Assessing Genetic Variation in New World Screwworm *Cochliomyia hominivorax* Populations from Uruguay

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ABSTRACT The New World screwworm *Cochliomyia hominivorax* (Coquerel) is an important parasitic insect pest in Neotropical regions. New World screwworm myiasis is caused by the larval stage of the fly infesting tissues of warm-blooded vertebrates. This species represents a serious threat to the livestock sector across its current distribution, which includes part of the Caribbean and all of South America (except for Chile). Knowledge of the extent and distribution of genetic variability of *C. hominivorax* is of great interest for the description of populations and for contributing to future strategies of control. This paper describes the analysis of genetic variability and structure of New World screwworm populations in Uruguay using two different molecular markers, mitochondrial DNA and microsatellites.

KEY WORDS New World screwworm, genetic differentiation, mitochondrial DNA, microsatellites

1. Introduction

The New World screwworm *Cochliomyia hominivorax* (Coquerel), one of the most important parasitic insect pests of warm-blooded vertebrates, causes invasive myiasis and is responsible for important economic losses to livestock rearing. The current distribution of the New World screwworm includes part of the Caribbean and all of South America (except for Chile). This species has been successfully eradicated from North and Central America using an area-wide approach involving the sterile insect technique (SIT) (Wyss 2000, Vargas-Terán et al. 2005). In 1988, the pest was introduced into Libya, but its spread to livestock and wildlife in the rest of Africa and the Mediterranean region was prevented by a successful SIT campaign using sterile flies shipped from the mass-rearing facility in Tuxtla-Gutiérrez, Mexico (Lindquist et al. 1992, Vargas-Terán et al. 1994).

In South America, however, this pest continues to affect the development of the livestock sector and wider economic development. An international effort is underway to evaluate the feasibility of eradicating the New World screwworm from endemic areas of the Caribbean and South America and to prevent invasions into screwworm-free areas. This involves *inter alia* collecting data on the damage and costs associated with control and on the distribution and density of the fly in these regions.

With respect to the latter, there have been speculations and conflicting reports about the existence of non-interbreeding populations and their possible effects on the control programme but to date there is no evidence that this situation exists (LaChance et al. 1982). To maximize the effectiveness of an eradication programme, it is essential to confirm that such populations do not exist in these new regions and to characterize the genetic variability of

Figure 1. *The* Cochliomyia hominivorax *collection sites in Uruguay.*

target populations. Knowledge of the genetic structure of New World screwworm populations will also be useful for identifying their actual and potential routes of gene flow and thereby improve the implementation of area-wide approaches to control this insect pest.

In the past, Krafsur and Whitten (1993) examined isozyme loci in 11 Mexican New World screwworm populations and their estimate of Wright's *F*-statistics (F_{ST}) (Wright 1965) was not significantly different from zero. They concluded, therefore, that screwworm populations in Mexico belonged to a single panmitic population. Taylor et al. (1996) also used isozyme loci to study two Brazilian populations and compared the results with previous data from Costa Rica (Taylor and Peterson 1994) and partial data from Mexico (Krafsur and Whitten 1993). They also concluded that New World screwworm forms a single panmitic population.

However, subsequent analyses of four Brazilian populations using different types of molecular markers in the mitochondrial and nuclear genomes, suggested a different pattern of substructuring. Restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) suggested that these

Table 1. *Field-collected samples of* Cochliomyia hominivorax *in Uruguay.*

Location	Number of individuals	Latitude	Longitude	Altitude (metres)
Bañados de Medina, Cerro Largo	24	32°23' 00 S	54° 21' 00 W	51
Cerro Colorado, Florida	29	33°52' 00 S	55° 33' 00 W	96
Colonia del Sacramento, Colonia	15	34°20' 00 S	57° 86' 67 W	213
Dayman, Paysandú	19	31°33' 00 S	57° 57' 00 W	27
Joaquín Suárez, Canelones	15	34°44' 01 S	56° 02' 12 W	203
Paso Muñoz, Salto	21	31°27' 00 S	56° 23' 00 W	55
San Antonio, Salto	15	31°24' 00 S	57° 58' 00 W	41

populations probably belonged to a single evolutionary lineage interconnected by reduced gene flow (Infante-Vargas and Azeredo-Espin 1995). The random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) technique was also used to detect genetic polymorphism and to select genetic markers to discriminate six Brazilian populations and one population from northern Argentina (Infante-Malaquias et al. 1999). In general, results from both mitochondrial and RAPD analyses were concordant in suggesting divergence among New World screwworm populations. Analysis of five Brazilian populations by means of isozyme loci revealed a high geographical differentiation across south-eastern Brazil with relatively low gene flow (Infante-Malaquias 1999).

