Molecular Ecology Resources (2008) **8**, 1436–1438 doi: 10.1111/j.1755-0998.2008.02253.x

PERMANENT GENETIC RESOURCES

Isolation and characterization of microsatellite markers in an endemic Hawaiian planthopper (*Nesosydne chambersi*: Delphacidae)

KARI ROESCH GOODMAN, VERONICA RUTH FRANCO MORRIS, STEPHEN C. WELTER and GEORGE K. RODERICK

Department of Environmental Science, Policy and Management, 137 Mulford Hall, University of California, Berkeley, Berkeley, CA 94720–3114, USA

Abstract

We have isolated and characterized 17 microsatellite loci for the endemic Hawaiian planthopper *Nesosydne chambersi* (Delphacidae), a member of a large Hawaiian *Nesosydne* radiation. Thirty individuals from one population and 10 individuals from two populations across the species' range were tested to investigate polymorphism. The observed loci contained two to nine alleles per locus. Expected heterozygosity within this species ranged from 0.2 to 0.85. These markers will be used to assess intraspecific differentiation and population structure within *N. chambersi*.

Keywords: Delphacidae, Hawaii, microsatellite, *Nesosydne*, planthopper, SSR

Received 7 April 2008; revision accepted 16 May 2008

The Hawaiian Islands have served as laboratories for evolutionary studies, in part because of rapid rates of diversification observed in many groups of species as a result of adaptive radiation (Wagner & Funk 1995; Gillespie & Roderick 2002). In many groups, this process has resulted in large, closely related lineages within the Hawaiian island chain: examples include the Hawaiian honeycreepers, plants in the silversword alliance, *Drosophila* flies, *Laupala* crickets, *Tetragnatha* spiders, and *Nesosydne* planthoppers. Population-level studies using multiple nuclear markers, such as microsatellites, have the potential to provide detailed information about the demographic and ecological factors that lead to divergence and ultimately speciation within individual species. *Nesosydne chambersi* is a planthopper (Delphacidae) that specializes on members of another Hawaiian radiation: plants in the silversword alliance (Zimmerman 1948; Swezey 1954; Roderick 1997). This planthopper species has a relatively widespread distribution across both major volcanoes (Mauna Kea and Mauna Loa) on the island of Hawaii. Here, we report the isolation of microsatellite markers, which will be used to assess intraspecific differentiation and population structure within *N. chambersi*.

Correspondence: Kari Roesch Goodman, Fax: 510.642.0477, E-mail: krgoodman@nature.berkeley.edu

Nesosydne chambersi specimens were collected from *Dubautia ciliolata* ssp. *glutinosa*, in the Waipahoehoe Exclosure in the Mauna Kea Forest Reserve at approximately 2800 m above sea level on the island of Hawaii (HI DOFAW Permit FHM07-143). Genomic DNA was extracted for the development of these primers using a QIAGEN DNeasy DNA extraction kit, following the manufacturer's protocol. DNA from one individual was used to construct a library enriched for microsatellite repeats, following the methods presented in Glen & Schable (2005) and briefly described below. Four hundred and fifty-two nanograms of extracted DNA was digested by RSAI (New England BioLabs), then ligated to superSNX linkers. This restriction–ligation mixture was hybridized to the following biotinylated oligonucleotides to probe for repeats: $(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$, $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$, $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, and $(AGAT)_8$. These hybridized repeat fragments were captured using Dynabeads (Dynal) and recovered using polymerase chain reaction (PCR). A second enrichment was performed on the PCR product, and this amplified, doubly enriched DNA was cloned using a TOPO TA kit (Invitrogen), following the manufacturer's protocol. Successful transformations were selected using ampicillin and screened for inserts using b-galactosidase, and 768 clones with inserts were isolated and grown in

Table 1 Characteristics of 17 microsatellite loci isolated from *Nesosydne chambersi*. Repeat motifs are those characterized from the cloned allele

