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Genetic make-up of arthropod vectors

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Summary
A better understanding of the molecular aspects of arthropod vector biology and the processes that determine pathogen transmission can lead to the development of novel or improved control methods for vectors and vector-borne diseases. The 'omics' era provides unprecedented opportunities to explore these aspects of vectors and the diseases which they transmit. This review aims to summarise recent developments in the field of vector genomics and to provide basic insight into the application of functional genetic tools such as RNA interference, RNA sequencing and genetic transformation in vector control development.

Keywords
Genome – Genomics – Reverse vaccinology – RNA interference – Transcriptome – Transformation – Vector.

Introduction

Vectors and vector-borne diseases are major causes of global morbidity and mortality in humans and animals, predominantly in developing countries in the tropics (1). Current control methods targeting arthropod vectors of disease rely principally on the use of insecticides and acaricides, but concerns about their impact on ecosystems, residues in animal products and the emergence of resistant arthropods undermine control efforts (2, 3, 4). Developments in the 'omics' era, such as high-throughput next-generation sequencing technologies and RNA interference, enable the analysis of the genetic make-up of vectors and vector-borne diseases in unprecedented detail. This review aims to provide insights into available genomic resources for arthropod vectors and an overview of how they are being used in vector-borne disease research and how this may contribute to the development of novel disease intervention strategies.

Arthropod vector genomes

The genome of arthropod disease vectors holds invaluable information concerning the molecular biology of vectors and vector–pathogen interactions. Unravelling the genetics of vector biology and pathogen transmission may lead to novel disease control methods and it is therefore not surprising that the first genome sequence of an arthropod vector to be published was that of *Anopheles gambiae*, the main mosquito vector of the most deadly vector-borne disease, malaria (5). This landmark was followed by the publication of several other vector genome sequences, including that of the mosquitoes *Aedes aegypti* (6) and *Culex quinquefasciatus* (7), the human body louse *Pediculus humanus* (8), the Neotropical mosquito *Anopheles darlingi* (9), the Asiatic mosquito *Anopheles sinensis* (10), and the tsetse fly *Glossina morsitans* (11). The number of available vector genomes will markedly increase with the imminent publication of the *Anopheles* 16 genomes project (12). An overview of other relevant ongoing and finished genome sequencing projects is presented in Table I. Detailed and updated information on vector genomes, which includes that of the snail *Biomphalaria glabrata*, an intermediate host for the transmission of *Schistosoma mansoni*, as well as a number of haematophagous arthropods, can be found at VectorBase (www.vectorbase.org), an online resource centre which organises and stores genomic data from invertebrate vectors (19). Genomes of vector-borne pathogens can be consulted through the National Center for Biotechnology Information (NCBI) Genomes portal (www.ncbi.nlm.nih.gov/genome).

There is a considerable difference in the size of vector genomes, i.e. the smallest known genome from a haematophagous arthropod, which is that of the human body louse, spans 1.1×10^8 base pairs (bp) (8), whereas the largest known arthropod vector genome is that of the cattle tick *Rhipicephalus (Boophilus) microplus,* with an estimated size of 7.1×10^9 bp (20). In addition, there may be significant

Table I
List of completed and ongoing genome sequencing projects on arthropod vectors
Source: www.vectorbase.org (accessed in July 2014)

Organism	Strain	Genome size (bp)	Gene count	Reference
Aedes aegypti	Liverpool	1,311,011,677	17,479	(6)
Anopheles darlingi	Coari	136,935 538	10,947	(9)
Anopheles gambiae	PEST	273,109,044	13,624	(5)
Anopheles sinensis	SINENSIS	267,735,131	16,766	(10)
Culex quinquefasciatus	Johannesburg	579,057,705	19,363	(7)
Glossina morsitans	Yale	363,107,930	12,963	(11)
Pediculus humanus	USDA	110,802,711	11,699	(8)
Anopheles albimanus	STECLA	170,508,315	12,335	(12)
Anopheles arabiensis	Dongola	246,567,867	13,678	(12)
Anopheles atroparvus	EBRO	224,290,125	14,230	(12)
Anopheles christyi	ACHKN1017	172,658,580	11,156	(12)
Anopheles coluzzii	Mali-NIH	224,455,335	14,703	(13)
Anopheles culicifacies A	A-37	202,998,806	14,882	(12)
Anopheles dirus A	WRAIR2	216,307,690	13,239	(12)
Anopheles epiroticus	Epiroticus2	223,486,714	12,602	(12)
Anopheles farauti	FAR1	180,984,331	13,703	(12)
Anopheles funestus	FUMOZ	225,223,604	13,756	(12)
Anopheles maculatus B	maculatus3	141,894,015	15,046	(12)
Anopheles melas	CM1001059	227,407,517	16,666	(12)
Anopheles merus	MAF	251,805,912	14,415	(12)
Anopheles minimus	MINIMUS1	201,793,324	13,128	(12)
Anopheles quadriannulatus A	SANGWE	283,828,998	13,857	(12)
Anopheles stephensi	Indian	221,324,304	12,350	(13)
Anopheles stephensi	SDA-500	225,369,006	13,650	(12)
Biomphalaria glabrata	BB02	755,615,849	14,351	(14)
Culicoides sonorensis	KC	~200,000,000	19,041	(15, 16)
Ixodes scapularis	Wikel	1,765,382,190	20,771	(17)
Lutzomyia longipalpis	Jacobina	154,229,266	10,429	(13)
Phlebotomus papatasi	Israel	347,840,937	12,678	(18)
Rhodnius prolixus	CDC	702,648,350	17,244	(13)

