Species, sub-species and hybrid populations of the blowflies *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae)

JAMIE STEVENS AND RICHARD WALL

Insect Biology Group, School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, U.K.

SUMMARY

The blowflies *Lucilia cuprina* Wiedmann and *Lucilia sericata* Meigen (Diptera: Calliphoridae) are facultative ectoparasites of warm blooded vertebrates, particularly domestic sheep. Despite being similar in morphology and ecology, the two species and different populations of each species, are known to vary in their importance as pests in different regions of the world. To elucidate the genetic basis of these species and population level differences, flies were collected from sites in Africa, Europe, Australasia, North America and the islands of Hawaii, and examined using a combination of morphology, the random amplified polymorphic DNA technique and a complementary mitochondrial DNA analysis. The results confirm the species integrity of *L. sericata* and *L. cuprina* and support the existence of intra-specific genetic variation in *L. cuprina*, but not *L. sericata*.

1. INTRODUCTION

The sheep blowflies *Lucilia cuprina* Wiedmann and *Lucilia sericata* Meigen (Diptera: Calliphoridae) are facultative ectoparasites. Their larvae infest and feed on the living tissues of warm blooded vertebrates, particularly the domestic sheep, *Ovis aries*. As a result, they are economically important pests in many parts of the world (Zumpt 1965; Hall & Wall 1995). Previous work has suggested that the original distribution of *L. cuprina* may have been either Afrotropical or Oriental, while *L. sericata* was probably endemic to the Palaearctic (Aubertin 1933). 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 cosmopolitan and, although *L. sericata* is generally cool temperate and *L. cuprina* largely warm temperate and sub-tropical in distribution, they are sympatric in many parts of their range (Zumpt 1965; Spradbery 1991).

The two species are extremely similar in appearance and can only be routinely separated using a small number of subtle morphological features, such as the colour of the fore femur, the shape of the male genitalia and the number of paravertical setae present on the back of the head (Aubertin 1933; Waterhouse & Paramonov 1950; Holloway 1991). Species identification is further complicated by the fact that *L. cuprina* is known to differ morphologically in various parts of its range; indeed, two distinct subspecies, *Lucilia cuprina cuprina* (Wiedmann) and *Lucilia cuprina dorsalis* Robineau-Desvoidy, have been described (Waterhouse & Paramonov 1950; Norris 1990). The former subspecies is believed to be distributed throughout the Neotropical, Oriental and southern Nearctic regions, while the latter is found throughout the sub-Saharan Afrotropical and Australasian regions (Waterhouse & Paramonov 1950; Spradbery 1991). The two putative subspecies interbreed readily in the laboratory, and intermediate forms are believed to be common in parts of Australia (Norris 1990). The existence of strains of *L. sericata* differing in their behaviour has also been proposed (Crombie 1944; Cragg & Cole 1956), but never related to morphological differences. Hybridization of *L. sericata* and *L. cuprina* has been achieved in the laboratory (Mackerass 1933; Ullyett 1945; Waterhouse & Paramonov 1950) with a male *L. cuprina* x female *L. sericata* cross producing offspring which were either intermediate (Ullyett 1945; Waterhouse & Paramonov 1950) or indistinguishable from *L. cuprina* (Mackerass 1933). In most cases, however, difficulty in achieving fertile crosses was apparent (Mackerass 1933; Waterhouse & Paramonov 1950) and hybrids have not been reported from the field.

The two species *L. cuprina* and *L. sericata* and populations of these species display different pathogenicities to livestock in various parts of the world. In Australia, production losses, prevention and treatment of sheep myiasis, largely by *L. cuprina*, were estimated to cost A$149 million in 1975 (Foster *et al.* 1975). In the absence of effective preventative measures, the sheep industry would be non-viable over much of its present range in Australia (Foster *et al.* 1975). Sheep strike by *L. cuprina* is also known to occur in South Africa (Zumpt 1965). However, despite its presence in North America (syn. *Phaenicia cuprina* = *Phaenicia pallescens* (Shannon)) it is not thought to be involved in sheep myiasis in this region (Williams *et al.* 1985). In northern Europe, *L. sericata* is the primary agent of sheep

