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Short Communication

Molecular genetic diversity in populations of the stingless bee *Plebeia remota*: A case study

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Abstract

Genetic diversity is a major component of the biological diversity of an ecosystem. The survival of a population may be seriously threatened if its genetic diversity values are low. In this work, we measured the genetic diversity of the stingless bee *Plebeia remota* based on molecular data obtained by analyzing 15 microsatellite loci and sequencing two mitochondrial genes. Population structure and genetic diversity differed depending on the molecular marker analyzed: microsatellites showed low population structure and moderate to high genetic diversity, while mitochondrial DNA (mtDNA) showed high population structure and low diversity in three populations. Queen philopatry and male dispersal behavior are discussed as the main reasons for these findings.

Keywords: mtDNA, Meliponini, microsatellites, philopatry, population genetics.

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Most of the Brazilian tropical flora is pollinated by bees, especially by those belonging to the tribe Meliponini (stingless bees) (Kerr *et al.*, 1996; Nogueira-Neto, 1997). The increase in habitat loss can lead to severe consequences for bee populations and species diversity (Foley *et al.*, 2005; Brown and Paxton, 2009). Studies focusing on Meliponini general biology, including genetic diversity, are still scarce in the literature. Genetic data are essential for a better understanding of macro- and micro-evolutionary processes and patterns in organisms, and provide support for conservation and managing programs (Moritz, 2002; Frankham *et al.*, 2004).

Recent studies have indicated a low genetic diversity in feral populations of Brazilian stingless bees (Costa *et al.*, 2005; Arias *et al.*, 2006; Tavares *et al.*, 2007; Borges *et al.*, 2010; Brito and Arias, 2010; Francisco and Arias, 2010). This low genetic diversity may have negative consequences for the long-term population survival rate and raises important questions related to conservation programs for the Meliponini.

An adequate understanding of how genetic diversity is distributed and maintained among stingless bee populations requires a consideration of behavioral components such as philopatry. Philopatry may restrict individual dispersion, leading to inbreeding, which consequently reduces heterozygosity; philopatry also increases the effects of genetic drift due to population subdivision and isolation. However, studies in a variety of organisms have shown that if one gender is philopatric then the other one normally mediates gene flow through dispersion (Whitehead, 1998; Nyakaana and Arctander, 1999; Kappeler *et al.*, 2002; Apio *et al.*, 2010). This behavioral mechanism minimizes the negative effects of philopatry.

It has already been demonstrated in some stingless bee species that the queen is philopatric (Nogueira-Neto, 1954; Engels and Imperatriz-Fonseca, 1990) and males are the dispersing sex (Carvalho-Zilse and Kerr, 2004; Cameron *et al.*, 2004). Despite this dispersal by males, studies based on allozyme analysis (Tavares *et al.*, 2007; Costa *et al.*, 2005), RAPD (Tavares *et al.*, 2007), mtDNA RFLP (Brito and Arias, 2010; Francisco and Arias, 2010) and microsatellites (Francisco *et al.*, 2006; Tavares *et al.*, 2007; Carvalho-Zilse *et al.*, 2009; Borges *et al.*, 2010) have revealed a low genetic diversity in Brazilian stingless bee populations.

The stingless bee *Plebeia remota* occurs in Bolivia and southeastern and southern Brazil (Camargo and Pedro, 2012). This species generally builds its nests in tree cavities, with colonies of up to 5,000 bees (van Benthem *et al.*, 1995); the workers are small (~0.5 cm in length) (Hilário *et al.*, 2007). In a previous investigation, Francisco and Arias (2010) described low intrapopulation mitochondrial polymorphism for this species. In the present work, we reanalyzed most of those samples to measure nuclear genetic diversity based on the amplification of microsatellite loci with specific primers and also by sequencing two mitochondrial genes.

