

annealing temperature X for 45 s, 72 °C for 45 s for two cycles each at X = 60 °C, 57 °C, 54 °C, 51 °C then 25 cycles at X = 48 °C, followed by 72 °C for 5 min. To optimize the PCR amplification of the loci found to be polymorphic, further PCRs consisted of one cycle at 95 °C for 3 min then 35 cycles at 94 °C for 1 min, annealing temperature (Table 1) for 30 s, 72 °C for 45 s, followed by 72 °C for 5 min. For the cross-species amplifications, a touchdown cycle was performed as above.

PCR products were visualized on a 0.8% agarose gel stained with ethidium bromide. When testing for polymorphism, PCR products were run on 6% polyacrylamide gels and visualized by staining with silver (Promega) or by autoradiography (after PCR with one of the primers end-labelled with [ $\gamma^{33}\text{P}$ ]-dATP; Sambrook *et al.* 1989).

We developed primers for 63 microsatellites, of which 50 were polymorphic in at least one of the tested species of *Sylviidae* (Table 1). Thirty loci were polymorphic, displaying up to five alleles, in a test panel of up to 25 unrelated Seychelles warblers. There was no significant difference at any locus between the observed and expected heterozygosity, though these comparisons were of limited power.

All 50 loci found to be polymorphic in the *Sylviidae* were tested for polymorphism in six unrelated individuals of the winter wren, *Troglodytes troglodytes* (M. Berg, personal communication). Fifteen of the loci that were also found to be polymorphic in the winter wren were selected and tested for utility in 16 other species, representing 15 passerine families (Table 2; following Sibley & Monroe 1990).

The high proportion of loci found to be polymorphic in the other *Sylviidae* will reduce or eliminate the need to develop new primers for future studies of these species. The cross-species amplification suggests that, after further testing, many of the primers presented here may also be useful for detecting polymorphic loci in other passerine families (Table 2).

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## Variable microsatellite loci in red swamp crayfish, *Procambarus clarkii*, and their characterization in other crayfish taxa

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The red swamp crayfish, *Procambarus clarkii*, is a temperate freshwater crayfish native to the south-eastern United States. It is heavily exploited as a fishery product and is used widely in aquaculture. Its economic importance led to widespread introductions on four continents. The species has been used extensively in laboratory studies, but studies of its population biology in the wild have been rare (Huner 1988). Previous population work using allozymes found low levels of genetic variation in two *Procambarus* species, including *P. clarkii* (Busack 1988). We developed two microsatellite libraries for *P. clarkii* (f. Cambaridae) from which 23 variable microsatellite loci were optimized. The 18 clearest markers were tested in representative taxa of the other two crayfish families (Parastacidae and Astacidae), as well as two cambarid species in Orconectes and one congeneric species; characterization is reported here.

Genomic DNA was extracted from frozen (–80 °C) tail muscle of a red swamp crayfish (Putah Creek, Yolo County California) using the Tris sodium chloride EDTA sodium dodecyl sulphate (SDS) (TNES)-urea buffer extraction protocol (Asahida *et al.* 1996) with the following modifications. Approximately 200 mg tissue were added to 700  $\mu\text{L}$  extraction buffer, containing 4 M urea and 0.5% SDS, and 0.035 mg Proteinase K. After overnight incubation (37 °C), samples were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated with 0.3 M sodium acetate pH 5.3 in a final ethanol concentration of 67%. The pellet was washed in 70% ethanol, air or vacuum dried, and resuspended in Tris low EDTA (TLE) buffer (10 mM tris + 0.1 mM EDTA, pH 8.0). Two subgenomic libraries were created by Genetic Identification Services (Chatsworth, CA) by partially digesting whole genomic DNA with a mixture of the following restriction enzymes: *BsrBR1*,

