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Development of polymorphic EST-SSR markers from *Bradysia odoriphaga* (Diptera: Sciaridae), a serious agricultural pest in China

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Abstract: 【Background】 The chive gnat, *Bradysia odoriphaga* Yang and Zhang (Diptera: Sciaridae), is a severe agricultural pest in China. Knowledge on the biology, dispersal, and other important aspects of this insect is limited. Filling this knowledge gap is hampered by the lack of suitable the genetic markers. The aim of the present study was to develop simple sequence repeat (SSR) markers from expressed sequence tags (ESTs) that can be used for genetic diversity and structure analysis of *B. odoriphaga*. 【Method】 The SSRs primers were designed and tested based on the ESTs of *B. odoriphaga* obtained in this study. 【Result】 A total of 3383 SSRs were identified from 42095 unigenes. Sixteen pairs of primers were designed and tested in 30 *B. odoriphaga* larvae, of which nine primer pairs produced polymorphic amplicons. Thirty alleles were identified from 30 larvae using the nine markers, with an average of 3.33 alleles per locus (ranged from 3 to 4). The range of observed and expected heterozygosity was 0.0000~0.6875 and 0.0370~0.6877, respectively. Five of the nine loci exhibited significant departure from Hardy-Weinberg equilibrium. 【Conclusion and significance】 The nine polymorphic microsatellite loci developed in this study can be used to research the genetic diversity and structure of *B. odoriphaga* populations.

Key words: *Bradysia odoriphaga*; expressed sequence tag; simple sequence repeat; genetic diversity; genetic structure

中国重要农业害虫韭菜迟眼蕈蚊多态性 EST-SSR 标记的开发

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摘要: 【背景】韭菜迟眼蕈蚊是我国重要的农业害虫,然而它的遗传资源有限。本研究旨在开发韭菜迟眼蕈蚊 EST-SSR 标记,为研究不同地区的韭菜迟眼蕈蚊种群结构和遗传多样性奠定基础。【方法】从韭菜迟眼蕈蚊的表达序列标签(EST 序列)中设计 16 对简单重复序列(SSR)引物,进一步筛选出 9 对具有多态性的 SSR 引物。【结果】从 42095 条 unigene 中确定了 3383 个 SSR 位点。利用查找到的 SSR 位点共设计出 16 对引物,进一步检测筛选发现 9 对引物具有多态性,引物的每个位点平均有 3.33 个等位基因。利用 9 对引物对 30 头韭菜迟眼蕈蚊进行检测,共获得 30 个等位基因,观测杂合度和期望杂合度的范围分别为 0.0000~0.6875 和 0.0370~0.6877;其中,9 个位点中有 5 个位点显著偏离 Hardy-Weinberg 平衡。【结论与意义】本研究成功从迟眼蕈蚊 EST 序列中筛选出 9 个具有多态性的微卫星位点,这为进一步分析该害虫种群的遗传结构和遗传多样性奠定了基础。

关键词: 韭菜迟眼蕈蚊; 表达序列标签; 简单重复序列; 遗传多样性; 遗传结构

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1 INTRODUCTION

The chive gnat, *Bradysia odoriphaga* Yang and Zhang (Diptera: Sciaridae), is one of the most important pest of chives in China (Li *et al.*, 2007; Tao *et al.*, 2015). This pest is mainly destructive to Chinese chives (*Allium tuberosum*) but can also attack other allium vegetables, as well as cabbage, radish, melon, celery, mushrooms, and various ornamentals (Mei *et al.*, 2003; Zhang *et al.*, 2015). The larvae of *B. odoriphaga* can feed on roots or stems underground, causing plants to be stunted or to even die and resulting in serious economic losses (Yang *et al.*, 2015). This pest can cause severe yield losses of Chinese chives every year (Dang *et al.*, 2001). Analysis of the genetic structure helps to elucidate the gene flow and genetic differentiation of many species (De Luca *et al.*, 2002). So far, the gene flow and genetic differentiation of *B. odoriphaga* remain poorly understood.

