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Research Article

Establishment of a Microfluidic Chip Based Rapid Detection Method for *Wasmannia auropunctata*

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Abstract

Invasive alien ants are one of the most aggressive, competitive, and widespread invasive alien species around the world. *Wasmannia auropunctata*, native to the neotropical zoogeographic area, has been inadvertently introduced all over the world. The first disclosed record of field establishment of *W. auropunctata* in Chinese mainland was reported in 2022. In this paper, we aim to establish a rapid detection method targeting the *W. auropunctata* polymorphic microsatellite locus Waur-275, based on the microfluidic chip technology, to contribute to an active response to the crisis caused by this invasive pest. Eight primer sets were generated using the PrimerExplorer v5 online service, and the fifth primer set selected had the lowest Ct in the volume ratio of 8 (FIP/BIP:F3/B3). Method validity with six *W. auropunctata* samples confirmed the efficiency of this assay. High specificity was demonstrated by the positive result for *W. auropunctata* out of 6 related insect samples tested. Sensitivity analysis showed that this microfluidic chip method could achieve the detection limit of 1.00×10^{1} copies/μL. A rapid screening method is essential for the whole process of interception and control of *W. auropunctata* in China. It can also help determine the origin of invasion, clarify the path of introduction to ensure biosecurity, and also uncover other possible undetected establishments in the wild.

Keywords

Rapid Screening, Microfluidic Chip, *Wasmannia auropunctata*, Invasive Alien Ants

1. Introduction

Invasive alien species (IAS), which persist a nuisance to ecosystems, are the main cause of biological diversity loss [1]. Invasive alien ants (IAAs) are one of the most dangerous IAS worldwide [2]. *Wasmannia auropunctata* (Roger, 1863), with aliases of ‘electric ant’ or ‘little or small fire ant’, is originated from the neotropical zoogeographic area [3]. It has been inadvertently introduced all over the world [4]. *W. auropunctata* has several traits in common with other IAAs, such as unicoloniality, and the corresponding polygyny [5]. Within its infested areas, it reduces the biodiversity of native ants and other invertebrates, vertebrates, and poses a non-negligible health risk to residents because of its venom-

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ous sting which can cause anaphylactic shock [6, 7]. This omnivorous pest has been able to expand rapidly worldwide because it can invade and establish in various biotopes, either disturbed or undisturbed, to build polygynous nests on diverse substrates, and to tolerate different climatic and environmental conditions [8]. It has been reported that organic packing material, ships, containers and natural transmission across borders are four dominant spread approaches for *W. auropunctata* [9]. Furthermore, *W. auropunctata* colonizes mainly in irrigated places or close to water sources with continual human activities, allowing it to survive in areas either warmer or drier [10].

In January 2022, *W. auropunctata* specimens were collected in the wild in Shantou City, Guangdong Province, China. This is the first formal record of field establishment of *W. auropunctata* in mainland China [11], representing an enormous potential crisis to economic, ecologic, and social welfare. Related potential geographical distribution estimation research showed that agricultural fields, urban and rural areas in the coastal places, the Yunnan-Guangxi border, as well as grasslands in southwest China face the greatest invasion risk [12, 13].

In this paper, we aim to establish a rapid detection method basing on the microfluidic chip technology, which will contribute to an active response to the crisis caused by this dangerous pest. Rapid detection method is essential for the whole process of interception and control of *W. auropunctata* in China. It can also help determine the origin of invasion, clarify the path of introduction to ensure biosecurity, and uncover other possible undetected establishments in the wild.

2. Materials & Methods

2.1. Sample Preparation & DNA Extraction

Twelve insect samples (Table 1) were employed in method establishment and corresponding analysis. Total DNA was extracted from 30-50 mg ground insect tissue of each sample by the UE Multisource Genomic DNA Minprep Kit (UElandy, China) based on the manual. The purity and concentration of DNA solutions were checked via Nanodrop 2000 (Thermo, USA). DNA solutions were stored at -20 ℃ for further use.

