and (ii) PCR products were run on an ABI 3730 automated sequencer (Applied Biosystems) and genotypes were assigned using GeneMapper version 3.7 (Applied Biosystems). Of the 46 primer sets screened, 24 exhibited polymorphism.

For each polymorphic locus, we calculated observed heterozygosity, expected heterozygosity and null allele frequencies using Cervus 1.0 (Marshall *et al*. 1998). GenePop version 3.4 (Raymond & Rousset 2000) was used to test for evidence of linkage disequilibrium and deviations from Hardy–Weinberg equilibrium. The number of alleles per locus ranged from two to 13, and single locus heterozygosities ranged from 0.032 to 0.871 (Table 1). None of the 24 loci were found to be out of Hardy–Weinberg equilibrium ($P < 0.05$). Evidence of null alleles was detected at seven loci (Table 1). We tested 276 pairwise comparisons and found no evidence for genotypic linkage disequilibrium between any set of paired loci after a sequential Bonferroni correction was applied ($P < 0.00004$).

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Isolation and characterization of nine microsatellite loci in an ant-tended treehopper *Publilia concava*

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Abstract

***Publilia concava* is an eastern North American membracid commonly occurring in large but spatially patchy aggregations, primarily on the host plant *Solidago altissima*. Like other**

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myrmecophiles, *P. concava* provides sugary excretions to ants in return for the various protective, competitive or even sanitary benefits that ants provide. We developed nine microsatellite loci from *P. concava*. Mean per locus allele number was 6.78, and observed heterozygosities ranged from 0.03 to 0.850. One locus exhibited significant heterozygote deficit, possibly due to the presence of null alleles. These markers provide important tools for future spatial ecological studies in this model system for the study of mutualism.

Keywords: membracids, ants, microsatellites, mutualism, spatial ecology, *Publilia*

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The treehopper *Publilia concava* Say (Hemiptera: Membracidae), is a sap-feeding insect that forms mutualistic associations with ants. *Publilia concava* individuals commonly form large aggregations on *Solidago altissima* and are tended primarily by ants of the genera *Formica* and *Myrmica* in the northeastern USA. Treehoppers excrete a sugary honeydew rich in carbohydrates that ants use as a food source. In turn, the ants offer protection from predators and facilitate feeding (Buckley 1987; Morales 2000a).

Ant-homopteran interactions have become model systems for understanding the role of ecological variation in determining the outcome of mutualism, and studies based on *Publilia* spp. have played prominently in these discoveries (Way 1954; Bristow 1984; Buckley 1987; Morales 2000a, b, 2002; Billick & Tonkel 2003; Morales & Beal 2006). For example, spatial variation in both abiotic and biotic factors can have strong effects on ant-homopteran interactions, leading to variation in population abundances, as well as mutualism strength and specificity (Yu & Davidson 1997; Parker 1999). Indeed, the spatial distribution of *P. concava* is dependent largely upon recruitment patterns of the ants that tend them (Morales 2000a, 2000b). Since interspecific mutualisms are often characterized by patchy spatial distributions (Yu & Davidson 1997; Doebeli & Knowlton 1998; Herrera 1998; Parker 1999), it is important to develop quantitative molecular methods to study the spatial dynamics of these interactions. However, to date, no markers have been available for studying the spatial ecology of membracids.

Here we report the isolation and characterization of nine variable microsatellite markers in *P. concava*. We also demonstrate their usefulness in assessing population genetic information.

Microsatellite enriched libraries for four different repeat motifs were obtained commercially through Genetic Identification Services (hitherto referred to as GIS, www.genetic-id-services.com). These included two libraries of dinucleotide repeats and one library each of tri- and tetranucleotide repeats. High molecular weight pooled genomic DNA from several *P. concava* individuals was partially restricted with a mixture of restriction endonucleases that are known to generate blunt end cuts in restricted DNA. These were *Rsa*I, *Hae*III, *BsrB*1, *Pvu*II, *Stu*I, *Sca*I and *Eco*RV. GIS isolated fragments ranging in size from 300–750 bp from these restriction digests and ligated them with adapters (sequences available from GIS) and were magnetically captured using biotinylated capture molecules. Polymerase chain reaction (PCR) was performed on captured fragments and adapters were removed by restriction digestion with *Hin*dIII endonuclease. Resulting fragments were inserted at *Hin*dIII restriction site in pUC19 vector which were then electroporated in *Escherichia coli* strain DH5α.