Simple sequence repeats (SSR), also known as microsatellites, have become one of the most important genetic markers in the analysis of population genetic structure (De Luca *et al.*, 2002). SSRs contain tandem repeat genetic loci of 1 ~ 8 bp (Richard *et al.*, 2008). Because of their abundance in genomes, codominant inheritance, and high level of polymorphism (Melnikova *et al.*, 2012; Meng *et al.*, 2012; Yakovin *et al.*, 2011), SSRs are useful for gene mapping and population genetic structure analysis (Jung *et al.*, 2006). Expressed sequence tags (ESTs) have been applied to discover SSR markers rather than using genomic sequences (Wang *et al.*, 2014). Compared with conventional genomic SSR markers, EST-based SSRs have several intrinsic advantages such as reduced costs, faster development of suitable markers, and broad transferability between species (Yu & Li, 2007).

The aim of the present study was to develop EST-SSRs that can be used for the analysis of genetic diversity and structure of *B. odoriphaga*. A total of 42095 *B. odoriphaga* unigene sequences were used firstly mined for analyzing SSR. Then the primer pairs were designed to select the polymorphic loci. Due to lack of suitable the genetic markers, knowledge on the biology, dispersal, and other important aspects of this insect is limited, and the polymorphic EST-SSRs obtained for *B. odoriphaga* could be helpful for genetic studies of this organism.

2 MATERIALS AND METHODS

2.1 Samples and EST mining

Samples collected from Tianjin, China in 2013 were used to construct the EST library of *B. odoriphaga*. A total of 42095 *B. odoriphaga* unigene sequences (data not presented) were mined for analyzing SSR. The DNAstar software (DNASTAR, Madison, WI, USA) was used to pretreat the *B. odoriphaga* EST sequences and the vector sequences and sequences shorter than 150 bp were removed. The software MISA (http://pgrc.ipk-gatersleben.de/misa/) was used to search SSRs with the criteria as follows: mononucleotide repeats at least 12 times, dinucleotide repeats at least six times, trinucleotide repeats at least five times, tetranucleotide repeats at least five times, and pentanucleotide and hexanucleotide repeats at least four times.

2.2 DNA extraction, primer design and testing

Genomic DNA was extracted from *B. odoriphaga* larvae collected using the lysis method described by Frohlich *et al.*(1999). The DNA-containing supernatant was stored at −20 ℃. Sixteen pairs of primers were designed using PRIMER PREMIER 5.0 software (Lalitha, 2000), and 30 *B. odoriphaga* larvae collected from Shouguang, Shandong Province, China in 2013 were used to test the validity. The samples were freshly collected and stored at −20 ℃. The DNA-containing supernatant of each individual larva was used for subsequent PCR amplification. PCR amplification was performed in 13 μL volumes containing 0.13 μL of Taq DNA polymerase (5 U · μL^{-1}), 1.3 μL of 10×Easy Taq PCR Buffer, 0.26 μL of each dNTP (10 μmol · L^{-1}), 0.26 μL of each primer (10 μmol · L^{-1}), and 2 μL of DNA. The PCR conditions were as follows: initial denaturation at 94 ℃ for 5 min; 35 cycles of 30 s at

94 ℃, 45 s at the primer-specific annealing temperature (50~56 ℃: the different primer sets had different PCR reactions), and 30 s at 72 ℃; and a final elongation step at 72 ℃ for 7 min. The allele size was determined according to Gao *et al.* (2014). Briefly, the products were run on an ABI 3730 xl DNA analyzer (Sangon, Shanghai, China) and the allele size was determined by comparing the mobility of the PCR products to the GeneScan™ 400HD size standard using GeneMapper software version 3.2 (Applied Biosystems, Shanghai, China).

