

# Mitochondrial DNA variation in two Brazilian populations of *Cochliomyia macellaria* (Diptera: Calliphoridae)

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## ABSTRACT

Restriction fragment analysis of mitochondrial DNA (mtDNA) was used to examine genetic variation of two populations of the blow fly *Cochliomyia macellaria* (Diptera: Calliphoridae) from southeastern Brazil. All 202 individuals were digested with 12 restriction endonucleases and probed with the entire mitochondrial genome of *Cochliomyia hominivorax*. Two endonucleases, *EcoRV* and *HindIII*, revealed polymorphism, yielding seven haplotypes. Individuals heteroplasmic for restriction patterns were found in the populations. Crosses between the two populations were fertile and produced fertile progeny.

## INTRODUCTION

*Cochliomyia* is an endemic New World genus containing four distinct species. Two of these, *Cochliomyia hominivorax* and *Cochliomyia macellaria* range from southern Canada to Argentina, but are especially abundant in the tropical regions (Ferreira, 1983; Baumgartner and Greenberg, 1985; Dear, 1985). The taxonomy of the species in this genus has been very confused, mainly because of misidentifications, and confusion of the screwworm fly, *C. hominivorax*, which is an obligatory wound parasite and *C. macellaria*, a carrion breeder which is a secondary agent of myiasis and a purely saprophagous species, that is perhaps the most widely distributed calliphorid in the Americas (Hall, 1948).

The adults of *C. macellaria* are attracted to a wide variety of substrates for food or reproduction, such as urban garbage, human and livestock feces and wounds infested with dipteran larvae or not (Zumpt, 1965; Paraluppi, 1992). This species has been implicated as a mechanical vector of human and animal diseases.

Recently, the Western Hemisphere has been invaded by four Old World blow flies of the genus *Chrysomya* (Robineau-Desvoid): *C. albiceps* (Wiedmann), *C. putoria* (Wiedmann), *C. megacephala* (Fabricius) and *C. rufifacies* (Macquart) (Guimarães *et al.*, 1978, 1979; Prado and Guimarães, 1982; Baumgartner and Greenberg, 1984; Laurence, 1986; Baumgartner, 1988). These species, particularly *C. albiceps* and *C. putoria*, occupy the habitats of the native species, including *C. macellaria*, providing interspecific competition, and have caused a population reduction and movement of *C. macellaria* in several regions of Brazil and Peru (Guimarães *et al.*, 1979; Linhares, 1981; Prado and Guimarães, 1982; Madeira *et al.*, 1982; Ferreira, 1983; Baumgartner and

Greenberg, 1984; Wiegand *et al.*, 1991; Paraluppi, 1992). Despite strong competition with these introduced species, *C. macellaria* remains at a low frequency (Ferreira, 1983).

At the genetic level, investigations of natural populations of *C. macellaria* have been limited to karyotypic characterization (Boyes, 1961; Boyes and VanBrink, 1965; Boyes and Shewell, 1975; Azeredo-Espin, 1982).

This fly is a Neotropical species, with sinantropic behavior and is important for public health. Investigation of genetic variability among populations of *C. macellaria* would help understand historical patterns of dispersal and current levels of gene flow.

## MATERIAL AND METHODS

Adult *C. macellaria* were collected directly on bovine carcass, near slaughter houses and local markets, with the help of entomological nets in Caraguatatuba (sample Ca-1), São Paulo State (Lat. 23°37'13"/Long. 45°24'47") and Itaboraí (sample RJ-1) on the campus of the Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro state (Lat. 22°44'40"/Long. 42°51'34") (Figure 1).

The flies were maintained in the laboratory in cages (34x50x26 cm) and provided a diet containing the same proportion of powdered milk and sugar and 10% of yeast ferment. The cages were placed in a room at 25 ± 1°C, 70% of relative humidity and a 12:12 hour photoperiod. The flies were allowed to oviposit on fresh

mouse carcass in a Petri dish inside the cage. The eggs were transferred to a plastic box with mouse carcasses and sawdust, and maintained at room temperature. After eclosion the larvae remained on the carcass, which was changed each 24 hours, until they reached the pupal phase. Nucleic acid extraction was made from pupae.

