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Amplified fragment length polymorphism used for inter- and intraspecific differentiation of screwworms (Diptera: Calliphoridae)

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Abstract

Morphologically, early immature stages of the economically important pest called screwworms, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), and non-pest secondary screwworms, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), are nearly indistinguishable. Correct identification is crucial to the ongoing eradication and exclusion program protecting the United States, Mexico and Central America from reinvasion of screwworms persistent in South America and the Caribbean. Amplified fragment length polymorphism (AFLP) polymerase chain reaction was used to differentiate populations of *C. hominivorax* and to discriminate them from *C. macellaria*. Ten primer pairs screened for interspecific discrimination of *C. hominivorax* from *C. macellaria* showed 52 discrete bands, allowing the two species to be readily distinguished; divergent branches on resulting dendrograms showed 100% bootstrap support. *C. macellaria* populations grouped at the 92% level; *C. hominivorax* populations grouped at the 68% level. Of the 52 bands, seven were monomorphic for both species, 22 were specific to *C. macellaria*, ten were present only in *C. hominivorax* and the remaining 13 bands differentiated *C. hominivorax* populations. Separate studies using ten strains of *C. hominivorax* showed a higher level of genetic similarity within than between populations. Analyses using 72 bands (19 monomorphic bands, 53 bands grouped all ten strains at the 58% similarity level) resolved seven mutant strains from Mexico (85% similarity level); all ten strains were resolved at the 72% similarity level. Diagnostic bands were identified for species and strain identification. We conclude that AFLP can be a valuable tool for studies of interspecific and intraspecific genetic variation in screwworm populations.

Keywords: AFLP, *C. hominivorax*, *C. macellaria*, genetic similarity

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Introduction

Larvae of the screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), are obligate parasites of endothermic vertebrates that caused economic losses estimated to exceed $1 billion annually before their eradication from the United States, Mexico and most of Central America by using the sterile insect technique (SIT) (Vargas-Terán

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et al., 2005). Screwworms remain a threat to domestic animals and wildlife, as well as humans, in regions of their current distribution, part of the Caribbean and all of South America (with the exception of Chile) (Wyss & Galvin, 1996). Major challenges to the eradication effort include monitoring the progress of eradication in zones under treatment with sterile flies, detecting reinfestations in screwworm-free zones and monitoring the area surrounding the mass production facilities for the escape of fertile flies. Consequently, effective monitoring, surveillance and quarantine methods should be adopted because sporadic outbreaks and reintroduction of this pest to the previously eradicated regions (southwestern United States and Mexico), as well as new geographical regions (Libya, Australia, and France), by livestock trade or human travel have threatened the efficacy of SIT (Chermette, 1989; Hall & Beesley, 1990; Searson *et al*., 1992; Narang & Degrugillier, 1995).

Additionally, the secondary screwworm, *Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae), shows morphological similarity and overlap in geographic distribution with *C. hominivorax*. *C. macellaria* also coexists with *C. hominivorax* in the same infested wound, thereby contributing to numerous misidentifications (Leite & Guevara, 1993; Litjens *et al*., 2001). *Cochliomyia macellaria* is a saprophagous species that feeds primarily on carrion and is a secondary agent of myiasis (Litjens *et al*., 2001). Therefore, it is important to investigate novel and reliable methods for correct identification of these two species because of the possibility of sabotage or fraud for outbreak samples (in one case, emergency release of sterile flies had been made on samples that proved negative on subsequent analysis: Anonymous, 1990). A rapid, simple and easily interpretable screening system that can distinguish closely related insect species would speed research on wild populations and improve insect control decisions. A blocking enzyme-linked immunosorbent assay, based on monoclonal antibodies against screwworm antigens, was developed that differentiated screwworm eggs, larvae, pupae and adults from those of the secondary screwworm, as well as *Phormia regina* (Meigen), *Lucilia sericata* Meigen, *Calliphora vicina* Robineau-Desvoidy and *Chrysomya rufifacies* (Macquart) (Figarola *et al*., 2001). However, apart from the development of a monoclonal antibody-based assay, the ability of DNA markers for identification also needs to be explored.

Currently, screwworm eradication programmes rely heavily on the use of SIT. A major constraint to the eradication effort is the limited amount of information regarding the population structure for implementing SIT. Therefore, assessment of the genetic population structure of the screwworm pest will be valuable to the success of the eradication program (Bram, 1985; FAO, 1992). It is not known whether the populations of *C. hominivorax* in South America are the 'same' as in Central America, i.e. whether the populations are related or isolated. These questions are important because, if there is mating isolation, eradication could not occur if an inappropriate strain was reared and released. Therefore, if the wrong strain was being reared the program would fail because of assortative mating and failure of sterile males to mate with native females.

The questions of population structure, identification and differentiation of *C. hominivorax* from *C. macellaria* and other related blow fly larvae can, to a large extent, be resolved using molecular genetic tools. Molecular genetic markers represent a powerful tool for identifying source populations in cases of reintroduction, outbreak, sabotage or biological warfare/terrorism. The overall objective of this study was to assess the genetic variability and to develop reliable DNA fingerprints for *C. hominivorax* (an obligatory pest) and *C. macellaria* (a facultative parasite) by using polymerase chain reaction (PCR)-based molecular markers.

