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Paraphyly in Hawaiian hybrid blowfly populations and the evolutionary history of anthropophilic species

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Abstract

Complementary nuclear (28S rRNA) and mitochondrial (COI + II) gene markers were sequenced from the blowflies, *Lucilia cuprina* and *Lucilia sericata*, from Europe, Africa, North America, Australasia and Hawaii. Populations of the two species were phylogenetically distinct at both genes, with one exception. Hawaiian *L. cuprina* possessed typical *L. cuprina*-type rRNA, but had *L. sericata*-type mitochondrial (COI + II) sequences. An explanation for this pattern is that Hawaiian flies are hybrids and comparison of observed levels of sequence divergence to possible introduction events, e.g. Polynesian colonization, suggests that Hawaiian *L. cuprina* may be evolving rapidly. Moreover, the monophyly of these flies also suggests that the *L. sericata* mtDNA haplotype was apparently fixed in Hawaiian *L. cuprina* by lineage sorting, indicating a population bottleneck in the evolutionary history of these island flies.

Keywords: *Lucilia*, hybrid, phylogenetic, 28S rRNA, cytochrome oxidase.

Introduction

The blowflies (Diptera: Calliphoridae) display a range of life-histories, from saprophagy to obligate parasitism. Two species in particular, *Lucilia cuprina* (Wiedmann) and *Lucilia sericata* (Meigen) are economically important agents of livestock – especially domesticated sheep – myiasis in many areas of the world (Zumpt, 1965; Hall & Wall, 1995). Despite close similarities in morphology and ecology, and extensive sympatry (Spradbery, 1991), genetic studies confirm the species integrity of *L. sericata* and *L. cuprina* (Gleeson & Sarre, 1997; Stevens & Wall, 1997a,b). In previous studies of evolutionary relationships between and within these two species, specimens from around the world have been examined on the basis of morphology, random amplified polymorphic DNA (RAPD) analysis and short stretches of 12S mitochondrial DNA (mtDNA) sequence (Stevens & Wall, 1995, 1996, 1997a,b). While results in the main confirmed the global species integrity of *L. sericata* and *L. cuprina*, genetic analysis of *L. cuprina* morphotypes from the Hawaiian island of Oahu, indicated a possibly hybrid population of blowflies (Stevens & Wall, 1996).

Given the findings of previous extensive laboratory crossing experiments (Waterhouse & Paramonov, 1950), which confirmed the low frequency with which these two species cross successfully, such a result is both unexpected and of potential importance to control efforts, e.g. the interspecific transfer of organophosphate resistance genes (Newcomb *et al.*, 1997). Blowfly genotypes are also of importance to forensic scientists who may use DNA to identify larvae collected from a corpse (Benecke & Wells, 2001). Experimental development data are commonly used to estimate the age of an insect specimen and therefore the minimum time since death of a victim. Among the reasons for proper identification is that species that are difficult or impossible to distinguish during the immature stages can display quite different development rates. For example, at 19 °C *L. cuprina* (= *Phaenicia pallescens*) required an average of 47 days to develop from oviposition to adult emergence, whilst *L. sericata* required an average of 84 days at the same temperature (Ash & Greenberg, 1975). Several authors have advocated identification methods based on the genes for cytochrome oxidase (Sperling *et al.*, 1994; Malgorn & Coquoz, 1999; Vincent *et al.*, 2000; Wells & Sperling, 2001) and ribosomal RNA (Stevens & Wall, 2001); such approaches may be invalid for any species not showing monophyly for the chosen DNA marker.

The purpose of the work reported here is to evaluate the genetic status of populations of *L. cuprina* from Hawaii in relation to other populations collected world-wide and to assess the possibility that hybridization of *Lucilia* species has occurred in the Hawaiian islands. Additionally, we aim