One possible explanation for the discrepancies between the different data is that different levels of substructuring were present in different locations. Infante-Malaquias et al. (1999) suggested that South America could be the centre of origin of this species, explaining the high variability and the population structure found there whereas the homogenous populations of North America were possibly formed by a founder effect.

It is clear from the above that the available information is insufficient to infer patterns of genetic variation and structure among New World screwworm populations throughout their geographical range. Therefore, an effort was made to add to the body of information available on these aspects by using mtDNA and microsatellites to analyse the genetic variability and structure of Uruguayan populations of the New World screwworm.

2. Materials and Methods

2.1. Sampling of New World Screwworm in Uruguay

New World screwworm samples were obtained from seven distinct geographic locations in Uruguay with distances between them ranging from 50 to 418 kilometres (Fig. 1, Table 1). Larvae were collected directly from infested wounds in sheep, cattle and dogs in January 2003. Sampling of related individuals was avoided by choosing wounds in different animals and/or farms and by classifying larvae from the same wound by instar.

Larvae were transferred to the laboratory and reared until the pupal stage under standardized conditions (Infante-Vargas and Azeredo-Espin 1995) or fixed in 100% ethanol. Genomic DNA was extracted either from single adults, pupae or larvae using a phenol-chloroform procedure (Infante-Vargas and Azeredo-Espin 1995).

2.2. Mitochondrial DNA as a Molecular Marker

Diverse aspects related to the structure and evolution of mtDNA, such as simple and uniform organization, lack of recombination, maternal inheritance and high rate of nucleotide sequence evolution, have made it a valuable marker for estimating intraspecific genetic variability (Avise 1994).

RFLP analysis of mtDNA was previously used for New World screwworm populations and revealed a high level of genetic variation (Infante-Vargas and Azeredo-Espin 1995, Taylor et al. 1996). However, restriction analysis of mtDNA polymerase chain reaction products (PCR-RFLP) provides a faster and simpler method and has successfully been used for population analyses (Ross et al. 1997, Dueñas et al. 2002).

2.2.1. Mitochondrial DNA Variability in New World Screwworm Populations in Uruguay

Lyra et al. (2005) used mtDNA PCR-RFLP to examine the genetic variability among the seven Uruguayan populations sampled. Two regions of the mtDNA, the control region (A+T/12S) and subunits 1 and 2 of the *cytochrome oxidase* (*cox*1/*cox*2), were amplified and digested with the restriction endonucleases *Dra* 1 (A+T/12S), *Ase* 1 and *Msp* 1 (*cox*1/*cox*2).

Among the populations, nine haplotypes were observed. The mean nucleotide diversity (π) was 0.0229 and the haplotype diversity (*Hs*)

was 0.6355, indicating high mtDNA variability. The similarity index (F) was high (96.7%) and the estimate of nucleotide divergence between populations (δ) was very low (0.00055), suggesting a high similarity among samples from the different locations. The analysis of molecular variance (AMOVA) showed no evidence of population differentiation, indicating that New World screwworm forms a single panmitic population in Uruguay. Lyra et al. (2005) suggested that the distribution of New World screwworm in the extreme south of the species' occurrence and the fact that there are no geographical barriers or important climatic differences between studied regions were responsible for the lack of differentiation in Uruguay.

2.3. Microsatellite Markers

Microsatellites, or simple sequence repeats, are short sequences made up of a single motif with no more than six bases that is tandemly repeated (Goldstein and Schlötterer 1999). They are found in large numbers and are relatively evenly spaced throughout the genome of every eukaryotic organism analysed so far.

