

## Research

# Development of Polymorphic Microsatellite Markers of *Obolodiplosis robiniae* (Haldeman) (Diptera: Cecidomyiidae), a North American Pest Invading Asia

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**ABSTRACT.** Microsatellite markers were developed for epidemiological studies on the black locust gall midge *Obolodiplosis robiniae* (Haldeman) (Diptera: Cecidomyiidae), a native North America pest introduced to Europe and Asia. Polymorphism at each locus was tested on 68 individuals from six populations reared from infected host leaves of *Robinia pseudoacacia* L. collected in China. Fourteen loci were found to be polymorphic, with the number of alleles ranging from 3 to 10. The observed heterozygosity varied evenly from 0.2667 to 0.6540. For populations, the observed heterozygosity ranged from 0.1429 to 1.000. The allele frequency of the predominant allele varied from 0.250 to 0.500. All loci with negative  $F_{ST}$  values indicated heterozygote excess in each locus with six populations. Of 14 loci, four were observed to have  $F_{ST}$  values up to 0.05, which indicated negligible genetic differentiation within the population. Significant deviations ( $P < 0.05$ ) from the expected Hardy–Weinberg equilibrium, as evaluated using the Markov chain algorithm for each locus and for all six populations, were observed, and genotypic linkage disequilibrium was clearly detected. These markers represent a useful tool to design strategies for integrated pest management and in the study of population evolution in this important introduced pest.

**Key Words:** black locust gall midge, population genetic, microsatellite polymorphism, invasive pest

The black locust gall midge *Obolodiplosis robiniae* (Haldeman) (Diptera: Cecidomyiidae) is an important introduced insect pest, a defoliator forming leaf margin roll galls on the species of genus *Robinia* (Fabaceae) in Europe and Asia. It damages its host's leaves, causing them to roll, which causes the plant to fall into decline and in turn leads to infestation by other pest insects that are dangerous to forests, such as longhorn beetles and jewel beetles. The pest was described by Haldeman (1847) in North America, but has been little known in the world except for biology and behavior data and a distribution limited to eastern North America (Buhl and Duso 2008, Kim et al. 2011) until 2002, when it was found in South Korea and Japan (Kodoi et al. 2003, Woo et al. 2003), then in 2003 in Italy (Duso and Skuhrava 2004). The pest was then detected gradually in many countries and regions of Europe and some areas of Asia, including Albania, Austria, Bosnia-Herzegovina, Croatia, the Czech Republic, Denmark, Corsica, France, Germany, Greece, Hungary, Luxembourg, Macedonia, the Netherlands, Poland, Romania, Serbia, Slovakia, Slovenia, Sweden, Switzerland, the United Kingdom, and Ukraine (Duso et al. 2005, Csoka 2006, Hoffmann et al. 2007, Mihajlovic et al. 2008, Roskam et al. 2008, Skrzypczynska 2008, Jorgensen 2009, Pernek and Matošević 2009, Balint et al. 2010, CABI/EPPO 2011), and China (Yang et al. 2006, Lin et al. 2007).

In China, it was first discovered on *Robinia pseudoacacia* L. in Qinhuangdao city, Hebei Province in 2004 (Lin et al. 2007). The pest was then found in four cities of Liaoning Province including Jinzhou, Anshan, Huludao and Chaoyang, and Beijing (Yang et al. 2006). Later it was detected in Shandong (Yan et al. 2007), Jilin (Zhang et al. 2008) and Gansu (Li et al. 2012) provinces. However, it is probably distributed through more areas in China because we collected the pest in other places in addition to the locations reported above, as listed in Table 1.

Prins et al. (2014) examined 11 hypotheses that were proposed to explain biological invasion. However, insects require rather complicated models for the knowledge of biological and ecological factors that influence some species' establishment and spread in new habitats

(Suarez et al. 2001, Boubou et al. 2011, Perdereau et al. 2011, Amouroux et al. 2013). Genetic variation has been regarded as an important factor in the colonization of invasive species (Sakai et al. 2001, Amouroux et al. 2013, Horst and Lau 2015). Molecular genetics techniques were recognized as a useful tool for understanding population structure (Kirk et al. 2013), especially microsatellite DNA simple sequence repeat markers, which have been widely used to analyze the population structure of invasive pest insects (Bonizzoni et al. 2000; Solano et al. 2000; Khemakhem et al. 2006, 2012; Pfunder and Frey 2006; Hinomoto et al. 2012; Amouroux et al. 2013).

