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PRIMER NOTE

Isolation and characterization of microsatellite loci from two inbreeding bark beetle species (*Coccotrypes*)

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Abstract

We developed 14 microsatellite markers in *Coccotrypes carpophagus* and 14 in *C. dactyliperda*. These loci will be used for studying genetic structure and the level of inbreeding in populations in the Canary Islands and Madeira. As a result of long-term inbreeding, genetic variability is relatively low in these bark beetle species. We found one to five alleles per locus in 29 *C. carpophagus* and 41 *C. dactyliperda* from various localities. Eleven of the markers developed for *C. carpophagus* amplified in *C. dactyliperda* and seven of the markers developed for *C. dactyliperda* amplified in *C. carpophagus*.

Keywords: bark beetle, *Coccotrypes*, cross-species amplification, genetic population structure, inbreeding, microsatellite

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Sib-mating inbreeding has evolved independently at least seven times in bark beetles (Scolytinae: Kirkendall 1993). Inbreeding in bark beetles is primarily a tropical phenomenon and there seems to be a strong inverse relationship between latitude and the proportion of species that inbreed (Kirkendall 1993). Small tropical islands have a disproportionately high frequency of inbreeders (Jordal *et al*. 2001).

Coccotrypes belongs to the largest clade (1400 species) of exclusively sib-mating species, containing the ambrosia fungi-feeding Xyleborini and three genera of Dryocoetini (Jordal *et al*. 2002). Most species of *Coccotrypes* breed primarily in seeds and fruits, particularly palm seeds. In seed breeders, outbreeding could occur by coalescence of galleries from different mothers breeding in the same seed but it has not been possible to estimate outbreeding rates. Markers with a high degee of resolution are needed to study the population structure of extreme inbreeders, since local genetic variability is presumably relatively low.

Coccotrypes carpophagus (Hornung) and *C. dactyliperda* (Fabricius) bark beetles were collected from the Canary Islands and Madeira. Genomic DNA was extracted from whole individuals using QIAamp Tissue Kits (Qiagen). For each species, genomic DNA was pooled from 15 individual female beetles and a hybridization selection protocol was used to create two genomic libraries enriched simultaneously for $(CA.GT)_n$, $(GA.CT)_n$, $(AT.TA)_n$, $(CAA)_{18}$, $(GCC)_{18}$, $(CTG)_{18}$, $(CAG)_{18}$, $(GATA.CTAT)_n$, $(TTTC.AAAG)_n$, $(GTTA.CAAT)_n$ and $(CTAA.GATT)_n$. The protocol used was essentially that described by Armour *et al*. (1994). To prevent duplicate clones, DNA fragments were not polymerase chain reaction (PCR) amplified before enrichment hybridization, as suggested by Gibbs *et al*. (1997). The plasmid-cloning vector was supplied predigested and dephosphorylated (pUC18-*Bam*HI/BAP; Amersham Pharmacia). Enriched fragments were ligated into pUC18-*Bam*HI/BAP and transformed into XL1-Blue competent cells (Stratagene).

Transformants were screened by hybridization to the same 11 probes as used for the enrichment. All probes were radiolabelled with [α^{32}P]-dCTP except $(AT.TA)_n$ which was radiolabelled with [α^{32}P]-dATP. Hybridization was performed overnight at 65 °C for probes consisting of di- and trinucleotide repeats and at 55 °C for tetranucleotide repeat or $(AT.TA)_n$ probes.

Positive clones were sequenced in both directions using M13 universal primers with an ABI 377 Sequencer with BIG DYE Terminators (Applied Biosystems) according to the manufacturer's protocol. Sequences were confirmed as unique using GENEJOCKEY sequence processor software (P.L. Taylor 1990; Biosoft) and submitted to the EMBL database (Accession nos AF549465–AF549494). After submission to the EMBL database, all sequences were confirmed unique using BLASTN 2.2.4 software (Altschul *et al*. 1997).

