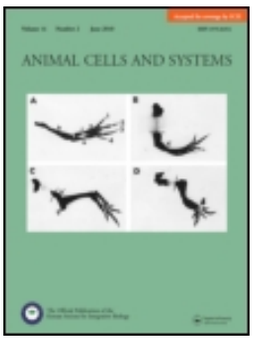

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Development and characterization of 15 microsatellite loci from *Lycorma delicatula* (Hemiptera: Fulgoridae)

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Lycorma delicatula (White 1845), which has been recently introduced into Korea, is a notorious pest on grapes. This invasive insect has rapidly spread throughout central and southern Korea. To date, we have no behavioral or population genetics information, such as invasion routes and subsequent dispersal rates in Korea, to help understand and control populations of *L. delicatula*. Here, we have developed 15 novel microsatellite loci for *L. delicatula*. The isolated loci were polymorphic, with 2 to 19 alleles in 42 individuals from a single population in Korea. The analyses revealed that all 42 individuals had different multilocus genotypes with heterozygosity ranging from 0.214 to 0.866. Eleven of the 15 loci did not deviate significantly from Hardy–Weinberg equilibrium. The isolated markers will facilitate population genetic studies of *L. delicatula*.

Keywords: insect pest; invasive species; polymorphic microsatellite; population genetics

Introduction

Lycorma delicatula has recently been introduced into Korea, where it has become a serious pest of grapes (Han et al. 2008; Lee et al. 2011). This species arrived suddenly in the country, where it now seriously damages grapevines and brings more economic losses each year (Han et al. 2008; Shin et al. 2010). Although the authenticity of previous Korean records of this species has been controversial, it now appears that the historical confusion derived from Doi's misidentification of another fulgorid, *Limois emelianovi* (Doi 1932a, b; Han et al. 2008). Continuing outbreaks of *L. delicatula* over the past several years also corroborate the argument that *L. delicatula* has not been present in Korea until the 2000s (Han et al. 2008). It obviously corresponds to the invasion history of other exotic insects such as the pine needle gall midge, *Thecodiplosis japonensis*, and the yellow locust midge, *Obolodiplosis robiniae*, both of which increased explosively immediately after their introductions into Korea (Jung et al. 1997; Woo et al. 2003).

L. delicatula causes serious damage to the host plant by sucking phloem sap and producing honeydew, causing sooty mold disease on leaves (Lee et al. 2009). Because disturbing the photosynthesis eventually reduces the quality of grapes, it is necessary to control this species rigorously in vineyards using chemical pesticides (Shin et al. 2010). According to a recent study (Park et al. 2009), this species can utilize 41 host plants, including 38 woody and four herbaceous species. It can, therefore, maintain very high population densities in the natural habitats where it is established. In addition, its main overwintering host, the native Chinese tree *Ailanthus altissima*, is already widely distributed in Korea along roadsides, which allows *L. delicatula* to proliferate everywhere (Lee et al. 2011). Based on distribution patterns observed to date, it is unlikely that it will be possible to eradicate this species in order to protect the grapevines. Instead, we must understand the biology of *L. delicatula* to control it (Choi et al. 2011).

As international trade expands, the probability of continent-to-continent or nation-to-nation introduction of exotic species by chance increases (Dalmon et al. 2008; Lozier et al. 2009). The outbreaks that follow biological invasions generally cause serious damage in the introduced regions (Miller et al. 2005). An important part of dealing with invasive species is to

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find the source (origin) locality (Guillemaud et al. 2010). This knowledge supports control strategies such as introduction of natural enemies (Torchin et al. 2003). Recently, microsatellite markers have been used in many studies aiming to clarify the origins of invasive species (Miller et al. 2005; Guillemaud et al. 2010). In particular, using multilocus genotypes based on a combination of multiple loci, researchers are able to precisely identify the migration route between source and invasive populations (Estoup and Guillemaud 2010).

As an unwelcome exotic insect pest, *L. delicatula* has rapidly spread throughout central and southern Korea over the past five years (Park et al. 2009). However, it is still not known from where the invasive population originated. Due to insufficient biological information about *L. delicatula*, its dispersal behavior is still unknown. A better understanding of its population genetic structures is also important for effective control. Conventional molecular markers such as the mitochondrial COI barcode do not seem relevant to resolving the *L. delicatula* populations rapidly dispersing in Korea (Han et al. 2008). Hence, to provide a tool for studying such factors as source of invasion, dispersal, and population genetics, we here report 15 polymorphic microsatellite loci.

