**Development of Microsatellite Markers and a Preliminary Assessment of
Population Structuring in the Rice Weevil, *Sitophilus oryzae* (L.)**

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Abstract

The rice weevil, *Sitophilus oryzae* is one of the primary pests of stored grains worldwide. To develop and implement an effective integrated pest management strategy, an understanding of the population structuring of this destructive pest is vital. In this study we used Illumina paired-end sequencing to develop *S. oryzae* species-specific microsatellite markers, and used these markers to conduct a preliminary assessment of population structuring in four populations of *S. oryzae* from three countries (Australia, China, and USA). 7,635,996 raw sequencing reads were produced, with 11,794 microsatellites detected and 214,257 primer options designed. 48 microsatellite markers were selected for further validation, with 10 markers amplifying consistently across the four *S. oryzae* populations. These markers displayed a high level of polymorphism overall (6.67 alleles/locus), though this was slightly lower within populations (3.10 - 4.88 alleles/locus). We used the markers to conduct a preliminary assessment of genetic structuring among the four *S. oryzae* populations: three laboratory cultures (New South Wales, Queensland, and Santai) and a field collected population from Kansas. Analyses suggest high levels of genetic differentiation between the sample locations, with a global F_{ST} of 0.239, and pairwise F_{ST} values ranging from 0.100 to 0.395. Bayesian clustering analyses suggest these four populations formed four distinct clusters, with a similar pattern identified by Principal Coordinate Analysis. These microsatellite markers, together with our preliminary population genetic analyses, will provide a valuable resource for population genetic research, and contribute to effective integrated pest management strategies in the future.

Keywords: *Sitophilus oryzae*, Microsatellite markers, Illumina paired-end sequencing, population genetics

1. Introduction

Sitophilus species (Curculionidae: Coleoptera) are considered to be the most serious insect pests of stored cereals in the world (Rees, 1996). One of these species, the rice weevil (*Sitophilus oryzae* (L.)), is particularly destructive, infesting numerous food grains including rice, wheat, maize, sorghum, oats, rye, barley and other cereals (Koehler, 1994; Grenier *et al.*, 1997). It mainly infests stored cereals but populations have also been recorded infesting grains in the field (Longstaff, 1981). Both the larvae and adults feed on whole grains causing significant losses of stored grains both quantitative and qualitatively, which subsequently reduces their market value (Lucas and Riudavets, 2000; Park *et al.*, 2003).

Conventional control of *S. oryzae* relies on fumigants and grain protectants. However, heavy and prolonged use of these chemicals can lead to the development of heritable resistance (Champ and Dyte, 1976; Daglish *et al.*, 2002; Wallbank and Collins, 2003). Integrated management is therefore considered the most effective method for the control of stored grain insect pests (Subramanyam and Hagstrum, 1995). To implement more effective integrated pest management strategies, knowledge of an insect's population structuring is vital, especially as affected by its movement (Kim and Sappington, 2013). Direct observations of the movement of stored grain insects using pheromone traps and other techniques is limited in both geographical scope and time scale, and occasional instances of movement may be ecologically significant yet easily missed (Chapman *et al.*, 2003; Jopp and Reuter, 2005). Population genetics provides an alternative, effective method for obtaining information about population structure and the short and long distance movement of insect pests (Kim and Sappington, 2013).

Microsatellites are the markers of choice for population genetics studies because of their high level of polymorphism and co-dominant features (Goldstein and Schlotterer, 1999). Traditionally microsatellite marker development has been a time consuming process involving enrichment of genomic DNA, followed by cloning and sequencing of the enriched DNA library by Sanger sequencing

(Santana *et al.*, 2009). More recently next generation sequencing approaches have made microsatellite development a more efficient and cost effective alternative. Initially 454 pyrosequencing was the preferred method for microsatellite development (Kang *et al.*, 2012), while more recently Ion semiconductor sequencing has also been evaluated (Gardner *et al.*, 2011). However, with the decreasing per-base cost of Illumina sequencing in recent years, coupled with increased read lengths, Illumina paired-end sequencing has become an attractive option for microsatellite marker development (Castoe *et al.*, 2012; Duan *et al.*, 2014).