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Table 1 Microsatellite markers for *Coccotrypes carpophagus* and *C. dactyliperda*

Locus	Species origin	Repeat motif*	Primer sequence (5′–3′)	EMBL no. and ID
*Ccarp*1	*C. carpophagus*	$(CTAT)_{12}$	F: TCCCGCGATTATATTTCGTC R: CTGCTTCATTCCGGTTTCTC	AF549465 C.c.1-186-CTAT
*Ccarp*2	*C. carpophagus*	$(CA)_{11}CG(CA)_{2}$	F: AACGTATGTTATCGCACGTGTT R: CATTACAGCACACGAGATGGA	AF549466 C.c.2-206-AC
*Ccarp*3	*C. carpophagus*	$(GA)_{4}$-$(GA)_{6}$-$(GA)_{2}$	F: GATCGCGAAAGAGTGAGTGA R: TCCCTTATTATCGTTCGTACCG	AF549468 C.c.3-162-GA
*Ccarp*4	*C. carpophagus*	$(TG)_{7}$-$(TGTGTC)_{4}(TG)_{7}C(GT)_{2}GC(GT)_{3}$	F: GCGCCTTCGTCGTTATTTAC R: TTGCCCCCTTTGTAGGTGTA	AF549468 C.c.4-181-GT
*Ccarp*5	*C. carpophagus*	$(AG)_{6}T(AG)_{3}AT(AG)_{9}$-$(AG)_{2}$-$(AG)_{4}$	F: AACCGTTCGATTCGCAAAA R: AGGTGTCGTGCGTCACTTTA	AF549469 C.c.5-152-GA
*Ccarp*6	*C. carpophagus*	$(GT)_{11}$	F: GTACCCGTCGCCCTGTATAA R: AACGGAAACGTCGAAAGAAA	AF549470 C.c.6-151-GT
*Ccarp*7	*C. carpophagus*	$(GA)_{4}(TA)_{4}(GA)_{2}(GATA)_{10}$-$(GATA)_{6}$- $(GA)_{4}$-$(GA)_{2}$-$(GA)_{2}(GATA)_{3}$-$(GA)_{2}$-$(GATA)_{4}$-$(GA)_{6}$	F: GATCGATACACAAAGATGTA R: GACTGTCGAATGACGAGAAT	AF549471 C.c.7-287-GATA
*Ccarp*9	*C. carpophagus*	$(CT)_{4}GT(CT)_{10}GT(CT)_{2}$-$(CT)_{5}GT(CT)_{2}$-$(CT)_{14}$	F: GAAAGTTCGAACGGGTCAA R: ATGACGTTTGTGACGGGATA	AF549472 C.c.9-207-CT
*Ccarp*10	*C. carpophagus*	$(CA)_{13}$	F: CTAAAGCGTAGGCTCCAAA R: ATCATCGGTCGACTCTCTG	AF549473 C.c.10-111-AC
*Ccarp*11	*C. carpophagus*	$(GT)_{10}AT(GT)_{4}AT(GT)_{8}$	F: GGAAAGTGCGGTGCATTTAT R: GCCTGGACGAATGTGCTAAG	AF549474 C.c.11-149-GT
*Ccarp*12	*C. carpophagus*	$(GTAA)_{2}$-$(GTAG)_{2}$-$(TAG)_{7}$	F: GATCAACCAGGGAGCTGTACT R: TCGAACGATGGATGACGAC	AF549475 C.c.12-185-GTA
*Ccarp*13	*C. carpophagus*	$(GATA)_{11}$	F: TTCGTAAGCTCACGTACAAC R: GATCTTTCACGTCCTAAGGT	AF549476 C.c.13-182-GATA
*Ccarp*16	*C. carpophagus*	$(AC)_{20}AT(AC)_{10}AT(AC)_{7}AT(AC)_{11}AT(AC)_{7}$ $GC(AC)_{10}$	F: ATCGGCTGACACACAAACG R: CCCAGTCCGACCCTGTTTAT	AF549479 C.c.16-200-AC
