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Identification, validation and application of molecular diagnostics for insecticide resistance in malaria vectors

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Abstract

Insecticide resistance is a major obstacle to control of *Anopheles* malaria mosquitoes in sub-Saharan Africa and requires an improved understanding of the underlying mechanisms. Efforts to discover resistance genes and DNA markers have been dominated by candidate gene and quantitative trait locus studies of laboratory strains, but with greater availability of genome sequences a shift toward field-based agnostic discovery is anticipated. Mechanisms evolve continually to produce elevated resistance yielding multiplicative diagnostic markers, co-screening of which can give high predictive value. With a shift toward prospective analyses, identification and screening of resistance marker panels will boost monitoring and programmatic decision making.

Emerging resistance to insecticides: a challenge to malaria control

Malaria remains a dominant cause of morbidity and mortality in many sub-Saharan African countries, but over the last decade anti-malaria interventions have achieved major successes [1]. These gains are strongly associated with a scale-up in application of neurotoxic insecticides via indoor residual spraying (see Glossary- IRS), insecticide treated bednets (ITNs) and long-lasting insecticidal nets (LLINs) [1]. Fast-acting insecticides are the mainstay of IRS and ITNs/ LLINs and will play a major role in malaria control for the foreseeable future. Unfortunately, the potency of such insecticides also represents their evolutionary Achilles heel because in large vector populations, strong insecticidal selection leads inexorably to insecticide resistance [2]. This situation is becoming acute in Africa with widespread resistance to the limited arsenal of available insecticide classes [3–5] (see accompanying article by Ranson and Lissenden [6]). However, whilst resistance to some compounds, such as dichlorodiphenyltrichloroethane (DDT) and class I pyrethroids, is near-ubiquitous in *Anopheles gambiae* and *Anopheles funestus*, the prevalence and levels of resistance to other insecticide classes remain highly variable [4, 5, 7]. Thus there is still an

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opportunity for malaria control programmes to employ insecticide resistance management (IRM) approaches to preserve or recover insecticide efficacy [8]. This is a key goal, equally important now and when new insecticide classes become available within the next decade.

In this article we discuss approaches to identify and validate insecticide resistance associated markers that we argue will be central to the sustained reduction of malaria transmission (Figure 1).

Discovery of insecticide resistance associated variants

Identification of DNA polymorphisms linked to insecticide resistance has taken two principal approaches (i) candidate gene studies, in which a specific gene or set of genes are targeted based on pre-existing information or hypotheses, and (ii) "hypothesis-free" or agnostic genomewide studies, in which no a priori candidate information is used; rather the genome or transcriptome is scanned for phenotype-associated variants.

Candidate gene studies

The simplest candidate gene studies involve sequencing the gene encoding the target site of an insecticide. Commonly, the next step is to associate DNA polymorphisms with resistance using resistant and susceptible mosquitoes selected by insecticide exposure or derived from characterized strains. However, given the wealth of data from a variety of taxa and the availability of predictive models (e.g. [9, 10]), simply identifying non-synonymous changes, novel to the species in question can be an important first step in the discovery process. This strategy works well for the loci which encode the proteins targeted by insecticide, because they are functionally-essential genes, subject to strong purifying selection and evolutionary conservation [9, 11, 12]. This strategy has been less successful for alternative mechanisms of resistance, such as cytochrome P450 mediated metabolic detoxification, in which gene families may be less highly conserved and closely related taxa may exhibit independent evolutionary radiations [13].

From the perspective of resistance marker discovery, if not sustained public health control, functional constraint on target loci is beneficial because: (a) variants in the target site conferring resistance are typically deleterious in the absence of insecticide [14, 15] and thus unlikely to have existed at appreciable frequency before insecticide deployment; (b) capacity to survive a generally effective, widely applied insecticide is likely to be a very strongly selected trait, even when generated by a mutation with serious side-effects [15]. Together these properties combine to generate strong signals of selection within the genome, evident as reduced diversity and enhanced linkage disequilibrium (LD), that localizes to target site mutations but extends throughout the gene [16] or even far beyond [12].

A candidate gene study that exploited this expectation of elevated LD around selected variants identified a novel mutation in the *Anopheles gambiae* voltage gated sodium channel (*Vgsc*), the target site of DDT and pyrethroid insecticides (Figure 2). The mutation discovered, *Vgsc*-N1575Y, has a single origin, and always occurs with the well-known *Vgsc*-1014F mutation [17]. Such mutations might compensate for the fitness costs of major variants, but both data from natural populations and subsequent functional validation [18]

demonstrated the effect of *Vgsc*-1575Y is synergistic, multiplying the resistance conferred by *Vgsc*-1014F [18, 19].