To date, little information is available about the population structuring and movement of *S. oryzae*. Only four microsatellite markers are currently available, with these markers having initially been developed for *Sitophilus zeamais* (Correa *et al.*, 2012). This is an insufficient number to assess the population structuring and genetic diversity of this key pest species. Here we report the development and characterization of additional microsatellite markers for *S. oryzae* and demonstrate their effective utility by conducting a preliminary assessment of population structuring and genetic diversity across four *S. oryzae* populations from three countries.

2. Materials and methods

2.1. Sampling

S. oryzae laboratory cultures originating from New South Wales (NSW), Queensland (QLD) and Santai (China), as well as a field-collected population (Kansas), were obtained from the Department of Agriculture and Fisheries (DAF), Brisbane, Queensland, Australia (Table 1). Samples were preserved in ethanol.

Table 1: *Sitophilus oryzae* sample details for population genetic analysis

Location	Latitude	Longitude	Collection date	Nature of sample
New South Wales, Australia	34.459	148.705	2012	Laboratory culture
Queensland, Australia	26.533	151.833	1994	Laboratory culture
Kansas, USA	38.500	98.000	2013	Field collected
Santai, China	31.120	148.705	1998	Laboratory culture

2.2. DNA extraction

Genomic DNA was isolated from *S. oryzae* adults using spin columns (Epoch Life Sciences). A single beetle was homogenized with 0.5 mm zirconium beads in a bullet blender (BioTools, NY, USA). 200 µl of lysis buffer (200mM Tris-HCl, 25mM EDTA, 250mM NaCl, 0.5% sodium dodecyl sulphate) and 2 µl of proteinase K were added. Tubes were centrifuged for 20 sec at 14,680 rpm (all centrifuge steps were conducted at this speed), and incubated at 55°C overnight. 100 µl of precipitation buffer (4M Ammonium acetate) was added and mixed gently by inversion, and tubes incubated at -20°C for 10 min. Tubes were centrifuged for 30 sec, then 400 µl of binding buffer (2M GuHCl; 50% Ethanol) added and mixed by shaking, then centrifuged for 30 sec. 500 µl of the supernatant was transferred into each spin column and centrifuged for 4 min. 500 µl of wash buffer (100mM Tris-HCl; 80% ethanol) was added, and tubes centrifuged for 4 min. A further 500 µl of wash buffer was added and the tubes were centrifuged for 15 min. The spin column was then placed in a new collection tube and 100 µl of elution buffer (10mM Tris-HCl) added, then centrifuged for 2 min. The isolated DNA was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Life Technologies, USA).

2.3. DNA library preparation and sequencing

A genomic library was prepared from a single field-collected sample of *S. oryzae* from Queensland, Australia. Genomic DNA (~ 50ng in 75 µl of TE buffer) was sheared for 3 min using an Ultrasonic cleaner (VGT-1620QTD) to obtain a fragment size distribution of about 300 bp to 1,000 bp. Sequencing libraries were generated using Illumina NEBNext Ultra DNA library preparation kit (New England Biolabs) following the standard Illumina sample preparation protocol with 10 cycles of PCR. Size selection was performed on a Blue pippin (Sage Science) with fragments between 350 and 600 bp selected. Libraries were checked by capillary microchip electrophoresis (MultiNA, Shimadzu) and sequenced by the Australian Genome Research Facility (AGRF) on Miseq P.E. 250bp as half of a run, with remaining libraries stored at -20°C.

2.4. Microsatellite loci search and primer design

Raw data were trimmed with Trimmomatic (Bolger *et al.*, 2014) using options HEADCROP 10, LEADING 3, TRAILING, SLIDING WINDOW 4:15 MINLEN 150 to remove adapter contamination (reading through fragment). Sequences where the paired-end reads overlap by at least 20bp were merged with the program FLASH (Magoc and Salzberg, 2011) to create single longer sequences (up to 480bp). Potential microsatellite markers were identified from the merged *S. oryzae* reads using QDD version 2.1. (Meglecz *et al.*, 2010). Primer pairs were designed for unique sequences with non-interrupted microsatellites using Primer 3 (Rozen and Skaletsky, 2000). To make scoring easier we restricted our choice to trinucleotides or longer and only included pure repeats. The 48 microsatellites with the highest repeat number were subsequently selected for preliminary screening.