*Ccarp*17	*C. carpophagus*	$(AGTT)_{9}$	F: ATTGAGTGAGAGACCGGAAT R: GATCGGGATCGTTAAAAGTA	AF549480 C.c.17-195-AGTT
*Cdact*1	*C. dactyliperda*	$(AC)_{13}$-$(GTT)_{4}$-$(AAC)_{3}$	F: CGTTGTATGCAATAATCACTCG R: AGACTCAGCCCTTAGCTTGG	AF549481 C.d.1-255-AC
*Cdact*2	*C. dactyliperda*	$(GT)_{21}(ATGT)_{2}$-$(GT)_{4}$	F: CACCCAGGGCTAATTGAATC R: TCCTACTCTGGACCGTAGTGC	AF549482 C.d.2-154-GT
*Cdact*3	*C. dactyliperda*	$(CTAA)_{3}$-$(CTAA)_{8}$	F: CGAACCAACTGATTGACTGG R: TCCTTCCCTTAGGAGACGAC	AF549483 C.d.3-204-CTAA
*Cdact*4	*C. dactyliperda*	$(AC)_{8}GC(AC)_{2}$-$(CA)_{4}$-$(GAG)_{4}$	F: TGACTGCTGCACTTTCCGTA R: CTCGCCGAAGAACCGTATT	AF549484 C.d.4-189-AC
*Cdact*5	*C. dactyliperda*	$(AGTT)_{12}$	F: GATCGGGGATGAAAGTTAGT R: CGCTCGTTTTGGCATTTATT	AF549485 C.d.5-176-AGTT
*Cdact*6	*C. dactyliperda*	$(GC)_{7}(GT)_{25}AC(GT)_{4}$	F: GATCTACGTACGTAGGCGCG R: CATGTCGGAAACCATCAGTG	AF549486 C.d.6-175-GT
*Cdact*7	*C. dactyliperda*	$(CA)_{13}$	F: GAAATGCACGATTCACGAAA R: GATCTTTCGAGTTGTCCAGTG	AF549487 C.d.7-235-AC
*Cdact*9	*C. dactyliperda*	$(TC)_{3}$-$(TC)_{3}$-$(TC)_{7}$-$(TC)_{4}$	F: TTAGCGAGATTAAACGAAAA R: GATTCATATAGTGGCAGAACG	AF549488 C.d.9-152-CT
*Cdact*10	*C. dactyliperda*	$(GT)_{5}A(GT)_{23}$	F: GTCGCAACAACGAAGGTTT R: CAGGTGACCGAAATGTACAGA	AF549489 C.d.10-151-GT
*Cdact*11	*C. dactyliperda*	$(CATA)_{3}(AC)_{16}$	F: GATATGGAAGGCGTTCTGGT R: GCACAAGTGTCCAAATGACG	AF549490 C.d.11-199-AC
*Cdact*12	*C. dactyliperda*	$(ATAG)_{9}$-$(ATAG)_{3}$-$(ATAG)_{4}$-$(ATAG)_{4}$	F: AATATGAATATAGGAGGTCACG R: TCGATCGATCAACACTGTAT	AF549491 C.d.12-354-GATA
*Cdact*13	*C. dactyliperda*	$(TA)_{4}(TC)_{19}$	F: GAAGAGACGAGTGGGTGGTC R: GGTTAAATTCGGCGGGTAGT	AF549492 C.d.13-199-CT
*Cdact*14	*C. dactyliperda*	$(CAA)_{5}(CAT)_{7}$	F: TCGTCGTGCCAACAATTAAG R: GTACTGCACACGGCAAGCTA	AF549493 C.d.14-181-CAA
*Cdact*15	*C. dactyliperda*	$(GT)_{2}(GTAT)_{2}(GT)_{13}AC(GT)_{4}(GTAT)_{2}(GT)_{12}AC(GT)_{15}$	F: CGGCGGTAGTTCCTTGACT R: AAGAGCTCAGTACGCGAGAA	AF549494 C.d.15-212-GT

