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Genetic Variation in Populations of the Blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae). Random Amplified Polymorphic DNA Analysis and Mitochondrial DNA Sequences

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Key Word Index—*Lucilia sericata*; *Lucilia cuprina*; blowfly; random amplified polymorphic DNA (RAPD); mitochondrial DNA (mtDNA); genetic variation.

Abstract—Intraspecific genetic variation in two species of calliphorid blowfly, *Lucilia sericata* and *Lucilia cuprina*, was studied by random amplified polymorphic DNA (RAPD) analysis and mitochondrial DNA (mtDNA) sequencing. These species are economically important facultative ectoparasites of sheep. Numerical analysis of RAPD fragment data was used to investigate genetic variation in *L. sericata* across Europe and in both *L. sericata* and *L. cuprina* worldwide. No evidence of genetic isolation within *L. sericata* was observed, despite the geographic separation of the populations studied. This finding was supported by a lack of variation in mtDNA sequences from a corresponding global sample of *L. sericata*. For *L. cuprina* distinct patterns of genetic variation, possibly related to geographical isolation, were detected in the RAPD data and the mtDNA sequences. However, while relationships between groups of *L. cuprina* defined by the two molecular methods were largely in agreement, at least one conflicting result was obtained. The significance of such conflict is explored. © 1997 Elsevier Science Ltd

Introduction

The blowflies *Lucilia cuprina* Wiedmann and *Lucilia sericata* Miegen (Diptera: Calliphoridae) are facultative ectoparasites. Their larvae infest and feed on the living tissues of warm blooded vertebrates (Hall and Wall, 1994). Economically, they are most important as pests of domesticated sheep, although a range of other wild and domestic animals, and occasionally humans, may also be attacked (Zumpt, 1965).

Previous work has suggested that *L. sericata* may have been Palaearctic in origin, while *L. cuprina* may have originated from the Afrotropical or Oriental regions (Aubertin, 1933; Stevens and Wall, 1996). However, as a result of natural patterns of movement and artificial dispersal by humans and livestock in the last few hundred years, both species are now found in temperate and sub-tropical habitats worldwide. During this distribution, populations of the two species may have experienced numerous genetic bottlenecks and periods of isolation and it might be expected that geographically isolated populations would be genetically distinct. Analysis of patterns of genetic variation may help to trace the evolutionary history and pattern of dispersal of these two species.

The existence of strains of *L. sericata* differing in their response to sheep wool has been proposed (Crombie, 1944; Cragg, 1950). In laboratory bioassays, *L. sericata* from Denmark were found to be less attracted to and less likely to oviposit on sheep wool

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than *L. sericata* from Britain (Cragg and Cole, 1956). A gradient of responses was observed, decreasing in the order: British > Danish (country) > Danish (city) > Australian. In Australia, *L. sericata* is generally restricted to urban habitats and only rarely strikes sheep. To account for this variation, Cragg and Cole (1956) suggested that there were geographically distinct strains of *L. sericata* differing in their oviposition behaviour in response to wool, resulting perhaps from historical selection pressures acting on flies in areas of high or low sheep density. Unfortunately, in subsequent years, little further work has been done to quantify or confirm the existence of these behavioural differences.

For *L. cuprina*, information on strain differences is even less readily available, although two morphologically-distinct subspecies, *Lucilia cuprina cuprina* (Wiedmann) and *Lucilia cuprina dorsalis* Robineau-Desvoidy, have been described (Waterhouse and Paramonov, 1950). The former subspecies is believed to be distributed throughout the Neotropical, Oriental and southern Nearctic regions, while the latter is found throughout the East and sub-Saharan Afrotropical and Australasian regions (Waterhouse and Paramonov, 1950; Spradbery, 1991; Bishop, 1995). The two putative subspecies interbreed readily in the laboratory, and intermediate forms are believed to be common in parts of Australia (Norris, 1990). Some authors have suggested that *L. cuprina cuprina* in the Nearctic should be described as a separate species, *Phaenicia pallescens,* on the basis of the absence of its involvement in ovine myiasis in that region (Hall and Townsend, 1977).

Among the many molecular methods currently available for taxonomic studies, the random amplified polymorphic DNA (RAPD) polymerase chain reaction technique (Welsh and McClelland, 1990; Williams *et al.*, 1990) appears particularly suitable for population studies of organisms for which only nanogram quantities of DNA are available for characterization. The RAPD technique has been used to study a range of insects including, aphids (Black *et al.*, 1992), the honey bee (Hunt and Page, 1992), grasshoppers (Chapco *et al.*, 1992), mosquitoes (Wilkerson *et al.*, 1993) and fruit flies (Haymer and McInnis, 1994). However, a number of RAPD characterization studies (reviewed by Black, 1993) indicate that the reproducibility of RAPD amplifications may be deleteriously affected by a range of factors. The relative concentrations of PCR reaction components, DNA template concentration, extraction method and preservation technique all appear potentially important. In addition, a relatively high molecular clock speed and the associated variability detected with RAPD further limit its use for characterizing more distantly-related groups. Consequently, the use of a complementary characterization technique such as DNA sequencing is to be recommended.

Particularly useful for phylogenetic analysis are sequence data from mitochondrial DNA (mtDNA; Kocher *et al.*, 1989; Avise, 1994), which includes regions with a range of different evolutionary rates (Simon *et al.*, 1994) suitable for studying different levels of phylogenetic divergence. Furthermore, the mtDNA molecule is maternally inherited and is passed intact to subsequent generations as a haploid genome, unaffected by recombination (Dawid and Blackler, 1972), while overall substitution rates in mtDNA have been estimated to be five to ten times greater than in nuclear DNA (Brown *et al.*, 1979), making it particularly useful for population level questions. These characteristics make it a powerful genetic marker, while widespread use of the technique in molecular taxonomy and evolutionary biology allows comparison with a range of other studies (Avise, 1994). In the current study, a portion of the 12S rRNA gene was selected as a

known conservative marker (Simon *et al.*, 1994) against which to compare the more variable RAPD results.