GenBank Accession no.	Primer	Sequence 5′– 3′	Repeat sequence	Forward primer label	Allele range (bp)	N_A	H_O	H_E
EU622228	Nc1	F: AACGCCTTCAGCCGTAATC R: GGCGACAGGGCTATTTTCTA	$(ACAG)_6$	NED	227–239	4	0.13*	0.59
EU622229	Nc2	F: GACCGAGCTAAGTGAGGTCAT R: TGAAGGGTATTCTTTAATTTTCCTTT	$(GCTC)_4$	PET	201–259	9	0.73	0.75
EU622230	Nc3	F: CATGAAACCAGCACTAGAGCAA R: TCTTCATGCCAAATCTCACG	$(GTCT)_5$	VIC	157–165	3	0.47	0.42
EU622231	Nc4	F: TCGCGCAGTTCAGAAAGTAA R: CGCCTCGAACTGGAATAGAA	$(GACA)_4$	VIC	223–231	3	0.30	0.33
EU622232	Nc5	F: CGTTGGGAACAGTCAGACAA R: TCGAGCAGTCCAAAAAGGAT	$(CTGT)_4$	VIC	191–207	5	0.40	0.50
EU622233	Nc6	F: TCGACGCACAGTTCAAAAAG R: TCCAAAAATTTTCCGAACAAAC	$(GACA)_7$	6FAM	168–192	6	0.75	0.64
EU622234	Nc7	F: TTCGAAAGTTATCGTCGAACA R: CCTTTGAATCCGGTGTGAAC	$(AACA)_5$	NED	161–165	3	0.48	0.40
EU622235	Nc8	F: AGCCGTTCGAAAGTTATCGT R: CACTTGAGGGTCCGCTGAT	$(CAAA)_4(CAGA)(CGGA)(CAAC)$	VIC	146–172	6	0.77	0.71
EU622236	Nc9	F: TACTGGCGTGTCTTGTGGTC R: GCGCAGTTCAAAAAGGAATC	$(GTCT)(GTTT)_3(GTCT)_4$	PET	196–216	5	0.38	0.38
EU622237	Nc10	F: TGCTTTCCTCCTCAATTCATC R: AACAAACAAGTGCCAATCACA	$(TTA)_5$	6FAM	96–99	2	0.15	0.20
EU622238	Nc11	F: ATGTGAACAGATCGGCCTTC R: GCCATTATCTCATATTCTGTTATTTGG	$(TGAG)_3$sequence$(GA)_3$ sequence$(GA)_3$	NED	195–207	5	0.27	0.30
EU622239	Nc12	F: AAGGTATAGCCGTCCCATGA R: TCCCAACAACACGACAACAT	$(CTCA)_3(CGCT)(CA)_3$ $(CTCA)(CT)_5$sequence$(CA)_3$	PET	184–191	3	0.77*	0.49
EU622240	Nc13	F: GCGATGTATCCACATGAAACTC R: TCCCAATCTGGGATCTAAGC	$(AGATT)_4$	VIC	171–181	3	0.20	0.21
EU622241	Nc14	F: TATCTACGCGTTTGGCCGTA R: TCGACAGAAGAAATCAGGCTAA	$(ACAG)_5(ACAA)_3$	PET	217–253	6	0.63	0.72
EU622242	Nc15	F: ATTTGCGATTCGAGGTGACT R: CATTCCGCCAAGAAGTTGAT	$(GT)_4(TGAGT)_3$	NED	147–157	4	0.55	0.64
EU622243	Nc16	F: TGGAGCTTTCGAGCTAGACC R: CGCGAATATTCAGGAACGAA	$(TTC)_6$sequence$(TTC)_{15}$ sequence$(TTC)_{16}$	PET	122–298	9	0.68	0.85
EU622244	Nc17	F: CGGGAGCTTTCCTTCTTATTTT R: TATTCCCTATGGTGCCAAGC	$(CTT)_{13}$	6FAM	136–183	9	0.67	0.68

N_A, number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; *significant deviation from Hardy–Weinberg equilibrium with $\mu = 0.05$ corrected for multiple comparisons using the sequential Bonferroni correction.

deep-well plates. Of these, 192 plasmids were sequenced with M13 forward and reverse primers using Big Dye version 3.1 (Applied Biosystems) on an ABI 3730 capillary sequencer.

