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Ancient and modern hybridization between *Lucilia sericata* and *L. cuprina* (Diptera: Calliphoridae)

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Abstract. There are important but inconsistent differences in breeding site preference between the blow flies *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) that have significance for medical and veterinary science. These inconsistencies might arise from hybridisation. The species are difficult to distinguish using external morphology, although the male genitalia are distinctive and there are reliable molecular markers. Molecular evidence of modern hybridisation, derived from a newly developed nuclear marker, the *period* (*per*) gene, is presented here. This has implications for identifications of these species based on mtDNA, and may lead to an explanation of the medical and veterinary anomalies noted in these species.

INTRODUCTION

The use of *Lucilia* blowflies for maggot debridement therapy (MDT) has become a topic of great interest in South Africa (Williams et al., 2008; F. Cronje & Du Plessis H.J.C, pers. comm). *Lucilia sericata* (Meigen, 1826) is the species of choice for MDT (Altincicek & Vilcinskas, 2009; Vilcinskas, 2011), but the misidentification of *Lucilia cuprina* (Wiedemann, 1830) and *L. sericata* for use in MDT and how best to supplement MDT colonies has raised the issue of species identification (Williams et al., 2008; Tantawi et al., 2010). *Lucilia cuprina* has recently been used successfully for MDT (Paul et al., 2009; Tantawi et al., 2010; Kingu et al., 2012) although this species is responsible for sheep-strike that causes losses to the wool and meat industries that amount to millions of dollars worldwide each year (Hepburn, 1943; Ullyett, 1945; Vogt & Woodburn, 1979; Heath & Bishop, 2006). Different populations of *L. sericata* show different degrees of *cuprina*-like attraction to sheep (Crombe, 1944; Cragg, 1956), but no clear pattern in this myiasis has been noted.

These two species have been suspected of interbreeding and producing fertile hybrids in South Africa (Ullyett, 1945). They have been shown to hybridise under laboratory conditions and to produce fertile hybrids, although there are no reports of this occurring naturally (Ullyett, 1945). *Lucilia cuprina* has consistently been found to be paraphyletic relative to *L. sericata* in studies of several mitochondrial genes (Table 1). If they are interbreeding, this leads to an explanation of the medical and veterinary anomalies noted in the biology of these species.

Several authors have suggested that these flies should be classified as three species or that *L. cuprina* should be classified as two subspecies – *Lucilia c. cuprina* (Wiedemann, 1830) and *L. c. dorsalis* Robineau-Desvoidy, 1830 (Waterhouse & Paramonov, 1950; Norris, 1990; Stevens & Wall, 1996; Stevens et al., 2002; Stevens, 2003; Wallman et al., 2005; Wells et al., 2007; DeBry et al., 2010). *Lucilia sericata* and *L. cuprina* are morphologically very similar and the adults are difficult to identify using the available keys based on morphological characters without using the male genitalia, which usually requires destructive sampling (Aubertin, 1933; Smith, 1986; Norris, 1990; Holloway, 1991). However, with some experience, the females can usually be reliably identified using the characteristics described by Holloway (1991a).

Molecular methods are useful in confirming the taxonomic status of these two species (Williams et al., 2008; Tourle et al., 2009; Tantawi et al., 2010). The use of more than one gene for taxonomic and phylogenetic studies is recommended as using only one gene may not give a true picture of relationships or patterns of gene flow (Sperling et al., 1994; Nelson et al., 2007; Whitworth et al., 2007; Tourle et al., 2009). Analysing both nuclear and mitochondrial genes simultaneously has highlighted introgression and the difference between gene trees and species trees (Page & Charleston, 1997; Nichols, 2001; Stevens et al., 2002; Stevens, 2003; Whitworth et al., 2007; Tourle et al., 2009; DeBry et al., 2010).

The purpose of this study was to test for evidence of hybridisation between these two species, shown by a difference between the gene trees produced from sequence data using nuclear, as opposed to mitochondrial, genes from these flies from different localities around South Africa and from sites in Africa, Europe, Australia, Asia, and North America.