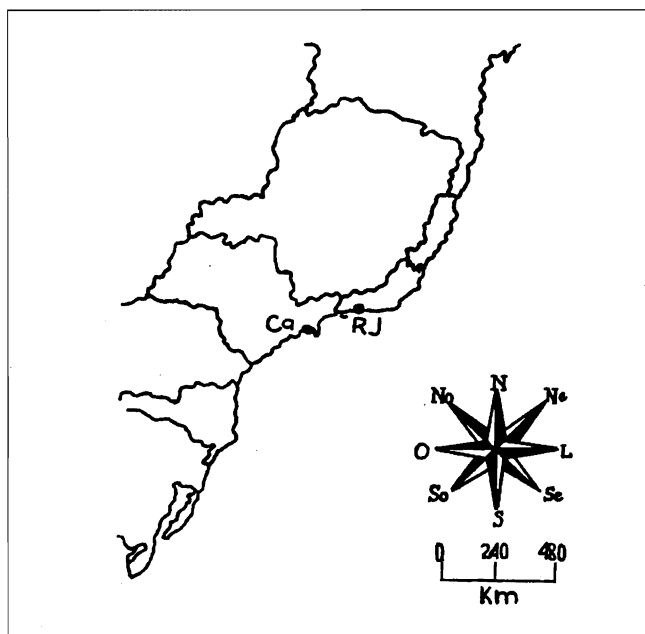
Reciprocal crosses were conducted to examine sexual compatibility between the samples of Ca-1 and RJ-1. Two sets of crosses were prepared, one cage with 20 females from Ca-1 and 20 males from RJ-1 and other cage with the reciprocal cross and the same number of individuals.

Mitochondrial DNA isolation: mtDNA was purified from individual larvae, pupae or adults as described by Azeredo-Espin *et al.*, 1991. One individual larva or pupa was gently homogenized in a 15 ml Corex tube with 1 ml of grinding buffer (10 mM TRIS, 60 mM NaCl, 300 mM sucrose, 10 mM EDTA). After this, 1 ml of lysis buffer was added (300 mM TRIS, 40 mM SDS, 20 mM EDTA) and 0.7% of DEPC. The resulting lysate was extracted once each with phenol, phenol:chlorophorm:isoamyl alcohol, and chlorophorm:isoamyl alcohol and centrifuged at 3,000 xg. The final supernatant was then ethanol precipitated at -20°C for two hours and spun at 10,000 xg to pellet the DNA. The pellet was dried under vacuum and resuspended in an appropriate amount of 1 x TE (1 mM TRIS.Cl, 0.1 mM EDTA pH 8.0).

Mitochondrial DNA restriction analysis: mtDNA was digested with restriction endonucleases according to manufacture's specifications (Bethesda Research Laboratories - BRL). The endonucleases used were: *Hind*III, *Eco*RV, *Eco*RT, *Hae*III, *Kpn*I, *Xho*I, *Xba*I, *Sst*I, *Cla*I, *Bam*HI, *Pvu*II and *Pst*I. After digestion, fragments were separated on 1% agarose gels and stained with ethidium bromide (0.1 g/ml). Phage λ DNA digested with *Hind*III and φX 174 digested with *Hae*III (BRL) were used as size standards.

Pure *C. hominivorax* mtDNA for use as a probe was obtained from pupae of an isofemale laboratory strain started from a field population (Ca sample) (Azeredo-Espin, 1993; Infante, 1994; Infante and Azeredo-Espin, 1995). mtDNA was obtained through the MIM (Mitochondrial Isolation Medium) technique according to Azeredo-Espin *et al.*, 1991, and purified via two subsequent equilibrium centrifugations in CsCl.

To determine which fragments present on the gels were mtDNA and for detecting small bands (0.5 Kpb), the fragments were transferred to nylon membranes (Hybond) through Southern Blot (Maniatis *et al.*, 1989) and hybridized with the whole mtDNA molecule of *C. hominivorax*, that was nick-translated to



**Figure 1** - Map showing geographic origin of the *Cochliomyia macellaria* populations sampled.

yield a  $^{32}\text{P}$  labeled probe. Hybridization was carried out under standard conditions (Maniatis *et al.*, 1989). Visualization of mtDNA fragments was conducted via autoradiographies based on Moritz *et al.*, 1987 and Arias, 1992.

The different restriction patterns of a given enzyme were designated by uppercase letters. The number of variant restriction sites required to account for the patterns of each enzyme was determined. The haplotypes of the sampled populations consider the two enzymes together and are denoted by letters, each corresponding to the pattern for *EcoRV* and *HindIII*, respectively.

