Parasites & Vectors

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Development of a multiplex SNaPshot assay for simultaneous detection of multiple insecticide-resistance mutations in *Anopheles sinensis*

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Abstract

Background *Anopheles sinensis*, the primary malaria vector in China, is a potential threat to the prevention of re-establishment of malaria transmission following imported cases. The extensive use of insecticides has led to widespread resistance. Conventional bioassays and molecular detection are limited in sensitivity, throughput, and efficiency, underscoring the need for a rapid and cost-effective genotyping tool for large-scale multiple insecticide-resistance markers surveillance.

Methods A multiplex SNaPshot assay was developed to simultaneously detect six SNPs (*kdr1014-F*, *kdr1014-R*, *Ace-1*, *RDL296*, *RDL327*, and *RDL345*) in *An. sinensis*. Positive plasmids carrying known resistance-associated genotypes and laboratory-reared *An. sinensis* samples were used to optimize and validate the reactions. Wild *An. sinensis* collected from Henan Puyang (HN-PY) and Anhui Shucheng (AH-SC) were used for bioassays with 0.05% deltamethrin, 0.15% beta-cyfluthrin, 5% malathion, 0.1% propoxur, and 0.06% flufenoxuron. Then samples randomly selected on the basis of 24 h survival status were genotyped via PCR–Sanger sequencing and the SNaPshot assay. Consistency was assessed using the Kappa testing, and genotype–phenotype correlations were evaluated by chi-squared tests.

Results Bioassays showed that the HN-PY *An. sinensis* population was resistant to deltamethrin, beta-cyfluthrin, malathion, propoxur, and flufenoxuron, with adjusted mortality rates of 5.2%, 24.4%, 9.1%, 24.3%, and 5.8%, respectively. In contrast, the AH-SC population remained susceptible to malathion (100% mortality) but exhibited resistance to deltamethrin, beta-cyfluthrin, propoxur, and flufenoxuron, with adjusted mortality rates of 71.6%, 78.6%, 44.9%, and 33.4%, respectively. Multiplex SNaPshot genotyping of 272 field-collected mosquitoes revealed high frequencies of resistance-associated mutations at the *kdr*1014-F, *kdr*1014-R, *Ace-1*, *RDL*296, *RDL*327, and *RDL*345 loci, with frequencies of 4.7%, 99.3%, 53.3%, 89.7%, 96.0%, and 53.3% in the HN-PY population and 25.5%, 78.0%, 55.3%, 89.8%, 89.8%, and 46.3% in the AH-SC population, respectively. Additionally, the SNaPshot assay showed strong agreement with PCR–Sanger sequencing (Kappa > 0.90 at all loci).

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Conclusions The multiplex SNaPshot assay provides a reliable, rapid, and economical tool for detecting multiple resistance mutations in *An. sinensis*. Its application in routine entomological surveillance will provide an alternative tool for early warning of insecticide resistance.

Keywords *Anopheles sinensis*, SNaPshot, Insecticide resistance, SNP

Background

Mosquito-transmitted diseases, such as malaria, have long been known to cause high disease burden worldwide [1, 2]. Malaria is a febrile illness caused by *Plasmodium* parasites, which are spread to people through the bites of infected female *Anopheles* mosquitoes [3]. In China, malaria was once highly prevalent, and the main malaria vector *Anopheles sinensis* distributes widely throughout almost all regions of China, particularly in rice-growing regions in flatlands [4, 5]. *An. sinensis* is regarded as a competent vector of *Plasmodium vivax*, being the only major vector species in central China where several *P. vivax* malaria outbreaks have historically occurred [6]. In addition to malaria, *An. sinensis* is also capable of transmitting *Wuchereria bancrofti*, Japanese encephalitis virus (JEV) and *Rickettsia felis* [4, 7].

Vector control using insecticides such as long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) remains one of the most effective strategies for mosquito-transmitted diseases control and prevention [3, 8]. However, extensive and long-term use of insecticides in both public health and agriculture has imposed strong selection pressure on mosquitoes, resulting in the emergence and spread of insecticide resistance [9]. The mechanisms of insecticide resistance in mosquitoes primarily include target-site resistance, metabolic resistance, and cuticular resistance [10]. Target-site resistance results from single nucleotide polymorphisms (SNPs) that alter critical amino acid changes, leading to structural changes in target proteins and reduced insecticide binding, and ultimately diminish mosquito susceptibility [11]. The major insecticide targets in mosquito vectors include the voltage-gated sodium channel (VGSC, targeted by pyrethroids and DDT) [12, 13], acetylcholinesterase (AChE, targeted by carbamates and organophosphates) [14], and the γ-aminobutyric acid (GABA) receptor–chloride channel complex (targeted by cyclodienes and pyrazoles) [15]. In *An. sinensis*, resistance-associated mutations that have been identified include nonsynonymous SNPs at codon 1014 of *vgsc* gene in the VGSC IIS6 region [16], a nonsynonymous SNP at codon 119 of the *Ace-1* gene [17], and nonsynonymous substitutions at codons 296, 327, and 345 of the *RDL* gene, which encode GABA receptor [18].

In China, *An. sinensis* populations also have developed widespread multiple resistance to several classes of commonly used insecticides, including pyrethroids, organophosphates, and organochlorines, with high levels reported in Anhui, Shanghai Guangxi, Yunnan, Henan, Hainan Provinces [19–21]. This is a potential threat to insecticide effectiveness for vector control, which will challenge the prevention of re-establishment of malaria transmission in China with the continued importation of malaria cases.