2.3 Data analysis

The average number of alleles per locus (N_a), the observed heterozygosity (H_o), and the expected heterozygosity (H_e) were calculated using POPGENE v.1.31 (Wang *et al.*,2014; Yeh *et al.*,1999). GENEPOP v.3.4 was used to test for deviation from Hardy-Weinberg equilibrium (Mortega *et al.*, 2015; Raymond & Rousset, 1995) and to calculate FIS values (Weir & Cockerham,1984).

3 RESULTS

3.1 Microsatellite loci identified

A total of 3383 SSR loci from 42095 EST sequences were identified. The 3383 SSRs mainly comprised mononucleotide (1408), dinucleotide (693), and trinucleotide (1210) repeats, with tetranucleotide (41), pentanucleotide (28) and hexanucleotide (3) repeats being uncommon (Table 1).

Table 1 Occurrence frequency of repeat motifs of EST-SSRs in *Bradysia odoriphaga*

Repeat type	Number of SSRs
Mononucleotide	1408
Dinucleotide	693
Trinucleotide	1210
Tetranucleotide	41
Pentanucleotide	28
Hexanucleotide	3
Total	3383

Among the mononucleotide repeats, A/T motifs were most common (Table 2). Among the dinucleotide repeats, AG/CT motifs dominated, followed by AC/GT and AT/AT (Table 2). Among the trinucleotide repeats, AAC/GTT motifs were most common, followed by ATC/ATG and AAG/CTT (Table 2). Tetranucleotide, pentanucleotide, and hexanucleotide repeats had relatively low occurrence frequencies (<1.00%).

Table 2 Occurrence frequency of main repeat motifs of EST-SSRs in *Bradysia odoriphaga*

Repeat type	Number	Percentage (%)	Repeat number	Repeat type	Number	Percentage (%)	Repeat number
A/T	1398	41.32	12~24	AAAG/CTTT	2	0.06	5
C/G	10	0.30	12~21	AAAT/ATTT	3	0.09	5~6
AC/GT	301	8.90	6~12	AACC/GGTT	1	0.03	5
AG/CT	349	10.32	6~12	AACG/CGTT	1	0.03	5
AT/AT	43	1.27	6~12	AACT/AGTT	1	0.03	5
AAC/GTT	505	14.93	5~8	AAGT/ACTT	3	0.09	5
AAG/CTT	169	5.00	5~8	AATC/ATTG	1	0.03	5
AAT/ATT	52	1.54	5~6	AATG/ATTC	1	0.03	5
ACC/GGT	97	2.87	5~8	ACAG/CTGT	9	0.27	5~6
ACG/CGT	37	1.09	5~6	ACAT/ATGT	1	0.03	5
ACT/AGT	29	0.86	5~8	ACGG/CCGT	1	0.03	5
AGC/CTG	106	3.13	5~8	ACTC/AGTG	1	0.03	5
AGG/CCT	21	0.62	5~6	ATCC/ATGG	1	0.03	5
ATC/ATG	188	5.56	5~8	ATCG/ATCG	1	0.03	5
CCG/CGG	6	0.18	5~6	Others	31	0.92	5~6
AAAC/GTTT	14	0.41	5				

3.2 Microsatellite primer pairs designed and testing

Sixteen primer pairs were designed using PRIMER PREMIER 5.0 software. We tested 16 primer pairs in the 30 *B. odoriphaga* larvae however, only nine primer pairs produced amplicons and revealed polymorphisms. We used the nine markers to identify 30 alleles, with 3 ~ 4 alleles detected per locus. The observed and expected heterozygosity was 0.0000 ~ 0.6875 and 0.0370 ~ 0.6877, respectively. Five loci (code: 3477, 3373, 2479, 2745, and 2312) exhibited significant departure from Hardy-Weinberg equilibrium (Table 3).