Beyond target sites: candidates for metabolic resistance

Independent studies, often using quite different designs, have converged upon a relatively small number of resistance associated variants which have been validated by several *in vitro* and *in vivo* models including recombinant protein assays [20] and heterologous expression in *Drosophila melanogaster* [21–23]. Gene expression studies have repeatedly identified cytochrome P450s as linked to pyrethroid resistance, especially those from the Cyp6 subfamily, such as: *Cyp6P3*, *Cyp6M2* and *Cyp6Z2* [24, 25] in *An. gambiae*; *Cyp6P4* in *Anopheles arabiensis* [26, 27]; *Cyp6P9a*,*b*, *Cyp6P4a*,*b*, and *Cyp6M7* in *An. funestus* [22, 28, 29]. Following identification of phenotypic links in microarray studies, *Cyp6P3* [23] and *Cyp6M2* [21, 23] have also been shown to play a role in resistance to carbamates, and to both carbamates and DDT, respectively. Resistance across unrelated insecticide classes caused by allelic and expression variation in P450s is extremely problematic for insecticide resistance management, limiting options for rotation or combination in a way that is currently difficult to predict. Functional involvement of epsilon class glutathione-S-transferase genes, via metabolism of DDT is well-established [30], with repeated implication of glutathione S transferase E2 (*GSTe2*) as the primary candidate in *An. gambiae* [31–33] and *An. funestus* [29]. In spite of convergence in identification of key metabolic candidate genes, the role of additional genes and gene families should not be discounted, though their exact mode of action in producing resistance may not be understood. For example, gene expression microarray studies comparing resistant and susceptible (control) mosquito strains have detected up-regulation of a range of transporters including aquaporins which regulate the flow of water and other small molecules across cellular membranes, and ATP-binding cassette (ABC) transporters, which move solutes across lipid membranes [21, 22, 29], with ABC transporters linked to both pyrethroid and DDT resistance in *An. funestus*. Coordinated expression of detoxification, sequestration, metabolite-conjugation and transporter genes, could form part of an integrated metabolic resistance response, and emphasises the need to obtain a more holistic view of resistance mechanisms.

Examples of larger-scale genotyping-based candidate studies in *Anopheles* are limited, likely due to the difficulty in identification of convincing candidates and the high cost of focal genotyping at an appropriate scale. Taking inspiration from the *Anopheles gambiae* ‘detox’ gene expression microarray chip [32], which targeted 265 genes primarily from, but not limited to, major detoxification gene families (P450s, GSTs, carboxylesterases, etc), we designed an Illumina Goldengate 1536 SNP genotyping array [34]. Although relatively well-powered, and with evidence from a complementary study which showed elevated expression of some of the candidate genes [35], the primary discovery was a replicated association of *Vgsc*-1014F with resistance to permethrin an insecticide widely used on ITNs [34]. This illustrates both the relative ease with which target site mutations can be identified and the greater difficulty in identification of markers associated with polymorphisms altering gene expression. This may be due to a combination of weaker selection, *trans*-regulation [29] or a qualitatively different nature of selective sweeps, the reduced variation in DNA polymorphisms surrounding a strongly selected locus, on the regulatory variants.

Agnostic discovery via genomewide studies

Quantitative trait locus *mapping*. Quantitative trait locus (QTL) mapping requires little a priori genomic information, and whilst there are now 16 *Anopheles* genomes sequenced [36] there are still numerous regionally important vectors without this resource where QTL mapping may remain the preferred approach. The basic QTL design is to cross strains differing in phenotype to produce F1 hybrids, which are then backcrossed to parental strains or inter-crossed to produce an F2 generation to separate loci generating the phenotype from their native genomic background. Ideally alleles at the markers (most commonly microsatellites and SNPs) can be traced unambiguously from offspring to both parents (fully-informative). The genomes of offspring from separate families are screened and their phenotype of interest scored, and the association between marker alleles and phenotype determined as a LOD (logarithm of odds) score, which describes the likelihood of co-inheritance of a marker and the causal genetic factor underpinning the phenotype. Genetically differentiated strains are used to increase the likelihood that markers will be informative and QTL analyses benefit from use of fully inbred lines of distinct phenotype of the kind readily available for laboratory models such as *Drosophila melanogaster* [37]. Unfortunately lines with such levels of inbreeding are extremely difficult to produce in *Anopheles* [38]. Therefore, partially-inbred laboratory colony strains, selected for divergent phenotypes, are usually used [39, 40]. However, a potential pitfall is the relevance of variants in strains to those circulating in nature [15]. Unfortunately the use of near field-strains [27] or natural pedigrees [41], whilst more likely to be field-relevant creates difficulties in obtaining sufficient family sizes and informative markers [41].

A more pervasive concern is the identification of huge QTL regions, which in F2 designs in *Anopheles* typically exceed 10 Mb [27, 42, 43]. These loci may harbour hundreds to thousands of genes, which makes it necessary to focus on known candidate genes within the region, with consequent reduction in the objectivity of the study. Production of advanced intercross lines (AILs), lines which are interbred for a greater number of generations, helps to narrow the QTL region by increasing the number of recombinant events. Wondji *et al*., though working with the marginally tractable species *A. funestsus*, produced F6 and F8 AIL generations, mapping of which halved the size of the original QTL and also identified two additional QTL [42]. Segments of the major and one minor QTL [44], as defined by their respective LOD scores, were incorporated into bacterial artificial chromosome (BAC) clones and sequenced with CYP6 P450 genes discovered therein proving to be strongly and repeatedly associated with pyrethroid resistance [22, 29].

