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MICROSATELLITE LETTERS

Isolation and characterization of 23 microsatellite loci in the stingless bee *Melipona subnitida* using next-generation sequencing

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Abstract We described the isolation and characterization of 23 microsatellite loci from the stingless bee (*Melipona subnitida*). Out of 52 microsatellite primer pairs screened, 17 loci displayed polymorphism and 6 were monomorphic. The analysis of variability was performed in 56 individuals. The number of alleles per locus ranged from 2 to 22 among populations; values for expected and observed heterozygosities ranged from 0.125 to 1.000 and from 0.121 to 0.923, respectively. These are the first microsatellite markers characterized for *M. subnitida* and they will be useful in obtaining estimates of population-level genetic diversity studies in a near future.

Keywords Genetic diversity · Microsatellite · Polymorphism · Stingless bee

Melipona subnitida Ducke is a stingless bee endemic to the semi-arid region of northeastern Brazil. The species has great ecological significance as a pollinator of the local native and cultivated flora. Their existence, however, is currently threatened by the progressive destruction of native vegetation and by the intensification of agriculture in the Caatinga biome. In response, populations of the stingless bee are declining, resulting in local extinction (Silva et al. 2014). Next-generation sequencing technology is a promising approach for species with limited genomic information (e.g. *M. subnitida*), the breeding programs of which would greatly benefit from the use of genomic tools, such as molecular markers, but for which these markers are not currently available.

Genomic DNA was extracted from the thorax of five individuals using standard proteinase-K digestion and phenol/chloroform method. An Illumina paired-end library was created using 1 ng of genomic DNA, following the standard protocol of the Illumina Nextera Sample Preparation Kit (Illumina Inc.). DNA sequencing was conducted using a MiSeq Benchtop Sequencer (Illumina Inc.). Contigs were created from the resulting paired-end sequence data using CLC Genomics Workbench 7.0.4 (Qiagen).

The library was loaded as 16 % of a MiSeq Reagent Kit v2 300 cycle sequencing run and produced 1,995,104 reads, which were assembled into 141,412 contigs. The program Msatcommander 0.8.2 (Faircloth 2008) identified 6,422 microsatellite loci, being in the majority dinucleotide repeats (5,998). For ease of imaging and scoring, we chose to examine only tri- and tetranucleotide loci. Of these, 52 loci were suitable for primer design and further testing. Microsatellites were individually amplified in 5 μL consisting of 2.15 μL of dd.H_2O, 0.5 μL of 10 × reaction buffer (Thermo Scientific Inc.), 2.5 mM of $MgCl_2$ (Thermo Scientific Inc.), 0.1 μM of fluorescently labeled CAG tag, 0.1 μM of un-tailed primer (either forward/reverse), 0.01 μM of CAG tailed primer (either forward/reverse), 200 μM dNTPs (New England BioLabs Inc.), 0.25 U *Taq* DNA Polymerase (Thermo Scientific Inc.), and ~15 ng of genomic DNA. Samples (n = 4) were initially amplified under the following thermocycling conditions: 95 °C for 5 min, followed by 30 cycles of 95 °C for 40 s, primer specific annealing temperature using gradient PCR (50–65 °C) for 30 s, 72 °C for 40 s, and a final extension at 72 °C for 7 min.

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Table 1 Characteristics of 23 microsatellite loci developed for *Melipona subnitida*

