Application of a targeted amplicon sequencing panel to screen for insecticide resistance mutations in Anopheles darlingi populations from Brazil

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Abstract

Large-scale surveillance and informed vector control approaches are urgently needed to ensure that national malaria programs remain effective in reducing transmission and, ultimately, achieving malaria elimination targets. In South and Central America, *Anopheles darlingi* is the primary malaria vector, responsible for the majority of *Plasmodium* species transmission. However, little is known about their molecular markers associated with insecticide resistance. Here we developed a low-cost, high throughput amplicon sequencing ("amp-seq") panel, consisting of 11 amplicons that target genes linked to mosquito species (*cox-1* and *its2*) and insecticide resistance (*ace-1*, *GSTe2*, *vgsc* and *rdl*). Used in tandem with dual index barcoding of amplicons, our approach permits high numbers of loci and samples to be sequenced in single runs, thereby decreasing costs and increasing efficiency. By screening 200 *An. darlingi* mosquitoes collected in Brazil, our amp-seq approach identified 10 point mutations leading to amino acid alterations in *ace-1* (V243I, N194H, S673N, S674N/T) and *GSTe2* genes (I114V, D128E, T166I, T179I, and T205A). Overall, our work has demonstrated the utility of amp-seq to provide insights into the genetic diversity of *An. darlingi* mosquitoes. The amp-seq approach can be applied as a wide-scale insecticide-resistance surveillance technique to better inform vector-control methods.

BACKGROUND

Malaria, caused by *Plasmodium* parasites and transmitted by *Anopheles* mosquitoes, is one of the most severe public health problems in the Americas, where ~ 597,000 cases were diagnosed in 2021 alone[1]. Approximately 1 in 5 of those malaria cases occurred in Brazil[1], where the number of cases rose by 3% between 2015 and 2020 (137,982 vs. 142,112)[2]. The transmission of *Plasmodium* spp. in Brazil predominantly occurs in the Amazon region[2,3]. *Plasmodium vivax* parasites cause the majority (~ 83%) of malaria cases, followed by *P. falciparum* (~ 17%), and then other *Plasmodium* spp. (0.1%). Over the last 7 years, the proportion of cases caused by *P. falciparum*, the species that causes most severe disease, has increased by 7%[2].

The primary malaria vector in Brazil, *Anopheles darlingi* (also called *Nyssorhynchus darlingi*), is highly susceptible to *Plasmodium* infection and can maintain malaria transmission even when parasites are at low densities[4–9]. This mosquito species is highly adaptable to recently anthropized environments and exhibits both exophagic and endophagic behavior. *An. darlingi* primarily feeds on humans and can occupy ecological niches left empty by other *Anopheles* spp[8,10]. Since 2017, deforestation has increased in the Brazilian Amazon, with previous studies showing these newly deforested areas or "frontiers" have higher *An. darlingi* abundance and increased malaria transmission[11]. The adaptability of *An. darlingi* mosquitoes is believed to be leading to year-round malaria transmission. Previously, forest populations of *An. darlingi* would peak during, and towards the end of the rainy season due to the increased availability of larval habitats in flooded areas near rivers. However, environmental changes produced by humans have created permanently available larval habitats, thereby supporting perennial malaria transmission[12,13]. These challenges represent a clear risk to Brazil to accomplish its National Elimination plan and achieve the World Health Organization (WHO) goal of reducing malaria cases by 90% within the next 7 years [14].

Typical malaria control practice in Brazil uses insecticides, especially indoor residual spraying (IRS) or long lasting insecticide treated nets (LLINs)[15,16]. Pyrethroids have been the insecticide class applied in recent years, but organophosphates (malathion) and carbamates are used on *Aedes aegypti* as part of arbovirus control. Since the reintroduction of dengue in Brazil and the occurrence of outbreaks of other arboviruses (e.g., Zika and chikungunya), *Ae. aegypti* has been part of a national insecticide resistance screening programme to optimise elimination strategies, but no such programme exists for *Anopheles* spp[17,18]. Resistance to pyrethroids has been reported across *Anopheles* spp. globally, and for *An. darlingi* in countries surrounding Brazil, including Bolivia, Peru, Colombia, and French Guiana[19–21]. Resistance to carbamates has also been reported in Peru and Bolivia, and organochloride resistance has been observed in Peru and Colombia[22,23]. No resistance has been reported for organophosphates in Brazil, and no data is available for pyrroles or neonicotinoids. The lack of reporting on insecticide resistance in *An. darlingi* is a cause for concern, due to the high levels of associated *Plasmodium* spp. transmission by this vector.

