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Research Article

Isolation and Characterization of Microsatellite Markers in *Canthon (Petelcanthon) Staigi* (Coleoptera: Scarabaeidae) and Cross-Amplification in Related Species

Abstract

The species *Canthon* (*Peltecanthon*) *staigi* is a Neotropical "dung beetle" from Brazilian Atlantic Rainforest, which plays a key ecological role reallocating organic material and in some cases being a seed disperser. Moreover, this species is considered a bio indicator once is sensible to environment changes like the habitat loss. Despite its' ecological importance nothing is known regarding the population structure of this dung beetle species. Molecular markers are informative tools to evaluate the extent and distribution of genetic diversity of the *C.* (*P.*) *staigi* populations remaining. Here we report the isolation and characterization of the first microsatellite markers for *C.* (*P.*) *staigi*. Four polymorphic microsatellite loci were characterized with allele numbers ranging from four to five per locus. The observed and expected heterozygosis ranged from 0.466 to 0.516 and 0.485 to 0.623, respectively. All loci were observed on Hardy Weinberg equilibrium. Linkage disequilibrium was not detected in any loci. From a subset of 24 loci it was observed positive transferability of six loci on four different tribes of Scarabaeidae. The loci will be used for studying population genetic structure of *C.* (*P.*) *staigi* and the cross-species amplification success extends the utility of these markers to be applied also on related species.

Introduction

Canthon is a neotropical genus which belongs to Scarabaeidae (Scarabaeidae) subfamily and comprises about 170 species, commonly known as "dung beetles". The species *Canthon* (*Peltecanthon*) *staigi* Pereira, 1953, is inserted in a well-defined telecoprid guilds of dung beetles and play a key ecological role in the reforestation, as part of process of reallocating organic material and seeds dispersal [1-3]. Besides that, *C.* (*P.*) *staigi* is a notable species due to its wide geographic distribution on different vegetation types of Atlantic forest between the states of Paraiba and Paraná in [4-8].

The *C. (P.) staigi* species is sensible to environmental changes like forest fragmentation, which disturb its population ecological dynamics on the level of guild structure, being considered a bio indicator [2,7,9-11]. Considering the high degree of Atlantic Forest fragmentation, the characterization of genetic diversity of *C. (P.) staigi* requires an understanding of the population genetic structure within and among the remaining forest fragments.

Co-dominant molecular markers such as microsatellites have been used as an informative tool to generate information on population genetics level of insects. However, there are no microsatellite markers developed for any species of *Canthon* genus. Here we report the isolation and characterization of four polymorphic microsatellite loci from *C. (P.) staigi*. Our data are informative for future studies of population genetic structure and also cross-amplification among related species of five tribes of Scarabaeidae subfamily.

Material and Methods

The genomic DNA was isolated from two individuals of *C. (P.) staigi* species according to [12]. The isolated DNA was submitted to a digestion process by restriction enzyme AfaI (Promega, Madison, Wisconsin, USA) and the resulted fragments were linked to adapters (Rsa21 5'-CTCTTGCTTACGCGTGGACTA-3' / Rsa25 5'-TAGTCCACGCGTAAGCAAGAGCACA-3'). The library was enriched for two monomers, (CT) 8 and (GT) 8, using biotinylated microsatellite probes. The capture of the target fragments was performed by the use of streptavidin-

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coated magnetic beads (Promega). The next step was to link the Microsatellite-enriched DNA fragments into a pGEM-T vector (Promega). After that the plasmids were introduced by electroporation into Escherichia coli XL-1 Blue strains. The transformed cells grew on petri dishes with LuriaeBertani (LB) agar medium containing X galactosidase (5-bromo-4-chloro-indolyl-B-D-galactoside) (20 mg/ml), IPTG (Isopropylthio-B-D-galactoside) (100 mM), ampicillin (50 mg/ml) and tetracycline (12,5 mg/ml).