Current surveillance of insecticide resistance in vector mosquitoes relies heavily on bioassays using WHO-defined diagnostic doses of insecticides [22]. While bioassays are simple and widely used, they require large numbers of mosquitoes, are time-consuming and prone to variability, and often lack sensitivity in detecting early resistance changes [23]. Molecular detection of known resistance alleles offers higher sensitivity and can identify these alleles at early stages, providing early warning of the emergence of resistance [24, 25]. PCR–Sanger sequencing is the most used molecular method for detecting insecticide resistance mutations in the *vgsc*, *Ace-1*, and *RDL* genes. Several PCR-derived techniques have also been developed for resistance genotyping in *An. sinensis*, particularly for detecting *kdr* L1014 mutations, including allele-specific PCR (AS-PCR) [26, 27], PCR-restriction fragment length polymorphism (PCR–RFLP) [12], real-time PCR [28] and TaqMan probe assays [29]. However, these methods generally detect only a single gene or a single mutation, making multi-site detection cumbersome and inefficient. Multiplex genotyping approaches such as SNaPshot-based assays and other high-throughput amplicon sequencing have been successfully applied in *Anopheles gambiae* and other mosquito vectors, demonstrating the feasibility of detecting multiple insecticide-resistance mutations [30–32]. However, comparable multiplex assays targeting multiple resistance loci are still lacking for *An. sinensis*.

Therefore, this study aimed to establish an economical, efficient, and rapid SNaPshot-based multiplex genotyping method capable of simultaneously detecting multiple resistance-associated SNPs in the *vgsc*, *Ace-1*, and *RDL* genes of *An. sinensis*. SNaPshot (Applied Biosystems, USA) is a medium-throughput method for multiplex SNP analysis. It enables the simultaneous detection of more than 20 SNPs per reaction with advantages of speed, accuracy, and low cost [33–35]. This Multiplex SNaPshot Assay provides an option for insecticide

resistance surveillance and offers a valuable technical supplement for strengthening resistance monitoring and vector control.

Methods

Samples collection

An. sinensis maintained in the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Chinese Center for Tropical Diseases Research) was used for method development. Meanwhile, wild female adult mosquitoes were collected from cattle sheds and sheepfolds near *An. sinensis* habitats in Puyang County, Henan Province (HN-PY), and Shucheng County, Anhui Province (AH-SC), from July to August 2025, with approximately 500–800 individuals captured per site. The captured mosquitoes were transported in mesh cages to the laboratory, maintained overnight with 8% glucose solution, and reared under suitable conditions (26–28 °C, 60–80% humidity) until the following morning for insecticide susceptibility bioassays. All adult females used in the tests were morphologically identified as *An. sinensis*.

Insecticide susceptibility bioassay

Insecticide susceptibility bioassays were conducted separately on *An. sinensis* populations from the two collection sites, following the standard World Health Organization (WHO) bottle bioassay for adult mosquitoes [22]. Five insecticides belonging to four insecticide classes were tested: two pyrethroids (0.05% deltamethrin, 0.15% beta-cyfluthrin), one organophosphate (5% malathion), one carbamate (0.1% propoxur), and one pyrazole (0.06% fipronil). For each insecticide, three replicates of 20–30 mosquitoes were tested, with an equal number of mosquitoes concurrently exposed to control papers. All insecticide-treated and control papers were provided by the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China. After 60 min of exposure, the mosquitoes were transferred to holding tubes and maintained on 8% glucose solution for 24 h. The mosquitoes were classified on the basis of their survival status after 24 h as susceptible phenotypes (dead within 24 h) or resistant phenotypes (those that survived after 24 h). A total of 272 specimens (150 from HN-PY and 122 from AH-SC) were selected for further analysis (five individuals of each phenotype from each replicate, and all available individuals were used if fewer than five). All specimens were individually transferred into 1.5-mL centrifuge tubes, placed in resealable plastic bags with desiccant silica gel and absorbent cotton, and stored at −20 °C for subsequent analysis.

DNA extraction and species identification

Genomic DNA was extracted individually from each mosquito using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. Extracted DNA was stored at −20 °C for further analysis. Species identification of *An. sinensis* was conducted by PCR based on ITS (Internal Transcribed Spacer) gene [36]. The reaction was performed in a total volume of 25 μL, containing 12.5 μL 2× Takara RR902A mix, 0.5 μL of universal *Anopheles* primers (ITS-UP: CCATGA CGTACACATACTTG, 10 μM), 0.5 μL of *An. sinensis*-specific primers (ITS-AS: GTTGTCCAGCCCGCTAAC AT, 10 μM), 1 μL DNA template, and 10.5 μL of nuclease-free water. To ensure experimental reliability and maintain strict control over PCR specificity and accuracy, both negative and positive controls were used in each PCR assay. The negative control contained nuclease-free double-distilled water (ddH_2O). The positive control utilized genomic DNA samples of laboratory-reared *An. sinensis*. PCR products were resolved on 2% agarose gels, and specimens showing a species-specific band of 425 bp were identified as *An. sinensis*.

SNP selection and plasmid construction

The resistance-associated mutation and corresponding genotypes of *An. sinensis* are summarized in Supplementary Table S1 of Additional file 1 [16–18, 26, 37–39]. Sequences of *vgsc* (GenBank accession no. DQ334052), *Ace-1* (GenBank accession no. ON257855.1), and *RDL* (GenBank accession no. MG190358.1) genes were retrieved from NCBI to identify SNP loci. To optimize and validate the Multiplex SNaPshot assay, plasmid controls carrying known resistance genotypes were constructed by Sangon Biotech Co., Ltd (Shanghai, China). Briefly, short gene fragments (*kdr* 1,160 + 130 bp, *Ace-1* 56 + 130 bp, *RDL*790 + 235 bp, *RDL* 293 + 130 bp) encompassing each target SNP were synthesized and cloned into the pUC57 vector. Each plasmid contained a single, sequence-verified resistance, or wild-type allele, confirmed by Sanger sequencing. These plasmid controls provided stable and homogeneous templates for assay optimization, enabling the accurate adjustment of primer concentrations and extension conditions without interference from mixed genotypes or variable DNA quality.

Conventional PCR–Sanger sequencing

PCR followed by Sanger sequencing was employed as the gold standard for genotyping validation of the field-collected *An. sinensis* samples. Four primer pairs (Supplementary Table S2 of Additional file 1) were used to amplify gene fragments encompassing the *kdr* 1014 site, the *Ace-1* 119 site, positions 296 and 327 in exon 7 of

RDL, and position 345 in exon 8 of *RDL* [18, 38, 40, 41]. Each reaction was performed in a total volume of 25 µL, containing 12.5 µL of 2× Takara RR902A mix, 0.5 µL of each primer (10 µM), 1 µL of genomic DNA template, and 10.5 µL of nuclease-free water. PCR products were verified by 1% agarose gel electrophoresis and subjected to Sanger sequencing (Qingke Biotech, China). Sequencing results were analyzed using SnapGene software to determine the genotypes of the target mutation sites.