Table 1. *Sample list.*

No.	Samples	Sources
1	Wasmannia auropunctata D1	Shantou, Guangdong, China
2	Wasmannia auropunctata D2	Shantou, Guangdong, China
3	Wasmannia auropunctata D3	Shantou, Guangdong, China
4	Wasmannia auropunctata D4	Shantou, Guangdong, China
5	Wasmannia auropunctata 1	Shantou, Guangdong, China
6	Wasmannia auropunctata 2	Shantou, Guangdong, China
7	Wasmannia auropunctata 3	Shantou, Guangdong, China
8	Messor barbarus	France
9	Pogonomyrmex barbatus	the USA
10	Atta mexicana	France
11	Solenopsis geminata	Mozambique
12	Solenopsis invicta	Japan

2.2. Target Region Selection & Primer Design

Depending on the particular sequence of *W. auropunctata* microsatellite Waur-275 from GenBank (accession number: AY779635.1) [14], the region located at 1-403 was selected, synthesized and inserted to the plasmid puc57 vector [15] (carried out by Sangon Biotech (Shanghai) Co., Ltd., China) as the positive control which was used for primer selection, method establishment, and parameter analysis. Web service PrimerExplorer v5 (http://primerexplorer.jp/lampv5e/index.html) was employed accordingly [16]. BGI Co., Ltd (Guangdong) helps with the primer synthesis and purification.

2.3. Establishment of Microfluidic Chip Assay

Primers were dissolved into 100 μM with ddH_2O initially, and then pre-fixed onto the reaction pores and heated at 65 ℃ for 10 min. Eight primer combinations (Supplementary Table 1) were analyzed at the fixed volume ratio of F3/B3:FIP/BIP=1:8. Then the primer volume ratio (FIP/BIP:F3/B3) was set at 2, 4, 6, 8, 10, 12, respectively. The best ratio was selected according to the Ct to obtain higher sensitivity.

Fluorescent isothermal amplification premix (Ningbo iGene Technology Co., Ltd, China) was employed in this study. The reaction mix (25 μL for 4 reactions, 5 μL per reaction, the rest for error) contained 10 μL buffer, and 15.0 μL DNA solution. Microfluidic fluorescence detector MA2000 (Ningbo iGene Technology Co., Ltd, China) was used for the temperature of 63.5 ℃ for 30-60 min.

2.4. Method Validity, Specificity and Sensitivity Analysis

Six reserved *W. auropunctata* samples (Table 1) were prepared for testing the efficiency of this new method. The positive plasmid was dissolved at the concentration of 1.00×10^6 copies/μL. It served as a parent solution which was serially diluted at the 10-fold gradient with ddH_2O. This operation generated 7 solutions ranging from 1.00×10^6 to 1.00×10^0 copies/μL, which were employed in later sensitivity test. Meanwhile, method specificity was analyzed using *W. au-*

ropunctata and related ant samples (Table 1). The ddH_2O continued to serve as a negative control.

3. Result & Analysis

3.1. Primer Design and Method Establishment

Eight primer sets were generated with the online service PrimerExplorer v5 (Table A1). These primer sets were subjected to microfluidic reactions with DNA solution extracted from *W. auropunctata*. Based on corresponding Ct (Tables A2-A3), the fifth primer set had the lowest Ct in the volume ratio of 8 (FIP/BIP:F3/B3), thus it was selected. The final reaction concentration was 2 μM for F3/B3, 16 μM for FIP/BIP, 8 μM for LP/LB. Six reserved *W. auropunctata* samples had typical curves and relatively low Ct (Table A4), suggesting a good efficiency of this new method.

3.2. Method Parameter Analysis

A total of seven solutions of positive puc57 plasmid in concentrations from 1.00×10^6 to 1.00×10^0 copies/μL were employed in sensitivity analysis. Each concentration had four repetitions. The Ct (Table 2) as well as amplification curves (Figure 1) demonstrated a detection limit of 1.00×10^1 copies/μL for this new microfluidic method.