*Repeat motifs derived from the sequenced clone; -, sequence interruptions between repeats.
*Ccarp*3 and *Ccarp*4 are derived from the same clone.

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Table 2 Characterization of 14 *Coccotrypes carpophagus* and 14 *C. dactyliperda* microsatellite loci

		C. carpophagus				*C. dactyliperda*			
Locus	Expected allele size (bp)	*T* (°C)	$MgCl_2$ conc. (mM)	*A* (*n* = 29)	Allele size range (bp)*	*T* (°C)	$MgCl_2$ conc. (mM)	*A* (*n* = 41)	Allele size range (bp)*
*Ccarp*1	186	58	2.5	3	178–186	55	3.0	2	186–190
*Ccarp*2	206	58	2.5	1	206	56	2.5	1	200
*Ccarp*3	162	58	2.5	3	158–164	58	3.0	4	162–170
*Ccarp*4	181	58	2.5	4	169–181	58	3.0	1	201
*Ccarp*5	152	58	2.5	3	142–152	58	3.0	2	138–144
*Ccarp*6	151	58	2.5	3	147–153	54	2.5	2	151–155
*Ccarp*7	287	58	2.5	3	287–307	48	3.0	2	299–307
*Ccarp*9	207	58	3.0	2	207–237	—	—	X	
*Ccarp*10	111	58	2.5	4	103–111	58	3.0	5	97–115
*Ccarp*11	149	58	2.5	4	147–157	—	—	X	
*Ccarp*12	185	58	3.0	3	177–185	55	3.0	2	177–181
*Ccarp*13	182	58	3.0	3	174–182	—	—	X	
*Ccarp*16	200	58	3.0	4	194–210	48	3.0	3	184–196
*Ccarp*17	195	60	3.0	2	195–199	48	3.0	2	199–208
*Cdact*1	255	56	2.5	2	251–255	58	2.5	4	255–265
*Cdact*2	154	56	2.0	4	164–174	58	2.5	3	144–156
*Cdact*3	204	—	—	X		58	2.5	4	200–216
*Cdact*4	189	58	2.5	2	191–203	60	2.5	2	189–179
*Cdact*5	176	58	2.5	2	176–180	58	2.5	2	176–180
*Cdact*6	175	56	3.0	3	155–165	54	2.5	5	161–175
*Cdact*7	235	—	—	X		58	2.5	3	235–243
*Cdact*9	152	—	—	X		54	3.0	2	142–152
*Cdact*10	151	54	3.0	3	145–157	54	3.0	2	147–151
*Cdact*11	199	—	—	X		54	3.0	2	199–207
*Cdact*12	354	—	—	X		52	3.0	2	346–354
*Cdact*13	199	—	—	X		54	3.0	2	199–201
*Cdact*14	181	54	3.0	1	184	62	3.0	1	181
*Cdact*15	212	—	—	X		52	3.0	2	208–212

*Allele sizes were scored on 6% denaturing polyacrylamide gels stained with silver.
Ccarp locus name indicates locus originates from *C. carpophagus*; *Cdact* locus name indicates locus originates from *C. dactyliperda*.
Expected allele size based on length in the original sequenced clone.
T (°C), Optimized annealing temperature; *A*, no. of alleles; *n*, no. of unrelated individuals tested; X, no amplification.

The PCR primers (Table 1) were designed with the assistance of the primer design program PRIMER 3 (Rozen & Skaletsky 2000). The PCRs were performed in a 10-µL volume using a Perkin Elmer 2700 thermal cycler (Applied Biosystems). An initial denaturing step of 3 min at 94 °C was followed by 35 PCR cycles of 94 °C for 30 s, *T* (°C; Table 2) for 30 s and 72 °C for 45 s and finished with a 7-min extension step at 72 °C. Each reaction consisted of approximately 10 ng genomic DNA, 0.5 µM of each primer, 200 µM of each dNTP, 2.0–3.0 mM $MgCl_2$ (Table 2), 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 0.25 U of *Taq* DNA polymerase (Ampli*Taq*, Perkin Elmer). The PCR products were run on a 6% denaturing polyacrylamide gel and visualized by silver staining (Bassam *et al*. 1991; Caetano-Anollés & Gresshoff 1994). Allele sizes were determined by comparison to PCR-amplified DNA from the original clone and 10-bp (Amersham Pharmacia) and 20-bp (Sigma) DNA ladders.

Of 4128 clones in *C. carpophagus* and 6336 clones in *C. dactyliperda*, 145 and 90 clones, respectively, were positive on the autoradiographs. From this, we developed 14 primers for *C. carpophagus* and 14 for *C. dactyliperda* (Table 1).

In a test panel of 29 unrelated *C. carpophagus* individuals and 41 unrelated individuals of *C. dactyliperda*, 13 of the 14 markers developed for *C. carpophagus* were polymorphic, as were 13 of the 14 markers developed for *C. dactyliperda* (Table 2). All 28 loci were also tested for cross-species amplification using a range of annealing temperatures (Table 2). In total, 11 of the markers developed for *C. carpophagus* amplified in *C. dactyliperda* and seven of the markers developed for *C. dactyliperda* amplified in *C. carpophagus*. Polymorphism was found in 19 of 21 amplifying markers in *C. carpophagus* and in 22 of 25 amplifying markers in *C. dactyliperda* with up to five alleles per locus (Table 2). Due to low genetic variation in these inbreeding

organisms, we chose to maximize the number of populations sampled (nine for each species) at the cost of within-population sample size; consequently, population sizes were too small for meaningful comparisons of observed and expected heterozygosities.

These microsatellite markers offer the means to study population structure and rates of inbreeding for natural populations of these sib-mating beetles. The high degree of successful cross-species amplification suggests that many of the primers presented here may also be useful for detecting polymorphic loci in other related *Coccotrypes* species.

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