Among the several classes of molecular markers, microsatellite loci stand out as co-dominant markers with a high number of alleles per locus, high polymorphism and a high heterozygosity value. Due to these features, variation in these co-dominant markers has been increasingly used as the marker of choice to investigate questions regarding population structure, gene flow and mating systems even in populations which have low levels of allozyme and mitochondrial variation. The recent isolation and characterization of polymorphic microsatellite markers for New World screwworm (Torres et al. 2004, Torres and Azeredo-Espin 2005) enables genetic variability and population structure of this pest in Uruguay to be investigated.

2.3.1. Microsatellite Amplification and Genotyping of New World Screwworm Populations in Uruguay

Ten previously characterized microsatellite markers (Torres et al. 2004) were used in this study. The primer sequences and the procedures for microsatellite amplifications and analyses of PCR products were described by Torres et al. (2004).

2.4. Data Analyses

The number and frequency of alleles, the allele size range and the observed (H_O) and the unbiased expected (H_E) (Nei 1978) heterozygosities under Hardy-Weinberg equilibrium were determined per locus for each location. The software Micro-checker 2.2.0 (Van Oosterhout et al. 2004) was used to test for technical artefacts such as null alleles, stuttering and large allele dropout. Each locus and population was tested for deviations from Hardy-Weinberg equilibrium expectations using exact tests implemented in GENEPOP, a population genetics software for exact tests and ecumenicism (Raymond and Rousset 1995). Genotypic linkage disequilibrium among all pairs of loci within each site was investigated using Fisher's exact test as implemented in GENEPOP. An unbiased estimate of the exact probability was obtained using the Markov chain algorithm (Guo and Thompson 1992). Two indices of genetic differentiation were estimated between the localities, F_{ST} and R_{ST}, the former based on the absolute frequencies of alleles (Weir and Cockerham 1984) and the latter estimated from the sum of the squared number of repeat differences (Slatkin 1995). An unbiased estimate of F_{ST}, θ was calculated using the FSTAT computer programme (Goudet 1995). The significance of pairwise F_{ST} estimates was tested by permuting genotypes among populations (Goudet et al. 1996). The overall estimate of R_{ST}, ρ_{ST} was calculated using RSTCALC, a PC-based programme for performing analyses of population structure, genetic differentiation and gene flow using microsatellite data (http:// helios.bto.ed.ac.uk/evolgen/rst/rst.html). Significance levels for simultaneous statistical tests were corrected using the sequential Bonferroni method (Rice 1989). The isolation-by-distance model of population genetic structure was tested by

linear regression of pairwise F_{ST} /(1 - F_{ST}) against the natural logarithm of the geographical distance between population pairs (Rousset 1997).

3. Results and Discussion

3.1. Microsatellite Variation

The number of alleles and the expected and observed heterozygosity per locus and per population are given in Table 2. Analysis of 138 New World screwworm genotypes revealed a moderate degree of polymorphism across the seven sampling locations. Ten loci were used in the first analysis, but the locus *CH02* presented some ambiguous, non-reproducible patterns. For this reason, it was excluded from the statistical analysis.

For the nine microsatellite loci analysed, the number of alleles detected per locus and per population ranged from 2 to 10, with an average of 6 (Table 2). The observed heterozygosity (H_O), varied from 0.19 to 0.91 and the expected heterozygosities (H_E) varied from 0.37 to 0.87 (Table 2).

Significant deviation from the Hardy-Weinberg equilibrium (exact probability test, $P < 0.05$) was recorded for all sampling localities. In all cases, departures from expectations were due to an excess of homozygotes. Among the possible factors that might account for these deviations is the Wahlund effect, since the samples were collected from different farms at each location. However, such effects should be apparent in most of the loci across populations, which was not the case for this data set. Another factor that could also have caused the observed deviations is the presence of null alleles. These result from mutations such as substitutions, insertions, or deletions in one or both priming sites preventing the binding of the DNA strand and primers (Callen et al. 1993) and non-amplification of the allele. At the population level this can lead to a misinterpretation of the number of heterozygotes and consequently of Hardy-Weinberg deviations. Only the locus *CH10* presented a significant number of null alleles and the analysis excluding this locus was not significantly altered. Furthermore, these results are being confirmed by the preliminary analysis of new populations using these loci and additional loci (Torres and Azeredo-Espin 2005). The occurrence of demographic changes that affected New World screwworm populations may therefore be the main cause of the observed homozygote excess. These in turn could have arisen from decreases in temperature and humidity in the Uruguayan winter and/or persistent insecticide treatment which can cause mass-population mortality and local extinction of New World screwworm populations.