MATERIAL AND METHODS

Adult flies of both *L. sericata* and *L. cuprina* were collected in Britstown, Bloemfontein, Cape Town, Durban, Grahamstown, Nelspruit, and Witbank in South Africa (Fig. 1 insert). *Lucilia* specimens originating from Welkom and Pretoria were

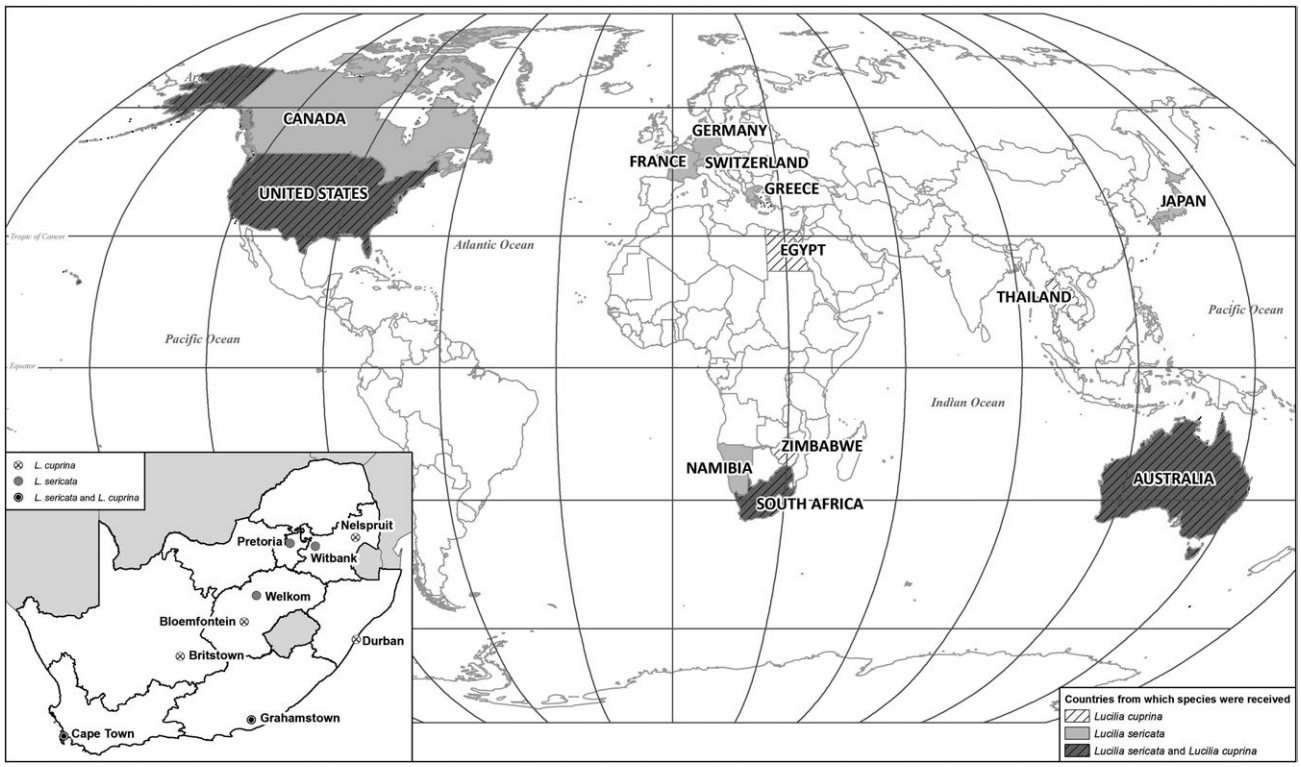

Fig. 1. World map showing the localities where flies were caught. Insert: map of South Africa showing the towns where flies were caught.

also obtained from a maggot debridement therapy colony at Eugene Marais Hospital in Pretoria. *Lucilia sericata* was also obtained from Australia, Canada, France, Germany, Greece, Japan, Namibia, Switzerland, and the United States of America (Fig. 1). Additional specimens of *L. cuprina* were obtained from Australia, Egypt, Thailand, the United States of America, and Zimbabwe (Fig. 1). A total of 84 flies were collected – 11 males and 73 females. They were identified by their morphology using published keys (Aubertin, 1933; Smith, 1986; Holloway, 1991a). Due to the biology of these flies, females are attracted to bait traps more than males and therefore characteristics identified by Holloway (1991a); specifically the distances and angles between setae on the vertex of females, the extent of metallic sheen on the parafrontal sclerites of females and the number of scutellar setulae were used to identify these flies.