2.5. Amplification of microsatellite markers

The 48 selected primer pairs were synthesized (IDT, Australia), with an M13 tail added to the forward primer for subsequent amplicon dye labelling (Schuelke, 2000). PCR amplifications were carried out as 12 μ l reactions containing 1X My Taq (Bioline) buffer, 0.1 μ M of forward primer, 0.2 μ M of reverse primer, 0.2 μ M of M13 dye (6-FAM, VIC, NED or PET; Applied Biosystems), 0.3 units of Taq polymerase and 2 μ l of template DNA. Reactions were performed using a Veriti 96-well Thermocycler (Applied Biosystems). PCR cycling conditions consisted of an initial denaturing step of 95°C for 10 min, followed by 35 cycles of 95°C for 25 sec, 57°C for 30 sec and 72°C for 45 sec, with an addition 10 cycles with the annealing temperature changed to 54°C, and a final extension step of 72°C for 10 min.

These 48 primer pairs were initially screened against eight individuals from the four populations to assess whether they amplified and were polymorphic, with the PCR products analyzed by capillary microchip electrophoresis (MultiNA, Shimadzu). Those microsatellite loci that showed strong amplification and no evidence of non-specific binding were subsequently screened against 24 individuals from the four populations. PCR products were then cleaned using ExoSAP (Glenn and Schable, 2005) and sent for genotyping (ABI, 3730, Applied Biosystems; Macrogen Inc., Seoul, South Korea).

2.6. Microsatellite genotyping and analysis

Microsatellite peaks were confirmed and binned manually using the Geneious 7.2.1 microsatellite plugin (Kearse *et al.*, 2012). Null allele frequencies (Dempster *et al.*, 1977), as well as pairwise and global F_{ST} (Weir, 1996) were estimated in FreeNA (Chapuis and Estoup, 2007) using the ENA algorithm. Allele size range, number of alleles, and observed and expected heterozygosity were calculated using GENEPOP 4.0.10. (Rousset, 2008). Loci that were monomorphic or had high null allele frequencies were excluded from further analysis (see section 3.1.).

An individual based Bayesian clustering algorithm, STRUCTURE 2.3.3. (Pritchard *et al.*, 2000) was used to investigate population structuring. Analyses were carried out under the non-admixture model with alleles correlated. 20 replicates for each value from K=1 to K=5 were conducted, with an initial burn-in of 100,000 iterations, followed by 1,000,000 iterations. The most likely value of K was determined with STRUCTURE HARVESTER (Earl and VonHoldt, 2012), based on the methods of Evanno *et al.* (2005). Results were visualized using CLUMPAK (Kopelman *et al.*, 2015).

A Principal Coordinate Analysis (PCoA) was computed using pairwise individual genetic distance in GenAIX 6.501 (Peakall and Smouse, 2006; 2012) so that genetic differentiation and clustering could be assessed without the assumptions that populations were in Hardy-Weinburg Equilibrium (HWE).

3. Results and discussion

3.1. Microsatellite and population characteristics

1.9 Gb of raw sequencing data, comprising 7,635,996 raw sequencing reads, was generated from the approximately 500 bp insert library. After quality trimming 7,120,305 (93.25%) clean reads remained and were used for further analysis. 11,794 microsatellites ranging from 10 to 76 bp in length, were identified. Dinucleotide repeats were the most abundant (69.69%), followed by trinucleotides (24.52%; Fig. 1). This high proportion of dinucleotide repeats is typical of arthropod genomes (Toth *et al.*, 2000; Correa *et al.*, 2012). 214,257 primer options were designed (Suppl. Table 1).

Fig. 1. Frequency of microsatellite classes in the genome of *S. oryzae*

Based on the results of the initial screen 8/48 primer pairs produced easily discernable products that could be accurately and consistently genotyped in all populations, with two additional primer pair amplifying consistently in at least half of the populations (Tables 2 & 3).