The main insecticide resistance mechanisms observed by mosquito species are target site, metabolic and cuticular, and behavioural avoidance[24]. Target site resistance is mediated by mutations in insecticide target genes, such as the acetylcholinesterase-1 (*ace-1*), γ-aminobutyric acid (GABA) receptor (*rdl*), and voltage-gated sodium channel (*vgsc*), as well as mutations in glutathione-s-transferase *epsilon* (*GSTe2*), which encodes an insecticide metabolising enzyme. Particularly well-studied are the knockdown resistance (*kdr*) mutations associated with Dichlorodiphenyltrichloroethane (DDT) and pyrethroid-based insecticides, including the L1014 mutation in *vgsc*[25,26]. Point mutations in the *GSTe2* gene are also associated with these insecticides, and the L119F and I114T mutations lead to resistance to permethrin in *An. funestus*[27,28]. Mutations and duplications in the *ace-1* gene result in organophosphate and carbamate resistance, and amino acid alterations in *rdl* have been associated with resistance to organochlorines, particularly dieldrin [29–31]. Molecular surveillance studies of insecticide resistance mutations in *An. darlingi* are scarce, with only a few investigations exploring *vgsc* and *ace-1* genes[32,33]. None of the single nucleotide polymorphisms (SNPs) previously found to be associated with insecticide resistance in other *Anopheles* species have been observed in *An. darlingi*, including in pyrethroid resistant populations[33]. However, only limited regions of these two genes have been analysed. It is possible that genetic variants in other gene regions are present, or other mechanisms are involved.

Whole genome sequencing (WGS) has been applied to many vectors to better understand their genomic landscapes and identify candidate genes to unravel mechanisms of insecticide resistance. However, *Anopheles* genomes are large (~ 300 Mbp), and WGS is an expensive method that requires high quantities of DNA to gain suitable genomic insights, meaning it is inappropriate as a high-throughput surveillance method. The application of next-generation sequencing to targeted PCR amplicons, in tandem with dual-index barcoding, has been successfully used in other *Anopheles* spp., *Aedes* spp., and *Plasmodium* spp., as a high-throughput and low-cost screening method for insecticide or drug resistance mutations in target *loci*[34–37]. Targeting several candidate genes in many samples permits the tracking of emerging resistance and spread of known mutations in the population. This approach also allows for an analysis of genotype-phenotype associations to identify novel mutations linked to insecticide phenotypic assays.

Here we have designed an amplicon-sequencing ("amp-seq") assay, consisting of a panel of 11 amplicons (each ~ 500bp) covering the 4 genes (*vgsc, ace-1, rdl,* and *GSTe2*) commonly associated with insecticide resistance, and a further 2 genes (*Its2* and *cox1*) used for species identification and phylogenetic analysis. The assay was used to screen 200 *An. darlingi* mosquitoes collected in Brazil, revealing new mutations. Our assay represents a cost-effective method to confirm mosquito species and conduct insecticide resistance surveillance, with the potential to inform control strategies for an understudied vector responsible for high levels of malaria transmission in South America.

METHODS

Sample Collection, Species Identification and DNA extraction

An. darlingi field mosquitoes were mostly collected from localities in Rondônia state (n = 171; Candeias do Jamari 116, Porto Velho 55) but also include four samples from the Amazonas state (n = 4; Manaus 1, Manacapuru 3) collected to established a *An. darlingi* colony[38,39]. The collections were performed during studies of vector density in malaria endemic areas of Rondônia in 2005–2006 and 2018-2019[38,39]. Mosquitoes from the *An. darlingi* colony of Porto Velho/Rondônia (colony generations : F2-F4, F9-F11, F21, F33-F35, F39, F40-F42) were also included in the study (n = 25) [40]. In total, 200 mosquitoes were screened. The field samples were selected based on whether the localities had used insecticides (Porto Velho) or had little/no recent insecticide usage (Candeias do Jamari). The specimens were initially identified by stereoscopic microscopy, using the established dichotomous keys [41]. Genomic DNA was extracted from whole mosquitoes using the Qiagen DNeasy ® Tissue and Blood kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, each mosquito was grounded with 30 µL and the DNA extract was resuspended in 50 µL elution buffer.

Primer Design

Amplicon primers were designed using Primer3 software, against sequences downloaded from VectorBase[42]. The primers aimed to amplify an approximate 500bp region containing SNPs previously described as associated with insecticide resistance in *Anopheles* or *Aedes* mosquito's species. This resulted in a panel of 9 primers targeting 4 genes: (i) *vgsc* (4 amplicons, targeting four domains); (ii) *rdl* (2 amplicons, targeting 3 SNPs); (iii) *ace-1* (2 amplicons, targeting two SNPs, including A280S[32]); and (iv) *GSTe2* (1 amplicon, targeting a single SNP). When possible, these primers were designed to bind to exonic regions. Two other amplicons were designed to target genes commonly used for species identification and phylogenetic investigation: the ribosomally encoded gene internal transcribed spacer 2 (*its2*); and cytochrome c oxidase I (*cox1*), a locus found in the mitochondria. This resulted in a final panel of 11 assays, covering 6 genes (Table 1). Each primer sequence was concatenated with a unique 5' barcode (8bp), to enable sample pooling during sequencing. Samples were assigned a unique forward and reverse barcode combination used for the generation of each amplicon. To identify amplicons suitable for multiplexing the ThermoFisher Scientific Multiple Primer Analyser was used with sensitivity for dimmer detection set to one.

