactivation can generate selection for increased dosage and favour the fixation of autosomal gene duplications, whether or not they are in fact sexually antagonistic.

Heteromorphic sex chromosomes have evolved independently multiple times in the Diptera. In the mosquito, *Anopheles gambiae*, males and females have fully differentiated heteromorphic sex chromosomes, whereas in *Aedes aegypti* the sex chromosomes are homomorphic and largely homologous (KRZYWINSKI et al. 2004; NENE et al. 2007). By classifying the origin of retrotransposition events between these species, Toups and Hahn (2010) found evidence for an excess of X-to-A turnover, primarily in the *Anopheles* lineage, indicating that their common ancestor had homomorphic sex chromosomes. Yet, in contrast to higher Diptera, they found no evidence that X-to-A duplications are associated with male-biased gene expression, leading to the conclusion that sex chromosome evolution is not driven by patterns of sex-specific gene expression in *Anopheles* (HAHN and LANZARO, 2005; TOUPS and HAHN, 2010). An important difference between *Anopheles* and other species investigated to date is that females only mate once during their lifetime (TRIPET et al., 2003); a key attribute affecting male testis size (HOSKEN and WARD, 2001). Whereas much of the sex-biased expression displayed by *Drosophila* and other polygonous species results directly from expression in the gonads (PARISI et al., 2003), in *Anopheles* the testis are proportionately much smaller compared to body mass, and consequently when transcription levels are assessed at a whole-body level, male-biased expression is less pronounced.

A recently published catalogue of tissue-specific expression in *Anopheles* (MozAtlas, [www.tissue-atlas.org](http://www.tissue-atlas.org)) reveals that fewer than 10% of testis-enriched genes are male-biased in expression because they are largely undetected in whole-body samples (BAKER et al., 2011). This contrasts with the ovaries in which over 50% of genes have female-biased expression, a finding which is likely to account for elevated levels of female transcription observed in whole-body samples (HAHN and LANZARO, 2005). As we have reported previously (BAKER et al., 2011), this dataset reveals there is also an under-representation of genes on the X chromosome expressed within the testis, consistent with studies in higher Diptera (PARISI et al. 2003).

In light of these findings, we have re-examined the relationship between *Anopheles* retrogene duplications and male-related gene expression (Suppl. File 1 for methods). Using recent orthology assignments (VILLELLA et al., 2009), and in agreement with Toups and Hahn (2010), we observe an excess of retrogene relocations out of the X chromosome after the Dipteran split (Fig. 1A). Importantly, while our dataset is more stringent, we also find that the majority of X-to-A duplication events occur within the *Anopheles* lineage (n=16/23; 53% Excess). However, in contrast to earlier findings, when male-related gene expression is compared with retrogene movement, we find that testis expression is a common feature of duplications derived from the X chromosome (X-to-A=17/18; 95%), whereas retrogenes originating from the autosomes are less likely to be expressed in the testis (A-to-A=18/37; 48%) ($\chi^2 = 9.08$, df = 1, P=0.002). Thus, we find support for a link between the movement of gene duplications and male-related gene expression.

One expectation for models involving X inactivation as the underlying cause of male-related genes moving off the X chromosome, is that ancestral X-linked genes should have low expression levels in the testis when compared to autosomal retrogenes. Indeed, we find that derived retrogenes
have a higher incidence of tissue-specific expression than ancestral genes (Fig. 1B; Unpaired Wilcoxon test; P<0.05). Unfortunately this difference in itself cannot differentiate the effects of X inactivation versus sexual antagonism, because the latter also predicts a spread of male beneficial dominant mutations to the autosomes (including testis-expressed genes). However, we find that testis enrichment, in particular, is higher for retrogenes in X-to-A relocations compared to retrogenes in A-to-A duplication events, whereas ancestral genes show the opposite effect, i.e. ancestral genes in X-to-A duplications have significantly lower testis enrichment in ancestral genes when compared to A-to-A duplication events (Table 1). Since sexual antagonism does not predict a difference in gene expression between autosomes based on where the ancestral gene was located, deviation in testis expression of X-to-A versus A-to-A duplicated genes suggests the involvement of X-inactivation (VIBRANOVSKI et al., 2009).