bp: base pairs

differences in genome size between different families within (sub)orders, species strains and sexes of the same species (21, 22). The observed differences in genome size cannot be explained by differences in the number of genes alone; it is non-coding genomic DNA of unknown functionality, such as repetitive and repeat-derived elements, which makes up the bulk of the larger arthropod genomes (6, 20). This high repetitive content makes it difficult to reconstruct the original genome sequence. It also complicates *de novo* genome sequencing projects focusing on arthropods with large genomes, such as *Ixodes scapularis* and *R. microplus*, whose genomes may have a repetitive DNA content as high as 66–69% (20, 23). The use of next-generation sequencing (NGS) methods, which have shorter read lengths (35–250 bp, depending on the platform) in comparison to more expensive capillary-based (Sanger) sequencing methods (650–1,000 bp), is a further computational challenge in the genome assembly process. Alternative strategies are thus required to obtain longer sequence reads or to filter out repetitive DNA prior to sequencing in order to obtain gene-enriched information (24, 25).

The increasing availability of large amounts of genetic data can also be used for phylogenetic studies to improve the resolution of evolutionary trees based on single-gene studies. In phylogenomics, entire genomes or large gene sets are compared to establish evolutionary relationships (26, 27). Its application in research on arthropod vectors is still constrained by the limited number and variety of species for which genomes are available, but this is likely to change in the future with NGS methods becoming more affordable.

Gene discovery

The sequencing of vector genomes is not an end in itself: the main challenge is to decipher the vast amount of genome-

sequence data and place it into useful biological context. A pivotal step in this process is the identification of protein-coding genes. This can be done using *ab initio* methods, which use statistical models to identify signals and features associated with protein-coding genes in the DNA sequence, or by evidence-based searches in which transcriptional evidence in the form of expressed sequence tags (EST), known proteins or full-length complementary DNAs (cDNAs) is used to detect and annotate similar sequences in the genome (28). *Ab initio* methods are quick, inexpensive and sensitive. However, they tend to predict false-positive genes, whereas evidence-based tools are quite accurate but fail to identify genes in the absence of transcriptional evidence or known homologues. Fine-tuning *ab initio* tools, by training them on reference gene collection sets from related organisms, and using consensus methods in which both *ab initio* and evidence-based methods are combined may result in more accurate gene predictions (28).

Once protein-coding genes have been identified, they are usually classified according to gene ontology terms. Gene ontology provides a controlled vocabulary for gene product annotation in a species-independent manner, covering three domains: molecular functions, cellular components and biological processes (29). Another tool which is frequently employed during the gene annotation process is the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (www.genome.jp/kegg/), which can also be used for the mapping of predicted genes on different biochemical and signalling pathways (30).

Transcriptomics

Transcriptome studies, in which the total complement of RNA molecules present in a cell under specific conditions is described, provide insight into the actively expressed genes in cells. The sequencing of ESTs, unique sequences of 100–800 bp in size derived from a cDNA library, has become an important resource for the reconstruction and exploration of the transcriptome. It has been instrumental for gene discovery in organisms for which whole genome sequencing is not immediately feasible, since EST databases are relatively cheap and easy to obtain in comparison to full genome sequences. This is why they have also been referred to as the 'poor man's genome' (31, 32). Expressed sequence tag datasets are available for a number of haematophagous arthropods for which whole genome sequences are not (yet) available. These include, for example, tick species of veterinary importance such as *Amblyomma americanum*, *Amblyomma variegatum*, *Rhipicephalus appendiculatus*, *Rhipicephalus (Boophilus) microplus* (33, 34, 35, 36, 37) and the mosquitoes *Armigeres subalbatus* and *Culicoides sonorensis* (38, 39).