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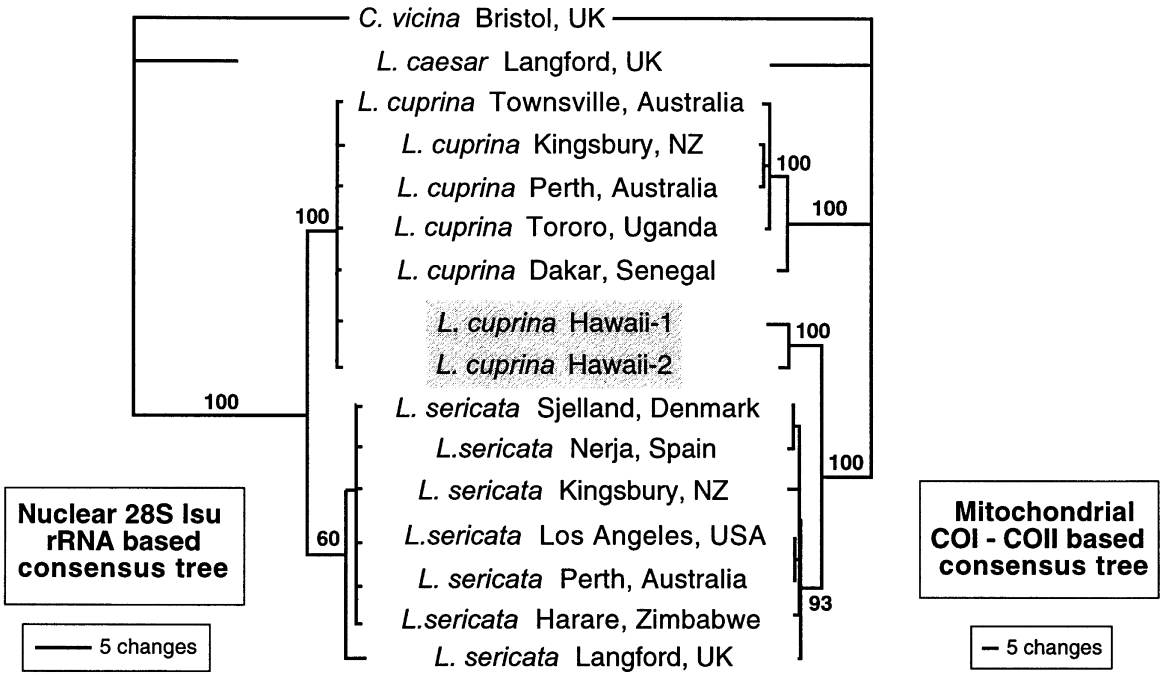

Figure 1. Phylogenetic trees derived by parsimony analysis of 28S rRNA (left) and mitochondrial COI + II (right) gene sequences. Relative bootstrap support analyses were performed using 1000 pseudoreplicates for each gene. The default options of PAUP were used: optimality criterion = maximum parsimony; initial upper bound computed step-wise; only minimal trees kept; addition sequence = furthest; zero length branches collapsed. See Table 2 for specimen details and accession numbers.

to explore the reasons and implications for such a discordant pattern, e.g. the effects of introgressive hybridization (Avise, 2000). Flies were examined using a combination of complementary nuclear – 28S subunit ribosomal RNA (28S rRNA) – and mitochondrial – cytochrome oxidase subunits I and II (COI + II) – molecular markers, and analysed using a range of phylogenetic tools.

Results

DNA sequence analysis

The degree of variation observed in individual flies across the 2193 nucleotides of the 28S rRNA gene varied according to species. All *L. cuprina* examined were identical, regardless of their geographical origin. In *L. sericata*, two rRNA sequence types were observed, a global-type and a sequence differing by three nucleotides, amplified from a laboratory colony of *L. sericata* from Bristol, UK. *L. cuprina* differed from the global-type *L. sericata* by eight nucleotide substitutions and from the Bristol, UK sample by nine substitutions.

The mitochondrial marker selected for this study was the 2.3 kb region coding for the cytochrome oxidase subunits I and II, together with tRNA-leucine (COI + II). COI + II was amplified from sixteen species/specimens; the degree of variation observed across the 2329 nucleotides of the mitochondrial COI + II gene varied widely across all specimens of *L. cuprina* and *L. sericata*. (COI-only sequences and complete 28S rRNA gene sequences have been analysed from four additional specimens of Hawaiian *L. cuprina*, confirming the pattern of phylogenetic relationships observed for the two specimens/populations presented in Figure 1.) All specimens were also well separated from the two outgroup species, *L. caesar* and *C. vicina*. *L. cuprina* sequences, excluding those from Hawaiian *L. cuprina*, showed between 2 and 11 intraspecific nucleotide differences. *L. cuprina* from Hawaii showed 53–59 nucleotide differences from other specimens of *L. cuprina*, but only 16–20 nucleotide differences from global *L. sericata* sequences; global, non-Hawaiian specimens of *L. cuprina* showed between 45 and 55 nucleotide differences with global *L. sericata* sequences. The global sample of *L. sericata* specimens showed 0–6 intraspecific nucleotide differences, of which sequences from only two specimens were identical.