Materials and methods

Genomic DNA isolation, enzyme digestion, and size fractionation

Genomic DNA was extracted using DNAzol® Genomic DNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Pooled genomic DNA was digested separately with *Nde*II (GATC) restriction enzyme, and DNA fragments between 250 and 600 bp were purified using Chroma spin-400 columns (Clontech, Mountain View, CA, USA).

Biotin enrichment strategy for isolating microsatellite loci

We followed the biotin enrichment methods of Kijas et al. (1994) and Sarno et al. (2000) with slight modifications as described by Kim and Sappington (2004). To find microsatellite loci, we constructed a genomic library with *Nde*II. The linker sequences, EP1 and EP2, having an *Nde*II compatible end described by Sarno et al. (2000) were incubated with the DNA digested with the respective restriction enzymes. Excess linker was removed through washing twice in Amicon® ultra-0.5 mL (Millipore, Billerica, MA, USA). Polymerase chain reaction (PCR) amplification was carried out in 30 μl reaction mixtures containing 5 U of Advantage® 2 Polymerase (Clontech), 10 × PCR buffer, 2.5 mM dNTP mix, 20 μM of the EP3 primer (designed by Sarno et al. (2000)), and 0.05 μg of DNA template. The biotinylated capture probe was annealed to the DNA fragments linked with an *Nde*II compatible end. We used four different types of biotinlayted dinucleotide or tri-nucleotide repeat sequences for capture hybridization: $(CA)_{15}$, $(CT)_{15}$, $(AGC)_7$, and $(ATT)_7$. After incubation with streptavidin-coated magnetic beads (Promega, Madison, WI, USA), the captured fragments were washed with 2 × SSC (150 mM NaCl, 15 mM sodium citrate pH 7.0) at an optimized temperature for the oligo repeat sequences (65°C for $(CA)_{15}$, 61°C for $(CT)_{15}$, 67°C for $(AGC)_7$, 50°C for $(ATT)_7$). The DNA was eluted from the beads after incubation for 5 min at 95°C and repeat with the elution with water. PCR amplification of DNA was performed with the EP-3 primer again. The PCR product was run on a 1.2% TAE agarose gel with low-range DNA ladder and the desired size fragment was purified.

Cloning and screening of microsatellite loci

PCR products were purified and ligated into pGEM®-T easy vector (Promega) following the manufacturer's protocol, and the plasmid transformed into DH5α chemically competent *Escherichia coli* (Enzynomics, Daejeon, Korea). Following transformation, white colonies were picked on the LB agar with Ampicillin (100 mg/ml), X-gal (25 mg/ml), IPTG, and then subjected to colony PCR amplification. All the selected colonies were preserved in a deep freezer at −70 °C, and an aliquot of each colony was prepared for the next step. Colony samples were screened to confirm the simple sequence repeats (SSR) motif using the PCR procedure of Wang et al. (2007). PCR products were amplified using AccuPower® PCR PreMix (Bioneer, Daejeon, Korea) in 20 μl reaction mixtures containing 0.8 μM forward & reverse M13 primers, 0.4 μM of each SSR motif primer, and 0.05 μg of DNA template. PCR was performed using a GS482 thermo-cycler (Gene Technologies, Essex) according to the following procedure: initial denaturation at 95°C for 5 min, followed by 34 cycles of 95°C for 30 sec; annealing at 56 °C for 50 sec; extension at 72°C for 30–60 sec, and a final extension at 72°C for 5 min. PCR products were visualized by electrophoresis on a 1.5% agarose gel. PCR products were sequenced directly on the ABI PRISM 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and aligned using MEGA 4.0 (Kumar et al. 2008) to check for the possible existence of duplicate sequences. The SSR motif on each microsatellite sequence was searched and analyzed

using FastPCR 6.1.5.beta 3 (Kalendar et al. 2009). To investigate possible matches with previously reported coding or non-coding regions, sequences were compared to the other reported sequences in GenBank using BLASTN 2.2.24+ (Zhang et al. 2000).