In this study, we identify 14 microsatellite markers that can be used to study the epidemiological dynamics, as well as revealing the genetic diversity of the geographic population of the black locust gall midge *O. robiniae* in the process of invasion.

## Materials and Methods

**Sample Collection.** We collected damaged rolled leaves of *R. pseudoacacia* in the field and took them to the lab, then put them into large jars (40 by 30 cm) and sealed them with gauze and a rubber band. All jars were placed in the insectary and treated with appropriate temperature (25°C) and humidity ( $\pm 70\%$ ). When adults emerged, they were moved into centrifuge tubes (1.5 ml) with 100% alcohol and stored at  $-20^\circ\text{C}$ . Sample collection data are reported in Table 1.

**Microsatellite Isolation.** Loci were isolated from the genomic DNA of 20 adult individuals for each location that emerged from rolled host leaves collected from Beijing, Qinhuangdao (Hebei province) and Tianjin, China (Table 1). Genomic DNA was extracted from each individual midge using the CATB genomic DNA kit (Aidlab, Beijing, China). Total genomic DNA was completely digested with the Sau3AI restriction enzyme (New England Biolabs, Ipswich, MA) for 4 h at 37°C. The 300- to 1,000-bp long fragments were recovered using the AxyPrep Gel Extraction Kit (Axygen, Union, CA) and then ligated to a Sau3AI adapter pair with T4 DNA ligase (New England Biolabs, Ipswich, MA) in a

**Table 1. Six populations of *Obolodiplosis robiniae* with collection data**

Samples	Sites	Location	Date	Altitude	Host
BJ	Beijing	N:40°00.184'E:116°14.363'	20 May 2013	76	<i>R. pseudoacacia</i>
BD	Baoding, Hebei	N:38°52.782'E:115°27.546'	21 May 2013	35	<i>R. pseudoacacia</i>
QHD	Qinhuangdao, Hebei	N:39°56.161'E:119°35.411'	27 May 2013	17	<i>R. pseudoacacia</i>
SY	Shenyang, Liaoning	N:41°50.438'E:123°25.690'	4 June 2013	51	<i>R. pseudoacacia</i>
TJ	Tianjin	N:39°07.935'E:117°08.818'	22 May 2013	5	<i>R. pseudoacacia</i>
XA	Xian, Shaanxi	N:34°15.474'E:108°58.938'	4 June 2014	428	<i>R. pseudoacacia</i>

**Table 2. Characteristics of 14 microsatellite loci from *Obolodiplosis robiniae***

Locus (accession no.)	Primer sequence(5'–3')	PCR Ta/°C	Repeat motif	Expected size	Allele size range	No	Ho (ave.)	He (ave.)
W3 (KP260518)	F: CGTCATATCGCAGTAGCAAT R: CGCCGCATTAAAAATACAT	50	(TC) <sub>52</sub>	249	234–252	8	0.6548	0.7161
W5 (KP260519)	F: ACCGGCGAAAAACAATTAT R: AGTTGATGAATGCGTTGTGTT	51	(AC) <sub>27</sub>	143	116–237	5	0.6560	0.7148
W8 (KP260521)	F: TTGAAGCGAAGCACTTGGGA R: AGCAACGAAAAAGAAACACAGAG	53	(CT) <sub>9</sub> (TC) <sub>17</sub>	193	187–197	4	0.3875	0.6104
W29 (KP260522)	F: TTGGGGGATCATAAAGTCTCG R: AGTTTTATGACTTGTTCGCCTC	51	(CA) <sub>9</sub> (AG) <sub>12</sub>	152	135–152	5	0.2713	0.5897
W31 (KP260523)	F: CACGCAGAAACCAATCATTATC R: AGCCAACTCAATGACGAACA	50	(AC) <sub>9</sub>	193	190–198	5	0.4792	0.6615
W33 (KP260524)	F: CCTTCGCGTACATTTGCA R: AACGCGTCTGATAAGCTGAA	49	(AG) <sub>16</sub>	166	160–195	10	0.5067	0.6832
W35 (KP260525)	F: TGTATGCTATCTCGTCACACTCA R: TCACACGCATTCAACCTCAG	51	(AC) <sub>36</sub>	207	167–215	10	0.5417	0.6833
W41 (KP260526)	F: TGCTATCTCTGGCGATTAT R: GAGACAGATTTATAGATAAATAAGA	47	(CT) <sub>20</sub>	152	150–154	3	0.2667	0.5388
W46 (KP260527)	F: GCTCATGCACTGACCAAGTTA R: TCGTACGCACTTGTCTTGTG	51	(TC) <sub>8</sub> (CT) <sub>5</sub> (AC) <sub>59</sub>	323	297–348	5	0.4200	0.6256
W82 (KP260528)	F: TGCACATCACTCACGAAACG R: TTGCACACGAAATGCATCAT	51	(CT) <sub>15</sub>	178	173–178	3	0.4046	0.5398
W83 (KP260529)	F: AAAGACTCAGCGTTTTTGCAG R: ACACACATACACGCAAAACA	50	(TG) <sub>6</sub>	133	130–194	7	0.5714	0.6540
W116 (KP260531)	F: AAGAGACGGATGGATGGTGA R: CATTGGTTCGAATAAATCGC	50	(TG) <sub>21</sub>	228	228–266	5	0.5292	0.6064
W126 (KP260532)	F: GGACGGGTGGCTGATGTA R: ATTGCTCTGGCTGTAAGTGG	52	(GA) <sub>5</sub> (TG) <sub>12</sub>	280	278–284	3	0.3533	0.5660
W132 (KP260533)	F: TTTGCGTGAACAAAAGTCAGA R: ACACGACTAAATACATGGCA	50	(GT) <sub>25</sub>	175	160–195	8	0.4417	0.6831