Studying the population structure of an insect species helps in understanding the influence of genetic processes such as mutation, migration and selection on insect biology, behavior, ecology and evolution (Roderick, 1996). In insects where autocidal biological control has been implemented to control pest populations, knowledge of insect population structure and distributional ecology can contribute to the development of elite strains for large-scale deployment. Population structure consists of deciphering the genetic relationships among individuals within and between subpopulations and, thus, assessing the genetic variation among individuals in a population (Leung *et al*., 1993). Advances in DNA marker technology in the past three decades have provided the necessary tools for systematists and population geneticists to use common approaches to study both intraspecific and interspecific variation in insect populations (Avise, 1994). Amplified fragment length polymorphism (AFLP) is a powerful technique that detects molecular genetic variations in DNA of any source or complexity without *a priori* knowledge of genome structure or sequence (Vos *et al*., 1995). The 'AFLP fingerprints' combine the reliability of restriction fragment length polymorphisms (RFLPs) and the power of PCR in a single technique, i.e. they are actually RFLPs visualized after selective PCR amplification of DNA restriction fragments (Vos *et al*., 1995). Therefore, analysis of AFLPs has the potential to become a powerful DNA fingerprinting technique for studying genetic relationships and genetic diversity in arthropods (Reineke *et al*., 1999; Bensch & Åkesson, 2005). AFLP analysis has been used to study geographic populations of gypsy moth, *Lymantria dispar* (Linnaeus) (Reineke *et al*., 1999), to assess the biodiversity of the midge *Orseolia oryzae* (Wood-Mason) (Katiyar *et al*., 2000), to determine differences in fall armyworm, *Spodoptera frugiperda* (J.E. Smith), based on host strains (McMichael & Pashley, 1999) and its genetic variation (Clark *et al*., 2007); and to study honey bee, *Apis mellifera* L., population genetics (Suazo & Hall, 1999). Here, we explore the utility of AFLP both to assess the degree of similarity and differentiate populations of *C. hominivorax* from different geographic regions and also to distinguish them from the morphologically similar calliphorid *C. macellaria*.

Materials and methods

Insect samples

All screwworm and secondary screwworm populations/strains used in this study were obtained from laboratory colonies reared at the USDA–ARS Midwest Livestock Insects Research Unit (MLIRU) Biosecure Screwworm Rearing Laboratory in Lincoln, Nebraska, USA. Specimens included third instars of *C. macellaria* samples from Nebraska, USA (CM), and third instars of *C. hominivorax* larvae representing four locations (Panama, PN and C9; Costa Rica, CR; Jamaica, JA; and Mexico, MX, CE, LH, LM, RL, and CN) (table 1). Samples were frozen and stored at -80°C before DNA extraction.

Table 1. Country of origin, approximate year of collection, code and important characteristics of screwworm populations studied.

Species	Country of origin	Year collected	Code	Comments
C. macellaria	Lincoln, NE, USA	1996–1997	CM	Secondary screwworm
C. hominivorax				
P95	Panama	1995	PN	Wild type
CR92	Costa Rica	1992	CR	Wild type
J98	Jamaica	1998	JA	Wild type
PA34	Mexico[a]	1984	MX	Orange eye mutant
CECH	Mexico[a]	1985	CE	Red eye mutant
C9	Panama	1995	C9	White eye mutant
LH	Mexico[a]	1984	LH	Yellow eye mutant
LIMON	Mexico[a]	1984	LM	Green eye mutant
RL	Mexico[a]	1984	RL	White eye mutant
CN	Mexico[a]	1984	CN	Wing mutant

[a] Flies from Mexico had the following geographic origins: PA34 – Chiapas, MX; CECH – Quintana Roo, MX; LH – Oaxaca, MX; Limon – Palenque, MX; RL – cross of LH and Limon; CN – cross between Chiapas and Oaxaca, MX.

Table 2. Oligonucleotide adapters and primers used for AFLP analysis.

Primer ID	Primer type	Sequence (5′–3′)
EcoRI-F	Forward adapter	CTCGTAGACTGCGTACC
EcoRI-R	Reverse adapter	AATTGGTACGCAGTCTAC
MseI-F	Forward adapter	GACGATGAGTCCTGAG
MseI-R	Reverse adapter	TACTCAGGACTCAT
E(N+0)	EcoRI Preamp primer	GAC TGC GTA CCA ATT C
M(N+1)	MseI Preamp primer	GAT GAG TCC TGA GTA A**C**
E(N+0+3)	EcoRI selective primer	GAC TGC GTA CCA ATT C **NNN***
M(N+1+2)	MseI selective primer	GAT GAG TCC TGA GTA A**CNN***

* N represents the position of (+3 and +2) selective nucleotides in the primer and may be either A, T, G or C.

DNA isolation

DNA was isolated from individually frozen insect samples by using a modified Black & Duteau (1997) cetyltrimethylammonium bromide (CTAB) extraction protocol. The gut was removed and the larvae were homogenized in 500 µl of extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 2% CTAB and 0.2% β-mercaptoethanol) by using a disposable micropestle. The homogenate was treated with proteinase K (200 µg ml^{-1} extraction buffer) for 2 h at 65°C, followed by RNase (500 µg ml^{-1}) treatment for another 2 h at 37°C. The homogenate was then centrifuged at 12,000 rpm for 4 min at room temperature, and the supernatant was extracted with an equal volume of chloroform : isoamyl alcohol (24 : 1) by centrifugation at 12,000 rpm for 10 min to separate the phases. The aqueous phase was transferred to a clean tube, and the chloroform:isoamyl alcohol step was repeated. DNA was precipitated by the addition of an equal volume of chilled isopropanol to the aqueous phase and incubation at 4°C for at least 30 min. The precipitate was collected by centrifugation at 12,000 rpm at 4°C for 15 min, rinsed with 70% ethanol, air-dried and dissolved in 100 µl of 1X TE buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA). DNA quality and concentration were determined on 0.8% Tris borate-EDTA agarose gel (Sambrook *et al*., 1989) by using a known λ concentration standard (Invitrogen, Carlsbad, California, USA).

Table 3. Selective primers used for AFLP analyses.