Linkage disequilibrium was found in only two of 252 comparisons among the loci and populations analysed, but no common pair of loci showed non-random associations in all the populations (data not shown).

3.2. Interpopulation Variability

Most variation was found within rather than between populations and the seven populations exhibited remarkably similar allele distributions. This is consistent with the results found by the PCR-RFLP of the mtDNA.

Two measures of interpopulation genetic differentiation were used in this study (F_{ST} and R_{ST}). The global multilocus estimate of R_{ST} was 0.015 and of F_{ST} was 0.031. Both estimates, although low, were numerically very similar and significantly different from zero ($P < 0.05$, for R_{ST} and $P < 0.001$, for F_{ST}), suggesting that little differentiation exists among these populations.

The relationship between local populations was tested by calculating pairwise F_{ST} estimates because it was demonstrated that F_{ST} yields the better estimate when the number of loci is small (< 10) or the sample size is small (Gaggiotti et al. 1999). F_{ST} estimates between populations ranged from -0.0005 to 0.0853 (Table 3) and for five of the ten population pairs were significantly different from zero at the 0.05 level.

These low levels of substructuring could be attributed to the high dispersal capacity of

Table 2. *Genetic diversity in* Cochliomyia hominivorax *from seven localities in Uruguay.*

Locus		Dayman 2N = 38	S. Antonio 2N = 42	Colonia 2N = 30	B. Medina 2N = 48	Suarez 2N = 30	C. Colorado 2N = 58	P. Muñoz 2N = 30
CH01	N_a	6	6	5	6	5	6	5
	H_O	0.3158	0.4762	0.5333	0.5833	0.6667	0.5517	0.5333
	H_E	0.6230*	0.7607*	0.7517	0.6809	0.6943	0.6031	0.7126*
CH05	N_a	5	5	6	7	4	7	5
	H_O	0.6316	0.5238	0.7333	0.4167	0.5333	0.5172	0.8000
	H_E	0.5874	0.6190	0.6667	0.6755*	0.4483	0.6636*	0.7747
CH09	N_a	4	4	4	7	2	6	5
	H_O	0.3333	0.4500	0.5333	0.7917	0.2000	0.5357	0.5333
	H_E	0.3762	0.4423	0.5724	0.7216	0.3701	0.6227	0.6299*
CH10	N_a	6	6	6	6	4	5	6
	H_O	0.3684	0.1905	0.2000	0.2917	0.6000	0.4000	0.4000
	H_E	0.6344*	0.5912*	0.7885*	0.7101*	0.6598	0.5167*	0.5011*
CH11	N_a	5	10	9	8	9	7	7
	H_O	0.6111	0.6190	0.7333	0.6364	0.6667	0.4286	0.4000
	H_E	0.6159	0.7317	0.7816	0.8245*	0.8276*	0.7247*	0.6943*
CH12	N_a	8	8	8	8	5	9	7
	H_O	0.8333	0.7143	0.6000	0.9167	0.6000	0.7241	0.8000
	H_E	0.8476*	0.8479*	0.8736	0.8475	0.6460	0.8100	0.7931
CH14	N_a	7	7	6	6	6	6	5
	H_O	0.5789	0.4762	0.6000	0.5217	0.6000	0.5714	0.5333
	H_E	0.8179*	0.6690*	0.7356	0.6135	0.6989	0.8013*	0.6713
CH15	N_a	7	7	6	6	3	6	5
	H_O	0.5556	0.4762	0.5333	0.3750	0.2667	0.2759	0.4000
	H_E	0.8302*	0.7607*	0.7862	0.7943*	0.5080*	0.7828*	0.7310*
CH20	N_a	7	7	5	7	5	6	4
	H_O	0.3684	0.4762	0.5333	0.5000	0.8000	0.6897	0.5333
	H_E	0.7084*	0.7120	0.6414	0.6835*	0.7540	0.7048*	0.6920
All loci	Mean N_a	55	59	55	61	43	58	49
	Mean H_O	0.5107	0.4892	0.5556	0.5592	0.5481	0.4816	0.5481
	Mean H_E	0.6723*	0.6816*	0.7331*	0.7279*	0.6230*	0.6922*	0.6889*

N_a, number of alleles

H_E, expected heterozygosity

H_O, observed heterozygosity.