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One worker bee from each of 65 nests was analyzed for nuclear and mitochondrial loci. The samples originated from four localities (referred to from here on as "populations"): Cunha in São Paulo state (n = 13), Curitiba (n = 6) and Prudentópolis (n = 34) in Paraná state and Blumenau (n = 12) in Santa Catarina state (Figure 1). Total DNA was extracted using Chelex® 100 (Bio-Rad) according to a protocol described by Walsh *et al.* (1991). All individuals were genotyped for 15 microsatellite loci (Francisco *et al.*, 2011): Prem03, Prem07, Prem57, Prem58, Prem70, Prem75a, Prem78, Prem79, Prem81a, Prem82, Prem83, Prem84, Prem87, Prem93 and Prem94. Microsatellite amplification and visualization were done as described by Francisco *et al.* (2011). Allelic richness (*A*), observed and expected heterozygosities (H_O and H_E, respectively) from Hardy-Weinberg proportions, percentage of polymorphic loci and allele frequencies were calculated for each population using Genalex v.6.41 (Peakall and Smouse, 2006). Due to differences in sample size, rarefaction was applied to allelic richness (*Ar*) by using the program HP-Rare 1.0 (Kalinowski, 2005). Log likelihood ratio statistics for linkage disequilibrium were computed using Genepop v.4.1.4 (Rousset, 2008). The Bonferroni correction (Rice, 1989) was applied when multiple comparisons were done. Population structure was analyzed with the program Structure v.2.3.3 (Pritchard *et al.*, 2000). The program was set up for 500,000 Markov chain Monte Carlo repetitions after an initial burn-in of 20,000 repetitions. The number of structured populations (K) was estimated based on 10 replications for each K (from 1 to 4). The estimate of the best K was calculated as described by Evanno *et al.* (2005) using Structure Harvester v.0.6.92 (Earl and VonHoldt, 2012). The program Clumpp v.1.1.2 (Jakobsson and Rosenberg, 2007) was used to align the 10 repetitions of the best K. The program Distruct v.1.1 (Rosenberg, 2004) was used to graphically display the results produced by Clumpp. Population structure was also analyzed using the D_{est} estimator (Jost, 2008) which was calculated for each population pair by the program SMOGD v.1.2.5 (Crawford, 2010).

Two mitochondrial genes, cytochrome c oxidase subunit I (*COI*) and cytochrome b (*Cytb*), were partially amplified by using the primers mtD06 + mtD09 (Simon *et al.*, 1994) and mtD26 (Simon *et al.*, 1994) + AMB16 (Arias *et al.*, 2008), respectively. PCR assays were done with 1 µL of DNA, 1x PCR buffer, 200 µM of each dNTP, 3 mM of $MgCl_2$, 0.8 µM of each primer, 1 M of betaine anhydrous (USB Corporation) and 1 U of *Taq* DNA polymerase (Invitrogen) in a final volume of 10 µL. The amplification conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 42 °C for 60 s and elongation at 64 °C for 80 s, and a final elongation step at 64 °C for 10 min. PCR products (2 µL aliquots) were analyzed by electrophoresis in 0.8% agarose gels stained with GelRed (Biotium) and visualized under UV light. About 100-200 ng of each product was purified with 0.5 µL of ExoSAP-IT(USB Corporation) and used for sequencing reactions according to the manufacturer's recommended protocols (BigDye Terminator v.3.1 Cycle sequencing kit, Applied Biosystems). The sam-