The aim of the present study, therefore, was to investigate genetic variation between populations of *L. sericata* and *L. cuprina* collected from around the world using RAPD analysis. The value of the RAPD analysis was then to be assessed by comparison of results with those from a complementary mtDNA analysis of a subset of the flies.

Materials and Methods

Blowflies. Specimens of *L. cuprina* and *L. sericata* were caught on sticky targets baited with liver and sodium sulphide solution (Wardhaugh *et al.*, 1984; Wall *et al.*, 1992). Traps were checked twice daily allowing flies to be collected alive. Previous work (Post *et al.*, 1993; Stevens and Wall, 1995) had indicated that DNA degradation in dried specimens may render RAPD results too inconsistent for further analysis. After removal from the target, *Lucilia* were placed in 100% ethanol and stored at 4°C prior to identification and DNA extraction (Stevens and Wall, 1995). *Lucilia sericata* from the University of Bristol colony were also used in the molecular analysis.

Flies collected from the field were identified as *L. cuprina* or *L. sericata* using the morphological characters described by Aubertin (1933) and Holloway (1991), including analysis of male genitalia. Details of all flies included in this study are presented in Table 1.

DNA extraction. To avoid contaminating sample DNA with DNA from eggs, ingested protein or gut parasites, only the head, legs and flight muscles of male flies were used as sources of DNA. Tissues were combined prior to DNA extraction. Previous work (Stevens and Wall, 1995) indicated that these tissues gave identical RAPD amplification patterns. DNA was extracted as total nucleic acid, by the cetyl trimethyl ammonium bromide (CTAB) method (see Towner, 1991), using 200 µl CTAB for the combined tissues from each fly. The quality of the DNA obtained, 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 chitin covered insect tissues (Stevens and Wall, 1995).

The yield (concentration) of DNA extracted from field caught flies was assessed using a spectrophotometer. UV absorbance measurements were taken at 260 nm. DNA quality was assessed by comparison with heat denatured Lambda Hind III marker on a 1% tris-glacial acetic acid-EDTA (TAE) agarose gel. The intensity of the upper band of high molecular weight in the sample DNA was compared with the 4.36 kb Lambda marker band which contained approximately 0.1 µg DNA. The discreteness of the sample high molecular weight band and the degree of streaking in each sample lane gave a measure of DNA quality, while also providing an alternative estimate of DNA yield.

RAPD analysis. The effect of template DNA concentration on RAPD amplification reproducibility was assessed at three concentrations: 2, 20 and 200 ng template DNA. The use of high quality DNA (see DNA extraction) from Bristol University colony flies ensured that amplification differences were due to changes in template concentration and not DNA quality. Amplification became inconsistent at or above 200 ng template DNA per reaction, and at or below 2 ng template DNA per reaction. Consequently, RAPD characterization reactions were performed with 20 ng template. Nevertheless, 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) and a negative control was run for each set of amplifications.

RAPD analysis was performed according to the protocol of Williams *et al.* (1990) using SuperTaq *Taq* polymerase (HT Biotechnology Ltd, Cambridge, U.K.). Twenty 10-mer primers (Operon kit E - "OPE"; Operon Technologies, U.S.A.) were screened. Nine primers (3, 4, 7, 9, 11, 12, 14, 18, 19) yielded RAPD patterns suitable for further numerical analyses (Stevens and Wall, 1995). All RAPD analyses were performed with a Hybaid TR1 thermal cycler. The resulting PCR-amplified DNA fragments were separated in 1.4% TAE gels at 50 mA for 4 h. Fragments were viewed under UV light after staining with ethidium bromide (10 min, 0.005% solution). Gels were then photographed and reproducibly amplified fragments between 0.4 and 1.5 kb were scored, each being identified by a unique ASCII code character (A, B, C, ... c, d, e) prior to numerical analysis.

mtDNA fragment amplification. A mtDNA fragment which included parts of the small rRNA (12S) gene and the non-conserved control, or AT-rich, region was targeted. Universal primer sequences with the potential to amplify such a fragment were obtained from Simon *et al.* (1994), using the mtDNA map of *Drosophila yakuba* (Clary and Wolstenholme, 1985) as a guide.

A pair of primers (29-mer TIN8X 5'-XCTATCAAGGTAACCCTTTTTATCAGGCA-3' and one 20-mer SRJ14612 5'-AGGGTATCTAATCCTAGTTT-3') were used to amplify a fragment of approximately 1.6 kb from *L. cuprina* and *L. sericata*. PCR reaction components per 50 ml reaction were as follows: 50 ng template DNA,

TABLE 1. CODE, ORIGIN AND YEAR OF COLLECTION OF SPECIMENS OF *LUCILIA SERICATA* AND *LUCILIA CUPRINA* CHARACTERIZED BY RAPD ANALYSIS. (Country codes: AU, Australia; DK, Denmark; FR, France; GM, Germany; HG, Hungary; KY, Kenya; NZ, New Zealand; SG, Senegal; SP, Spain; UG, Uganda; UK, United Kindom; US, United States of America; ZB, Zimbabwe)