Table 1
Primers and targets of *An. darlingi* amp-seq panel

Anopheles darlingi Primers								
Aim	Target Gene	Amplicon	Accession ID	Target SNP*	Exon Span	Forward primer	Reverse Primer	Pro Siz (bp
Insecticide Resistance	*vgsc*	VGSCI	ADAC011160	V416L	9–10	GCCTTTCGTCTAATGACTCAAGA	GCCAAGATTAAATTTACAAGGTAAAAC	500
		VGSCII		L1014F	20–21	ACCGTTTCCCCGATAAAGAC	ACGGACGCAATTTGACTTGT	450
		VGSCIII		F1511C/ N1552Y	30	TTTTCCAGGTTGCCACTTTC	ATTGCTTGTGGCCTCCACT	475
		VGSCIV		D1739Y	32–33	AAAATATTTCGTTTCCCCAACA	TCCCAGGATAACCTTTGTCG	447
	ace-1	ACE1_1	ADAC000377	G305S	2	TAAGAAGGTGGACGTGTGGC	AGAGCAAGGTTCTGATCGAA	450
		ACE1_II		N642I	4–5	GACGGGGTACGTCGACAA	AAGGCGCTACTTTCACACG	500
	GSTe2	GSTe2	ADAC008205	L119F	3	TTCGAATCCGGTGTGATCTT	TGGTCACGATCATCTTTATTGG	471
	rdl	RDL1	ADAC005672	A296S/ V371I	7	CACCAACACCAGTCTGATCG	TGGCAAATACCATGACGAAG	490
		RDL2		T345S	8	TGGTTTTTCCCAATCGTTTT	CTGCCCATCTGCTGCTTC	492
Phylogeny	*cox-1*	COX-1	HM022406.1	n/a	n/a	TCTCCAGGGATTACTTTAGATCG	GCTGGGCTGTATGTTAATTGAG	494
	ITS2	ITS2	KF436940.1	n/a	n/a	GACTCAGTGCGAGGTACACA	GAGGCCCACTTGAGATCCTA	455

*Target SNP loci in *An. darlingi*

Amplicon Generation

Multiplex Polymerase chain reactions (PCR) were performed using NEB Q5 hot start polymerase (New England BioLabs, UK) with a total volume of 25ul per reactions. Sample volume of 1µL (~ 2ng/µL) was used, with an average final primer concentration of 0.5µm in each PCR. The amplification was conducted as

follows: hot-start polymerase activation for 3 minutes at 95ºC, followed by 30 cycles of 95ºC for 10 seconds, 58ºC for 30 seconds and 72ºC for 45 seconds, followed by a final elongation step of 72ºC for 5 minutes. Post-multiplex PCR reaction, amplicons were visualised on a 1% agarose gel to confirm amplification, alongside band size and intensity. The multiplex PCR amplicons consisting of 11 amplicons were first pooled by sample, and subsequently pooled with other samples that had different 5' barcode tags. Sample pools were purified using Roche Kapa beads following manufacturer's instructions. A bead to sample ratio of 0.7:1 was used to remove excess primers and PCR reagents. The Qubit 2.0 fluorimeter HS DNA kit was used to quantify the pool concentration. Illumina adaptors and barcodes were added as part of the indexing PCR to the sample pool as a part of the Illumina-based Amplicon-EZ service (Genewiz, UK). Pools contained a maximum of 200 amplicons to maximise coverage. Each indexed pool was sequenced using a 2 x 250bp (paired-end) configuration on an Illumina MiSeq. A minimum of 50,000 paired-end reads were attained per pool, which equates to at least 450 reads per amplicon in a pool of 110 amplicons, at a cost of < US$0.5 per amplicon.

Amplicon Analysis

Raw fastq files were de-multiplexed using the unique barcode combination assigned to each sample, using an in-house python script (https://github.com/LSHTMPathogenSeqLab/amplicon-seq). The resulting sample fastq files were then analysed using another in-house pipeline, where files are first trimmed using Trimmomatic software, then mapped to the reference sequence idAnoDarlMG_H_01 (from NCBI) using the bwa-mem package, and reads are then filtered using Samclip software[43–45]. GATK HaplotypeCaller (v4.1.4.1, default parameters) and Freebayes (v1.3.5, --haplotype-length – 1) software were used to call variants [46,47]. The SNPs and small insertions/deletions (INDELs) identified were then filtered using bcftools[48]. To pass quality control checks, a minimum depth of 30 reads, phred score of > 30 per base, and a minimum allele depth of 10 was required. Variants had to be present in > 1 sample, and across > 1 of the sample pools sequenced. The SnpEff tool was applied to annotate variants using a database built from the idAnoDarlMG_H_01 reference genome[49]. Variants were then genotyped based on the proportion of alternative allele to total depth coverage, called as homozygous reference (< 20% alternate allele reads), heterozygous (20–80% alternate allele), or homozygous alternate (> 80% alternate allele reads)[34].