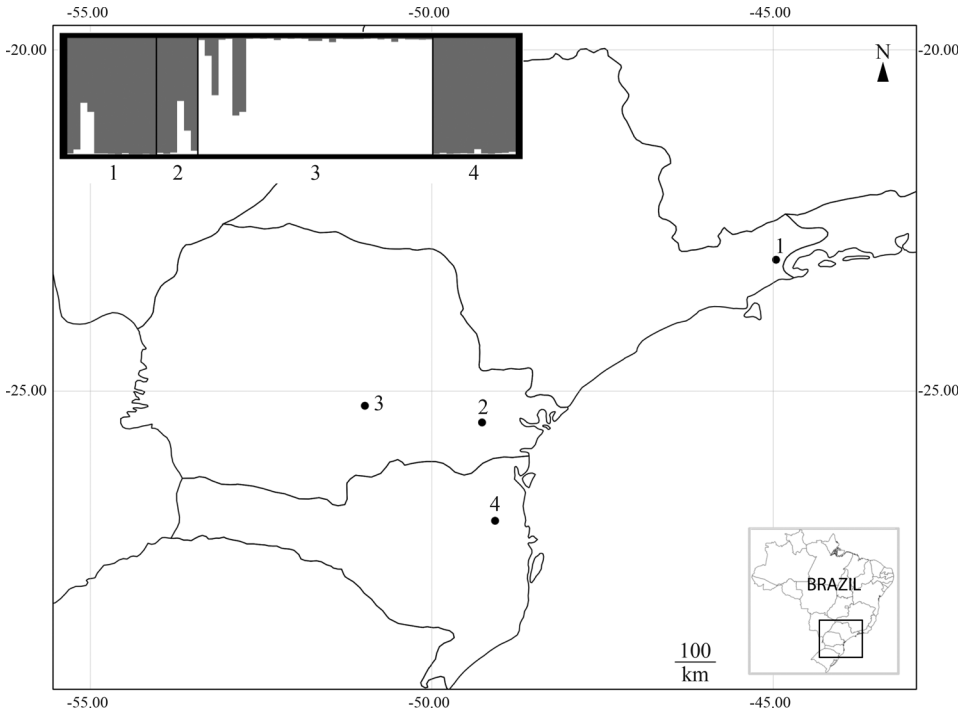

Figure 1 - Geographic location of *Plebeia remota* populations and graphic display of the Structure results. 1: Cunha (n = 13), 2: Curitiba (n = 6), 3: Prudentópolis (n = 34) and 4: Blumenau (n = 12).

ples were analyzed in an automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). DNA sequences were edited using the Geneious v.5.1.6 software package (Drummond *et al.*, 2010). The alignment was done using the algorithm Muscle (Edgar, 2004) from Geneious, with a maximum number of eight iterations. DnaSP v.5.10.01 software (Librado and Rozas, 2009) was used to identify individual haplotypes and their frequencies. A haplotype network was generated with the software Network v.4.6.1.0. Exact tests between pairs of populations were done using Arlequin v.3.5.1.3 (Excoffier and Lischer, 2010).

All microsatellite loci analyzed were polymorphic. The allele frequencies for each locus and for each population are included in the Supplementary material to this paper (Table S1). The intrapopulation genetic diversity indices ranged from moderate to high (Table 1). No significant linkage disequilibrium was detected after Bonferroni correction for each pair of loci tested. Population structure results divided the four populations into two clusters: [Cunha-Curitiba-Blumenau] and [Prudentópolis] (Figure 1, Table S2).

A total of 794 bp (415 from *COI* and 379 bp from *Cytb*) was obtained for all individuals (GenBank accession numbers JQ517144-JQ517273). Ten haplotypes were identified, most of which were exclusive to a specific population, except for one (h04) (Table 2). Table 2 also shows the haplotype and nucleotide diversity indices; they were not correlated to sample size. Figure 2 shows the network built to represent the associations between haplotypes and the genetic differentiation among populations. The maximum number of nucleotide differences between two haplotypes was 14 (1.8%). Exact tests based on haplotype frequencies showed differentiation between all population pairs (all $p < 0.0005$).

Population structure and genetic diversity varied, depending on the molecular marker analyzed. The microsatellite data showed a low population structure and moderate to high genetic diversity, whereas the mtDNA data showed a high population structure and low diversity in three populations. The mtDNA data suggested an absence of female gene flow among the populations, and reinforced the philopatric behavior of queens and its strong influence on the genetic differentiation observed. These findings agree with previous data obtained by RFPL of mtDNA that also showed no gene flow through females (Francisco and Arias, 2010).

Table 2 - Frequency and distribution of mtDNA haplotypes identified in *Plebeia remota.*

Haplotype	Cunha (13)	Curitiba (6)	Prudentópolis (34)	Blumenau (12)
h01	-	1	-	-
h02	-	1	-	-
h03	-	2	-	-
h04	13	1	-	-
h05	-	1	-	-
h06	-	-	23	-
h07	-	-	1	-
h08	-	-	8	-
h09	-	-	2	-
h10	-	-	-	12
NH	1	5	4	1
h	0.00	0.93	0.50	0.00
π	0.00000	0.00420	0.00068	0.00000

The number of individuals analyzed is indicated in parentheses. NH: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity.

The level of intrapopulation nuclear diversity was moderate to high. The Prudentópolis population had the lowest genetic diversity index, which suggested genetic isolation. The nuclear data also indicated a low population structure among the Cunha, Curitiba, and Blumenau populations. An absent or low genetic structure can be attributed to homoplasy in microsatellite size but should be accompanied by a decrease in genetic variability (Estoup *et al.*, 2002), which was not the case here.

Since female philopatry was detected in these populations, the absence of genetic structure can be explained by male dispersal. The few genetic studies of Meliponini male congregations have demonstrated the presence of males from distant areas, with more than 100 colonies acting as male donors (Paxton, 2000; Cameron *et al.*, 2004; Kraus *et al.*, 2008; Mueller *et al.*, 2012).

Table 1 - Genetic diversity for each population of *Plebeia remota* based on microsatellite data.