Seven microsatellite loci were polymorphic in all populations, with three markers monomorphic in one or more populations (*Sit_05*, *Sit_07*, *Sit_10*; Table 3). The number of alleles per locus ranged from 3 (*Sit_7*, *Sit_10*) to 22 (*Sit_08*), with an average of 6.67 alleles/locus, indicating a high level of polymorphism, though the allelic diversity within each population was notably lower. Within populations there were between 1 and 17 alleles per locus, with the average number of alleles per locus ranging from 3.10 to 4.88 (Table 3), a range of values similar to that observed in field collected populations of the congeneric *S. zeamais* (3.44 – 4.11) (Correa *et al.*, 2012). Finding similar levels of allelic diversity between these two studies was unexpected given three of the four populations in our study were lab cultures, as beetles that have been in culture for many generations often display reduced allelic richness (Nowak *et al.*, 2007; Lagisz *et al.*, 2011). The sole field collected strain in our study (Kansas) was found to have the highest allelic richness (4.88 alleles/locus), but there did not appear to be a direct correlation between time spent in culture and allelic richness in the lab strains, with the

oldest strain (QLD) displaying the second highest allelic diversity (4.11 alleles/locus). This indicates other factors, for example allelic diversity of populations prior to culture, may have a greater influence on allelic diversity than the time spent in culture.

Table 2: Primer sequences and characteristics of 14 microsatellite markers developed in *S. oryzae*

Locus	Primer sequence (5'-3')	Repeat motif	Size range (bp)	Dye	GenBank accession
Sit_01	F: ACTCTTCCTTGCGACCATTTG	AAAGAG	282-366	VIC	xxxxxx
	R: GAAACATCGCAGTATCCAGACA				
Sit_02	F: TGCAAGGCTGAACAGTGCTA	ACT	151-250	NED	xxxxxx
	R: TCTAGATTACATTCTTCCATTGTATCG				
Sit_03	F: CAGGGTGAGAAGGAAGGTCA	AAC	250-307	PET	xxxxxx
	R: CCGGTGAAATGGAAGGGTAT				
Sit_04	F: GGCTCGACCTGATGGTATGT	ACCGC	138-168	6-FAM	xxxxxx
	R: CCATTTCCGCGACTGATTT				
Sit_05	F: CGAAGGTCCTTTGAAGCAGA	ACT	148-181	VIC	xxxxxx
	R: CGGAAGCATTACCTCATCA				
Sit_06	F: AGCAGCATTGATCGGATTGT	ACAT	180-216	NED	xxxxxx
	R: GTGCATCACCGGGATTTAGT				
Sit_07	F: AACCAAGGCCAAGACCAAGT	AACCAG	189-243	PET	xxxxxx
	R: GATGGCGTTGACTTCTTTG				
Sit_08	F: GAAACACCTAGTTAACAATAGAGACCG	ACCGAG	164-388	6-FAM	xxxxxx
	R: GCAACTTTCTAGAGCAATTCGT				
Sit_09	F: CCCTCTTGAATGGATGGATG	AAAG	186-214	PET	xxxxxx
	R: CAAGGAAGGAGGATGGGATT				
Sit_10	F: AAATCCGAGGACATCCGTCT	AAACC	152-182	NED	xxxxxx
	R: TCACACGCTAGTTCGATAGAGG				

The observed heterozygosity of individual polymorphic loci ranged from 0.05 to 0.91 across populations, with mean population values ranging from 0.30 – 0.45 (Table 3). The Santai population on average had notably lower observed heterozygosity than the other populations ($H_O = 0.30$), but surprisingly the oldest strain (QLD) had the highest ($H_O = 0.45$), though the field collection Kansas strain had a similar level of observed heterozygosity ($H_O = 0.43$).

Table 3: Results of initial primer screening in four populations (24 individuals per population) of *S. oryzae*.

**** Indicates less than 50% of the samples amplified from a population. ^ Indicates null alleles frequencies are high (>0.15) in all populations. N_A = number of alleles, H_E = Expected heterozygosity, H_O = Observed heterozygosity.