Locus	Primer sequence (5′-3′)	Repeat motif	T_a (°C)	N_a	Size range (bp)	N	GenBank accession no.
Msub02	F: GCCCAAAGATGGTATGCCG	$(ACG)_{14}$	60	6	146–173	56	KM494946
	R: ACGAGGCGGATTCAACGAG						
Msub03	F: CTCGGCGCACAATTCGAG	$(CGTT)_{11}$	60	6	171–191	56	KM494947
	R: GGTTATTTCGCCGGCAAGC						
Msub04	F: AAACTTGGTTCAGGTGCCC	$(CTT)_{15}$	60	7	146–173	56	KM494948
	R: CAGGGCGGGTTCAATACC						
Msub07	F: ACACCAACCCTAATGCTCATCGC	$(ACTG)_{13}$	60	4	160–180	56	KM494949
	R: CTCGCTTGTAAAGCTGCCC						
Msub08	F: GGAAGAAGTCTCTCGAGTAAAG	$(AAG)_{14}$	60	7	146–185	56	KM494950
	R: TATCTCTGGCAGGTTCGCC						
Msub09	F: TGGTCTTCTTTATGGCAGCG	$(CTT)_{17}$	60	8	155–188	56	KM494951
	R: GCATCCGACAAGTTGGCTC						
Msub11	F: TCTCGCGCATACCTAACC	$(CTT)_{13}$	60	3	175–181	56	KM494952
	R: GCTGACTCGGAACAATGGC						
Msub18	F: TCCCGATTTCCACCGATCC	$(ACG)_{18}$	62	17	154–232	56	KM494953
	R: GCCGACCTCTTCGACGG						
Msub26	F: CAACACCTCCTGCTTTATCGT	$(ATGT)_{12}$	60	8	171–203	56	KM494954
	R: CACACTCACTTTGTTTCCCTTT						
Msub30	F: CCTGTTATTTGCTCCTCGAAAT	$(CCTT)_{12}$	60	5	142–170	56	KM494955
	R: AACTCAAGGTTTCCCCGAAC						
Msub31	F: TTACCGTCTGTGCTACTGATCC	$(AGAT)_{14}$	60	12	162–210	56	KM494956
	R: TGTCTGTCTGTCTGTCTATCTTTCTG						
Msub37	F: AAATGCAGGCAGAAATGG	$(ACGC)_{14}$	60	17	150–226	56	KM494957
	R: TTGGACGAAAGTCAAATGC						
Msub38	F: AATACTCTGTTTCTTCCAGGGG	$(AAAG)_{15}$	60	7	134–184	56	KM494958
	R: CTGAAATTGCTTTCGTGCC						
Msub41	F: ATCTCCTTCCTTGCACTCACTC	$(ACTC)_{15}(TC)_{11}$	60	22	146–218	56	KM494959
	R: GTGGACAGAGGTTGGAAAAGAC						
Msub46	F: CACTGTTTCTCCAGTTGCTGTC	$(AAAG)_{12}$	60	6	118–146	56	KM494960
	R: GTTTCGTTCGCGTGATTTC						
Msub48	F: AAAGAGCGTAGGACTTCCACAG	$(GGAT)_{10}$	60	2	181–185	56	KM494961
	R: CATCCATCTATCCGTACATCCA						
Msub51	F: GGCGTTACAAAGGGGAGAA	$(AGAA)_9$	62	4	147–159	56	KM494962
	R: AGTTGACAGCGTTTCCTACCTC						
Msub01	F: GGGCGTGGACTAAGTAGC	$(CTT)_{11}$	60	1	161	56	KM494963
	R: GTGAGGAGAAACGTCGCAG						
Msub20	F: GCAGAGTTGACAGCGTTTCC	$(CTTT)_9$	60	1	100	56	KM494964
	R: TGTATAAGAGACAGGCTCGGC						
Msub32	F: AAAACTCGAAGAAAACGAGGG	$(AACG)_{14}$	60	1	158	56	KM494965
	R: GCTTGTCGTAAGGCACCG						
Msub35	F: GGCTTAGATACAGATCGGGTGT	$(ACGC)_{16}$	60	1	134	56	KM494966
	R: GGGTGGGGTGATGGG						
Msub49	F: AAACACCGTCGAGAGCCAT	$(CCTT)_{13}$	60	1	120	56	KM494967
	R: CTGACAAGCAAAGAAGCAAAGA						
Msub52	F: ATCAGGCATCAGACACAATCC	$(CCTT)_9$	60	1	110	56	KM494968
	R: ACTACTGAAATATGGCGTCGTG						

T_a, annealing temperature; N_a, number of observed alleles; N, number of individuals