Population	N	*A*	*Ar*	H_O	H_E	PPL
Cunha	13	7.667 (± 0.866)	5.731 (± 0.447)	0.749 (± 0.044)	0.772 (± 0.029)	100.00%
Curitiba	6	5.933 (± 0.679)	5.933 (± 0.679)	0.644 (± 0.067)	0.692 (± 0.049)	100.00%
Prudentópolis	34	6.600 (± 1.041)	4.110 (± 0.522)	0.551 (± 0.078)	0.582 (± 0.076)	93.33%
Blumenau	12	6.400 (± 0.920)	4.799 (± 0.629)	0.600 (± 0.090)	0.605 (± 0.082)	86.67%
Mean	16.3 (± 1.4)	6.650 (± 0.439)	5.143 (± 0.297)	0.636 (± 0.036)	0.663 (± 0.032)	95.00% (± 3.19%)

A: allelic richness; *Ar*: allelic richness after rarefaction for six individuals; H_O and H_E: observed and expected heterozygosity from Hardy-Weinberg proportions, respectively; N: sample size; PPL: percentage of polymorphic loci. Values in parentheses are standard errors.

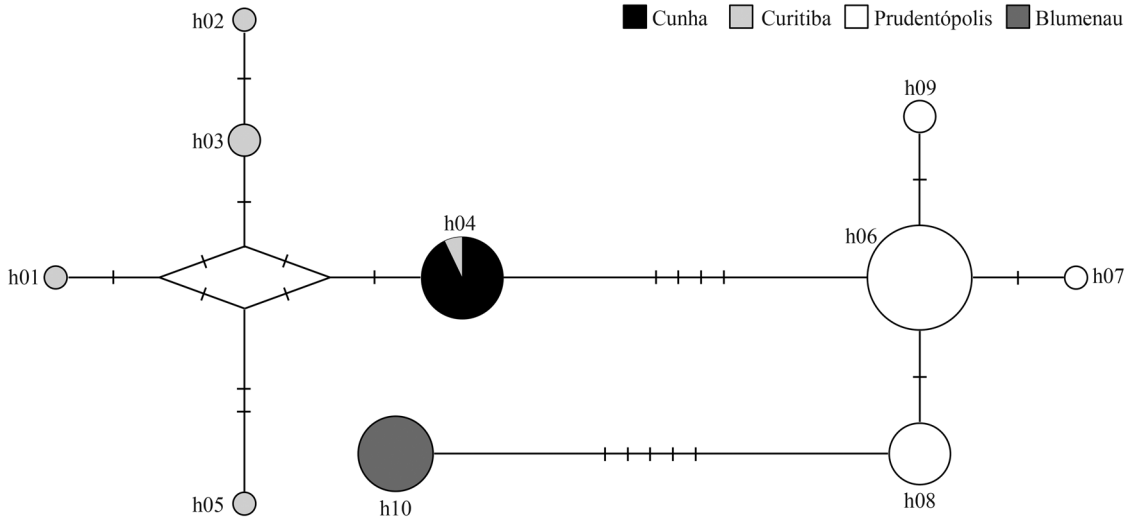

Figure 2 - mtDNA haplotype network for *Plebeia remota*.

Thus, the divergence between mitochondrial and nuclear data is a consequence of the reproductive behavior of *P. remota*. The low mtDNA variability indicates a low dispersal capability of females, *i.e.*, queen philopatry. In contrast, the high nuclear genetic variability is maintained by male dispersal. This male behavior is crucial to avoid inbreeding and to keep the population genetically healthy. Since Meliponini species show queen philopatry (Nogueira-Neto, 1954; Engels and Imperatriz-Fonseca, 1990), we expect a similar genetic scenario in other species.

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Supplementary Material

The following online material is available for this article:

Table S1 – Allele size and frequency for each locus scored in four populations of *Plebeia remota*.

Table S2 – D_{est} values for each population pair of *Plebeia remota*.

This material is available as part of the online article from http://www.scielo.br/gmb.

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Table S1 - Allele size and frequency for each locus scored in four populations of *Plebeia remota*.

