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A new DNA extraction protocol for screwworm fly *Cochliomyia* species (Diptera: Calliphoridae)

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Modifications to the DNA isolation protocol from *Cochliomyia* spp., are suggested based on the Chelex® 100 reactive. To apply the sterile insect technique (SIT) program it is necessary to study the molecular variations of endemic populations with efficient, fast and low costs techniques. The test samples were collected in the Pichincha province of Ecuador. The isolation protocol had 3 steps: (a) pretreatment (optional), (b) mechanic and chemical lysis, (c) two incubations; then the supernatant were separated by centrifugation. Furthermore, variations in concentrations of magnesium chloride present in the master mix were evaluated. Results showed a high efficiency in isolation with approximately 1.20 h of manipulation (without pretreatment). Additionally, the quality of the amplicon that was visualized on 2% agarose (w/v) showed that the magnesium chloride concentration was influential in the PCR reaction mix.

Keywords: *Cochliomyia* spp., DNA extraction, magnesium chloride

INTRODUCTION

Cochliomyia hominivorax (Coquerel) (Diptera: Calliphoridae), is a New World screwworm (NWS) which in its larval stage is an obligate parasite that causes parasitic and zoonotic disease known as traumatic myiasis in warm-blooded animals including humans (Forero et al., 2008; Bárcenas, 2010). The illness is included in the list of multiple species diseases, infections and infestations and their presence is notifiable (OIE, 2013). The NWS is also considered part of Transboundary Diseases in the Americas (GF-TADs, 2007).

NWS is endemic to tropical and subtropical regions of America, where it is widely distributed, mainly in South America and the Caribbean (Wyss and Galvin, 1996; Wall and Shearer, 1997). The annual losses caused by screwworm infestations in the United States, Mexico and Central America were significant; while in some Caribbean islands and South America it remains as a latent problem (Forero et al., 2007; Rodríguez et al., 2011). The programs based on the sterile insect technique eradicated the NWS caused by *C. hominivorax* from the southern United States, Mexico, Central America and some Caribbean islands (Robinson et al., 2009). For the period 2012–2021 the FAO has provided strategies for collecting information about the incidence of populations of screwworm and the impact of NWS on public health, in order to establish the eradication program in South America (FAO, 2011).

In Ecuador, two studies surveyed on animals from tropical areas reported presence of screwworm larvae in 4132 and 830 animals, respectively (Miño et al., 2005; Arteaga et al., 2012). Besides, studies carried out at the International Centre for Zoonosis of the Central University of Ecuador (CIZ-UCE) on adult *Cochliomyia* spp. have shown difficulties in the morphological differentiation of *C. macellaria* and *C. hominivorax* (unpublished data).

Griffiths et al. (2009), Torres and Azeredo-Espin (2009), Robinson et al. (2009) and Lyra et al. (2009) have reported the use of different molecular techniques and several modifications of the DNA isolation protocols for *Cochliomyia* spp. The principal changes were based on the type of sample (legs, abdomen and complete specimen) to be analyzed and focused to phenol-chloroform or commercial kit methods, but the quantification and purity was not reported. The aim of this study was to prove a fast and user-friendly protocol of DNA isolation, that will allow to study the genetic variability and molecular taxonomy of the species in Ecuador. In order to contribute to an efficient biological control method with more genetic information based on a large number of samples in a short period of time (Klassen and Curtis, 2005; McDonagh et al., 2009).

MATERIALS AND METHODS

SAMPLE

The specimens were collected as larvae from animals with myiasis and bovine corpses, which were *in-vitro* cultured in the laboratory until adult stage. All specimens were from the northwest of the Pichincha province. All specimens were labeled and deposited in the bank of specimens of CIZ-UCE; each specimen was preserved dry individually in a tube of 1.5 ml at—20°C for ≈3 months until laboratory process. Details of 9 adults specimens selected

for this analysis are shown in **Table 2**. Additionally, all flies were previously identified morphologically as *Cochliomyia* spp., using dichotomous keys published by FAO (1993).

ISOLATION AND QUANTIFICATION OF DNA

Modified and standardized according to Musapa et al. (2013), Golczer and Arrivillaga (2008), Nagdev et al. (2010), Arrivillaga et al. (2002) (**Table 1**). *Pretreatment—Hydration of the sample* (optional): The section of the specimen (**Table 2**) was suspended and incubated in 250 µl of Buffer H [100 mM Tris-HCl (Invitrogen), 50 mM EDTA (Invitrogen), 100 mM NaCl (Schalau), 0.5% SDS (Sigma), 200 mM sucrose (Santa Cruz Biotechnology)] at room temperature overnight if the samples are drying or 30 min if the samples are fresh.

