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The use of random amplified polymorphic DNA (RAPD) analysis for studies of genetic variation in populations of the blowfly *Lucilia sericata* (Diptera: Calliphoridae) in southern England

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Abstract

The use of the random amplified polymorphic DNA (RAPD) polymerase chain reaction to characterize individual *Lucilia sericata* Meigen from southern England was evaluated. Some simple techniques which allowed the preservation and extraction of DNA to be optimized without the complications of transporting liquid nitrogen were investigated. The RAPD results show that closely related *L. sericata*, including those from a single strike, can be readily distinguished from each other on the basis of their RAPD profiles resolved using electrophoretic analysis; profiles were defined with ten random primers. Analysis of these RAPD data using a similarity coefficient method and a recently developed randomization test to detect the non-random association of alleles at different loci, allowed the genetic homogeneity of *L. sericata* within southern Britain to be explored. This study shows that while a number of factors can complicate the use and interpretation of RAPD fragments as genetic markers, RAPD fingerprinting can be a valuable technique for studies of intraspecific genetic variation in *L. sericata*.

Introduction

The random amplified polymorphic DNA (RAPD) polymerase chain reaction technique (Welsh & McClelland, 1990; Williams *et al.*, 1990) has been used previously for population genetics studies of a number of insects including, aphids (Black *et al.*, 1992), grasshoppers (Chapco *et al.*, 1992) and fruit-flies (Haymer & McInnis, 1994). In the current study RAPD analysis was used to investigate genetic variation within populations of the blowfly *Lucilia sericata* Meigen (Diptera: Calliphoridae) from southern Britain. This species is a primary agent of cutaneous myiasis in many parts of the world, largely affecting sheep, although a range of other wild and domestic animals and humans may also be infested (Hall & Wall, 1995).

The RAPD technique appears particularly suitable for population studies of organisms, such as insects, for which only nanogram quantities of DNA can be extracted. However, a number of recent studies (reviewed by Black, 1993) have indicated that, in addition to template concentration, the manner in which DNA is extracted and preserved can also affect RAPD banding patterns (Post *et al.*, 1993). The aims of the present work, therefore, were to develop a protocol to standardize the yield and quality of extracted blowfly DNA and then to use RAPD data to consider the extent to which *L. sericata* within southern England freely interbreed using similarity coefficient analysis and a randomization method to test for linkage (Stevens & Tibayrenc, 1995).

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Methods and materials

DNA extraction

To avoid contaminating sample DNA with DNA from eggs, ingested protein or gut parasites, only the head and legs of male *L. sericata* were used as sources of DNA. After verifying that these tissues gave identical amplification patterns, tissues were combined when extracting DNA from field-caught individuals. DNA was extracted, as total nucleic acid, by the cetyl trimethyl ammonium bromide (CTAB)

method (see Towner, 1991) using 50 µl CTAB per tissue and 100 µl for combined extractions for each individual. The quality of DNA yielded, the suitability of CTAB extraction for material rich in polysaccharides, and the small number of extraction steps required, make this method ideal for preparation of the small quantities of DNA present in chitinous insect tissues. Between 1000 and 4000 ng of DNA was obtained separately from the head and legs of individual *L. sericata*, effectively providing an independent control for each individual.

The yield and quality of DNA extracted from field caught *L. sericata* was assessed on a 1% tris-glacial acetic acid-EDTA (TAE) agarose gel prior to RAPD characterization. Samples (2 µl of total nucleic acid) were run against 10 µl of heat denatured (65°C, 5 minutes) Lambda Hind III marker. The intensity of the upper band of high molecular weight in the sample DNA was then compared with the 4.36 kb marker band which contained approximately 0.1 µg DNA; this allowed the DNA yield of each sample to be readily assessed. The discreteness of the high molecular weight band and the degree of streaking in each sample lane provided a measure of DNA quality; RNA appeared as an indiscrete broad band near the bottom of the gel.