**Table 1** Summary of locus data for 23 microsatellite loci developed for *Procambarus clarkii*. GenBank Accession nos are AF290219-AF290941.  $n$  is the number of individuals screened; individuals were drawn from two or three (where  $n \leq 10$ ) to four (where  $n > 10$ ) populations.  $H_O$  and  $H_E$  are the observed and expected heterozygosities, respectively, calculated across all populations due to small sample sizes (Genes in Populations version 2, May *et al.* 1995); sample sizes precluded reasonable inference of the presence of null alleles

Locus ID	Primer sequences (5'-3')	Repeat (cloned allele)	Product size range (bp)	$n$	No. of alleles	$H_O$	$H_E$	MgCl <sub>2</sub> (mM)	Primer ( $\mu$ M)
PclG-02	F: CTC CCC ATG CAC TCT GGC TCT GT R: TGG CGA ATT TTG CCT GTT TCT GTC	(GATA) <sub>3</sub> GAGAA(GATA) <sub>5</sub>	216–224	25	3	0.56	0.61	1.5	0.5
PclG-03	F: CTC TCC ACC AGT CAT TTC TT R: AAG CTT ACA ATA AAT ATA GAT AGA C	(TCTA) <sub>20</sub>	216–420	26	12	0.73	0.89	2.0	0.5
PclG-04	F: TAT ATC AGT CAA TCT GTC CAG R: TCA GTA AGT AGA TTG ATA GAA GG	(TCTA) <sub>3</sub> ... (TCTA) <sub>2</sub> ... (TCTA) <sub>29</sub> ... (TCTA) <sub>2</sub>	170–290	26	15	0.77	0.89	2.0	0.5
PclG-07	F: CCT CCC ACC AGG GTT ATC TAT TCA R: GTG GGT GTG GCG CTC TTG TT	(TCTA) <sub>8</sub>	100–160	19	11	0.84	0.85	1.5	0.5
PclG-08	F: ACG ATA AAT GGA TAG ATG GAT GAA R: CCG GGT CTG TCT GTC TGT CA	(GATA) <sub>16</sub>	148–220	18	11	0.56	0.82	1.0	0.3
PclG-09	F: TAT GCA CCT TTA CCT GAA T R: TGT TGG TGT GGT CAT CA	(TCTA) <sub>14</sub>	80–160	20	8	0.35	0.85	1.5	0.5
PclG-10	F: TGC TCA CGC AAA CTT GTA TTC AGT R: CAA TGG TCC TTG ATT TGG TGT TCT	(TAGA) <sub>2</sub> TA(TAGA) <sub>16</sub>	90–176	10	6	0.40	0.65	1.5	0.5
PclG-13	F: CTC TCC TGG CGC TGT TAT TTA GC R: TGA AGA GGC AGA GTG AGG ATT CTC	(TCTA) <sub>12</sub>	130–150	17	3	0.53	0.54	1.5	0.5
PclG-15	F: GGC GTG ACG CCA ACG TGT CTT R: GGC TGG CCA CTT TGT TAG CCT GAG	(TATC) <sub>2</sub> TGTC(TATC) <sub>17</sub> TATT(TATC) <sub>3</sub>	150–185	18	12	0.78	0.85	1.5	0.5
PclG-16	F: CTC GGA ATG TCC ACC TGA GA R: TCA TTA TGG ATT TTG TCA ATC TAT	(TCTA) <sub>18</sub> TCTC(TATC) <sub>3</sub>	80–160	19	11	0.95	0.86	1.5	0.5
PclG-17	F: GTC GGG AAC CTA TTT ACA GTG TAT R: AAG AGC GAA GAA AGA GAT AAA GAT	(TCTA) <sub>14</sub>	156–190	19	8	0.84	0.78	1.5	0.5
PclG-24	F: CAA GGC ATT GAG GGG GTG AGA T R: CCG CGC CAC AGA ATT ACG AGT	(GATA) <sub>3</sub> AATA(GATA) <sub>24</sub> ... (AC) <sub>8</sub> T(CA) <sub>31</sub>	280–290	3	3	1.00	0.61	1.5	0.5
PclG-26	F: ATA TAG CCT CGC CCT TTT ACC C R: TCG TGT TCA CAT CAG CAG GAG A	(CT) <sub>5</sub> (CA) <sub>41</sub>	210–300	16	9	0.75	0.85	1.5	0.5
PclG-27	F: AAT CTT AAG ATC ATG AAA AAG GTA R: TTT AAG GAA CGT ATA AGA AAA GAC	(TATC) <sub>4</sub> CATC(TATC) <sub>8</sub>	80–150	8	11	0.63	0.84	1.5	0.5
PclG-28	F: CTC GGC GAG TTT ACT GAA AT R: AGA AGA AAG GGA TAT AAG GTA AAG	(GATA) <sub>22</sub> (GA) <sub>5</sub>	210–270	20	8	0.65	0.82	1.5	0.5
PclG-29	F: GAA AGT CAT GGG TGT AGG TGT AAC R: TTT TTG GGC TAT GTG ACG AG	(TATC) <sub>9</sub>	95–165	19	7	0.58	0.82	1.5	0.5
PclG-32	F: CCC CCA CTC GTC TCT GTG TAT G R: TGT GCT TGC GGG AGT GAG C	(CT) <sub>7</sub> ... (TC) <sub>37</sub> ... (CA) <sub>15</sub> ... (CA) <sub>5</sub>	150–250	19	14	0.74	0.91	1.5	0.5
PclG-33	F: TTC GAG GCG TTG CTG ATT GTA AGT R: CAA GGA AGC GTA TAG CCG GAG TCT	(GT) <sub>21</sub>	120–180	19	11	0.63	0.85	1.5	0.5
PclG-34	F: CAG TCC ATG TGA TCA ATA CTG ACC R: CTC AGG TGG AAC ACT CAT AAA CAA	(CA) <sub>4</sub> CG(CA) <sub>22</sub> TA(CA) <sub>15</sub>	80–160	4	6	0.75	0.75	1.5	0.5
PclG-35	F: TCC TCA CGT TTC TTT CCC ATC ATT R: TGC CTT TTC GAT CTC CAC CTT C	(GT) <sub>6</sub> AA(GT) <sub>8</sub> AA(GT) <sub>11</sub> AA(GT) <sub>5</sub>	152–190	18	6	0.56	0.68	1.5	0.5
PclG-37	F: TAA ATA AGT GGC GTG TAA GAC GAG R: TAA CTA AGC CAG GGT GGT CTC CAG	(CA) <sub>4</sub> CG(CA) <sub>15</sub> CG(CA) <sub>13</sub>	80–180	20	12	0.85	0.90	1.5	0.5
PclG-45	F: ATA TAA ACC GGT GTC GGT GTA G R: CTT TGA CTT CAC CTT TTC TCT TAT	(CA) <sub>3</sub> ... (GA) <sub>6</sub>	96–98	16	2	0.25	0.43	1.5	0.5
PclG-48	F: CTG TTG GTG ATT TCC GTC AAT TTT R: AGA TTC AAC GCT GTG TTC CTG ATC	(CA) <sub>12</sub>	146–190	17	8	0.59	0.84	1.5	0.5