Table 2 Variability of 17 microsatellite loci and *F*-statistic in *Melipona subnitida* populations

Locus	Ceara (N = 21)			Piaui (N = 15)			Rio Grande do Norte (N = 20)			F_{ST}
	H_o	H_e	P_{HWE}	H_o	H_e	P_{HWE}	H_o	H_e	P_{HWE}	
Msub02	0.563	0.688	0.022	0.909	0.558	0.025	0.789	0.679	0.500	0.144
Msub03	0.882	0.804	0.940	0.467	0.370	0.527	0.947	0.721	0.034	0.156
Msub04	0.722	0.717	0.855	0.643	0.627	0.074	0.727	0.675	0.051	0.037
Msub07	0.316	0.474	0.144	0.667	0.515	0.414	0.600	0.483	0.562	−0.009
Msub08	0.611	0.675	0.355	0.933	0.828	0.004	0.474	0.568	0.292	0.100
Msub09	0.800	0.650	0.040	0.500	0.632	0.063	0.600	0.687	0.295	0.104
Msub11	0.750	0.578	0.460	0.600	0.683	0.010	0.650	0.606	0.030	0.060
Msub18	1.000	0.863	0.052	1.000	0.869	0.003	0.867	0.862	0.060	0.069
Msub26	1.000	0.656	0.006	0.571	0.794	0.001	0.842	0.819	0.000	0.089
Msub30	0.611	0.570	1.000	0.667	0.653	0.005	0.450	0.383	1.000	0.063
Msub31	0.947	0.868	0.021	0.929	0.828	0.050	0.889	0.852	0.003	0.040
Msub37	0.842	0.898	0.035	0.769	0.886	0.002	0.750	0.798	0.098	0.096
Msub38	0.789	0.757	0.190	0.786	0.767	0.036	0.471	0.526	0.092	0.142
Msub41	0.842	0.923	0.001*	0.643	0.810	0.000*	0.737	0.865	0.000*	0.094
Msub46	0.368	0.367	0.457	0.333	0.287	1.000	0.625	0.597	0.455	0.083
Msub48	0.143	0.136	1.000	0.200	0.480	0.034	0.200	0.185	1.000	0.142
Msub51	0.125	0.121	1.000	0.308	0.280	1.000	0.417	0.467	0.117	0.069
Mean	0.665	0.632	–	0.643	0.639	–	0.649	0.634	–	0.083

*Locus that deviated significantly from *HWE* after Bonferroni correction (adjusted critical $P < 0.0029$)

H_o, observed heterozygosity; H_e, expected heterozygosity; P_{HWE}, probabilities of departure from Hardy–Weinberg Equilibrium

Imaging of PCR products was conducted using Li-COR 4200/4300 DNA analyzers (Li-COR Biosciences). For polymorphic markers, a subsequent set of 56 individuals from three Brazilian populations (Ceara, Piaui and Rio Grande do Norte) were chosen for further testing in order to obtain estimates of basic population level statistics.

The genotyped data was analyzed using Micro-checker 2.2.3 (van Oosterhout et al. 2004) to test for the presence of null alleles or other possible scoring inconsistencies. Tests for Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium were conducted using Arlequin 3.5.1.2 (Excoffier and Lischer 2010). Bonferroni-corrected *P*-values were used to assess the significance ($P < 0.05$).

Of the 52 tested loci, 17 were identified as polymorphic with allele numbers ranging between 2 (Msub48) and 22 (Msub41). A further six microsatellites were identified as monomorphic (Table 1) and the remaining exhibited unclear allelic pattern and/or unspecific products. Observed and expected heterozygosities ranged from 0.125 to 1.000 and from 0.121 to 0.923, respectively (Table 2). No evidence of null alleles or scoring error was detected using Micro-checker. A departure from HWE was detected for locus Msub41 (adjusted *P* value < 0.0029).

The first microsatellite loci for *M. subnitida* will be used for genetic analyses of distinct populations across the northeast region of Brazil, and to reveal how environmental degradation affects this endangered species.

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