SAMPLE HOMOGENIZATION AND CHEMICAL LYSIS

Mechanical lysis was divided into three steps: (a) grinding of the sample with scissors for 15 s; (b) homogenization with battery micromortar (Kontes, Vineland, NJ) for 15 s; and (c) centrifugation for 5 min at 13,000 rpm to separate the Buffer H from the crushed sample. Then, the supernatant was discarded and 200 µl of TEX buffer [10 mM Tris pH 8.0 (Invitrogen), 0.5 mM EDTA (Invitrogen), 1% Triton X-100 (Promega)] with Chelex ® 100 (Biorad) at 20% was added. 35 µl of proteinase K (20 mg/ml in PK buffer −50 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$) was added to the tube and the solution was incubated first for 1 h at 60°C with shaking of 1300 rpm and followed by 10 min at 100°C with shaking of 1000 rpm. Finally, the solution was centrifuged for 5 min at 13,000 rpm the supernatant was transferred to a new tube. A measurement of 2 µl of each sample was performed with spectrophotometry as per the Nanodrop 2000c program, by following the supplier's instructions. The results obtained were presented in **Table 2** and the samples were stored at -20 °C till further us.

POLYMERASE CHAIN REACTION

A genetic mitochondrial DNA (COI) fragment was amplified based on modifications to Arrivillaga et al. (2002) using the primers CIJ1632 (5′-TGATCAAATTTATAAT-3′—eurofins mwg operon) and CIN2191 (5′-GGTAAAATTAAAATATAAACTTC-3′—eurofins mwg operon). The concentrations were adjusted

Table 1 | Main steps of DNA extraction.

Protocol/steps	Time hours	1	2	3	4	5	6	7
Hydration (optional)	1–12	X				X		X
Homogenization (scissors)	0.015							X
Homogenization (pipette)	0.015						X	
Homogenization (Polytron Tissue Homogenizer)	0.015			X				
Homogenization (micromortar)	0,015	X						X
Chemical lysis	1–12		X	X		X		X
Salts methods		X		X				
Alcohol washes	1.20	X	X					
Phenol:cloroform						X		
Chelex (10% water)							X	
Chelex (20% in TEX buffer)	0.1		X					X
Commercial kit					X			
TOTAL TIME		3.15	2.0	2.30	2.0	2.0	1.0	1.20

X, step used in different protocols. 1.- Golczer and Arrivillaga (2008); 2.- Nagdev et al. (2010) [with bacterial colonies]; 3.- Aljanabi and Martinez (1997); 4.- Griffiths et al. (2009); 5.- Infante-Vargas and Azeredo-Espin (1995); 6.- Musapa et al. (2013); 7.- New protocol.

Table 2 | Results of DNA quantifications from samples of *Cochliomyia* spp., collected in Pichincha—Ecuador.

#	Sample	Sex	Nucleic acids [ng/µl]	A260	A280	260/280	260/230	Observations
1	1 leg	Female	216	4.32	7.71	0.56	0.25	Larvae cultured *in-vitro* from bovine corpses.
2	1 leg	Male	193.6	3.872	6.43	0.6	0.21	
3	1 leg	Female	139.3	2.786	4.35	0.64	0.16	
4	2 legs	Male	213.1	4.263	7.78	0.55	0.26	Larvae cultured *in-vitro* from Myiasis of cow—hip lesion.
5	2 legs	Female	197.9	3.959	7.27	0.54	0.23	
6	2 legs	Female	189	3.78	6.65	0.57	0.22	
7	Midabdomen	Male	277.4	5.547	9.89	0.56	0.36	
8	Midabdomen	Female	281	5.62	9.69	0.58	0.35	
9	Midabdomen	Female	301.6	6.032	10.33	0.58	0.41	

260/280, 260/230: absorbance rate.

to obtain the appropriate master mix reagents quantities (see **Table 3**). To assess the optimal concentrations, the quality of the band (sharpness and intensity), the presence of non-specific bands and primers excess were considered. The amplification conditions were as follows: initial denaturation step of 95°C for 3 min was followed by 35 cycles of 95°C for 0.3 min, 45°C for 1 min and 72°C for 1 min, and final elongation step of 72°C for 7 min. Finally, the amplicons were separated by electrophoresis on a 2% agarose gel (w/v) (**Figure 1**).

RESULTS AND DISCUSSION

The application of several steps and buffers permitted to obtain suitable DNA extracts with better PCR amplification in a short period of time. Resulting in, less cost in reagents compared with other methods, due to the correct sequence of the steps of the different protocols. Previous reports about DNA isolation from samples of *Cochliomyia* spp., using phenol-chloroform and salts reported an average of 2–3 h (Infante-Vargas and Azeredo-Espin, 1995; Aljanabi and Martinez, 1997). Although, the method 6 had 1 h because the authors used a complete specimen without chemical lysis, the results present more inhibition components and less efficient. Due to the characteristics of the preserved specimens, a hydration step is required to perform a pre-treatment and prepare the samples for cell lysis. The use of TEX buffer with 20% of Chelex® 100 provided the conditions to chemical attraction between the components of the sample with the chelating ion. Chelex contained paired ions to pH 7–12, which act as chelating groups in binding polyvalent ions. The resin is highly selective for positive ions and it is much higher bond strength with the components (Walsh et al., 1991). Although variations were found in the absorbance that did not relate with the standards, the PCR's performance was not affected. DNA quantification is shown in **Table 2**. The analysis of the data provided a reference for the state of the samples and final results showed in the PCR and with an efficient sequencing. The absorbance at 260 nm (0.1–1 optimum values according to the Lambert-Beer law and the wavelength of maximum absorption of the components) refers to a good quality of DNA. The results obtained present high values compared with the standards due to presence of double and single stranded DNA and RNA because RNase or alcohols purification were not used. In 260/280 (1.8–2.0 optimum values) (Chen et al., 2010), the results obtained (0.54–0.64) demonstrated the presence of DNA but with the presence of proteins that does not interfere with the PCR reaction. Chelex® 100 reagent could not have a total efficiency to capture the excess of proteins due to the saturation of the chelating resin. Finally, 260/230 (2.00–2.2, optimum values), the results obtained were between 0.16 and 0.41 that indicated the presence of reaction inhibitors (e.g., carbohydrates, peptides, phenols, degraded molecules of RNA) (Linton et al., 2001), these contaminants did not interfere with the PCR amplification.