Primer ID	Code	Sequence
E(N+0+3)	E1	GAC TGC GTA CCA ATT C **AGG**
E(N+0+3)	E2	GAC TGC GTA CCA ATT C **ACA**
E(N+0+3)	E3	GAC TGC GTA CCA ATT C **ACT**
M(N+1+2)	M1	GAT GAG TCC TGA GTA A **CTA**
M(N+1+2)	M2	GAT GAG TCC TGA GTA A **CTT**
M(N+1+2)	M3	GAT GAG TCC TGA GTA A **CTG**
M(N+1+2)	M4	GAT GAG TCC TGA GTA A **CTC**
M(N+1+2)	M5	GAT GAG TCC TGA GTA A **CAG**
M(N+1+2)	M6	GAT GAG TCC TGA GTA A **CAC**
M(N+1+2)	M7	GAT GAG TCC TGA GTA A **CAA**
M(N+1+2)	M8	GAT GAG TCC TGA GTA A **CAT**

AFLP–PCR

Template preparation and AFLP assay were performed using a modified protocol of Vos *et al*. (1995) with the incorporation of infrared fluorophore (IRD-700) (LI-COR, Lincoln, Nebraska, USA) labeled EcoRI primers in the PCR.

Template preparation

One microgram of genomic DNA was incubated with restriction endonucleases EcoRI (Invitrogen) and MseI (New

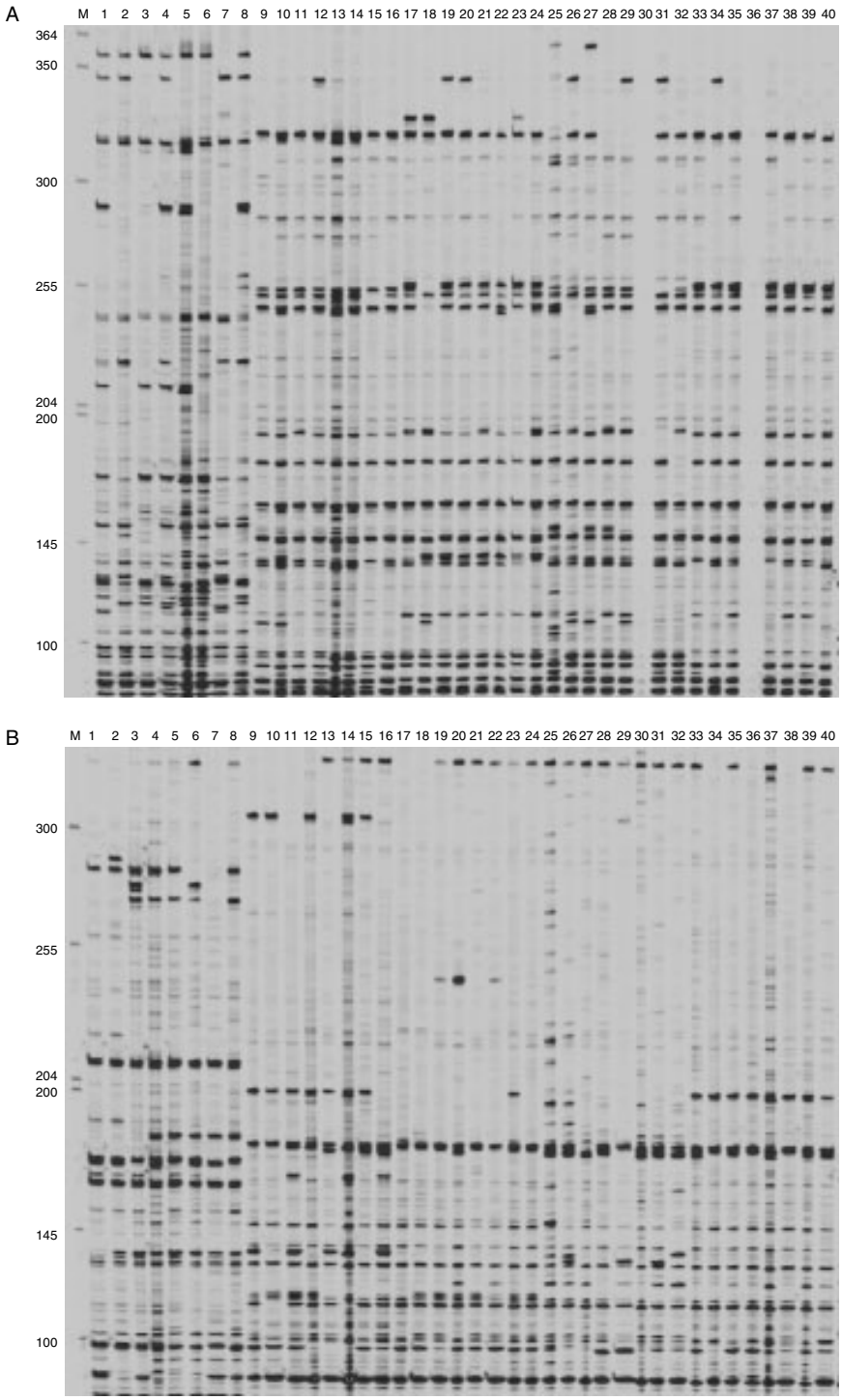

Fig. 1. AFLP fingerprints generated using primer combinations (a) E-AGG + M-CAA and (b) E-AGG + M-CAC from the genomic DNA of one population of *C. macellaria*, lanes (1–8) CM, and four populations of *C. hominivorax*, lanes (9–16) PN, (17–24) CR, (25–32) JA and (33–40) MX. Lane M contains the IRD-700 ladder (50–700 bp).

England Biolabs, Beverly, Massachusetts, USA) for 2.5 h at 37°C in a total volume of 12.5 µl that contained 1.25 µl of 10X One-Phor-All *PLUS* buffer (GE Healthcare, Little Chalfont, Buckinghamshire, UK), 0.32 µl of 4 U µl^{-1} MseI enzyme (1.25 U per reaction), 0.08 µl of 15 U µl^{-1} EcoRI enzyme (1.25 U per reaction), and 0.125 µl of 10 mg ml^{-1} bovine serum albumin (New England Biolabs); autoclaved nanopure water was added to make up the final volume of 12.5 µl.