Multiplex SNaPshot assay

Multiplex PCR

To enable the simultaneous detection of multiple SNP sites in a single reaction, the target fragments containing the SNPs were first amplified by multiplex PCR with the four primers used in conventional PCR–Sanger sequencing (Supplementary Table S2 of Additional file 1). Laboratory-reared *An. sinensis* mosquitoes were used to optimize the multiplex PCR conditions. A gradient of annealing temperatures (65 °C, 63.3 °C, 61.4 °C, 59 °C, 57 °C, and 55 °C) was tested on the basis of the predicted melting temperature (T_m) values of the four primer pairs to determine the optimal annealing temperature for each primer. Touchdown PCR was employed to enhance specificity while maintaining efficient amplification. The 20 µL PCR reaction contained 1× GC-I buffer (Takara, Japan), 3.0 mM Mg^{2+} (Takara, Japan), 0.3 mM deoxynucleotide triphosphate (dNTP) (Generay Biotech, Shanghai), 1 U Hot Star Taq polymerase (Qiagen, Germany), 1 µL of template DNA, and 1 µL of primer mix (each at 1 µM). PCR products were subsequently treated with 5 U shrimp alkaline phosphatase (SAP; Promega, USA) and 2 U exonuclease I (EXO I; Epicentre, USA) at 37 °C for 1 h, followed by 75 °C for 15 min to remove excess dNTPs and primers, respectively.

SNaPshot single-nucleotide extension reaction

Designation of probes First, 18–30-nucleotide-long SNaPshot extension probes (Supplementary Table S3 of Additional file 1) were designed using Oligo7 software to anneal immediately adjacent to the target nucleotide site on either the sense or antisense DNA strand. Each extension primer was synthesized with a distinct poly (dT) tail length to enable the size-based separation of SNaPshot products. All primers and probes were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Initially, extension reactions were firstly performed with equal primers (1:1:1:1) and probes (1:1:1:1:1:1) concentrations, and these concentrations were subsequently adjusted to obtain more equivalent peak heights.

Single-nucleotide extension reaction The SNaPshot reaction was conducted using the Applied Biosystems SNaPshot Multiplex Kit (Applied Biosystems, USA). Extension reactions were performed in a volume of 10 µL containing 5 µL of SNaPshot Ready Multiplex Ready Reaction Mix, 1 µl of extension probes mix, 2 µL of purified production of multiplex PCR, and 2 µL of ddH_2O. Cycling conditions were: 96 °C for 1 min, followed by 28 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. Extension products (10 µL) were purified with 1 U shrimp alkaline phosphatase (SAP) at 37 °C for 1 h and 75 °C for 15 min. Purified products (0.5 µL) were mixed with 0.5 µL Liz120 size standard and 9 µL Hi-Di™ formamide, denatured at 95 °C for 5 min, and analyzed on an ABI 3730XL DNA Analyzer. Step-by-step operational workflow and functional principle of Multiplex SNaPshot assay are shown in Fig. 1 (created in https://BioRender.com). Genotyping at each locus was determined by analyzing SNaPshot electropherograms. Alleles were identified on the basis of peak colors within predefined size ranges: G (blue), C (black), A (green), and T (red). For forward probes (*kdr*1014-F,

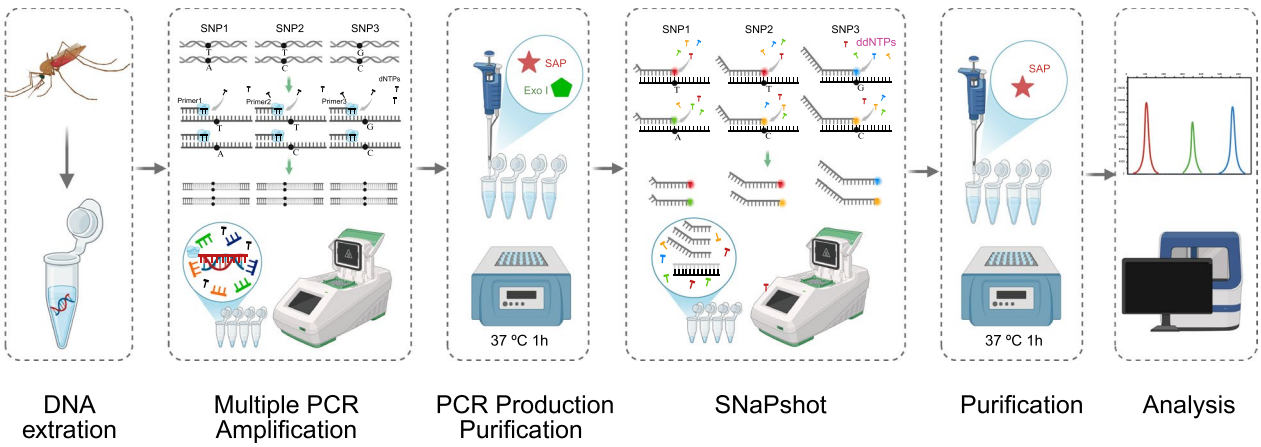


Fig. 1 Step-by-step operational workflow and functional principle of multiplex SNaPshot assay

*RDL*296-F, *RDL*327-F), genotypes were directly inferred from the observed peak colors. For reverse probes (*kdr*1014-R, *Ace-1*−119-R, *RDL*345-R), genotypes were assigned according to the complementary base of the detected color signal. Data were processed using GeneMapper v4.1 (Applied Biosystems, USA).