To fully exploit the benefits of increased recombination in AIL, designs incorporating larger numbers of markers are required than for F2 crossing designs. At this scale microsatellites are better replaced by SNPs, although direct genotyping of hundreds of SNPs is expensive. Promising alternative approaches are the use of reduced representation sequencing (RAD-seq) of individuals or whole genome sequencing of pools of individuals representing family level phenotypes [45]. At some point in the localization process, however, the balance will shift from the advantage of further generations of intercrossing to fine mapping using natural *Anopheles* populations within which there is little linkage disequilibrium [34, 46].

Association studies in natural populations

The major advantage of association studies is their capacity to screen wild populations in which functional variants are segregating and, if conducted at a sufficient scale as genomewide association studies (GWAS), yield a truly agnostic discovery pipeline. GWAS involves screening large numbers of markers (typically 10^4–10^6) to identify those where allele or genotype frequencies differ significantly between phenotype groups; whilst conceptually straightforward, in practice GWAS suffer from several limitations. Cryptic population subdivision, a common observation in *Anopheles* mosquitoes, can be a major source of false positive associations where allele frequency differences primarily reflect population subdivision, rather than association with the target phenotype [34]. Especially relevant to studies of insecticide resistance, is the instability of the frequencies of causal variants for traits under strong selection [47]. Rare variants, i.e. those where the frequency of the minor allele is low, are statistically difficult to detect [34]. Given the low background LD in *Anopheles*, the cost of genotyping the hundreds or even thousands of samples required at sufficient markers to approach true genomewide coverage has precluded individual-based approaches. However GWAS studies of insecticide resistance using pooling of samples genotyped by whole genome sequencing are currently ongoing in *Anopheles*. Analogous studies in *Drosophila* have provided highly accurate estimates of allele frequencies and have good statistical power at moderate cost [48]. The principal limitation of the pooling approach is that with large pool sizes, recovery of haplotype information is very difficult, making a replication stage with individual mosquitoes especially important. Individual based GWAS are becoming feasible as sequencing costs continue to fall.

Validation of resistance associated variants

Once a candidate gene has been identified, either *in vitro* or *in vivo* experiments can be performed to provide a functional explanation for the association with insecticide resistance (Figure 3. If the candidate gene encodes an enzyme thought to metabolize an insecticide, recombinant expression in *E. coli* followed by an enzymatic assay can be used to measure activity. Expression in *Drosophila melanogaster* followed by insecticide bioassays can be an efficient method to screen several candidates *in vivo* [22]. An analogous approach using *Xenopus* eggs is available for some membrane transporters [49] and ion channels [18] but there are many candidates for which there is no heterologous expression-based, functional validation platform. Candidate gene expression in *A. gambiae* can be time-consuming, but offers the opportunity to characterize the pleiotropic functions of a gene i.e. for both general mosquito physiological processes and insecticide resistance phenotype. For example, RNAi-mediated gene silencing can be used to transiently suppress candidate gene expression in mosquitoes prior to insecticide exposure. An additional tool for manipulating gene expression is the Gal4-UAS transgenic system, which may be used to direct ubiquitous or tissue-specific transgene expression in *A. gambiae* (Figure 3) [50]. Lastly, a genome-engineering tool which shows great promise for studies of gene function is the CRISPR-Cas9 system (Figure 3) [51]. A recent study detailed the function of CRISPR-Cas9 in the mosquito *Aedes aegypti*, and provided a practical guide which could be adapted for use in *A. gambiae* [52]. Once the expression of a candidate gene has been altered, its impact upon fitness parameters such as development, reproduction, aging, and insecticide resistance can

be tested. Functional validation can elucidate the mechanism underlying insecticide resistance and thereby yield new strategies for combating the spread of resistance genes.

Opportunities and challenges to the application of markers for resistance monitoring and diagnosis

Despite major advances in identification and characterization of resistance mechanisms [17, 21, 23, 33] and the availability of resistance DNA diagnostics [17, 33, 53], positive impacts on disease control programmes to date have primarily been limited to retrospective analyses of changes in insecticide application [54, 55]. A change toward prospective analyses which directly guide management strategies is urgently required; this will need geographically tailored data not only on the presence of resistance mechanisms in *Anopheles* populations, but on their quantitative predictive value for phenotypic resistance. Although a variety of phenotypic resistance ‘diagnostics’ exist (see accompanying article by Ranson and Lissenden [6]), DNA markers offer ready calibration for predictive value, sensitivity and specificity and can be highly diagnostic.

Not surprisingly, the power to detect significant association, and thus the direct utility of DNA markers as diagnostics for resistance depends strongly on effect size. This effect size may be consistently strong for markers at the **a**cetylcholinesterase (Ace-1) target site, but can be much more variable for some *Vgsc* mutations (Table 1). However, whether a marker is intended for use as a diagnostic or monitoring tool or is in the process of post-discovery replication validation, study sample sizes must be considered carefully to avoid false rejection of association due to lack of statistical power. Moreover, variability in effect sizes among studies (Table 1), which may be in part methodological but probably also reflects true geographical variation, argues against any strict threshold of epidemiological significance at present. Consequently we suggest to retain markers demonstrated as significantly associated as part of screening panels, with integrated analyses applied (e.g. stepwise logistic regression or haplotype association testing) to avoid the statistical penalty of multiple testing and estimate their relative importance. Efforts are underway to associate resistance diagnostics with epidemiological impacts [56] or transmission proxies such as sporozoite rates, which may aid development of epidemiological thresholds for significance.