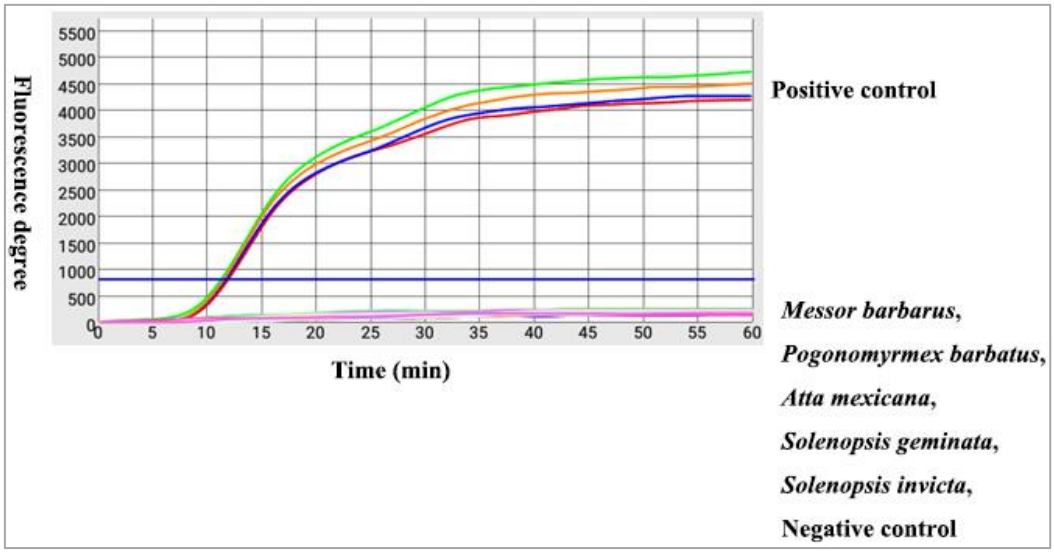

Figure 1. *Specificity test results.*

Table 2. *Sensitivity analysis results.*

Concentration	Repetition	Ct
106copies/ul	1	10.35
	2	9.92
	3	10.16
	4	9.94
105copies/ul	1	8.93
	2	8.79
	3	8.5
	4	8.74
104copies/ul	1	16.35
	2	16.16
	3	16.37
	4	16.34

Concentration	Repetition	Ct
103copies/ul	1	12.74
	2	12.73
	3	12.86
	4	12.83
112copies/ul	1	15.67
	2	15.53
	3	15.85
	4	15.9
111copies/ul	1	15.35
	2	15.79
	3	16.21
	4	15.38
110copies/ul	1	15.64
	2	23.04
	3	17.68
	4	22.65
Negative Control	1	0
	2	0
	3	0
	4	0

DNA samples from 6 insects were used to describe the specificity of this new method. Concentrations of these DNA solutions were diluted near 5.00 $\times 10^5$ copies/μL. Reconstructed puc57 plasmid (1.00×10^4 copies/μL) and ddH_2O were used as positive and negative controls, respectively. The result confirmed that only the positive control showed typical amplification curve and a low Ct. Other treatments gave negative results (Table 3 & Figure 2).

Table 3. *Specificity test results.*

Treatment	Repetition	Ct
Positive Control	1	11.82
	2	11.19
	3	11.59
	4	11.42
Messor barbarus	1	0
	2	0
	3	0
	4	0
Pogonomyrmex barbatus	1	0

Treatment	Repetition	Ct
Atta mexicana	2	0
	3	0
	4	0
	1	0
	2	0
	3	0
	4	0
Solenopsis geminata	1	0
	2	0
	3	0
	4	0
Solenopsis invicta	1	0
	2	0
	3	0
	4	0
Negative Control	1	0
	2	0
	3	0
	4	0