Validation of SNaPshot assay Because of the influence of fluorescent dyes, the reported peak positions in capillary electrophoresis may deviate slightly from the expected probe lengths. To address this, single nucleotide extension was first performed individually for each of the six SNP sites to determine peak positions and validate genotype calling. For each of the six SNP loci, plasmids representing different genotypes were first mixed at an equimolar (1:1) ratio to generate locus-specific mixtures. These six mixtures were firstly subjected to individual SNaPshot reactions at to ensure that every probe could perform the extension reaction efficiently. The lengths of the extension products for all six probes were determined to prevent overlapping signals during capillary electrophoresis, ensuring unambiguous interpretation of each SNP. The six plasmids' mixtures were then combined at equal ratios (1:1:1:1:1:1) and subjected to multiplex SNaPshot single-nucleotide extension with equal concentrations of probes (1 μM, 1:1:1:1:1:1). This step aimed to evaluate whether multiplexed probes generate overlapping signals when used simultaneously and to establish well-separated locus windows for analysis with GeneMapper software. Probe concentrations were adjusted according to the peak heights at different loci to achieve balanced signal intensities. To validate the specificity and accuracy of the established Multiplex SNaPshot assay, eight laboratory-reared *An. sinensis* specimens were genotyped, confirming the absence of non-specific signals.

Data statistics

Data were entered in Microsoft Excel 2010 and analyzed using IBM SPSS Statistic 20. Methodological consistency was evaluated using the kappa consistency test in SPSS, where a kappa value > 0.75 was considered to indicate good agreement and Kappa value > 0.90 to indicate excellent agreement. The association between phenotypic resistance bioassays and genotypic mutations was analyzed using the chi-squared test, with statistical significance set at $P < 0.05$.

Results

Optimization of multiplex SNaPshot assay

Optimization of multiplex PCR

A temperature gradient PCR was performed to determine the optimal annealing temperature for each of the four primer pairs, which were identified as 55 °C, 57 °C, 57 °C, 61.4 °C, respectively (Fig. 2a). Based on these results, a touchdown PCR protocol was optimized as follows: 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 62 °C (−0.5 °C per cycle) for 40 s, and 72 °C for 1 min; 24 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1.5 min; with a final extension 72 °C for 2 min. The final concentration of each primer was 1 μM.

Optimization of SNaPshot single nucleotide extension reaction

Representative electropherograms of the plasmids representing different genotypes (at an equimolar, 1:1) at each locus are shown in Fig. 2b. When the different genotype-specific plasmids of six loci were mixed at equal ratios and subjected to multiplex SNaPshot reactions, all six loci generated distinct, nonoverlapping peaks, allowing the unambiguous discrimination of each SNP and genotype (Fig. 2c). On the basis of signal intensities of each SNP, concentrations of probes were adjusted to balance peak heights, and final concentration of the *Ace-1*-R probe was 2 μM, and the rest were 1 μM. All eight laboratory-reared *An. sinensis* specimens yielded distinct and unambiguous genotyping profiles using the multiplex SNaPshot assay. No nonspecific signals or aberrant peaks were observed, confirming the high specificity and accuracy of the established method (Fig. 2d).

Detection of insecticide resistance in field-collected samples

Insecticide susceptibility bioassay

The HN-PY population showed high resistance to all five tested insecticides with adjusted mortality rates of 5.19% (0.05% deltamethrin), 24.4% (0.15% beta-cyfluthrin), 9.1%

(See figure on next page.)

Fig. 2 Optimization of multiplex SNaPshot assay. **a** Temperature gradient PCR of four primer pairs. 1, 2: Lab-reared *An. Sinensis* samples. N, Negative control. **b** Electropherograms of genscan analysis of the single SNaPshot reaction of each probe, and alleles are indicated at the bottom. Nucleotides are shown as: G, guanine (blue); C, cytosine (black); A, adenine (green); T, thymine (red). Orange peak: size standard. **c** Electropherograms of genscan analysis of the Multiplex SNaPshot reaction. and alleles are indicated at the bottom. Nucleotides are shown as: G, guanine (blue); C, cytosine (black); A, adenine (green); T, thymine (red). Orange peak: size standard. **d** Validation of the multiplex SNaPshot assay using laboratory-reared *An. sinensis*, and alleles are indicated at the bottom. Nucleotides are shown as: G, guanine (blue); C, cytosine (black); A, adenine (green); T, thymine (red)

