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Alelle Number and Heterozigosity for Microsatellite Loci in Different Stingless Bee Species (Hymenoptera: Apidae, Meliponini)

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Número de Alelos e Heterozigose para Locos de Microssatélites em Diferentes Espécies de Abelhas sem Ferrão (Hymenoptera: Apidae, Meliponini)

RESUMO - No presente trabalho compararam-se as características de locos de microssatélite, como diversidade alélica e taxa de heterozigose observada, de três espécies de abelhas sem ferrão (*Plebeia remota* Holmberg, *Partamona mulata* Moure In Camargo e *Partamona helleri* Friese), amplificados com oligonucleotídeos heteroespecíficos originalmente desenhados para *Melipona bicolor* Lepeletier e *Scaptotrigona postica* Latreille. Foram analisados 360 indivíduos de *P. remota* de 72 ninhos, 58 indivíduos de *P. mulata* de 58 ninhos e 47 indivíduos de *P. helleri* de 47 ninhos. As três espécies apresentaram baixo nível de polimorfismo para locos amplificados com oligonucleotídeos derivados de *Melipona bicolor*. Entretanto, para os locos amplificados com oligonucleotídeos derivados de *S. postica*, somente *P. remota* apresentou baixo nível de polimorfismo.

PALAVRAS-CHAVE: *Plebeia remota*, *Partamona mulata*, *Partamona helleri*, taxa de heterozigose, polimorfismo

ABSTRACT - In the present study we compare genetic characteristics (allele diversity and observed heterozygosity) of microsatellite loci, from three stingless bee species (*Plebeia remota* Holmberg, *Partamona mulata* Moure In Camargo and *Partamona helleri* Friese), amplified by using heterospecific primers originally designed for *Melipona bicolor* Lepeletier and *Scaptotrigona postica* Latreille. We analyzed 360 individuals of *P. remota* from 72 nests, 58 individuals of *P. mulata* from 58 nests, and 47 individuals of *P. helleri* from 47 nests. The three species studied showed low level of polymorphism for the loci amplified with primers derived from *M. bicolor*. However, for the loci amplified with primers derived from *S. postica*, only *P. remota* presented low level of polymorphism.

KEY WORDS: *Plebeia remota*, *Partamona mulata*, *Partamona helleri,* heterozygosity, polymorphism

Currently, the term microsatellite is widely accepted to assign sequence repeats involving a small number of bases (Hancock 1999). These repeated sequences are randomly distributed along the euchromatic regions (Schlötterer & Wiehe 1999), but are rare in coding regions (Hancock 1999). Microsatellites have been described in all organisms studied (Hancock 1999), and are also present in plasmids, mitochondrial, and plastidial genomes (Filutowicz *et al*. 1994, Powell *et al*. 1995, Soranzo *et al*. 1999).

Usually microsatellites are PCR amplified by using primers located in conserved regions flanking the repeats (Weber & May 1989, Strassmann *et al*. 1996). According to Goldstein & Schlötterer (1999), they can be classified as: perfect (sequence of bases repeated with no interruption - CTCTCTCTCT); imperfect (one or more repeats having a different base pair of the repeat structure - CTCTCACTCT); interrupted (insertion of a small number of base pairs different from the repeat structure - CTCTCAAACTCT); and compound (two or more adjacent microsatellites with different repeat sequences - CTCTCTCTCTGATGATGATGAT).

It has been postulated that interrupted microsatellites are more stable than the perfect ones, and in consequence they present less alleles (Chung *et al*. 1993, Pépin *et al*. 1995). This is explained by the presence of extra bases in the repeat, which would contribute to diminish the possible error during the DNA replication (Weber 1990). However the mutation rate of microsatellites is highly variable and depends either on the number and type of repeats and on their base composition (Chakraborty *et al*. 1997, Estoup & Cornuet 1999). Moreover, the genome type and organism where the microsatellites are located also seem to interfere. Microsatellites in nuclear chromosomes showed more stable

than identical microsatellites in plasmids (Henderson & Petes 1992, Eisen 1999); in humans the mutation rate can reach 1.5 $\times 10^{-2}$ per locus per gamete per generation (Aşicioğlu *et al*. 2004) or even 3.5×10^{-3} (Nikitina *et al*. 2005), and in *Drosophila*, 6.3×10^{-6} (Schug *et al*. 1997). Although different types of mutation may occur in the microsatellite sequence, changes in the number of repeats are the most frequent (Eisen 1999).

Microsatellite is a molecular marker considered codominant, selectively neutral, highly polymorphic, and show mendelian inheritance (Strassmann *et al*. 1996). Due to these characteristics they have been extremely useful in gene mapping studies, relatedness, parentage, intraspecific variation, species hybridization, population dynamic, and phylogeography (Moritz & Hillis 1996, Chakraborty & Kimmel 1999). Also they have been used to evaluate the impact of the reproductive behavior, social structure, and dispersion in endangered populations (Beaumont & Bruford 1999). At population level the microsatellite high polymorphism is considered as a consequence of new mutations, genetic drift, and selection in genes linked to the repetitive sequences (Schlötterer & Wiehe 1999).