* denotes a significant ($\alpha = 0.05$) deviation from Hardy-Weinberg equilibrium

New World screwworm, since migration is assumed to prevent genetic differentiation at neutral markers (Agis and Schlötterer 2001). However, the analysed populations showed no isolation by distance ($P = 0.6115$). Since restricted migration results in positive correlation between geographical and genetic distance (Slatkin 1993), simple migration models may not be sufficient to explain the low differentiation between New World screwworm populations. One factor that could be responsible for this pattern of genetic differentiation

Table 3. F_{ST} *estimates for all* Cochliomyia hominivorax *population pairwise comparisons.*

Study area	Salto	Colonia	B. Medina	Suarez	C. Colorado	Paso Muñoz
Dayman	0.0080^{NS}	0.0171^{NS}	0.0194^{NS}	0.0497*	0.0209^{NS}	0.0281^{NS}
Salto		0.0112^{NS}	0.0111*	0.0709*	0.0201*	0.0240*
Colonia			-0.0005^{NS}	0.0675*	0.0200^{NS}	0.0378*
B. Medina				0.0853*	0.0163^{NS}	0.0334*
Suarez					0.0664*	0.0533*
C. Colorado						0.0399*

NS, not significant
* significant at the 5% nominal level after standard Bonferroni corrections

is the passive migration of larvae by the movement of infested animals. However, an alternative explanation can be considered as responsible for the low differentiation and the lack of isolation by distance. It was noted (Slatkin 1993) that the absence of isolation by distance could be indicative of a recent recolonization event. Considering the hypothesis of mass-mortality by climatic conditions or insecticide treatment, a recolonization by a large founder population could cause a demographic turnover if this population spread rapidly over Uruguay during climatically favourable seasons. In this case, a very similar allele distribution would be expected over the country. To test this hypothesis it is necessary to compare Uruguayan New World screwworm samples collected during different hot/rainy seasons, as well as samples from intermediate and central populations which can be acting as stable sources of New World screwworm for recolonization events.

4. Conclusions

Information about patterns of genetic variation, structure and gene flow is needed before investing in large-scale efforts to control insect pests. This information can, to a large extent, be assessed using modern molecular techniques. Mitochondrial and microsatellite markers have helped to provide this information for New World screwworm populations in Uruguay.

The results presented here and elsewhere by Lyra et al. (2005) suggest that the seven populations from Uruguay are very similar, sharing homogenous haplotype (for mtDNA) and allele (for microsatellites) distributions. Although the mtDNA data indicate that this species forms a single panmitic population in Uruguay, results from microsatellite analysis yielded low, but significant, levels of subdivision between populations. These results can be explained by differences in the modes of inheritance of the two markers since the effective population size of mtDNA is only one quarter that of nuclear DNA (Sanetra and Crozier 2003). These differences, however, can also be explained by sex-biased gene flow among these populations. This would suggest that levels of female-mediated gene flow are slightly higher than male levels; consequently, mtDNA markers showed less structuring than the microsatellite polymorphisms. While Mayer and Atzeni (1993) described higher dispersal rates for New World screwworm females, this should be further investigated since microsatellite data also suggested that restricted migration might not play a significant role in population differentiation.

The results presented here provide some baseline data on genetic variation to which other New World screwworm populations can be compared. Analysis of other populations throughout its geographical distribution would determine if similar patterns of genetic variation and gene flow are observed and lay

the groundwork for future control strategies against this livestock pest.

5. Acknowledgements

The authors thank R. Rodrigues for valuable technical assistance and J. Dargie and two anonymous reviewers for helpful discussions and comments on an earlier draft of this manuscript. This work was supported by grants to AMLAE from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant 03/01458-9), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, grant 471132/01-2) and the International Atomic Energy Agency (IAEA, grant 11822/RO). TTT was supported by a fellowship from FAPESP (grant 02/00035-4).

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