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myiasis; in England and Wales, over 80% of sheep farms are affected by blowfly strike and about 750000 sheep are infested per year, of which approximately 2% die (French *et al.* 1992, 1995). However, mortalities of 20–30% among animals infested by *L. sericata* have been recorded (Liebisch *et al.* 1983; Mashkei 1990). Although present in Australia, *L. sericata* is largely a synanthropic species and is rarely implicated in myiasis of sheep (Waterhouse & Paramonov 1950; Foster *et al.* 1975). In contrast, for many years *L. sericata* has been the primary agent of sheep strike in New Zealand. In 1976, it was estimated that about 1.7% of sheep were struck each year by *L. sericata* on the North Island and about 0.7% on the South Island, at an annual cost of about $NZ 1.7 million (Tenquist & Wright 1976). However, recently, *L. cuprina* has been inadvertently introduced into New Zealand from Australia (Heath *et al.* 1991) where it is increasingly prevalent in sheep strike.

To examine the genetic variation among populations of *L. sericata* and *L. cuprina*, fresh specimens were collected from sites in Europe, Africa, Australasia, continental North America and the island of Oahu, Hawaii. Flies were examined using a combination of morphological characters, the random amplified polymorphic DNA (RAPD) technique (Welsh & McClelland 1990; Williams *et al.* 1990) and a more conservative, but complementary analysis of mtDNA sequence data (Kocher *et al.* 1989; Simon *et al.* 1994).

2. MATERIALS AND METHODS

(a) *Fly collection and DNA extraction*

Specimens of *L. cuprina* and *L. sericata* were caught at a range of sites in Europe, Africa, North America and Australasia using 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. After removal from the target, *Lucilia* were placed in 100% ethanol and stored at 4 °C. Flies were initially identified to species–subspecies using the morphological characters described by Aubertin (1933), Norris (1990) and Holloway (1991), including analysis of male genitalia. DNA was extracted as total nucleic acid, by the cetyl trimethyl ammonium bromide (CTAB) method according to the protocol described by Stevens & Wall (1995). Details of all flies included in this study are presented in table 1.

Table 1. *Code, origin and year of collection of specimens of* Lucilia sericata *and* Lucilia cuprina *characterized by* RAPD *analysis and mtDNA sequence analysis*

(Country codes: AU, Australia; DK, Denmark; KY, Kenya; NZ, New Zealand; SG, Senegal; UG, Uganda; UK, United Kingdom (UBC, University of Bristol colony); US, United States of America; ZB, Zimbabwe.)

Lucilia cuprina	
AU-Canberra-1, AU-Canberra-3	Canberra, A.C.T., Australia, 1995
AU-Perth	Serpentine, Perth, W.A., Australia,1995
AU-Townsville-1, AU-Townsville-3	Townsville, Queensland, Australia, 1994
KY-Nairobi	Nairobi, Kenya, 1994
NZ-Blenheim, NZ-Dorie	South Island, New Zealand, 1994
SG-Dakar	Dakar, Senegal, 1994
UG-Tororo	Tororo, Uganda, 1994
US-Oahu-1, US-Oahu-2, US-Oahu-3	Oahu Island, Hawaii, U.S.A., 1994
Lucilia sericata	
AU-Perth	Glendalough, Perth, W.A., Australia, 1995
DK-Hilerod	Hilerod, Sjelland, Denmark, 1994
NZ-Dorie	South Island, New Zealand, 1994
NZ-Rotorua	North Island, New Zealand, 1994
US-Sacramento-1, US-Sacramento-3	Sacramento, California, U.S.A., 1994
UK-Uckfield	Uckfield, East Sussex, U.K., 1994
UK-Bristol	Wrington, Bristol, U.K., 1994
UK-UBC	University of Bristol colony, U.K., 1994
ZB-Harare	Harare, Zimbabwe, 1994
Calliphora Vicina	University of Bristol colony, U.K., 1995

(b) RAPD *analysis*

RAPD analysis (Welsh & McClelland 1990; Williams *et al.* 1990) was done according to the protocol described by Stevens & Wall (1995). Briefly, nine primers from Operon kit E (primers 3, 4, 7, 9, 11, 12, 14, 18, 19; Operon Technologies, U.S.A.) yielded fragment patterns suitable for numerical analyses. RAPD data were analysed with a phenetic distance measure, Jaccard's coefficient (Dunn & Everitt 1982). Similarity values were calculated using RAPD bands scored for each primer, between each fly. 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. A tree was derived from the distance matrix by the Neighbour-Joining method (Saitou & Nei 1987) using the computer program NEIGHBOR, available in PHYLIP 3.5c (Felsenstein 1993). Neighbour-Joining is believed to be one of the better performing distance measures currently available (Nei 1991).