Phylogenetic Analysis

For the *Its2* and *cox1* amplicons, SNP calls with > 50-fold read depth were converted to fasta files using an in-house pipeline (https://github.com/LSHTMPathogenSeqLab/fastq2matrix). Only sequences with SNP calls that reached this depth were included in phylogenetic analysis. For each gene, sequence data was aligned using the MAFFT tool. Sequences from the NCBI other countries were included in the resulting alignments[50]. For *cox-1*, 62 sequences were added from Brazil, Honduras, Belize, Colombia, Panama, Ecuador, and Peru. For *its2*, an additional 27 sequences were aligned from Brazil, Colombia, Belize, and Bolivia. For tree generation, sequences from both genes were concatenated, and the resulting alignments were viewed and trimmed using Aliview[51]. Phylogenetic trees were constructed using RAxML software[52]. The trees were built using a maximum-likelihood method, with the GTRGAMMA option. This approach assumes a GTR model of nucleotide substitution, and a gamma model of rate heterogeneity. A bootstrap value of 1000 was used for tree construction, and the resulting tree was visualised using iTOL software[53].

Haplotype networks and Maps

To construct the haplotype network, fasta sequences for each gene were aligned for all samples, and then the Pegas package in R[54] was applied. The same package was used to calculate nucleotide diversity, haplotype diversity, Tajima's D statistic, fixation indel (Fst), heterozygosity and linkage disequilibrium.

RESULTS

Detection of novel SNPs in genes associated with insecticide resistance

A total of 200 *An. darlingi* samples were sequenced, with the resulting average amplicon coverage ranging from 171.17- to 5621.32-fold (Table 2). From the alignments, 246 SNPs and 20 INDELs passed all quality control measures, the majority of which were either synonymous (37.8%) or intronic variants (45.9%). Ten SNPs and one INDEL were annotated as non-synonymous and resulted in an amino acid change (Table 3). Missense SNPs were only found in *ace-1* and *GSTe2* genes, and all were present in at least two samples and in two or more populations (Table 3). These SNPs have not been previously reported. In the *ace-1* gene, 111 SNPs were found across the two amplicons, including five missense SNPs (V243I, N194H, S673N, S674N, and S674T), with S674N occurring at the highest frequency (22.3%). Three of these five mutations (V243I, N194H and S674T) occurred only in field populations, while S673N and S674N appeared in field populations and colony samples (Table 4). A further five non-synonymous SNPs were found in the *GSTe2* gene. Three of the missense SNPs (I114V, T166I, and T179I) occurred at frequencies below 23%, and two others (D128E and T205A) appear to be at or approaching fixation as no samples were genotyped as homozygous reference, and over 90% were homozygous alternate for both amino acid alterations. Two of these amino acid alterations in the *GSTe2* gene were found only in field populations (I114V and T166I), and the remaining three (D128E, T191I, and T205A) were also observed in colony samples.

Table 2
Average amplicon coverage, and number of genetic variants identified

Amplicon	Average coverage	Number of SNPs	Number of Non-synonymous SNPs	Number of INDELs	Number of Non-synonymous INDELs
ACE1_I	3190.40	43	2	0	0
ACE1_II	1415.81	68	3	12	0
COI	1996.52	20	0	0	0
GSTe2	3196.05	40	5	1	0
ITS2	5621.32	6	0	0	0
VGSCI	272.17	9	0	3	1
VGSCII	216.81	4	0	0	0
VGSCIII	780.34	4	0	0	0
VGSCIV	1620.89	1	0	2	0
Rdl1	171.17	16	0	1	0
Rdl2	895.84	35	0	1	0

Table 3
Locations and allelic frequencies of detected non-synonymous variants

Chromosome	Amplicon	Position	Sample Number	Annotation	Genotype Frequencies			Allele Frequencies	
					0/0	0/1	1/1	0	1
SNPs									
NC_064874.1	ACE1_I	15679573	191	Val243Ile	92.67	6.81	0.52	96.07	3.93
		15679726	191	Asn294His	97.38	2.62	0	98.70	1.30
	ACE1_II	15681121	149	Ser673Asn	95.30	4.03	0.67	97.32	2.68
		15681124	148	Ser674Asn	77.70	19.60	2.70	87.50	12.50
		15681124	145	Ser674Thr	98.64	0.68	0.68	98.97	1.03
	GSTe2	89825807	129	Ile114Val	95.35	4.65	0	97.67	2.33
		89825922	129	Asp128Glu	0	7.75	92.25	3.88	96.12
		89826035	128	Thr166Ile	87.50	12.50	0	93.75	6.25
		89826074	128	Thr179Ile	78.91	19.53	1.56	88.67	11.33
		89826151	129	Thr205Ala	0	6.20	93.80	3.10	96.90
INDELs									
NC_064875.1	VGSCI	35317107	40	Ile422del	67.5	30.0	2.5	82.5	17.5

