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Comparison of K^+-channel genes within the genomes of *Anopheles gambiae* and *Drosophila melanogaster*

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Abstract

Background: Potassium channels are the largest and most diverse type of ion channel found in nature. The completion of the sequencing of the genomes of *Drosophila melanogaster* and *Anopheles gambiae*, which belong to the same order, the Diptera, allows us to compare and contrast K^+-channel genes and gene families present within the genomes of two dipterans.

Results: This study identifies at least eight voltage-gated K^+-channel genes in *Anopheles*, as well as three Slo-family, three Eag-family and six inward rectifier K^+-channel genes. The genomic organization of K^+-channel genes from *Drosophila* and *Anopheles* is well conserved. The sequence identity of the most similar K^+-channel gene products between these two species ranges from 42% to 98%, with a mean value of 85%. Although most K^+-channel genes in *Drosophila* and *Anopheles* are present in a 1:1 ratio, *Anopheles* has more genes in three K^+-channel types, namely KQT, Kv3, and inward rectifier channels. Microsynteny between the genes flanking K^+-channel genes in *Drosophila* and *Anopheles* was seldom observed; however, most of the K^+-channel genes are indeed located at positions which a previous genome-wide comparison has designated as homologous chromosomal regions.

Conclusions: The *Anopheles* genome encodes more voltage-gated and inward rectifier K^+-channel genes than that of *Drosophila*. Despite the conservation of intron-exon boundaries, orthologs of genes flanking K^+-channel genes in *Drosophila* are generally not found adjacent to the *Anopheles* K^+-channel orthologs, suggesting that extensive translocation of genes has occurred since the divergence of these two organisms.

Background

The rapid rate of sequence acquisition has revolutionized molecular biology. The sequencing of entire genomes, in addition to new computer-based search tools has allowed us to identify and analyze large sets of data very rapidly. The acceleration of data acquisition, in fields such as whole-genome sequence determination and genome-wide gene-expression profiling, has opened the door for the study of model organisms and organisms of importance to the study of medicine and disease states by allowing for the analysis of the entirety of genetic information in a given organism. The recent completion of the sequencing of the *Anopheles gambiae* genome provides us with the entire genetic makeup of this organism. Furthermore, the completion of the sequencing of both the *Drosophila melanogaster* [1] and *Anopheles gambiae* [2] genomes provides the first opportunity for genome-wide comparisons from two metazoans from the same order (Diptera). This presents new opportunities to

detect synteny groups and facilitates the comparison of splicing patterns and orthologous sequences between these two organisms.

The first K^+ channel gene identified was cloned from *Drosophila*. The *Shaker* gene was isolated by positional cloning of a gene for which a mutation causes a leg-shaking phenotype in anesthetized flies [3,4]. This gene encodes a six-transmembrane protein (Figure 1) subunit which assembles as a tetramer. This gene provided a molecular probe by which other K^+ channel genes could be isolated by hybridization, and later, by computer-based homology search. This led to the cloning of different K^+ channel subunits and the discovery of different K^+ channel types [5]. Subsequent to the cloning of *Shaker*, K^+ channel genes from the *Shab*, *Shaw* and *Shal* families (later renamed Kv2, Kv3, and Kv4, respectively, for clarity [6]) were identified in *Drosophila*. These sequences are shown in the alignment in Figure 2a and a tree is shown in Figure 3a. Later, other types of K^+ channel subunits were identified by hybridization, with the conserved pore region generally used as a probe, or by positional cloning using neurological mutants in *Drosophila melanogaster* and other organisms. Among these channel types were KQT channels, calcium-activated K^+ channels, inward rectifier K^+ channels, and the two-pore K^+ channels [7]. The sequencing of the *Drosophila* genome provided evidence that the vast majority of K^+ channel genes in the fruit fly have been identified, since certain domains within K^+ channels, particularly the pore region, are readily identifiable by homology.

Other K^+ channel types possess the same conserved pore domain sequence as the Kv and KQT channels. Among the six-transmembrane channels, there are two additional families. The Eag gene family consists of *eag*, *erg* (*seizure*) and *elk*; one of each is present in the *Drosophila* genome [8]. The other 6TM K^+ channel gene family is the Slo family. These genes encode Ca^{2+}-activated K^+ channels of large conductance, intermediate conductance and small conductance: these are thought to be mediated by Slo, slack and SK channels, respectively. These K^+ channels are shown in Figure 2b.

Another family of two-transmembrane K^+ channels called inward rectifier K^+ channels exists as well. Although these channels lack a voltage-sensor domain they play an important role in controlling resting potential and K^+ homeostasis. Between the two transmembrane domains these channels possess a pore sequence homologous to the pore domain found in Kv, Eag, and Slo channel types. Three Kir genes have been reported in *Drosophila* [9]. Two of these genes, *Irk2* and *Irk3*, are quite similar at 54% amino acid sequence identity while a third member is roughly 27% identical to the other two. Finally, although they will not be investigated in this study, a group of four-transmembrane, two-pore K^+ channels exists. These tandem-pore channels may be involved in a wide range of physiologic processes but are generally thought to mediate leak conductances which influence resting membrane potential. All the K^+ channel genes mentioned here contribute to K^+ channel conductance in excitable and/or non-excitable cells. In the nervous systems of insects and other metazoans K^+ channels are known to play an important role in perception, learning and locomotion. This paper will investigate the genes encoding K^+ channels of two distantly related Diptera now that their entire genomes have been made public.

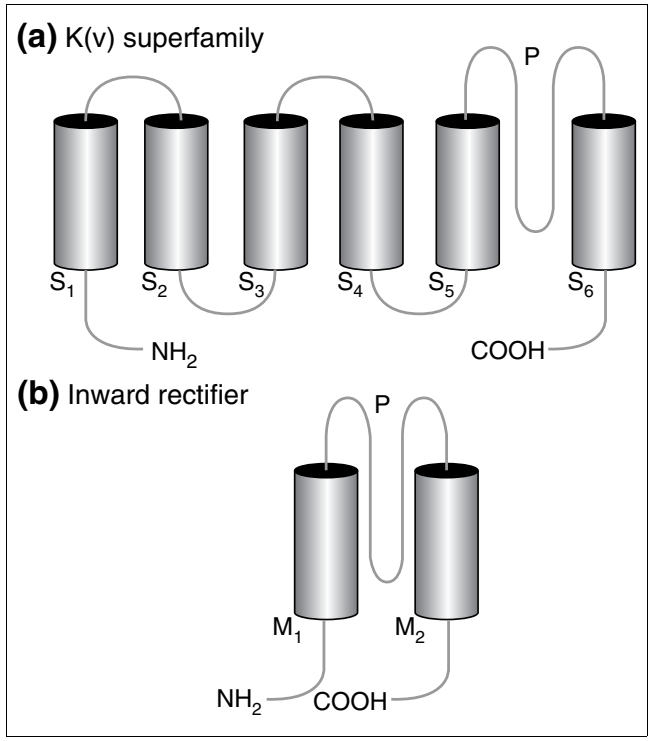

Figure 1
Membrane topology of K^+-channel subunits. The membrane topology of the Kv-superfamily **(a)** and inward rectifier **(b)** channel subunits are illustrated. The letter P is shown at the conserved pore domain and the cytoplasmic amino and carboxyl termini for both types of channel subunits are shown.