European samples—*L. sericata*	
UK-UBC-13	University of Bristol colony, since 1990
UK-UBC-15	
UK-UBC-17	
UK-Uckfield-A	Uckfield, East Sussex, 1994
UK-York	University of York, 1994
UK-Edinburgh-C	St Boswell's, Edinburgh, Scotland, 1994
FR-Agon-A	
SP-Nerja	Nerja, Andalucia, Spain, 1994
DK-Hilerod-A	Hilerod, Sjelland, Denmark, 1994
GM-Celle-C	Celle, Niedersachsen, Germany, 1994
GM-Busum-D	Büsum, Schleswig Holstein, Germany, 1994
HG-Budapest	Budapest, Hungary, 1994
Worldwide samples—*L. cuprina*	
AU-Canberra-1	Canberra, ACT, Australia, 1995
AU-Canberra-2	
AU-Canberra-3	
AU-Perth-c1	Serpentine, Perth, Western Australia, 1995
AU-Townsville-1	Townsville, Queensland, Australia, 1994
AU-Townsville-2	
AU-Townsville-3	
NZ-Blenheim	South Island, New Zealand, 1994
NZ-Dorie-c1	
NZ-Leeston	
NZ-Bulls	North Island, New Zealand, 1994
NZ-Kaikohe	
US-Oahu-1	Hawaii, U.S.A., 1994
US-Oahu-2	
US-Oahu-3	
SG-Dakar-1	Dakar, Senegal, 1994
SG-Dakar-2	
SG-Dakar-3	
KY-Nairobi-11	Nairobi, Kenya, 1994
KY-Nairobi-12	
KY-Nairobi-15	
UG-Tororo	Tororo, Uganda, 1994
Worldwide samples—*L. sericata*	
AU-Perth-s1	Glendalough, Perth, Western Australia, 1995
AU-Perth-s2	
AU-Perth-s3	
NZ-Dorie-s1	South Island, New Zealand, 1994
NZ-Dorie-s2	
NZ-Kaiwaka	North Island, New Zealand, 1994
NZ-Gisborne	
NZ-Rotorua	
US-Sacramento-1	Sacramento, northern California, U.S.A., 1994
US-Sacramento-2	
US-Sacramento-3	
US-LA-1	Los Angeles, southern California, U.S.A., 1994
US-LA-2	
ZB-Harare-1	Harare, Zimbabwe, 1994
ZB-Harare-2	
ZB-Harare-3	
UK-CVL	Grange Farm, Central Vet. Lab., Surrey, 1993
UK-Uckfield	Uckfield, East Sussex, 1994
UK-Bristol	Wrington, Bristol, 1994
UK-Edinburgh-1	St Boswell's, Edinburgh, Scotland, 1994
UK-Edinburgh-2	
FR-Agon-1	Agon-Coutanville, Normandy, France, 1994
FR-Agon-2	
DK-Hilerod	Hilerod, Sjelland, Denmark, 1994

0.2 mM primer TIN8X, 0.2 mM primer SRJ14612, 1.0 U SuperTaq *Taq* polymerase, dNTPs 0.2 mM, 1.5 mM $MgCl_2$, 1x reaction buffer (concentrations as recommended by manufacturer, HT Biotechnology Ltd, Cambridge, U.K.). PCR reaction conditions were as follows: 3 min at 94°C for 1 cycle; 1 min at 94°C, 1 min at 51°C, 1 min 30 s at 72°C for 30 cycles; 5 min at 72°C for 1 cycle. To avoid PCR amplification errors, which could be carried through to the sequencing stage, twelve 50 ml PCR reactions are performed in parallel for each sample. Samples were then gel purified in a 1.2% TAE agarose gel using a suitable size marker; having verified the size of the fragment it was then cut out. Following centrifugal extraction, reactions were pooled; any amplification errors were thus diluted 12-fold, such that they would be negligible in the aliquot of DNA used in the sequencing reaction.

mtDNA sequencing. A portion of the 12S rRNA gene was sequenced using the solid-phase sequencing technique described by Hultmann *et al.* (1989). Addition of a biotin molecule at the 5' of the TIN8X primer allowed the amplified fragment to be single-stranded and sequenced on streptavidin magnetic beads (Dynabeads, Dynal, Norway) after gel purification. Labelling reactions were performed with 35S by the T7 DNA polymerase dideoxy base-specific termination method using a T7Sequencing kit (Pharmacia Biotech, U.S.A.). Sequence fragments were then run on acrylamide gel. Manual sequencing is preferred for AT-rich material, where sequences of ten or more identical bases are not uncommon.

Genetic distance analysis. RAPD data were analysed by a phenetic distance measure, Jaccard's coefficient (Dunn and Everitt, 1982). For each primer, each reproducible RAPD 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 fly, for any one primer. Similarity values were calculated using all bands scored for each primer, between each fly. Distance matrices were calculated from the RAPD data using a program developed by Stevens and Cibulskis (1990). Dendrograms were derived from each matrix by the unweighted pair-group method using arithmetic averages (UPGMA) available in SPSS/PC+.

Using the package PHYLIP 3.5c (Felsenstein, 1993) a genetic distance analysis of the mtDNA sequence data was performed. To provide a measure of support for the clades identified, a majority-rule consensus tree was constructed from 100 bootstrapped data sets. Distance matrices were produced with the program DNADIST. Distances were calculated using the nucleotide substitution model of Kimura (1980). Neighbour-Joining (Saitou and Nei, 1987) cluster analysis was performed using NEIGHBOR. Neighbour-Joining is believed to be one of the better performing distance measures now available (Nei, 1991). The data were bootstrapped using SEQBOOT and the majority-rule consensus tree was derived using the program CONSENSE. The tree was rooted with the *Drosophila yakuba* sequence.

Detection of linkage disequilibrium. The nonrandom association of alleles at different loci in *L. sericata* and *L. cuprina* was studied using the method of Stevens and Tibayrenc (1995), a modified Mantel test (Mantel, 1967), to detect linkage disequilibrium based on correlations between independent genetic markers (RAPD primers). While the composition of individual RAPD genotypes, in terms of alleles and loci, remains unknown, such associations can provide evidence that gene flow is restricted in the population under study, irrespective of the reasons for this (Tibayrenc, 1995). The technique is a generalized Monte-Carlo test for linkage, independent of mating system or ploidy, and is suitable for use with RAPD data (Stevens and Tibayrenc, 1995; Mathieu-Daudé *et al.*, 1995).