Table 4
Genotype frequencies of NS SNPs in the four collection sites

Amplicon	SNP Position	Amino Acid Change	State of Rondonia						State of Amazonas					
			Porto Velho* (50)			Candeias do Jamari (n = 113)			Manacapuru (n = 3)			Manaus* (n = 1)		
			0/0	0/1	1/1	0/0	0/1	1/1	0/0	0/1	1/1	0/0	0/1	1/1
ACE1_I	15679573	Val243Ile	90.0%	8.0%	2.0%	93.8%	6.2%	0.0%	66.7%	33.3%	0.0%	0.0%	100%	0.0%
	15679726	Asn294His	96.0%	4.0%	0.0%	97.3%	2.7%	0.0%	100%	0.0%	0.0%	100%	0.0%	0.0%
ACE1_II	15681121	Ser673Asn	100%	0.0%	0.0%	96.4%	2.4%	1.20%	66.7%	33.3%	0.0%	0.0%	100%	0.0%
	15681124	Ser674Asn	78.3%	17.4%	4.3%	77.4%	20.2%	2.40%	100%	0.0%	0.0%	100%	0.0%	0.0%
	15681124	Ser674Thr	97.7%	0.0%	2.3%	98.8%	1.2%	0.0%	100%	0.0%	0.0%	100%	0.0%	0.0%
GSTe2	89825807	Ile114Val	97.6%	2.4%	0.0%	93.3%	6.7%	0.0%	100%	0.0%	0.0%	100%	0.0%	0.0%
	89825922	Asp128Glu	0.0%	4.9%	95.1%	0.0%	10.7%	89.3%	0.0%	0.0%	100%	0.0%	0.0%	100.0%
	89826035	Thr166Ile	90.2%	9.8%	0.0%	86.5%	13.5%	0.0%	66.7%	33.3%	0.0%	100%	0.0%	0.0%
	89826074	Thr179Ile	80.5%	17.1%	2.4%	77.0%	23.0%	0.0%	66.7%	0.0%	33.7%	100%	0.0%	0.0%
	89826151	Thr205Ala	0.0%	4.8%	95.2%	0.0%	8.0%	92.0%	0.0%	0.0%	100%	0.0%	0.0%	100%
*indicates insecticides are regularly used in this locality														

For v*gsc*, the analysis revealed a INDEL caused by a 3bp deletion, resulting in an isoleucine deletion at position 422 in the first domain. This mutation has not previously been reported and occurred at a frequency of 25% across field and colony samples. Sanger sequencing confirmed these mutations, ruling out sequencing artifacts. No missense polymorphisms were detected in the *gaba* gene (*rdl*).

Genetic diversity of An. darlingi populations in Brazil

Sequences for mitochondrial *cox-1* gene and ribosomal *its2* were generated for genetic diversity analysis. In the *its2* gene, six SNPs were identified. Originally a total of 91 SNPs were identified in the *cox-1* amplicon, but upon further inspection, 71 of these SNPs were present in one sample (AnDar600), which was subsequently identified as an *An. peryassui* isolate (Blast score: 99.2% identity) and excluded from further analysis. Twenty SNPs were identified in the *cox-1* gene, 19 of which appeared in the Candeias do Jamari population, 13 in colony samples, 10 in the Porto Velho populations, and six in the State of Amazonas samples. A fixation index analysis for each SNP revealed no significant population differentiation across these genes (Fst < 0.032).

Phylogenetic analysis with the *its2* gene reflected the small number of SNPs shown in these populations, and very little differentiation was observed between Brazilian isolates and those from other countries (Fig. 1). The tree separated into two main clades, the first of which contains three publicly available samples from Brazil, Belize, and Bolivia. The second clade contained several subclades that included all sequences generated in this study (n = 198) along with the remaining publicly available Brazilian and Colombian samples (n = 26). No differentiation between the different Brazilian populations was observed, which was supported by low nucleotide diversity (π = 0.00536) (Table 6).

For the *cox-1* gene, a total of 128 sequences from this study alongside 67 publicly available samples were analised. (Fig. 2). Using this gene, it was possible to see clusters separating most Colombian, Honduran and Brazilian samples. Although, sequences from other countries can be seen interspersed particularly within the Brazilian *cox-1* sequences. There was both low genetic and nucleotide diversity (0.00697) in *Cox-1* (Table 5), similar to the *its2* results.