The homology of comigrating RAPD fragments was investigated by Southern hybridization–alkaline blotting (Southern 1975; Reed & Mann 1985) using Zeta-Probe membranes (Bio Rad). Template fragments were ^{32}P labelled using a ^{T7}QuickPrime kit (Pharmacia LKB) as per the manufacturers instructions.

(c) *mtDNA sequence analysis*

The 12S rRNA gene was targeted as a conservative mtDNA marker (Simon *et al.* 1994) by using the mtDNA map of *Drosophila yakuba* (Clary & Wolstenholme 1985) as a guide. The fragment was amplified by using a pair of universal primers (29-mer TIN8X 5′-XCTATCAAGGTA-ACCCTTTTTATCAGGCA-3′ and 20-mer SRJ14612 5′-

AGGGTATCTAATCCTAGTTT-3′; Simon *et al.* 1994) to one of which a molecule of biotin had been added at the 5′ end. Polymerase chain reaction (PCR) components per 50 µl reaction were as follows: 50 ng template DNA, 0.2 µM primer TIN8X, 0.2 µM primer SRJ14612, 1.0 U SuperTaq *Taq* polymerase, dNTPs 0.2 mM, 1.5 mM $MgCl_2$, 1 × reaction buffer (concentrations as recommended by manufacturer, HT Biotechnology, Cambridge, U.K.). PCR reaction conditions were as follows: 3 min at 94 °C; 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. A total of 12, 50 µl PCR reactions were done in parallel for each fly DNA to be sequenced; reactions were then pooled. Any amplification errors, which could be carried through to the sequencing stage, were thus diluted 12-fold, such that they would be negligible in the aliquot of DNA sequenced. Solid-phase sequencing as described by Hultmann *et al.* (1989) was done using streptavidin magnetic beads (Dynabeads, Dynal A. S., Norway). Labelling reactions were done with ^{35}S by the T7 DNA polymerase dideoxy base-specific termination method (Sanger *et al.* 1977) using a ^{T7}sequencing 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.

Parsimony analysis was performed using the program DNAPARS, available in the package PHYLIP 3.5c (Felsenstein 1993). A measure of support for the clades identified was provided by constructing a majority-rule consensus tree from 100 bootstrapped data sets, using the programs SEQBOOT and CONSENSE. Data for *Calliphora vicina*, representative of a closely related genus, were included for comparative purposes. The tree was rooted with the *Drosophila yakuba* sequence (Clary &Wolstenholme 1985).

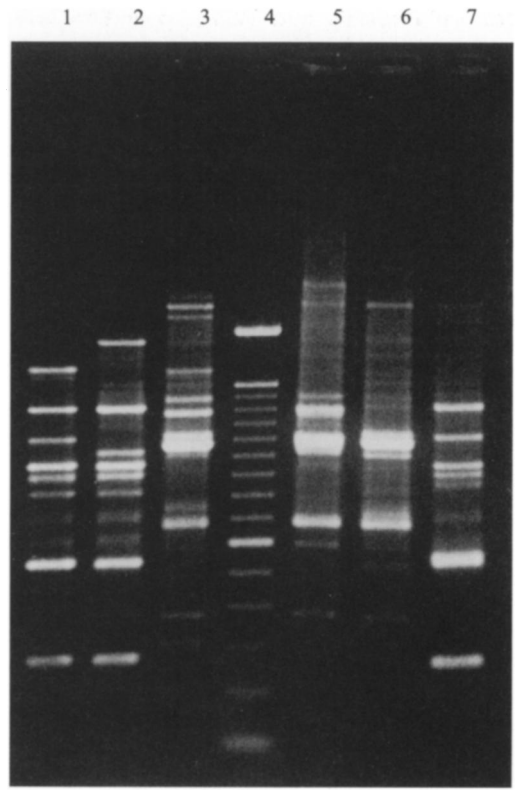

Figure 1. RAPD amplification patterns obtained with primer OPE-7, separated in a 1.4% TAE gel stained with ethidium bromide. Left to right, lane 1: *L. sericata*, NZ-Dorie; 2: NZ-Rotorua; 3: *L. cuprina*, AU-Canberra-1; 4: 100 bp marker; 5: US-Oahu-1; 6: US-Oahu-3; 7: *L. sericata*, US-Sacramento-1.

