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Resistance and Genetic Differentiation of *Rhyzopertha dominica* to Phosphine among Different Geographical Populations in China: a Preliminary Study

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Abstract: In this article, the mortality effect of the most world's used fumigant, phosphine, was studied on one of the most important species of stored-product insect, the lesser grain borer *Rhyzopertha dominica* (Fabricius). In parallel, genetic differentiation among various resistant populations of *R. dominica* was investigated using amplified fragment length polymorphism (AFLP). The LC_{50} values from 6 different geographical populations, showed values between 0.05 mg/L and 3.25 mg/L. The populations Banan, Chengdu, Shayang, Zhucheng, Yangchun, Xuchang were, respectively, the most sensitive to the most resistant. In addition, AFLP results using *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I primers, separated populations into a phylogenetic tree. The dendogram obtained with the primers *Eco*R I/*Msp* I could be easily explained by a geographical hypothesis but the pattern obtained with *Eco*R I/*Hpa* II primers could not. Two populations (Yangchun and Zhucheng) with distant geographical origins but with a high resistance to phosphine were clustered together. The influence of the phosphine treatment, and therefore, resistance of *R. dominica* to this insecticide, on the genes is one hypothesis resulting from this preliminary work.

Key words: *Rhyzopertha dominica*, geographical populations, phosphine, resistance, AFLP, genetic diversity

Introduction

The lesser grain borer, *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae). *R. dominica.* is a harmful grain pest originating from tropical areas but actually distributed throughout the world. Both larvae and adults cause serious damage to grain such as rice, maize, wheat, paddy. It is described as the most important pest of stored wheat in Brazil [11] and it attacks the storage of paddy rice too [13]. Several resistant strains of *R. dominica* have been described [10].

The main method used for controlling stored-product insects world-wide, including China, is the fumigation with phosphine gas [1]. It is commonly used against all species of stored-product insects including *R. dominica.* Resistance of stored-grain insects to phosphine was detected in 33 of 82 countries surveyed[3] and stored product insects are described as resistant to the compound in more than 45 countries[1,4]. Resistance to phosphine has been reported occurringin several economically important species, including*Sitophilus oryzae* (F.), *Sitophilus zeamais* (Motschulsky), *Tribolium castaneum* (Herbst) and *R. dominica*[3,9,11].

Several studies have dealt with the problem of the resistance to insecticides, particularly to the phosphine. But few of them studied the genetic resistance[5,16]. In these articles, authors observed the involvement of resistance genes, and also their inheritance. Contrary to other disciplines where genetic resistance and population genetics are strongly described, no data exist on a possible relationship between resistance evolution and genetic polymorphism in stored-product species. Amplified fragment length polymorphism (AFLP) is a technique which could help to understand this resistance [17]. AFLP is used to analyze genetic diversity, to identify biotypes, to construct linkage genetic maps, to study population genetics, gene location and genetic polymorphism[12,8,2,7,14].

The objective of this research was to test the hypothesis of genetic variation among six populationsof *R. dominica* populations in China. Using both classical measures of mortality

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and AFLP, we aimed to described a possible genetic differentiation among populations with different levels of resistance.

Material and Methods

Insects and Rearing Conditions

Populations of *R. dominica* were collected between 14 May and 4 September, 2007 in six cities of China: Banan (Chongqing province), Chengdu (Sichuan), Shayang (Hubei), Yangchun (Guangdong), Zhucheng (Shandong) and Xuchang (Henan).

Xuchang, Shayang, Zhucheng and Yangchun populations were collected from central storages. Banan population came from a rice mill And the Chengdu population was from Chengdu Grain Storage Research Institute. Populations were then reared in our laboratory with cracked wheat at a moisture content of 13 1%. Rearing temperature was maintained at 30 ± 1℃ and relative humidity was 75 5%. Adult *R. dominica* 14 days old were used in phosphine resistance assays.

Phosphine Bioassays

Phosphine was obtained by a reaction of zinc phosphide (Jining City Yimin Chemical Plant. Concentration) and sulfuric acid. The Lethal Concentration 50 (LC_{50}) values of *R. dominica* to the fumigant phosphine were detected using an FAO method. After 20h treatment, each sample of tested insects was transferred into glass tubes with creaked wheat. Mortality was recorded 2 weeks later after the transfer of adult insects. Each treatment was undertaken three times.

DNA extraction

We adopted phenol-chloroform extraction method and tested OD 260/280 from 1.6 – 1.9[18]. Seven adults of *R. dominica* were suitable for DNA extraction.

Amplified Fragment Length Polymorphism (AFLP)

DNA was digested with *Hpa* II, *Msp* I and *Eco*R I restriction endonucleases (MBI Fermentas, EU). Then, for the ligation, used adapters were prepared by the mixing of two adapters (MBI Fermentas, EU): *Hpa* II/*Msp* I adapter was prepared by mixing 5' – GACGATGAGTC TAGAA – 3' and 5' – CGTTCTAGACTCATC – 3' adapters and *Eco*R I was prepared by mixing 5' – CTCGTAGACTGCGTACC – 3' and 5' – AATTGGTACGCAGTC – 3' adapters. DNA fragments were ligated to *Hpa* II/*Msp* I and *Eco*R I.