Gene expression profiling studies using hybridisation-based approaches, e.g. microarrays and sequence-based approaches such as sequencing of EST libraries, have been used to generate insight into various aspects of vector biology. A tool which is becoming increasingly popular for transcriptome profiling is RNA sequencing (RNA-seq). In this NGS-based method, RNA is converted to an adapter-ligated cDNA library which can be amplified and subsequently sequenced using high-throughput NGS. The sequence reads can then be aligned to a reference genome or transcriptome or, when these are not available, a *de novo* transcriptome assembly. In contrast to hybridisation-based approaches, RNA-seq is not limited to detecting only transcripts that are known from existing genomic databases, which makes this method particularly attractive for studies in organisms for which there is limited genomic information available (40). RNA-seq can also be used for quantifying expression levels with a large dynamic range and is finding increased application in studies on arthropod vectors, including studies on the molecular mechanism of insecticide resistance (41, 42, 43).

Insecticide resistance may be caused by changes in behaviour which reduce insecticide exposure (behavioural resistance), a reduced uptake through the cuticle or digestive tract linings (reduced penetration), mutations in the target proteins (target-site insensitivity) and/or biodegradation of the insecticide due to increased detoxification activities (metabolic resistance) (41). Comparison of the transcriptome from sensitive and resistant strains for sequence polymorphisms and gene transcript levels may elucidate the mechanisms underlying insecticide resistance. This knowledge can, for instance, be used in the development of metabolic enzyme inhibitors such as piperonyl butoxide, which can be used as an add-on to insecticides to block the resistance pathway and thereby extend the insecticides' effective life span. Other examples of recent RNA-seq studies include:

– the *de novo* assembly of the *Culicoides sonorensis* biting midge transcriptome and expression profiling of blood- and sucrose-fed midges (15)

– *de novo* transcriptome assembly of the tiger mosquito *Aedes albopictus* (44)

– the detection of sex- and tissue-specific differences in gene expression related to chemoreception in *Anopheles gambiae* mosquitoes (45)

– the detection of differences in the innate immune response of human head and body lice (*Pediculus humanus capitis* and *P. humanus humanus*) upon challenge with the causal agent of trench fever, *Bartonella quintana* (46).

The saliva of ectoparasites contains a complex mixture of proteins which counteract the hosts' haemostasis and immune response, in order to ensure successful feeding and survival of the parasite. It thus forms a key element in the interaction between haematophagous arthropods

and their vertebrate host, and biologically active saliva molecules may find potential use as pharmaceuticals (47). In addition, modulation of the vertebrate immune response by saliva molecules may potentiate the infectivity of vector-borne pathogens, as demonstrated for *Leishmania* and sandflies (48), West Nile virus and mosquitoes (49) and several pathogens transmitted by ticks (50). Other salivary proteins may directly contribute to pathogen transmission. *Borrelia burgdorferi*, for example, the causal agent of Lyme disease, upregulates the expression of the tick salivary gland protein salp15 and coats itself with this protein through interaction with one of its outer surface proteins (OspC). This in turn protects the spirochete from antibody-mediated killing and facilitates its survival in the mammalian host (51). Vaccination of mice against salp15 conferred protection against *B. burgdorferi* infection, which demonstrates the potential of vaccines based on salivary gland proteins to interrupt disease transmission (52). RNA-seq, protein analysis and haemostasis assays demonstrated that the composition of saliva from *G. morsitans* tsetse flies infected with *Trypanosoma b. brucei* differs from the saliva of uninfected tsetse. The haemostatic activity of the saliva of infected flies is reduced, which prolongs their feeding time and increases the number of blood meals that have to be taken to reach engorgement. This in turn could lead to an increase of transmission of *T. b. brucei* to a larger number of hosts (53, 54). These and other examples explain why the salivary glands of vectors have attracted particular attention in organ-specific transcriptome studies, and the sialotranscriptomes (from Greek *sialo*, saliva) have become available for an increasing number of important haematophagous arthropods, including mosquitoes, biting midges, sandflies, black flies, tsetse flies, fleas, kissing bugs, and ticks (55, 56, 57, 58, 59, 60, 61, 62, 63). Such datasets typically contain many proteins of unknown function, whose biological relevance has yet to be determined. A reverse genetics tool which has proven to be particularly efficient for the functional characterisation of genes in arthropods is RNA interference (RNAi) (64).