Table 5
Nucleotide and haplotype diversities of *cox-1* per population

Population	Nucleotide diversity	Haplotype diversity
All (n = 129)	0.00653	0.856
Amazonas (n = 4)	N/A	N/A
Rondônia (n = 105)	0.00688	0.867
Colony (n = 20)	0.00611	0.837

Table 6
Nucleotide and haplotype diversities of ITS2 per population

Population	Nucleotide diversity	Haplotype diversity
All (n = 198)	0.00536	0.893
Amazonas (n = 4)	N/A	N/A
Rondônia (n = 172)	0.00607	0.883
Colony (n = 23)	0.00639	0.913

A phylogenetic based on concatenated *its2* and *cox-1* sequences revealed little differentiation between samples across the geographical regions (**Supplementary Fig. 1**). Both loci demonstrated high haplotype diversity (Table 5, Table 6), with 44 haplotypes identified for the c*ox-1* gene, and 31 for *its2*. Most of the haplotypes, 77.3% for *cox-1* (n = 34), and 54.8% for *Its2* (n = 17), were present in only one sample (singletons). The high number of singleton haplotypes reflects the high proportion of SNPs occurring at low frequency in the populations. Haplotype 34, representing the *cox-1* gene, was the most frequent, and present in samples from the colony, and states of Amazonas and Rondônia (**Supplementary Fig. 2**). A higher number of *its2* haplotypes (n = 14, 45.2%) were present in more than one sample, compared to *cox1* (n = 10, 22.7%). Haplotypes 1 and 9 were the most frequent, present in both colony and state of Rondônia populations (**Supplementary Fig. 3**). The samples from the state of Amazonas shared haplotypes with both colony and state of Rondônia samples. The networks revealed shared haplotypes for both genes across the three populations, and included several other samples available from other Brazilian states.

DISCUSSION

The application of our amp-seq panel to Brazilian field and colony *An. darlingi* samples has demonstrated its potential utility for species identification, and the discovery of SNPs in genes associated with insecticide resistance. Whilst no previously reported SNPs associated with insecticide resistance in other *Anopheles* species were found in this study, ten other non-synonymous SNPs were detected. Of the five SNPs found in the *GSTe2* gene, all except one (D128E) are either in amino acid positions that are highly variable across *Anopheles* spp. or the mutation results in a change to an amino acid that is present in the reference of another *Anopheles* spp. The I114V substitution is in the same location as a previously reported amino acid alteration in *An. gambiae*[56]. In *An. gambiae*, the mutation results in a I114T alteration, which is hypothesised to cause resistance through the introduction of hydroxyl (-OH) group on the substituted threonine. The hydroxyl group decreases product affinity in the hydrophobic DDT binding site, thereby increasing metabolic turnover of the insecticide. In this instance the valine substitution does not introduce this same hydroxyl group, and valine is present as a reference amino acid in *An. atroparvus.* The D128E mutation occurs at a highly conserved site across *Anopheles* spp., with aspartic acid (D) present as the reference for all. The alteration to glutamic acid results in a similar amino acid structure with the addition of an extra carbon. This mutation appears to be near fixation as 92% of samples were genotyped as homozygous alternate, and the remaining 8% as heterozygous .

Pyrethroids are the predominant insecticide class being applied for malaria-focussed vector control in Brazil[16]. This implies that if target site alterations were to arise, they would predominantly occur in *vgsc* and *GSTe2* genes, as these are the proteins pyrethroids interact with. However, in Brazilian dengue control programmes, *Aedes* spp. are targeted with both pyrethroids and organophosphates, and this usage could impact *Anopheles* spp also present. This could explain the three non-synonymous SNPs found in the *ace-1* gene, a target for organophosphates. The previous reported target-site mutations G119S and N485I (positions in *Torpedo califonica*, G305S and S642I) alter susceptibility to the organophosphate and carbamate classes of insecticide[29]. Resistance to carbamates has been reported in Bolivia, close to the Brazilian border[22]. Of the five amino acid alterations detected here in *Ace-1* (V243I, N294H, S673N, and S674N/T), none have been previously reported. The S673N and S674N/T *ace-1* mutations occur near one of the three catalytic sites (H440; H625 in *An. darlingi*), so may impact the binding of insecticides. The N294H alteration occurs 9 amino acids upstream of the G119S (G305S) mutation, and results in a change from an amino acid with a polar uncharged side chain (asparagine) to one with a charged side chain with an aromatic imidazole ring. For all five of these amino acid alterations, the reference amino acid is conserved across other *Anopheles* species. Futher studies, which include bioassays, are needed to confirm if these mutations result in organophosphate or carbamate resistance. It is not possible at present to exclude potential candidate SNPs on the basis of their presence in the colony samples, as these have demonstrated resistance to both carbamates and pyrethroids, data that is currently being confirmed with bioassays.