## RESULTS AND DISCUSSION

Two out of twelve enzymes used were suitable to detect mtDNA variation between the sampled *C. macellaria* populations. A total of four restriction patterns for *EcoRV* and three for *HindIII* were observed (Figure 2). For each of the patterns obtained for these enzymes, the number of restriction fragments observed and the corresponding size of each one are shown in Table I.

The estimated size of the mtDNA of *C. macellaria* was close to 16.5 Kbp. This is similar to that obtained for *C. hominivorax* (16.3 Kbp, Roehrdanz and Johnson, 1988; Roehrdanz, 1989; Azeredo-Espin, 1993).

The populational distribution of the variant patterns for each of the enzymes are shown in Table II.

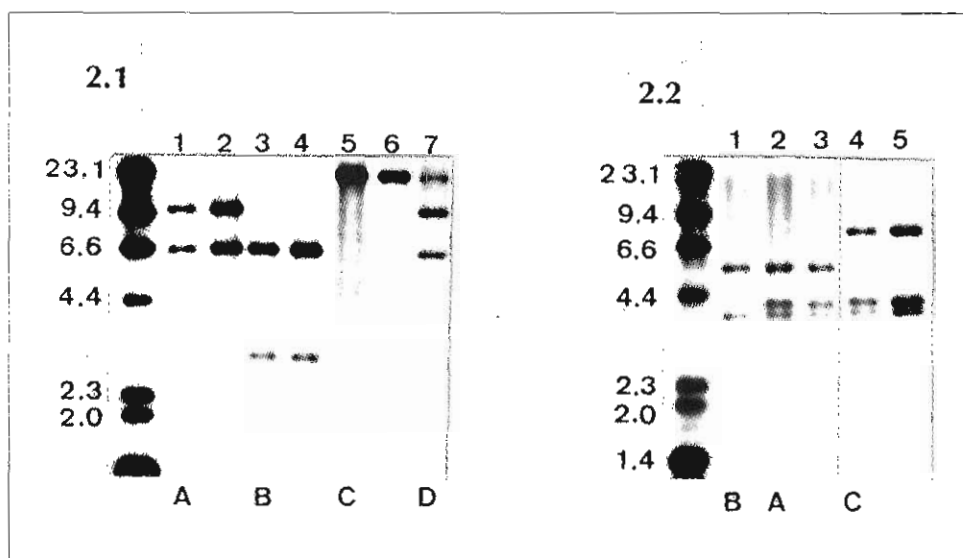
For *EcoRV*, four patterns were observed in Ca-1 (patterns A, B, C and D) and three for RJ-1 (patterns A, C and D). For *HindIII*, two patterns were found in Ca-1 (patterns A and C) and two for RJ-1 (patterns A and B).

The fragment patterns for each of the two enzymes, *EcoRV* and *HindIII*, are related to one another by simple gain or loss of a single site. Transformation series reflecting probable steps for interconversion of the restriction patterns for the two enzymes (*EcoRV* and *HindIII*) produced three pattern types for each (Figure 3).

**Table I** - Approximate sizes (in kilobases) of fragments generated by digestion of *Cochliomyia macellaria* mtDNA with restriction endonucleases. Letters designate haplotypes observed with *EcoRV* and *HindIII*.

Enzyme and pattern	Recognition site	Fragment size (Kbp)
<i>EcoRV</i>	GAT/ATC	
A		10.1, 6.6
B		6.6, 6.6, 3.0
C		17.6
D*		17.6, 10.1, 6.6
<i>HindIII</i>	A/AGCTT	
A		5.5, 4.1, 3.8, 1.3
B		5.5, 3.8, 2.4, 1.8, 1.3
C		7.0, 4.1, 3.8

\*The individuals with pattern D were considered heteroplasmic for this restriction site.



**Figure 2** - Autoradiograms of Southern Blot showing *Cochliomyia macellaria* mtDNA of the sampled populations digested with *EcoRV* (2.1) and *HindIII* (2.2). 2.1: lane 1 = Ca-1 (pattern A); lane 2 = RJ-1 (pattern A); lanes 3 and 4 = Ca-1 (pattern B); lane 5 = Ca-1 (pattern C); lane 6 = RJ-1 (pattern C); lane 7 = RJ-1 (pattern D). 2.2: lane 1 = RJ-1 (pattern B); lane 2 = Ca-1 (pattern A); lane 3 = RJ-1 (pattern A); lanes 4 and 5 = Ca-1 (pattern C). *HindIII* cut lambda DNA and *HaeIII* cut phiX 174 DNA are the size standards used. Numbers at left denote sizes of fragment patterns in kilobase pairs.

