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Isolation and characterization of eight microsatellite loci from *Lycorma delicatula* (White) (Hemiptera: Fulgoridae) for population genetic analysis in Korea

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Abstract *Lycorma delicatula* (White) is native to China but is becoming an important insect pest in Korea. Polymorphic DNA markers like microsatellites are widely used for characterizing dispersal patterns and capacity of invasive insect pests which can contribute to designing effective management of the species. To facilitate such population genetic studies of *L. delicatula* in Korea, we isolated and characterized eight microsatellite loci for *L. delicatula* using a hybridization-biotin enrichment method. We further used these novel microsatellite loci to determine population genetic parameters for 33 *L. delicatula* specimens collected from Cheonan, South Korea where outbreaks of this species were first reported in Korea. The number of alleles per locus ranged from three to ten, with an average of 6.25. The mean expected (H_E) and observed heterozygosities (H_O) were 0.575 and 0.626, respectively. The eight loci showed no deviation from Hardy–Weinberg equilibrium according to the adjusted significance threshold ($P = 0.00625$), and there was no linkage disequilibrium between each pair of these eight markers. Bayesian cluster analysis using the program structure revealed no evidence of genetic structuring in *L. delicatula* samples from Cheonan. These new microsatellite markers will be widely applicable to future ecological genetic studies of *L. delicatula*, including assessment of the level of gene flow and genetic connectivity among populations that are necessary for effective management and monitoring of the species.

Keywords *Lycorma delicatula* · Invasive species · Microsatellite · Population genetics · Genetic structure

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Introduction

Lycorma delicatula (White) (Hemiptera: Fulgoridae), first reported to have invaded Korea in 2004 based on the collection of a few adults at Cheonan [1], has became an important insect pest of some fruit trees, especially grapevines, after dispersing widely throughout South Korea. In Korea, *L. delicatula* has one generation per year. The first instar nymphs appear in May and then molts four times, becoming adults in July. After mating and ovipositing, *Lycorma delicatula* adults dies before winter. Eggs are the overwintering stage. It feeds on a wide range of host plants including *Vitis vinifera* L. (Vitaceae) and *Ailanthus altissima* Swingle (Simaroubaceae) and it has different host plant preferences in the nymph and adult stages [2].

Lycorma delicatula was first reported in China in 1939 [3], establishing successful colonies in Shanxi, Shandong and Hubei provinces. The species subsequently spread to adjacent countries, including India and Vietnam [4]. Although the geographic source of *L. delicatula* introduction to Korea remains unclear, its establishment may be associated with global climate change and increasing winter temperatures that allow it to successfully overwinter [5].

In the recent past, many researchers have tried to trace the origins of migratory invasive insects by studying their long distance dispersal behavior. Mark, release, and recapture methods have been widely used to determine the movement pathways and abilities of insect species [6, 7]. These

methods, however, have the disadvantages of high cost and low efficiency to detect long-distance dispersal events.

To understand the invasion ecology of *L. delicatula* in Korea, it is important to determine the geographical location source populations and to understand patterns of dispersal and gene flow in regions where outbreaks of the species have been reported. Highly polymorphic DNA markers such as microsatellites have been successfully used to characterize the genetic structure and level of genetic diversity among populations of migratory insects [8, 18–20]. In this study, we report the isolation and characterization of eight polymorphic loci from *L. delicatula* and describe the genetic diversity of the *L. delicatula* population in Cheonan, Korea.