Sequences were edited in Sequencher 4.0 (GeneCodes Corporation). Microsatellite repeats were identified both visually and with the aid of Microsatellite Repeats Finder (Bikandi 2006). Primers were designed using Primer 3 (Rozen & Skaletsky 2000). Finally, IDT's (2007) Oligo Analyser 3.0 (www.idtdna.com/analyser/Applications/OligoAnalyser) was used to check for secondary structure and primer–dimer formation. A total of 60 unlabelled primers were ordered. These were tested for consistency in amplification and polymorphism on two to three individuals from four populations each by PCR amplification and visualization on 1.8% agarose gels stained with ethidium bromide, yielding 19 suitable primers.

Flourescent-labelled forward primers were ordered for each of these 19 and were tested using 30 individuals from a population on Mauna Kea (Pu'u Kanakaleonui), as well as on five individuals from two populations across its range (Saddle Road and Mauna Loa Trail). PCR solutions contained 2.5 μL of extracted DNA (10 ng/μL), 2.5 μL 10× PCR gold buffer (Applied Biosystems), 4 mM $MgCl_2$, 25 μg/mL BSA, 0.52 μM each primer, 150 μM dNTPs, 10.325 μL ddH2O and 1 U *Taq* polymerase (Invitrogen). Thermal cycling conditions consisted of an initial activation cycle at 95 °C for 10 min. This was followed by two cycles of 1 min denaturing at 94 °C, 1 min annealing at 60 °C, and

35 s extension at 70 °C. Next were 18 cycles of 45 s denaturing at 93 °C, 45 s annealing through a touchdown series starting from 59 °C and stepping down 0.5 °C per cycle, with 45 s extension at 70 °C. This was followed by 20 cycles of 30 s denaturing at 92 °C, 30 s annealing at 50 °C and 1 min extension at 70 °C. Thermal cycling was completed by a final extension for 5 min at 72 °C. All loci amplify well using the same PCR conditions.

This PCR product was mixed in a solution of 0.5 μL PCR mixture, 9.215 μL of HiDi formamide and 0.285 μL of Liz500 size standard (Applied Biosystems), then run on an ABI 3730 automated capillary sequencer. The genotypes were analysed using GeneMapper version 3.0 (Applied Biosystems).

Testing of the 19 primers on 30 individuals of *N. chambersi* from Pu'u Kanakaleonui on Mauna Kea yielded 17 primer pairs that were polymorphic within the populations examined here and produced at most two alleles per individual per locus (Table 1). Numbers of alleles, allele size ranges and observed and expected heterozygosities were calculated using Genepop (Raymond & Rousset 1995). The number of alleles per locus ranged from two to nine and the expected heterozygosities ranged from 0.20 to 0.85 (Table 1). Genepop was also used to test for deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Two loci (NC1 and NC12) were found to be significantly out of HWE after a correction for multiple comparisons (sequential Bonferroni correction: Rice 1989). Departure from HWE in locus NC1 may be due to the presence of null alleles. No locus pairs were in significant LD after applying the sequential Bonferroni correction (Rice 1989). Analysis of the five individuals each from the Saddle Road and Mauna Loa populations indicates that these loci amplify reliably and are polymorphic among populations.

In summary, the 17 loci reported here will be useful for investigating the population genetic structure among populations of *N. chambersi*, allowing a detailed assessment of the intraspecific differentiation within a diversifying lineage.

Acknowledgements

Access to the land and permission to collect was granted by the State of Hawaii's Department of Natural Resources and Hawaii Volcanoes National Park. Funding was provided by the UC Pacific Rim Research Program, the Walker Fund, the Steinhaus Memorial Fund, Sigma Xi (UC Berkeley), NSF, and UC Berkeley. We thank Ellen Simms for providing additional laboratory space, Caroline Lee and Abby Moore for helpful discussion, and Ryan Lew and Justin Lin for technical assistance.

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