RANDOM AMPLIFIED POLYMORPHIC DNA-POLYMERASE CHAIN REACTION APPROACHES TO *LUCILIA SPP.* (DIPTERA: CALLIPHORIDAE) IDENTIFICATION

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INTRODUCTION

Blowflies are among the earliest recognized insect plagues of mankind. Many species are associated with dermal and secondary traumatic myiasis, and a few species are agents of primary traumatic myiasis (Hall, 1948). The sheep blowflies *Lucilia sericata* (Meigen) and *Lucilia cuprina* (Wiedemann) cause myiasis (sheep strike) of sheep in the temperate zone of the northern hemisphere and throughout Australia (Anonymous, 1933; Murray, 1980; Watts *et al.*, 1979; and Zumpt, 1956). Infestation of sheep with *L. sericata* and *L. cuprina* constitute one of the major problems of sheep industry throughout the world. The parasitic role of the flies on the sheep reduces the wool clip. Infested sheep suffer from anemia, alteration in protein and mineral metabolism as well as decrease in weight gain and wool production (Abdel-Meguid *et al.*, 2001).

The traditional methods used to detect and differentiate closely related species include mating incompatibility, morphometric, polytene chromosomes analysis and isozyme electrophoresis (Coluzzi *et al.*, 1979; Collins *et al.*, 1988; Rosa Freitas *et al.*, 1990; Coetzee *et al.*, 1993; Foley and Bryan, 1993).

Recently, many molecular genetic techniques as RAPD-PCR were described by Williams *et al.* (1990); Kambhampati *et al.* (1992) and Raich *et al.* (1993) for the differentiation of subspecies and species in insects, for the detection of genetic polymorphism and for the selection of genetic markers with no prior knowledge of DNA sequence.

The objective of the present work is to use modern molecular genetic technique to determine the genetic divergence in the two species of *Lucilia* and to identify the taxonomic status of *Lucilia sericata* and *Lucilia cuprina* in Egypt.

MATERIAL AND METHODS

Insect collection and DNA extraction:

Colonies of *Lucilia sericata* (Meigen) and *Lucilia cuprina* (Wiedemann) were initially established in the laboratory by collecting gravid females of *L. sericata* from Borg El-Arab, 45 Km West of Alexandria Governorate, Egypt. While gravid females of *L.cuprina* were supplied from the Department of Entomology, Cairo University.

Collected female adults of *L.sericata* were transferred to the laboratory, stored, sexed and identified according to Zumpt (1965) and Tantawi and El-Kady (1997). Stock colonies were reared under laboratory conditions of 25±2°C and 40-60%R.H. according to Adham *et al.*, (2003) and El-Bassiony (1996) for *L.sericata* and *L. cuprina*, respectively.

Samples of third instars larvae of *L.sericata* and *L.cuprina* were frozen directly and kept at -20° C until use for DNA extraction. Genomic DNA for both *Lucilia* species was extracted using the procedure described by Infante and Azeredo Espin (1995). Genomic DNA was extracted by digestion of body cells with proteinase-K in the presence of EDTA and SDS. Extraction and isolation of DNA was carried according to the method of Gross-Bellar *et al.* (1972).

RAPD-PCR:

A primary screen for RAPD- PCR conditions was used according to West and Black (1998) procedure. Amplification was performed in the thermal cycler (Perkin-Elmer, Gene Amp PCR system 9700) for 40 cycles. The cycling parameters of Williams *et al.* (1990) were used with some modifications: An initial denaturation step was performed at 94°C for 1min., annealed at 34°C for 2min., extended at 72°C for 3min. and finally a post-extension step was applied for 10 min. at 72°C and then stored at 4°C until use.

Amplification products of PCR and DNA size markers (QX174-Hind III markers Promega Biotech Inc.) were loaded onto a 1.5% agarose gel for genomic and 3% for mtDNA (Sambrook *et al.*,1989) and run at 50 volts for 2h. Gels were stained with ethidium bromide and photographed on an UV transilluminator. The bands were analyzed through a computer program to reveal the Nm (migration flow), the molecular size and the density of bands. The similarity index (S) compares patterns within, as well as, between different species. This index reflects

the extent of band sharing and was calculated according to Nei&Li (1979) as follows:

$$\text{Similarity index (S)} = (2Nab / Na + Nb)$$

Where Nab is the number of bands common to individuals a and b; Na and Nb are the total number of bands in individual a and b, respectively. The value produced by this index ranges from zero, (respecting no bands sharing) to 1 (respecting complete identity), while the genetic distance (G) between two species was estimated as follows:

$$\text{Genetic distance (G)} = 1 - \text{similarity index}$$

Primers that didn't produce well-amplified bands in the two species together were eliminated to reduce ambiguity.

