Research Article

Development of Microsatellite Markers for the Nipa Palm Hispid Beetle, Octodonta nipae (Maulik)

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Received 29 November 2017; Accepted 18 April 2018; Published 7 June 2018

Academic Editor: Taoyang Wu

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The nipa palm hispid beetle, Octodonta nipae (Maulik) (Coleoptera: Chrysomelidae), is an important invasive pest on palm plants in southern China. Based on existing transcriptome data, polymorphisms simple sequence repeat (SSR) loci were identified. In total, 1274 SSR loci were identified from 49919 unigenes. The majority of them contained mononucleotide, dinucleotide, and trinucleotide motifs (43.56%, 26.14%, and 28.18%), in which A/T (41.21%) and AT/TA (15.86%) were the most abundant motifs. 104 pairs of the SSR primers produced amplification bands of expected sizes in O. nipae, 80 pairs of SSR primers were tested randomly for polymorphism, 9 loci of them were validated to be polymorphic markers, and the number of alleles ranged from 2 to 3, with an average of 2.56 per locus. The population of Zhangzhou and Fuzhou was analyzed by the 9 loci (On1–On9). These SSR transcriptome data can provide invaluable resource for SSR development, population genetics research, invasion and expansion mechanism, paternity testing, and other research on O. nipae and its related species.

1. Introduction

The nipa palm hispid beetle, Octodonta nipae (Maulik) (Coleoptera: Chrysomelidae), which was originated in Malaysia, is an alien pest on palm plants in southern China [1]. The beetles attack young leaves of various palm plants, which poses a great threat to palm plants industry, economic palm planting, city landscaping, and ecological safety [2]. Much efforts have been devoted to research on O. nipae, including external morphology, physiology characteristic, biological habits, raising management, disease and pest control technique [3–6], multiple mating [7], transcriptome immune analysis [8], and rapid identification of species based on cytochrome c oxidase subunit I (COI) and internal transcribed spacer (ITS) [9]. Genetic markers are largely lacking for O. nipae; in recent years, microsatellites, also called simple sequence repeats (SSRs), have been widely used in studies of intraspecies and interspecies variation and genetic structure in biological populations because of their high levels of polymorphism, codominant Mendelian inheritance, and ease of detection by polymerase chain reaction [10]. Recently, less microsatellite DNA has been reported in O. nipae and its closely related species, excepting eight microsatellite DNA loci described for use in Brontispa longissima [11]. However, the lack of microsatellite markers developed specifically for O. nipae has caused a bottleneck in the study of population genetics in O. nipae.

In this study, a series of microsatellite markers for O. nipae were developed. The utility of the newly developed nine markers were tested in two Fujian populations (Zhangzhou and Fuzhou). These polymorphism microsatellite markers would be powerful tools for pests’ population genetic and dispersal studies in the future. It is the first time that microsatellite markers have been utilized in O. nipae.

2. Materials and Methods

2.1. Insects Sampling and DNA Extraction. O. nipae collected from canary date palm Phoenix canariensis in Zhangzhou
2.2. Microsatellite Development. Based on the existing transcriptome data of *O. nipae* [4], TRINITY software was used to screen all perfect microsatellites [13]. The evaluation criterion used for mono-, di-, tri-, tetra-, penta-, and hexanucleotide repeats was the default setting in TRINITY, with a minimum length of 10bp for mono-, dinucleotide, 20bp for tri-, tetra-, and 30bp for penta- and hexanucleotide repeats. The total DNA of adults from two populations (Zhangzhou and Fuzhou, Fujian Province), were extracted individually using the DNA extraction kit (SK8252, Sangon Biotech Co., Ltd., Shanghai, China) according to the manufacturer’s instructions.

### Table 1: Frequency of SSRs based on the number of repeat units in *Octodonta nipae* transcriptome.

| Repeat type    | Number of repeats |
|----------------|------------------|
|                | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | ≥12   | Total | Percentage |
| Mononucleotide | —     | —     | 555   | 555   | 43.56 |
| Dinucleotide   | —     | 159   | 71    | 18    | 20    | 22    | —     | —     | 333   | 26.14 |
| Trinucleotide  | 255   | 78    | 15    | 11    | —     | —     | —     | —     | —     | 359   | 28.18 |
| Tetranucleotide| 6     | 3     | —     | —     | —     | —     | —     | —     | 17    | 1.33  |
| Pentanucleotide| 6     | 14    | 3     | —     | —     | —     | —     | —     | —     | —     | 0.78  |
| Hexanucleotide | 4     | —     | —     | —     | —     | —     | —     | —     | —     | —     | 0.31  |
| Total          | 10    | 269   | 240   | 86    | 54    | 18    | 20    | 22    | 555   | —     | —     |
| Percentage     | 0.78  | 21.11 | 18.84 | 6.75  | 4.24  | 1.41  | 1.57  | 1.73  | 43.56 |

Allele sizes were determined using GENEMAPPER version 4.0 (Applied Biosystems), LIZ-500 as size standard.