However, a third mechanism we must consider which could deplete male-related genes on the X chromosome is the action of dosage compensation systems. Dosage compensation acts to balance gene expression between the X chromosome and autosomes of the heterogametic sex. When the X chromosome is hyper-activated in males, increased expression in the testis may be harder to achieve for single X-linked genes than autosomal genes if transcription rates are limited, thus reducing the accumulation of male-biased genes on the X chromosome (VISCO and CHARLESWORTH, 2009; BACHTROG et al., 2010). We find that while male and female somatic tissues are expressed at the same level on autosomes and the X chromosome (Soma: \(X_{\text{female}}/A_{\text{female}}: 1.07-1.23; X_{\text{male}}/A_{\text{male}}: 1.08-1.24\); Suppl File 1), male germline expression on the X chromosome is half of the autosomal levels, as would be expected in the absence of dosage compensation (Germline: \(X_{\text{female}}/A_{\text{female}}: 1.03; X_{\text{male}}/A_{\text{male}}: 0.48\); Unpaired Wilcoxon test \(P<8.22\times10^{-13}\)). Subsequently, unlike Drosophila (GUPTA et al., 2006), we only detect X chromosome hyper-activation in the mosquito male soma, indicating that an absence of male germline dosage compensation may contribute to a deficiency of male-related genes on the X chromosome in Anopheles.

Taken together, these observations suggest retrogene movement, as a result of expression in the male testis, may be a general feature of sex-chromosome evolution in the Diptera. In the future it will be of considerable interest to verify whether X chromosome inactivation or dosage compensation occurs in the male germline of Anopheles, to help resolve which mechanisms contribution to this trend. Since Y chromosome degeneration appears to have evolved rapidly in Anopheles after its divergence from Aedes, further study is likely to yield important insight into how these processes and modes of sexual antagonism contribute to sex chromosome evolution in monandrous species like the mosquito.

For example, if we extend our original analysis of germline and somatic expression to consider the chromosomal distribution of genes only expressed in single tissues, unlike D. melanogaster (MUELLER et al., 2005), we find an excess of genes detected solely in the male accessory glands of Anopheles (Fig 1C). Male Accessory Gland (MAG) proteins are an essential component of seminal fluid deposited into the female reproductive tract during copulation, often associated with male reproductive success (KLOWDEN and RUSSELL, 2004; ROGERS et al., 2008). While the relative importance of such proteins to sexual conflict in the mosquito is still debated, an over-
representation of MAG proteins on the X chromosome suggests that at least some components of male reproductive biology are potentially antagonistic in this species. Indeed, it is predicted that recessive genes conferring a male advantage at the expense of females should be located on the X chromosome (RICE, 1984). However, if sexually antagonistic mutations are often dominant, this could explain a deficiency of male-biased genes on the X chromosome, including somatically expressed gene.

REFERENCES


Figure 1.

A. Dipteran retrogene events. We find an excess of X-to-A retrogene events occurring within the *Anopheles* lineage, but not on the branch prior to the *Anopheles* and *Aedes* split. (X) X chromosome; (A) Autosomes; Excess = [(O – E)/E] x 100.

B. Tissue-specificity of retrogene and progenitors. Retrogene show a higher incidence of tissue-specific gene expression. Unpaired Wilcoxon test; P<0.05.

C. X Chromosome enrichment of genes expressed in reproductive tissue. Consistent with previous studies, there is a deficiency of testis-specific genes on the *Anopheles* X chromosome, but an over-representation of male accessory gland genes (Expected X : 8.5%) (Chi-squared Test; *P < 0.05).

Table 1. Testis gene expression enrichment. Testis enrichment is significantly higher for retrogene in X-to-A relocations when compared to A-to-A duplications across branches, whereas testis enrichment of ancestral genes (progenitors) in X-to-A relocations is significantly lower than in A-to-A duplications. (X) X chromosome; (A) Autosomes. See Supplementary File 2 for expression values and significance tests.

<table>
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<th>Progenitors</th>
<th>Retrogene</th>
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<td>Direction</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enriched</td>
</tr>
<tr>
<td>X-to-A</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>A-to-A</td>
<td>8 (32%)</td>
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Fisher’s Exact test P = 0.016

Fisher’s Exact test P = 0.006
A. gambiae

Ae. aegyptii

D. melanogaster

### Significance of X-to-A

<table>
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<td>9.3</td>
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<td>-24.4%</td>
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<tr>
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<td>7.4</td>
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\[ X^2 = 5.80, \text{ d.f.} = 2, P = 0.055 \]

### Significance of A-to-A

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<td>-76.1</td>
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\[ X^2 = 7.84, \text{ d.f.} = 2, P = 0.0197 \]