Phylogenetic analysis

Two partitions corresponding to the two genes were defined within the 4523 characters of the combined data file using an incongruence-length difference (ILD) test. The ILD test showed that sequence data for the two genes were not homogeneous and that they should not be combined for subsequent phylogenetic analysis. Of the 4523 characters, 125 were parsimony-informative. Analysis yielded a tree of length 345 for the original partition, which was significantly

Table 1. Matrix of absolute character distances between taxa based on phylogenetic analysis of 2329 base pairs of COI + II gene sequences. Country abbreviations: NZ = New Zealand, UK = United Kingdom, USA = United States of America

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *C. vicina* Bristol, UK	–															
2 *L. caesar* Langford, UK	161	–														
3 *L. cuprina* Hawaii-2	165	111	–													
4 *L. cuprina* Hawaii-1	166	114	5	–												
5 *L. cuprina* Kingsbury, NZ	165	116	57	59	–											
6 *L. cuprina* Perth, Australia	165	116	57	59	2	–										
7 *L. cuprina* Dakar, Senegal	166	113	53	54	11	11	–									
8 *L. cuprina* Townsville, Australia	163	114	57	59	3	3	10	–								
9 *L. cuprina* Tororo, Uganda	164	115	56	57	4	4	9	3	–							
10 *L. sericata* Sjelland, Denmark	162	111	16	19	53	53	45	53	52	–						
11 *L. sericata* Kingsbury, NZ	165	114	19	22	54	54	48	54	55	5	–					
12 *L. sericata* Langford, UK	161	111	16	19	53	53	45	53	52	4	5	–				
13 *L. sericata* Los Angeles, USA	163	112	17	20	54	54	46	54	53	3	4	3	–			
14 *L. sericata* Perth, Australia	163	112	17	20	54	54	46	54	53	3	4	3	0	–		
15 *L. sericata* Nerja, Spain	161	112	17	20	52	52	46	52	51	1	6	5	4	4	–	
16 *L. sericata* Harare, Zimbabwe	163	112	17	20	54	54	46	54	53	3	4	3	2	2	4	–

shorter than trees derived from 999 pseudoreplicate partitions (*P*-value = 0.001). Removal of the two Hawaiian taxa resulted in a non-significant ILD test.

Subsequent independent phylogenetic analyses using PAUP yielded two trees (Fig. 1) with significantly different topologies, as confirmed by a Kishino-Hasegawa test ($P < 0.0001$). Removal of the Hawaiian specimens resulted in no significant difference between tree topologies for the two genes.

Substitution distribution analysis

For COI + II sequences, pair-wise comparisons of all combinations of *L. sericata*, *L. cuprina* and Hawaiian *L. cuprina* were performed (Table 1).

Analysis of the distribution of phylogenetically informative characters (nucleotide changes) within trees showed that, while variation in the 28S rRNA gene was largely confined to three distinct regions within D domains: D2, D6 and D7a (Hancock *et al*., 1988), nucleotide variation in the COI + II sequences was evenly spread across the length of the gene sequences, with no obvious gene associated clusters, as might be expected if discrete parts of the mtDNA genome had undergone recombination. Moreover, nucleotide variation in the COI + II sequences was largely confined to non-coding sites (1st position: 37/287 = 13%; 2nd position: 9/287 = 3%; 3rd position: 241/287 = 84%), suggesting that substitutions are predominantly neutral and that variation is non-adaptive; such a finding justifies the application of molecular clocks to explore these data.

Discussion

The 28S rRNA marker, has an inherently diverse rate of genetic evolution that facilitates its use across a broad phylogenetic range. Hence it is suitable for elucidating both higher (older) evolutionary relationships and, by analysis of the divergent 'D' domains, relationships between more recently diverged species (Larson, 1991). The COI + II mitochondrial marker offers another relatively rapidly evolving complementary marker suitable for investigating recently diverged species.