Two simple preservation methods were evaluated as alternatives to the use of liquid nitrogen for the short-term storage of field collected material; methods were evaluated using male *L. sericata* from the University of Bristol colony. *Lucilia sericata* were stored for 8 weeks in 100% alcohol at 4°C, and over silica gel at ambient temperature; 8 weeks was considered to be the maximum length of time that collected *L. sericata* would remain stored prior to DNA extraction. Extracted nucleic acid samples were resuspended in 12 µl of tris-EDTA (TE) buffer. DNA concentration was determined by measuring UV absorption; quality was assessed on an agarose concentration gel. The suitability of 100% alcohol for preservation was further evaluated by storing *L. sericata* for 0, 2, 4, 6, and 8 weeks in 100% ethanol at 4°C. DNA was diluted (1:100) and the concentration measured by examining UV absorbance using a spectrophotometer; measurements were taken at 260 nm (fig. 1). DNA quality was assessed by comparison with heat denatured Lambda Hind III marker on a 1% TAE concentration gel, which also provided a second alternative estimate of DNA yield.

Table 1. Origin, codes and year of field collection of individual *Lucilia sericata* from southern England.

Fly	Origin
CVL 22-2	Hall Farm, Central Veterinary Laboratory, Weybridge, Surrey 1992
CVL 28-1	Grange Farm, Central Veterinary Laboratory, Weybridge, Surrey, 1993
CVL 30-3	"
CVL 31-8	"
UBC 1-13	University of Bristol colony, 1990
UBC 1-15	"
UBC 1-17	"
WRN-1	Wrington, near Bristol, Avon, 1994
LANG-1	Langford, near Bristol, Avon, 1994
LANG-2	"
LANG-3	"
LANG-4	"
LANG S1-A	Flies collected as larvae from a single sheep-strike Langford, near Bristol, Avon, 1994
LANG S1-B	
LANG S1-C	
LANG S1-D	

RAPD analysis

For the study of genetic variation, RAPD analysis of *L. sericata* was carried out; details of the individuals analysed are given in table 1. Four of these (CVL) came from colonies which had been maintained at the Central Veterinary Laboratory, one since July 1992 and the other three since July/September 1993. The CVL colonies originated from

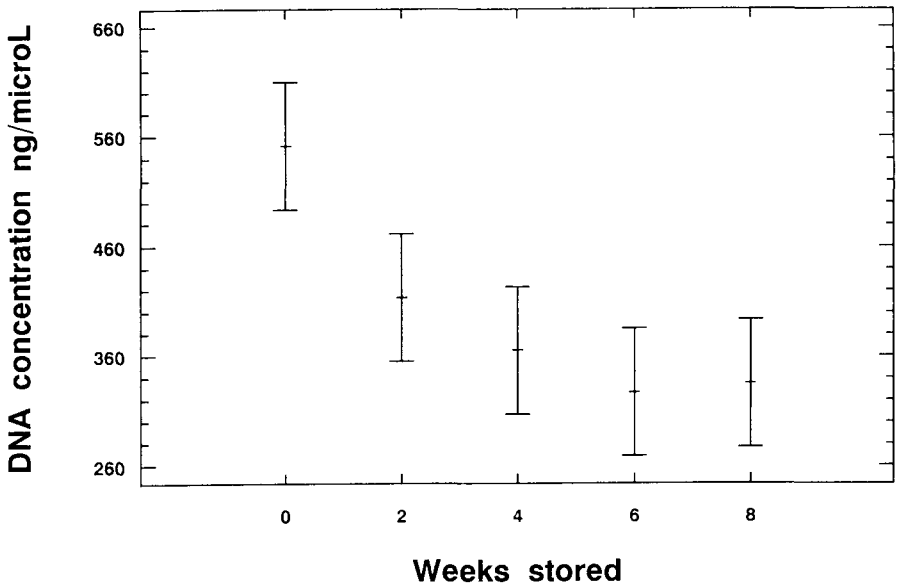


Fig. 1. DNA yield (total nucleic acid) from groups of three *Lucilia sericata* at 0, 2, 4, 6 and 8 weeks of storage in 100% ethanol at 4°C. Concentrations (ng/µl resuspended DNA) calculated from UV absorption at 260 nm; extracted, precipitated pellets from the combined head and legs of each fly were resuspended in 12 µl of TE buffer. 95% confidence intervals around the mean are indicated by bars.

larvae collected from four different sheep strikes from farms in the Weybridge area in Surrey. Three of the individuals used came from the Bristol University colony which originated from larvae removed from a single strike in the Weybridge area and which had subsequently been maintained at Bristol since 1991 (UBC). Five individuals were collected as adults from farms near the Bristol University Veterinary School in south-west England (LANG) and four were reared from larvae collected from a single sheep strike from a farm near Bristol (LANG S1).