In the absence of geographically relevant data, marker application tends to be limited to research groups documenting changes in resistance allele frequencies as they accelerate towards fixation [16], at which point they may attract attention of programmatic decision makers [54]. One of the unfortunate impacts of this delayed knowledge transfer is that once a resistance marker approaches fixation in a population, the power to detect a significant association with resistance phenotype declines, although the marker can retain its predictive power at an inter-population level [47]. Under strong selection, resistance will continue to evolve, for example via synergistic mutations or copy number variation (Figure 2), and our capacity to track such temporal changes must follow suit.

Whilst there exists a number of markers which are reliably associated with a resistance phenotype, a substantial proportion of variance in susceptibility remains unexplained. For example, in a recent study of *A. gambiae* from West Africa three DNA markers were only

able to explain approximately 50% of the variance in DDT susceptibility [33]. A combination of environmental variation [56], interactions between resistance markers [7, 23], and expression-based resistance mechanisms [21, 23] may account for the remaining variation in susceptibility. This highlights the need for markers for regulators of the key metabolic resistance genes discussed above. Fortunately, novel metabolic and target-site resistance associated variants are likely to emerge from the *Anopheles gambiae* 1000 Genomes (*Ag*1000G) project (http://www.malariagen.net/projects/vector/ag1000g). To date this project has whole-genome sequenced over 1600 samples from 13 countries with an aim of producing a comprehensive reference database of genetic variation in *A. gambiae* from sub-Saharan Africa. Data have already been released in advance of publication as a service to the community (http://www.malariagen.net/data/ag1000g-phase1-preview), and plans for developing similar resources for other vector species are well advanced. The challenge will then be to work with intervention programmes to use these diagnostics predictively and at the fine temporal and spatial scales that IRM requires.

Concluding Remarks and Future Directions

While insecticide resistance is viewed as a threat to the efficacy of LLIN and IRS interventions, its effects on entomological outcomes and disease transmission have proven difficult to quantify [57, 58]. It cannot be assumed that a resistant mosquito will exacerbate malaria transmission, as the genetic variants which confer resistance may also alter mosquito survival and vectorial capacity. While there are few studies of the life history traits of resistant *Anopheles* mosquitoes, target site mutations have been associated with a fitness burden, reducing reproductive competitiveness and the survival of pupae [59, 60]. Another factor that may influence the vectorial capacity of resistant mosquitoes is the developmental regulation of resistance gene expression [61]. For instance, a resistance gene whose expression decreases with increasing age may enable a young mosquito to survive insecticide exposure, yet offer no protection for older, potentially infective females. Finally, resistance genes may alter mosquito physiology in a way which directly or indirectly affects parasite development. A study of mosquitoes bearing the *Ace1*-119S or *kdr* mutations revealed a higher prevalence of *P. falciparum* infection in the resistant strains [62].

In summary, insecticide resistance may impact mosquito lifespan, vary with mosquito age, and perturb parasite development—all factors which confound the measurement of its impact upon malaria transmission. Use of diagnostic markers to relate such variables to specific mechanisms, rather than general phenotypic resistance should provide more precise quantification (Outstanding questions box). Improved standardization of assays is required to elucidate marker effect sizes, however, some degree of geographical variation clearly exists and needs to be taken into account to optimize marker application in monitoring programs. It is essential that the development and implementation of diagnostics keeps pace with the ineluctable evolution of resistance and expands to encompass both well-characterized and novel mechanisms such as copy number variations, epigenetic modifications and microRNAs. Finally, once new insecticides become available for disease vector control it is crucial that we adopt a pre-emptive framework to identify resistance and resistance mechanism before they become important in natural populations.

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Glossary

Acetylcholinesterase 1	A catalytic enzyme involved in neurotransmission, and the target of carbamate-class insecticides.
Effect size	A measurement of the size of the difference between two groups. One measurement of effect size, an odds ratio, compares the odds that a treatment will produce an outcome relative to a control group.
Genomewide association studies	A method which detects associations between genetic variants, such as single-nucleotide polymorphisms, and an observed phenotype.
Indoor residual spraying	Coating the indoor wall surfaces of a house with an insecticide, such as DDT or bendiocarb, for the purpose of killing mosquitoes that rest upon these surfaces after feeding.
Insecticide treated bednets	Pyrethroid-treated nets that form a protective barrier against mosquitoes when suspended over a sleeping person.
Insecticide resistance management	The global plan for insecticide resistance management in malaria vectors, as defined by the World Health Organization, is a strategy which includes goals such as slowing the spread of resistance, improving understanding of insecticide resistance, and developing new vector control tools.
Linkage disequilibrium	The non-random association of alleles at different loci. It determines power of association mapping in a species.
Long-lasting insecticidal nets	A class of pyrethroid-treated bednet that is designed to resist wear and tear for multi-year use without insecticide retreatment.
Quantitative trait locus mapping	A method in which strains, differing in phenotype, are crossed to separate loci generating the phenotype from their native genomic background. Markers such as microsatellites and SNPs can be traced from offspring to parents, permitting identification of the genomic region associated with the phenotype.
Voltage gated sodium channel	Transmembrane proteins that transmit electrical signals in excitable cells, and the target of both DDT and pyrethroid insecticides.