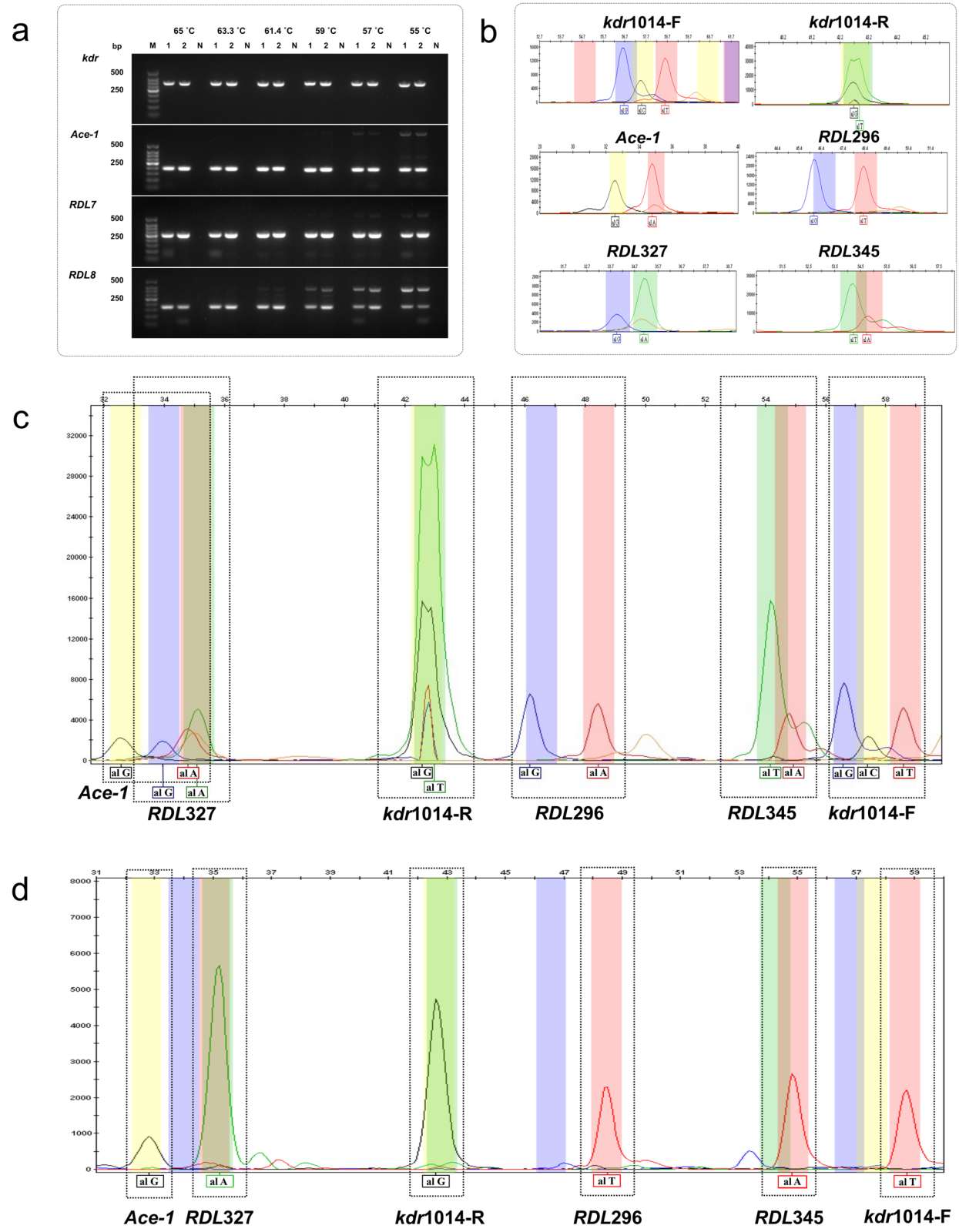


Fig. 2 (See legend on previous page.)

Table 1 Genotypes of multiple insecticide-resistance SNPs in 272 field-collected samples via two methods

Population	Method	*kdr*−1014-F					*kdr*−1014-R						*Ace-1*−119				*RDL* 296				*RDL* 327			*RDL*345			
		T/T	T/G	G/G	F	K	G/G	G/T	T/T	T/C	C/G	K	G/G	G/A	A/A	K	G/G	G/T	T/T	K	G/A	G/G	K	A/A	T/A	T/T	K
HN-PY	PCR–Sanger	136	14	0	0	1	0	5	130	15	0	0.91	11	123	16	0.90	1	29	120	1	12	138	1	29	82	39	1
	SNaPshot	136	14	0	0		0	2	133	15	0		11	118	21		1	29	120		12	138		29	82	39	
AH-SC	PCR–Sanger	66	49	7	0	0.99	7	52	59	2	2	0.97	8	96	18	0.93	0	25	97	1	25	97	1	33	65	24	1
	SNaPshot	66	48	7	1		7	50	61	2	2		8	93	21		0	25	97		25	97		33	65	24	

F failed, *K* kappa value; $P < 0.01$

(5% malathion), 24.3% (0.1% propoxur), and 5.8% (0.06% fipronil), respectively. The AH-SC population exhibited resistance to the four insecticides with adjusted mortality rates of 71.6% (0.05% deltamethrin), 78.6% (0.15% beta-cyfluthrin), 44.9% (0.1% propoxur), and 33.4% (0.06% fipronil), while remaining susceptible to 5% malathion (with adjusted mortality rates of 100%). The difference in malathion susceptibility between the two populations was statistically significant ($P < 0.01$). Detailed mortality rates and resistance status for all insecticides are summarized in Supplementary Table S4 of Additional file 2.

PCR–Sanger sequencing

All 272 field-collected samples were confirmed as *An. sinensis* by molecular identification (Supplementary Fig. S1 of Additional file 2). The genotyping of 272 wild *An. sinensis* samples via conventional PCR–Sanger sequencing revealed mutation profiles across the two populations (Table 1). In the HN-PY population,frequencies of *kdr*1014-F, *kdr*1014-R, *Ace-1*, *RDL*296, *RDL*327, *RDL*345 mutations were 4.7%, 98.3%, 51.7%, 89.7%, 96.0%, and 53.3%, respectively; the AH-SC population exhibited frequencies of 25.8%, 72.1%, 54.1%, 89.8%, 89.8%, and 46.3%, accordingly.

Three *kdr* genotypes were detected in the HN-PY population: L/F (TTG/TTT or TTG/TTC), F/F (TTT/TTT or TTT/TTC), and F/C (TTT/TGT). The homozygous F/F genotype (TTT/TTT) was the most prevalent, with a frequency of 87.3%. In the AH-SC population, a total of six *kdr* genotypes were observed. In addition to the L/F, F/F, and F/C genotypes found in HN-PY, three other genotypes—the susceptible wild-type L/L (TTG/TTG) and the mutant types L/C (TTG/TGT) and C/C (TGT/TGT)—were identified, exclusively in AH-SC. The heterozygous L/F genotype was the most frequent in the AH-SC population, accounting for 30.3%. For the *Ace-1* gene, the G119S mutation was identified, with the heterozygous G/S (AGC/GGC) genotype being dominant. It accounted for 82.0% (132/150) of the detected variants in HN-PY and 78.7% (97/122) in AH-SC. In the *RDL* gene, the A296S (TCA), V327I (ATA), and T345S (TCA) mutations were detected. Both populations showed high frequencies of the homozygous S/S (TCA/TCA) genotype at the A296S locus and the homozygous I/I (ATA/ATA) genotype at the V327I locus. In contrast, the T345S mutation occurred predominantly in the heterozygous form (T/S, ACA/TCA) in both populations. The genotypic distributions for all three genes are summarized in Fig. 3.

Multiplex SNaPshot assay

The SNaPshot assay revealed frequencies of *kdr*1014-F, *kdr*1014-R, *Ace-1*, *RDL*296, *RDL*327, and *RDL*345 mutations in the HN-PY population as 4.7%, 99.3%, 53.3%, 89.7%, 96.0%, and 53.3%, respectively. In the AH-SC population, the corresponding frequencies were 25.5%, 78.0%, 55.3%, 89.8%, 89.8%, and 46.3%, accordingly (Table 1). Compared with conventional PCR–Sanger sequencing, the SNaPshot results were consistent except for one sample from the AH-SC population, in which the genotype at *kdr*−1014-F loci was not detected.