Materials and methods

Microsatellite marker development

Polymorphic microsatellites were isolated from *L. delicatula* genomic DNA based on the biotin-enrichment methods described by Ronald et al. [9] and Kim and Sappington [10]. Genomic DNA was extracted using a Puregene Core Kit (QIAGEN, Germany). Extracted genomic DNA was digested with *Nde*II (Promega, USA), and fragments larger than 400 bp were screened using Chroma Spin-400 columns (Clontech, USA). One microgram of the *Nde*II linkers EP-1 (CCC CCA CCT CCT GCC CAT CAT AAA AAA TC) and EP-2 (GAT CGA TTT TTT ATG ATG GGC AGG AGG TGG GGG, 5′-phosphorylated, for *Nde*II), described in Ronald et al. [9], were ligated to DNA fragments by adding 20 µl of T4 DNA ligase (Promega, USA). These attached linkers provide a priming site for EP-3 (CCC CCA CCT CCT GCC ATC AT) in the initial polymerase chain reaction (PCR) amplification. PCR reaction mixtures contained 1× PCR buffer, 2 µM $MgCl_2$, 0.2 µM each dNTP, 1.6 µM EP-3 primer, 1.5 U i-Star *Taq* DNA polymerase (iNtRON Inc. Korea), and 20 µg of genomic DNA template in a final volume of 30 µl. PCR was performed with an initial denaturation for 2 min at 94°C, followed by 30 cycles of 1 min at 65°C, 30 s at 72°C, 2 min at 72°C; this was followed by a final extension step of 5 min at 72°C. Unattached residual linkers less than 100 bp in size were discarded by running the completed PCR reactions through Microcon 100 columns (Millipore Corporation, USA). One microliter each of biotinylated capture probe, 5′biotin $(CA)_{15}$, 5′biotin $(CT)_{15}$, and 5′biotin $(AGC)_7$ was annealed to 10 µl of linker-ligated DNA in 89 µl of 5× SSC, and then this mixture was heated at 95°C for 10 min, cooled on ice for 30 s, and incubated for 5 min at room temperature. To bind the biotinylated probes, we added 100 µl of washed magnetic beads (1 mg/µl) to the DNA, followed by a 15 min incubation at room temperature. Residual unattached fragments were removed through three washes with 200 µl 2× SSC at room temperature and then three washes with 200 µl of 1× SSC at an optimized temperature for 3 min (65°C for $(CA)_{15}$, 61°C for $(CT)_{15}$, and 67°C for $(AGC)_7$). The DNA was eluted from the beads into 50 µl of water after incubation for 5 min at 95°C. The repeat sequences were amplified with the EP-3 primer. PCR products were ligated into the pGEM®-T Vector (50 ng/µl) and transformed into *Escherichia coli* JM109 competent cells (Promega, USA). Positive clones were screened with M13 forward and reverse primers using the method described in Schuelke [11]. Amplification of the presumptive microsatellite markers was conducted in a 10 µl reaction volume containing 2.5 µM $MgCl_2$, 0.2 µM dNTPs, 1 µM each primer (M13 forward, M13 reverse, one of the internal repeat primers; $(CA)_{12}$, $(CT)_{12}$, $(AGC)_6$), 0.25 U of i-Star *Taq* DNA polymerase (iNtRON Inc., Korea), and 10–50 ng of template. The PCR was performed under the following conditions: initial denaturation for 5 min at 96°C, followed by 35 cycles of 96°C for 1 min, 50°C for 1 min, and 72°C for 1 min; a final extension step was performed at 72°C for 1 min. PCR products were electrophoresed on agarose gels to select repeated sequence inserts (products with a smeared band pattern), and these products were sequenced using either a forward or reverse M13 primer. Primers for microsatellite amplification were designed using the program Primer 3 [12]. To screen for microsatellite markers, the primers used to PCR amplify template genomic DNA from 3 to 4 individuals from each of four geographic locations: Gwang-Ju (N35°8′33″ E126°54′46″), Yeong-Gwang (N35° 18′21″ E126°32′49.9″), Cheonan (N36°53′50.2″ E127° 10′55.6″), and Suwon (N37°16′43.1″ E126°58′53.5″). PCR reaction mixtures contained 6.15 distilled water, 1.0 µM $MgCl_2$, 0.8 µM each dNTP, and 0.05 U of i-Star *Taq* DNA polymerase (iNtRON Inc., Korea). The PCR was performed under the following conditions: initial denaturation for 5 min at 94°C, followed by 20 cycles of 94°C for 20 s, annealing at 60–50°C decreased by 0.5°C per cycle, 72°C for 20 s, followed by 20 cycles of 94°C for 20 s, 50°C for 20 s, 72°C for 20 s, a final extension was performed at 72°C for 7 min. PCR products were electrophoresed on agarose gels to verify the presence of PCR bands, indicating successful amplification of genomic template across all samples tested. The Forward primer of selected primer sets was labeled with the fluorescent dye for further study.

DNA samples and genotyping

All *L. delicatula* samples (thorax, $n = 33$) were collected from Cheonan, Korea in 2010. All of the samples were frozen at −20°C until used for DNA extraction. Genomic DNA was extracted from the thoraces of individuals using the Puregene Core Kit (QIAGEN, Germany).

To genotype the samples, genomic DNA was amplified using the microsatellite primers following the touchdown PCR amplification profile mentioned above with only the change of annealing at 65–55°C. PCR reaction mixtures contained 1.0 μM $MgCl_2$, 0.8 μM each dNTP, 0.05 U of i-Star *Taq* DNA polymerase (iNtRON Inc., Korea), 0.5 μM each of the fluorescently labeled forward primer and unlabeled reverse primer, and 10–50 ng of template DNA. For fluorescent detection, the forward primer of each primer pair was labeled with either Hex, 6Fam or Tamra dyes. Amplified PCR products (alleles) were separated and electrophoresed on an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems Inc., USA) using the GENESCAN-500 [Rox] size standard, and the genotype data were analyzed using GeneMapper version 3.7 (Applied Biosystems Inc., USA).