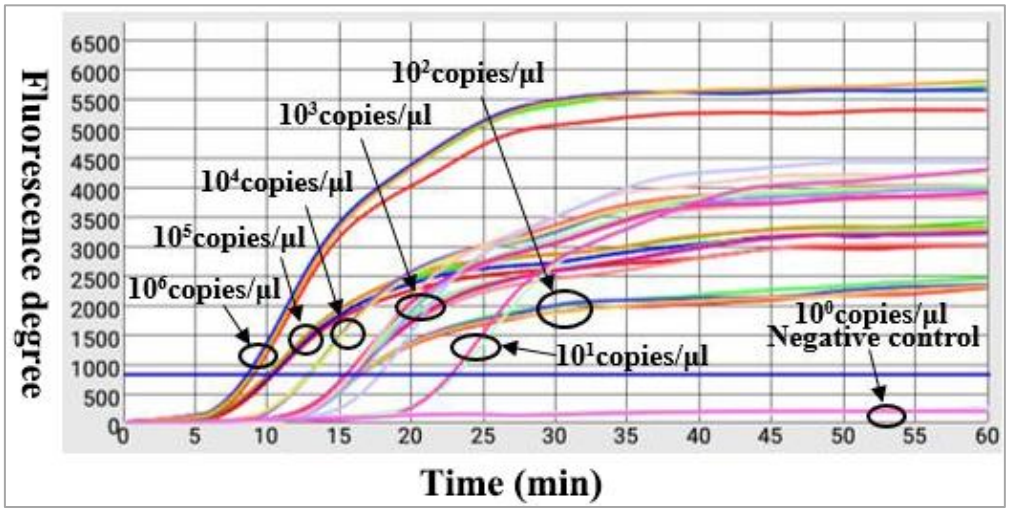

Figure 2. *Sensitivity test results.*

4. Discussion

Insects are the most serious competitors for food, the main cause of crop quality degradation and productivity reduction, and also vectors of some serious diseases [17]. To date, there has been no efficient way to monitor insect activity, abundance, and diversity in a cost-effective, and standardized pattern. Work that requires taxonomic information faces some challenges, such as struggling identification of many taxa and is labor intensive, relying heavily on well trained taxonomists [18]. The accuracy of identification is also limited by the

growth stages and integrity of samples. Eggs, nymphs, sibling species, polymorphic insects, and even insect debris may not be correctly identified based on only morphological features. Recently, technologies based on the online database, like the AntWeb (www.antweb.org), and machine learning algorithms to identify insects directly from images occurs. Those machine learning algorithms include artificial neural networks (ANN), support vector machine (SVM), k-nearest neighbors (KNN), naive bayes (NB) and convolutional neural network (CNN) model and so on [19]. The classification rate could reach as high as 91.5% using the CNN model [18]. Molecular methods are the most efficient among those used for detection and identification. DNA barcoding theory has been widely applied in plant phylogeny and taxonomy since 2003 [20]. Commonly used DNA barcoding regions, such as cytochrome oxidase I(COI), 28S ribosomal DNA, and long-wavelength rhodopsin, have been widely applied in ant taxonomic analysis [21, 22]. The mitochondrial gene COI is considered one of the most acceptable genetic markers, while coamplification, especially nuclear mitochondrial pseudogenes (Numts) must be avoided. Yet those barcoding primers have an amplification rate ranging from 60% to 70% [23]. Ant invasions pose a serious threat to local biodiversity, agriculture, and other human well-being. DNA barcoding assists in uncovering the origin and monitoring the spread of *W. auropunctata*. Polymorphic microsatellite regions are supposed to reveal significant information on the colony evolution and population genetic diversity after initial establishment in a new environment. In this study, the polymorphic *W. auropunctata* microsatellite locus Waur-275 was used for rapid screening method development.

Microfluidic chip technology is an emerging tool that offers several advantages, including reduced time and reagent consumption, and the ability to perform multiple reactions simultaneously. A microfluidic chip consists of several microchannels carved on specific materials (glass, silicon, or polymers). With a pump, it assists in determining the behavioral variation of microfluids [24]. The miniaturized size accelerates the reaction due to the increase of surface area. It has been applied in various fields such as food safety examing, peptide analysis, medical analysis, DNA extraction, PCR performance, and glucose test [25, 26]. The technology revolution has resulted in many progresses such as increased throughput, high sensitivity, accelerated analytical capability, more parallelization, and less reagent volumes. Microfluidic chip analytical platforms hold great promise for enabling high-throughput and precise inspection along with reduced consumption, ease of use, light weight, parallel detection, and mobility. Today, plant quarantine and biosecurity are facing a revolution in methodology with the blossoming of omics techniques. The future of bio-diagnostics is moving towards "doing more with less". Thus development of effective methods for rapid and accurate on-site plant pest screening with reduced costs and analysis time is essential.