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Trends box

- Insecticide resistance in African *Anopheles* malaria vectors is a growing problem. Diagnosis and monitoring for insecticide resistance management would be aided by wider application of DNA markers.
- Protein altering mutations in the conserved genes encoding insecticide target sites are often associated with insecticide resistance and are readily diagnosed. Emerging studies suggest that mutations accumulate in these genes and can act synergistically.
- Convergent results identify cytochrome P450s as crucial in metabolic resistance. Some of these genes confer resistance across multiple insecticides but few DNA markers are available.
- New genomic resources and functional validation approaches will shortly yield additional resistance associated variants for monitoring and evaluation.

Outstanding Questions Box

- How important are individual metabolic resistance genes for resistance in the field? Target site resistance is clear and readily quantifiable, but current paucity of markers for metabolic resistance genes limits predictions to correlations and laboratory predictions.
- How many resistance markers are required for an informative diagnostic panel? Some existing markers such as *Ace1* G119S are highly predictive, but will be decreased by increasing population frequency and evolution of additional resistance mechanisms. The answer to this question is likely to change over time and it is crucial that marker discovery pipelines keep pace with vector evolution.
- Will resistance mutations drop in frequency once insecticides are withdrawn? This question has proved difficult to answer to date owing to limitations in insecticide replacement, markers for metabolic resistance and knowledge of mutation fitness costs, but is crucial for insecticide resistance management.
- On what geographic scale do insecticide resistance mechanisms vary? Regionally or between adjacent villages? Variation in the type and frequency of resistance mechanisms, especially target site, is well known in East vs. West African comparisons, recent studies suggest more fine-scale heterogeneities but more studies are urgently required.
- How will currently unknown resistance mechanisms be discovered and validated? This will require a shift to truly agnostic studies with sufficient statistical power and replication for confident identification of genes and mutations, coupled with increased application of promising but largely untested methods for gain and loss of function validation approaches.
- What pre-existing mechanisms may impact novel insecticides? The answer to this will hopefully be none, but it is essential that novel compounds are screened for interactions with known resistance mechanisms prior to wide-scale field implementation.