Data analysis

Three measures of genetic diversity, the number of alleles (A) per locus, observed heterozygosity (H_O), and expected heterozygosity (H_E), were calculated using the program GenAlEx version 6.1 [13]. F_{IS}, the inbreeding coefficient at each locus, represents the reduction of H_O from heterozygosity expected under Hardy–Weinberg equilibrium (HWE). A significant difference between H_O and H_E results in a significant F_{IS} value, and may indicate non-random mating, the presence of null alleles, the Wahlund effect or some other anomaly. Deviations from HWE and linkage equilibrium between loci were tested using GENEPOP version 4.0.10 [14]. The sequential Bonferroni correction was applied to the significance level for multiple comparisons [15]. The plausible occurrence of null alleles was tested using the program MICROCHECKER [16]. The presence of null alleles is expected when excess homozygotes are evenly distributed across all alleles at a locus. The population structure of *L. delicatula* in Cheonan was investigated via structure simulation using the Bayesian clustering procedure implemented in Structure 2.3.2 [17]. Assuming that the data could be represented as K separate clusters, the log posterior probability of the data for a given K, Ln Pr(X/K), was generated for each of the five STRUCTURE runs at K values of 1–5 for the *L. delicatula* sampled. The initial burn-in period was 100,000, followed by 200,000 replications. The mean posterior probability for each K was also calculated. The software was further used for the graphical display of Q-matrix output data from the analysis.

Results

A total of 541 positive (white) colonies—214 for the CA repeat probe, 177 for the AGC repeat probe, and 150 for the CT repeat probe—were obtained after microsatellite cloning. Of these, a total of 195 (36%) colonies [99-CA (46%), 49-AGC (28%) and 47-CT (31%)] were considered to have inserts of repeat units as determined by a smearing band pattern on agarose gels. Out of 195 colonies, 150 were sequenced with the M13 forward primer and at least 96 (64%) were confirmed to have repeat sequences. We selected 36 unique sequences (21 CA, 12 AGC, and three CT repeat sequences) with more than five repeats for each sequence unit to design primers for PCR amplification. Of these, 20 primer pairs (12 CA- and eight AGC-targeting primer pairs) produced positive PCR results based on the test panel of four *L. delicatula* DNA samples. Among these, 16 primers pairs that amplified discernable PCR products were further selected, and the forward primer of each primer pair was labeled with one of three different fluorescence dyes (6Fam, Hex or Tamra) for multiplex PCR analysis to reduce the time and cost of genotyping. Finally, eight polymorphic microsatellite markers for *L. delicatula* were selected. Two subsets of microsatellite markers, LD-T1, LD-T3, LD-D4, LD-5 and LD-D1, LD-D2, LD-T2, LD-D3 were successfully amenable to multiplexing conditions.

A total of 33 adult individuals collected from Cheonan were genotyped using the eight microsatellite loci developed in this study. All microsatellite markers were polymorphic, with the A per locus ranging from three to ten (mean = 6.25) (Table 1). The mean H_O and H_E values were 0.575 (0.267–0.903) and 0.626 (0.369–0.837), respectively (Table 1). The F_{IS} ranged from −0.242 in LD-T1 to 0.405 in LD-T2, with a mean of 0.103 across loci. The eight loci did not show significant deviation from Hardy–Weinberg proportions at the adjusted significance threshold ($P = 0.00625$) for multiple testing. However, when the unadjusted significant threshold ($P < 0.05$) was applied, three of eight loci, LD-D2, LD-D3, and LD-T2, showed significant deviation from HWE in the direction of heterozygote deficiency. Of these, two loci, LD-D2 and LD-T2, appeared to harbor a null allele as confirmed by the program MICROCHECKER (Oosterhout's null allele frequency; 0.188 for LD-D2, 0.163 for LD-T2). No significant linkage disequilibrium was found among any of the loci pairs. Structure 2.3.3 software was employed to determine the population structure of the 33 *L. delicatula* individuals sampled from Cheonan in 2010. The highest likelihood values in all runs were obtained for K = 1 (Table 2), implying that the *L. delicatula* from Cheonan constitute a single genetic population. Therefore, it is concluded that the 33 *L. delicatula* specimens collected from "Cheonan" where the outbreak of the species was reported in Korea have genetically similar background with no apparent genetic structuring among them.