The four study locations had varying insecticide usage, with Porto Velho (State of Rondônia), and Manaus (State of Amazonia) sites having a history of intensive use of pyrethroids and carbamates previously. Whilst Candeias do Jamari (State of Rondônia) and Manacapuru (State of Amazonas), had little to no insecticide spraying. All missense SNPs identified in this study were found in locations with intensive insecticide spraying, and locations with little to no insecticide usage. Additional studies, combining phenotypic and molecular surveillance data, are needed to understand the impact of the SNPs detected here on insecticide resistance in *An. darlingi*.

The lack of SNPs found in the *rdl* gene is unsurprising. Mutations in *gaba* result in dieldrin resistance, an organochloride that has been prohibited from use for at least the past decade, due to its adverse effects on human health. In relation to the *vgsc* gene, no *kdr* mutations were detected in the populations surveyed here. This observation was also reported in pyrethroid resistant populations of *An. darlingi* from Colombia[33]. The lack of *kdr* mutations suggests that SNPs in v*gsc* may play a reduced role in *An. darlingi* insecticide resistance. An INDEL resulting in isoleucine deletion at position 422 was detected in 25% of sample, across both field and colony sources. Further studies are needed to understand the involvement of this deletion in insecticide resistance.

The lack of known resistance associated SNPs in *An. darlingi* could be due to the amount of gene flow between geographically close populations. The mixing of possible resistant urban populations with insecticide-sensitive forest populations on the borders of urban areas may minimise the frequency of resistant alleles[19]. An alternative hypothesis is other genetic variants are important for this species, or the involvement of other mechanisms, such as differential expression of genes.

In relation to the genetic diversity across populations, there was little differentiation between *An. darlingi* from Brazil and other regions using *its2* gene data. However, the phylogenetic tree constructed using *cox-1* gene data revealed that samples generally grouped by country. It has been hypothesised that physical barriers like the Atlantic forest mountain range or the Amazon River, prevent the mixing of these populations and so they appear genetically distinct[58,59]. Data from the *cox-1* gene revealed an outlying sample, which coincided with another species (*An. peryassui*).

Within the Brazilian population, low nucleotide diversity was seen in tandem with high haplotype diversity for both *cox-1* and *its2* genes, indicating many low frequency variants. This observation is consistent with previous studies conducted on *cox-1* across Central and South American, and within Brazilian and

Colombian *An. darlingi* populations [13,57,60]. The geographical proximity of the collection sites of these mosquitoes may also contribute to the low genetic diversity observed here. The inclusion of study sites that are more geographically distant or from other countries may give greater resolution to the population dynamics of this species.

Overall, our amp-seq panel provides a tool for the better understanding of the genomic landscapes of this understudied *An. darlingi* vector. It is a high-throughput, low-cost assay for species identification and the detection of novel SNPs in insecticide resistance associated genes. Further investigation is required to identify whether these SNPs contribute to insecticide resistance in *An. darlingi*. A clear limitation of this method is that it currently only targets known loci, however the panel is easily adaptable to new targets, including metabolic markers. The panel can be used in tandem with phenotypic assays to identify SNPs that result in functional changes. Large-scale surveillance methods are urgently needed to inform malaria vector control methods in Brazil, particularly to assist initiatives to reduce malaria transmission. Our panel represents the first steps towards a molecular surveillance method to track known and identify potencial markers of resistance.

Declarations

Data Availability

All raw sequence data is listed in the European Nucleotide Archive (Project ID: PRJEB61194, Accession numbers: ERR11204754 to ERR11204953).

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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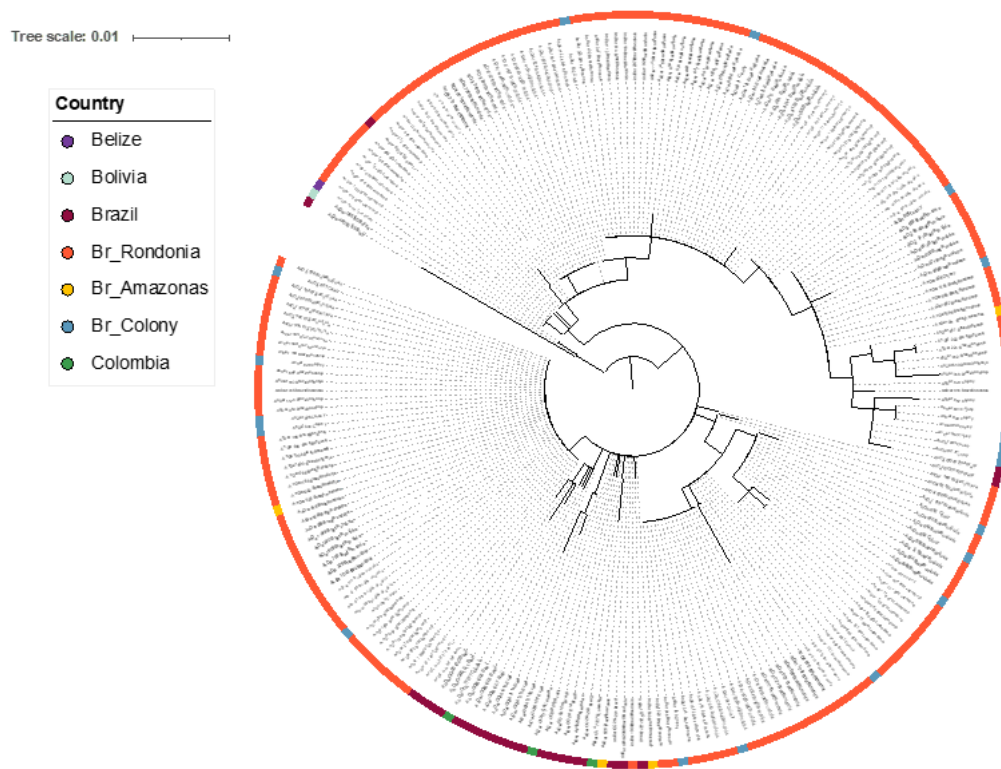
Figures