Analysis of the data using an ILD test showed the two sets of sequence data to be significantly phylogenetically incongruent. However, this incongruence disappears if the putative hybrid flies from Hawaii are excluded from the analysis (results not presented). Examination of the independently constructed phylogenetic trees (Fig. 1) shows that the Hawaiian *L. cuprina* specimens (as defined by morphological analysis) possess typical *L. cuprina*-type 28S rRNA sequence, but have *L. sericata*-type mitochondrial (COI + II) DNA.

The mtDNA molecule is generally maternally inherited and unaffected by recombination, therefore our findings lead us to suppose that a female *L. sericata* and a male *L. cuprina* crossed to produce an F1 generation with *L. sericata* mtDNA, a mix of nuclear DNA, and very likely an *L. cuprina* phenotype. Over time descendants mated with wild-type *L. cuprina* individuals would have led to a dilution and/or stochastic loss of *L. sericata* nuclear alleles, while at the same time lineage sorting would have produced fixation of the *L. sericata* mtDNA haplotype. Indeed, in previous laboratory crosses between *L. cuprina* and *L. sericata* the majority of hybrids were either indistinguishable from *L. cuprina*, or possessed characters intermediate between *L. cuprina* and *L. sericata* (Mackerass, 1933; Ullyett, 1945; Waterhouse & Paramonov, 1950). However, while such crosses have been seen in the laboratory, there are no previous records of their existence in the field. Lack of any evidence for such an event from nuclear DNA markers and the monophyly[2] of Hawaiian *L. cuprina* in the phylogenetic analysis of mtDNA (Fig. 1) suggests that if hybridization

has occurred (a) it happened sufficiently long ago that all detectable traces within the nuclear genome have been over-written, and (b) the *L. sericata* mtDNA haplotype was apparently fixed in Hawaiian *L. cuprina* by lineage sorting, suggesting a population bottleneck; successive bottlenecks associated with recent human-aided expansion have been previously postulated to explain stochastic mtDNA lineage survival in two species of mosquito introduced to Hawaii (Kambhampati & Rai, 1991; Guillemaud *et al.*, 1997).

The level of intraspecific mtDNA variation can be interpreted in several ways. The Hawaiian *L. cuprina* mtDNA lineage could simply be an otherwise undetected *L. sericata* haplotype. If this is the case, the only implied time period since hybridization necessary to account for these data is that required for mtDNA lineage sorting and introgression of the *L. sericata* haplotype. The 0.9% COI + II sequence divergence between the Hawaiian *L. cuprina* and global *L. sericata* is only slightly greater than the 0.8% intraspecific variation observed in a world-wide survey of the blowflies *Chrysomya albiceps* and *C. rufifacies* (Wells & Sperling, 1999). However, it seems unlikely that the Hawaiian haplotypes are simply a sample of *L. sericata* diversity given that we found only 0.2% sequence variation among specimens identified as *L. sericata*. If the phylogram in Fig. 1 represents the true phylogeny then we would interpret the divergence between haplotypes of the Hawaiian *L. cuprina* and global *L. sericata* as being the amount of evolutionary change that has occurred since the putative hybridization event and the divergence within Hawaiian *L. cuprina* as mutations that have accumulated since that species reached Hawaii. Of course, such a finding also indicates that the relationship between sequence divergence and time for these blowflies may not follow the same pattern as observed in some other insects (Brower, 1994).