RNA interference

RNAi plays a central role in the innate antiviral immunity of arthropods. It is a selective gene-silencing strategy in which any messenger RNA (mRNA) with sequence identity to the double-stranded RNA (dsRNA) trigger is degraded via intermediate small interfering RNA (siRNA) or piwi-interacting RNA (piRNA) in the RNAi pathway (reviewed by 64, 65). The dsRNA can be virus-generated, but synthetic dsRNA or siRNA can also be introduced in arthropods – either by injection, ingestion or immersion – to selectively silence the expression of specific genes of interest. The effects of this gene knockdown can subsequently be studied to decipher gene function. RNAi also has other applications, such as RNAi-based parasite control (66, 67) and the screening of potential vaccine antigens in reverse vaccinology (68).

Key components of the RNAi pathway such as Dicer, an enzyme which cleaves dsRNA into siRNA, appear to be conserved among arthropods. Other components, such as the molecular mechanisms of dsRNA uptake and transitive amplification of the initial dsRNA signal, differ between arthropods and this may help to explain differences in the sensitivity to RNAi among organisms (64, 69).

Of particular concern in terms of the specificity of RNAi studies are off-target effects, which may occur when there is sequence identity between siRNA and random mRNA transcripts, resulting in the down-regulation of unintended targets. Off-target effects can be prevented by avoiding the use of dsRNA or siRNA sequences present in multiple genes (70), but this is not possible in organisms for which the genome sequence is not available and alternative strategies are required to limit off-target effects (71).

Reverse vaccinology

Proof of principle for vaccination against ectoparasites was presented by the development of recombinant Bm86 vaccines against *R. microplus* ticks in the 1990s, and Bm86 vaccines remain the only commercialised vaccines targeting an ectoparasite to date (72). The inefficacy of Bm86 vaccines against some tick species and the absence of a direct knock-down effect justify the development of improved vaccines, but a bottleneck in the development of novel efficacious vaccines is the identification of effective antigens. The 'omics' era offers promising new possibilities for this identification step using reverse vaccinology approaches. This was illustrated by a recently published study in which *R. microplus* transcriptome data was used for the design of a microarray chip, which allowed for the identification of genes expressed during different life stages and in different tissues. Through downstream *in silico* analysis for properties such as predicted antigenicity and cellular location, a subset of potential vaccine candidates was identified for further evaluation in vaccine trials (73).

Transgenesis

The genomes of some arthropod vectors can be manipulated through the insertion of foreign DNA into their germline, using transgene vectors such as transposons and site-specific recombinases (74). The first arthropod vector to be efficiently transformed was *Aedes aegypti* (75, 76), and successful transformation has since been reported for other mosquitoes, including *Anopheles* spp. (77, 78) and *Culex quinquefasciatus* (79). Transformation of non-mosquito disease vectors has not yet been reported, but cell lines of the ticks *R. microplus* and *I. scapularis* could be transformed to express DsRed, a red fluorescent protein (80, 81). This suggests that transformation of live ticks

may also be feasible in the future, but additional studies on suitable transformation vectors, promoters and techniques to introduce the transgenic constructs into preblastoderm eggs will be required. In arthropods where microinjection of eggs is not feasible, e.g. in tsetse flies which are viviparous, alternative methods such as paratransgenesis may be used. In this approach, genetically modified endosymbionts of arthropods are engineered to express proteins which may, for instance, interfere with vector competence (82, 83).

The development of genetically modified vectors has focused mainly on the creation of transgenic mosquitoes which are incapable of pathogen transmission and on the development of methods to suppress target populations, but some studies have also focused on the creation of transgenic insects that express vaccine antigens in their saliva which are delivered via blood feeding to their host.

The feasibility of these approaches was first demonstrated in *Anopheles stephensi*. Genetically modified mosquitoes were engineered to express salivary and midgut peptide 1 (SM1) in their midgut following the uptake of a blood meal and secrete SM1 into the midgut lumen. The peptide binds to an ookinete receptor on the luminal surface of the midgut epithelium and thereby inhibits *Plasmodium* ookinete midgut invasion (84, 85). In another study, *A. aegypti* mosquitoes were transformed with a transposable element that expresses an inverted-repeat (IR) RNA derived from a Dengue virus premembrane protein-coding region, also under the control of a midgut specific carboxypeptidase promoter. Genetically modified mosquitoes express the IR RNA dsRNA in their midgut after they imbibe a blood meal and this interferes with Dengue virus replication via an RNAi-mediated mechanism, thus making the mosquito resistant against Dengue virus (66).