In brief, a single data set is considered and the fragment data for each RAPD primer (locus) are shared into two groups at random. Next, genetic distance matrices are calculated, one based on each group of primers (loci). 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 fly) are randomly reassorted a given number of times (e.g. 1000 times), each time creating a 'new' set of flies of randomly mixed genetic composition. A new r-value is calculated for each random reassortment. Following the required number of genotype reassortments and r-value calculations, a frequency distribution of r-values can be constructed, allowing a probability value to be attached to the observed correlation coefficient. The process then begins again and the primers are again randomly shared into two, probably different, groups. This is repeated for the required number of primer combinations. When the number of primers (loci) is small all combinations may be evaluated. However, with large numbers of primers a random sample of reshuffles is performed. For each analysis in this study 40 combinations of primers were generated and 1000 random reassortments of each combination were run to construct each distribution of r-values. A significance level of $P<0.01$ was used for each combination.

Results

RAPD-PCR patterns

The two species, *L. cuprina* and *L. sericata*, were readily distinguished by their RAPD patterns with all primers, although certain primers gave more species-specific fragments within patterns than did others (e.g. OPE-18; Fig. 1).

Cluster analysis of European L. sericata

All samples produced distinct RAPD electrophoretic types using nine primers (OPE-3, 4, 7, 9, 11, 12, 14, 18, 19). British flies (UK-) formed a distinct cluster, grouped together at the 60% level (Fig. 2). Within this cluster Bristol colony flies (UK-UBC 1-13, UK-UBC 1-15, UK-UBC 1-17) were greater than 93% similar. The relationship of these colony flies to other wild flies from southern England has been explored more fully in a separate study (Stevens and Wall, 1995).

Flies from central and eastern Europe (GM-Celle-C, GM-Busum-D, HG-Budapest and DK-Hilerod-A) grouped together at the 62% level, being slightly more similar to each other than British flies. German flies (GM) showed a high degree of similarity to each other (82%), while flies from France (FR-Agon-A) and Spain (SP-Nerja) showed a high level of dissimilarity from others in this analysis.

Cluster analysis of worldwide L. sericata *and* L. cuprina

The RAPD analysis of samples of *L. sericata* and *L. cuprina* collected worldwide (Fig. 3) unambiguously separated the flies of each species into two major clusters which were only 14% similar, with few RAPD fragments common to the two species with the nine OPE primers used.

Within *L. sericata* groupings of flies from Britain and Australasia were apparent, separated at the 40% level. However, overall the level of definition within *L. sericata* was not good, the degree of relatedness within subgroups being only slightly less than that between subgroups. For example, while Australasian *L. sericata* were separated from a group of European, African and American flies at the 48% level, the minimum within group similarity (for Australasian *L. sericata*) was only 51%. Overall, the *L. sericata* studied appeared as a group of relatively heterogeneous, but undifferentiated species by RAPD analysis.

Within the *L. cuprina* some distinct subdivision was apparent (Fig. 3). In particular flies from Oahu, Hawaii were well separated from other *L. cuprina* at the 34% level. Australasian *L. cuprina* displayed some apparent separation from African flies, the majority being only 55% similar, while *L. cuprina* from Canberra, Australia (AU-Canberra-) formed a further group, being at most only 45% similar to other *L. cuprina*. Nevertheless, for the most part the majority of *L. cuprina*, as with the *L. sericata*, appear from the RAPD analysis to represent a largely heterogeneous, but again, undifferentiated species (Fig. 3).

Analysis of linkage disequilibrium

Initial linkage disequilibrium tests on the European *L. sericata* RAPD data showed significant linkage in 39 of the 40 primer combinations. However, removal of any two of the three flies from the inbred Bristol University colony (UK-UBC 1-13, 15 or 17) reduced the number of combinations of primers which exhibited significant departures ($P < 0.01$) from random mixing to 4 out of 40.

Tests for linkage disequilibrium within the global data were performed using all data and three sub-groupings. Sub-groups were selected with reference to the groupings identified in the numerical analysis (Fig. 3). Analysis of all flies ($n = 46$) showed significant linkage in all 40 primer combinations. Such a result, combining RAPD results from two separate species, is not unexpected, but was performed to provide a baseline for the subsequent linkage tests of the subgroups. Analysis of *L. sericata* only (24 flies)