3. RESULTS

(*a*) *RAPD analysis*

The two species, *L. cuprina* and *L. sericata*, were readily distinguished by their RAPD patterns (e.g. OPE-7; figure 1). Few RAPD fragments were common to both species, the non-homology of principal comigrating fragments having been determined by Southern hybridization. Similarly, the number of species–specific fragments amplified varied between primers. Neighbour-Joining cluster analysis of the RAPD data (figure 2) from *L. sericata* and *L. cuprina* unambiguously separated the flies of each species into distinct clusters, separated by a distance of 0.26. Within these groupings, only limited further differentiation was apparent and *Lucilia sericata* and the majority of *L. cuprina* appeared as separate, relatively heterogeneous, but undifferentiated groups. The one exception to this pattern, however, were flies from Oahu, Hawaii which had been identified morphologically as *L. cuprina* and which appeared to be somewhat apart from other *L. cuprina* (figure 2, distance 0.13).

(*b*) *mtDNA sequences*

The degree of variation observed in the 329 bases sequenced in the 12S rRNA gene of individual flies varied according to species (data available from authors). All *L. sericata* examined were identical, regardless of their geographic origin (see figure 3). In contrast, four different sequences were obtained from *L. cuprina* (figure 3). *Lucilia sericata* and most *L. cuprina* differed by two nucleotide substitutions. The majority-type *L. cuprina* differed from the *L. cuprina* collected on Oahu, Hawaii by three substitutions and from *L. cuprina* collected near Townsville (Queensland, Australia) by only one substitution. The *L. cuprina* from Townsville and Hawaii each differed from *L. sericata* by only one substitution and from each other by two nucleotide substitutions. The majority-type *L. cuprina* differed from *L. cuprina* collected in Senegal by two single nucleotide insertions. The cluster analysis of *L. cuprina* and *L. sericata* mtDNA sequences (see figure 3) showed *L. sericata* and Hawaiian *L. cuprina* to be the most closely related of the four *Lucilia* mtDNA types detected and *L. cuprina* from Townsville also appeared distinct from the majority-type *L. cuprina*. Further support for these relationships was provided by the bootstrap values, despite the limited number of informative nucleotides (see figure 3). All *Lucilia* were approximately 75% homologous to *Drosophila yakuba* (Clary & Wolstenholme 1985) and were well separated from the blowfly *Calliphora vicina* and *D. yakuba* by parsimony analysis (figure 3).