Table 1 Characteristics of the eight *L. delicatula* microsatellite loci tested in 33 *L. delicatula* specimens from Cheonan, South Korea

Locus	Primer sequence (5′–3′)	Repeat motif	N	No. alleles	Size range (bp)	H_O	H_E	*P* value	F_{IS}	Genbank accession
LD-D1	F:(6FAM)-CCCAACATATGTCAGCTCCA R:CCCCTGAGTGAATTTTCCAA	CA	31	5	266–276	0.839	0.773	0.8188	−0.0692	JF913272
LD-D2	F:(6FAM)-GAAACCCAACAAATCGGAAG R: CGGTTTAGTGAGTCTTACACCAA	CA	29	10	108–142	0.517	0.837	0.0063	0.3966	JF913273
LD-D3	F:(TAMRA)-GGTCAAAACCGGTCCAGTAG R:AAGGAATCCAGAAAACCGGA	CA	30	6	122–168	0.267	0.369	0.0313	0.2938	JF913274
LD-D4	F:(6FAM)-TTAAATCATCAGCCTTATCCACT R:CGGGTAGTTCGGGGATATTT	CA	28	8	96–158	0.786	0.819	0.3188	0.0586	JF913275
LD- D5	F:(HEX)-CCTGGAGGTAGGTGATTCCA R:TTGATAGTGTTCATGAGAATGCG	CA	31	8	168–236	0.903	0.76	0.9688	−0.1724	JF913276
LD-T1	F:(6FAM)-CCGACCTCTACCACTCCTCA R:CTTGTGGCTCCGGTGTATTT	AGC	32	3	202–211	0.500	0.398	0.9563	−0.2416	JF913277
LD-T2	F:(6TAM)-GAATCCAGACCTCTGCTGGT R:CGGGTAGTTCGGGGATATTT	AGC	31	4	181–190	0.290	0.477	0.0125	0.4053	JF913278
LD-T3	F:(TAMRA)-GCACGCGTGAGTGTTATGAT CGCGCTACACTCAACCGTA	AGC	30	6	155–218	0.500	0.576	0.1688	0.1487	JF913279
Across loci				6.25		0.575	0.626	0.4102	0.1025	

Microsatellite primer sequences with fluorescent labeled dyes, repeat motifs, number of individuals (*N*), number of alleles (*A*), size of PCR products in base pairs (bp), expected heterozygosity (H_E), observed heterozygosity (H_O), *P* value of the HW test, inbreeding (F_{IS}) and GenBank accession numbers are shown

HW test Hardy–Weinberg exact test (Raymond and Rousset 1995) with a sequential Bonferroni correction ($P = 0.00625$)

Table 2 Likelihood values, Ln Pr(X/K), from Structure analyses (Pritchard et al. 2000) to determine the genetic structure of the 33 *L. delicatula* specimens sampled from Cheonan in 2010

Run	K = 1	K = 2	K = 3	K = 4	K = 5
1	−655.6	−694.8	−679.3	−727.2	−771.2
2	−655.9	−656.9	−670.4	−742.8	−728.8
3	−655.7	−695.6	−656.5	−752.2	−767.7
4	−655.7	−696.2	−690.7	−697	−748.1
5	−655.9	−657	−698.1	−717	−718.7
Mean	−655.76	−680.1	−679	−727.24	−746.9

The highest mean likelihood value (over five runs at 200,000 replications per run) was for K = 1 indicating that the sample of individuals most likely represents a single genetic population

Discussions

Eight polymorphic microsatellite markers developed in this study have been successfully applied to obtain preliminary population genetics parameters for 33 *L. delicatula* specimens from a location in Korea. Although three of eight loci showed significant deviation from HW proportion at the unadjusted significant threshold with the plausible occurrence of a null allele at two of these loci, these markers can still be used for population genetics studies if analytical methods are used to correct for null alleles [8, 21]. These new microsatellites will facilitate the study of the population and ecological genetics of *L. delicatula* and closely related species in Korea. For example, these microsatellites will be available to trace the unknown origin of this species in Korea using a population assignment strategy successfully employed to identify the origin of cotton boll weevils captured in eradication zones of North America [18, 19]. These markers also could be applicable to elucidate invasion routes of the insects from China to Korea using the Approximate Bayesian Computation method as was done to reveal frequent and ongoing introductions of western corn rootworm from North America to Europe [20]. In addition, these markers will be useful to characterize the dispersal patterns, genetic connectivity among populations, effective population size, population dimensions, and spatial and temporal variation among geographic populations of *L. delicatula* in Korea and adjacent countries, as has been done with European corn borer [8]. Successful outcomes of these population and ecological genetics studies eventually will contribute to designing effective strategies for controlling and monitoring strategies for this species.

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