For each locus, the number of alleles, observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) are based on a sample of 68 individual midges.

**Table 3. Observed heterozygosity ( $H_o$ ) for each locus with each population**

Locus	Ho for loci in each population (numbers of individuals)					
	BJ(15)	BD(20)	QHD(7)	SY(10)	TJ(6)	XA(10)
W3	0.6667	0.7000	0.4286	1.0000	0.3333	0.8000
W5	0.8000	0.5500	0.2857	0.8000	0.5000	1.0000
W8	0.2000	0.3500	0.0000	0.4000	0.1667	0.6000
W29	0.4667	0.2000	0.2857	0.2000	0.1667	0.2000
W31	0.5333	0.6500	0.4286	0.6000	0.3333	0.3000
W33	0.5333	0.4000	0.1429	0.7000	0.1667	0.9000
W35	0.2667	0.7000	0.0000	0.7000	0.5000	0.3000
W41	0.2000	0.3000	0.0000	0.1000	0.3333	0.2000
W46	0.4667	0.4000	0.0000	0.4000	0.3333	0.5000
W82	0.5333	0.5000	0.2857	0.4000	0.0000	0.3000
W83	0.5000	0.7000	0.4286	0.5000	0.8333	0.3000
W116	0.6667	0.3500	0.1429	0.5000	0.0000	0.6000
W126	0.3667	0.5000	0.1429	0.2000	0.5000	0.3000
W132	0.4667	0.6000	0.0000	0.3000	0.1667	0.4000

**Table 4. Expected heterozygosity ( $H_e$ ) for each locus with each population**

Locus	He for loci in each population (numbers of individuals)					
	BJ(15)	BD(20)	QHD(7)	SY(10)	TJ(6)	XA(10)
W3	0.8050	0.7422	0.6112	0.7950	0.6250	0.7181
W5	0.7327	0.7433	0.6250	0.7104	0.7224	0.7550
W8	0.7224	0.6021	0.0000	0.5938	0.6250	0.5087
W29	0.6943	0.5938	0.5000	0.6250	0.5000	0.6250
W31	0.7961	0.7424	0.6112	0.7078	0.5000	0.6112
W33	0.8353	0.7500	0.5000	0.8164	0.5000	0.6976
W35	0.6250	0.6783	0.0000	0.7245	0.7776	0.6112
W41	0.5000	0.5692	0.0000	0.5000	0.6250	0.5000
W46	0.7417	0.6324	0.0000	0.5938	0.5000	0.6600
W82	0.5938	0.6050	0.5000	0.5000	0.0000	0.5000
W83	0.7400	0.7525	0.6112	0.7400	0.5800	0.5000
W116	0.6500	0.6218	0.5000	0.5800	0.0000	0.6804
W126	0.6250	0.6600	0.5000	0.5000	0.6112	0.5000
W132	0.7349	0.7081	0.0000	0.7224	0.5000	0.7500

40  $\mu$ l reaction mixture overnight at 16°C. A polymerase chain reaction (PCR) Purification Kit (Tiangen, Beijing, China) was used to purify the above mixture, and the solution was finally diluted with 20  $\mu$ l ddH<sub>2</sub>O. The 10-fold diluted digestion–ligation mixture was

amplified with adaptor-specific primers in 50  $\mu$ l reactions with the following conditions: 5 min denaturation at 95°C; followed by 25 cycles of 1 min denaturation at 95°C, 1 min annealing at 60°C, and 2 min extension at 72°C; with a final extension of 72°C for 10 min.