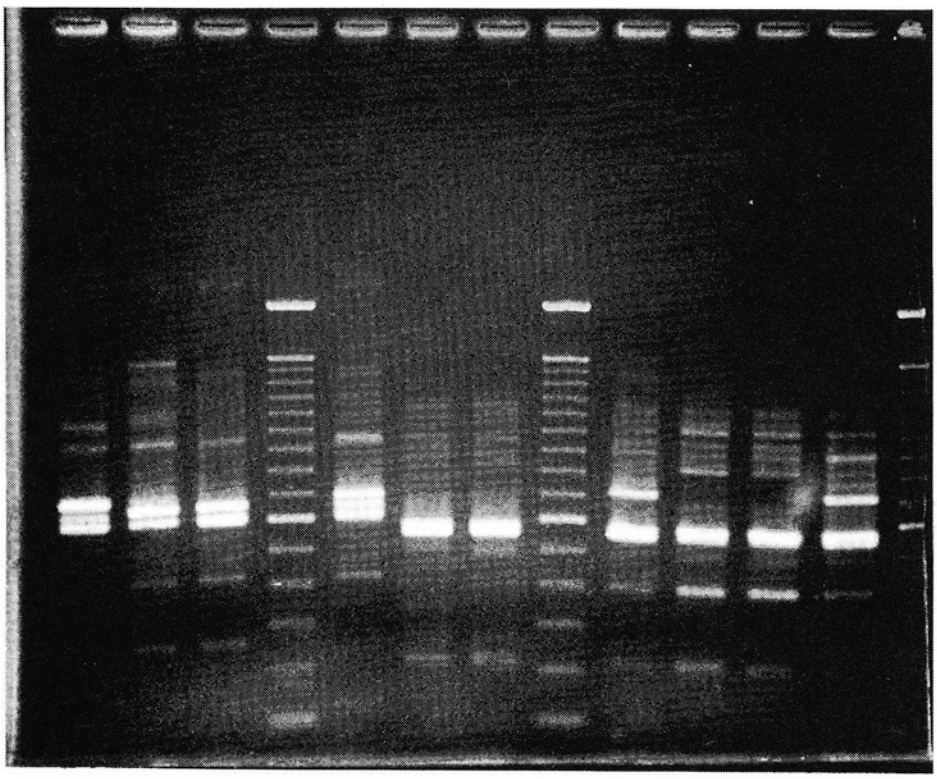

FIG. 1. RAPD AMPLIFICATION PATTERNS OBTAINED WITH PRIMER OPE-18, SEPARATED IN A 1.4% TAE GEL STAINED WITH ETHIDIUM BROMIDE. Left to right, lane **1**: *L. cuprina*, US-Oahu-2; **2**: AU-Canberra-2; **3**: AU-Canberra-3; **4**: 100 bp marker; **5**: AU-Townsville-1; **6**: *L. sericata*, AU-Perth-s1; **7**: AU-Perth-s2; **8**: 100 bp marker; **9**: AU-Perth-s3; **10**: NZ-Dorie-s1; **11**: NZ-Dorie-s2; **12**: NZ-Kaiwaka.

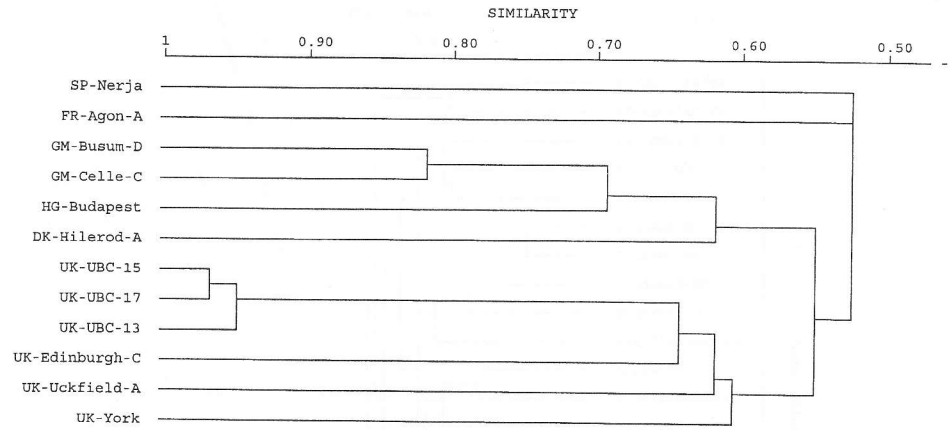

FIG. 2. DENDROGRAM SHOWING RELATIONSHIPS BETWEEN 12 EUROPEAN SPECIMENS OF *LUCILIA SERICATA* BASED ON RAPD DATA FROM NINE RANDOM PRIMERS. Similarity values were calculated using the Jaccard coefficient; UPGMA clustering. (Country codes: DK, Denmark; FR, France; GM, Germany; HG, Hungary; SP, Spain; UK, United Kingdom.)

gave non-significant linkage in 20% of primer combinations, while analysis of *L. cuprina* only (22 flies) gave non-significant linkage in only 2.5% of primer combinations. *Lucilia cuprina* was then subdivided further by removing the three Hawaiian flies (US-Oahu-), which were well separated from the majority of *L. cuprina* in the RAPD based numerical analysis (Fig. 3). Non-significant linkage was detected in 43% of primer combinations for this subgroup.

mtDNA sequences

As described, a mitochondrial DNA fragment which included parts of the small rRNA (12S) gene and the non-conserved control, or AT-rich, region was targeted. However, analysis of the first, conserved part of the fragment within the 12S gene yielded sequence data variable enough to distinguish between and within *L. cuprina* and *L. sericata* for a subsample of the flies characterized by RAPD analysis (Table 2). The results presented in this study are thus based on sequence data (271 bases) from this first part of the mtDNA fragment (Table 3).

The degree of variation observed in the mtDNA sequences of individual flies varied according to species. All *L. sericata* were identical, while three different sequences were obtained from *L. cuprina*, although the majority were of a single sequence type (Table 3). *Lucilia sericata* and the majority of *L. cuprina* differed by two nucleotides out of 271 sequenced and were 99% homologous. For *L. cuprina*, the majority of flies differed from Hawaiian *L. cuprina* by three nucleotides (98% homology) and from the Townsville (Australia) *L. cuprina* by only one nucleotide (99% homology). The Townsville and Hawaiian *L. cuprina* each differed from *L. sericata* by only one nucleotide, and from each other by two nucleotides. *Lucilia cuprina* and *L. sericata* were 75 and 74% homologous to the published *Drosophila yakuba* sequence (Clary and Wolstenholme, 1985).