Hawaiian *Drosophila* (DeSalle & Hunt, 1987) reportedly reached the Hawaiian archipelago around 40 million years (Myrs) ago when the 'newer' islands such as Oahu were still in the process of being formed; similarly, the ancestor of the endemic blowfly – *L. graphita* – also presumably arrived in the islands unaided by humans[3]. However, *L. sericata* is endemically Palearctic in distribution, whereas *L. cuprina* is Afrotropical and Oriental; it is difficult therefore to envisage a meeting of these two species in the mid-Pacific by natural dispersal. Both *L. sericata* and *L. cuprina* are commonly associated with humans and their domestic animals. Hence, if it is assumed that one or both species were carried by colonizing humans, the molecular clock can be calibrated by reference to known historical events. Calibrating the hybridization of *L. sericata* and *L. cuprina*, and the subsequent divergence of Hawaiian *L. cuprina*, against the arrival of the first Polynesians in Hawaii in approximately 500 AD yields a rate of divergence of 6.3×10^{-6} substitutions per site per year across COI + II. Calibrating the hybridization event with respect to the arrival of Europeans in 1778 yields a rate of divergence of 4.4×10^{-5} substitutions per site per year for COI + II. Such rates, however, appear unrealistically fast. Using the alternative approach and employing an independently estimated rate of mtDNA divergence of 2.3% per million years (Brower, 1994), the 0.9% divergence of Hawaiian *L. cuprina* from *L. sericata* (see above) suggests the hybridization event happened around 390 000 years ago. The appropriateness of using a mtDNA divergence rate (2.3% per Myrs) calculated from a different insect order (Lepidoptera) is of course questionable. Nevertheless, use of such an estimate indicates that the *L. cuprina* × *L. sericata* cross-mating event which gave rise to contemporary Hawaiian *L. cuprina* predates the arrival of humans in Hawaii, probably by several hundred-thousand years. The major question now, is whether such introgressant hybrids are particular to Hawaii or whether they are found elsewhere; for example, would the hypothesis of a relatively recent date for hybridization be supported if, for example, Tahitian *L. cuprina* fit the Hawaiian pattern? Taxonomically, while the existence of such an apparently purely introgressant form does not warrant the recognition of a new species, it may be appropriate, given the distinct genetic profile and geographical isolation of this form, to recognize it as a new subspecies, *L. cuprina hawaiiensis*.

Finally, the evidence for interspecific hybridization presented in this paper may have important consequences across a number of applied disciplines. For example, blowfly control: if frequent and recurring, hybridization raises the possibility of horizontal transfer of organophosphate resistance genes in sheep rearing regions – Australia and New Zealand – where both species exist sympatrically; forensic entomology: species molecular identification protocols are commonly based on mitochondrial gene markers, e.g. COI + II (Sperling *et al.*, 1994; Malgorn & Coquoz, 1999; Vincent *et al.*, 2000), especially when identifying larvae collected from a corpse (Benecke & Wells, 2001). The results reported here suggest that under some circumstances the use of a nuclear locus for species identification may be more robust, highlighting the importance of a multigene approach.

Experimental procedures

Fly collection and DNA extraction

Specimens of *L. cuprina* and *L. sericata* were caught at a range of sites in Europe, Africa, North America, Australasia and the Hawaiian island of Oahu; specimens of *Lucilia caesar* (L.) and *Calliphora vicina* Robineau-Desvoidy were included as outgroup species. Flies were identified to species/subspecies using the morphological characters described by Aubertin (1933), Norris (1990) and Holloway (1991); *L. sericata* and *L. cuprina* were distinguished by analysis of male genitalia (Fig. 2) and the paravertical setulae present on the back of the head. Details of all flies included in this study are presented in Table 2. DNA was extracted following standard protocols (Stevens & Wall, 1995, 1996, 2001; Wells & Sperling, 2001).