The number of alleles per locus (NA), expected heterozygosity (*H₀*), observed heterozygosity (*Hₑ*), and polymorphic information content (PIC) were assessed using Microsatellite Tools for Excel [14]. Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium between pairs of microsatellites were calculated with GENEPOP V3.4 [15]. This method precludes the false positive in multiple tests. When the loci deviated from HWE, tests were performed using GENEPOP V3.4 to find out whether deviations were the result of a deficit or an excess of heterozygotes.

### 3. Results

3.1. Characterization of SSR Distribution. 1274 SSR loci were identified from the 49919 unigenes. These SSRs have 555 mono-, 333 di-, 359 tri-, 17 tetra-, 6 penta-, and 4 hexanucleotide repeats, which corresponded to 43.56%, 26.14%, 28.18%, 1.33%, 0.47%, and 0.31% of total SSRs, respectively. Frequency of SSRs based on the number of repeat units in *Octodonta nipae* transcriptome is provided in Table 1.

The 10 most frequent motif types in transcriptome were A/T (41.21%), AT/TA (15.86%), AAG/CTT (7.61%), AAT/ATT (7.22%), AC/CT (5.49%), AG/CT (4.79%), AAC/GTT (3.45%), ACC/GGT (3.14%), ATC/ATG (2.43%), and C/G (2.35%). All the possible cases were included, while calculating the frequency of each motif type. In a repeat sequence, for example, AC contained AC and CA, and AAC have AAC, ACA, and CAA. The frequency and number of repeats of all microsatellite motif types are given in Table 2.

3.2. Microsatellite Characteristics. By analyzing the 1274 SSR loci by the software Primer Premier 5, we found 157 loci were suitable for designing 788 pairs of primers. According to the evaluation of Oligo 7.0, the primers of 124 loci were up to standard, namely, 40 for dinucleotide repeats, 80 for trinucleotide repeats, and 4 for tetranucleotide repeats, respectively. In total, 124 pairs of primers were designed and synthesized, one optimized pair of primers for one locus.

Out of the 124 primer pairs, 104 primer pairs amplified the expected products. The 9 loci that appeared to be polymorphic and easy to amplify were chosen to genotype 24 individuals from 2 different populations (12 individuals from Zhangzhou
population and 12 individuals from Fuzhou population). The number of alleles per locus ranged from 2 to 3, with an average of 2.56. The primer sequences and other summarized data for each locus are given in Table 3.

In Zhangzhou population, the expected and observed heterozygosity ranged from 0.083 (On1) to 0.681 (On2) and from 0.083 (On1) to 0.667 (On1), respectively. Out of the 9 loci, three (On1, On3, and On8) were significantly deviated from HWE. Further analyses revealed that On1 was caused by excess of homozygotes, and On3 and On8 were due to excess of heterozygotes. The PIC (polymorphic information content) of alleles per locus ranged from 0.077 to 0.584, with an average of 0.372 (Table 3).

In Fuzhou population, the expected and observed heterozygosity ranged from 0 (On4 and On6) to 0.591 (On2) and from 0 (On4 and On6) to 0.583 (On18), respectively. Three of the 9 loci (On2, On4, and On6) were significantly deviated from HWE. Further analyses revealed that those 3 loci were caused by excess of homozygotes. The PIC of alleles per locus ranged from 0 to 0.501, with an average of 0.256 (Table 3).

4. Discussion

In this study, we found that the SSR loci of *O. nipae* had some difference with those of other insects, which can be observed from Table 4, and the ratio of mononucleotide repeat was mostly in the transcriptome data of the four known insects, ranging from 37.59% to 89.44% with significant difference. In general, except for mononucleotide repeat, trinucleotide repeat was dominant. Because the codon was constituted of 3 nucleotides, the loss and increase of one trinucleotide repeat would not result in frameshift mutations while the loss and increase of mononucleotide, dinucleotide, and tetranucleotide repeat would result in severe frameshift mutations, which further affected the expression and regulation of genes [16].