Recombinant vector harbouring *E. coli* cells were grown for all four motif libraries on Luria–Bertani agar media. The cells were selected for antibiotic resistance with ampicillin and for blue-white screening with X-GAL. White or light blue clones were selected for PCR using vector primers (GIS-A and GIS-B). Resulting PCR products were sequenced on an ABI PRISM 377 genetic analyser (Applied Biosystems, Inc.) with the oligo GIS-A (5′-AGGAAACAGCTATGACCATG-3′). Ninety-six sequences were obtained for each library and were screened for presence of microsatellites. Duplicate and nonmicrosatellite-containing sequences were discarded. Primers were designed using Primer 3 software (http://primer3.sourceforge.net) (Rozen & Skaletsky 2000). Each primer pair was tested on eight *P. concava* genomic DNA samples using PCR and electrophoresis was performed on 1% agarose gel (Fisher Reagents). Gels were stained using ethidium bromide and visualized by fluorescing with ultraviolet light. Primer pairs that produced consistently amplifiable fragments in the expected size range were then tested for the same eight individuals using a modified PCR protocol. For each potential microsatellite primer pair, the forward primer was 5′ modified by attaching a M13 prefix sequence (5′-CACGACGTTGTAAAACGAC-3′). The reverse primer was left unmodified. An independent M13 primer with above sequence was also designed with two different fluorescent modifications (6-FAM and TET) at 5′ end. All primers were obtained from Invitrogen Corporation. The modified forward primer, reverse primer and fluorescently labelled M13 primer (FAM or TET) was used in an economical 'three primer PCR' as described in Schuelke (2000). PCR amplification was performed in a 10 μL reaction containing 10 ng of template DNA, 10× PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, a limiting amount of modified-forward primer (empirically determined, but usually in the range of 1/100 of the other primers), 5 μM each of reverse and labelled M13 primer, and 0.5 U of *Taq*

Table 1 Details of microsatellite markers in *Publilia concava*

Locus	Accession no.	Primer sequence (5′–3′)	Repeat motif	N_a	N	Size ranges	H_O	H_E	P
D117	FJ595223	F: TGTTTTGTTTATGAGGGATTCG TTAGTGGTCCACATTATCAAAGG	$(GATA)_8$	2	35	260–264	0.03	0.03	1
D129	FJ595224	F: CCAGTGGAGGAGGTCATGC TATGGCGTTACCAAACTCAGG	$(TAGA)_9$	8	38	244–276	0.68	0.68	1
D164	FJ595225	F: TCCATTAACACGGGACTCG TTTATTTGGATCATCTAGACGAAGAC	$(CTAT)_{10}$ ... $(CTAT)_3$ ... $(CTAT)_4$ ... $(TCTA)_7$	10	33	289–361	0.85	0.77	0.34
D174	FJ595226	F: AAAGCCCAATTGAAATAAACG CTGTTAGACCAGGCTGATGG	$(TAGA)_7$	9	30	252–300	0.77	0.82	0.54
D305	FJ595227	F: CAAAAATTGCCATCGTTTATCC GGTATTTGTCGTCCTCTTTTTCC	$(TCTA)_8$	3	36	238–250	0.06	0.06	1
D317	FJ595228	F: TCGATAACATTTCTGTTTCAAAGC TCTGTTAGACCAGGCTGATGG	$(GAGT)_2GAGA(GATA)_6$*$(GA)_{19}$*$(CTAT)_3$	12	32	272–324	0.75	0.87	0.078
D359	FJ595229	F: AAAAATAGTTCAGAAGGGAGAAAAG TGCAACTTCTACTTCGTGGTC	$(GATA)_7(GA)_3CA(GA)_3$	7	31	254–270	0.32	0.76	< 0.001
D385	FJ595230	F: CAGGAGTTCCAGGTAATTAGGTTC GTGTGACATGTCGTCTCCAATG	$(CTAT)_7$	7	37	250–278	0.51	0.59	0.32
D392	FJ595231	F: ATCCCTTCAAACACCTTTCC TCTCCCACTGTGATGTCAGG	$(CTAT)_7$	3	38	248–260	0.16	0.16	1

Genbank Accession numbers, number of alleles (N_a), number of individuals (N), size range of amplified products, observed (H_O) and expected (H_E) heterozygosities, χ^2 test probability (P) at 95% that a given locus is in Hardy–Weinberg equilibrium. All markers amplified at an annealing temperature of 54 °C. More details are available in the text.

polymerase (Invitrogen Corp.). The PCR profile included an initial denaturation at 95 °C for 3 min., followed by 30 cycles of a denaturation step at 94 °C for 30 s., annealing at 54 °C for 30 s., extension at 72 °C for 30 s., and a final extension at 72 °C for 6 min. Genotyping was performed using MJ Research Basestation DNA Fragment Analyser (Bio-Rad). Alleles were scored in Cartographer software version 1.2.6 (MJ Research).