In bees, primers for microsatellite loci and respective amplifications were first described for *Apis mellifera* L. and *Bombus terrestris* L. (Estoup *et al*. 1993). Later, microsatellite primers for three stingless bee species, *Melipona bicolor* (Peters *et al*. 1998), *Scaptotrigona postica* (Paxton *et al*. 1999a), and *Trigona carbonaria* Smith (Green *et al*. 2001) were described. Subsequently, population, ecological and evolutionary studies were developed (Paxton *et al*. 1999b, Tóth *et al*. 2003, Cameron *et al*. 2004, Franck *et al*. 2004).

Most of the biological inferences derived from microssatelite data are based on allele and genotype diversity and heterozygosity index. In this work we compared the number of microsatellite alleles and observed heterozygosity (H_O) among three stingless bee species (*Plebeia remota* Holmberg, *Partamona mulata* Moure In Camargo and *Partamona helleri* Friese) and also with data from other Meliponini species already described in the literature (Peters *et al*. 1998, Paxton *et al*. 1999a). Considering that most of the loci are amplified by heterospecific primers, although all from bees, the level of information differs from locus to locus and also among the species. The compilation of these data provides guidance for the use of these loci in other bee species.

Material and Methods

The species studied were *P. remota* (360 individuals from 72 nests), *P. mulata* (58 individuals from 58 nests), and *P. helleri* (47 individuals from 47 nests). The collecting sites are presented in Table 1. DNA template for PCR reactions was extracted by Chelex method (Walsh *et al*. 1991).

The bees were scored using heterospecific microsatellite primers described for *M. bicolor*: *Mbi28AAG*, *Mbi32GAG*, *Mbi33AAG*, *Mbi201AAG*, *Mbi215AAG*, *Mbi218AAG*, *Mbi254AAG*, *Mbi259AAG*, *Mbi278AAG*, *Mbi522CAG* (Mbi set) (Peters *et al*. 1998), and *S. postica*: *T3-32*, *T4-171*, and *T7-5* (T set) (Paxton *et al*. 1999a).

Amplifications were performed through PCR in 10 µl reaction volume using 2 µl of DNA template, 1 µl of PCR buffer, 0.3 µl of $MgCl_2$ (50 mM), 0.2 µl of each primer (20µM), 0.5 µl of dNTPs (2 mM each), 1 U of *Taq* DNA polymerase (Gibco-BRL) and sterile water to achieve the final volume. The PCR amplifications consisted of an initial denaturation at 93°C/4 min, followed by 30 to 40 cycles at 93°C/40 s for denaturing the DNA, 50 s at the appropriate temperature for annealing (Table 2) and 72°C/40 s for elongation. An additional final extension step of 72°C/5 min was performed. The PCR products for Mbi and T primer sets were separated by electrophoresis on 5.6% and 9% polyacrylamide gels, respectively. Size of the amplified microsatellite alleles was estimated by comparison with standard molecular weight marker (10 bp ladder).

Results and Discussion

Table 3 presents the observed heterozygosity (H_O) values and number of alleles, for the loci amplified with primers described for *M. bicolor*, verified in *P. remota*, *P. mulata*, *P. helleri*, and in other four meliponini species (Peters *et al*. 1998). It is worth to point out that *Mbi28AAG* and *Mbi33AAG* heterozygotes were reported only in *M. bicolor* and *P. helleri*, respectively. All seven species showed heterozygotes for the locus *Mbi259AAG* and no heterozygotes were found for the locus *Mbi522CAG*. *P. mulata* presented $H_O = 0$ for all loci but *Mbi201AAG* and *Mbi278AAG*. The highest number of alleles (six) was found in *P. remota* for the loci *Mbi259AAG* and *Mbi278AAG*.

Table 4 shows H_O values and number of alleles observed in *P. remota*, *P. mulata*, *P. helleri*, and in other seven meliponini species (Paxton *et al*. 1999a) for the loci derived

Table 1. Collecting sites and nest number sampled (in parenthesis) for *Plebeia remota*, *Partamona mulata*, and *Partamona helleri*.