5. Conclusion

Invasive alien ants are one of the most aggressive, competitive, and widespread invasive alien species around the world. *Wasmannia auropunctata*, native to the neotropical zoogeographic area, has been inadvertently introduced all over the world. The first disclosed record of field establishment of *W. auropunctata* in Chinese mainland was reported in 2022. In this paper, we aim to develop a rapid detection method targeting the *W. auropunctata* polymorphic microsatellite locus Waur-275, based on the microfluidic chip technology, to contribute to an active response to the crisis caused by this dangerous pest. Eight primer sets were generated using the PrimerExplorer v5 online service, and the fifth primer set selected had the lowest Ct in the volume ratio of 8 (FIP/BIP:F3/B3). Method validity with six *W. auropunctata* samples confirmed the efficiency of this assay. High specificity was demonstrated by the positive result for *W. auropunctata* out of 6 related insect samples tested. Sensitivity analysis showed that this microfluidic chip method could achieve the detection limit of 1.00×10^{1} copies/μL. A rapid screening method is essential for the whole process of *W. auropunctata* interception and control in China. It can also help determine the origin of invasion, clarify the path of introduction to ensure biosecurity, and also uncover other possible undetected establishments in the wild.

Abbreviations

IAS: Invasive alien species
IAA: Invasive alien ant
FIP: Forward inner primer
BIP: Backword inner primer
ANN: Artificial neural networks
SVM: Support vector machine
KNN: K-nearest neighbors
NB: Naive bayes
CNN: Convolutional neural network
COI: Cytochrome oxidase I
Numt: nuclear mitochondrial pseudogene

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Conflicts of Interest

The authors declare no conflicts of interest.

Appendix

Table A1. *Primer list.*

No.	Primer	Sequence (5'-3')
1	Waur-F3-1	AGCGTGCTCTCTCTCTCTC
	Waur-B3-1	CTTGAGTCGCGCTGACAAT
	Waur-FIP-1	CGGGAATTACAGGCTTTGAACTTCC-TCTCTCTCTCTCTCTCGATGGA
	Waur-BIP-1	CAAATTTCATTCAGCGTGTGTGCGT-TCGTAGAAGATAATGTCTCGGC
	Waur-LB-1	GCGTGTGTATGTGTGTGTGG
2	Waur-F3-2	TCTCTCTCTCTCTCTCTCTCGA
	Waur-B3-2	CGCGTCGTATATTCTCAGAACG
	Waur-FIP-2	GCACACACGCTGAATGAAATTTGCA-GGAAAAACAACAAAGCTTTCGC
	Waur-BIP-2	TGTGTATGTGTGTGTGGCGCG-CCACTTAATCCCTTGAGTCGC
	Waur-LF-2	CAGGCTTTGAACTTCCTGGTTAAT
3	Waur-F3-3	TCTCTCTCTCGATGGAAAAACAAC
	Waur-B3-3	TCTCAGAACGAGCCGCT
	Waur-FIP-3	GCACACACGCTGAATGAAATTTGCA-AAAGCTTTCGCATTAACCAGGA
	Waur-BIP-3	TGTGTATGTGTGTGTGGCGCG-CCACTTAATCCCTTGAGTCGC
	Waur-LF-3	CGGGAATTACAGGCTTTGAACT
	Waur-LB-3	CGCGCGCCGAGACATTA
4	Waur-F3-4	GGAAAAACAACAAAGCTTTCGC
	Waur-B3-4	TCTCAGAACGAGCCGCT
	Waur-FIP-4	CGCACGCACACACGCTGAA-TTAACCAGGAAGTTCAAAGCCT
	Waur-BIP-4	TGTGTATGTGTGTGTGGCGCG-CCACTTAATCCCTTGAGTCGC
	Waur-LB-4	GCGCGCCGAGACATTATC
5	Waur-F3-5	ACAAAGCTTTCGCATTAACCAG
	Waur-B3-5	TCGTCGGCGCTTATTATGC
	Waur-FIP-5	TACACACGCACGCACACACG-AGTTCAAAGCCTGTAATTCCCG
	Waur-BIP-5	ATCTTCTACGAATTGTCAGCGCGA-CGCGTCGTATATTCTCAGAACG
	Waur-LB-5	CTCAAGGGATTAAGTGGAGC
6	Waur-F3-6	TCGCATTAACCAGGAAGTTCA
	Waur-B3-6	TCGTCGGCGCTTATTATGC
	Waur-FIP-6	GCCACACACACATACACACGC-AAGCCTGTAATTCCCGTCTTTG
	Waur-BIP-6	ATCTTCTACGAATTGTCAGCGCGA-CGCGTCGTATATTCTCAGAACG
	Waur-LF-6	GCACACACGCTGAATGAAATTTG
7	Waur-F3-7	CCTGTAATTCCCGTCTTTGCA
	Waur-B3-7	TGATGAAGAGAAAGATGCTCGTC
	Waur-FIP-7	GAAGATAATGTCTCGGCGCGCG-AATTTCATTCAGCGTGTGTGC
	Waur-BIP-7	TGTCAGCGCGACTCAAGGGATTA-GGCGCTTATTATGCGCGTTA