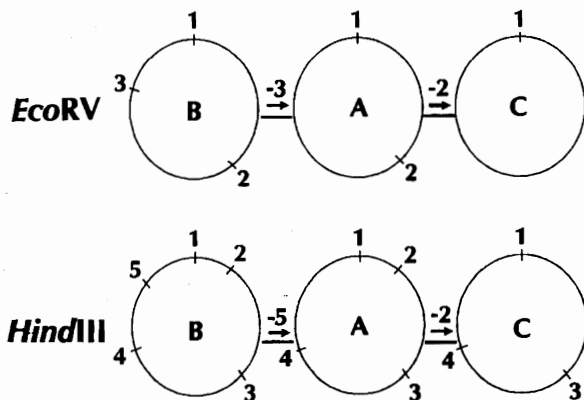
**Table II** - Frequencies of alternative mtDNA restriction patterns for two enzymes (*EcoRV* and *HindIII*) obtained for *Cochliomyia macellaria* populations and hybrids.

Haplotypes	Population			
	Ca-1	RJ-1	H-1*	H-2**
<i>EcoRV</i>				
A	59.67	82.0	95.5	-
B	8.06	-	-	-
C	30.64	8.0	-	100.0
D <sup>†</sup>	1.61	10.0	4.50	-
<i>HindIII</i>				
A	96.7	74.0	50.0	100.0
B	-	26.0	50.0	-
C	3.22	-	-	-
	n=62	n=50	n=44	n=46

<sup>†</sup>The individuals with pattern D were considered heteroplasmic for this restriction site.

\*Progeny of crosses between RJ-1 females and Ca-1 males.

\*\*Progeny of crosses between RJ-1 males and Ca-1 females.



**Figure 3** - Diagram of restriction patterns obtained with *EcoRV* and *HindIII* in *Cochliomyia macellaria*. The diagnostic sites between each pattern are shown. Arrows indicate the direction of loss of a single site. Locations for the restriction sites are arbitrary.

Heteroplasmy, the occurrence of more than one mtDNA genotype in individuals, followed by germ line segregation and selection, was observed. Individuals that presented the restriction pattern D for *EcoRV* were considered heteroplasmic (Figure 2). The number of heteroplasmic individuals was low (n=8 individuals) and their mtDNA differed in restriction enzyme recognition sites (pattern D) rather than size. The heteroplasmy observed in mtDNA of *C. macellaria* was a combination of two distinct restriction enzyme patterns that also occurred separately within RJ-1 and Ca-1 populations. The individuals were considered heteroplasmic for

restriction site, possessing a combination of mtDNA with patterns A and C in their cells (Table I). Heteroplasmy for size variants is common and has been reported for many animal groups (Harrison, 1989; Solignac *et al.*, 1983; Bermingham *et al.*, 1986; Boyce *et al.* 1989). Heteroplasmy for restriction site is considered rare (Fauron and Wolstenholme, 1980; Bentzen *et al.*, 1988). This may be less a consequence of the absolute rarity of the phenomenon than of two biases that affect restriction endonucleases analysis of mtDNA (Awise and Lansman, 1983). First, examples of heteroplasmy involving only a single restriction site are much less likely to be detected than those associated with major length polymorphisms, since the former manifest themselves with only one enzyme whereas the latter can only be detected with many enzymes. Second, cases of restriction site heteroplasmy are liable to be misinterpreted as instances of incomplete digestion, since the patterns of substoichiometric bands that result from phenomena may be similar. Unfortunately, details of the structure of *C. macellaria* mtDNA are not available, and using only restriction site variation we cannot locate heteroplasmy precisely in the variable regions on mtDNA. It seems that in *C. macellaria*, heteroplasmy represents a transient state between haplotypes after a mutational event and should be rare. In fact, in the whole sample of 202 individuals, only eight heteroplasmic individuals were found, all of which were A+C, giving pattern D for *EcoRV* (Figure 2.1). The observation that RJ-1 and Ca-1 *C. macellaria* populations had heteroplasmic individuals, and that both populations were found to have fixed homoplasmic haplotypes, supports this interpretation.