RAPD analysis was performed according to the protocol of Williams *et al.* (1990) using SuperTaq *Taq* polymerase (HT Technology Ltd). Twenty 10-mer primers (Operon kit E; Operon Technologies, USA) were screened. All RAPD analyses were performed with a Hybaid TR1 thermal cycler, placed in a constant temperature room (15°C) to eliminate potential differences in cooling rate. The resulting PCR-amplified DNA fragments were separated in 1.4% TAE gels at 50 mA for 4 hours. Fragments were viewed under ultra-violet light after staining with ethidium bromide (10 minutes, 0.005% solution). Gels were photographed for scoring and numerical analysis. The presence of some faint bands complicated the scoring procedure and necessitated repeat amplification of certain samples; only bands reproducible between amplifications for each data set were scored (Graham *et al.*, 1994). RAPD patterns from ten primers (3, 4, 7, 8, 9, 11, 12, 14, 18, 19) proved suitable for inclusion in numerical analyses.

Numerical analysis

Analysis of variance, t-tests and a Tukey multiple range test were performed using Statgraphics V.5 (STSC Inc. USA).

Reproducible RAPD fragments detected by electrophoresis were scored for presence or absence, prior to the calculation of a phenetic distance measure based on Jaccard's method (1908). For RAPD data, where the total number of fragments is unknown, a similarity coefficient is preferred that does not allow the joint absence of a character to contribute to similarity (e.g. Jaccard's coefficient). For each RAPD pattern, each fragment was coded with a letter up to a maximum of 30 bands in the case of primer OPE-4; a maximum of 10 scorable bands were recorded for any one *L. sericata*, for any one primer. Similarity was calculated between each pair of *L. sericata* for each primer using the RAPD bands scorable in the two patterns for each primer in turn. A distance matrix (table 2) was produced using the program of Stevens & Cibulskis (1990). A dendrogram was derived from each matrix by the unweighted pair-group method using arithmetic averages (UPGMA), using SPSS/PC+.

The non-random association of alleles at different loci in *L. sericata* was studied using the method of Stevens & Tibayrenc (1995) to detect linkage disequilibrium. While the composition, in terms of alleles and loci, of individual RAPD genotypes remains unknown, such associations can provide evidence that gene flow is restricted in the population under study, whatever the reasons for this (Tibayrenc, in press). The test (see Stevens & Tibayrenc, 1995 for full details) is a generalized test for linkage independent of mating system or ploidy and suitable for use with RAPD data. In brief, the RAPD patterns observed for all primers are shared into two groups at random; a correlation coefficient (r) is then calculated between the two groups of distances for this, the observed arrangement of genotypes. Next, the genotypes (the RAPD patterns for each primer for each individual) are randomly reassorted, creating a 'new' set of individuals of randomly mixed genetic composition; this is repeated enough times to build up a randomized distribution of r values (e.g. 2000 times), a new r value being calculated for each reassortment. After the required number of random reassortments of the observed genotypes, a significance value can be attached to the observed correlation coefficient. The process is then repeated for the required number of random combinations of primers. In this study 20 combinations of primers were generated and 2000 random reassortments of each combination were run to construct each distribution of correlation coefficients.

Results

DNA storage and extraction

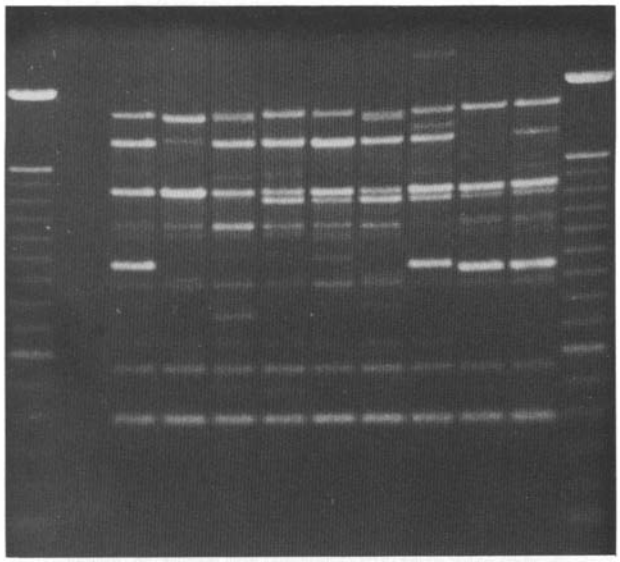
Combined head and leg tissues from *L. sericata* stored in 100% ethanol at 4°C yielded significantly more DNA (mean=335.0 ng/μl TE buffer, SD=13.2 ng/μl, n=3) after 8 weeks (t-test, 4 DF, $P < 0.004$) than DNA stored over silica gel (mean=201.7 ng/μl TE buffer, SD=36.2 ng/μl, n=3). The yield of DNA from *L. sericata* stored for 0, 2, 4, 6 and 8 weeks in 100% ethanol at 4°C decreased significantly between 0 and 2 weeks in storage (fig. 1; ANOVA, $F=12.47$, $P < 0.001$; Tukey multiple range test, $P < 0.05$). However, from 2 weeks onwards the change in yield did not alter significantly ($P > 0.05$) and remained at approximately 340 ng DNA/μl TE buffer (fig. 1). The quality of DNA, as assessed on a 1% TAE gel, did not appear to be affected by storage in ethanol at 4°C for up to 8 weeks. In consequence, ethanol preservation was used for all field-collected *L. sericata* prior to extraction. Attempts to extract DNA from dried specimens and individuals preserved in lower concentrations of alcohol, proved unsatisfactory; similar findings have been reported for Simuliidae (Post *et al.*, 1993).