Results

The entire set of predicted protein sequences from *A. gambiae* was downloaded from the National Center for Biotechnology Information [10]. A Perl script was written to search for proteins containing the conserved GYGD (single-letter amino-acid code) K^+-channel pore/selectivity filter motif. To reconcile the fact that computer-generated open reading frame (ORF) predictions might be imperfect I also used TBLASTN to screen for proteins containing this pore region using the amino-acid sequence of pore regions from the major K^+-channel families from *Drosophila*. Although a definitive sequence analysis of full-length proteins cannot be accomplished until the cloning of cDNAs and ESTs is carried out, the high degree of similarity between the genes from

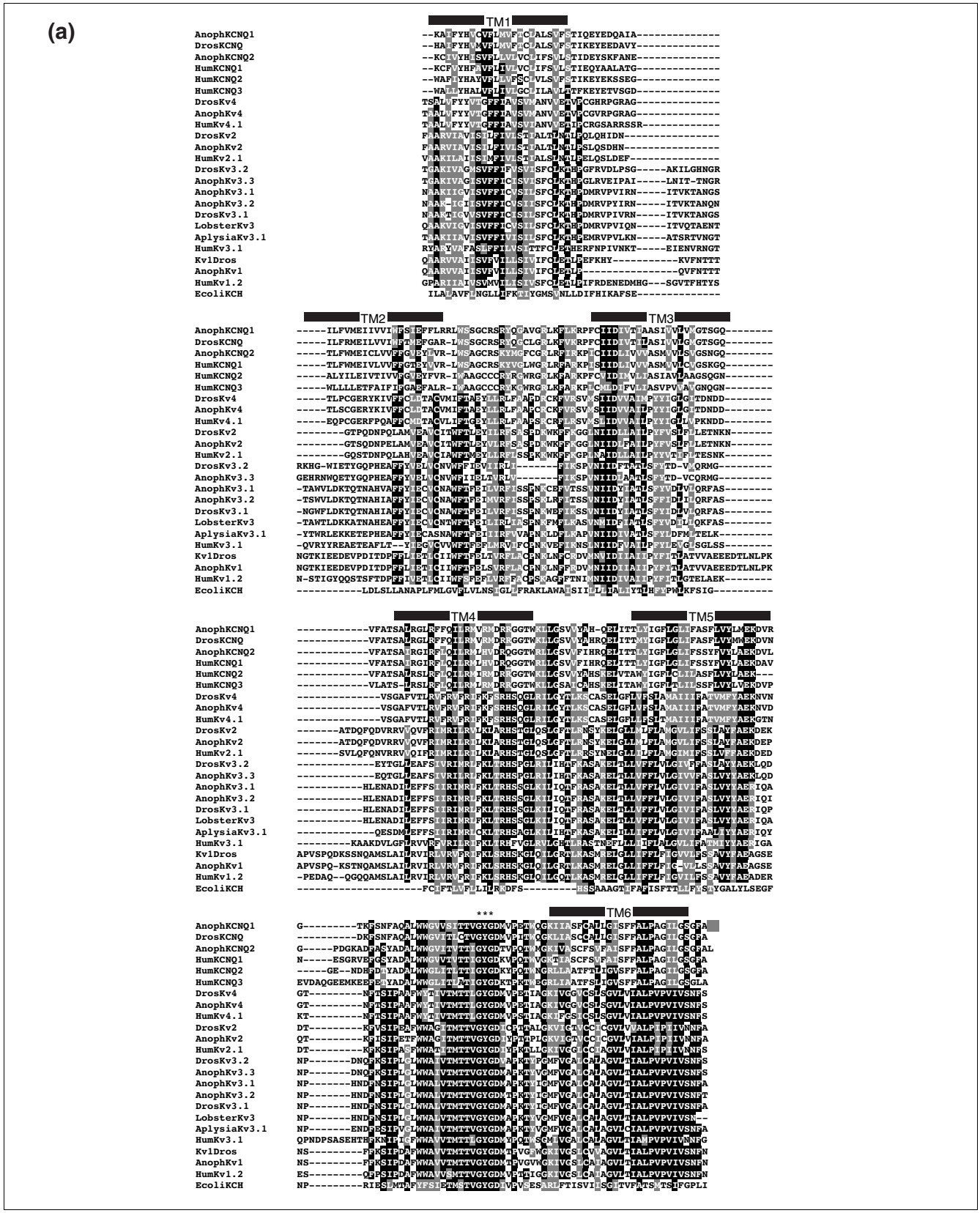

Figure 2 *(see the legend on the page after next)*

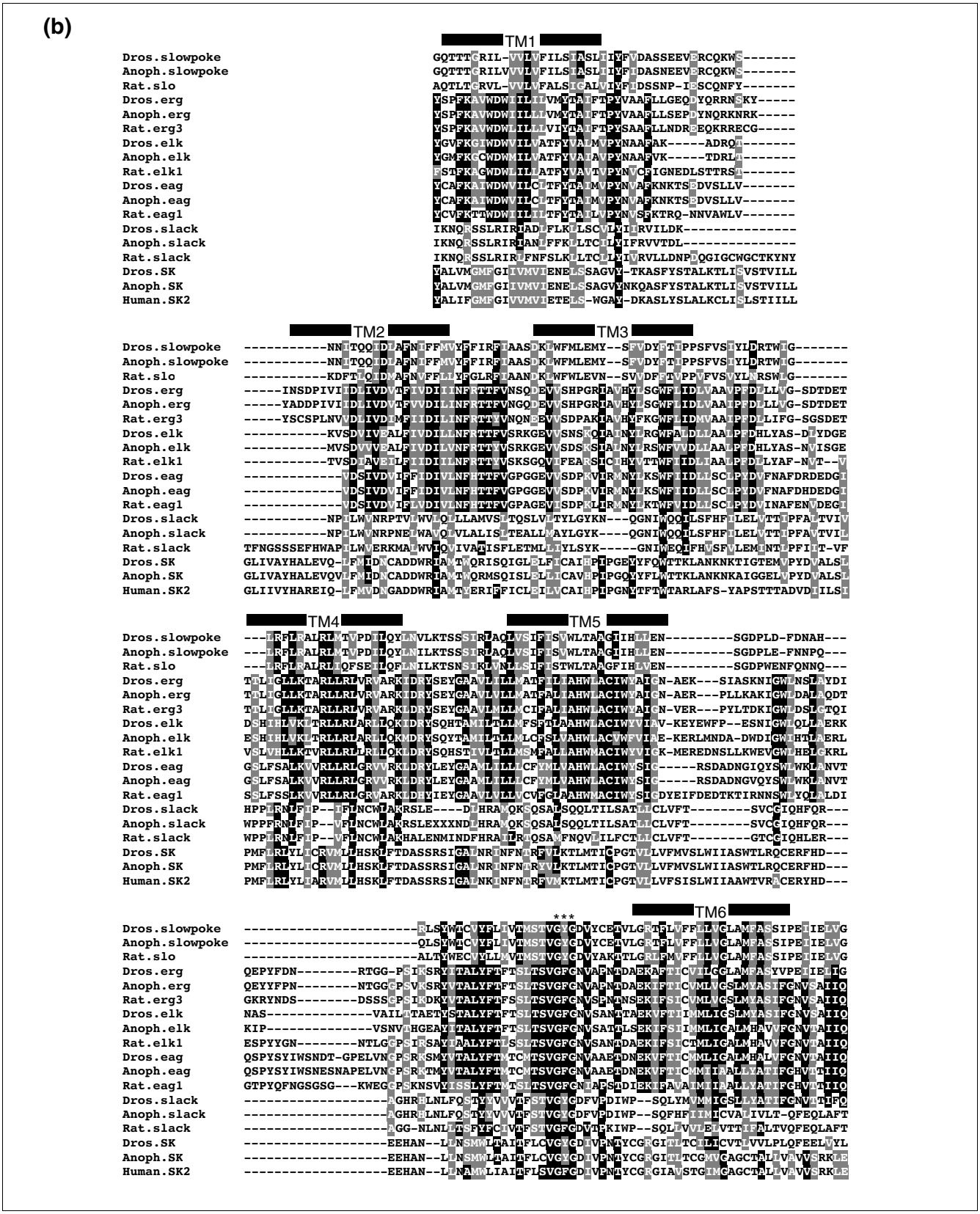

Figure 2 *(continued from the previous page, see the legend on the next page)*

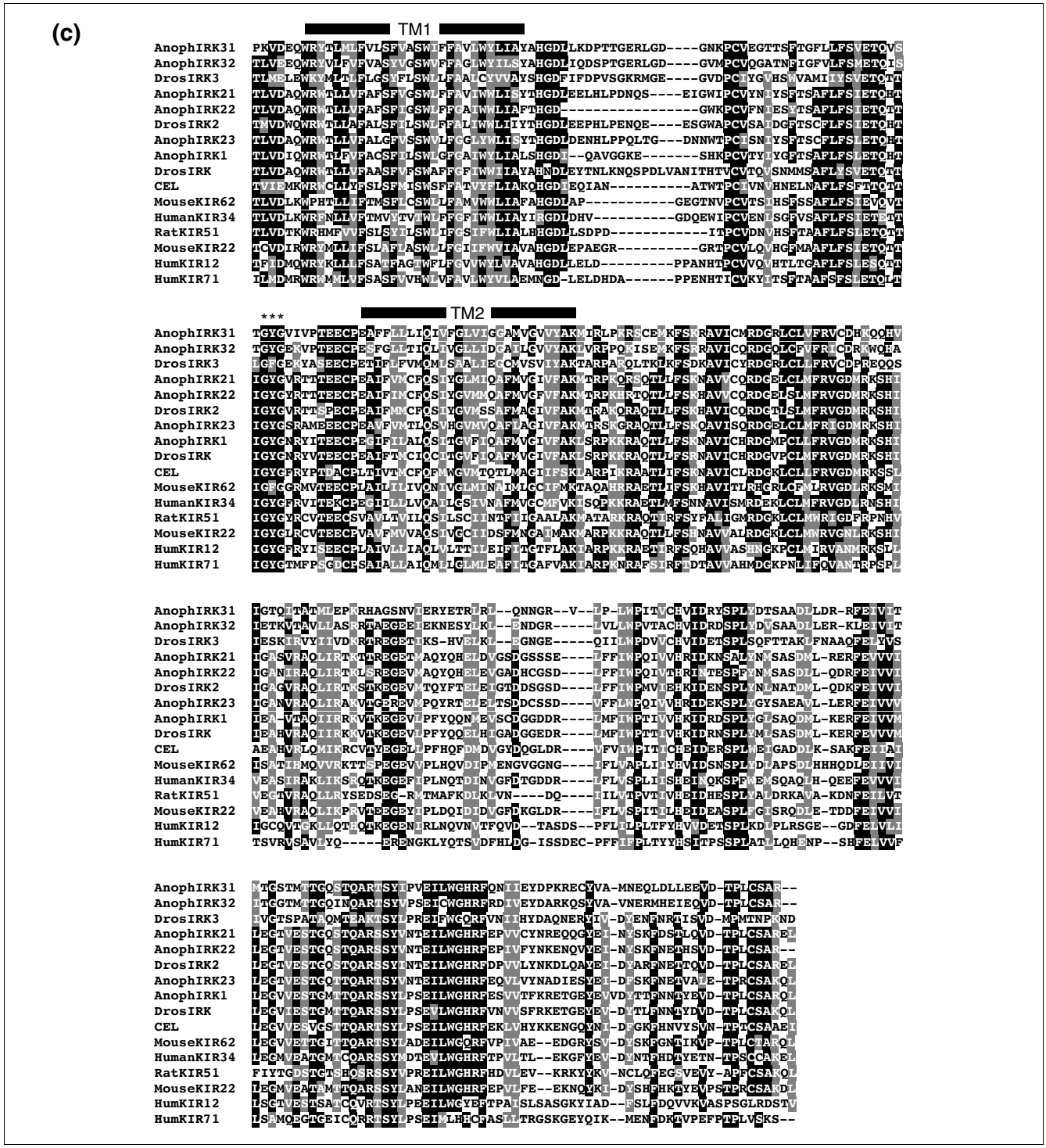

Figure 2
Multiple alignment of channel sequence from *Anopheles gambiae* and other organisms. **(a)** Voltage-gated K^+-channel sequences; **(b)** Slo and Eag family K^+ channels; **(c)** inward rectifier K^+ channels. Alignments were generated with the ClustalX program, and highlighted with BOXSHADE. Sequences were chosen to illustrate diversity of *Drosophila* and mammalian K^+-channel types, in addition to the *Anopheles* sequences. Transmembrane domains are labeled with a horizontal bar and the conserved GYG