Table 4. Diagnostic AFLP bands for (a) interspecific and (b) intraspecific identification of screwworms.

(a)

Primer ID	Band size	*C. macellaria*	*C. hominivorax*			
			PN	CR	JA	MX
E1+M6	160 bp	8	0	0	0	0
	165 bp	8	0	0	0	0
	172 bp	0	8	8	8	8
	200 bp	0	8	0	0	8
	210 bp	8	0	0	0	0
	325 bp	0	4	8	8	5
E2+M3	150 bp	8	0	0	0	0
	165 bp	0	8	0	0	0
E2+M6	210 bp	0	8	8	8	8
	225 bp	0	8	8	8	8

(b)

Primer ID	Band size bp	*C. hominivorax* Strains									
		PN	CR	JA	MX	CE	C9	LH	LM	RL	CN
E2+M1	206	1	0	0	4	0	0	4	0	0	0
	214	3	3	3	0	2	4	0	4	0	3
E2+M2	240	2	2	3	3	0	0	4	4	4	4
	275	2	3	4	0	0	0	0	4	4	0
E2+M3	162	4	0	0	0	0	0	0	0	0	0
	280	4	2	4	4	1	4	1	3	0	4
	292	0	0	0	0	0	4	0	0	0	0
E2+M6	190	2	4	0	0	4	4	4	0	0	4
	206	1	4	3	2	0	4	0	0	4	0
E2+M4	178	1	2	3	0	4	0	4	0	3	2
	204	4	0	4	4	4	3	2	4	0	4

bp represents band size in base pairs; values below each strain represent the number of individuals displaying a band.

The restriction fragments were incubated for 6 h at room temperature (~25°C) with 5 µl of ligation mixture containing 0.5 µl of 10X T4 DNA ligase buffer (Invitrogen), 0.5 µl of 5 pmol μl^{-1} EcoRI adapter, 0.5 µl of 50 pmol μl^{-1} MseI adapter, 0.150 µl of T4 DNA ligase (catalog no. M0202S, New England Biolabs) and 3.350 µl autoclaved nanopure water for ligating the adapters. Double-stranded adapters for the corresponding restriction enzymes were prepared by incubating equimolar amounts of both strands (EcoRI-F and EcoRI-R for EcoRI adapter and MseI-F and MseI-R for MseI adapter) (QIAGEN Operon, Alameda, CA) (table 2; sequences taken directly from Vos *et al*., 1995) for 10 min at 65°C, 10 min at 37°C and 10 min at 25°C.

AFLP assay

Ligation was followed by 20 cycles of preselective PCR amplification (30 s at 94°C, 1 min at 56°C and 1 min at 72°C) by using 1 µl of adapted DNA diluted 10-fold with 1X TE buffer (10 mM Tris-Cl and 0.1 mM EDTA, pH 8.0), 10 µl of pre-amp primer mix II (contains two oligonucleotide primers, one primer corresponding to the EcoRI adapted-ends and one primer corresponding to the MseI-ends) (Invitrogen), 1.25 µl of 10X PCR buffer plus 15 mM $MgCl_2$ and 0.25 µl of 5 U μl^{-1} Ampli*Taq* DNA polymerase (1.25 U per reaction) (Applied Biosystems, Foster City, California, USA). The oligonucleotide primers in the pre-amp primer mix II are complementary to the adapter/restriction site with MseI primer containing one selective nucleotide (M+1 primer; table 2.) and EcoRI primer containing no selective nucleotide (E+0 primer; table 2). Products from preamplification reactions were diluted 20-fold with autoclaved nanopure water and were used as templates for further selective amplifications by using IRD-700 fluorophore-labeled EcoRI primer (LI-COR) with three selective nucleotides at the 3′ end (E+0+3) and MseI primer with two additional nucleotides added at the 3′ end of M+1 primer used earlier (M+1+2) (table 3).

Selective PCR amplifications were performed in a 12-µl volume and contained 2.5 µl of diluted preamplification product, 5.8 µl of autoclaved nanopure water, 1.2 µl of 10X PCR buffer containing 15 mM $MgCl_2$ and 0.06 µl of 5 U μl^{-1} Ampli*Taq* DNA polymerase (Applied Biosystems), 2.0 µl of MseI primer (M+1+2) (6.7 ng μl^{-1}; dNTPs) (Invitrogen) and 0.5 µl of 1.0 pmol μl^{-1} IRD-700-labeled EcoRI primer (LI-COR). Selective amplifications were performed in a DNA thermal cycler 9600 (Applied Biosystems) by using the following touch-down PCR profile (Vos *et al*., 1995): one cycle of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C, 12 cycles of subsequently lowering annealing temperature (65°C) by 0.7°C per cycle while keeping denaturation (30 s at 94°C) and extension (1 min at 72°C) temperature constant, 23 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, and soak at 4°C. After amplification, reactions were stopped by adding 2.5 µl of blue stop solution (LI-COR), and the samples were denatured at 94°C for 5 min and flash cooled on ice immediately before electrophoresis. One microliter of the sample along with 1 µl of IRD-labeled 50–700-bp size marker (LI-COR) was electrophoresed through KB^{Plus} 6.5% ready-to-use gel matrix (LI-COR), and the infrared fluorescent bands