Pre-amplification was performed with preselective primers *Hpa* II/*Msp* I (5' – GATGAGTCTAGAACGGT – 3') and *EcoR* I(5' – GTAGACTGCGTACCAATTCA – 3') (Shanghai Generay Biotech Co, China). Pre-amplification was performed with 0.1 μL of each *Eco*R I and *Hpa* II/*Msp* I preselective primers (100μM), 0.5 μL dNTPs (10 mM), 2 μL Mg^{2+} (20 mM), 2.5 μL 10X PCR buffer (500 mM KCl; 100 mM, Tris – HCl; pH 8.3; 15 mM $MgCl_2$) and 0.2 μL Taq DNA polymerase (5U/μL) and 5μL of ligation solution in a total volume of 25 μL. The PCR reaction was performed at 94℃ for 3 min, following by 20 cycles of 94℃ for 30 s, 56℃ for 1 min, 72℃ for 1 min, and a final elongation of 72℃ for 5min.

Selective amplification used two selective primers *Hpa* II/*Msp* I(5' – GATGAGTCTAGA ACGGT – 3') and *Eco*RI + ATA (5' – GTAGACTGCGTACCAATTCATA – 3'), each containing three selective nucleotides. Selective amplification was performed with 0.05 μL of each selective primers (100μM), 0.3 μL dNTPs (10mM), 1.2 μL Mg^{2+} (20mM), 1.5μL 10X PCR buffer (500 mM KCl; 100 mM, Tris – HCl; pH 8.3; 15 mM $MgCl_2$), 0.2μL Taq DNA polymerase and 3 μL of preamplification solution in a volume of 15μL. The PCR reaction was performed with a first denaturation cyle at 94℃ for 3 min, following by 13 cycles (94℃ for 30 s, 56℃ for 1 min, and 72℃ for 1 min) during which the annealing temperature was decremented 0.7℃ each cycle. The PCR continued with 23 cycles (94℃ for 30 s, 56℃ for 1min, and 72℃ for 1 min) following by a final elongation of 72℃ for 5 min.

Amplifications were carried out in PTC – 200 DNA machine (MJ, USA). The reaction products were separated on denaturing 6% polyacylamide gels adopting at 70W power with a DYCZ – 20C electrophoresis machine (Beijing Liuyi Instrument Factory, Beijing, China).

Data Analysis

Linear regressions of mortality and LC_{50} values were obtained with SPSS 10.0 (Statistical Package for Social Science, Chicago, USA). AFLP results were analyzed by a cluster analysis. Each strain was scored 1 for the presence or 0 for the absence of each band. A dendrogram was obtained using the Unweighted Pair – Group Method using the Arithmetic Averages (UPGMA) with NTSYSpc version 2.10p (Applied Biostatistics, Inc., New – York, USA).

Results

Mortality of *R. dominica*

The six populations of *Rhyzopertha dominica* showed a range of sensitivities to phosphine. Mortality values, expressed as LC_{50}, fluctuated between 0.05 mg/L and 3.30 mg/L (Table 1). The highest resistance factor was almost 66. Populations could be ordered by sensitivity to the phosphine in 3 groups. The most sensitivewere the populations from Banan and Chengdu presenting almost the same low LC_{50}, almost 0.05 mg/L. Then one population, from Shayang, had an intermediate mortality value with a 0.59 mg/L LC_{50}. Then, the three populations from Yangchun, Xuchang and Zhucheng had the highest LC_{50} values (3.19 – 3.29 mg/L).

Table 1. Analysis of mortality of *Rhyzopertha dominica* populations to phosphine. Linear regression was performed for each population. The bioassays were performed in triplicate.

Origins	Regression of the linear regression of mortality	LC_{50}/mg/L	95% Confident limit
Banan	Y = 2.27 X + 4.61	0.0495	0.207 – 16.7
Chengdu	Y = 2.24 X + 2.91	0.0498	0.00271 – 0.184
Shayang	Y = 2.73 X + 0.629	0.585	0.0167 – 2.03
Yangchun	Y = 2.52 X + 0.379	3.19	1.447 – 107.0
Xuchang	Y = 5.84 X – 2.99	3.25	0.633 – 4.90
Zhucheng	Y = 4.85 X – 2.51	3.29	0.0735 – 4.08

AFLP Analysis

The tree obtained with both *Msp* I and *Hpa* II bands clustered populations into two groups (Fig. 1). The first group included the Banan and Chengdu populations. The second group was itself separated in two groups of two populations: on the one hand Shayang and Xuchang populations, on the other hand, Yangchun and Zhucheng populations.