A total of 96 recombinant colonies were obtained and sequenced using the Big Dye terminator version 3.1 Cycle Sequencing Kit (Applied Bio systems), T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') sequencing primers and 3100 DNA Analyzer (Applied Bio systems). For clones containing SSR motifs, forward and reverse sequences were aligned to obtain the consensus sequence using Chromatogram Explorer 3.2 (Heraclea Bio Soft S.R.L., Romania). Microsatellite amplification-specific primers were designed from 24 loci of applicable flanking sequences with the software PRIMER3 [13]. For the polymorphic loci see table 1.

The polymerase chain reactions (PCR) were performed in a Bicycler 96-Well Thermal Cycler (Applied Bio systems) in a reaction volume of 10 µL containing 5 ng of DNA template, 10x PCR buffer, 0.50 mM $MgCl_2$, 2.5 mM dNTP, 5 U/µL Taq polymerase (Invitrogen), 10 pmol forward primer and 10 pool reverse primer. A touchdown cycle program was used as follow: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, the annealing temperature for each primer for 45 s (The optimum annealing temperature of different primers was screened by gradient temperature PCR) and 72°C for 45 s, and a final extension of 72°C for 10 min. The amplicons were checked on 1,8% agarose gel stained with Gel Red (Biotium, Hayward, California, USA) and were genotyped in 7% polyacrylamide denatured gel stained with silver nitrate. The genotyping was performed using a 100bp DNA ladder (Invitrogen) as size standard marker. Sixty individuals from two populations collected on Atlantic forest fragments on the states of Paraiba and Pernambuco, Brazil, were analyzed regarding genetic diversity (Table 2).

The 24 microsatellite loci were tested in seven species belonging to four tribes of Scarabaeidae subfamily. The PCR conditions and amplicons checking were performed as previously described. The bands amplified on the expected size were subsequently sequenced by Macrogen Inc (Seoul, Rep. of Korea), in order to check the presence of microsatellite motifs.

The genetic diversity per locus and population were evaluated on the basis of different indexes: allelic richness (R), number of alleles (A), observed (H_O) and expected (H_E) heterozygosis and inbreeding coefficient (F_{IS}) on MSA program [14]. It was also calculated the polymorphic information content (PIC) [15] (Table 1). The Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested for all loci using Arlequin 3.5 [16]. Regarding the results robustness genotyping errors were calculated in relation to null alleles, stuttering, and allele dropout using Micro-Checker 2.2.3 [17].

Results and Discussion

The microsatellite enrichment library of 96 recombinant colonies resulted on 45 microsatellite repeat motifs, of which the most frequently encountered repeat motifs were $(A)_n$, $(AATT)_n$, $(CTTTC)_n$, $(TTGTG)_n$ and $(TGTGCA)_n$. The penta-nucleotides (71.11%) and hexa-nucleotides (17.77%) were frequently observed, followed by mono-nucleotides (6.66%) and tetra-nucleotides (4.44%). The di-nucleotides and tri-nucleotides were not found. The hexa-nucleotides $(TTAAAA)_n$ and $(TGTGCA)_n$ were found on the repetitive portion of the *C.*(*P.*) *staigi* genome related with a transposable element Tiger of the family Tc1/mariner, which is the most common on insect genomes [18].

From the 45 microsatellite 24 presented applicable flanking regions for primer design. From these four were polymorphic

Table 1: Characterization of four polymorphic microsatellite markers developed for C. (P) staigi (Scarabaeidae). Locus name, GenBank accession numbers, primer sequence (F: forward, R: reverse), anneling temperature (Ta), repeat motif, fragment size range in base pairs (bp), number of alleles per locus (A), observed heterozygosity (HO), expected heterozygosity (HE), and polymorphic information content (PIC).