Table 3 Expressed sequence tag-simple sequence repeats used to identify polymorphisms in one *Bradysia odoriphaga* population

Locus	SSR	Primer sequence (5′-3′)	T_a(℃)	Size (bp)	N_a	H_o	H_e	F_{IS}	P
3477	$(TTG)_5$	F: ACGAATGACTTGGCAAAA	51	151	3.000	0.0445	0.1321	0.6613	0.0233
		R: TCTGGTGAAGAAAGAAAGAATA							
3373	$(GT)_6$	F: CGGCAGTCGTATCACAGT	55	124	2.000	0.0000	0.0754	1.0000	0.0004
		R: TTCCATCAGATCACATATTAAG							
9830	$(AG)_{11}$	F: GTTGTTTGCGAGTTGTAT	50	94	6.000	0.3333	0.5119	0.3017	0.0992
		R: AAGTTGTTGGTTCGTAAG							
2479	$(GTA)_5$	F: GAGGCACCTGTTTGACGG	56	150	2.000	0.0000	0.3522	1.0000	0.0000*
		R: CGATACCCAAGCTCCATT							
8825	$(AAC)_5$	F: ACTGTGACGGTTGGAAAC	55	121	2.000	0.0370	0.0370	0.0000	–
		R: GCATTGTAACTACCACCAGA							
2745	$(CGA)_5$	F: TCGGAAGATGTGGCTATT	52	99	5.000	0.5294	0.5722	0.0769	0.0038
		R: AGTGACCCCATCAACAAT							
2312	$(TGG)_5$	F: ACCGCATTACCATTGTCA	55	139	5.000	0.1429	0.2610	0.4573	0.0269
		R: ACATGGTCAAGAGCCTGC							
4081	$(AC)_7$	F: GAGCAAGCGCCTAACTTT	54	110	2.000	0.6875	0.4980	−0.3983	0.1590
		R: GCGTGCACATTTATTCGT							
4481	$(TTG)_5(AG)_6$	F: CCAACATGCCAAGAAAGG	56	101	7.000	0.5714	0.6877	0.1716	0.2076
		R: GAGATGGGATGACGGAGA							

SSR: Simple sequence repeat; F: Forward; R: Reverse; T_a: Annealing temperature; N_a: Number of detected alleles; H_o: Observed heterozygosity; H_e: Expected heterozygosity; F_{IS}: Estimator of the fixation index; P: Probability value of Hardy-Weinberg equilibrium (* Significance at $P<0.05$).

4 DISCUSSION

In China, *B. odoriphaga* is an important pest in agricultural ecosystems. However, due to lack of suitable the genetic markers, knowledge on the biology, dispersal, and other important aspects of this insect is limited. The population spread and dynamics in the field, which is important for the sustainable regional management of this pest. The potential spread model of a pest insect can be revealed via genetic markers including microsatellite loci. The design and selection of polymorphic microsatellite loci are important to reveal the gene flow, genetic differentiation, and the population dynamics of insect pests. Using microsatellite markers, Gao *et al.* (2014) demonstrated that the spread of the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), in some regions of China was through secondary introduction from regions where it was initially introduced.

In this study, the constructed ESTs of *B. odoriphaga* showed that the proportion of different motifs of SSRs in this species varied greatly. The most common motifs were mononucleotide repeats, similar to the western flower thrips, *Frankliniella occidentalis* (Duan *et al.*, 2012). However, the proportion of the repeat motif types differs according to the species (see Duan *et al.*, 2012), which indicates that the proportion of motif types is species-specific.

Among the 42095 *B. odoriphaga* unigene sequences, a total of 3383 SSR loci were revealed, which suggests that SSR loci are distributed widely in the ES-

Ts of this species. Compared with conventional genomic SSR markers, EST-based SSRs have several intrinsic advantages (Yu & Li, 2007). Previous study showed that the ESTs can be useful for the development of SSR markers (Wang *et al.*, 2014). The new SSR markers developed could be helpful to reveal the gene flow and genetic differentiation of *B. odoriphaga* in China.

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