Assessment of applicability for microsatellite loci

Pairs of primers on the sequences applicable for each microsatellite were designed using PRIMER 3 (Rozen and Skaletsky 2000). Then, microsatellite loci were finally characterized using 42 individuals collected from a single population in Cheonan, Chungnam Prov., Korea. PCR was performed using GeneAll® *Taq* DNA Polymerase Premix (GeneAll, Seoul, Korea) in 10 μl reaction mixtures containing 0.5 μM forward & reverse primers and 0.05 μg of DNA template. After amplification tests, the forward primers were labeled with a fluorescent dye (6-FAM or HEX). Automated fluorescent fragment analyses were performed on the ABI PRISM 377 Genetic Analyser (Applied Biosystems), and allele sizes of PCR products were calibrated using the molecular size marker, ROX labeled-size standard (GenScan™ ROX 500, Applied Biosystems). Raw data on each fluorescent DNA product were analyzed using GeneMapper® version 3.7 (Applied Biosystems).

Statistical analysis

The number of alleles, observed and expected heterozygosity values, test for Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium among loci were estimated using GENEPOP 4.0.7 (Raymond and Rousset 1995) and ARLEQUIN 3.0 (Excoffier et al. 2005). Checking for the existence of null alleles was performed using MICRO-CHECKER 2.2.3 (Oosterhout et al. 2004). Allelic frequency, multilocus genotype, and principal components were analyzed using GeneAlEx 6.3 (Peakall and Smouse 2006).

Results

In all, 650 positive colonies were obtained from the genomic DNA libraries enriched with the oligo repeat sequences $(CA)_{15}$, $(CT)_{15}$, $(AGC)_7$, and $(ATT)_7$ were incubated in LB solution at room temperature for 24 hours. From these, 351 colonies were screened to confirm the SSR motif (Wang et al. 2007), and 330 (94%) of these appeared to have the SSR motif. After screening, products were directly sequenced, and alignment of these sequences using MEGA confirmed that there were no duplicate sequences among them. In addition, no sequences matched other sequences reported in GenBank. From these, we chose 37 designed pairs of primers using PRIMER 3 (Rozen and Skaletsky 2000). After the tests of amplification and visualization by gel loading, the forward primers of 27 loci were labeled with a fluorescent dye (FAM or HEX). After the initial tests to evaluate the applicability and efficiency as microsatellite markers, 15 loci were chosen for further investigation. The 12 other loci were monomorphic or badly stuttering.

Variability of the 15 polymorphic microsatellites was characterized in 42 individuals of *L. delicatula* collected from a single population in Cheonan, Chungnam Prov., Korea (Table 1). We found 42 different multilocus genotypes, and the number of alleles per locus ranged from two to 19, with expected heterozygosity values ranging from 0.214 to 0.866. Allele frequencies showed that there were 45 rare alleles with frequency $< 5\%$ among a total of 100 alleles from all loci. Pairwise comparison of 105 locus pairs showed no significant evidence of linkage disequilibrium based on Fisher's method using the genotype disequilibrium option. Of the 15 loci, Lde02, Lde03, Lde05, and Lde10 displayed significant deviations from HWE ($P < 0.05$), and Lde02 and Lde05 also indicated the existence of null alleles. After subsequent tests for heterozygote excess or deficiency, these loci showed a significant deficiency of heterozygote (Rousset and Raymond 1995). The deviation from HWE might be explained by the presence of null alleles or small sample size of 42 individuals. Nevertheless, the principal components analysis based on codominant genotypic distances between individuals showed that the first two principal components (PC1 and PC2) accounting for 41% of total variance displayed widely scattered plots, which means that each individual sample could be identified separately by these multilocus genotypes based on 15 microsatellite loci (Figure 1).

Discussion

It is highly likely that *L. delicatula* was introduced from somewhere in China, but this working hypothesis has not yet been tested using a population genetics approach. In an earlier study, Korean and Chinese populations of *L. delicatula* could not be discriminated using the COI barcode region, in which their sequences were identical (Han et al. 2008). Moreover, we found no difference between sequences of two internal transcript spacers (ITS1, ITS2) in our preliminary tests (unpublished data). It seems that other nuclear or mitochondrial markers are not sufficient to resolve genetic differences at this level. Therefore, it is necessary to develop microsatellite markers to study the population genetics of *L. delicatula*.