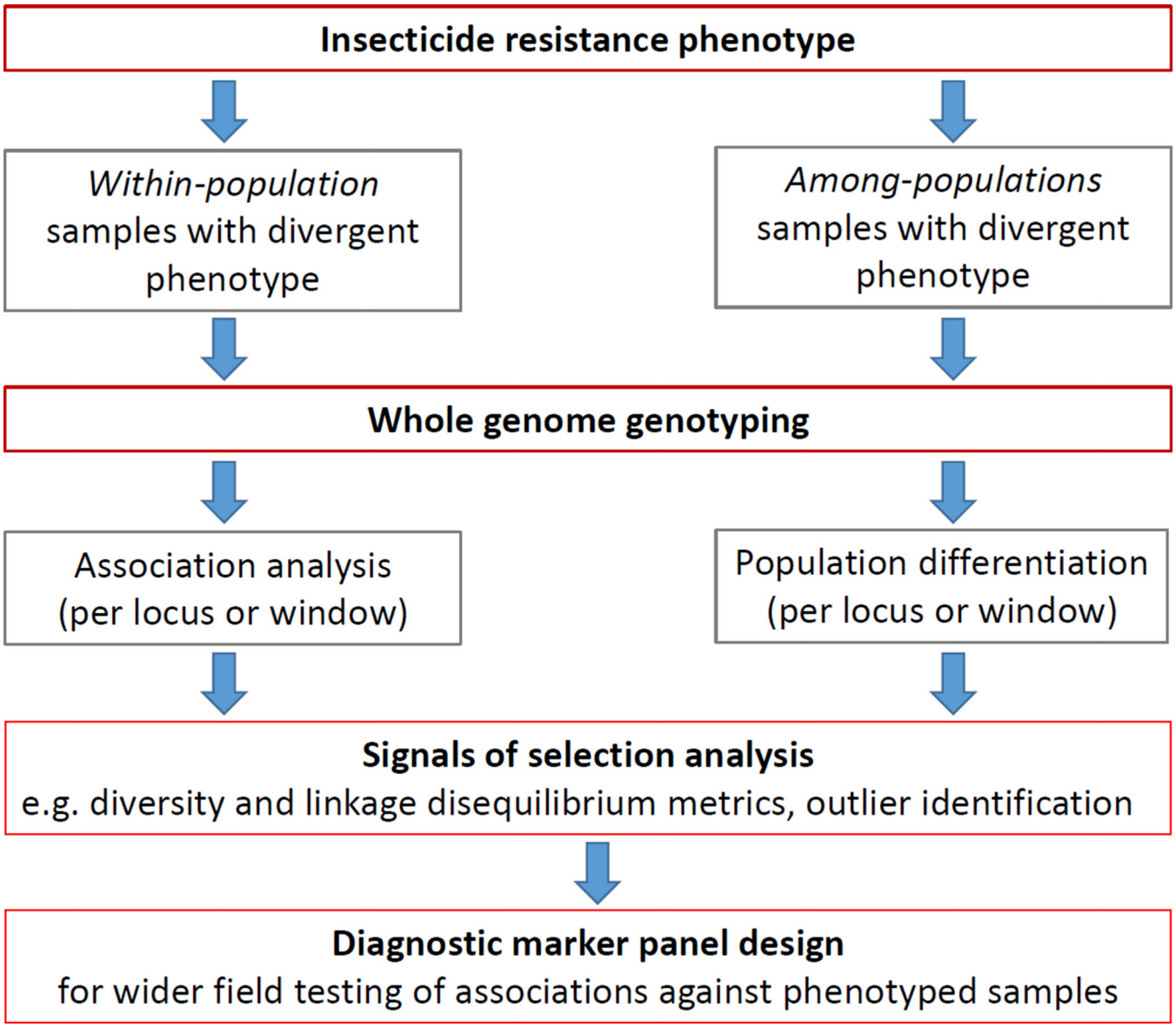

Figure 1. DNA-based resistance marker discovery pipeline

The discovery pipeline begins with an observation of a decreased insecticide sensitivity phenotype in one or more *Anopheles* populations. Possible options for discovery then diverge. On the left hand side mosquitoes are tested for insecticide resistance (typically using bioassays) from within a single population, yielding resistant and susceptible mosquitoes for whole genome genotyping. On the right hand side mosquitoes from separate populations of known phenotype are compared (the samples themselves may or may not be phenotyped) by whole genome genotyping. The former lessens the risk of false positives but at a possible cost of reduced sensitivity. In both cases, the next step involves comparison of allele frequencies between the groups of different resistance status, though the exact analyses and metrics may be different for within and among population analyses. Collection-appropriate population genetic analyses are conducted to localise signatures of selection and inter-population divergence to genomic regions, and representative markers from these

regions are used for replication of genotype:phenotype associations in independent samples. Figures 2 illustrates a different, but overlapping approach for discovery from a known candidate gene, whereas Figure 3 illustrates the alternative functional validation pathway.