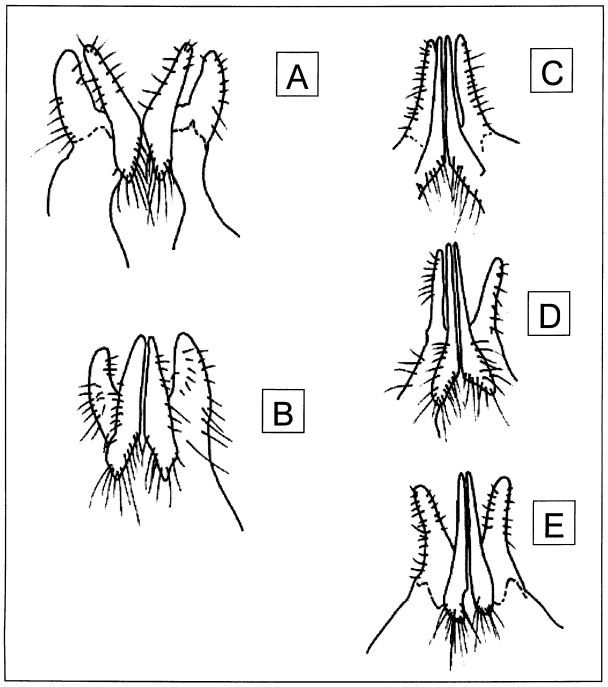

Figure 2. Forceps (dorsal view) of the male genitalia of: (A) *Lucilia sericata* of UK origin maintained in a colony (note: forceps slightly splayed in mounting); (B) *L. sericata* from California, USA; (C) *Lucilia cuprina* from Oahu, Hawaii; (D) *L. cuprina* from Townsville, Australia; and (E) *L. cuprina* from Kenya. Note the shorter and broader structure of the *L. sericata* forceps relative to those of *L. cuprina*. The forceps of the flies collected in Hawaii were used in identifying them as *L. cuprina* because they were indistinguishable from those of *L. cuprina* collected elsewhere. Drawings were prepared using permanently mounted slide preparations and a camera lucida.

Phylogenetic markers

Preliminary data from RAPD analysis of genomic DNA and short (329 bp) stretches of 12S mitochondrial DNA (Stevens & Wall, 1996, 1997a) indicate that atypical *L. sericata*-like mitochondrial sequence signatures occur in *L. cuprina* from the Hawaiian islands. Accordingly, evolutionary relationships in a global sample of *L. cuprina* and *L. sericata* were studied by sequencing complementary nuclear and mitochondrial gene markers. The nuclear sequence marker was the 2.2 kb D1–D7 region of the 28S large subunit (lsu) ribosomal RNA gene (28S rRNA) and the mitochondrial marker was the 2.3 kb region coding for the cytochrome oxidase subunits I and II, together with tRNA-leucine (COI + II).

In the case of the 28S rRNA marker, its inherently diverse rates of genetic evolution facilitate its use across a broad phylogenetic range, so that it is suitable for elucidating both higher (older) evolutionary relationships and, by analysis of the divergent 'D' domains, relationships between more recently diverged species (Larson, 1991). The COI + II mitochondrial marker offers another relatively rapidly evolving complementary marker suitable for investigating recently diverged species.

28S rRNA: PCR amplification and DNA sequencing

A fragment of 2.2 kb spanning the D1–D7 regions of the 28S lsu rRNA gene was amplified from sixteen species/specimens of blowflies, including seven specimens each of *L. sericata* and *L. cuprina*, *L. caesar* and *C. vicina* (Table 2), by PCR using conserved primers as published (Table 3). The 2.2 kb fragment was amplified in two overlapping sections of approximately 0.8 kb (D1–D2) and 1.45 kb (D3–D7), giving an overlap of ~50 bp to facilitate sequence assembly.

PCR amplification mixtures (25 μl volume) were prepared as follows: 50 ng template DNA, reaction buffer (final concentration 75 mM Tris/HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 0.01% TWEEN-20, 1.5 mM $MgCl_2$; Hybaid-AGS), dNTP mix at a final concentration of 0.2 mM per dNTP, 2 × primers at a final concentration of 2 μM, 1 U AGS*Gold* DNA polymerase (Hybaid UK), sterile water to a final volume of 25 μl. Amplification reactions were performed in a Hybaid thermal cycler for an initial denaturation step of 94 °C for 3 mins, followed by thirty cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min (D1–D2 fragment)/1 min 30 s (D3–D7 fragment), followed by a final elongation step of 68 °C for 15 mins.

PCR products were separated by electrophoresis in 0.5% TBE agarose gels, stained with ethidium bromide and visualized under

Table 2. Specimen details: identity, origin and DNA sequence accession numbers

Specimen	Collection location	Date	Accession no. 28S rRNA	Accession no. COI + II	Reference
Calliphora vicina	University of Bristol colony, UK	1995	AJ300131	AJ417702	1,3
Lucilia caesar	Langford, nr. Bristol, Somerset, UK	1994	AJ300138	AJ417703	3
Lucilia sericata	Langford, nr. Bristol, Somerset, UK	1994	AJ300139	AJ417714	3
	Hilerod, Sjelland, Denmark	1994	AJ300140	AJ417712	1,2,3
	Glendalough, Perth, W.A., Australia	1995	AJ300140*	AJ417715	1,2
	Los Angeles, California, USA	1994	AJ300141	AJ417715*	2,3
	Dorie, South Island, New Zealand	1994	AJ300140*	AJ417713	1,2
	Nerja, Andalucia, Spain	1994	AJ300140*	AJ417716	2
	Harare, Zimbabwe	1994	AJ300140*	AJ417717	1,2
Lucilia cuprina	Townsville, Queensland, Australia	1994	AJ417709	AJ417710	1,2
	Hawaii-1: Waianae, Oahu, Hawaii, USA	1994	AJ417709*	AJ417705	1,2
	Hawaii-2: Honolulu, Oahu, Hawaii, USA	1998	AJ417709*	AJ417704	–
	Dorie, South Island, New Zealand	1994	AJ417709*	AJ417706	1,2
	Serpentine, Perth, W.A., Australia	1995	AJ417709*	AJ417707	1,2
	Dakar, Senegal	1994	AJ417709*	AJ417708	1,2
	Tororo, Uganda	1994	AJ417709*	AJ417711	1,2