*EcoRV*, *HaeIII*, *PvuII*, *ScaI*, and *StuI*. An oligonucleotide linker containing a *HindIII* site was ligated to fragments in the range of 300–700 bp. Magnetic beads were used to capture fragments containing (CA)<sub>n</sub> or (TAGA)<sub>n</sub>. These were ligated into the *HindIII* site of pUC19; the products were used to transform competent *Escherichia coli* DH5 $\alpha$ . Of the positive clones initially screened, 82% ( $n = 11$ ) (CA)<sub>n</sub>, and 58% ( $n = 12$ ) (TAGA)<sub>n</sub> contained microsatellites. We plated additional clones and amplified approximately 300 recombinant clones by colony polymerase chain reaction (PCR) using the following protocol. We added a toothpick stab of each colony to 10  $\mu$ L of 24 mM Tris-HCl (pH 8.4), 60 mM KCl, 0.075 mM each dNTP, 7.5 mM MgCl<sub>2</sub>, and 0.6 mM pUC19 forward and reverse sequencing primers. We incubated the mixture at 100 °C for 10 min then placed the tubes on ice. Five  $\mu$ L *Taq* solution (12 mM Tris-HCl, pH 8.4, 30 mM KCl, 0.5 U *Taq* DNA polymerase, recombinant, GIBCO) were added to each tube. Fifteen  $\mu$ L reactions (final conditions: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.05 mM each dNTP, 5 mM MgCl<sub>2</sub>, 0.4 mM each primer, 0.5 U *Taq* DNA polymerase) were placed in a preheated thermal cycler (MJ Research PTC 100) set to cycle as follows: 94 °C for 4.5 min, 25 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s, then 72 °C for 2 min. Approximately 1  $\mu$ L product was run on a 3% TAE agarose gel made with 0.03 $\times$  GelStar nucleic acid stain (BioWhittaker Molecular Products) to identify inserts of 300–800 bp. Colonies containing these inserts were grown overnight in Luria broth from which plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen). More than 150 clones were sequenced using the Big Dye™ Terminator cycle sequencing protocol and visualized on an ABI 377 DNA sequencer (Applied Biosystems) by Davis Sequencing (Davis, CA). Fifty-four primer pairs were designed from approximately 100 unique sequences using 'PrimerSelect' (DNASar, Inc.). Ten to 20 ng DNA from up to four crayfish populations sampled within the Sacramento Valley, California, were combined with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each dNTP and 0.5 U *Taq* DNA Polymerase in a 10  $\mu$ L reaction volume; MgCl<sub>2</sub> and primer concentrations are indicated in Table 1. Cycling conditions were 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, then 72 °C for 5 min. Amplification products were mixed 1:1 with 98% formamide loading dye, denatured for 3 min at 95 °C, placed on ice, then run on 5% denaturing acrylamide gels and stained by agarose overlay containing 0.5  $\mu$ L SYBR GreenI nucleic acid stain (BioWhittaker Molecular Application). Staining otherwise followed Rodzen *et al.* (1998). Products were visualized on a Molecular Dynamics FluorImager 595. Locus details are reported in Table 1. Eighteen primer pairs were also tested on *P. zonangulus*, *Orconectes virilis*, *O. rusticus*, *Pacifasticus leniusculus*, and *Cherax quadricarinatus*. Amplification success is reported in Table 2. These results indicate the utility of these microsatellite loci for genetic studies involving *P. clarkii*, and their potential utility in related species.