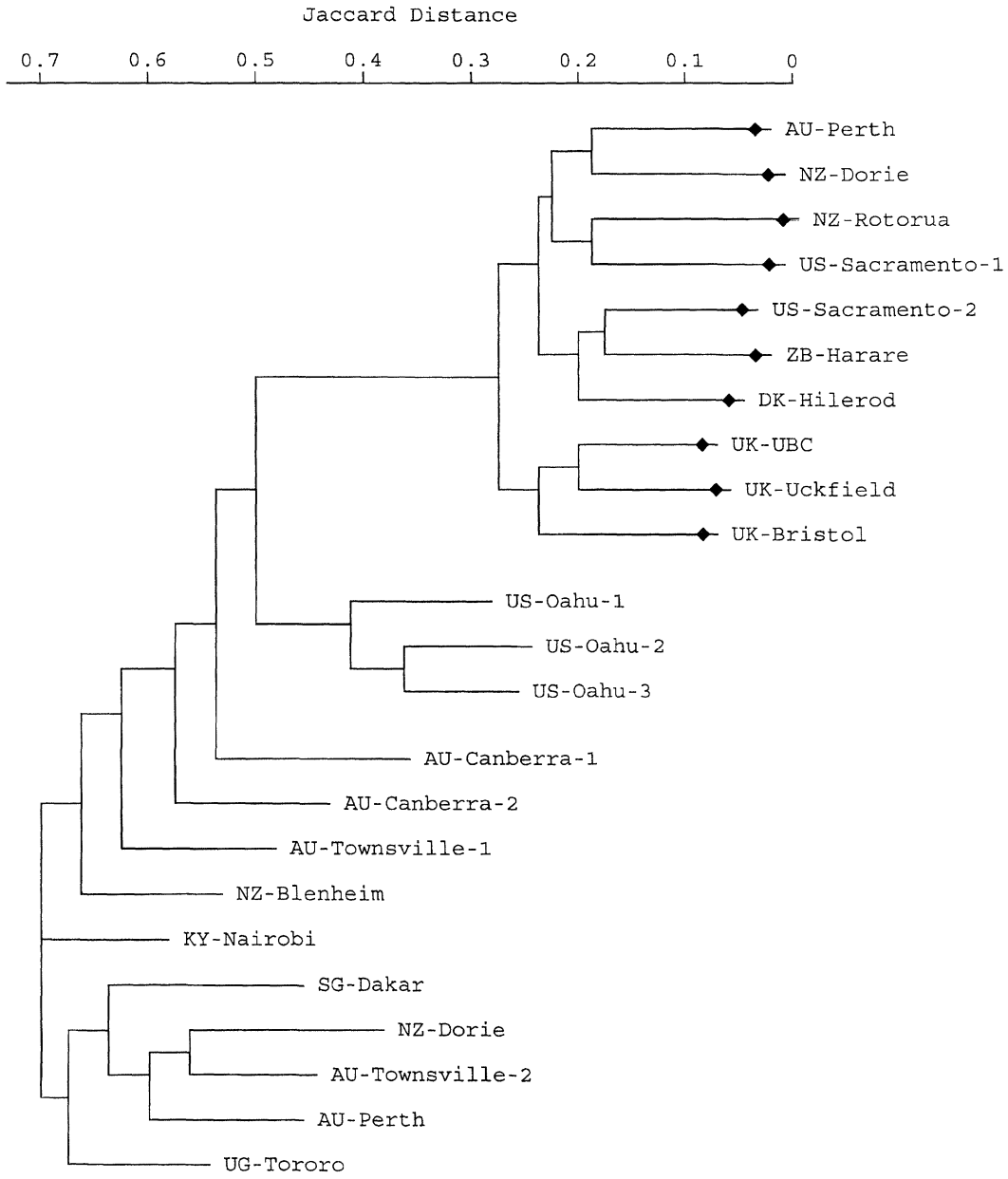

Figure 2. A Neighbour-Joining tree showing the genetic relationships between *Lucilia sericata* and *Lucilia cuprina* (23 specimens) collected from a range of sites and characterized by RAPD analysis using nine random primers. *Lucilia sericata* are represented by diamonds. Flies are identified by a country code and the town nearest to the collection site. Country codes: AU, Australia; DK, Denmark; KY, Kenya; NZ, New Zealand; SG, Senegal; UG, Uganda; UK, United Kingdom (UBC, University of Bristol colony); US, United States of America; ZB, Zimbabwe.

4. DISCUSSION

The present study employed two molecular techniques with very different 'clock' speeds to uncover genetic relationships within these two closely related species of *Lucilia* blowfly. The RAPD technique appears particularly suitable for population studies of organisms for which only nanogram quantities of DNA are available for characterization and RAPDs have been used previously to study a range of insects including, aphids, honey bees, grasshoppers, mosquitoes (reviewed by Black 1993) and *L. sericata* (van der Leij 1995; Stevens & Wall 1995). However, these and other studies indicate that a number of factors including the relative concentrations of PCR reaction components and DNA template concentration and quality may affect the reproducibility of RAPD amplifications (Black 1993; Stevens & Wall 1995). In addition, the extremely A+T rich, non-conserved, repetitive regions found in the genome of many insects (Meyer 1994) can cause a high degree of variability and a large number of possibly similarly sized amplification products leading to the comigration of non-homologous fragments which in turn may affect the interpretation of RAPD data (Smith *et al.* 1994; Rieseberg 1996). A relatively high molecular clock speed and the associated fine level of variability detected further limit the use of RAPDs for studying more distantly related groups. The use of complementary characterization techniques is thus recommended (Weller *et al.* 1994; Walker *et al.* 1995). Particularly useful in this respect are mtDNA sequence data which include regions with a range of evolutionary