of the pore is marked by asterisks. The letter X represents regions at which amino-acid prediction was particularly difficult in *Anopheles* sequences, generally because of short exons in coding regions. *Anopheles* sequences are predictions based on regions homologous to *Drosophila*, as opposed to confirmed sequence data.

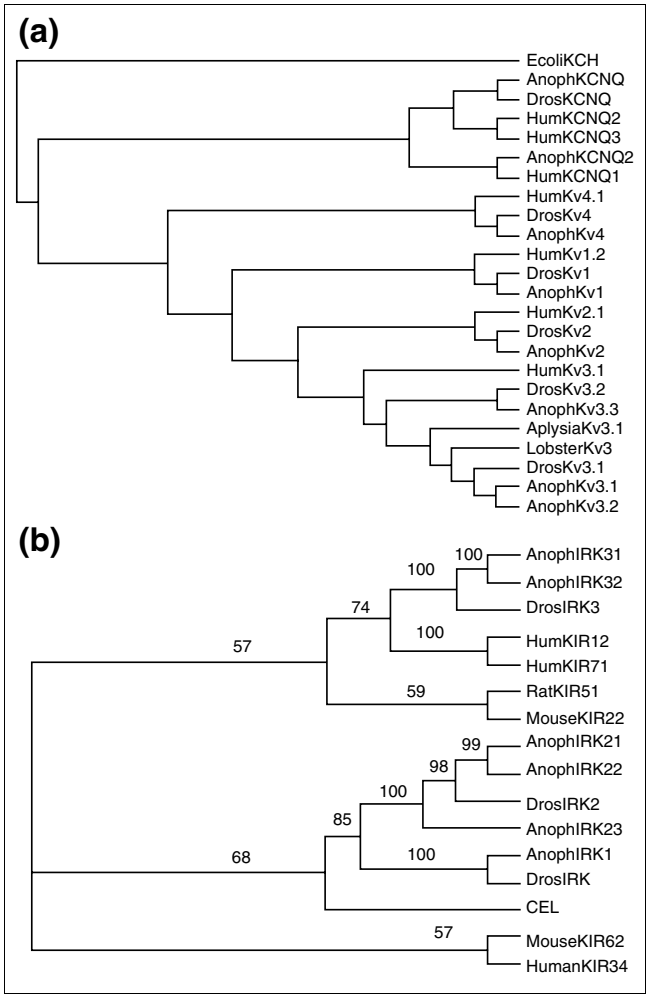

Figure 3

Phylogenetic trees of K^+-channel types. **(a)** Voltage-dependent K^+ channels; **(b)** inward rectifier K^+ channels. Sequences were aligned using ClustalX. Six transmembrane channel sequences are confined to the region spanning the first through the sixth transmembrane domain, so as to remove highly variable sequence. Similarly, variable amino-terminal sequences of inward rectifier K^+ channels were removed in order to exclude highly variable sequence. The culled alignments were then used to construct a maximum likelihood tree in (a) and a neighbor-joining tree in (b). The tree in (a) uses the *Escherichia coli* KCH K^+ channel homolog (Genbank accession number 808903) as an outgroup. For the tree in (b), the bootstrap values above the branch before each node are based on 1,000 replicates and are a measure of robustness at each node. The Jones-Taylor-Thornton amino-acid substitution matrix was used in the maximum likelihood calculations from PHYLIP. The sequences in (b) are shown without the punctuation such that MouseKIR22 = Mouse KIR2.2, and so on. The sequence named CEL is *Caenorhabditis elegans* inward rectifier K^+ channel (gi 7511460).