**Table 5. Allele frequencies (most frequent alleles) of the polymorphic SSR markers amplified in different gall midge populations**

Locus	Number of alleles and frequency of most frequent alleles(size, bp) in each population(numbers of individuals)											
	BJ(15)		BD(20)		QHD(7)		SY(10)		TJ(6)		XA(10)	
	No. of alleles	Frequency (size)	No. of alleles	Frequency (size)	No. of alleles	Frequency (size)	No. of alleles	Frequency (size)	No. of alleles	Frequency (size)	No. of alleles	Frequency (size)
W3	6	0.250(248,254)	5	0.393(242)	3	0.500(234)	6	0.300(234)	3	0.500(248)	5	0.357(234)
W5	4	0.333(128)	4	0.273(122,126,128)	3	0.500(126)	4	0.375(116)	4	0.333(126,137)	5	0.350(137)
W8	4	0.333(191,197)	3	0.500(191)	—	0.000	3	0.500(191)	2	0.500(187,191)	3	0.500(191)
W29	4	0.357(149,152)	3	0.500(149)	2	0.500(149,152)	3	0.500(147)	2	0.500(147,149)	3	0.500(147)
W31	5	0.250(196)	5	0.385(192)	3	0.500(196)	4	0.417(196)	2	0.500(194,196)	3	0.500(196)
W33	8	0.250(190)	5	0.375(190)	2	0.500(166,172)	7	0.286(177)	2	0.500(160,166)	4	0.389(177)
W35	3	0.500(194)	5	0.429(190)	—	0.000	4	0.357(194)	5	0.333(215)	3	0.500(194)
W41	2	0.500(150,154)	3	0.417(154)	—	0.000	2	0.500(150,154)	3	0.500(150)	2	0.500(150,154)
W46	4	0.313(297)	4	0.500(297)	—	0.000	3	0.375(299)	2	0.500(299,334)	4	0.400(297,334)
W82	3	0.500(178)	3	0.500(178)	2	0.500(175,178)	2	0.500(175,178)	—	0.000	2	0.500(173,178)
W83	5	0.350(180)	6	0.357(180)	3	0.500(130)	4	0.300(175,180)	3	0.500(130)	2	0.500(175,180)
W116	4	0.450(264)	4	0.429(228,264)	2	0.500(261,264)	3	0.500(266)	—	0.000	4	0.417(264)
W126	3	0.500(282)	3	0.400(278)	2	0.500(278,282)	2	0.500(278,282)	3	0.500(282)	2	0.500(278,282)
W132	5	0.357(195)	5	0.375(166)	—	0.000	4	0.333(160,166)	2	0.500(189,195)	5	0.375(172)

**Table 6. F-Statistics and gene flow for all loci**

Locus	F index and gene flow			
	$F_{IS}$	$F_{ST}$	$F_{IT}$	Nm
W3	-0.2723	0.0778	-0.1732	2.9634
W5	-0.3060	0.0390	-0.2551	6.1603
W8	-0.5229	0.0365	-0.4673	6.5993
W29	-0.4577	0.0208	-0.4274	11.7692
W31	-0.3215	0.0288	-0.2834	8.4306
W33	-0.2346	0.0586	-0.1622	4.0162
W35	-0.3875	0.0687	-0.2922	3.3890
W41	-0.7419	0.1642	-0.4559	1.2725
W46	-0.3876	0.0616	-0.3021	3.8084
W82	-0.7188	0.0945	-0.5562	2.3955
W83	-0.3672	0.1128	-0.2129	1.9663
W116	-0.5151	0.1026	-0.3596	2.1866
W126	-0.5549	0.0023	-0.5513	108.4457
W132	-0.3088	0.0751	-0.2105	3.0789