Publilia concava samples were collected from *S. altissima* near Putnum, Connecticut (Windham County). Genomic DNA was extracted from 38 individuals using a modified DNA extraction protocol (Bender *et al.* 1983). All 38 individuals were genotyped at nine loci that were isolated and optimized from a single tetranucleotide motif library. Multilocus genotypes from all individuals were analysed to estimate heterozygosities (observed vs. expected), number of alleles, allele size range, linkage disequilibrium in GenAlex software version 6.1 (Peakall & Smouse 2006) and GenePop version 3.2 (Raymond & Rousset 1995). Consistency with Hardy–Weinberg expectations was checked in HW-QuickCheck version 1.0 (Kalinowski 2006). Table 1 contains details of the loci and the results from the population genetic analysis.

The number of alleles ranged from two to 12, with an average of 6.78. The observed heterozygosity ranged from 0.03 (D117) to 0.850 (D164). One locus exhibited an extreme heterozygote deficiency and deviation from Hardy–Weinberg equilibrium, suggesting the possible presence of null alleles. We performed a null allele analysis using Micro-Checker program (van Oosterhout *et al*. 2004), which indicated the likely presence of null alleles for this locus. Linkage disequilibrium was detected between two pairs of loci (D174 × D392; D129 × D385). Further sampling will be necessary to determine if the observed heterozygote deficiencies and linkage disequilibrium are artifactual, rather than biological attributes of the sampling population. These markers open the door to developing the appropriate spatial framework for future studies of geographical variation in the *P. concava*–ant mutualism.

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Development of microsatellite markers for *Euchresta japonica* and *E. formosana* (Leguminosae)

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Abstract

We isolated 13 microsatellite loci from *Euchresta japonica*, an endangered shrub species that grows in warm-temperate forests in East Asia. Of these 13 loci, only one was codominant and polymorphic with five alleles. Cross-species amplification in a related species, *E. formosana*, detected nine of these loci, all of which were codominant and polymorphic with 2 to 9 alleles. These markers will facilitate further studies on the genetic characteristics of these two *Euchresta* species.

Keywords: *Euchresta formosana*, *Euchresta japonica*, insect-pollinated, low genetic diversity, SSR, understorey plant

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Euchresta japonica, an evergreen shrub belonging to the family Leguminosae, grows on the forest floor of evergreen broad-leaved forests throughout southwestern Japan and southern China (Ohashi 1978). This species is endangered at the local level in Japan. *Euchresta formosana*, a congener of *E. japonica*, grows on subtropical evergreen forest floors in southern Japan, Taiwan and the Philippines (Valkenburg 2003). Detailed genetic data are required to elucidate the ecological characteristics of these species. Therefore, we developed microsatellite markers for *E. japonica* and *E. formosana*.

Two methods were used to isolate simple sequence repeat (SSR) markers: a dual-suppression polymerase chain reaction (PCR) technique (Lian & Hogetsu 2002) and an improved technique for isolating compound SSR markers (Lian *et al*. 2006). Total DNA was extracted from fresh leaves using a modified cetyltrimethyl ammonium bromide method (Lian *et al*. 2003). Adaptor-ligated, restricted DNA libraries were constructed according to the method described by Siebert *et al*. (1995). Briefly, DNA was digested separately with *Acc*II *Afa*I, *Alu*I, *Eco*RV, *Hae*III and *Ssp*I blunt-end restriction enzymes. The fragments were then ligated to a specific 48-mer adaptor (5′-GTAATACGACTCACTATAGGGCACGCG TGGTCGACGGCCCGGGCTGGT-3′) and to an 8-mer adaptor with the 3′-end capped by an amino residue (5′-ACCAGCCCNH$_2$-3′) using a DNA Ligation kit (TaKaRa Shuzo Co.). To completely block polymerase-catalysed extension of the 8-mer adaptor strand, the ligated fragments were treated with ddGTP using AmpliTaq Gold kit (Perkin Elmer). As a first step for isolating microsatellite

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