Anopheles and the well-characterized K^+-channel genes in *Drosophila* allows us to compare the genomic organization and to predict coding regions with a high degree of confidence.

A series of BLAST searches [11,12] was carried out using the amino-acid sequence at the *Drosophila melanogaster* Shaker, Shab, Shaw, and Shal (Kv1, Kv2, Kv3, and Kv4) K^+-channel pore region (from SwissProt P08510, P17970, P17971, P17972) as the query sequence against the DNA of the *Anopheles* genome. In addition, a probabilistic ancestral sequence (the most recent ancestor of the four major K^+-channel families) was used as a query sequence with the hope that more divergent sequences (for example, specialized K^+-channel types) might be identified. The first search, using the amino acids spanning the *Shaker* K^+-channel pore sequence from *Drosophila* as the query, revealed that the *Shaker* ortholog in *Anopheles* is located at chromosome X:3D (see Figure 4) and has 86% identity to the *Drosophila* gene product (see Table 1). The *Shaker* gene in *Drosophila* is also located on chromosome X, at 16F4. The Kv1 gene in *Anopheles* had two 'pore' domains in close proximity on chromosome X within genomic scaffold CRA_x9P1GAV59NY_261. These exons code for the amino acids 411-448 of the *Drosophila* Shaker sequence. Closer scrutiny, and the observation that other functionally critical segments of the coding region (such as the voltage-sensor) were not redundant, led us to conclude that these were splice variants, rather than separate genes. This splice variant matched an exon already reported in the spiny lobster *Shaker* K^+ channel [13]. Another example of a splice variant occurs at position 450-514 in *Drosophila*, amino acids adjacent to the aforementioned exons at the pore region, though it was not possible to find more than one homologous sequence at this locus of the *Anopheles* genome (see Figure 5). The organization of the gene in terms of intron-exon boundaries was highly conserved between the two species, with exons spanning DNA coding for amino-acid positions 103-159 (110-159 in AG), 191-227, 257-297, 297-348, 411-448, 450-513 observed in both species.

The *Drosophila* Shab sequence (SwissProt P17970) was used as a query against the *Anopheles* genome. The *Drosophila* gene *Shab* is located at chromosome 3L:63A1. The *Anopheles Shab* (Kv2) ortholog lies at chromosome 2L:23C (see Table 2). The exon encoding the ORF spanning residues 256 to 438 of the Shab protein is conserved in *Anopheles*, though finding an *Anopheles* sequence homologous to the amino-terminal 250 amino acids was not accomplished, perhaps because the sequence is repetitive, particularly with respect to poly-glutamine stretches, and, perhaps, species specific. These homologs were found in *Drosophila* scaffold 142000013386045 section 10 (DNA sequence spanning 166595-194840) and *Anopheles* scaffold CRA_x9P1GAV5CJS_391 and _392. At least two other exons, spanning residues 436-717 and 931-968, were also conserved in both species. To look for microsynteny, CG9970, CG9972 and CG2077, putative genes products found directly upstream and downstream of *Shab* in *Drosophila*, were run against the *Anopheles* genome as queries in a TBLASTN search. No homologous sequences in the mosquito genome

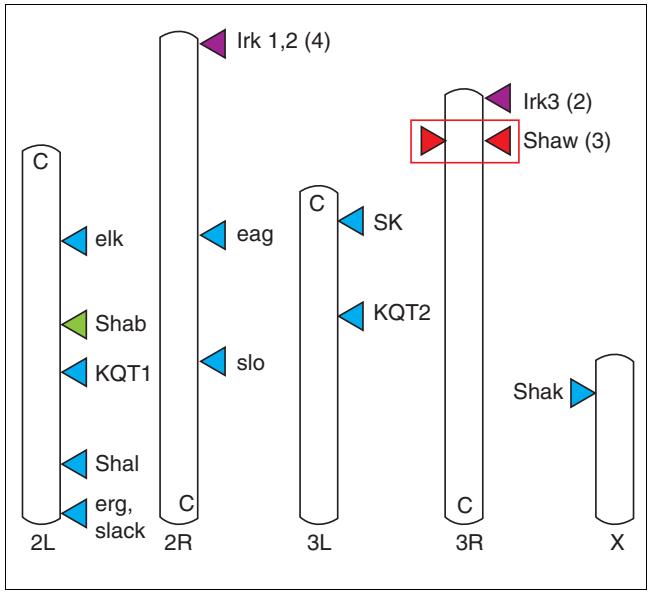

Figure 4
Chromosomal locations of K+-channel genes of *Anopheles gambiae*. Triangles are colored red for highest sequence identity, green for intermediate sequence identity, blue for lower sequence identity, and purple for the inward rectifier genes. The 'hits' are based on sequence similarity, with the *Drosophila* Shaw protein used as the query sequence against the *A. gambiae* genome. The rectangular box indicates the location of the highest score, reserved for the *Anopheles* ortholog of the query sequence. C indicates the location of the centromeres.

were found at the same locus as *Shab* (*Anopheles* chromosome 2L:23).

I used the *Drosophila* Shaw (SwissProt P17972) sequence as a query for homologs in *Anopheles*. The *Shaw* gene in *Drosophila* is located at chromosome 2L:24A3-4. Not one but three genes encoding K+-channel subunits of the Kv3 family were present in the genome of *Anopheles*; these genes, oriented in the same direction, were clustered at chromosome 3R:29, near the telomere (Figure 4), within a genomic segment of roughly 150,000 bases. The first was located at region CRA_x9P1GAV5CRW_227 and showed 85% amino-acid identity, scaffold CRA_x9P1GAV5CRW_225 showed approximately 85% identity, and a third gene located at CRA_x9P1GAV5CRW_222 showed roughly 80% identity. I called these genes Kv3.1, Kv3.2, and Kv3.3, respectively. Similar regions of protein sequence from a TBLASTN suggested that the genes, particularly Kv3.1 and Kv3.2, have intron-exon boundaries similar to those of the *Drosophila Shaw* gene. As the sequence identity comparisons of *Ag* Kv3.1 vs *Dm* Kv3.1 and *Ag* Kv3.2 vs *Dm* Kv3.1 are nearly the same, the assignment of the *Anopheles* 'ortholog' of *Dm* Kv3.1 is not trivial: the ancestral sequence at the node which represents the divergence of these two *Anopheles* genes is the actual ortholog of *Dm* Kv3.1. The recent divergence of *Ag* Kv3.1 and *Ag* Kv3.2 is supported by neighbor-joining, parsimony and maximum-likelihood trees. The exons spanning amino-acid positions 1-70, 109-175, 175-248, and 249-447 are present in the *Drosophila Shaw* gene, as well as the two most similar *Anopheles* genes, Kv3.1 and Kv3.2. The *Anopheles* Kv3.3 gene has similar intron-exon boundaries compared to the *Drosophila* K+-channel ortholog. Exon-coding regions for amino-acid positions 25-72, 72-116, 117-176, and 254-322 are present in both the fly and mosquito, though other exons are more variable between the two organisms. To look for microsynteny in this region, I used *Drosophila* gene products CG3513, CG10019, CG10020, and cutlet, which flank the *Shaw* locus in *Drosophila*, as queries to search for homologs in the *Anopheles* genome. No homologous sequences mapped to *Anopheles* chromosome 3R:29A.