**Table 7. HWE test (P-values) for each locus with each population**

Locus	HWE for loci in each population (numbers of individuals)					
	BJ(15)	BD(20)	QHD(7)	SY(10)	TJ(6)	XA(10)
W3	0.1876	0.0165	0.4000	0.1588	0.6667	0.1099
W5	0.0393	0.0685	0.6667	0.1051	0.6667	0.0894
W8	0.6667	0.0373	0.0000	0.2286	0.0000	0.0693
W29	0.1332	0.2286	0.6667	0.6667	0.0000	0.6667
W31	0.2855	0.0248	0.4000	0.1847	0.6667	0.4000
W33	0.3947	0.1448	0.0000	0.3915	0.0000	0.0606
W35	0.2286	0.0039	0.0000	0.1758	0.7968	0.4000
W41	0.4000	0.0693	0.0000	0.0000	0.6667	0.6667
W46	0.4539	0.0199	0.0000	0.2286	0.6667	0.2286
W82	0.0199	0.0055	0.6667	0.2286	0.0000	0.4000
W83	0.0763	0.0227	0.4000	0.3937	0.1270	0.4000
W116	0.0172	0.0693	0.0000	0.1270	0.0000	0.1616
W126	0.2286	0.0277	0.0000	0.6667	0.4000	0.4000
W132	0.1850	0.0235	0.0000	0.6667	0.0000	0.5106

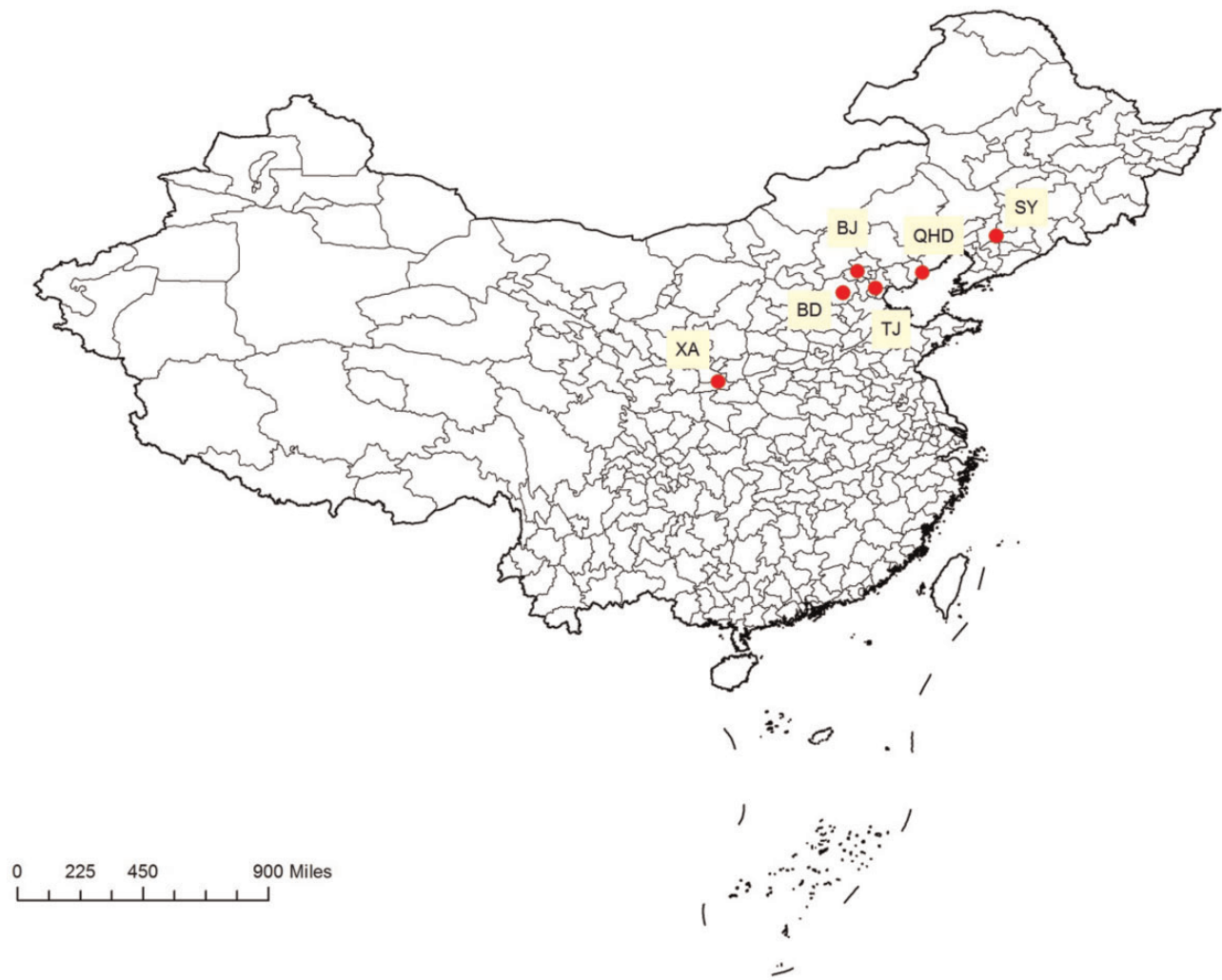
Microsatellite-containing sequences were captured using four different 5'-biotinylated probes, (AC)<sub>15</sub>, (AG)<sub>15</sub>, (AGAT)<sub>8</sub>, and (ACG)<sub>12</sub>. Each probe was used to hybridize a sequence size. Bead enrichment for microsatellite-containing DNA was performed. From the hybridized adapter-ligated genomic DNA plus probe mixture, the DNA fragments with microsatellite repeats were captured using Streptavidin MagneSphere Paramagnetic Particles (Promega, Madison, WI), which were separated magnetically from the hybridization mixture (Paetkau 1999). The hybridization library was amplified again using adaptor-specific primers and purified using a PCR Purification Kit (Tiangen, Beijing, China).