For the *vgsc* gene, six genotypes were all successfully detected. However, five samples originally identified as L/F by PCR–Sanger sequencing (three from HN-PY and two from AH-SC) were determined as F/F by SNaPshot. For the *Ace-1* gene, the AGC (G119S) mutant allele was detected. Among these, eight samples initially characterized as A/S by PCR–Sanger sequencing (five from HN-PY and three from AH-SC) were identified as S/S by SNaPshot. For the *RDL* gene, the results for all three mutation sites were consistent between SNaPshot and PCR–Sanger sequencing. The genotypic distributions for all three genes are summarized in Fig. 4. The raw data of detections of six SNP loci in 272 field-collected samples using PCR–Sanger sequencing and the multiplex SNaPshot assay are recorded in Supplementary Table S5 of Additional file 3.

Association between insecticide resistance phenotypes and target-site gene mutations in field-collected samples

A total of 272 field-collected *An. sinensis* mosquitoes were analyzed to evaluate the association between insecticide resistance phenotypes and corresponding genetic mutations. For pyrethroids (Supplementary Table S6 of Additional file 3), all resistant and susceptible mosquitoes in HN-PY carried the *kdr* L1014F or L1014C mutation, while no significant association was detected in AH-SC (for 0.05% deltamethrin: $\chi^2 = 0.63$, $P = 0.429$; for 0.15% beta-cyfluthrin: $\chi^2 = 0.22$, $P = 0.636$). For organophosphates and carbamates (Supplementary Table S7 of Additional file 3), all resistant HN-PY mosquitoes carried the *Ace-1* G119 mutation, whereas a small number of susceptible individuals (3 out of 15 for each insecticide) carried the nonmutant allele, showing no significant association in HN-PY ($\chi^2 = 3.33$, $P = 0.068$). In the AH-SC population, resistance to 5% malathion was not observed, but all 15 susceptible samples selected for molecular detection carried the G119S mutation. No significant association was detected for 0.1% propoxur in AH-SC population ($\chi^2 = 0.00$, $P = 1.000$). For pyrazoles (Supplementary Table S8 of Additional file 3), all mosquitoes were mutant at *RDL* A296 and V327 loci, while *RDL*345 showed no significant correlation with resistance in either population (HN-PY: $\chi^2 = 1.68$, $P = 0.195$; AH-SC: $\chi^2 = 0.16$, $P = 0.690$). In this study, we found no statistically significant difference of resistance phenotypes

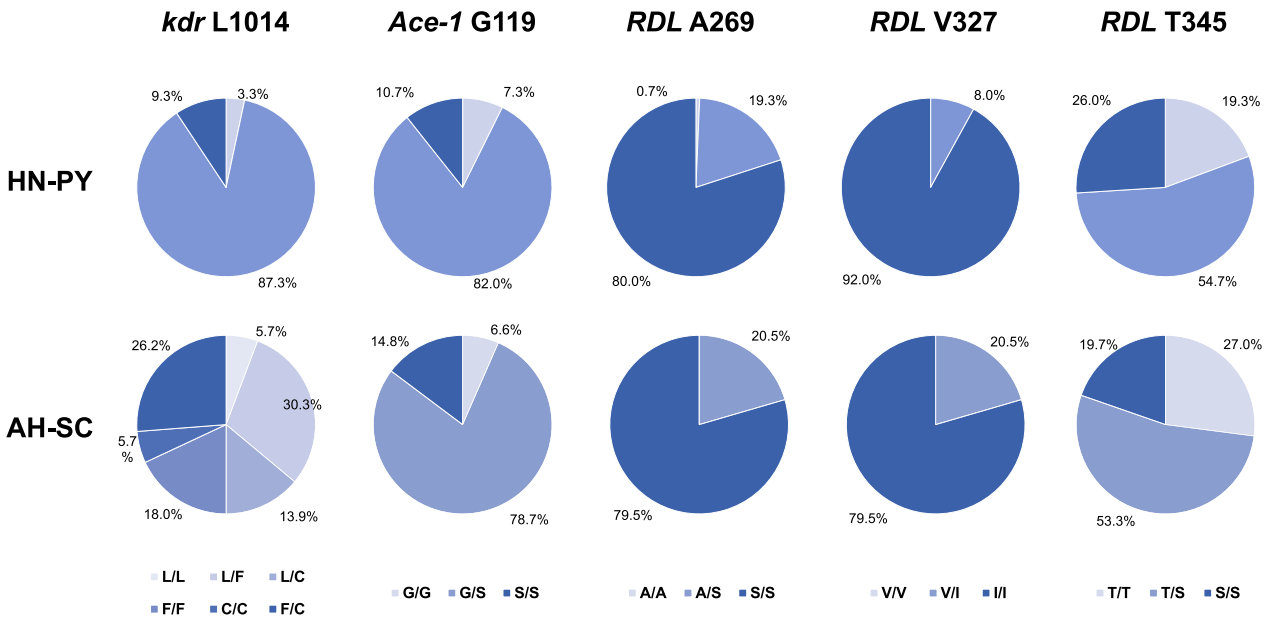


Fig. 3 Genotypic frequencies at *vgsc*, *Ace-1*, and *RDL* genes in *An. sinensis* populations from HN-PY and AH-SC, determined by PCR–Sanger sequencing