Cluster analysis of mtDNA sequences

The cluster analysis of *L. cuprina* and *L. sericata* mtDNA sequences (Fig. 4) showed *L. sericata* and Hawaiian *L. cuprina* to be the most closely related of the five mtDNA types detected. In turn, these two appeared closely related to Townsville *L. cuprina*, while all

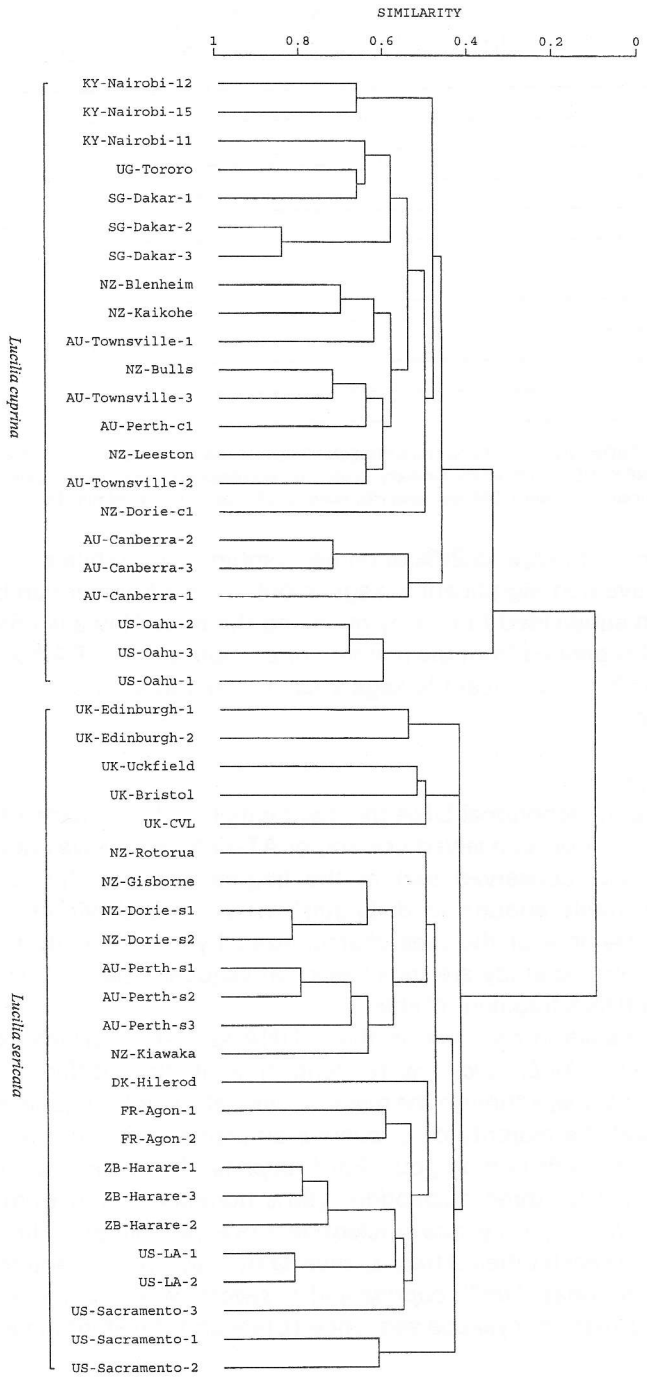

FIG. 3. DENDROGRAM OF RELATIONSHIPS BETWEEN *LUCILIA CUPRINA* (22 SPECIMENS) AND *LUCILIA SERICATA* (24 SPECIMENS) COLLECTED WORLDWIDE, BASED ON RAPD DATA FROM NINE RANDOM PRIMERS. Similarity values were calculated using the Jaccard coefficient; UPGMA clustering. (Country codes: AU, Australia; DK, Denmark; FR, France; GM, Germany; HG, Hungary; KY, Kenya; NZ, New Zealand; SG, Senegal; SP, Spain; UG, Uganda; UK, United Kindom; US, United States of America; ZB, Zimbabwe.)

TABLE 2. SAMPLES OF *LUCILIA CUPRINA* AND *LUCILIA SERICATA* FOR WHICH MITOCHONDRIAL SEQUENCE DATA WAS OBTAINED. All flies were also characterized by RAPD analysis and full details of each specimen are given in Table 1. (Country codes: AU, Australia; NZ, New Zealand; UG, Uganda; UK, United Kindom; US, United States of America)

L. cuprina	AU-Canberra-1
	AU-Canberra-3
	AU-Perth-c1
	NZ-Blenheim
	UG-Tororo
L. cuprina—Hawaii	US-Oahu-1
	US-Oahu-2
	US-Oahu-3
L. cuprina—Townsville	AU-Townsville-3
L. sericata	UK-Bristol
	UK-Uckfield
	NZ-Rotorua
	US-Sacramento-1
	US-Sacramento-2

three types appear to be derived from the majority-type *L. cuprina*. Further support for these relationships was provided by the bootstrap values, which despite the limited number of informative nuclotides were above 55%. All *Lucilia* were well separated from the *D. yakuba* outgroup.

Discussion

Given the different evolutionary characteristics associated with different parts of the genome (Hoelzel and Dover, 1991), the use of complementary molecular techniques can be valuable in determining evolutionary relationships (e.g. Weller *et al.*, 1994; Walker *et al.*, 1995). In the present study, conclusions drawn from RAPD analysis were supported by mtDNA sequence data.

Initial analysis of the RAPD data from European *L. sericata* suggested that intra-specific divisions may exist. However, while British *L. sericata* were separated from German, Hungarian and Danish flies, and flies from France and Spain also appeared distinct, the numbez of non-random associations between alleles at different loci, which could indicate the existence of barriers to gene flow, was of only borderline significance. Numerical analysis of the worldwide RAPD data for *L. sericata*, while separating British and Australasian flies, identified no clear subgroupings within the species. This finding was confirmed by linkage disequilibrium analysis of the *L. sericata* RAPD data, in which a large proportion (20%) of primer combinations failed to evidence linkage.

The findings of the present study are in general agreement with the conclusions of most other molecular-based studies of variability in *L. sericata*. A RAPD study of *L. sericata* from across the Netherlands by van der Leij (1995) detected no significant genetic differences between populations of *L. sericata* collected from a range of sources including sheep, carrion and as larvae from commercial angling stores. Sperling *et al.* (1994), using restriction fragment analysis (RFLP), reported finding no differences among five *L. sericata* from Vancouver, Canada. In contrast, however, RFLP analysis of *L. sericata* from New Zealand has yielded a number of different restriction patterns (D. Gleeson, personal communication). In addition, parsimony analysis of RFLP data (Gleeson, 1995a) indicated differences between *L. sericata* from Australia and *L. sericata*

TABLE 3. MITOCHONDRIAL DNA SEQUENCE DATA FOR *LUCILIA CUPRINA* AND *LUCILIA SERICATA* COLLECTED WORLDWIDE; DETAILS OF SPECIMENS INCLUDED ARE GIVEN IN TABLE 2. The *Drosophila yakuba* sequence is from Clary and Wostenholme (1985), from which the nucleotide identification numbers are taken (i.e. 14651, 14701, etc.). Insertions are represented by '↓' and the appropriate character, deletions by '↑'.