I ran a BLAST search using the *Anopheles* Kv3.3 as the query against the *Drosophila* genome, and this revealed a K+-channel sequence belonging to the Kv3 (*Shaw*) family located on chromosome 2L:30A8. This gene encodes a protein with 69% amino-acid identity to the previously reported Shaw K+-channel sequence and 91% identity to the predicted *Anopheles* Kv3.3 protein sequence, with a large percentage of amino-acid differences confined to the short loop between the first and second transmembrane domain, compared to the latter sequence. For the purposes of this paper I will refer to the previously published Shaw sequence (SwissProt P17972) as

Table 1

K+ channel gene number and amino-acid sequence identity of orthologs in *Drosophila* and *Anopheles*

Channel protein	*Drosophila*	*Anopheles*	Amino-acid identity (%)
Kv1	1	1	86
Kv2	1	1	94
Kv3	2	3	87
Kv4	1	1	96
KQT	1	2	75
Eag	1	1	98
Erg	1	1	90
Elk	1	1	92
Slo	1	1	96
Slack	1	1	91
SK	1	1	94
Irk1	1	1	78
Irk2	1	3	65
Irk3	1	2	43

The middle two columns show the numbers of each gene for a given K+-channel family in fruit fly and mosquito. The right-hand column gives the percent amino-acid identity of the predicted gene product for each gene in *Drosophila* against its ortholog in *Anopheles*. In cases for which gene expansion in *Anopheles* made necessary two identity comparisons (for example, *Dm* Kv3.1 vs *Ag* Kv3.1 and *Ag* Kv3.2), an average value was used.

```
VVLFSSAVYFAEAGSENSFFKSIPDAFWWAVVTMTTVGYGDMT   DrosEx1
VVLFSSAVYFAEAGSDNSFFKSIPDGFWWAVVTMTTVGYGDMR  DrosEx2
VVLFSSAVYFAEAGSEKSYFKSIPDAFWWAVVTMTTVGYGDMT  LobstEx1
VVLFSSAVYFADAGSERSFCKSIPDAFWWAVVTMTTVGYGDMR  LobstEx2
VVLFSSAVYFAEAGSENSFFKSIPDAFWWAVVTMTTVGYGDMT  AnophEx1
VVLFSSAVYFAEAGTEMSFFKSIPDAFWWAVVTMTTVGYGDMT  AnophEx2
```

Figure 5
Conserved exon boundary for the pore domain of Kv1 (*Shaker*) K^+-channel genes. The splice variants are shown for Dros (*Drosophila melanogaster*), Anoph (*Anopheles gambiae*) and Lobst, the spiny lobster, *Panulirus interruptus*. Variations in sequence are boxed for emphasis.

Kv3.1 and the one I report here as Kv3.2. *Dm* Kv3.2 and *Ag* Kv3.3 appear to define a new subfamily within the Kv3 K^+-channel family. The FlyBase GadFly Genome Annotation Database [14] predicts that Kv3.2 (CG54450) spans 8,000 nucleotides and comprises at least 10 exons.

The Shal K^+-channel sequence (SwissProt P17971) from *Drosophila* was next used as a query sequence against both the *Anopheles* and *Drosophila* genomes. The *Shal* gene in *Drosophila* is located at chromosome 3L:76B. The *Shal* ortholog was found at a region on chromosome 2L near 26C (Figure 4) from *Anopheles*. *Shal* is found at *Drosophila* genomic scaffold 142000013386050 section 52 and *Anopheles* scaffold CRA_x9P1GAV591D_309. There is considerable conservation of intron-exon boundaries between *Drosophila* and *Anopheles* for these orthologs. An exon encoding amino acids 1-372 is present in *Anopheles*, but this region is split into two exons in *Drosophila* - from amino-acid position 1 to position 68 and another spanning amino-acid position 68 to position 372. Another exon spanning amino acids 440-488 is located over 10 kilobases (kb) downstream in *Anopheles*, although in *Drosophila* the corresponding exon is found approximately 1 kb downstream. An exon spanning the coding region for amino acids 491-540 was found for both species. Evidence of microsynteny was evident for the *Shal* locus between *Drosophila* (chromosome 3L:76B5) and *Anopheles* (chromosome 2L:26). Gene products CG9231, CG9299, CG9300 and CG9268, which lie in close proximity to *Shal* between 3L:76B3 and B5, showed regions of homologous sequence on *Anopheles* chromosome 2L:26.

A highly conserved carboxy-terminal segment of KvLQT was used as a query sequence for genes encoding K^+ channels of the KQT family. The carboxy-terminal sequence of mouse KQT2 and the *Caenorhabditis elegans* KQT channel (gi7511689) were run against the *Anopheles* genome. Homologous sequences were detected on *Anopheles* chromosome 3L:41A (scaffold AAAB00108816_186) and twice on chromosome 2L:25 on adjacent scaffolds AAAB001008960_650 and _651. Regarding the chromosome 2L homologous sequences, the proximity of the two carboxy-terminal sequences and lack of redundant sequence at other regions of this predicted protein suggest that these are splice variants rather than separate genes. These same queries were run against the *Drosophila* genome and just one homolog was found at genomic scaffold 142000013386047 section 13. This *Drosophila* gene product KCNQ is roughly 75% identical to the *Anopheles* chromosome 2L gene product and 50% identical to the chromosome 3L gene product, suggesting that the gene on chromosome 2L is the *Anopheles* ortholog of *Drosophila KCNQ*. I will refer to the chromosome 3 homolog as *KCNQ2* (or *KQT2*) and the chromosome 2 homolog as *KCNQ1* (or *KQT1*).

Drosophila Slowpoke (gi17738179, chromosomal location 3R:96A) was used as a query against the *Anopheles* genome using TBLASTN. There is a highly similar region at chromosome 2R:16A which is roughly 90% identical at the amino-acid level on *Anopheles* genome scaffolds AAAB01008888_131 and AAAB01008888_132. The exons were short for these genes (typically encoding 12-50 amino acids maximum) in *Anopheles* compared to what was observed for the Kv channel genes. Slack and SK from *Drosophila* revealed orthologs at chromosomal positions 2L:28D and 3L:38C, respectively. I assembled the predicted sequences from the exons. The *Anopheles* Slo, Slack, and SK amino-acid sequences were 96, 91, and 94% identical, respectively, to the *Drosophila* orthologs between the first and sixth transmembrane domains.