No.	Primer	Sequence (5'-3')
8	Waur-LF-7	GCCACACACACATACACACG
	Waur-LB-7	AGTGGAGCGGCTCGTTC
	Waur-F3-8	GCAAATTTCATTCAGCGTGTGT
	Waur-B3-8	GACGATGGCGCGTGATG
	Waur-FIP-8	AGTCGCGCTGACAATTCGTAGAAG-GCGTGCGTGTGTATGTGT
	Waur-BIP-8	GGATTAAGTGGAGCGGCTCGTTC-AAGAGAAAGATGCTCGTCGG
	Waur-LB-8	GACGCGCGATATAACGCGCATA

Table A2. *Primer selection results.*

No.	Repetition	Ct
Primer set 1	1	23.8
	2	22.46
	3	24.14
	4	24.82
Primer set 2	1	43.6
	2	43.94
	3	49.31
	4	44.95
Primer set 3	1	38.94
	2	39.95
	3	37.27
	4	36.94
Primer set 4	1	54.04
	2	50.36
	3	48.35
	4	57.07
Primer set 5	1	12.84
	2	13.85
	3	12.18
	4	12.85
Primer set 6	1	16.54
	2	17.89
	3	17.89
	4	17.23
Primer set 7	1	39.04
	2	35.02
	3	34.35

No.	Repetition	Ct
Primer set 8	4	38.04
	1	45.43
	2	46.44
	3	49.78
	4	44.77
Negative Control	1	0
	2	0
	3	0
	4	0

Table A3. *Primer ratio selection results.*

Ratio	Repetition	Ct
1:2	1	37
	2	37.67
	3	33.65
	4	30.97
1:4	1	21.12
	2	21.8
	3	22.47
	4	21.8
1:6	1	13.12
	2	12.79
	3	12.46
	4	12.8
1:8	1	12.14
	2	12.14
	3	12.15
	4	12.49
1:10	1	15.44
	2	15.78
	3	15.78
	4	15.45
1:12	1	30.27
	2	31.28
	3	29.94
	4	30.96
Negative Control	1	0

Ratio	Repetition	Ct
	2	0
	3	0
	4	0

Table A4. Method validity results.

Specimen No.	Repetition	Ct
D1	1	12.84
	2	13.85
	3	12.18
	4	12.85
D2	1	13.07
	2	14.08
	3	13.42
	4	13.42
D3	1	14.1
	2	14.44
	3	14.11
	4	15.45
D4	1	15.81
	2	13.8
	3	13.8
	4	14.14
1	1	15.82
	2	16.5
	3	18.52
	4	26.9
2	1	13.85
	2	14.18
	3	13.85
	4	14.19
3	1	15.87
	2	20.57
	3	14.21
	4	20.92
Negative Control	1	0
	2	0
	3	0

Specimen No.	Repetition	Ct
	4	0

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