RESULTS AND DISCUSSION

Table (1) revealed the seven primers OPA-14, OPB-08, OPB-09, OPC-03, OPC-04, OPH-16 and OPH-20 chosen for analysis, in order to detect differences between the two species and to produce banding patterns that could be scored. Only two primers OPH-16 (5`-TCT CAG CTG G-3`) and OPH-20 (5`- GGG AGA CAT C-3`) for PCR amplifications of genomic DNA from *L. sericata* and *L. cuprina* larvae were chosen.

TABLE (I)

RAPD-PCR primers used in each of the two species *Lucilia.*

Primer	Sequence	Species
OPA14	5`TCT GTG CTG G 3`	*L. cuprina*
OPB08	5`GTC CAC ACG G 3`	*L. cuprina*
OPB09	5`TGG GGG ACT C 3`	*L. cuprina*
OPC03	5`GGG GGT CTT T 3`	*L. cuprina*
OPC04	5`CCG CAT CTA C3`	*L. cuprina*
OPH16	5`TCT CAG CTG G3`	*L. cuprina, L. sericata*
OPH20	5`GGG AGA CAT C3`	*L. cuprina, L. sericata*

TABLE (II)

Molecular size (Mol. size) in bp, band percentage (Band %) and migration flow (Nm) of *L.sericata* and *L. cuprina* genomic DNA using OPH16 and OPH20 primers.

Parameters / Band no.	OPH16						OPH20					
	L.sericata			*L.cuprina*			*L.sericata*			*L.cuprina*		
	Mol.size	Band%	Nm	Mol.size	Band%	Nm	Mol.size	Band%	Nm	Mol.size	Band%	Nm
1				2000	5.21	0.052						
2				1800	4.85	0.048				1800	4.84	0.048
3				1500	11.5	0.12				1600	8.29	0.083
4				750	11.3	0.11				1000	12.3	0.12
5										600	14.6	0.15
6							450	14.9	0.15	450	16.6	0.17
7							350	22.5	0.22			
8				300	17.8	0.18						
9	200	21.7	0.22									
10	150	29.2	0.29				150	15.8	0.16	150	11.5	0.12
11				120	21.6	0.22						
12	50	5.53	0.055	50	1.96	0.02	50	6.43	0.064	50	4.05	0.04

PCR-amplification of *Lucilia* DNA with the two primers of arbitrary DNA sequence results in the amplification of a series of discrete fragments (Fig. 1). Primer OPH-16 generated 3 and 7 bands for *L. sericata* and *L. cuprina*, respectively. Primer OPH-20 generated 4 and 7 bands for *L.sericata* and *L.cuprina*, respectively.

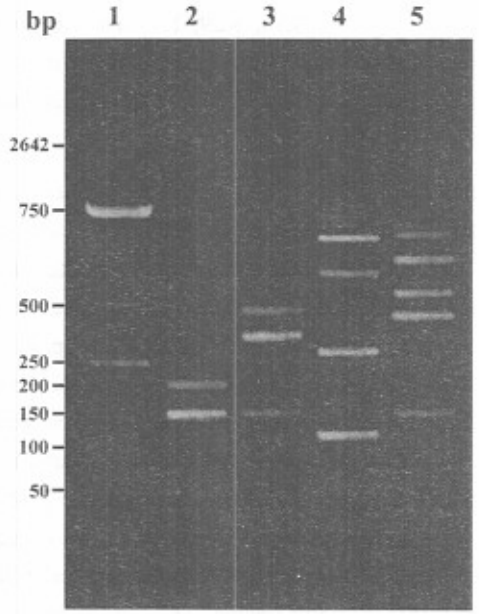

Figure (1): RAPD – PCR products from *L. sericata* and *L. cuprina* larvae. Reading lanes left to right: Lane 1, bp-size marker ; 2, *L. sericata* with primer OPH16 ; 3, *L. sericata* with OPH20 ; 4, *L. cuprina* with OPH16 ; 5. *L. cuprina* with OPH20.