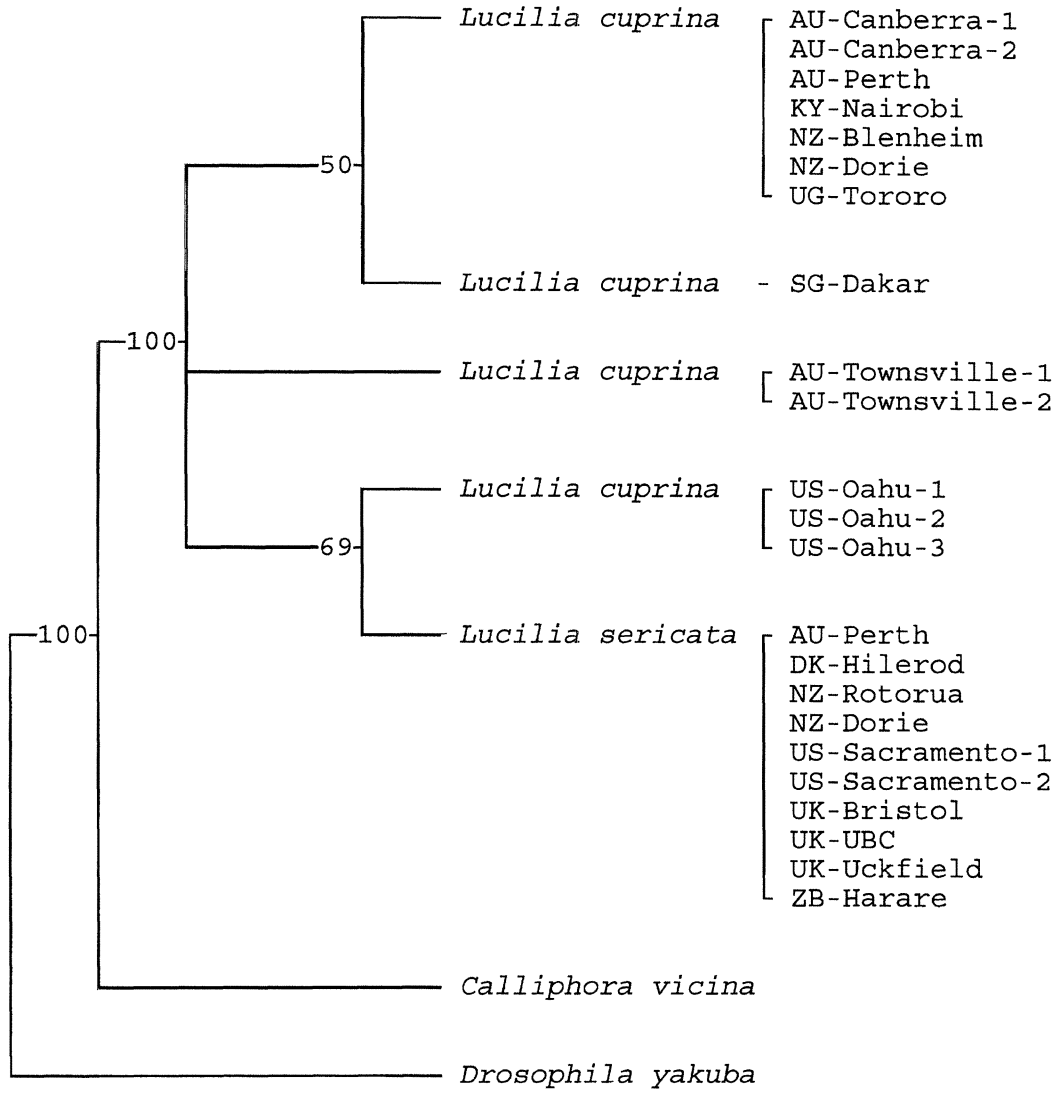

Figure 3. A majority-rule consensus tree derived from parsimony analysis of mtDNA sequence data for *Lucilia sericata* and *Lucilia cuprina* (23 specimens) collected from around the world. Flies are identified by a country code and the town nearest to the collection site. Country codes: AU, Australia; DK, Denmark; KY, Kenya; NZ, New Zealand; SG, Senegal; UG, Uganda; UK, United Kingdom (UBC, University of Bristol colony); US, United States of America; ZB, Zimbabwe.

rates suitable for studying different levels of phylogenetic divergence (Kocher *et al.* 1989; Simon *et al.* 1994). These characteristics make the mtDNA molecule a powerful genetic marker, and 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 region 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 identification of little RAPD and no mtDNA variation within or between populations of *L. sericata* worldwide is in general agreement with the limited molecular-based studies of variability in *L. sericata* that have been done previously (Sperling *et al.* 1994; van der Leij 1995). For *L. cuprina*, however, the results were somewhat different. Based on the RAPD data, phylogenetic analysis (figure 2) identified two distinct groupings within *L. cuprina*: the majority-type and those from a population on Oahu, Hawaii. Furthermore, while the RAPD patterns of Hawaiian flies were characteristic of *L. cuprina* (see figure 1) the phylogenetic analysis indicated that, of all the *L. cuprina* included in this study, they (the Hawaiian *L. cuprina*) were the most similar to *L. sericata*. Most of the *L. cuprina* collected from Australia, New Zealand and East Africa appeared to conform on morphological grounds to descriptions of *L. cuprina dorsalis*, whereas the flies collected on Oahu, Hawaii appeared to be *L. cuprina cuprina*.