Locus	Allele	Cunha	Curitiba	Prudentópolis	Blumenau
Prem03	**107**	0.154	0.000	0.000	0.000
	115	0.192	0.000	0.000	0.000
	117	0.077	0.000	0.000	0.000
	119	0.385	0.833	0.632	1.000
	121	0.192	0.167	0.368	0.000
Prem07	**330**	0.038	0.000	0.000	0.000
	332	0.077	0.000	0.000	0.000
	334	0.115	0.000	0.000	0.000
	336	0.038	0.000	0.000	0.042
	338	0.038	0.083	0.000	0.000
	340	0.154	0.000	0.000	0.000
	344	0.115	0.083	0.000	0.000
	346	0.077	0.000	0.015	0.167
	348	0.000	0.000	0.059	0.000
	350	0.000	0.000	0.103	0.000
	352	0.038	0.000	0.000	0.042
	354	0.038	0.167	0.015	0.083
	356	0.000	0.000	0.029	0.208
	358	0.038	0.000	0.000	0.125
	359	0.038	0.083	0.162	0.083
	360	0.000	0.000	0.015	0.000
	361	0.038	0.000	0.074	0.042
	362	0.077	0.083	0.044	0.042
	364	0.038	0.000	0.221	0.000
	366	0.000	0.083	0.132	0.000
	368	0.000	0.083	0.132	0.000
	370	0.000	0.000	0.000	0.083
	372	0.000	0.083	0.000	0.000
	374	0.000	0.083	0.000	0.000
	376	0.000	0.083	0.000	0.000
	378	0.000	0.083	0.000	0.042
	380	0.038	0.000	0.000	0.000
	393	0.000	0.000	0.000	0.042
Prem57	**152**	0.038	0.000	0.191	0.042
	154	0.192	0.000	0.000	0.000
	156	0.000	0.083	0.044	0.042
	162	0.077	0.000	0.000	0.000
	164	0.077	0.000	0.000	0.000
	166	0.000	0.000	0.015	0.042
	168	0.000	0.250	0.015	0.042
	170	0.077	0.000	0.000	0.000

	172	0.038	0.167	0.000	0.000
	174	0.077	0.083	0.074	0.083
	176	0.115	0.000	0.176	0.083
	178	0.038	0.167	0.000	0.250
	180	0.000	0.000	0.015	0.250
	182	0.077	0.083	0.044	0.083
	184	0.038	0.000	0.265	0.083
	186	0.000	0.083	0.088	0.000
	188	0.077	0.000	0.044	0.000
	190	0.000	0.000	0.029	0.000
	192	0.000	0.083	0.000	0.000
	196	0.077	0.000	0.000	0.000
Prem58	**160**	0.000	0.000	0.029	0.000
	162	0.000	0.083	0.000	0.542
	164	0.000	0.000	0.000	0.042
	166	0.038	0.000	0.000	0.000
	168	0.154	0.083	0.059	0.292
	170	0.231	0.500	0.000	0.042
	172	0.269	0.167	0.118	0.083
	174	0.192	0.083	0.721	0.000
	176	0.115	0.000	0.059	0.000
	178	0.000	0.083	0.015	0.000
Prem70	**106**	0.346	0.000	0.000	0.042
	108	0.038	0.000	0.000	0.000
	110	0.115	0.000	0.191	0.042
	112	0.077	0.417	0.206	0.500
	114	0.077	0.583	0.603	0.333
	116	0.269	0.000	0.000	0.083
	118	0.077	0.000	0.000	0.000
Prem75a	**185**	0.000	0.000	0.000	0.125
	187	0.154	0.000	0.000	0.000
	189	0.115	0.000	0.000	0.000
	191	0.000	0.167	0.412	0.000
	193	0.269	0.083	0.000	0.042
	195	0.231	0.250	0.176	0.083
	197	0.038	0.167	0.029	0.167
	199	0.000	0.000	0.044	0.250
	201	0.000	0.000	0.015	0.083
	203	0.077	0.083	0.147	0.000
	205	0.000	0.000	0.000	0.083
	209	0.038	0.000	0.029	0.042
	214	0.038	0.000	0.000	0.000
	215	0.000	0.000	0.132	0.042