With OPH-16 primer, the 200, 150 and 50 bp fragments were present in variable intensities and migration flows in *L.sericata* while in *L.cuprina* the 2000, 1800, 1500, 750,300,120 and 50bp fragments were also present as well in different intensities and migration flows. The species specific amplification bands of *L.sericata* were 200 and 150 bp fragments with band percentage 21.7 and 29.2 and migration flow 0.22 and 0.29, respectively. However, species specific bands of *L. cuprina* were 2000, 1800, 1500, 750, 300 and 120 bp fragments with band percentage 5.21, 4.85, 11.5, 11.3, 17.8 and 21.6 and migration flow 0.052, 0.048, 0.12,0.11,0.18 and 0.22, respectively. The similarity index and the genetic distance between *L. sericata* and *L. cuprina* were 0.2 and 0.8, respectively.

With OPH-20 primer, the 450, 350, 150 and 50 bp fragments were present in relatively variable intensities and migration flows in *L. sericata* while in *L. cuprina* the 1800, 1600, 1000, 450, 150 and 50 bp fragments were also present as well in different intensities and migration flows. Species specific amplification bands of about 450 bp fragment; 14.9 band percentage and 0.15 migration flow were produced in *L. sericata* larvae while species specific amplification band of 1800, 1600, 1000 and 600 bp fragment; 4.84, 8.29, 12.3 and 14.6 band percentage and 0.048, 0.083, 0.12 and 0.15 migration flow were produced in *L.cuprina* larvae. The similarity index and the genetic distance between *L. sericata* and *L. cuprina* were 0.54 and 0.46, respectively.

The primers used to differentiate between the species in this study produced apparently different patterns. Some of these differences were in fact quantitative. The quantitative differences observed might have resulted from variable copy members of target DNA sequences with more intense bands representing multiple copies. Meanwhile 5 primers out of the 7 primers used produced well-amplified bands with *L.cuprina* and did not amplified with *L. sericata* indicating that there is variation among individuals within the two species; however, some DNA fragments were unique to a given species and are present in all individuals of that species. Such conserved fragments can be used to generate a diagnostic profile (Table 2). This could be explained "that if the employed primer amplifies conserved region (s) in the genome, it is assumed that closely related species are more likely to share those regions than those that are distantly related" (Kambhampati *et al.*, 1992). RAPD data should reflect the ancestral relationships. It is possible that the primers we employed, although resulting in the amplification of species-specific DNA fragments, did not result in the amplification of lineage-specific DNA fragments.

Our results indicate that, based on RAPD patterns, the degree of similarity between *L. sericata* and *L. cuprina* is low and they are easily distinguished by RAPD-PCR. The genetic distance between *L.sericata* and *L. cuprina* was so great; it reached with the primer OPH16 to about 0.8 indicating that the two species are genetically distinct. These results confirm the findings of Hepburn (1943) and Waterhouse and Paramonov (1950) regarding *L.sericata* and *L.cuprina* as quite distinct species.

It is concluded that RAPD-PCR is best suited for the identification of *L.sericata* and *L. cuprina*. This method will be particularly useful in cases where the morphological characters do not permit an unambiguous or a rapid identification of species. This suggests that these relatively simple techniques may become

invaluable in vector surveillance and control operations by providing species and population genetic markers.

SUMMARY

The sheep blowflies *Lucilia sericata* and *Lucilia cuprina* are of great medical significance as primary causal agent of myiasis in sheep. To distinguish between these two species RAPD-PCR techniques were used. Results obtained showed that 2 primers only out of the 7 primers used, readily distinguish between the two species. Similarity index and genetic distance calculated from RAPD bands indicated that these two species of blowflies are different from each other. They share only one band with OPH16 and 3 bands with OPH20. When OPH16 was used 2 diagnostic bands in *Lucilia sericata* and 6 diagnostic bands were detected in *Lucilia cuprina*. On the other hand, when using OPH20 only one diagnostic band appeared in *Lucilia sericata* and 4 diagnostic bands appeared in *Lucilia cuprina*.

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