Partial restriction digests, or contamination were considered in the analysis of the heteroplasmic *EcoRV* D pattern. The reproducibility of pattern D under conditions of excess of the enzyme and long incubation periods (5-8 hs) discarded the hypothesis of partial restriction digests. On the other hand, if the heteroplasmy was caused by contamination with mtDNA from different individuals, patterns for other restriction endonucleases should show heterogeneity for any site variants for which the contaminating flies may have differed. None of the individuals that were putatively heteroplasmic for site variant were found to be heterogeneous for site variants for other enzymes. This fact ruled out the possibility of contamination.

The heteroplasmy in *C. macellaria* could have resulted from biparental inheritance, or from incomplete segregation of the variant forms of mtDNA involved. We favor the latter possibility, since all available evidence in this study and others indicates that at least within

the limits of detection, metazoan mtDNA is inherited solely through maternal lineages (Awise and Lansman, 1983; Awise *et al.*, 1984).

Combined restriction patterns for *EcoRV* and *HindIII* comprised the mtDNA haplotypes of each of the individuals. Based on the fragment patterns obtained for these enzymes a total of seven mitochondrial haplotypes were detected among the 112 individuals representing the two locations analyzed (Table III). Haplotype 1 (AA) was the most frequent in both populations, occurring in 56% of the analyzed individuals of Ca-1 and in 60% of RJ-1. The haplotype distributions indicated two unique haplotypes in Ca-1 (haplotypes 3 and 4) and three unique haplotypes in RJ-1 (haplotypes 2, 6 and 7) (Table III). The presence of unique haplotypes demonstrates the existence of different maternal lineages.

**Table III** - Distribution of mtDNA haplotypes in sampled populations of *Cochliomyia macellaria*. The haplotypes are represented by two uppercase letters. The first letter represents the restriction pattern for *EcoRV* and the second represents the restriction pattern for *HindIII*.

Haplotype <i>EcoRV/HindIII</i>	Population			
	Ca-1	RJ-1	H-1*	H-2**
1-AA	35	30	20	-
2-AB	-	11	22	-
3-AC	2	-	-	-
4-BA	5	-	-	-
5-CA	19	4	-	46
6-DA	1	3	2	-
7-DB	-	2	-	-
Total	n=62	n=50	n=44	n=46

\*Progeny of crosses between RJ-1 females and Ca-1 males.

\*\*Progeny of crosses between RJ-1 males and Ca-1 females.

Mating tests were conducted to verify sexual compatibility. The reciprocal interstrain crosses were denominated H-1 for the progeny of crosses between males Ca-1 and females RJ-1, and H-2 for the progeny of crosses between females Ca-1 and males RJ-1. The progenies resulting from both crosses were fertile and produced fertile progenies. The restriction endonucleases analysis of mtDNA of H-1 individuals (F1) revealed two patterns for *EcoRV* (patterns A and D) and two patterns for *HindIII* (patterns A and B). H-2 individuals had a single pattern for *EcoRV* (pattern C) and for *HindIII* (pattern A) (Table II). The combination of restriction patterns resulted in three mitochondrial

haplotypes for H-1 (haplotypes 1, 2 and 6) and only one haplotype for H-2 (haplotype 5) (Table III).

One of the most frequent haplotypes obtained for H-1 (haplotype 2) was exclusive for the parental population that provided the females (RJ-1), an indication of the maternal inheritance of mtDNA in this species. Two other mitochondrial haplotypes were also found in the F1 of H-1, indicating that more than one female, with different mitochondrial haplotypes, contributed to the hybrid progeny (Table III). A different result was obtained in the reciprocal cross, H-2, which all gave haplotype 5 (n=46), Table III.

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## RESUMO

A análise do padrão dos fragmentos de restrição do DNA mitocondrial (mtDNA) foi utilizada para examinar a variabilidade genética em duas populações de *Cochliomyia macellaria* (Diptera: Calliphoridae) do sudeste do Brasil (Caraguatatuba e Rio de Janeiro). O mtDNA de 202 indivíduos foi analisado com 12 endonucleases de restrição e hibridizados com sonda de mtDNA de *Cochliomyia hominivorax*. Duas endonucleases, *EcoRV* e *HindIII* detectaram polimorfismo e produziram sete haplótipos mitocondriais. Testes de cruzamento entre Ca-1 e RJ-1, foram conduzidos para verificar a compatibilidade sexual entre as duas linhagens e o padrão de restrição do mtDNA da progênie. Estes cruzamentos produziram híbridos férteis. Indivíduos heteroplásmicos para padrões de restrição foram encontrados nas populações analisadas.

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