	217	0.038	0.000	0.000	0.083
	219	0.000	0.167	0.000	0.000
	221	0.000	0.083	0.000	0.000
	225	0.000	0.000	0.015	0.000
Prem78	**176**	0.269	0.000	0.426	0.125
	178	0.192	0.167	0.000	0.125
	180	0.077	0.250	0.000	0.125
	184	0.154	0.000	0.000	0.000
	186	0.000	0.167	0.000	0.000
	190	0.000	0.000	0.000	0.042
	192	0.000	0.167	0.015	0.167
	194	0.000	0.083	0.103	0.167
	196	0.115	0.000	0.279	0.083
	198	0.000	0.000	0.059	0.083
	200	0.192	0.000	0.118	0.000
	202	0.000	0.083	0.000	0.083
	207	0.000	0.083	0.000	0.000
Prem79	**209**	0.000	0.000	0.000	0.042
	216	0.231	0.750	1.000	0.958
	218	0.269	0.083	0.000	0.000
	220	0.308	0.083	0.000	0.000
	222	0.192	0.083	0.000	0.000
Prem81a	**123**	0.038	0.000	0.059	0.000
	127	0.000	0.000	0.147	0.000
	129	0.038	0.000	0.088	0.500
	131	0.038	0.000	0.029	0.208
	133	0.077	0.167	0.103	0.125
	135	0.000	0.250	0.235	0.000
	137	0.154	0.083	0.044	0.000
	139	0.346	0.333	0.000	0.000
	141	0.038	0.000	0.015	0.000
	143	0.269	0.167	0.132	0.000
	145	0.000	0.000	0.088	0.125
	147	0.000	0.000	0.044	0.042
	151	0.000	0.000	0.015	0.000
Prem82	**145**	0.115	0.417	0.162	0.083
	149	0.115	0.083	0.000	0.000
	150	0.000	0.000	0.162	0.000
	151	0.077	0.083	0.000	0.042
	153	0.385	0.083	0.044	0.292
	154	0.077	0.167	0.059	0.583
	155	0.115	0.083	0.529	0.000

	157	0.115	0.083	0.044	0.000
Prem83	**208**	0.000	0.000	0.015	0.083
	217	0.000	0.083	0.000	0.000
	221	0.115	0.000	0.015	0.000
	223	0.038	0.000	0.000	0.125
	225	0.231	0.000	0.044	0.042
	227	0.038	0.000	0.147	0.042
	229	0.038	0.417	0.147	0.083
	231	0.077	0.167	0.324	0.208
	233	0.115	0.250	0.074	0.167
	235	0.077	0.083	0.044	0.042
	237	0.192	0.000	0.015	0.042
	239	0.077	0.000	0.044	0.125
	241	0.000	0.000	0.103	0.042
	243	0.000	0.000	0.029	0.000
Prem84	**166**	0.308	0.083	0.000	0.167
	168	0.231	0.583	0.441	0.292
	170	0.192	0.000	0.235	0.292
	172	0.000	0.083	0.000	0.042
	174	0.000	0.167	0.000	0.000
	176	0.038	0.083	0.000	0.083
	178	0.000	0.000	0.029	0.000
	180	0.038	0.000	0.191	0.125
	182	0.077	0.000	0.000	0.000
	186	0.077	0.000	0.000	0.000
	188	0.038	0.000	0.103	0.000
Prem87	**126**	0.038	0.000	0.000	0.000
	128	0.000	0.000	0.162	0.000
	130	0.077	0.000	0.235	0.000
	132	0.577	0.167	0.015	0.000
	134	0.115	0.250	0.147	0.000
	136	0.077	0.167	0.294	0.083
	138	0.038	0.083	0.103	0.458
	140	0.000	0.000	0.000	0.125
	142	0.000	0.167	0.015	0.125
	144	0.000	0.000	0.000	0.042
	146	0.000	0.000	0.000	0.125
	154	0.077	0.000	0.000	0.000
	156	0.000	0.000	0.029	0.000
	162	0.000	0.083	0.000	0.000
	164	0.000	0.000	0.000	0.042
	176	0.000	0.083	0.000	0.000

Prem93	**106**	0.692	0.167	0.000	0.000
	108	0.269	0.667	0.912	0.000
	110	0.038	0.167	0.088	1.000
Prem94	**189**	0.269	0.167	0.971	0.542
	191	0.038	0.083	0.015	0.125
	193	0.346	0.083	0.000	0.000
	195	0.115	0.000	0.000	0.000
	197	0.115	0.000	0.000	0.000
	199	0.115	0.000	0.015	0.125
	201	0.000	0.083	0.000	0.042
	207	0.000	0.167	0.000	0.042
	210	0.000	0.000	0.000	0.042
	214	0.000	0.000	0.000	0.042
	216	0.000	0.000	0.000	0.042
	220	0.000	0.083	0.000	0.000
	222	0.000	0.083	0.000	0.000
	224	0.000	0.083	0.000	0.000
	225	0.000	0.083	0.000	0.000
	226	0.000	0.083	0.000	0.000

Table S2 - D_{est} values for each population pair of *Plebeia remota*.

	Cunha	Curitiba	Prudentópolis	Blumenau
Cunha	0.00			
Curitiba	0.25	0.00		
Prudentópolis	0.49	0.21	0.00	
Blumenau	0.43	0.20	0.32	0.00