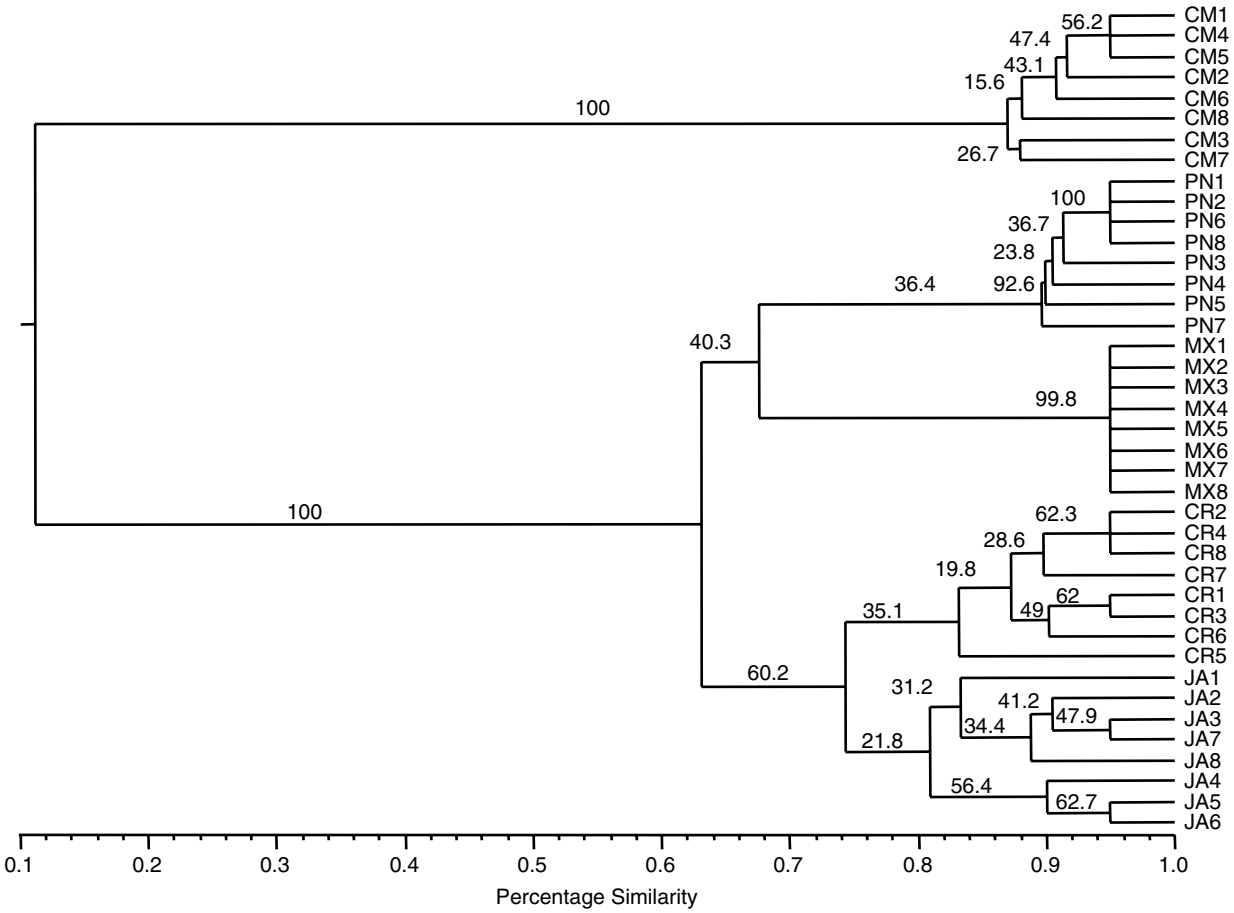

Fig. 2. Dendrogram showing interspecific relationship between screwworms, *C. macellaria* (CM) and *C. hominivorax* (PN, CR, JA and MX) based on data from ten AFLP primer combinations. Similarity values were calculated using the Jaccard coefficient, UPGMA clustering. Values on branches indicate bootstrap values.

were detected by the laser scanning system (model 4200S-2, LI-COR).

Data scoring and analysis

For statistical analysis of inter- and intraspecific genetic variation, we acknowledge the following assumptions: the genotypes at marker loci are in Hardy–Weinberg proportions; AFLP products segregate as dominant alleles in a Mendelian manner, with most variation at the AFLP loci being biallelic (+/−) with one band allele and one bandless allele (Haymer, 1994; Borowsky, 2001). Image data (16-bits) were automatically collected and simultaneously recorded during electrophoresis. Sizes of AFLP fragments were estimated by comparing to IRD-700-labeled 50–700-bp AFLP ladder (LI-COR). AFLP fragments were scored and analyzed using Gene ImagIR software, version 3.52 (LI-COR) and converted into numerical data as a matrix by assigning 1 (band present) and 0 (band absent).

Data were analyzed with the software NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, version 2.02) (Rohlf, 1997). Similarity, matrices were generated according to the methods of Jaccard (=Grower's; $a/(a+b+c)$), as well as Dice (=Nei and Li; $2a/(2a+b+c)$) – where 'a' is the number of fragments shared by two populations, 'b' is the number of fragments present only in one population and 'c' is the number of fragments present only in the other population – and results were compared (Sneath & Sokal, 1973; Rohlf, 1997). Coefficients were computed using the SIMQUAL (similarity based on qualitative differences) program and a phenogram was generated by unweighted pair group method [UPGMA] by using arithmetic averages (Sokal & Michener, 1958). The resulting cluster was represented as a dendrogram by using the sequential, agglomerative, hierarchical and nested (SAHN) clustering method. Bootstrapping (1000 replications) was performed using the Winboot program (Yap & Nelson, 1996) to assess the strength of the clusters generated by the tree-making program and to test the consistency of the estimated relationships among individuals and populations (Felsenstein, 1985).