The cleaned DNA fragments were ligated into the pEASY-T1 vector using the pEASY-T1 cloning kit (Promega, Madison, WI), then transformed into Trans10 Chemically Competent Cells (TrashGen, Beijing, China). Individual clones were screened for the presence of a microsatellite by PCR with the universal primer M13 (-21) (5'-TGTTAAACGACGCCAGT-3') in a 10 µl reaction mixture, with PCR conditions as follows: 95°C denaturation for 5 min; then 35 cycles at 94°C for 30 s, annealing at 57°C for 30 s, 72°C for 30 s; and final extension at 72°C for 12 min. The PCR product was electrophoresed in 1.2% agarose gels, and clones over 200 bp were bidirectionally sequenced using an ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster, CA).

Sequences were analyzed by the DNAMAN software version 6.0 and the vector sequence removed using the Vector Search and Trim

tool in Sequin on the Genbank website (<http://www.ncbi.nlm.nih.gov/tools/vecscreen>) for alignment. Primers for sequences containing simple sequence repeats (SSRs) were designed using Primer version 5.0 (Lalitha 2000). All primer pairs were assayed in individual PCR utilizing fluorescently labeled M13-tailed primers: the forward primer of each pair added a universal M13(-21) tail to the 5'-end (Schuelke 2000). The 15 µl volume PCR mix contained 5 pmol reverse primer and forward primer, 4 pmol TP-M13(-21) primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 0.5 U DNA polymerase (KAPA, Wilmington, MA), and 10 ng template DNA. PCR amplification was performed as follows: 95°C for 5 min; then 10 cycles at 94°C for 30 s, 63 °C (-1°C/cycle) for 45 s, 72°C for 45 s; followed by 32 cycles at 94 °C for 30 s, 53°C for 45 s, 72 °C for 45 s; and a final extension at 72°C for 12 min. The PCR product was electrophoresed in 1.2% agarose gels, and 16 primer pairs showed clear bands and expected sizes.

**Loci Polymorphism Analysis.** Sixteen primers were tested in 68 adult individuals who emerged from rolled host leaves originally collected in Beijing (15 individuals), Tianjin (6 individuals), Baoding (20 individuals), and Qinhuangdao (7 individuals), Hebei province; Xian (10 individuals), Shaanxi province and Shenyang (10 individuals), and Liaoning province (Table 1). Genomic DNA was extracted using the cetyltrimethyl ammonium bromide method (Doyle and Doyle 1987). Microsatellite PCRs were conducted in a final volume of 15 µl containing 1 µl (10 ng/µl) of genomic DNA, 0.3 µm microsatellite-specific