1 Stevens & Wall (1996); 2 Stevens & Wall (1997a); 3 Stevens & Wall (2001). *Sequence analysed equivalent to accession number given.

Table 3. Amplification and sequencing primers for 28S rRNA regions D1–D7 and mitochondrial cytochrome oxidase I and II subunits

28S rRNA D1–D2 fragment		
Amplification primers:		
D1.F:	5′-CCCCCTGAATTTAAGCATAT-3′	(20-mer)[1]
D2.R:	5′-GTTAGACTCCTTGGTCCGTG-3′	(20-mer)[2]
Internal sequencing primers:		
D1.R:	5′-CTCTCTATTCAGAGTTCTTTTC-3′	(22-mer)[1]
D2.F:	5′-GAGGGAAAGTTGAAAAGAAC-3′	(20-mer)[3]
28S rRNA D3–D7 fragment		
Amplification primers:		
D3-5.F:	5′-GACCCGTCTTGAAACACGG-3′	(19-mer)[4]
D7.R:	5′-CGACTTCCCTTACCTACAT-3′	(19-mer)[1]
Internal sequencing primers:		
D3-5.R:	5′-TTACACACTCCTTAGCGGA-3′	(19-mer)[4]
D3-5.486.R:	5′-TCGGAAGGAACCAGCTACTA-3′	(20-mer)[5]
D3-5.742.F:	5′-TCTCAAACTTTAAATGG-3′	(17-mer)[4]
D7.F:	5′-GACTGAAGTGGAGAAGGGT-3′	(19-mer)[1]
COI + II CO1–CO5 fragment		
Amplification primers:		
TYJ-1460:	5′-TACAATTTATCGCCTAAACTTCAGCC-3′	(26-mer)[6]
TL2N-3014:	5′-TCCATTGCACTAATCTGCCATATTA-3′	(25-mer)[7]
Internal sequencing primers:		
C1N-2191:	5′-CCCGGTAAAATTAAAATATAAACTTC-3′	(26-mer)[8]
UEA7:	5′-TACAGTTGGAATAGACGTTGATAC-3′	(24-mer)[9]
C1J-1751 A:	5′-GGATCACCTGATATAGCATTCCC-3′	(23-mer)[8]
C1N-1840F:	5′-AGGAGGATAAACAGTTCATCC-3′	(21-mer)[10]
COI + II CO3–CO8 fragment		
Amplification primers:		
TKN-3775:	5′-GAGACCATTACTTGCTTTCAGTCATCT-3′	(27-mer)[8]
C1J-2495:	5′-CAGCTACTTTATGAGCTTTAGG-3′	(22-mer)[6]
Internal sequencing primers:		
C2J-3138:	5′-AGAGCCTCTCCTTTAATAGAACA-3′	(23-mer)[7]
C2J-3408F:	5′-CAATGATATTGAAGTTATGA-3′	(20-mer)[11]

Modified from: [1]Friedrich & Tautz (1997a), [2]Hoelzel & Green (1992), [3]Hoelzel & Green (1992), [4]Friedrich & Tautz (1997b), [5]Friedrich & Tautz (1997b), [6]Sperling *et al.* (1994), [7]Simon *et al.* (1994), [8]Bogdanowicz *et al.* (1993), [9]Lunt *et al.* (1996), [10]Sperling *et al.* (1995), [11]Wells & Sperling (1999).

[1]COI-only sequences and complete 28S rRNA gene sequences have been analysed from four additional specimens of Hawaiian *L. cuprina*, confirming the pattern of phylogenetic relationships observed for the two specimens/populations presented in Fig. 1.