Figure 1

Maximum-likelihood tree constructed using Its2 gene sequences generated in this study (total=198, Br_Rondonia=171, Br_Amazonas=4, Br_Colony=23), alongside other publicly available *An. darlingi* ITS2 sequences (n=26, Brazil, Colombia, Belize, and Bolivia). The tree was built using the maximum-likelihood method assuming GTR model of nucleotide substitution, with the gamma model of heterogeneity rate.
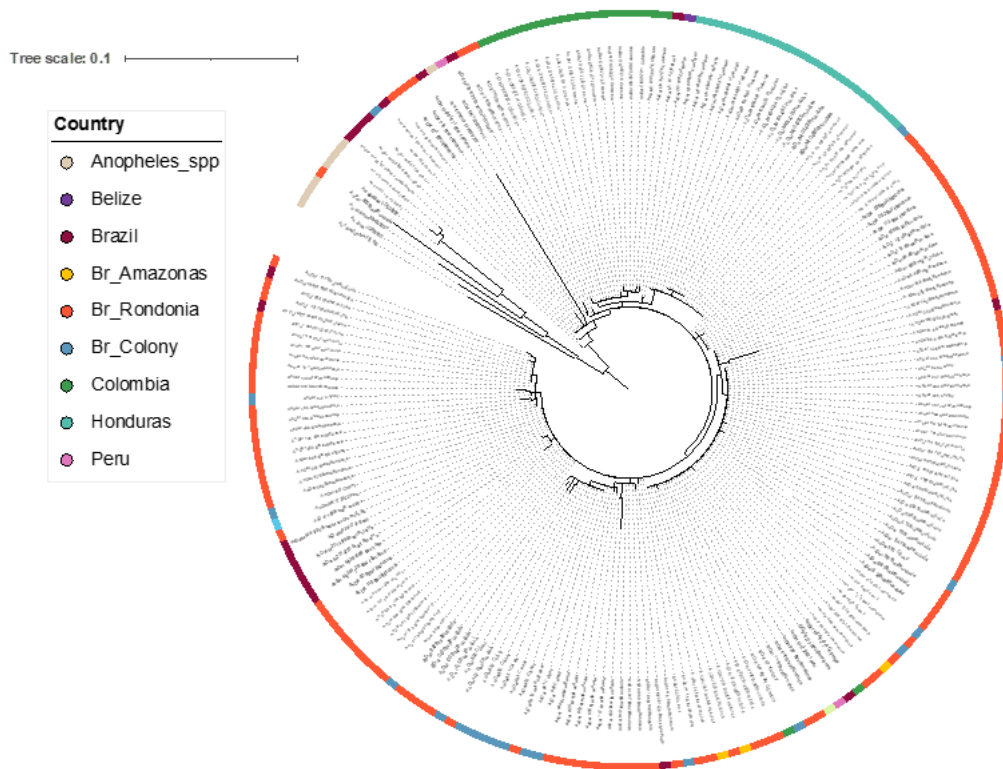
Figure 2

Maximum-likelihood tree constructed using *Cox-1* gene sequences generated in this study (total=129, Br_Rondonia=106, Br_Amazonas=3, Br_Colony=20), alongside other publicly available *An. darlingi cox-1* sequences (n=67, Brazil, Honduras, Belize, Colombia, Panama, Ecuador, and Peru). This tree also has a group of *Anopheles spp.* including *An.albimanus, An.arabiensis, An.coluzzi, An.dirus, An.funestus,* and *An.gambiae.* The tree was built using the maximum-likelihood method assuming GTR model of nucleotide substitution, with the gamma model of heterogeneity rate.

Supplementary Files

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