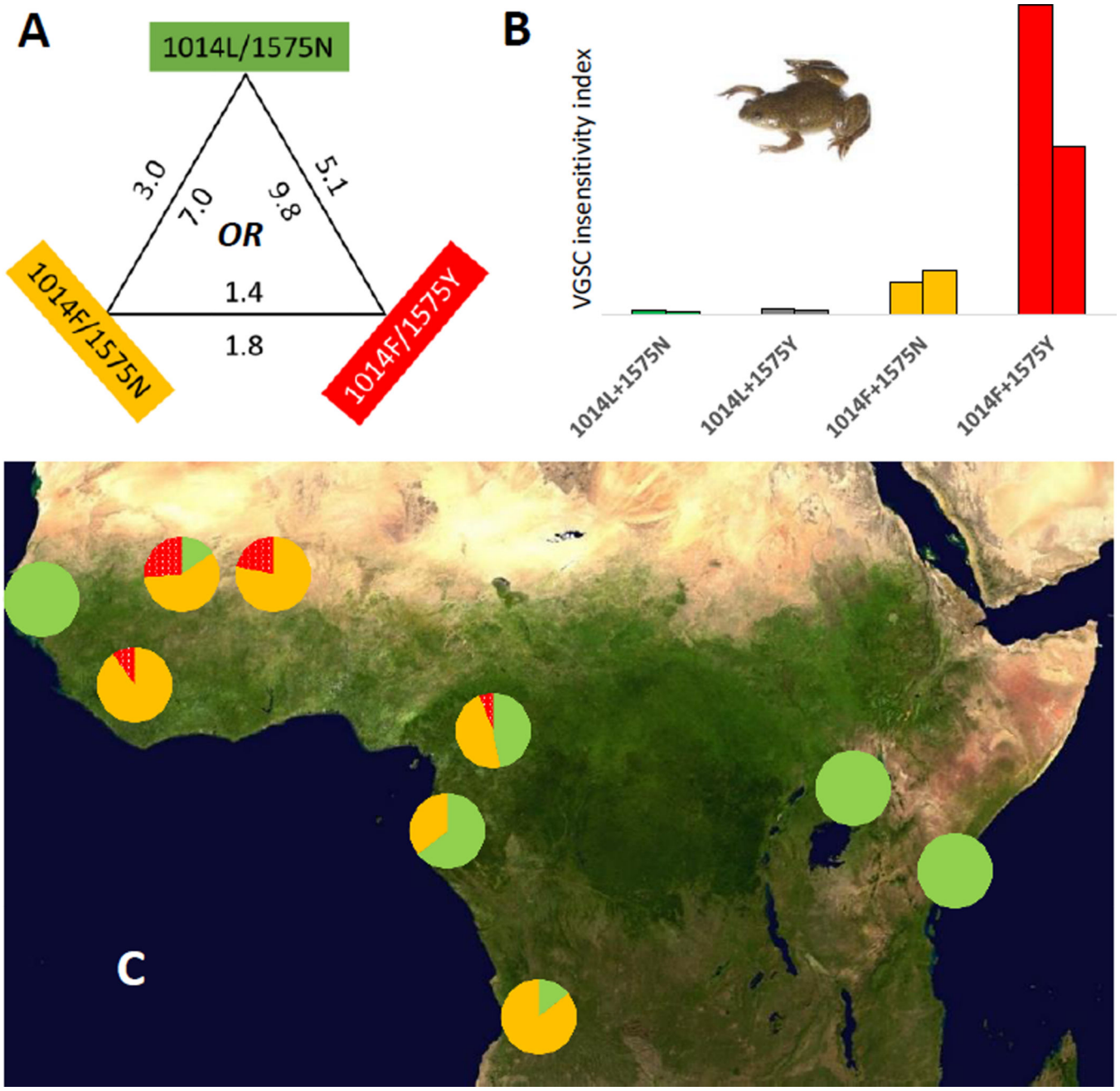

Figure 2. Discovery, assessment and validation of a novel target site mutation

(A) Sequencing of the insecticide target site gene, *Vgsc*, detected a non-synonymous polymorphism, N1575Y. Genotyping of resistance-phenotyped mosquitoes from Burkina Faso revealed that for dichlorodiphenyltrichloroethane (DDT) and two pyrethroid insecticides (average odds ratios, (OR) across insecticides are shown) 1575Y significantly added to the resistance conferred by 1014F, in both *Anopheles gambiae* (OR inside triangle) and *Anopheles coluzzii* (OR outside triangle) [17]. (B) Functional validation of the synergistic effect of 1014F and 1575Y on pyrethroid resistance was provided via expression in *Xenopus* oocytes which demonstrated that 1575Y alone conferred no resistance.

Quantitatively, the y-axis shows the concentration of insecticide required to activate 20% of sodium channels on a linear scale of 0–10μM [18]. (C) *Vgsc*-1575Y is widespread across West and West-Central Africa (pie chart colours correspond to haplotype colours in A) but not in East Africa to date (data from [17] and http://www.malariagen.net/projects/vector/ag1000g).