Table 2

Chromosomal location and interarm homology of K^+ channel genes

	Drosophila	*Anopheles**
Kv1 *Shaker*	**X:16F**	**X:3D**
Kv2 *Shab*	**3L:63A**	**2L:23**
Kv3 *Shaw*	**2L:24C**	**3R:29 (x3)**
	2L:30A8	3R:29 (x3)
Kv4 *Shal*	**3L:76B**	**2L:26**
KQT 1	**2R:46F**	**2L:25**
KQT 2	-	3L:41
Slowpoke	**3R:96 A**	**2R:16**
Slack	**2R:47 A**	**2L:28D**
SK	X:4F	3L:38C
Eag	**X:13A**	**2R:13E**
Erg (seizure)	**2R:60B5**	**2L:28D**
Elk	**2R:55A1**	**2L:21F**
Hyperkinetic	X:9B	3R:37
Irk1	**3R:94E**	**2R:7A**
Irk2	**3R:95A**	**2R:7A (x3)**
Irk3	**2L:37A**	**3R:29A (x2)**

The locations of the different K^+-channel genes are shown for *Anopheles gambiae* and *Drosophila melanogaster*. Bold type is used for those cases in which inter arm homology is conserved for the orthologs. *The *Anopheles* chromosomal maps are still in their early stages of annotation, making designations more general (estimated) than for *Drosophila*.

A sequence nearly identical at the amino-acid level to *Drosophila* Eag (gi17530941, chromosomal location X:13A) was found at chromosome 2R:13E on scaffold AAAB01008859_213. The *Drosophila* Erg and Elk protein sequences were used as queries and revealed orthologs in *Anopheles* at chromosomal locations 2L:28D and 2L:21F, respectively. The predicted Eag, Erg and Elk amino-acid sequences from *Drosophila* were 98, 90, and 92% identical to the *Drosophila* orthologs, respectively. Like the three genes mentioned in the preceding paragraph, Eag, Elk and Erg were encoded by exons much shorter than those observed for the Kv K^+-channel genes. The alignment for Slo- and Eag-family K^+ channels is shown in Figure 2b.

Each of the three Irk inward-rectifier K^+-channel sequences was used as a query against the *Anopheles* DNA database. Four homologous genes were clustered very close together near the telomere of chromosomal arm 2R at 2R:7A. Three of these genes encode protein sequences very similar (nearly 70% amino-acid identity) to *Drosophila* Irk2. The fourth gene, oriented in the opposite direction, was most similar to *Drosophila Dir* (or *Irk*) (Figure 2c). Two additional genes were located near the telomere of chromosomal arm 3R at 29A. These two genes, which have in common a large exon encoding an ORF homologous to amino acids 144-437 of *Drosophila* Irk3, were clustered very close to one another on the chromosome; *Irk3.1* and *Irk3.2* from *Anopheles*, as I have named these genes, are separated by no more than 1 kb of intronic sequence. The predicted sequences share roughly 40% amino-acid identity to each of the three *Drosophila* inward rectifier channels. It was necessary to consider whether these two ORFs might constitute one two-pore channel but reciprocal BLAST searches using the *Anopheles* sequences suggest that these sequences are most similar to inward rectifier genes from *Drosophila*, and the presence of carboxy-terminal signature sequences such as EILWGHRF suggest the genes encode inward rectifier channels. The analysis of ORFs flanking *Irk* genes in the *Drosophila* genome revealed that they were not in close proximity to the *Irk* orthologs in *Anopheles*, again providing evidence of reshuffling of genes in these two organisms.

A BLAST search using *Drosophila* Hyperkinetic (gi 902000, chromosomal location X:9B) as the query revealed the presence of a K^+ channel β-subunit ortholog in *Anopheles gambiae*. This sequence was located at chromosome 3R:37D, on scaffold AAAB01008980_497. This sequence showed roughly 78% amino-acid identity to the *Drosophila* ortholog and 40-50% identity to the mammalian homologs. The amino terminus of the *Drosophila* sequence is similar to the *Anopheles* sequence of amino acids 162-200, upstream of the conserved aldo-keto reductase core. Furthermore, the *Anopheles* sequence, like *Drosophila*, has a histidine residue at the position at which mammalian *Shaker* β-subunits have the putative catalytic tyrosine.

Neutral evolutionary distance values

In addition to amino-acid identity we looked at neutral evolutionary distance (NED) values. Values for f2 (the percentage of identical codons for conserved twofold-redundant amino acids - Cys, Asp, Glu, Phe, His, Lys, Asn, Gln, and Tyr) between two aligned proteins are calculated by looking at the codons' third position in positions at which amino acids with twofold degeneracy occur. These values may be more useful for evaluating divergence dates than amino-acid sequence identity because they are silent and mutation occurs in a clocklike fashion, rather than in the bursts that are thought to accompany rapid environmental changes. The f2 values for Kv1, Kv2 and Kv4 orthologs in the fruit fly and mosquito were as follows: 0.69 for *Anopheles* Kv1 vs *Drosophila* Kv1, 0.73 for *Anopheles* Kv2 vs *Drosophila* Kv2, and 0.69 for *Anopheles* Kv4 vs *Drosophila* Kv4. The f2 values for *Shaw* vs *Shaw* and other K^+-channel genes were calculated, as shown in Table 3. *Anopheles* Kv3.1 vs *Anopheles* Kv3.2 gave a f2 value of 0.74. *Anopheles* Kv3.1 vs *Anopheles* Kv3.3 gave an f2 value of 0.75, whereas *Anopheles*. Kv3.2 vs *Anopheles* Kv3.3 gave a value of 0.69. The f2 value of *Anopheles* Kv3.3 vs *Drosophila* Kv3.2 gave a value of only 0.52. The f2 value for the two *Shaw* genes in *Drosophila*, Kv3.1 and Kv3.2, was 0.60.

Discussion

Anopheles gambiae is the most important vector of *Plasmodium falciparum* malaria in Africa, where nearly 90% of the world's malaria-specific mortality occurs. DDT has been used extensively to control this mosquito. Because the target of DDT and pyrethroid insecticides is the voltage-gated Na^+ channel [15], and considering that the anti-malarial quinine blocks K^+ channels, insights into the ion channels in the genomes of this mosquito and other insects may be useful for investigating how DDT and other pesticides may be used with greatest efficacy and safety. Using the conserved K^+-channel pore as a probe, I screened the entire *A. gambiae* genome for the presence of voltage-gated K^+ channels, Ca^{2+}-activated K^+ channels and inward rectifier K^+ channels, as all these channels possess a homologous pore domain. I have identified eight voltage-gated K^+ channels, three Eag-family, three Slo-family and six inward rectifier channel genes using this search. A greater number of genes within a given family in *Anopheles* compared to *Drosophila* can be a result of gene expansion in *Anopheles* or, alternatively, gene loss in *Drosophila*. I considered the likelihood of either possibility for these cases, based on the trees that were constructed using neighbor-joining, parsimony, and maximum-likelihood algorithms.