Results

Interspecific discrimination of Cochliomyia *spp.*

Four populations of *C. hominivorax* (PN, CR, JA and MX) and one population of *C. macellaria* (CM) (table 1) were used to screen ten primer combinations (E1+M7, E1+M6, E2+M7, E2+M2, E2+M3, E2+M1, E2+M4, E2+M6,

Table 5. Comparison of the genetic similarity levels in populations of *C. macellaria* (CM) and *C. hominivorax* (PN, CR, JA, MX, CE, C9, LH, LM, RL and CN) as calculated by the Jaccard and Dice coefficients for the interspecific (a) and intraspecific (b) studies.

(a)

Species	Jaccard coefficient (%)	Dice coefficient (%)
C. macellaria	92	96
C. hominivorax (all populations)	68	81
PN	94	97
CR	88	94
JA	86	92
MX	100	100
Between CM and *C. hominivorax*	16	28

(b)

Strain	Jaccard coefficient (%)	Dice coefficient (%)
PN	75	86
CR	73	84
JA	78	88
MX	89	94
CE	88	94
C9	92	96
LH	90	94
LM	89	94
RL	89	94
CN	85	92
Among all strains	58	74

E2+M5 and E3+M8) (table 3). The two species *C. hominivorax* and *C. macellaria* were readily distinguished by their well-resolved AFLP banding patterns generated by all of the ten primer combinations tested (fig. 1). Two kinds of parameters were considered for this study: (i) the presence or absence of diagnostic bands (particularly those present only in *C. macellaria* and absent in *C. hominivorax* and/or vice versa); and (ii) the existence of a characteristic, reproducible pattern of AFLP–PCR products, revealing a potentially diagnostic profile for a particular geographical population. The ten primer combinations used generated a large number of fragments (10–35 bands per individual) that depended on the type of selective nucleotides used. For statistical analyses, 52 consistent and discrete, scorable bands were used: seven bands were monomorphic and were found in both species; 22 bands were specific for *C. macellaria* populations; of the remaining 23 bands, ten were found only in *C. hominivorax* and 13 bands were diagnostic for different geographical populations of *C. hominivorax*. The species-specific, as well as population-specific, diagnostic bands for *Cochliomyia spp.* discrimination and identification are listed in table 4. Cluster analysis of similarity matrix (Jaccard coefficient) by UPGMA unambiguously separated the flies of each species into two major clusters that were only 16% similar. Also, bootstrap analyses showed 100% support for the branch separating the two species (fig. 2). Although both Jaccard and Dice coefficients generated similar clustering patterns, the resulting similarity values were slightly different (table 5a). *Cochliomyia macellaria* samples formed a distinct cluster, grouped together at the 92% level. Within this cluster, the similarity values between individuals in the population varied (range 92–100%). *Cochliomyia hominivorax* samples formed four distinct clusters, and bootstrap values among individuals within a population and between populations were variable (fig. 2). Populations from PN grouped together at the 94% level (range 94–100%), populations from MX grouped at the 100% level, CR samples clustered together at the 88% level (range 88–100%) and samples from JA grouped together at the 86% level. Within the *C. hominivorax* groupings, flies from PN and MX at the 72% level and CR and JA at 79% level were apparent. Populations of *C. hominivorax* from the four geographical locations grouped at the 68% level. Fly specimens from MX showed a high degree of similarity to each other (100%), whereas flies from JA showed relatively higher heterogeneity compared with the other flies (fig. 2).

Intraspecific discrimination of C. hominivorax *strains*

PCR amplification of DNA from the ten *C. hominivo*rax strains (table 1) produced a discrete banding pattern (fig. 3) for each of the ten primer combinations tested (E2+M1, E2+M2, E2+M3, E2+M6, E2+M4, E2+M5, E2+M7, E2+M8, E3+M7 and E3+M8) (table 3). The number of bands generated by each primer pair ranged between ten and 20 and depended largely on the selective nucleotides used. For estimating intraspecific relationships among different screwworm strains, statistical analyses were performed using 72 bands composed of 19 monomorphic fragments (found in all *C. hominivorax* strains) and 53 polymorphic bands. Cluster analysis of similarity matrix (Jaccard coefficient) by UPGMA grouped all of the strains with a similarity level of 58%. At the 85% similarity level, seven clusters (MX, LM, CN, LH, C9, CE and RL), representing mutant strains, were resolved; and, at the 72% similarity level, all ten clusters (representing ten strains) were resolved (fig. 4). PN flies grouped at the 75% similarity level, JA flies at the 78% level, MX flies at the 89%, LM flies at the 89% level, CN flies at the 85% level, LH flies at the 90% level, C9 flies at the 92% level, CR flies at the 72% level, CE flies at the 88% level and RL flies at 89% level. Within the *C. hominivorax* strains, PN, JA and CR flies seemed to have a relatively lower level of similarity compared with the mutant strains from MX (table 5b) that had similarity levels greater than 85%. Bootstrap values are presented for each strain and not for individuals within a strain (fig. 4). The bootstrap values for clusters representing individual strains were high, suggesting a strong cluster. However, clusters of different strains had a lower bootstrap value (fig. 4), suggesting a weak cluster. Additionally, compared with the previous study of interspecific relationships in screwworms, here we observed that JA strain flies grouped with MX (and not CR strain) at the 72% similarity level; and these two groups further clustred with PN flies at the 67% level. Strain-specific marker bands generated by AFLP primers are presented in table 4b.