The Sterile Insect Technique (SIT) is a control method that involves the sterilisation of male insects, usually by DNA-damaging γ-radiation, followed by their release in large numbers. The sterilised males compete with wild males for females and the offspring of females that mate with sterilised males are not viable, which ultimately suppresses the population (86). Although SIT has been successful in certain vector control programmes, for instance in the eradication of tsetse flies from Zanzibar (87), a disadvantage of this approach is that irradiated males may have a reduced fitness for competition in mating with females. This can be overcome by using genetically modified males which carry a dominant lethal gene (termed RIDL: Release of Insect with Dominant Lethality) instead of irradiated males. This approach has been evaluated in field trials with the OX513A *A. aegypti* mosquito. The stably transformed OX513A mosquitoes carry a transposon which encodes for the tetracycline-repressible transcription activator (tTA) protein and a fluorescent marker. The transgenic mosquitoes are mass-reared in the presence of tetracycline and sexed at the pupae stage, then the non-biting males are released into the wild (88). Breeding of the OX513A males with wild-type females results in the high-level expression of tTA in the offspring, which accumulates in the cell and is lethal to the larvae (89, 90). In another recent study, researchers genetically modified *A. gambiae* mosquitoes, which have X and Y chromosomes, by germline transformation with a modified I-PoI endonuclease that cuts the X chromosome during spermatogenesis. As a consequence, the large majority of the functioning sperm carries only the male Y chromosome and the offspring of the transgenic mosquitoes in laboratory experiments were almost exclusively male. This effect can be self-sustaining, since the male transgenic progeny also produce a distorted sex ratio when mated with females (91).

The secretion of proteins with saliva can also be exploited for the delivery of protective antigens by haematophagous insects. A proof of principle for these 'flying vaccinators' was demonstrated using transgenic *A. stephensi* mosquitoes which expressed vaccine candidates in their salivary glands. Mice which were repeatedly bitten by the transgenic mosquitoes developed antibodies against the vaccine candidates, indicating that the antigens were delivered via blood feeding (92, 93).

The use of genetically modified insects for vector and pathogen control holds great promise, but remains controversial. Scientific, ethical and regulatory issues regarding the release of transgenic vectors in the field and its impact need to be carefully addressed following a case-by-case approach.

Outlook

The last decades have seen a steep rise in the amount of available genomic data for arthropod disease vectors, in particular for mosquito species affecting human health, as well as the discovery and application of functional genetic tools such as RNA-seq, RNAi, and genetic transformation. These developments allow the study of vectors and vector-borne pathogens in unprecedented detail to elucidate the molecular mechanisms of complex processes such as insecticide resistance and vector–pathogen interactions. The number of available high-quality reference genomes of important arthropod vectors is steadily increasing, aided by improved and more affordable NGS methods. High-quality reference genomes will facilitate (re)sequencing projects of the same or related species to study genetic variation without the need for *de novo* assembly. The sheer amount of genomic data does pose challenges to the information-technology infrastructure, and resources will be required to analyse and explore genomic data for the development of control strategies for vectors and vector-borne diseases in the best possible way.

Configuration génétique des arthropodes vecteurs

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Résumé
Dans le domaine du contrôle des vecteurs et des maladies à transmission vectorielle, l'approfondissement des connaissances sur les aspects moléculaires de la biologie des arthropodes vecteurs et des processus à l'œuvre dans la transmission des agents pathogènes permettra de mettre au point des méthodes innovantes et de perfectionner celles qui existent déjà. L'aire de la génomique ouvre des voies absolument nouvelles pour explorer ces aspects. L'auteur expose brièvement les récentes évolutions intervenues dans le domaine de la génomique vectorielle et apporte un éclairage précieux sur l'application d'outils fonctionnels faisant appel à la génétique, par exemple l'interférence de l'ARN, le séquençage de l'ARN et les transformations génétiques applicables au contrôle des vecteurs.

Mots-clés
Génome – Génomique – Interférence par l'ARN – Transcriptome – Transformation – Vaccinologie inverse – Vecteur.

Configuración genética de los vectores artrópodos

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Resumen
El camino para dar con métodos novedosos o perfeccionados de lucha contra los vectores y las enfermedades que transmiten pasa por una comprensión más profunda de los aspectos moleculares de la biología de los vectores artrópodos y de los procesos que determinan la transmisión del patógeno. La aparición en nuestra época de las disciplinas «ómicas» abre posibilidades sin precedentes para explorar esa dimensión de los vectores y las enfermedades que acarrean. El autor resume los adelantos más recientes en el ámbito de la genómica de vectores y arroja luz sobre la aplicación de herramientas genéticas funcionales, como las de interferencia de ARN, secuenciación de ARN o transformación genética, al desarrollo de la lucha contra los vectores.

Palabras clave
Genoma – Genómica – Interferencia de ARN – Transcriptoma – Transformación – Vacunología inversa – Vector.

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