The ratio of dinucleotide repeat in *O. nipae* was about 26.14% similar to the ratio of trinucleotide repeat (28.18%) which was different with the situation in other insects. This kind of situation may be caused by the different screening criteria of SSR and transcriptome cover.

A/T repeat unit was abundant in the SSR repeat unit of *O. nipae*: A/T, AT/TA, and AAG/GTT/AAT/ATT were the dominant types of repeat unit of mononucleotide, dinucleotide, and trinucleotide, respectively. It can be seen from this that the SSR had a preference of the sequence enriched of A or T; therefore, the frequency of SSR sequence enriched of G or C was relatively low, and thus, our results are in line with the previously published research results of transcriptome [17]. As the SSR of transcriptome was located in the transcribed region, the sequence of SSR would affect the translation to perform different function. The study of the SSR enriched of A or T would deepen the understanding of

| SSR | Number of repeats | Total | Percentage |
|-----|------------------|-------|------------|
| A/T | 4 5 6 7 8 9 10 11 ≥12 | 525 | 525 | 41.21 |
| C/G | — — — — — — — — | 30 | 30 | 2.35 |
| AC/GT | 28 17 14 5 4 2 | 70 | 70 | 5.49 |
| AG/CT | 28 9 10 4 4 6 | 61 | 61 | 4.79 |
| AT/TA | — — 103 45 19 9 12 14 | 202 | 202 | 15.86 |
| AAC/GTT | — 33 9 1 1 — — — — | 44 | 44 | 3.45 |
| AAG/CTT | — 63 23 6 5 — — — — | 97 | 97 | 7.61 |
| AAT/ATT | — 70 17 2 3 — — — — | 92 | 92 | 7.22 |
| ACC/GGT | — 28 9 3 — — — — | 40 | 40 | 3.14 |
| ACG/CGT | — 2 1 1 — — — — | 4 | 4 | 0.31 |
| ACT/AGT | — 8 2 1 2 — — — — | 13 | 13 | 1.02 |
| AGC/CTG | — 14 5 1 — — — — | 20 | 20 | 1.57 |
| AGG/CCT | — 11 1 — — — — — | 12 | 12 | 0.94 |
| ATC/ATG | — 24 7 — — — — — | 31 | 31 | 2.43 |
| CCG/CGG | — 2 4 — — — — — | 6 | 6 | 0.47 |
| AAAC/GTTT | — 1 — — — — — — | 1 | 1 | 0.08 |
| AAAG/CTTT | — 1 — — — — — — | 1 | 1 | 0.08 |
| AAAT/ATT | — 6 — — — — — — | 6 | 6 | 0.47 |
| AACC/GGTT | — 3 — — — — — — | 3 | 3 | 0.24 |
| AAGT/ACCT | — 1 1 — — — — — — | 2 | 2 | 0.16 |
| AATC/ATT | — 1 — — — — — — | 1 | 1 | 0.08 |
| ACTG/AGTC | — 2 — — — — — — | 2 | 2 | 0.16 |
| AGAT/ATCT | — 1 — — — — — — | 1 | 1 | 0.08 |
| AAAA/ATT | 3 — — — — — — — | 3 | 3 | 0.24 |
| AAAC/AGTTT | 1 — — — — — — — | 1 | 1 | 0.08 |
| AACT/GAGTT | 1 — — — — — — — | 1 | 1 | 0.08 |
| AATT/GACTT | 1 — — — — — — — | 1 | 1 | 0.08 |
| AATTTC/AATTG | 2 — — — — — — — | 2 | 2 | 0.16 |
| AATTG/ATT | 1 — — — — — — — | 1 | 1 | 0.08 |
| AACCT/AGGTT | 1 — — — — — — — | 1 | 1 | 0.08 |
| AATGG/AATTG | 1 — — — — — — — | 1 | 1 | 0.08 |
| AAACCT/AGGTTT | 1 — — — — — — — | 1 | 1 | 0.08 |
| AATGGG/AATTCCC | 1 — — — — — — — | 1 | 1 | 0.08 |

**Table 2: Frequency of SSRs based on motif types in Octodonta nipae transcriptome.**
According to this, we speculated that the polymorphism of dinucleotide repeat and trinucleotide repeat, respectively. 103 and 11, which possessed the ratio of 30.9% and 3% in conditions of dinucleotide repeat and trinucleotide repeat was mentioned criteria, the number of SSR loci meeting with the above-mentioned conditions of dinucleotide repeat and trinucleotide repeat in repetition of 8 bases or more may be higher, according to the above-mentioned criteria, the number of SSR loci meeting with the expected heterozygosity; PIC, polymorphic information content; HWE, Hardy–Weinberg equilibrium.