Discussion

Genome fingerprinting with arbitrary primers is a versatile method for obtaining highly informative genetic markers in geographical populations of insects, and the information generated from such studies can be used for

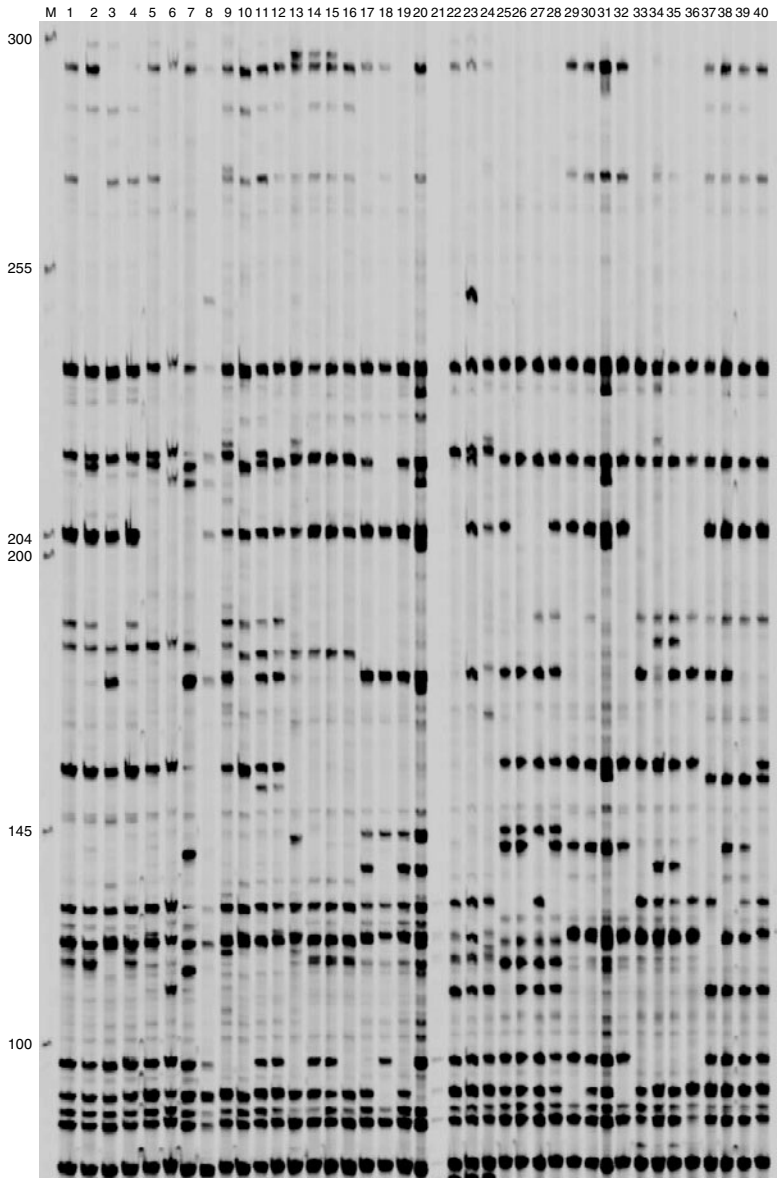

Fig. 3. AFLP fingerprints generated using primer combination E-ACA + M-CTC from the genomic DNA of ten strains of *C. hominivorax*: lanes (1–4) PN, (5–8) CR, (9–12) JA, (13–16) MX, (17–20) CE, (21–24) C9, (25–28) LH, (29–32) LM, (33–36) RL and (37–40) CN. Lane M contains IRD-700 ladder (50–700 bp).

tracking individuals, genes or both (Parker *et al*., 1998). As with random amplified polymorphic DNA (RAPD) (Welsh & McClelland, 1990; Williams *et al*., 1990), the AFLP technique generates individual specific profiles from template DNA by amplifying anonymous fragments from sites scattered through the genome (Borowsky, 2001). However, AFLP differs from the RAPD method in technical detail and has several advantages, such as higher temperature stringency, resulting in more reliable, reproducible data (Majer *et al*., 1996; Maugham *et al*., 1996; Sanchez *et al*., 1998; Reineke *et al*., 1999). Similar to RAPD markers, AFLP produces dominant markers and also is affected by small changes in reaction conditions, such as buffer concentrations, quality and concentration of DNA, source and concentration of *Taq* polymerase enzyme, and amplification conditions (Cervera *et al*., 2000); therefore, we optimized several parameters as defined in 'Materials and methods'.

A primary requirement for the screwworm eradication program is the correct identification of species, because facultative and scavenger flies in the families Calliphoridae and Sarcophagidae may be attracted to necrotic wounds of animals, thereby confounding the identification dilemma in the early instars, especially by morphological observations (Hall & Wall, 1995). On average, we identified at least 5–10 diagnostic bands per primer pair to discriminate *C. hominivorax* from *C. macellaria*. Observed differences in the banding patterns, low levels of similarity (16%) and 100% support for the branch separating *C. hominivorax* from