All flies were kept in separate 1.5 ml Eppendorf tubes in 96% ethanol and deposited with the Durban Natural Science Museum after analysis. One hind leg of each fly was used for DNA analysis. DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions (Qiagen 07/2006).

Three genes were chosen for sequencing – *28S* rRNA (*28S*), a nuclear gene that has been used in previous studies and would allow comparison with other studies (Table 1); *period* (*per*), a second nuclear gene that is faster-evolving than *28S* to give better resolution; and cytochrome oxidase I (*COI*), that has been used in previous studies (Table 1). A region of approximately 650 bp in domain 1–2 of the *28S* gene was amplified using the primers 5'-CCCCCTGAATTTAAGCATAT-3' and 5'-GTTAGACTCCTTGGTCCGTG-3' (Stevens et al., 2002). A region of approximately 600 bp of the *COI* gene was amplified using the primers C1-J1709 (5'-AATTGGGGGGTTTGGAA ATTG-3') and C1-N2353 (5'-GCTCGTGTATCAACGTCTA TTCC-3') (Simon et al., 2006). This region overlaps the "barcoding" region for approximately 300 base pairs. A region of approximately 730 bp of the *per* gene, was amplified using the primers *per*5 (5'-GCCTTCAGATACGGTCAAAC-3') (G. Warman, pers. comm.) and *per* reverse (5'-CCGAGTGTGGTTTG

TABLE 1. Genes used in studies of *Lucilia sericata* and *Lucilia cuprina.*

Source	Mitochondrial		Nuclear		
	CO1	12S rRNA	*28S* rRNA	*per*	RAPDs
Stevens & Wall, 1996	–	329 bp	–	–	X
Stevens et al., 2002	2300 bp (*CO1* & *2*)	–	2193 bp	–	
Stevens, 2003	2300 bp (*CO1* & *2*)	–	2200 bp	–	
Wallman et al., 2005	3008 bp (*CO1* & *2* & *ND4-ND4L*)	–	–	–	
Wells et al., 2007	1545 bp	–	–	–	
Harvey et al., 2008	1167 bp	–	–	–	
Williams et al., 2008	601 bp	–	654 bp	–	
Tourle et al., 2009	439 bp	–	678 bp	–	
DeBry et al., 2010	1200bp	–	2100 bp	–	
Tantawi et al., 2010	576 bp	–	656 bp	746 bp	
This study	576 bp	–	654 bp	722 bp	

TABLE 2. Specimen locality data for sequences included from GenBank.

Species	Locality	Country	Accession Number		
			28S	*per*	*COI*
L. sericata	Langford	UK	AJ300139		
	Hilerod	Denmark	AJ300140		
	Hilerod	Denmark			EF531193
	Kingsbury	UK			AJ417713
	Nerja	Spain			AJ417716
	Harare	Zimbabwe			AJ417717
	–	China			DQ345086
L. cuprina	Townsville	Australia	AJ417709		AJ417710
	Wallaceville	New Zealand		Y19108.1	
	Tororo	Uganda			AJ417711
	–	Taiwan			AY097335
	–	China			DQ345087
	Oahu	Hawaii			DQ453496
	Honolulu	Hawaii			AJ417704
	Waianae				AJ417705

GAGATT-3') (designed by the authors). Polymerase chain reaction (PCR) amplification was performed using 1 µL of DNA in a 25 µL reaction. Amplification times were 94°C for 5 min denaturation, followed by 36 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 30 s and a final extension period at 72°C for 7 min. PCR products were confirmed by gel electrophoresis stained in ethidium bromide.

PCR products were then sequenced using an ABI 3730l Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and the primers used in amplification. Additional DNA sequences for these two species were obtained from GenBank (www.ncbi.nlm.nih.gov) for comparative analysis (Table 2). The sequences were aligned and edited using the BioEdit v7.0.9 software (Hall, 1999).