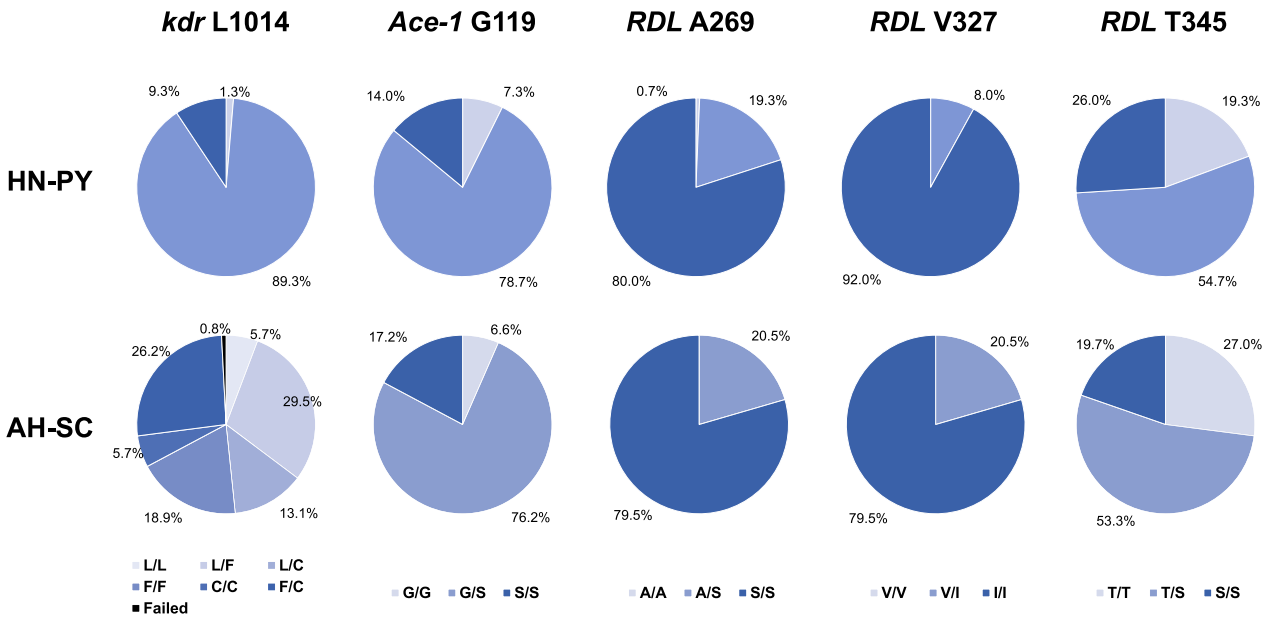


Fig. 4 Genotypic frequencies at *vgsc*, *Ace-1*, and *RDL* gene in *An. sinensis* populations from HN-PY and AH-SC, determined by multiplex SNaPshot assay

and the examined target-site mutations, although high mutation prevalence and limited sample sizes may have reduced statistical power.

Evaluation of multiplex SNaPshot assay

Using the multiplex SNaPshot assay, genotyping was successfully performed for two mosquito populations. All six SNP loci (*kdr*−1014-F, *kdr*−1014-R, *Ace-1*−119, *RDL* 296, *RDL* 327, *RDL* 345) showed high concordance with reference sequencing, with kappa values were 1, 0.91, 0.90, 1, 1, 1 in HN-PY and 0.99, 0.97, 0.93, 1, 1, 1 in AH-SC ($P < 0.01$), indicating excellent reproducibility and reliability (Table 1).

Moreover, the SNaPshot assay shows more efficiency and economical for large-scale surveillance in *An. sinensis*. Conventional PCR–Sanger sequencing requires four times independent PCR amplifications and sequencing runs, which take more than 24 h (at least 6 h per reaction) to cover all six SNP loci. The SNaPshot assay can simultaneously genotype all six SNP loci in a single reaction per sample within 8 h. Moreover, the cost of the SNaPshot assay (approximately ¥48 per sample) was substantially lower than that of PCR–Sanger sequencing (approximately ¥80 per sample).

Discussion

An. sinensis, the dominant malaria vector in China, challenges the prevention of re-establishment of malaria transmission following imported cases [5]. Intensive and prolonged insecticide use in both agriculture and public health has exerted strong selection pressure on mosquito populations, resulting in widespread and complex resistance [42]. However, among conventional detection methods of insecticide resistance, bioassays have limited sensitivity, and several molecular detection techniques are typically restricted to detecting single resistance genes, which limits their application to large-scale screening of multiple mutations [24, 43]. Previous studies have demonstrated the utility of multiplex SNaPshot or other high-throughput genotyping platforms for detecting resistance-associated mutations in *An. gambiae*, enabling simultaneous interrogation of multiple loci in a single assay [30–32]. In this study, we developed a multiplex SNaPshot assay to simultaneously detect six target-site mutations associated with insecticide resistance in *An. sinensis*.

Molecular assays can identify resistance-associated alleles, providing an early warning of insecticide resistance emergence [43]. In *An. sinensis,* prior efforts to determine *kdr*, *Ace-1*, and *RDL* mutation status have relied on PCR–Sanger sequencing, which remains the gold standard for discovering known and novel mutations but is low-throughput and costly [39]. Although other methods such as AS-PCR for the *kdr*1014 mutations [44] and PCR–RFLP applied for the *Ace-1* G119S mutation [45] are simple and inexpensive, they are prone to false positives and limited to single loci. In addition, the use of TaqMan/AllGlo probe-based quantitative PCR (qPCR) assays for *kdr*1014 detection provides high sensitivity and specificity but is hampered by reliance on specialized equipment, high cost, and low throughput [46]. In contrast, the SNaPshot assay used in this study can interrogate multiple SNPs at known locations on the basis of single-base extension in a single-tube reaction [47]. This approach has proven well-suited for applications such as forensic analysis, tumor gene genotyping (such as POLE, PIK3CA, and KRAS) [48, 49], and population and association genetics [50], providing a rapid, accurate, and cost-effective genotyping approach. These advantages were also supported by the results in this study. First, the developed multiplex SNaPshot assay demonstrated high accuracy, with validation using 272 field-collected samples showing strong concordance with Sanger sequencing (kappa > 0.9 for all six loci). Moreover, the multiplex SNaPshot assay simultaneously genotypes all six SNPs in a single reaction, substantially reducing labor, time, and cost compared with PCR–Sanger sequencing, which requires separate amplification and sequencing for each gene. Collectively, this multiplex SNaPshot assay offering a robust, rapid, and cost-effective alternative for simultaneously detection of multiple resistance associated mutations in *An. sinensis*.