Drosophila yakuba		5'TTTAA	CTTCAATTAT	TATTTTATAA	
L. sericata		.CA.G	.....C.AA..A..		
L. cuprina		..A.G	.....C.AA..A..		
L. cuprina —Townsville		..A.G	.....C.AA..A..		
L. cuprina —Hawaii		.CA.G	.....C.AA..A..		
14651	AATAATTTAA	ATATAAAATT	TCACTTAATA	TATTTAATTT	TATTTTTAAA
sericata	..ATT.A...	..T.....	....C.....	A.....T...	.....AT..
cuprina	...TT.A...	..T.....	....C.....	A.....T...	.....AT..
Towns.	..ATT.A...	..T.....	....C.....	A.....T...	.....AT..
Hawaii	..ATT.A...	..T.....	....C.....	A.....T...	.....AT..
14701	TAAATCAATT	TAA↓TTCATAC	T↓AAAAAAATT	TATTTGTATT	ATTGGTATAA
sericata	AT..A.....	...C....↑..	.A.....	.....C...	...C.....
cuprina	AT..A.....	...C....↑..	.A.....	.....C...	...C.....
Towns.	AT..A.....	...C....↑..	.A.....	.....C...	...C.....
Hawaii	AT..A.....	...C....↑..	.A.....	.....C...	...C.....
14751	CCGCGACTGC	TGGCACCAAT	TTGGTCAATA	CTTTTTAATA	TTGCTATTTC
sericata	.....G....	.....A...	..A.C.....	..C....G..	..A.....
cuprina	.....G....	.....A...	..A.C.....	..C....G..	..A.....
Towns.	.....G....	.....A...	..A.C.....	..C....G..	..A.....
Hawaii	.....G....	.....A...	..A.C.....	..C....G..	..A.....
14801	TAAATTTCTT	TAATTAATAA	TATTAATTAC	TGCGAATAAA	TAATTTATAA
sericata	...G....C.	.....	.....	....G.....	A..AAAT..T
cuprina	...G....C.	.....	.....	....G.....	A..AAAT..T
Towns.	...G....C.	.....	.....	....G.....	A..AAAT..T
Hawaii	...G....C.	.....	.....	....G.....	A..AAAT..T
14851	TATATTTATT	TTTTAAATAA	ATATAAATTC	ACACAAAAAT	TTAC3'
sericata	.TAT.A.TAA	AA.A..TA..	TAT.C.TA.A.A	.ATTT.CA	.ATA
cuprina	.TAT.A.TAA	AA.A..TA..	TAT.C.TA.A.A	.ATTT.CA	.ATA
Towns.	.TAT.A.TAA	AA.A..TA..	TAT.C.TA.A.A	.ATTT.CA	.ATA
Hawaii	.TGT.A.TAA	AA.A..TA..	TAT.C.TA.A.A	.ATTT.CA	.ATA

from New Zealand/South Africa/Canada, although the cladogram was largely unresolved with generally low bootstrap values. The significance of these conflicting assessments of genetic variation within *L. sericata* is at present unknown and it remains to be seen how the RFLP variation reported in *L. sericata* from New Zealand relates to the approximately equal levels of genetic heterogeneity detected by RAPD analysis in the current study. As this study has shown, the detection of genetic variation alone is generally of limited value in evolutionary studies, rather it is the pattern of variation which is most informative.

For *L. cuprina*, the RAPD data were somewhat different. Numerical analysis showed that the majority grouped together at approximately equivalent levels, however, Hawaiian flies appeared well separated from other *L. cuprina*. The significance of this subgrouping is underlined by the results of the linkage disequilibrium analysis of *L. cuprina* RAPD data. When all *L. cuprina* are analysed together, significant linkage was

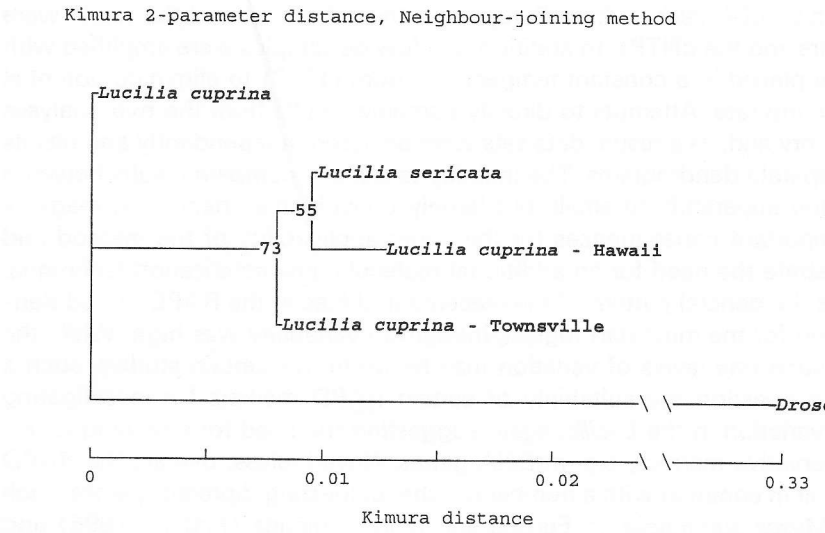

FIG. 4. NEIGHBOUR-JOINING TREE OF RELATIONSHIPS BETWEEN THE FOUR MTDNA TYPES OBTAINED FROM *LUCILIA CUPRINA* AND *LUCILIA SERICATA* IN THIS STUDY (SEE TABLE 3). Genetic distances were calculated using the nucleotide substitution model of Kimura (1980). Bootstrap values (Majority rule consensus, 100 replicates) are given at the nodes. The tree is rooted on the outgroup, *Drosophila yakuba*, which was included for comparative purposes.

obtained in 98% of primer combinations suggesting population substructuring. However, when RAPD data for Hawaiian flies are removed from the analysis, 43% of tests for linkage are non-significant indicating no genetic substructuring within the majority type of *L. cuprina* found worldwide, but suggesting significant association between loci within Hawaiian *L. cuprina*.