K^+ channels are dispersed throughout the genomes of both *Drosophila* and *Anopheles*, although multiple members of a given family are most often clustered. Comparing the gross homology of the two species, both the *Anopheles* and *Drosophila* have two major metacentric autosomes and an X chromosome (five chromosomal arms in total). Of the

Table 3

Third position (f2) values for the Kv3 K^+ channels

Spec 1	Spec 2	f2	c2	n2
Mouse3.1	Aptero3.3	0.71	74	105
	Hum3.1	**0.85**	116	136
	LobstKv3	0.51	34	67
	Dros3.1	0.58	40	69
	Agam3.1	0.69	47	68
	Agam3.2	0.75	47	63
	Dros3.2	0.58	32	55
	Agam3.3	0.66	41	62
Hum3.1	LobstKv3	0.54	65	121
	Dros3.1	0.60	71	119
	Agam3.1	0.52	60	115
	Agam3.2	0.56	49	88
	Dros3.2	0.54	53	99
	Agam3.3	0.68	42	62
LobstKv3	Dros3.1	0.54	65	121
	Agam3.1	0.60	71	119
	Agam3.2	0.52	60	115
	Dros3.2	0.56	49	88
	Agam3.3	0.54	53	99
Dros3.1	Agam3.1	**0.61**	79	129
	Agam3.2	**0.65**	81	124
	Dros3.2	*0.61*	53	89
	Agam3.3	0.62	62	100
Agam3.1	Agam3.2	*0.74*	93	126
	Dros3.2	0.55	47	85
	Agam3.3	*0.75*	72	96
Agam3.2	Dros3.2	0.57	47	83
	Agam3.3	*0.69*	63	92
Dros3.2	Agam3.3	**0.52**	52	101
Dros Kv1	AgamKv1	**0.69**	69	100
Dros Kv2	Agam Kv2	**0.73**	73	101
Dros Kv4	Agam Kv4	**0.69**	80	114

Calculations are based on alignments spanning the first through the sixth transmembrane domain. The n2 value indicates number of twofold degenerate amino acids, c2 indicates the number of twofold degenerate amino acids with conserved third-position nucleotides, and f2 signifies the percentage of conserved third-position nucleotides at these positions (that is, c2/n2). Interspecies orthologs between fly and mosquito (and between mouse and human, near top) are shown in bold, and intraspecies paralogs are shown in underline and italics. Comparisons of lower f2 values (for example, 0.55 vs 0.50) are not as meaningful (with respect to estimating divergence dates) as higher-value comparisons owing to equilibration within the lower ranges. Agam, *A. gambiae*; Dros, *D. melanogaster*; Lobst, *Panulirus interruptus*; Hum, *Homo sapiens.*

channels focused on here, only *Shaker* is located on the same arm in both species, namely the X chromosome; however, the locations of other K^+ channel genes in *Anopheles* and *Drosophila* are consistent with previously reported regions of major interarm homology between these species (Table 2). This was true for *Shab* (*Dm* 3L, *Ag* 2L), *Shaw* (*Dm* 2L, *Ag* 3R), *Shal* (*Dm* 3L, *Ag* 2L), *KCNQ* (*Dm* 2R, *Ag* 2L) and *Slowpoke* (*Dm* 3R, *Ag* 2R), as well as for *Slack*, *eag*, *erg*, *elk*, and the three inward rectifier genes, as shown in Table 2. The translocations between autosomes and chromosome X, observed for *eag* and *Hyperkinetic*, are notable: these examples raise questions about dosage compensation which will need to be addressed in future studies.

There is 78-98% amino-acid sequence identity between the six-transmembrane K^+-channel gene products in *Drosophila* and their orthologs in *Anopheles*, a value significantly greater than what other studies have calculated (62% identity and 56% in separate studies [16,17]) as a mean value for sequence identity between orthologs in these two organisms. Amino-acid sequence identity of 78-98% is an impressive figure, given that these two organisms are thought to have diverged 250 million years ago [16]. Although this value may be slightly higher than the true value, as uncertainties resulting from splicing boundaries led us to disregard the more variable amino-and carboxy-terminal extreme ends, the sequence identities for the *Drosophila* and *Anopheles* K^+-channel orthologs over the core regions for K^+-channel sequences are well above the mean values calculated by the other groups for orthologs between these species. It suggests that K^+-channel genes are subject to a stricter selection pressure than other genes in these organisms. This is consistent with the observation that transporters and channels are among the proteins with highest sequence similarity between *Anopheles* and *Drosophila* [17].

Of the four voltage-gated K^+-channel types Kv1-4, the *Shaker*, or Kv1 channel gene is, from a genomic perspective, arguably the most complex. *Shaker* from *Drosophila* is a gene with at least 11 exons and spanning over 16 kb. Exons are short in the *Anopheles* ortholog of *Shaker* as well, as it was not possible to find an exon encoding more than 75 amino acids in this gene. The presence of more than one pore region in *Anopheles Shaker* suggests that sequence diversity can be generated in an integral part of the internal segments of the channel, rather than what has been reported for *Drosophila Shaker* - splicing at the 5' and 3' ends [18,19]. Alternative splicing at the pore region occurs in another arthropod, the lobster *Panulirus interruptus*. Functional channels translated from genes with either of the two splice variants were expressed and exhibited different electrophysiological and pharmacological properties [13]. It is tempting to assume that the two transcripts with the two variable pore-regions in *Anopheles* would encode channels with different properties as well, although this would be premature until it is shown that both exons are transcribed. An exon encoding the region containing the pore exists in *Drosophila*, yet no transcripts could be found containing this putative exon [20].

In the coding region, the *Shal* and *Shab* genes from *Anopheles* and *Drosophila* are made up of longer exons than the *Shaker* gene. The lack of more than one splice variant at central regions of the Shal protein suggests that splicing may be confined to the 5' and 3' regions of this gene. Although evidence of microsynteny was found for the region surrounding *Shal*, flanking genes of the other channels did not provide evidence of microsynteny between *Anopheles* and *Drosophila* at these regions.

The identification of three Kv3 (*Shaw*) family K^+-channel genes in *Anopheles* (but only two in *Drosophila*) is intriguing. In mammals, this family of K^+ channels activates at potentials considerably more positive than observed in other K^+ channel types; these channels have the ability to produce currents that can specifically enable fast repolarization of action potentials without compromising spike initiation or height [21]. Also, these channels are localized at specialized regions in mammalian brain associated with higher-order cognitive functions, such as the thalamus and cortex [22]. Furthermore, Kv3 channel sequence identities are lower between *Drosophila* and mammals than are other K^+-channel types.

The identification of multiple Kv3 channel genes, but only single members of the Kv1, Kv2 and Kv4 families, in Diptera (two in *Drosophila* and three in *Anopheles*) raises questions about the evolutionary history of Kv3 K^+ channel genes. In some organisms with very primitive nervous systems, such as *Polyorchis penicillatus* (jellyfish, phylum Cnidaria) at least two *Shaker* (Kv1)-family genes exist [23]; moreover, in the electric fish *Apteronotus* the Kv1 (Shaker) family is the most diverse, with at least 10 members [24]. One can predict from the protein and DNA similarity that Kv3.1 and Kv3.2 from *Anopheles* diverged recently. The previously published *Drosophila* Kv3 (Shaw) protein is 88% identical to the *Anopheles* Kv3.1 and Kv3.2 sequences, but only 70% identical to the *Anopheles* predicted Kv3.3 gene product. Regarding the relationships between *Dm* Kv3.1 and the two *Anopheles* genes *Ag* Kv3.1 and *Ag* Kv3.2, this paper has already stated that the ancestral sequence at the node representing the divergence of these two *Anopheles* genes is the true ortholog of *Dm* Kv3.1 (gi 158460). Given the awkwardness of comparing an extant gene (for example *Dm* Kv3.1) to its ancestral ortholog, it may suit the genomics and/or evolutionary community to devise new terminology for such cases. In relation to the *Drosophila* Kv3.1, the terms 'novolog' (corresponding to *Ag* Kv3.1 or *Ag* Kv3.2) and 'archaelog' (corresponding to the ancestral gene represented by the node from which the two *Anopheles* genes diverged) might be useful; these terms, as presented here, would apply to cases in which contemporaneous orthologs do not exist between two organisms, as opposed to the general phenomena of duplication and divergence.