Phylogenetic reconstruction by maximum parsimony analysis was performed using PAUP*4b10 (Swofford, 2003) using the best-fitting model (HKY) from MrModelTest v2.2 (Nylander, 2004) applied in MrMTgui (Nuin, 2005). Statistical support for nodes was assessed by bootstrapping with 100 replicates retaining a maximum of 10,000 trees. Bayesian inference analysis was performed using one cold and three hot chains and the HKY model. Analysis was run for 5,000,000 generations, sampling every 1,000 generations with burn-in of 1,000 samples. All phylogenetic analyses used *Calliphora vicina* and *Lucilia infernalis* as outgroups. Incongruence length difference (ILD) tests (Farris et al., 1994) were run in PAUP* 4b10 (Swofford, 2003) to quantify the differences in topology between trees for *28S*, *COI* and *per*. Analysis was then conducted on the partitioned data sets (*28S* and *per*; *28S*, *per* and *COI*) with the parameters as above.

When hybridization is involved, a single dichotomising phylogenetic tree will often not be a suitable representation of the phylogenetic history (Huson & Bryant, 2006). This may make it necessary to use a more general graph, such as a network to represent the data. NeighborNet computes a set of splits from the data. If splits are compatible, the resultant graph will be a dichotomous tree, but when the splits are not compatible, it results in a network diagram with multiple parallel branches representing a single split (Huson & Bryant, 2006). Network diagrams were created using NeighborNet in SplitsTree4 (Huson & Bryant, 2006) using the uncorrected P-method for distance.

RESULTS

A total of 654 base pairs for *28S*, 576 bp for *COI* and 722 bp for *per* (a total of 1952 bp) were sequenced and aligned. There were no indels in the aligned sequences. A total of 77, 83 and 76 specimens were sequenced respectively for *28S*, *COI* and *per* (Table 3).

The ILD test showed *28S* and *per* to be congruent (P = 0.99), and the ILD test for *28S* and *COI* was not statistically significant (P = 0.08). *per* and *COI* were significantly incongruent (P = 0.01) as was the combination of *28S*, *per* and *COI* (P = 0.01). Due to the high level of congruence between *28S* and *per*, these two data sets were concatenated and used for the analyses and network diagrams. Despite the incongruence between the nuclear (*28S* and *per*) and mitochondrial (*COI*) data, these data sets were also concatenated and an analysis run on the total evidence.

The Bayesian Inference trees (Fig. 2A) for the nuclear genes (*28S* and *per*) show both *L. sericata* and *L. cuprina* to be monophyletic clades with strong support (Fig. 2A). The Bayesian Inference tree for *COI* (Fig. 2B) shows that *L. cuprina* is paraphyletic with respect to *L. sericata*, with good posterior probability support. The first *L. cuprina* clade (Fig. 2B) exhibits both nuclear and mitochondrial sequences (and morphology) of "pure *cuprina*", while the second clade exhibits nuclear DNA (and morphology) of *L. cuprina* but mitochondrial DNA of *L. sericata* – a "hybrid" clade. The *L. cuprina* sequences from GenBank from Hawaii, Taiwan and China grouped with the "hybrid" clade (Fig. 2B).

Out of 42 specimens with the morphology of *L. cuprina*, five have mitochondrial genes that are typical of the *L. sericata* clade (Fig. 2B), but not of the "ancient hybrid" clade. The maximum parsimony trees were topologically compatible with the Bayesian Inference trees but the trees were less well resolved (trees not shown).

The network diagrams of the nuclear genes (*28S* and *per*) (Fig. 3) indicate a clear and simple split between the *L. sericata* specimens and the *L. cuprina* specimens. The *COI* network diagram (Fig. 4) shows two clear splits between a cluster of *L. sericata* specimens, and two clusters of *L. cuprina* specimens. The "hybrid" cluster of *L. cuprina* specimens lies closer to the *L. sericata* cluster than to the "pure" *L. cuprina* cluster, but is distinctively monophyletic. The five *L. cuprina* specimens that group

TABLE 3. Specimen locality data for sequences from this study added to GenBank (* indicate identical sequences that are represented by one sequence in the Bayesian Inference tree, M – Male, F – Female).