Locus	GenBank	Sequence (5' 3')	T_a (°C)	Repeat motif	Size range (bp)	A	H_O	H_E	PIC
Cstms01	KY794604	F: GGACTGTTTTGAGCGTTACCAT R: GGCCCCTCCAGTATAGAAAATC	54.5	(TTGTG)2	161 - 196	5	0.533	0.572	0.312
Cstms02	KY794605	F: TTCTTATTGTAGCTTCGTTCATGC R: GACTAACACATCTCACGGGGAA	52	(TGTCGA)2	397 - 361	5	0.499	0.583	0.339
Cstms04	KY794606	F: GCTGGTTTCATCAACTGCATTA R: ACATACCACCCAGTCCTTTGTC	55.6	(TTATT)2	242-267	5	0.566	0.580	0.338
Cstms09	KY794607	F: AATCGCTTTAATCTTGTCTGGG R: CTCAAAATCCCTAGAAGAACACG	55.6	(ATTT)3(ATGAAT)2	178 - 198	4	0.533	0.479	0.368

All loci departed significantly from HWE at the 0.05 level.

Table 2: Location of sampled populations and parameters of genetic diversity in C. (P) *staigi*.

Population	Latitude S	Longitude W	N	R	H_O	H_E	F_{IS}
Guaribas - GUA	06°36'44"	35°17'34"	30	2.7	0.516	0.623	0.168
Pesqueira - PES	08°21'28"	36°41'47"	30	2.0	0.466	0.485	0.034

Number of individuals sampled (N), allelic richness (R), observed heterozygosity (HO), expected heterozygosity (HE), and inbreeding coefficient (FIS). Significant departure from HWE (P < 0.00250).

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while 20 were monomorphic. The polymorphic loci generate a total of 19 alleles, ranging from four to five per locus. For monomorphic loci see table S1. The H_O ranged from 0.466 to 0.516 while H_E ranged from 0.485 to 0.623, per locus. The PIC index ranged from 0.312 to 0.368 per locus (Table 1). All loci were in HWE equilibrium and no linkage disequilibrium was detected. Furthermore, no evidence of scoring error was found.

Regarding the two populations, GUA presented higher values of allelic richness, H_E and H_O than the PES population (Table 1). Interestingly, the observed F_{IS} value in PES was lower than GUA, which means that population GUA could be suffering greater inbreeding depression, likely caused by geographic isolation on the range margin of the *C.* (*P.*) *staigi* spatial distribution.

The cross-species amplification tests revealed eight primer loci (Cstms02; Cstms06; Cstms10; Cstms12; Cstms14; Cstms15; Cstms19; Cstms20) which amplified satisfactorily among all species from four tribes. However, just six loci presented microsatellite motifs, indicating that are possibly useful for population genetics analysis of other beetle species (Table 3).

The four polymorphic loci isolated for *C.* (*P.*) *staigi* will be used to study the genetic diversity and population genetic structure of *C.* (*P.*) *staigi* providing knowledge which will be important for conservation strategies of this species.

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Table S1: List of loci which were monomorphic in *Canthon (P) staigi* (Scarabaeidae).