Table 4: Frequency of SSRs based on the number of repeat units in four insects’ transcriptome.

| Insect               | Mononucleotide | Dinucleotide | Trinucleotide | Tetranucleotide | Pentanucleotide | Hexanucleotide |
|----------------------|----------------|--------------|---------------|-----------------|-----------------|---------------|
| Occtodonta nipae     | 43.56          | 26.14        | 28.18         | 1.33            | 0.47            | 0.31          |
| Phenacoccus solenopsis | 89.44          | 2.31         | 7.52          | 0.67            | 0.06            | —             |
| Nilaparvata lugens   | 37.59          | 17.28        | 37.38         | 2.09            | 0.52            | 0.21          |
| Tenebrio molitor     | 44.44          | 11.21        | 41.15         | 1.68            | 0.96            | 0.56          |

The function of SSR. Moreover, the polymorphism of SSR in animals and plants was positively correlated with the length of SSR [18], generally the polymorphism of low-grade units was higher than that of high-grade units [19], the polymorphism of dinucleotide repeat and trinucleotide repeat in repetition of 8 bases or more may be higher, according to the above-mentioned criteria, the number of SSR loci meeting with the conditions of dinucleotide repeat and trinucleotide repeat was 103 and 11, which possessed the ratio of 30.9% and 3% in dinucleotide repeat and trinucleotide repeat, respectively. According to this, we speculated that the polymorphism detection efficiency oddinucleotide repeat of O. nipae would be higher than that of trinucleotide. Our experimental results verify the speculation that 7 out of 9 polymorphic sites are higher than that of trinucleotide. Our experimental results verify the speculation that 7 out of 9 polymorphic sites are higher than that of trinucleotide.

The 9 loci that appeared to be polymorphic and easy to amplify were chosen to genotype 24 individuals from two species: (1) The genome, even some transcriptome SSRs have no polymorphism, because the polymorphism of SSR is related to the times of repetition of core sequence, but the times of repetition of transcriptome SSR is not high. (2) The SSR obtained from transcriptome is cDNA without introns, and it is different from the corresponding genome SSR; thus, the length of PCR products may be longer than expected; the PCR amplification may be inefficient or the PCR amplification may fail. (3) Defect still exists in high-throughput sequencing assembly which can lead to many SSRs with more repetitions fracturing or missing in the assembly process, leading to the transcriptome data incomplete and less reliable.

Setting more effective criteria to screen SSR based on the characters of SSR was suggested, for instance, assessment of flank sequence length and similarity could be added into and sites with more repetitions could be chosen in the process of site selection [23]. The development efficiency could be improved greatly by means of strict biological information method. Besides, it is very meaningful to explore the SSR function of molecular markers, through gene location and sequence alignment conduct function annotation to get a deeper understanding of the biological meaning of these genes [24]. As part of the transcriptome SSR can be used in the same genus and even within the same family to conduct cross-species amplification, those chosen sites can be used to construct an evolutionary tree model to analyze evolution and classification and other issues.
different populations (Fuzhou and Zhangzhou populations), and their PICs had large differences in those two populations. Some loci were discovered to be significantly deviated from HWE ($P < 0.05$), which may be due to population bottleneck effect or null alleles or small sample size, considering the fact that the species invaded Fujian Province several years ago. The relatively short time for the species invasion into Fujian may result in the low allele diversity in this study. Although the polymorphisms of 9 loci were not very high, they should be powerful tools for investigating the genetic diversity and the invasion route in $O$. nipae.

**Conflicts of Interest**
The authors declare that they have no conflicts of interest.

**Authors’ Contributions**
Zhiming Chen and Jun Chen contributed equally to the work.

**Acknowledgments**
This work was supported by grants from the National Key R&D Program of China (2017YFC1200600), Fujian Province Major Science and Technology Project (2017NZ0003-1-6), and the National Natural Science Foundation of China (31471829).

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