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**Table 2** Cross-species amplification with 18 of the primers listed in Table 1.  $n$  indicates number of individuals tested unless otherwise indicated in parentheses in each cell. Numbers in cells indicate the number of observed (presumed) alleles; '-' indicates amplification but unclear; '0' indicates no amplification or smear only

Species	PdG-02	PdG-03	PdG-04	PdG-07	PdG-08	PdG-09	PdG-13	PdG-15	PdG-16	PdG-17	PdG-27	PdG-28	PdG-29	PdG-32	PdG-37	PdG-45	PdG-47	PdG-48
<i>Procambarus zonangulus</i> ( $n = 4$ )	7	1	5	3	—	1	0	1	0	0	2(2)	1	1	3	—	2	—	2(3)
<i>Orconectes virilis</i> ( $n = 2$ )	3	0	1	1	2	1	0	1	0	0	0	0	0	1	1	1	0	—
<i>Orconectes rusticus</i> ( $n = 2$ )	2	0	3	1	2	1	0	1	0	0	0	0	0	1	1	1	1	—
<i>Pacifasticus leniusculus</i> ( $n = 4$ )	1(2)	0	1	1(3)	1(1)	1(3)	1(2)	1	1(3)	0	0	0	0	1	1(1)	1	2(3)	1(3)
<i>Cherax quadricarinatus</i> ( $n = 4$ )	—	0	0	1	1	1	0	1	0	1(2)	0	0	0	1	—(2)	0	0	1

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