Species	Collecting site and nest number
P. remota	Cunha - SP (18), Prudentópolis - PR (35), Curitiba - PR (7), Blumenau - SC (7), Ibirama - SC (4), and Leoberto Leal - SC (1)
P. mulata	Cáceres - MT (13), Cuiabá - MT (12), Poconé - MT (14), Santo Antônio do Leverger - MT (6), and Campo Grande - MS (13)
P. helleri	Blumenau - SC (8), Luiz Alves - SC (2), Biritiba Mirim - SP (2), São Paulo - SP (11), Porto Firme - MG (3), Viçosa - MG (8), São Miguel do Anta - MG (1), Colatina - ES (9), Ilhéus - BA (1), and Coaraci - BA (2)

Table 2. PCR conditions for the microsatellite loci analyzed and their respective repeats. Nc: number of cycles. Ta: annealing temperature.

Loci	Repeat	*Plebeia remota*		*Partamona mulata*		*Partamona helleri*	
		Nc	Ta (°C)	Nc	Ta (°C)	Nc	Ta (°C)
[1]*Mbi28AAG*	$(TCC)_6ACC(TCC)_3$	-		30	60.0	30	60.0
[1]*Mbi32GAG*	$(GGA)_4(GGAGAA)_5$	-		30	65.0	30	65.0
[1]*Mbi33AAG*	$TTC(TCC)_2TCTTCC$ $(TCT)_2(TCC)_3$	30	64.0	-		30	65.0
[1]*Mbi201AAG*	$(CTT)_{10}CTC(CTT)_5$ $CCT(CTT)_2$	-		40	65.0	30	65.0
[1]*Mbi215AAG*	$(TTC)_6$	30	65.0	30	65.0	30	65.0
[1]*Mbi218AAG*	$(CCT)_3(TCT)_7$	-		30	65.0	30	65.0
[1]*Mbi254AAG*	$(AAG)_{11}$	30	55.0	-		-	
[1]*Mbi259AAG*	$(AGG)(AGA)_5(GGA)_2$ $GAA(GGA)_2$	35	63.0	-		-	
[1]*Mbi278AAG*	$CTT(CTC)_2CTTCTCTGCT$ $TCC(TCT)_9CCTTCG(TCT)_2$	30	65.0	30	65.0	30	65.0
[1]*Mbi522CAG*	$(TGC)_4TGT(TGC)_3$	-		35	65.0	45	64.0
[2]*T3-32*	$(CT)_{21}$	35	62.5	30	65.0	-	
[2]*T4-171*	$(CT)_{19}$	-		40	55.0	40	55.0
[2]*T7-5*	$(CT)_{20}$	30	65.0	-		-	

[1]Peters *et al*. 1998; [2]Paxton *et al*. 1999a; -: not attempted.

from *S. postica* (Paxton *et al*. 1999a). All individuals of *P. helleri* were heterozygotes for the locus *T4-171*. The locus *T7-5* was monomorphic in *P. remota* and, in general, no high number of alleles was found in any species but in *S. postica* and *P. mulata*.

The number of individuals and nests did not interfere in the number of alleles found (Tables 3 and 4), although, according to Nei (1987), a high number of alleles is detected when high number of individuals is analyzed. In general, the number of alleles and the H_O values were higher on the species for which the microsatellite primers were designed from.

As already mentioned, perfect microsatellites have more alleles than interrupted ones with a similar number of repeats (Chung *et al*. 1993, Pépin *et al*. 1995). The interruptions seem to stabilize the microsatellites, diminishing the error possibility during the replication (Weber 1990). However, two loci (*Mbi259AAG* and *Mbi278AAG*) classified as compound and interrupted, presented the highest number of alleles in *Plebeia remota.* In this species, the loci *Mbi254AAG* and *T7-5* (perfects in the original species) were monomorphic.

The alleles from loci *T3-32*, *T4-171*, and *T7-5* are two bases repeat. According to Chakraborty *et al*. (1997), this type of repeat evolves in a higher rate than those of three and four bases. This means that these loci should have more alleles than the others analyzed. In fact this is observed for *P. helleri* and *P. mulata*, and also in *S. postica* (Paxton *et al*. 1999a).

In a general view, none of the three species studied here showed high level of polymorphism for the primers designed for *M. bicolor*. Nonetheless for the primers derived from *S. postica*, *P. remota* was the only species that did not present high level of polymorphism.

One factor that leads to a low level of polymorphism or to a heterozygote deficit would be the occurrence of null alleles (Callen *et al*. 1993). The use of heterospecfic primers may contribute to this (Pépin *et al*. 1995). According to Callen *et al*. (1993), null alleles and population subdivision are the main factors that lead to a heterozygotes deficiency under the premises of Hardy-Weinberg equilibrium.

Size homoplasy can lead to polymorphism reduction either. Viard *et al*. (1998) observed that in *A. mellifera* and *B. terrestris*, size homoplasy is more common between than within populations. These authors showed also that the presence of size homoplasy can modify phylogenetic reconstructions. According to Estoup *et al*. (1995) not perfect microsatellites would be less susceptible to size homoplasy and more polymorphic than the perfect ones. This latter evidence contradicts other authors (Chung *et al*. 1993, Pépin *et al*. 1995). Considering that the loci amplified for the species studied here present the same repeat characteristics as described in the original species, the results obtained from *P. remota* are in agreement with postulated by Estoup *et al*. (1995), and the results from *P. mulata* and *P. helleri* are in concordance with Chung *et al*. (1993) and Pépin *et al*. (1995).