RAPD-PCR analysis

Twenty random primers (Operon 10-mer Kit E) were screened; of these, ten produced discrete, scorable band patterns suitable for subsequent numerical analyses of genetic variation in *L. sericata* (e.g. primer, 14; fig. 2). Certain primers, e.g. OPE-2 and OPE-6, identified so much variation that they were of little use for broader numerical analyses. The reproducibility of results between *L. sericata* tissues (head and legs) was verified by RAPD analysis of DNA extracted separately from these two tissues. DNA from colony *L. sericata* was analysed using all OPE primers, except OPE-10 which did not amplify. In all instances RAPD fragment patterns for the two tissue types were identical within individuals. The effect of template DNA concentration on amplification pattern reproducibility was assessed at three concentrations. Amplification became inconsistent at or above 200 ng template DNA per reaction, and at or below 2 ng template DNA per reaction. Characterization reactions, therefore, were performed with a 20 ng template.

Table 2. Lower triangular similarity matrix computed using Jaccard's coefficient (1908).

	92/cvl 22-2	93/CVL 28-1	93/CVL 30-3	93/CVL 31-8	90/UBC 1-13	90/UBC 1-15	90/UBC 1-17	94/WRN-1	94/LANG-1	94/LANG-2	94/LANG-3	94/LANG-4	94/LANG S1-A	94/LANG S1-B	94/LANG S1-C	94/LANG S1-D
92/CVL 22-2	1.00															
93/CVL 28-1	0.60	1.00														
93/CVL 30-3	0.52	0.45	1.00													
93/CVL 31-8	0.41	0.38	0.43	1.00												
90/UBC 1-13	0.42	0.42	0.51	0.45	1.00											
90/UBC 1-15	0.40	0.40	0.49	0.43	0.94	1.00										
90/UBC 1-17	0.41	0.41	0.48	0.43	0.93	0.97	1.00									
94/WRN-1	0.38	0.32	0.45	0.43	0.44	0.42	0.41	1.00								
94/LANG-1	0.42	0.33	0.39	0.44	0.40	0.43	0.42	0.44	1.00							
94/LANG-2	0.41	0.38	0.45	0.45	0.46	0.48	0.50	0.47	0.43	1.00						
94/LANG-3	0.47	0.48	0.46	0.47	0.46	0.47	0.48	0.43	0.50	0.49	1.00					
94/LANG-4	0.41	0.44	0.42	0.38	0.50	0.48	0.49	0.54	0.44	0.57	0.53	1.00				
94/LANG S1-A	0.50	0.49	0.43	0.49	0.48	0.47	0.48	0.44	0.57	0.53	0.65	0.50	1.00			
94/LANG S1-B	0.44	0.50	0.43	0.44	0.47	0.45	0.47	0.45	0.45	0.52	0.53	0.62	0.60	1.00		
94/LANG S1-C	0.47	0.47	0.47	0.50	0.49	0.48	0.50	0.50	0.53	0.61	0.59	0.60	0.70	0.67	1.00	
94/LANG S1-D	0.44	0.47	0.45	0.51	0.50	0.49	0.51	0.53	0.52	0.65	0.59	0.59	0.68	0.67	0.87	1.00



1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2. RAPD fragment patterns for *Lucilia sericata* using primer OPE-14, lanes 3-11. 100 base-pair marker, lanes 1 and 12; PCR negative control, lane 2.