**Fig. 1.** Sampling locations of six populations.

primer pair with M13 tail, 1.5 mM  $MgCl_2$ , 0.2 mM dNTP, 1 U Taq DNA polymerase, and  $1 \times$  Taq buffer (KAPA, Wilmington, MA). PCR conditions were as follows:  $95^\circ C$  for 10 min; 35 cycles of  $95^\circ C$  for 30 s,  $56^\circ C$  for 30 s, and  $72^\circ C$  for 30 s; followed by 10 cycles of  $94^\circ C$  for 30 s,  $53^\circ C$  for 30 s, and  $72^\circ C$  for 30 s; and final followed at  $72^\circ C$  for 7 min. PCR products were tested for polymorphism using an ABI PRISM 3730 Genetic Analyzer and the results analyzed using the GeneMapper software version 4.0 (Applied Biosystems, Foster, CA). The numbers of alleles per locus, observed and expected heterozygosities, and deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were examined using GenePop software version 4.3 (Rousset 2008).

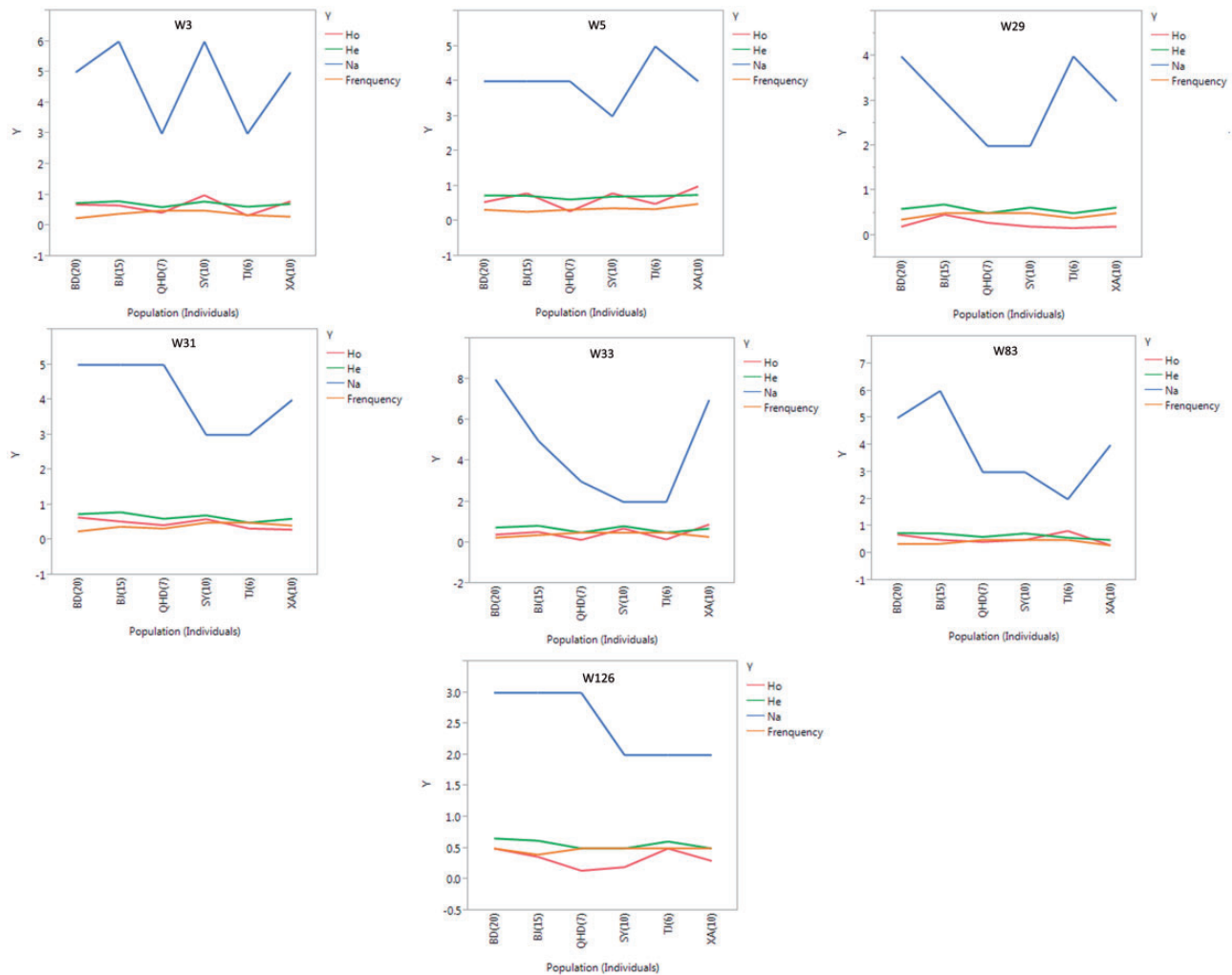
## Results and Discussion

Out of the hundreds of clones, 35 included repeats of varying lengths. Of these 35, 16 microsatellite loci were selected, and primers were designed using Primer version 5.0 (Lalitha 2000). Of the 16 loci studied, the majority contained single dinucleotide repeat motifs (68.5%), followed by double dinucleotide (25%), and higher (6.7%) repeat motifs. Dinucleotide repeats mostly consisted of AC (32.5%) repeats, followed by TC repeats (22.5%). The significantly higher percentage of dinucleotide repeats found was in contrast to other SSRs discovered in the family Cecidomyiidae (Khemakhem et al. 2006, 2012; Pfunder and Frey 2006; Hinomoto et al. 2012; Amouroux et al. 2013).

As a result, 14 loci were found to be polymorphic, with the observed number of alleles per locus ranging from 3 to 10, and the observed and

expected heterozygosity varying evenly from 0.2667 to 0.6548 and 0.5388 to 0.7148, respectively (Table 2). For each population, the observed and expected heterozygosity ranged from 0.1429 to 1.000 and 0.5 to 0.805, respectively (Tables 3 and 4). The frequency of alleles varied in different populations despite the multiple alleles. The allele frequency of the predominant allele for each population varied from 0.25 to 0.5 (Table 5). The structure of a subdivided population was also analyzed using  $F$ -statistics (Table 6). All loci had negative  $F_{IS}$  values, indicating heterozygote excess (outbreeding) in each locus with six populations.  $F_{ST}$  values up to 0.05 suggested negligible genetic differentiation within the population, as observed for locus W5, W8, W29, W31, and W126. The number of migrants per subpopulation ( $N_m$ ) reached a maximum of 108.4457 for locus 126.