Locus	NSW			QLD			Kansas			Santai		
	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E	N_A	H_O	H_E
Sit_01^	7	0.55	0.87		****			****		5	0.25	0.62
Sit_02	2	0.11	0.10	5	0.91	0.77	3	0.65	0.57	2	0.36	0.51
Sit_03	2	0.55	0.41	5	0.48	0.54		****		3	0.45	0.44
Sit_04	3	0.55	0.66	2	0.50	0.46	2	0.54	0.49	3	0.30	0.43
Sit_05	2	0.05	0.05	2	0.05	0.05	1	0.00	0.00	1	0.00	0.00
Sit_06	5	0.78	0.76	9	0.67	0.83	9	0.62	0.77	6	0.47	0.66
Sit_07	3	0.33	0.41	2	0.06	0.17	2	0.30	0.43	1	0.00	0.00
Sit_08	11	0.39	0.78	7	0.56	0.74	17	0.82	0.91	4	0.48	0.42
Sit_09	3	0.41	0.43	3	0.50	0.39	3	0.32	0.35	3	0.26	0.24
Sit_10	1	0.00	0.00	2	0.29	0.25	2	0.17	0.22	3	0.46	0.37
Mean	3.90	0.37	0.45	4.11	0.45	0.47	4.88	0.43	0.47	3.10	0.30	0.37

A single locus (*Sit_01*) showed strong evidence of null alleles (null allele frequencies > 0.15 for all populations), and was hence excluded from the subsequent analyses of population structuring.

3.2. Population structure

Strong genetic structure was identified between the populations of *S. oryzae* (9 loci; global F_{ST} = 0.239, $p < 0.01$). All pairwise F_{ST} comparisons were significant even after sequential Bonferroni correction, with values ranging from 0.100 (QLD versus Kansas) to 0.395 (NSW versus Santai; Table 4). The Santai population appeared the most distinct, with two of the three of the highest pairwise F_{ST} values occurring between the Santai population and other populations. Similar high pairwise F_{ST} values were observed between globally collected laboratory strains of *Tribolium castaneum* (0.231 – 0.489; Lagisz *et al.*, 2011). The high global and pairwise F_{ST} values are perhaps unsurprising given the populations originated from wild populations at several distinct geographical locations, and it is possible the high level of molecular divergence reflects the genetic structuring of wild populations.

However, it is also likely that any such divergence would be magnified by genetic drift acting over many generations in the isolated laboratory populations (Wade, 1991), so extrapolating these findings to field populations should be done with caution.

Table 4: Population pairwise F_{ST} values between four *S. oryzae* populations based on 9 microsatellite markers. All F_{ST} values are significant at $P < 0.01$.

	Santai	Kansas	NSW	QLD
Santai	0.000			
Kansas	0.291	0.000		
NSW	0.395	0.230	0.000	
QLD	0.212	0.100	0.204	0.000

Bayesian clustering analysis of microsatellite genotypes indicated that the most likely number of populations (K) was four (Suppl. Fig. 1), with the four geographic populations forming the four distinct populations (Fig. 2). This was expected given the high pairwise F_{ST} values observed between populations, and further reinforces the suggestion that these populations are genetically distinct.

Fig. 2. Results of the clustering analyses performed using STRUCTURE. Each bar represents the posterior probability that the individuals concerned belong to one of four clusters.

Principal Coordinate Analysis (PCoA) similarly suggested population genetic structuring, with four clusters identified (Fig. 3). There was, however, a small degree of overlap between several of the populations, which was surprising given the high pairwise F_{ST} values observed between these populations.

Fig. 3. Principal Coordinate Analysis showing ordination of genetic identity between the four *S. oryzae* populations, as assessed from 9 microsatellite loci. Axis 1 explains 16.30% of the variation, with axis 2 explaining 13.80%.

4. Conclusions

The microsatellite markers developed in this study will provide a valuable resource for future population genetic assessments of this key pest species worldwide. Most of these markers are in HWE and display a moderate to high degree of polymorphism. We have demonstrated the effective utility of these markers by conducting a preliminary assessment of population structuring of beetles collected from three countries, with our preliminary results suggesting a high level of population structuring on a global scale.

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