Numerical analysis

All *L. sericata* showed distinct RAPD electrophoretic types using the ten primers. The three individuals from the Bristol University colony (UBC 1-13, UBC 1-15 and UBC 1-17) were identical by six primers while, overall, numerical analysis of the RAPD fragments showed them to be more than 93% similar (fig. 3).

The four individuals derived from a single strike (fig. 3, LANG S1-A, B, C, D) were grouped together, somewhat less tightly, at the 64% level. However, two of these (LANG S1-C and D) were identical by five primers and 88% similar overall (fig. 3). *Lucilia sericata* from three of the four CVL strikes (CVL 22-2, CVL 28-1 and CVL 30-3) group together at the 48% similarity level, while the fourth (CVL 31-8) is 46% similar to other wild *L. sericata* from the Bristol area. Indeed, all wild caught *L. sericata* and those from three of the CVL strikes are only 46-58% similar.

Further analysis of the RAPD data were performed using a randomization method to detect linkage disequilibrium, where such linkage, being due to non-random associations between alleles at different loci, can be interpreted as a measure to which gene flow in the population under study is restricted; such evidence can provide an indication of barriers to gene flow in the population. Analysis of RAPD data for all *L. sericata* showed extreme linkage in all 20 combinations ($P < 0.001$). However, removal of any two of the three *L. sericata* from the same inbred Bristol colony (UBC 1-13, 15 or 17) reduced the number of combinations of primers which exhibited significant departures ($P < 0.01$) from random mixing to 2 out of

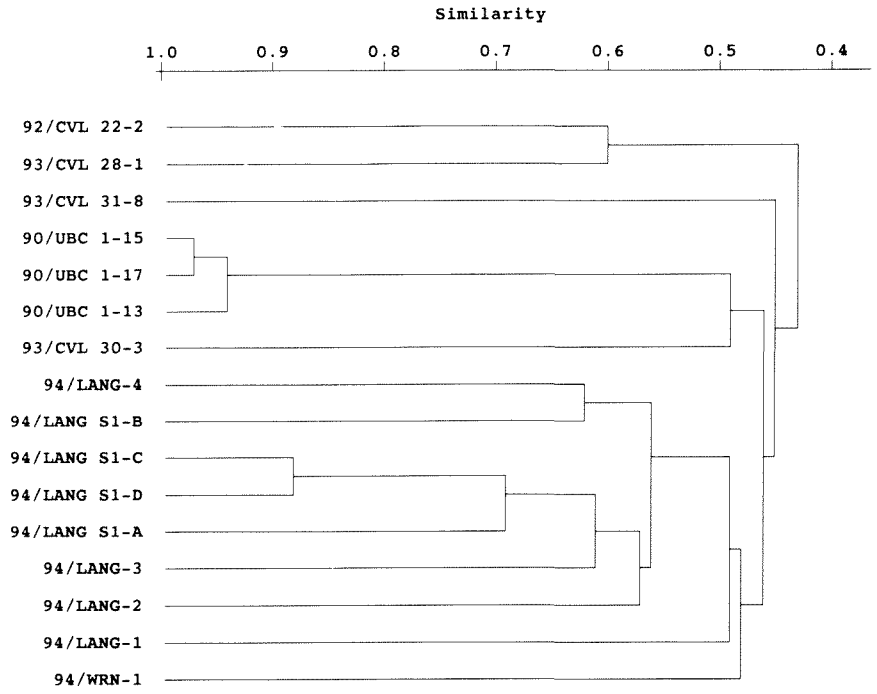


Fig. 3. Dendrogram of relationships between *Lucilia sericata* from southern England based on RAPD fragment pattern analysis. Similarity values calculated using the Jaccard coefficient; UPGMA clustering.

20. Finally, removal of either one of the two *L. sericata* reared from larvae removed from a sheep-strike, and which are presumed to have come from the same oviposition, leaves a data set composed of unrelated blowflies which exhibit only zero (LANG S1-C removed) or one (LANG S1-D removed) significant departures ($P < 0.01$) from random mixing out of 20 combinations of the primers.

Discussion

In this study, the use of RAPD analysis has allowed individual *L. sericata* to be characterized on the basis of their distinct electrophoretic types permitting closely related individuals, including those from a single strike, to be readily distinguished from each other. Analysis of these RAPD data using suitable numerical methods has provided an indication of the genetic homogeneity of *L. sericata* within southern Britain. Thus, although still much in debate (Black, 1993), RAPD fingerprinting does appear to be valuable for population studies (Hadrys *et al.*, 1992).