Table 1. Characteristics of polymorphic microsatellites from *Lycorma delicatula*.

Locus	Repeat motif	Primer sequences (5′–3′)	T_{a+} (°C)	5′ Fluoro label	Size range (bp)	N_A	N_{IA}/N_{IS}	H_O	H_E	HWE *P* values	Null allele frequency	GenBank Accession no.
Lde01	$(AGC)_7$	F: TGCTGCTCAGCAAATGAATC R: GAGTCAGCTTTTGTCTTTTCTGC	56	HEX	183–194	2	42/42	0.262	0.228	0.240	0.048	HQ644424
Lde02	$(GA)_8TT(GA)_{17}$	F: AGCGTAATTATAAATATTTCTTGCTGT R: GGCATTTCCAGCACCTATTG	56	FAM	226–267	19	42/42	0.548	0.866	0*	0.171	HQ644425
Lde03	$(CAG)_9$	F: AGAGTGACCAGTTTTGGAGCA R: TCGAAACAATTCCACTTCCA	56	FAM	178–181	2	42/42	0.310	0.375	0.022*	0	HQ644426
Lde04	$(CGT)_9(GTT/GCT)_{16}$	F: GCTGATTCGGTGGTTGAAGT R: GCTCCATCCAATACCCAAAA	56	HEX	186–195	4	42/42	0.714	0.597	0.802	0.004	HQ644427
Lde05	$(CT)_{33}$	F: TCCCAATAGAAAGCGTTAAGTT R: CGGGCTGAAATAAGCACGTA	56	FAM	210–249	5	42/42	0.262	0.488	0*	0.152	HQ644428
Lde06	$(CAG)_7$	F: TACCAGCACGGTACAGCAAG R: CGGCGAATTCTCTTTCTCTG	56	FAM	143–179	4	42/42	0.452	0.458	0.167	0	HQ644429
Lde07	$(GT)_5(CT)_{19}$	F: GGTGAAGCATACCGATGTTG R: CCCAGAGGATACCTGCAAAG	56	HEX	191–211	10	42/42	0.833	0.750	0.375	0	HQ644430
Lde08	$(CA)_8$	F: GAACATGGTCAAATCACTCATCA R: GGTCCCTCCCGCTATTATTAC	56	HEX	211–213	2	42/42	0.476	0.408	0.810	0.028	HQ644431
Lde09	$(GCT)_{15}$	F: AACATGGGAGAAGTCGGTGA R: TCAGCAACAAGTCCAGCAAC	56	FAM	237–243	3	42/42	0.238	0.214	0.112	0.035	HQ644432
Lde10	$(CT)_{14}$	F: TGTCTGCATGAAATTTTTACCG R: ACCGGAGGCTAAAAAGGAAA	56	FAM	174–221	11	42/42	0.786	0.855	0.033*	0.059	HQ644433
Lde11	$(AC)_{18}$	F: CGGCAGCAGCACATAGTAAA R: TCGAATAGCAAGAAGCACCA	56	HEX	153–201	9	42/42	0.476	0.569	0.815	0	HQ644434
Lde12	$(CAG/CAA)_{22}$	F: TAACATGCAGCCTTCAGCAC R: TGGTTGATGAACGCAGTACC	56	FAM	218–233	5	42/42	0.500	0.430	0.454	0	HQ644435
Lde13	$(GCA)_7$	F: CTCTAACACCCGGATTGCTC R: GGGATGTGCGATAGAAAAGC	56	FAM	215–224	4	42/42	0.524	0.567	0.860	0.037	HQ644436
Lde14	$(GT)_{18}$	F: ACGCCCTCTCTACCTGTGTG R: GATTGAGAGGAGGGGAGAGAT	56	FAM	170–199	8	42/42	0.524	0.504	1.000	0	HQ644437
Lde15	$(CACT)_6(CT)_{19}$	F: CGGTCGTTCTTTCTCACTCA R: TTCCACAACACCGCTAAAGA	56	FAM	151–189	12	42/42	0.738	0.801	1.000	0	HQ644438

T_a, optimal annealing temperature; N_A, number of alleles; N_{IA}, number of individuals assayed; N_{IS}, number of individuals successfully genotyped; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, significance of departure from Hardy–Weinberg equilibrium; * loci that significantly deviated from HWE.