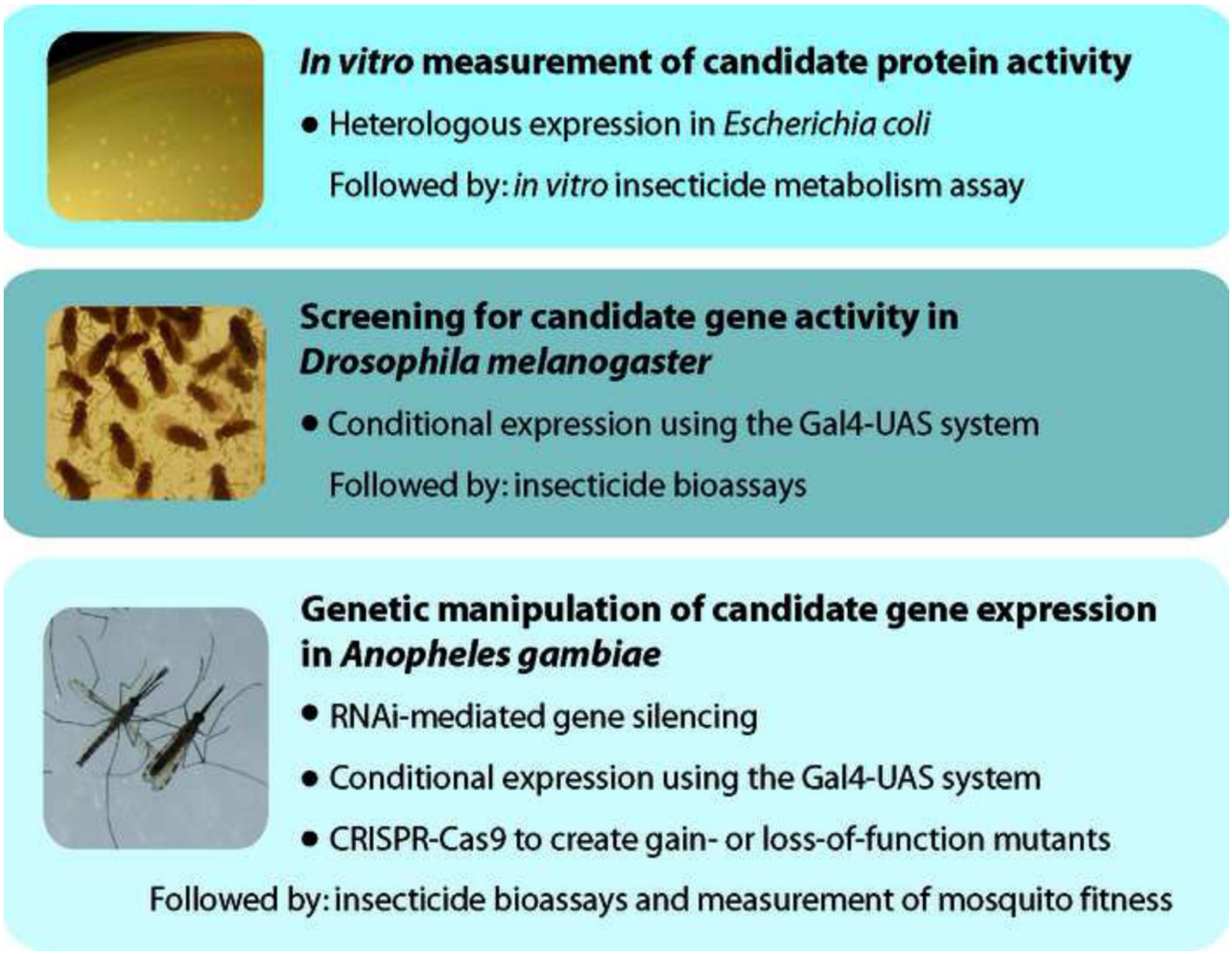

Figure 3. Strategies for functional validation of novel resistance-associated candidate genes or allelic variants

Validation of a resistance candidate is often performed using multiple *in vitro* and *in vivo* strategies, however the choice of organism may depend upon cost, timescale, and the availability of insect culturing facilities. Two transformation systems that have the potential for high-throughput screening of candidates are Gal4-UAS and CRISPR-Cas9 [50, 51]. Gal4-UAS allows for conditional transgene expression, as your gene of interest is under the transcriptional control of Gal4 binding sites. The Gal4 yeast transactivator is encoded by a separate transgene containing user-selected regulatory sequences. Your gene of interest is expressed only when these two transgenes are joined in a single organism through genetic crosses. Alternatively, the CRISPR-Cas9 transformation system can be used if the desired outcome is a site-specific mutation or transgene insertion. When provided either in vivo or in vitro, the Cas9 nuclease creates double-stranded breaks in genomic DNA sequences complementary to a single guide RNA. These breaks are most commonly repaired through non-homologous end joining, which results in short insertions and deletions. If a plasmid

DNA donor containing regions of homology is provided, homology-directed repair can result in insertion of donor sequence.

Table 1

Summary of effect sizes for resistance variants.

Species	Insecticide group	Marker	Geographic location	Effect size with respect to wildtype[a].	Refs
Anopheles gambiae and Anopheles coluzzii	Pyrethroids	Vgsc-1014F	Cameroon Tanzania Burkina Faso Ghana Cameroon/Ghana Cote d'Ivoire	15 – 40 1.38 2.7 0 – 19 5.8 – 6.6 0.8 – 1.23	[63] [64] [65] [66] [34] [4]
Anopheles gambiae and Anopheles coluzzii	XPyrethroids	Vgsc-1014S	Uganda Tanzania Uganda	17 62[b] 1.1 – 2.3	[67] [64] [5]
Anopheles gambiae and Anopheles coluzzii	Pyrethroids	Vgsc-1575Y	Burkina Faso	3 – 12	[17]
Anopheles gambiae and Anopheles coluzzii	DDT	Vgsc-1014F	Cote d'Ivoire Burkina Faso	4 2.8	[4] [65]
Anopheles gambiae and Anopheles coluzzii	DDT	Vgsc-1014S	Uganda	62	[67]
Anopheles gambiae and Anopheles coluzzii	DDT	Vgsc-1575Y	Burkina Faso	8 – 15	[17]
Anopheles gambiae and Anopheles coluzzii	DDT	Gste2-114T	Ghana	1.5 –1.6	[21]
Anopheles gambiae and Anopheles coluzzii	Carbamates	Ace1-119S	Cote d'Ivoire Ghana Ghana	100 15.7 17–18.6	[4] [7] [12]
Anopheles gambiae and Anopheles coluzzii	Organophosphates	Ace1-119S	Cote d'Ivoire Ghana	9.1 28.8	[4] [7]
Anopheles coluzzii	Carbamates	Ace1-CNV	Cote d'Ivoire	18.8[c]	Estimated from data in [23]
Anopheles gambiae	Dieldrin/cyclodienes	Gaba-296G	D.R. Congo	31[b]	[68]
Anopheles coluzzii	Dieldrin/cyclodienes	Gaba-296S (*A. coluzzii*)	Burkina Faso	39	[65]
Anopheles arabiensis	Pyrethroids	Vgsc-1014F	Sudan	6.1 – 14.7	[69]
Anopheles arabiensis	DDT	Vgsc-1014F	Sudan	7.7 – 8.3	[69]
Anopheles funestsus	DDT	Gste2-119F	Cameroon	5.7	[70]

[a] Effect sizes are allelic odds ratios.

[b] Odds ratios were infinite due to absence of data from one cell of the contingency so table so a single score was moved between classes.

[c] Odds ratios estimated from predictive classification by logistic regression