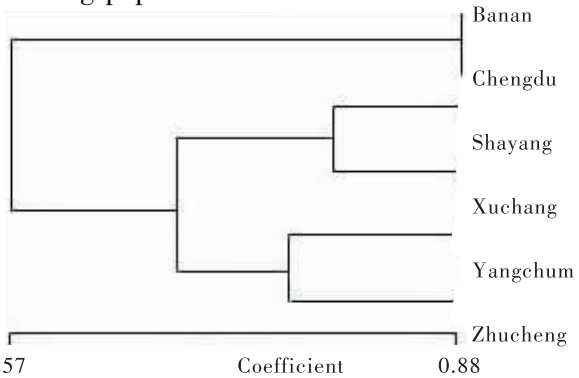

Fig. 1 Dendrogram of *Rhyzopertha dominica* populations based on amplified fragment length polymorphism (AFLP) analysis using the number of bands, of *Msp* I and *Hpa* II, coding by absence/presence. Analysis of bands, performed in duplicate, was based on the analysis of 50 bands for *Eco*R I/*Hpa* II and 52 bands *Eco*R I/*Msp* I.

The dendogram obtained with *Msp* I bands alone showed similar relationships, although the one obtained with *Hpa* II bands showed a different pattern (Fig. 2). The tree obtained with Hpa II, like the other two, divided *R. dominica* populations into two major clusters. The major difference was in the subdivision of the second cluster, the first cluster included Banan and Chengdu populations. For the four other populations, Zhucheng population was nearest with Shayang and Xuchang populations. Yangshun population was by itself.

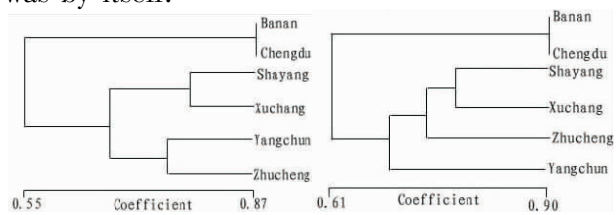

Fig. 2 Dendrogram of *Rhyzopertha dominica* populations based on amplified fragment length polymorphism (AFLP) analysis with bands *Hpa* II (on the left) and bands of *Msp* I (on the right). Each band analysis, performed in duplicate, was coding by absence/presence. Analysis is based on the analysis of 50 bands for *Eco*R I/*Hpa* II and 52 bands *Eco*R I/*Msp* I.

Discussion

The dendograms obtained from analysis of *Hpa* II/*Eco*R I and *Msp* I/*Eco*R I bands, showed three final clusters: Benan – Chengdu, Shayang – Xuchang and Yangchun – Zhucheng. We propose a geographical explanation for this pattern. Indeed, Chengdu and Banan (the first cluster) are cities close together. Similarly, the cluster Shayang – Xuchang could be explained by the proximity of these two cities. However, a geographical hypothesis cannot be used to explain the cluster Yangchun – Zhucheng, as Yangchun is in the south of China and Zhucheng on the East coast and closer to

Xuchang or Shayang. In contrast, the pattern obtained with *Msp* II primers can be explained with a geographical hypothesis alone. However, the results obtained with both primers or only with *Hpa* I cannot be explained by geography alone. Looking at both dendograms and the mortality data, two points need to be highlighted: the differences in mortality values between the two close (genetically and geographically) populations, Shayang and Xuchang, and the relatedness of the Yangchun and Zhucheng populations in spite of their geographical remoteness. The influence of phosphine treatment and/or the resistance of *R. dominica* to this insecticide, on the *Eco*R I/*Hpa* I restriction sites is one hypothesis that could explain the relatedness of Yangchun/Zhucheng .

These two particular last points are encouraging although we didn't reach our major objective. Indeed, none of the studied bands showed specifically a region involved in phosphine resistance. Several explanations could explain this failure. Compared with classical samples in AFLP studies (e. g. 38 *Aedes aegypti* samples from Mexico[14] or 133 pollen beetles samples from Sweden[6]), there was a lack of the diversity in the sample. Concerning the method, while in this study only two pairs of primers were used without combination, four primer combinations were used on pollen beetles [6] and three on *Aedes aegypti* individuals[14].

However, we managed to obtain some interesting results with this preliminary experiment using AFLP method on a species of stored-product insects,. Knowing that both molecular and classical studies demonstrated that resistance can occur by a genetic change at a single locus[15], we are currently developing the method to study more populations within China. The final objective is to study genes involved in resistance in *R. dominica* populations internationally. In the same way, the influence of generation time, the role of spatial factors and insecticide treatment on population differentiation[6] can be studied. Moreover, we are currently completing this phylogeographic study with experiments based on mtDNA sequence analysis from cytochrome oxidase I and II.

Acknowledgements

This work was partially supported by a China Postdoctoral Science Foundation, China National Science and Technology Project of the11th Five-Year Plan (2006BAD02A18 – 03 and 2006BAI09B04 – 06) and Hubei Key Project of Science and Technology.

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