[2]Although our analysis provides strong support for placing Hawaiian *L. cuprina* mtDNA in the *L. sericata* clade, the basal position of these haplotypes leaves open the possibility that they are not descended from an *L. sericata* female, but rather from a recent ancestor of that species.

[3]*Lucilia graphita* is substantially morphologically different from either *L. cuprina* or *L. sericata* and is confined to the northern-most islands of the Hawaiian archipelago several hundered miles distant; it has never been recorded on the principal islands such as Oahu (Hardy, 1981; N. Evenhuis, personal communication).

UV illumination. The products of four to eight separate PCR reactions per specimen were excised from the gel with a clean scalpel and pooled prior to purification with Sephaglas BP beads (Amersham Pharmacia Biotech). The pooling of amplicons reduces (dilutes) the possibility that random PCR amplification errors in individual reactions are carried forward and sequenced; in addition, all output traces from automated sequencing (see below) were checked for possible hybrid sites and ambiguities.

Fragments were sequenced in both directions at 350–500 base pair intervals, depending on the location of suitable primer sites, using the four original amplification primers and six additional internal primers (Hoelzel & Green, 1992; Friedrich & Tautz, 1997a,b; Table 3). Between 30 and 90 ng of pooled, purified template were used per sequencing-PCR reaction; reactions were run on a Perkin-Elmer ABI 377 automated sequencer. A consensus sequence was assembled from the individual internal primer sequences for each specimen using the program AutoAssembler (ABI).

COI + II: PCR amplification and DNA sequencing

A fragment of 2.3 kb spanning the mitochondrial COI and COII subunits, as well as tRNA-leucine, was amplified from the sixteen species/specimens using published primers and protocols (Wells & Sperling, 2001; Table 3). The 2.3 kb fragment was amplified in two overlapping sections of approximately 1.55 kb (CO1–CO5) and 1.3 kb (CO3–CO8), giving an overlap of ~500 bp to facilitate sequence assembly.

PCR amplification mixtures (25 μl volume) were prepared and amplified as for the 28S rRNA gene (see above). Amplification times were 94 °C for 3 mins, followed by thirty cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 2 mins 10 s (CO1–CO5 fragment)/1 min 30 s (CO3–CO8 fragment), followed by a final elongation step of 68 °C for 15 mins.

PCR products were separated by electrophoresis as described for 28S rRNA fragments (see above). Fragments were sequenced in both directions at 350–500 base pair intervals, depending on the

location of suitable primer sites, using the four original amplification primers and six additional internal primers (Table 3). Again, 30 and 90 ng of pooled, purified template were used per sequencing-PCR reaction and sequencing was performed as for the 28S rRNA gene (see above).

Phylogenetic analysis

Sequences were aligned using the program Clustal W (Thompson *et al*., 1994), before final adjustments were made by eye. Alignments comprised 2193 (28S rRNA) and 2329 (COI + II) nucleotide positions and are available on request from J.R.S.

Incongruence-length difference (ILD) tests (Farris *et al*., 1995) were performed to test the homogeneity of the sequence data and to assess the combinability of the data from the two genes. While the ILD test is used to assess combinability of data, it also serves as an indicator of phylogenetic congruence (Cunningham, 1997) between genetic markers; it is implemented in PAUP* as a partition-homogeneity test. Two partitions corresponding to the two genes were defined within the 4523 characters of the combined data file.

Phylogenetic analyses were performed using the program PAUP*, version 4.0b6 (Swofford, 2001). Parsimony analysis was performed separately for each set of gene sequences. Relative bootstrap support analyses were performed using 1000 pseudoreplicates for each gene. The default options of PAUP were used: optimality criterion = maximum parsimony; initial upper bound computed step-wise; only minimal trees kept; addition sequence = furthest; zero length branches collapsed.

Differences between the mitochondrial (COI + II) and nuclear (28S rRNA) gene tree topologies were assessed using a Kishino-Hasegawa test (Kishino & Hasegawa, 1989) as implemented in PAUP*.

Substitution distribution analysis

The program MacClade (Maddison & Maddison, 1992) was used to explore the distribution of phylogenetically informative characters (nucleotide changes) within trees. The 'Character Steps' option within the 'Chart' menu was used to evaluate coding position variation and to map the distribution of variable sites along genes.

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