High frequencies of resistance-associated mutations across all six loci were successfully detected by developed multiplex SNaPshot assay, highlighting the prevalence of cross-resistance in two populations. For the codon 1014 of the *vgsc* gene, the high frequencies of mutation were comparable to previous reports from Anhui, Henan, Jiangsu, and Hubei provinces [51], suggesting that *kdr* mutations have stabilized in *An. sinensis* populations in these regions. Two amino acid substitutions (1014F and 1014C) were identified, with homozygous L1014F predominant in HN-PY and heterozygous L1014F most frequent in AH-SC, aligning with previous surveys in central and eastern China [21, 52, 53] For the *Ace-1* G119S mutation, frequencies in two populations were both high, consistent with its widespread distribution in Asian *An. sinensis* [54, 55]. However, this genotypic prevalence did not translate to a resistant to 5% malathion phenotype in the AH-SC population, which showed 100% mortality. The discrepancy may be attributed to the dominance of heterozygotes (78.7%), which often confer incomplete resistance [56, 57], and the potential lack of co-occurring metabolic resistance mechanisms [58]. Furthermore, variables such as the physiological status (age, blood-feeding status, and activity et.al) of field-collected mosquitoes and bioassay conditions can influence mortality rates, potentially obscuring true resistance patterns [59, 60]. For the *RDL* gene, A296S and V327I were predominantly as homozygotes, nearly fixed implying strong resistance to cyclodiene and phenyl pyrazole insecticides. This pattern resembles that reported in Guangxi and Sichuan populations of *An. sinensis* and may reflect historical selection from banned dieldrin use or increasing application of GABA receptor-targeting insecticides in agriculture such as ethiprole [41, 61].

In this study, we also identified certain discrepancies in the obtained results. First, minor discrepancies were observed in the genotyping results at three loci

(*kdr*−1014-F, *kdr*−1014-R, and *Ace-1*−119) between PCR–Sanger sequencing and the multiplex SNaPshot assay. These inconsistencies may result from suboptimal DNA quality leading to preferential amplification bias in the multiplex reactions, or copy number variation (CNV) at the *kdr* and *Ace-1* loci—a mechanism documented in *An. gambiae* [62–66] but not yet confirmed in *An. sinensis*. Moreover, the absence of significant associations between insecticide resistance phenotypes and target-site mutations might be explained by the high mutation frequencies,which could have limited statistical power to detect correlation between individual genotypes and resistance phenotypes, or by the coexistence of multiple resistance mechanisms, particularly metabolic detoxification mediated by cytochrome P450 monooxygenases, esterases, and glutathione *S*-transferases [67]. These results further suggest that phenotypic bioassays may underestimate actual resistance risk, as their outcomes are influenced by mosquito age, physiological condition, and environmental factors [68].

A primary limitation of the established SNaPshot assay is the restriction to known target-site mutations, as it cannot detect novel or undefined resistance alleles, but this limitation does not diminish the utility of the assay for routine, high-throughput monitoring of established resistance markers. Consequently, complementary surveillance approaches, such as periodic PCR–Sanger or next-generation sequencing, remain important to identify emerging mutations and to ensure comprehensive resistance monitoring. Although only two field populations from Henan and Anhui provinces were included in this study, the assay targets well-characterized resistance-associated SNPs with conserved flanking sequences in the *vgsc*, *Ace-1*, and *RDL* genes [69]. These loci have been widely reported across geographically distinct *An. sinensis* populations [19–21, 37–41], suggesting that the multiplex SNaPshot assay is expected to perform robustly across different genetic backgrounds of *An. sinensis*. Nevertheless, further validation using samples from a broader geographic range will be necessary to fully assess its applicability across the species' distribution. Furthermore, this study was constrained using wild-caught adult females without age or blood-feeding history control, which may affect bioassay-based resistance phenotyping [70]. Future studies should aim to clarify the presence of CNVs in the *vgsc* and *Ace-1* genes of *An. sinensis*, and link genotypic and phenotypic data to provide a more comprehensive resistance profile.

Conclusions

This study developed a rapid, accurate, and cost-effective tool for simultaneous detection of multiple resistance mutations in *An. sinensis* based on a multiplex SNaPshot assay. Its scalability and practicality make it a valuable complement to conventional assays for routine monitoring insecticide resistance-associated mutations in *An. sinensis* in the malaria post-elimination setting.

Abbreviations

WHO	World Health Organization
VGSC	Voltage-gated sodium channel
GABA	γ-Aminobutyric acid
Kdr	Knockdown resistance
Ace-1	Acetylcholinesterase-1
RDL	Resistant to dieldrin
P450s	P450 monooxygenase system

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-026-07250-2.

Additional file 1: Table S2 Major insecticide targets and resistance-associated gene mutations in An. sinensis. Table S3 Primers for PCR. Table S4 Probes for SNaPshot

Additional file 2: Table S4 Bioassay results of resistance to five insecticides in HN-PY and AH-SC populations. Fig S1 Gel electrophoresis image of species identification of *Anopheles sinensis* samples collected from field sites. M: DNA Marker; P: Positive control; N: Negative control. Table S5 Raw data of genotyping results of six insecticide resistance–associated SNP loci in 272 field-collected *An. sinensis* samples detected by PCR–Sanger sequencing and the Multiplex SNaPshot assay.

Additional file 3: Table S6 Association between *kdr* L1014 mutation and pyrethroid resistance phenotypes in field-collected *An. sinensis* populations. Table S7 Association between *Ace-1* G119 mutation and organophosphate/carbamate resistance phenotypes in field-collected *An. sinensis* populations. Table S8 Association between *Rdl* gene mutationsand pyrazole resistance phenotypes in field-collected *An. sinensis* populations.

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Author contributions

YX, BZ, JY, and BX conceived this study. YX and BX conducted sample collection and preparation. YX, ZY, SY, and ML performed the laboratory works. CY, SY, and RX interpretation of data. YX performed the result analysis. YX wrote the manuscript. BZ, JY, and BX revised the manuscript. All authors read and approved the final manuscript.

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Data availability

The original contributions presented in the study are included in the article or supplementary materials.

Declarations

Ethics approval and consent to participate

For mosquito collection, consent was obtained from field and house owners in each location. These locations were not protected land, and the field studies did not involve endangered or protected species.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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