$P$ -value deviations from the expected HWE were evaluated using the Markov chain algorithm (10,000 steps) for each locus and for all six populations. Significant deviation from HWE was observed for W5 ( $P = 0.039$ ), W82 ( $P = 0.020$ ), and W116 ( $P = 0.017$ ) in the population of Beijing and for W3 ( $P = 0.014$ ), W8 ( $P = 0.037$ ), W31 ( $P = 0.025$ ), W35 ( $P = 0.006$ ), W46 ( $P = 0.020$ ), W82 ( $P = 0.006$ ), W83 ( $p = 0.028$ ), W126 ( $P = 0.028$ ), and W132 ( $P = 0.020$ ) in the population of Baoding (Table 7). Genotypic LD was tested between all pairs of loci across populations based on a permutation procedure ( $P < 0.05$ ) in the same program (10,000 permutations). A significant LD was detected between three pairs of loci ( $P = 0.049$ , W5/W83;  $P = 0.017$ , W5/W116;  $P = 0.004$ , W83/W116) in Beijing and two others ( $P = 0.026$ , W5/W132;  $P < 0.01$ , W31/W35) in Baoding.



**Fig. 2.** The relationship between populations and observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ), number of alleles ( $N_a$ ), frequency of most frequent alleles in each locus.

Here, a total of 68 individuals of six populations were used to test the new loci, but the number of individuals of each population varied from 6 to 20 (Fig. 1). As a result, some data are missing in certain populations, especially ones with a low number of individuals, as listed in Tables 2–4, with a maximum of 57% data missing (Table 6) in the Qinhuangdao population (7 individuals). Fortunately, however, the data on populations with low numbers of individuals did not deviate seriously from the others, as shown in Fig. 2, which includes seven loci without missing examples analyzed by JMP Pro software version 11: observed heterozygosity was clearly reduced in small populations in loci W3, W5, W31, and W33, but expected heterozygosity ( $H_e$ ) was reduced slightly in all loci pictured except W126. However, alleles number significantly changed in each population even in the same locus, and frequency seem to be unrelated to the number of individuals in the population.

Those new microsatellites exhibit moderate to high polymorphism with 3–10 alleles per locus and population, and the loci in each population exhibit distinctly different characters, which indicates that all of these markers can be used in the analysis of population genetic diversity and structure.

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