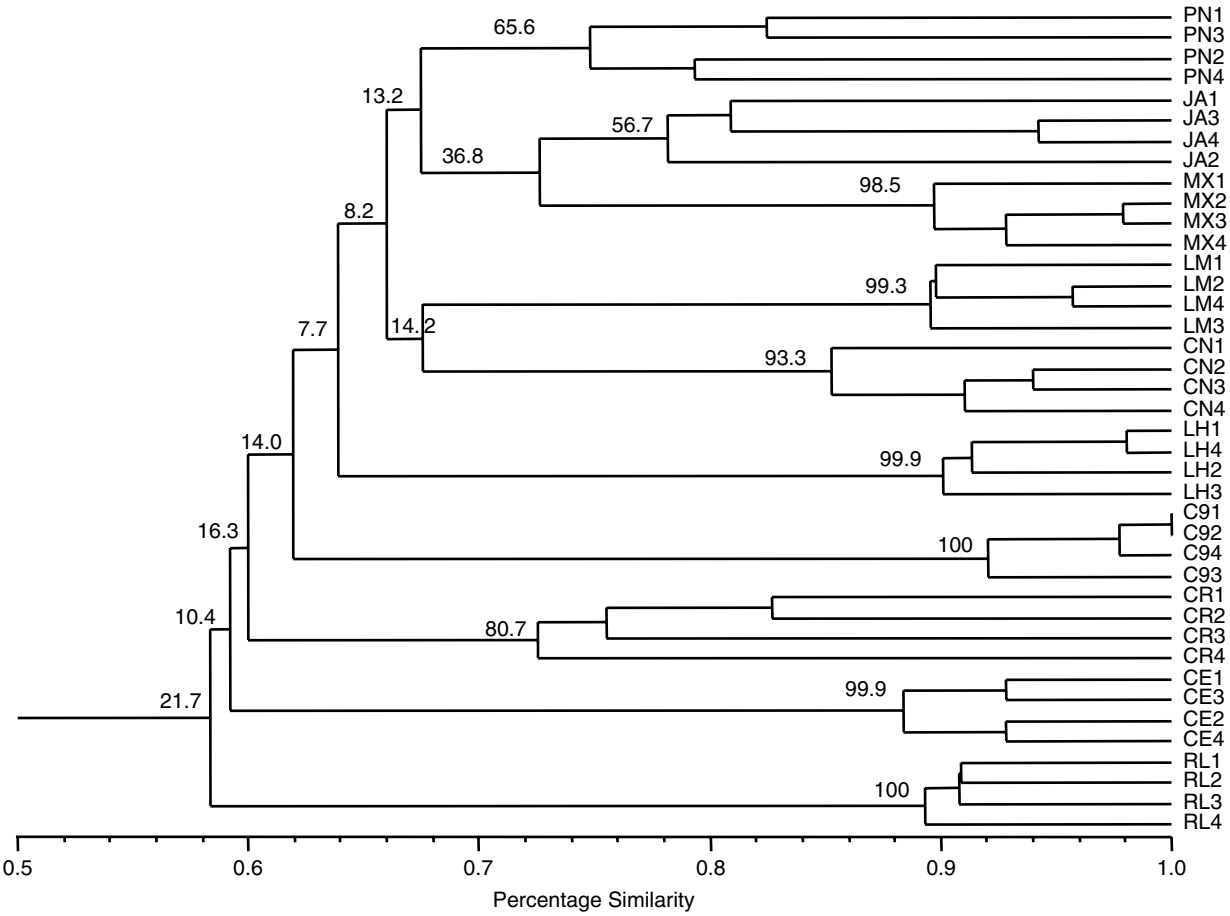

Fig. 4. Dendrogram showing intraspecific relationship among *C. hominivorax* populations (PN, CR, JA, MX, CE, C9, LH, LM, RL and CN) based on data from ten AFLP primer combinations. Similarity values were calculated using the Jaccard coefficient, UPGMA clustering. Values on branches indicate bootstrap values.

C. macellaria indicate that the two species have considerable genetic differences and that AFLP is a good tool for rapid identification at the interspecific level (figs 1 and 2). Species-specific diagnostic bands were found (table 4a) that provide for discrimination of these species based on the presence or absence of some AFLP–PCR products. AFLP analysis yielded 10–35 bands per individual per primer combination, more than those detected either by RAPDs (Skoda *et al*., 2002) or PCR–RFLP of mitochondrial DNA (mtDNA) (Taylor *et al*., 1996), many diagnostic bands that may ultimately be used for characterizing *C. hominivorax* strains (table 4b).

The population genetics of screwworms explored in the intraspecific studies showed that flies from MX were more homogenous and had high percent similarity values and bootstrap support (>95%). For example, MX flies grouped at the 100% similarity level (fig. 2) and 89% similarity level (fig. 4) in both studies, respectively. Also, other mutant strains originating from Mexico (CE, LH, LM, RL and CN) had >85% similarity level, and the branches were well supported (>95%) by bootstrap analysis, indicating low genetic variability (fig. 4). These results are consistent with the strains from MX (table 1) being the oldest of the screwworm strains examined (established as laboratory cultures in 1984–1985). Consistent with this observation is the relatively higher variability (lower percent similarity) of JA flies that represented the more recent (1998) establishment of the population from Jamaica as laboratory colonies. AFLP patterns for the earlier, interspecific studies indicated similarity between two populations, PN and MX; whereas CR showed similarity with JA populations (fig. 2). However, in the intraspecific studies clustering between populations was not strong (fig. 4). This difference in the clustering may be caused by the small sample size for intraspecific studies as well as the difference in the primer pairs screened.

In general, within-population variability was low compared with between-population variability, suggesting population substructure in *C. hominivorax*. The relatively higher variability between populations probably occurred because different alleles were fixed in different populations during culture. These results are similar to results reported using RAPD–PCR (Skoda *et al*., 2002) on laboratory populations, as well as by Infante-Malachias *et al*. (1999) in screwworms from southeastern Brazil and Argentina. Population genetic studies of screwworms using protein electrophoresis indicated a low genetic differentiation in flies (Krafsur & Whitten, 1993; Taylor & Peterson, 1994), whereas mtDNA analyses revealed high genetic variability and low gene flow (Roehrdanz & Johnson, 1988; Roehrdanz, 1989). As

suggested by Taylor *et al*. (1996), the low level of genetic variation observed within screwworm populations can be caused by bottlenecking, especially when samples used in the analyses are obtained from colonies that were in the laboratory for ≥4 years, as in this case. Microsatellite markers for screwworms, co-dominant and potentially highly polymorphic (Torres & Azeredo-Espin, 2005), used along with AFLP, dominant but providing high numbers of markers, may provide additional resolution for assessing genetic variability in screwworms.

In conclusion, our study demonstrates that, despite certain limitations, AFLP–PCR analysis is a good tool for differentiating species and has tremendous potential for studies of intraspecific genetic variation; it is capable of elucidating relationships and may provide diagnostic markers that identify geographical populations of *C. hominivorax*. Also, diagnostic bands could be isolated from the gels and sequenced so that specific primers can be generated and then used to determine the geographical origin of an outbreak or infestation. In addition, these markers can be used effectively to screen strains for quality control in mass-rearing facilities.

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