In the PCR results, the variation of final concentrations of the chemical components allowed the observation of amplicons

Table 3 | Final concentrations of the different master mixes tested and qualitative results obtained for samples *Cochliomyia* spp., collected in Pichincha—Ecuador.

	$[]_0$	$[]f_1$	$[]f_2$	$[]f_3$	$[]f_4$	$[]f_5$	$[]f_6$	$[]f_7$	$[]f_8$	$[]f_9$	$[]f_{10}$	$[]f_{11}$	$[]f_{12}$
H_2O	–	–	–	–	–	–	–	–	–	–	–	–	–
Buffer [X](Promega)	5	1	1	1	1	1	1	1	1	1	1	1	1
$MgCl_2$[mM] (Promega)	25	4	2	2	1,5	2	2,5	1,8	2,5	1,8	3	2,5	3
dNTPs [mM] (Promega)	10	0,2	0,2	0,3	0,2	0,2	0,2	0,2	0,2	0,2	0,2	0,3	0,3
CI-J-1632 [mM]	100	0,8	0,6	0,5	0,7	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6
CI-N-2191 [mM]	100	0,8	0,6	0,5	0,7	0,6	0,6	0,6	0,6	0,6	0,6	0,6	0,6
Polymerase [U/μl] (G2-Promega)	5	0,1	0,4	0,03	0,04	0,04	0,04	0,04	0,05	0,05	0,04	0,04	0,04
Bands		+++	+	–	–	+	+	–	++	+	++	++	++
Non-specific Bands		+	–	–	–	–	–	–	+	–	+	–	–
Primers excess		–	–	–	–	–	–	–	–	–	+	–	+

$[]_0$: Initial concentration; $[]f_{1–12}$: final concentration.

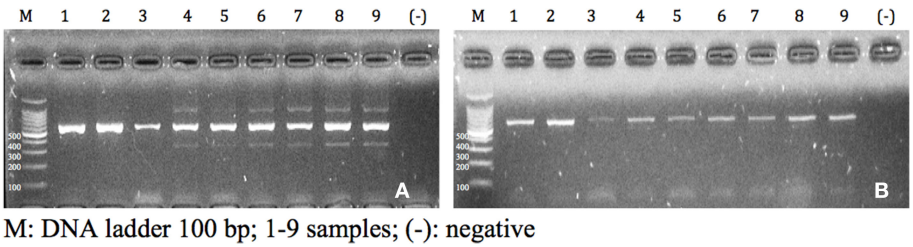

FIGURE 1 | Amplicons visualized in agarose gel of 2% (w/v) of samples of *Cochliomyia* spp., collected in Pichincha-Ecuador. (A) f_1 concentration; **(B)** f_2 concentration.

with good quality according to the parameters evaluated (quality of bands, non-specifc bands, and primer excess). Variation in absorbance values showed interference or relation of the different components of the sample. Further studies are necessary to detect possible inhibitors that intergrade the sample.

The qualitative assessments demonstrated variations among assays, as shown in **Table 3**. These variations were in the reaction and concentration of $MgCl_2$/polymerase. The highest concentration of magnesium chloride, i.e., 3.0 mM, had the best results, discarding problems for primers and total amount of polymerase. The appropriate concentration of $MgCl_2$ improves the catalytic activity of the polymerase (ref). Due to the characteristic of the sample could be precipitate the reagent induce negative PCR. The results showed that f_{12} test was the best (**Table 3**). Additionally, the reproducibility of the test was assessed by different laboratory technicians and with more samples.

CONCLUSIONS

Contrary to reported protocols, this assay permitted to obtain suitable molecular results in a short period of time (≈1.2 h, except hydration) using few reagents, friendly and with simple steps. Properties of Chelex® 100 reagent facilitated the purification of DNA from the complex molecules present in the initial sample. The results of the method shows good quality of the bands in agarose, despite not removing all necessary components shown in absorbance allowing adequate amplifications. It was feasible to demonstrate that chemical variations of the PCR and the relative concentration of magnesium chloride/polymerase are proportional to the quality of the band, the presence of non-specific fragments and primers excess.

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