Nevertheless, in common with a number of recent studies (Levi *et al.*, 1993; Smith *et al.*, 1994) the present work indicates that while RAPD fragments are useful as genetic markers, a number of factors can complicate their use and interpretation, e.g. DNA quality, primer sensitivity and co-migration of non-homologous fragments. Problems of template quality and concentration were overcome by use of the cold ethanol preservation technique and the extraction protocol described. However, the over-sensitivity of those primers which identified so much variation between closely related *L. sericata* that they were of no use for broader numerical studies was of particular concern; each unusable primer reduced the number of available markers. In view, however, of the high degree of variability associated with the extremely A+T rich non-conserved, repetitive regions found in the genome of many insects (Meyer, 1994), it is perhaps to be expected that primers annealing in or around such regions will produce a large number of possibly similarly sized amplification products of uncertain homology. The use of such primers is thus to be avoided and a broad preliminary screening of primers is essential for each new taxonomic group to be studied. Consequently, the genetic variation described in this study is, and should be regarded as, intraspecific, particularly in view of the findings of Black (1993) and Smith *et al.* (1994) concerning the co-migration of non-homologous fragments. Nevertheless, recent work by the authors of this paper (Stevens & Wall, unpublished data) suggests that certain random primers will also be suitable for studies of interspecific variation, at least between closely related species, e.g. *L. sericata* and *L. cuprina* (Wiedemann) (Stevens & Wall, in press).

Male *L. sericata* only were characterized in this study. This avoided contamination of adult DNA with DNA from eggs and reduced the possibility of contamination from ingested protein since, unlike females, males do not require a proteinaceous meal prior to reproduction. While the use of males alone could have limited the degree of genetic variation observed, work by Foster *et al.* (1980) indicates that more than 95% of the genetically variant loci detected in *Lucilia cuprina dorsalis* Robineau-Desvoidy are autosomal. Hence, any study of one or other sex still has the potential to cover the significantly major proportion of all genetic variation.

RAPD polymorphisms serve as dominant genetic markers which are inherited in a Mendelian fashion (Williams *et al.*, 1990) and heterozygotes are not normally detectable; results are not readily usable for the calculation of Hardy-Weinberg gene frequencies or Nei's standard genetic distance (Lynch & Milligan, 1994). Consequently, in the current study, RAPD polymorphisms were analysed with a phenetic distance measure (Jaccard's coefficient) from which a dendrogram was constructed, providing an indication of the base line diversity present within *L. sericata* in southern England. Thus, the high degree of relatedness observed between inbred colony flies (UBC 1-13, 1-15, 1-17) in comparison with that detected among the other samples, appears logical since they had been reared through 75-100 generations, indicating the extent to which genetic variation may be lost over time due to inbreeding.

Similarly, a high degree of similarity was detected between two of the *L. sericata* derived from a single strike (LANG S1-C and S1-D). Given the lower levels of similarity between these and other presumably unrelated *L. sericata* from the same area (between 48-69%), it seems probable that these two may have come from the same oviposition, while the others analysed from the strike did not. Notably, the wound from which these individuals originated was a breech strike of over 1000 larvae, the result of oviposition by several female *L. sericata* (N.P. French, pers. comm.). Such a result indicates the potential use of RAPD analysis in determining the number of female blowflies contributing to a strike.

In conclusion, this study demonstrates that despite certain limitations RAPD analysis is of value for studies of intraspecific genetic variation and is capable of elucidating relationships between even closely related populations of *L. sericata*. In particular, analysis of the data with novel numerical methods allows investigation of results beyond the purely descriptive level presented in many RAPD studies. The approximately equal levels of genetic similarity identified by RAPD analysis between *L. sericata* originating from around Bristol and Weybridge, and the results of the limited tests for linkage disequilibrium, provide no evidence of genetic barriers between these two groups of flies, suggesting that *L. sericata* in southern England form a more or less freely mixed population. The methods described in this paper will be used in future work for wider studies of global genetic variation in *L. sericata*; such information may help provide an insight into the causes of reported geographic differences in behaviour between populations of *L. sericata* (Cragg & Cole, 1956; Liebisch *et al.*, 1983) with respect to blowfly strike of sheep (Hall & Wall, 1995).

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