In the light of the high amino-acid identity, roughly 87%, the low f2 value of 0.51 for *Anopheles* Kv3.3 and its ortholog in *Drosophila* (as opposed to an f2 value of 0.69 for *Anopheles* Kv1 vs *Drosophila* Kv1, 0.73 for *Anopheles* Kv2 vs *Drosophila* Kv2, or 0.70 for *Anopheles* Kv4 vs *Drosophila* Kv4) suggests these two genes diverged longer ago than would be predicted by the amino-acid identity alone, and that selective pressure has prevented the two sequences from diverging; homoplasy may explain their high amino-acid identity and low third-position (f2) identity. The f2 value comparing Kv3.1 vs Kv3.3 from the mosquito is 0.75, higher than expected, considering that amino-acid identity between the two (64%) is significantly lower than that observed between *Anopheles* Kv3.3 and its *Drosophila* ortholog.

It is likely that different K+ channel subunits within the same family would provide the potential to generate many K^+-channel tetramer combinations. This would allow greater variation and specificity of Kv3 channels, as K^+ channel subunits within a family can readily form functional heteromultimeric channels [25]. The number of XXR repeats of the voltage-sensor (where X is a hydrophobic residue and R represents arginine within the voltage-sensor) in non-vertebrate Kv3 K^+ channels is of interest. The presence of four such repeats in invertebrate Kv3 channels and six in vertebrate Kv3 K^+ channels may help explain the difference in voltage-dependence observed between the mammalian and fly Kv3 channels, as even single amino-acid mutations in this domain can affect voltage-dependence of K^+ channels considerably [26]. The greater PAM distance between *Drosophila* and mammalian Kv3 channels (PAM distance 65) compared to Kv1, Kv2, or Kv4 (for which the intra-family PAM distances between *Drosophila* and mammalian channel sequences range from 25-40) shows that Kv3 channels have undergone more extensive adaptation than other K^+-channel families. It can be inferred that the greater complexity of the vertebrate brain made necessary a rapidly deactivating, high-threshold K^+-channel type which has not evolved in protostomes; indeed, given the biophysical properties of Kv3 channels in mammals, the amino-acid replacements that have occurred in mammalian Kv3 channels seem to have provided exactly this.

Like the Kv3 (*Shaw*) family, KQT (KCNQ) K^+-channel genes are more abundant in *Anopheles* than in *Drosophila*. Sequence analysis suggests these channels evolved before other classical voltage-gated K^+ channels (Kv1-K4). The neighbor-joining (Figure 3a) and maximum-likelihood trees we constructed, in combination with the fact that mammalian KCNQ1 and *Anopheles* KCNQ2 gene products share a striking 75% identity (despite the divergence of protostomes and deuterostomes close to 700 million years ago), suggest that gene loss in *Drosophila*, specifically loss of an ancestral *KCNQ2* (mammalian *KCNQ1*), is the cause of this difference, rather than gene expansion in *Anopheles*, which may be the case for the Kv3 and Irk3 (Figure 3b) gene families. Alternatively, lateral transfer of *KCNQ1* from mammals to *Anopheles* must be considered, given the intimate relationship of these organisms. Although the genome size of *Anopheles* is twice the size

of the *Drosophila* genome, the numbers of genes in both organisms are nearly equivalent [17], suggesting that gene duplication depends on the advantage of additional genes in distinct families, rather than a general consequence of possessing a larger genome. Unlike other K^+-channel types, for which amino and carboxyl termini are highly variable, KQT channel sequences are even more highly conserved in some regions of the cytoplasmic carboxy-terminal region than in the conserved pore region. The presence of two potential splice variants within the carboxy-terminal tail raises questions about the role of this domain in channel function. Although the physiologic significance of this region is not yet known, evidence suggests it may be involved in calmodulin binding [27]. For this region one homologous gene product can be found in *Drosophila*, KCNQ, which raises questions about whether products of this gene mediate the M-current, as has been postulated for KCNQ2 and KCNQ3 in mammals [28].

The greater number of inward rectifier K^+-channel genes in *Anopheles* compared to *Drosophila* is striking, given that these organisms belong to the same order. Our analysis, based on maximum-likelihood and neighbor-joining algorithms, suggests that gene duplication in *Anopheles* is the most likely explanation for the greater number of *Irk3* genes in mosquito. This also appears to be the case for *Irk2.1* and *Irk2.2*; however, from the tree (Figure 3b) it appears that the divergence of *Irk2.3* from *Irk2.1* and *Irk2.2* in the mosquito occurred earlier than the divergence of *Drosophila Irk2* and the two *Anopheles* genes *Irk2.1* and *Irk2.1*, suggesting that gene loss in *Drosophila* may have occurred. The same tree topology was supported by both neighbor-joining and maximum-likelihood trees, though lack of a clear ortholog from a more distant organism (for example, a deuterostome) makes this type of assessment, regarding gene history, more difficult. Future studies may help explain why the mosquito has twice as many of the inward rectifier genes as the fruit fly. Gene expansion in *Anopheles* has been observed for genes involved in hematophagy and insecticide resistance; it is unclear to what extent these two factors are involved here, although ion channels are clearly targets of insecticides. The overall compositions of K^+-channel genes in *Anopheles* and *Drosophila* are strikingly similar in some respects, such as conservation of sequence and intron-exon boundaries of orthologs, and strikingly different in others, such as the number of *Irk* homologs and lack of microsynteny. The genome projects of other insects, such as *Manduca sexta* and *Bombyx mori*, will help paint a broader picture of the composition of ion-channel genes within the genomes of these related organisms.

Conclusions

Within the *Anopheles* genome there are orthologs for the four major voltage-dependent K^+-channel gene families in *Drosophila*: Kv1, Kv2, Kv3 and Kv4 (*Shaker*, *Shab*, *Shaw* and *Shal*, respectively). In addition we have identified genes that encode the Shaker β-subunit, two members of the KQT family of K^+ channels, as well as three Slo-family genes, three Eag-family genes, and six inward rectifier K^+-channel genes. In *Anopheles*, the *Shaw* family is more diverse than in *Drosophila*: three genes from this family are located next to one another along chromosome 3R, in contrast to two Kv3-family genes in *Drosophila*. The greater number of genes for three K^+-channel types, inward rectifier, KQT, and Kv3 (Shaw), in *Anopheles* is intriguing, given that these organisms have roughly the same number of genes: both gene expansion in *Anopheles* and gene loss in *Drosophila*, in separate cases, may account for these differences. The high level of amino-acid sequence identity, as well as the conservation of intron-exon boundaries, in combination with the chromosomal proximity of these genes in *Anopheles* and *Drosophila*, provides a greater understanding of the molecular diversity and evolutionary history of K^+-channel genes in the order Diptera.

Materials and methods

I used BLAST [11] and PSI-BLAST at the NCBI website to find K^+-channel homologs using the Shaker K^+-channel pore sequence as a query initially and then other, longer, K^+-channel family-specific query sequences for verification. The predicted splice sites were compared with results of the TBLASTN to help confirm intron-exon boundaries. Increases in nucleotide position number from one putative exon to the next were used to deduce the size of introns.

This study utilized the Ensembl *Anopheles gambiae* server [29] to search for homologs of various K^+-channel types and to identify and visualize their respective chromosomal locations. The DARWIN server [30] was used to calculate the f2 values for the sequences, as well as a phylogenetic tree for the *Shaw* sequences, along with PAM distances and ancestral sequences. Figures were visualized and optimized using Adobe Photoshop.

Sequences were aligned using ClustalX version 1.81. Phylogenetic trees were generated using ClustalX (for neighbor joining) and PHYLIP (for neighbor joining, parsimony and maximum likelihood using Protdist, Protpars, and ProML, respectively). The resulting trees were then visualized and evaluated using Treeview. Bootstrap values were calculated using ClustalX and PHYLIP.

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