Over and above the RAPD variation, mtDNA sequence analysis identified four genetic types: the majority-type, flies from Senegal, flies from Townsville, Australia and flies from Oahu, Hawaii. Thus, on the basis of this marker (mt DNA sequence data) Hawaiian *L. cuprina* appeared more closely related to *L. sericata* than to other *L. cuprina*, while the *L. cuprina* from Townsville, Australia were intermediate between the majority-type and the Hawaiian *L. cuprina*. The *L. cuprina* collected from Senegal and Townsville, Australia also appeared to conform on morphological grounds to the description of the subspecies *L. cuprina*

cuprina (Norris 1990). 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).

The apparent conflict in the data can be interpreted in two ways. The Hawaiian population of *L. cuprina* may be *L. cuprina cuprina* and the majority-type *L. cuprina* collected in Australia, New Zealand and East Africa are *L. cuprina dorsalis*. In this case, the *L. cuprina* from Townsville and Senegal, although appearing morphologically to be *L. cuprina cuprina*, may be genetic variants of *L. cuprina dorsalis* or crosses between the subspecies *L. cuprina cuprina* and *L. cuprina dorsalis*. Such intermediate populations have been described in Australia by Norris (1990). An alternative, although perhaps less plausible explanation, is that the *L. cuprina* collected from Senegal and Townsville, Australia are true *L. cuprina dorsalis* while the flies from Oahu, Hawaii represent the result of a successful cross-breeding event between a male *L. cuprina* and female *L. sericata* at some time in the past. This latter possibility is supported by the fact that RAPD analysis (figures 1 and 2) grouped the Hawaiian flies with *L. cuprina* while the mtDNA sequence data (figure 3) indicated that these specimens were more closely related to *L. sericata*. As the mtDNA molecule is maternally inherited and is passed intact to subsequent generations as a haploid genome, unaffected by recombination (Dawid & Blackler 1972), such a cross may then have given rise to a population with morphology and nuclear DNA resembling *L. cuprina* and mtDNA more closely related to the maternal *L. sericata*. Indeed, in previous laboratory crosses between *L. cuprina* and *L. sericata* most hybrids were either indistinguishable from *L. cuprina*, or possessed characters intermediate between *L. cuprina* and *L. sericata* (Mackerass 1933; Ullyett 1945; Waterhouse & Paramonov 1950). As described previously, while such crosses have been seen in the laboratory, there are no previous records of their existence in the field.

This study has shown that the population genetics of *L. cuprina* is complex and, at present, poorly understood. The simple division of *L. cuprina* into two subspecies may be an inadequate description of these heterogeneous, geographically isolated populations. Such heterogeneity may have arisen following the global spread of this species or may reflect its spread from already reproductively isolated ancestral populations. Further resolution of these questions will require genetic analysis of a greater number of populations of *L. cuprina*, particularly from the Oriental and Nearctic regions where *L. cuprina cuprina* is believed to be indigenous, and analysis of flies representative of the range of *L. cuprina* morphologies described (Norris 1990).

The data obtained in the present study do not suggest any clear genetic relationships with pathogenicity of *L. sericata* or *L. cuprina*. For the most part, populations of the two species appeared as relatively heterogeneous, but geographically undifferentiated groups. For example, *L. sericata* from Europe and Australia, where clear differences in myiasis behaviour are believed to exist, had identical mtDNA sequences and showed little geographic differentiation according to the RAPD analysis. It may be that the myiasis habit has arisen relatively recently and independently in geographically isolated populations of *L. sericata* and *L. cuprina*. Hence, if differences in pathogenicity do have a genetic basis, rather than being simply related to differences in sheep husbandry practice and climate, more suitable markers intermediate between the fine-grained RAPD analysis and the conservative mtDNA sequence analysed here will be required for future studies.

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