The utility of RAPD analysis is still much in debate for a variety of reasons (Hadrys *et al.*, 1992; Black, 1993). Firstly, RAPD polymorphisms serve as dominant genetic markers which do not lend themselves readily to the calculation of gene frequencies with respect to the Hardy–Weinberg rule and Nei's standard genetic distance (Lynch and Milligan, 1994). Thus, the choice and power of suitable phylogenetic methods is limited, prompting the use of a phenetic distance measure (Jaccard's coefficient) in the current study. Furthermore, in common with a number of recent studies (Levi *et al.*, 1993; Smith *et al.*, 1994), the work reported here indicates that a range of other factors can complicate the use and interpretation of RAPD fragment patterns. Certain random primers are inappropriate for the analysis of *Lucilia* DNA as they identify so much variation between closely related flies that they are of little use in broader numerical analyses. The extremely A+T rich, non-conserved, repetitive regions found in the genome of many insects (Meyer, 1994) appear to cause a high degree of variability and a large number of possibly similarly sized amplification products with primers annealing in or around such regions; the significance of co-migration of non-homologous fragments in relation to phylogenetic analysis is discussed by Smith *et al.* (1994). A broad preliminary screening of primers is thus essential for each new taxonomic group to be studied. Again, in common with a number of previous studies (reviewed by Black, 1993), results obtained between runs using different sources of reagents and reaction conditions (e.g. ambient temperature) also proved variable. For example, stocks of materials which changed

between the analysis of *L. sericata* from Europe and those from around the world were the 10-mer primers and the dNTPs. In addition, worldwide samples were amplified with the PCR machine placed in a constant temperature room (15°C) to eliminate potential differences in cooling rate. Attempts to directly combine results from the two analyses were not satisfactory and, as a result, data sets were analysed independently and results summarized in separate dendrograms. The inability to directly compare results between RAPD studies, due apparently to small, but largely unavoidable changes in reagents over time, has important consequences for the wider applicability of the method and serves to demonstrate the need for an additional molecular characterization technique. In addition, while the general pattern of the placement of flies in the RAPD-based dendrograms appeared for the most part logical, intragroup variability was high. While the ability to detect such fine levels of variation may be useful for certain studies, such a result leads us to question the suitability of certain RAPD markers for investigating population level variation in the *Lucilia*, again suggesting the need for a technique utilizing more conservative markers, e.g. mtDNA genes. Nevertheless, overall, the RAPD results suggest that in common with a number of other colonizing dipteran species, such as the face fly, *Musca autumnalis*, in Europe and North America (Krafsur, 1995) and *Drosophila melanogaster* (Singh and Rhomberg, 1987) and *Culex pipiens* (Raymond *et al.*, 1991) worldwide, genetic isolation to date has not disrupted the underlying pattern of global panmixia in *L. sericata* or *L. cuprina*, with the exception of *L. cuprina* from Hawaii. However, the importance of localized variation, e.g. in New Zealand (Gleeson, 1995b), remains to be explored.

The mtDNA sequence data for all *L. sericata* examined were identical, further supporting the hypothesis of global homogeneity in this species. Sequence data from the majority of *L. cuprina* analysed from around the world were also identical, while Hawaiian *L. cuprina* were, as by RAPD analysis, again shown to be genetically distinct. However, also different according to mtDNA sequence data, was the single specimen of *L. cuprina* from Townsville, north-east Australia, a difference not detected by RAPD analysis. Sequence data for this specimen identified it as being intermediate between *L. cuprina* and *L. sericata* (Table 3) and suggested, therefore, that the Hawaiian and the Townsville *L. cuprina* were somewhat more closely related to *L. sericata* than the majority of the *L. cuprina* analysed. Indeed, the mtDNA data presented here would appear to suggest that *L. sericata* and the closely associated Hawaiian and Townsville *L. cuprina* are derived from the majority-type *L. cuprina* (Fig. 4).

Reasons for the differences between Hawaiian and Townsville *L. cuprina* and the other *L. cuprina* cannot be assessed from this limited mtDNA study. However, intriguingly, both Hawaiian and Townsville *L. cuprina* appeared to conform on morphological grounds to the description of *L. cuprina cuprina* given by Norris (1990), whereas all other *L. cuprina* in the analysis appeared to be *L. cuprina dorsalis*. Although *L. cuprina dorsalis* is present throughout most of Australia, populations of *L. cuprina cuprina* have been reported most commonly from along the north-eastern coast of Queensland, particularly near Townsville (Norris, 1990). If it is accepted that the specimen collected in the present study from Townsville was *L. cuprina cuprina*, the results of the mtDNA analysis may suggest that *L. cuprina cuprina* is more closely related to an evolutionary ancestor, linking *L. cuprina dorsalis* and *L. sericata*. Alternatively, the genetic differences seen between the *L. cuprina* populations in the present study could be simply the result of the fixation of random mutations in small isolated populations. Whatever the

explanation, the result points to evolutionary relationships within *L. cuprina* and between *L. cuprina* and *L. sericata* which are of considerable interest, but which will require more detailed studies to elucidate.

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