Locus	Sequence (5'→3')	Repeat motif	T_a (°C)	Expected allele size (bp)
Cstms03	F: TGCCACAACTCGTTTATTCCTA R: CGAAAACAACTGTAACGCCATA	(AACGT)2	56.8	151
Cstms05	F: TCAGTGGTGAAACTCGTCTTTT R: GCGTGGACTAACAGCAAGTAAT	(T)10	58	235
Cstms06	F: AATCCCGAGCTTATGTGCTG R: AGCTGCAACACTCCTTCTAATTG	(TGCACA)2	53.2	386
Cstms07	F: CGTGGACTACCCACTTGAAATAA R: TGGATCGAAGCGAGATCAAT	(ATAAG)2 (AGACA)2	56.4	374
Cstms08	F: GGCCCCTCCAGTATAGAAAATC R: GGACTGTTTTGAGCGTTACCAT	(AAATA)2 (CACAA)2	52.6	161
Cstms10	F: GTCTCAATAACAACGGATGGC R: AGCTAACACTTGCTGGTGGATT	(ATGAAT)2	52	132
Cstms12	F: TTGCCATCTTCTTCTTCAACTG R: GCTTTATGACCACTAGCCGATT	(TTAAAA)2	58	293
Cstms13	F: AATCGGCTAGTGGTCATAAAGC R: GGCAATCCCTTTGAACCTAAAT	(ATTAT)2	55	262
Cstms14	F: TTGCTGAATGATCGAAGTGATG R: TTCTTCTAAGTCCTCACCAGCG	(ATAGC)2	51	231
Cstms15	F: GTGTTTCTGACAAGAGCAAGGA R: CTGTATTTGTTGACCTAGCAATCG	(GCCAG)2 (AAAAAG)2	55	344
Cstms17	F: GTTGGCTCGAATTTTAACCTGT R: CGCGTGGACTAACTCAATGTAA	(AATT)3 (ATACA)2	56.4	337
Cstms18	F: CCTAATCTTCTAAACAAACCGC R: CGCGTGGACTAGCTTTATG	(TAATT)2 (CTAAC)2 (TTTCT)2	54.5	400
Cstms19	F: TCCTTTGTTCTTATCGCCCTTA R: GTAGGATCGTTTTGCTCTTGCT	(TAAAAG)2 (CTTTC)2	51	254
Cstms20	F: AGCTGCAACACTCCTTCTAATTG R: AATCCCGAGCTTATGTGCTG	(TGTGCA)2	55	386
Cstms21	F: TGGATCGAAGCGAGATCAAT R: CGTGGACTACCCACTTGAAATAA	(TGTCT)2 (TCTTA)2	55.5	374
Cstms22	F: GGACTGTTTTGAGCGTTACCAT R: GGCCCCTCCAGTATAGAAAATC	(TTGTG)2 (TTTTA)2	53.6	161
Cstms23	F: CTGTATTTGTTGACCTAGCAATCG R: GTGTTTCTGACAAGAGCAAGGA	(TCTTTT)2 (CTGGC)2	55	344
Cstms24	F: GCACAAATAACGAAGAGGAAGG R: ATGCTGTGAATGTTGCTGAACT	(ACTAA)2 (AATTT)2	54.5	367
Cstms25	F: GGTTTCGAGTATTTGGATTTAAGCG R: AGTGTCCTTTGAGTGTGATTCCTGT	(TTGTA)2 (AATT)3	54.5	235
Cstms26	F: CGCGTGGACTAGCTTTATG R: CCTAATCTTCTAAACAAACCGC	(GGTTA)2 (AGAAA)2 (AATTA)2	54.5	400

Note: Ta = anneling temperature

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Table 3: Transferability of eight microsatellite markers developed for C. (P) *staigi* accross five different tribes of Scarabaeinae (Scarabaeidae).

Tribes/Species	Cstms02*	Cstms06	Cstms10	Cstms12	Cstms14	Cstms15	Cstms19	Cstms20
Canthonini								
Canthon nigripenne	+	+	+	+	-	-	+	+
Coprini								
Coprophanaeus ensifer	-	-	+	-	-	-	-	-
Dichotomiini								
Dichotomius bos	-	-	-	-	-	-	-	-
Dichotomius geminatus	-	-	-	-	-	-	-	+
Eurysternini								
Eurysternus caribaus	-	-	-	-	-	-	-	-
Eurysternus hyrtelus	-	-	+	-	-	-	-	-
Phanaeini								
Phanaeus splendidus	-	-	+	-	-	-	-	-
Total	1	1	4	1	0	0	1	2

'+' indicates successful amplification and presence of microsatellite motif; '-' indicates absence of microsatellite motif
*Polymorphic locus on C. (P) *staigi* species

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