Nowadays, microsatellites are being applied in a great variety of studies mainly because of their high

Table 3. Observed heterozygosity (H_O) and number of alleles (in parenthesis) per *Mbi* microsatellite loci (Peters *et al*. 1998) for seven stingless bee species. Ni: Number of individuals analyzed. Nn: Number of nests analyzed.

Species	*Mbi28AAG*	*Mbi32GAG*	*Mbi33AAG*	*Mbi201AAG*	*Mbi215AAG*	*Mbi218AAG*
[1]*Plebeia remota*	-	-	0.00 (2)	-	0.41 (2)	-
[1]*Partamona mulata*	0.00 (1)	0.00 (1)	-	0.01 (2)	0.00 (1)	0.00 (1)
[1]*Partamona helleri*	0.00 (1)	0.04 (2)	0.02 (2)	0.00 (1)	0.34 (2)	0.00 (2)
[2]*Melipona bicolor*	0.63 (5)	0.63 (4)	0.00 (1)	0.75 (5)	0.50 (3)	0.12 (3)
[2]*Melipona quadrifasciata*	0.00 (2)	0.00 (1)	0.00 (1)	0.75 (2)	0.00 (1)	0.25 (2)
[2]*Scaptotrigona postica*	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	-
[2]*Tetragona clavipes*	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	0.00 (1)	-

Species	*Mbi254AAG*	*Mbi259AAG*	*Mbi278AAG*	*Mbi522CAG*	Ni	Nn
[1]*Plebeia remota*	0.00 (1)	0.65 (6)	0.16 (6)	-	360	72
[1]*Partamona mulata*	-	-	0.06 (2)	0.00 (2)	58	58
[1]*Partamona helleri*	-	-	0.49 (2)	0.00 (1)	47	47
[2]*Melipona bicolor*	0.38 (3)	0.12 (2)	0.86 (5)	0.00 (1)	8	8
[2]*Melipona quadrifasciata*	1.00 (3)	0.50 (2)	0.01 (1)	0.00 (1)	4	2
[2]*Scaptotrigona postica*	0.50 (5)	0.50 (3)	0.25 (2)	0.00 (1)	4	4
[2]*Tetragona clavipes*	0.00 (1)	0.75 (3)	0.25 (2)	0.00 (1)	4	4

[1]This work; [2]Peters *et al*. 1998; -: not attempted.

polymorphism, however the effectiveness of this marker depends on H_O values. Several evolutionary inferences like extinction probability or effective population size are made based on these values. Therefore, it is important to be aware if heterozygote lack is a reflex of the natural population history or just an "artifact" due to the marker chosen.

We conclude that heterospecific primers can be used but with caution since the genetic variability found here, for the three stingless bees, was low when compared to the polymorphism detected in other organisms by using homospecific primers (Simonsen *et al*. 1998, Widmer *et al*. 1998, Franck *et al*. 2001). Despite the low variability detected, this molecular marker is already contributing to our understanding of genetic structure and dynamics of natural populations of stingless bees (data not shown). The design of homospecific primers, which implies in genomic library construction, and also additional data from other molecular markers such as RFLP of mitochondrial DNA, would certainly improve the knowledge of population dynamics and evolution of stingless bees.

Table 4. Observed heterozygosity (H_O) and number of alleles (in parenthesis) per T microsatellite loci (Paxton *et al*. 1999a) for ten stingless bee species. Ni: Number of individuals analyzed. Nn: Number of nests analyzed.

Species	*T3-32*	*T4-171*	*T7-5*	Ni	Nn
[1]*Plebeia remota*	0.34 (2)	-	0.00 (1)	353	71
[1]*Partamona mulata*	0.57 (5)	0.64 (6)	-	58	58
[1]*Partamona helleri*	-	1.00 (4)	-	47	47
[2]*Scaptotrigona postica*	0.72 (5)	0.94 (6)	0.78 (6)	18	18
[2]*Scaptotrigona pectoralis*	(2)	(2)	(2)	2	1
[2]*Scaptotrigona tubiba*	(2)	(2)	(2)	2	1
[2]*Lestrimellita limao*	(2)	(1)	(?)	2	1
[2]*Melipona beechei*	(?)	(2)	(3)	99	10
[2]*Nannotrigona* sp.	(1)	(1)	(?)	2	1
[2]*Trigona nigra*	(1)	(1)	(1)	2	1

[1]This work; [2]Paxton *et al*. 1999a; -: not attempted; (?): no product, or multiple bands.

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