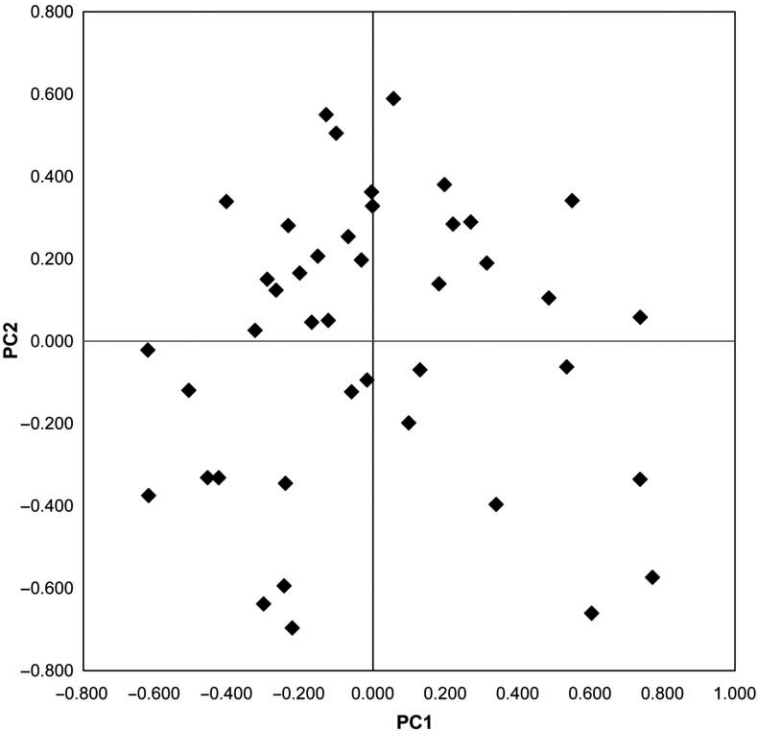

Figure 1. Plot of the principal component analysis based on the first two principal components, PC1 and PC2, for the 42 individuals of *L. delicatula*.

The biotin enrichment strategy was used in this study for developing microsatellites (Kijas et al. 1994; Sarno et al. 2000). This enrichment procedure using magnetic bead hybridization has been most widely applied for acquiring highly informative microsatellite loci in a large variety of taxa, including plants, invertebrates, and vertebrates (Zane et al. 2002; An et al. 2010). In our study, efficiency was very good, because we successfully obtained 94% colonies with the SSR motif among those screened. Although using the four genomic DNA libraries enriched with CA, CT, AGC, and ATT repeat sequences seems time- and labor-consuming, it ensured that we were able to easily select polymorphic microsatellite loci in the screening procedures. Of 15 microsatellite loci, the ten loci were especially highly polymorphic with the number of alleles per locus ranging from 4 to 19, and with expected heterozygosity values ranging from 0.430 to 0.866 (Table 1). As seen in the results, these markers are sufficiently powerful to resolve the 42 individuals collected from one single colony (Figure 1), and, hence, appropriate to trace the invasion route or dispersal even within Korean populations.

Microsatellites are particularly reliable for studying recent biological invasions (Zygouridis et al. 2009) and are powerful to detect the origin and invasion route of exotic insect species (Fonseca et al. 2010). As a representative case, Miller et al. (2005) revealed that the western corn rootworm, *Diabrotica virgifera*, was introduced into several European regions from North America through at least three independent transatlantic invasions. For other examples, Lozier et al. (2009) provided evidence for multiple invasions of the mealy plum aphid, *Hyalopterus pruni*, into North America from Europe. In addition, there is some evidence that the Asian longhorned beetle, *Anoplophora glabripennis*, in North America may be derived from China (Carter et al. 2010). Considering the results of these previous studies, we expect that the invasion route and origin of *L. delicatula* can be clarified in the near future using our 15 new microsatellite loci.

Furthermore, these microsatellite loci are the first that have been developed for the family Fulgoridae. It is hoped that studying the population genetics of this fulgorid species can contribute to other fulgorid research by providing basic biological information.

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