Species	Specimen	Locality		Accession Number		
				28S	*per*	*COI*
Calliphora vicina	CV_FRC_01	Montferrier-Sur-Lez	France	JN792781		
Lucilia caesar	Ca_FRC_01	Montferrier-Sur-Lez	France	JN792782	JN792858	
Lucilia infernalis	In_RWN_01	Nyungwe Forest Reserve	Rwanda	JN792780	JN792857	JN813094
	C_AUS_01* (M)	Sydney	Australia			JN792622
	C_AUS_02* (F)	Sydney	Australia			JN792623
	C_AUS_03 (F)	Hornsby Heights	Australia	JN792705	JN792783	JN792624
	C_EGT_01 (F)	Alexandria	Egypt	JN792706	JN792784	JN792625
	C_EGT_02 (F)	Alexandria	Egypt	JN792707	JN792785	JN792626
	C_SA_BFN_01(F)	Bloemfontein	South Africa	JN792708	JN792786	JN792627
	C_SA_BFN_02 (F)	Bloemfontein	South Africa	JN792709	JN792787	JN792628
	C_SA_BRT_01 (F)	Britstown	South Africa	JN792710	JN792788	JN792629
	C_SA_BRT_02 (F)	Britstown	South Africa	JN792711	JN792789	JN792630
	C_SA_CT_01*(M)	Cape Town	South Africa	JN792712	JN792790	JN792631
	C_SA_CT_02 (F)	Cape Town	South Africa	JN792713	JN792791	JN792632
	C_SA_CT_03*(F)	Cape Town	South Africa	JN792714	JN792792	JN792633
	C_SA_CT_04 (F)	Cape Town	South Africa	JN792715	JN792793	JN792634
	C_SA_CT_05 (F)	Cape Town	South Africa	JN792716	JN792794	JN792635
	C_SA_CT_06 (F)	Cape Town	South Africa	JN792717	JN792795	JN792636
	C_SA_CT_07 (F)	Cape Town	South Africa	JN792718	JN792796	JN792637
	C_SA_CT_08 (F)	Cape Town	South Africa	JN792719	JN792797	JN792638
	C_SA_CT_09*(F)	Cape Town	South Africa	JN792720	JN792798	JN792639
	C_SA_CT_10 (M)	Cape Town	South Africa	JN792721	JN792799	
	C_SA_CT_11*(F)	Cape Town	South Africa	JN792722	JN792800	JN792640
	C_SA_CT_12*(F)	Cape Town	South Africa	JN792723	JN792801	JN792641
	C_SA_DBN_01*(F)	Durban	South Africa	JN792724	JN792802	JN792642
	C_SA_DBN_02 (F)	Durban	South Africa	JN792725	JN792803	JN792643
Lucilia cuprina	C_SA_DBN_03(M)	Durban	South Africa	JN792726	JN792804	JN792644
	C_SA_DBN_04 (F)	Durban	South Africa			JN792645
	C_SA_DBN_05 (F)	Durban	South Africa			JN792646
	C_SA_DBN_06 (F)	Durban	South Africa	JN792727	JN792805	JN792647
	C_SA_DBN_07*(F)	Durban	South Africa	JN792728	JN792806	JN792648
	C_SA_DBN_08 (F)	Durban	South Africa	JN792729	JN792807	JN792649
	C_SA_DBN_09 (F)	Durban	South Africa	JN792730	JN792808	JN792650
	C_SA_DBN_10*(F)	Durban	South Africa	JN792731	JN792809	JN792651
	C_SA_DBN_11*(F)	Durban	South Africa	JN792732	JN792810	JN792652
	C_SA_DBN_12 (F)	Durban	South Africa	JN792733	JN792811	JN792653
	C_SA_DBN_13 (F)	Durban	South Africa	JN792734	JN792812	JN792654
	C_SA_DBN_14*(F)	Durban	South Africa	JN792735	JN792813	JN792655
	C_SA_GHT_01(M)	Grahamstown	South Africa	JN792736	JN792814	JN792656
	C_SA_GHT_02 (F)	Grahamstown	South Africa	JN792737	JN792815	JN792657
	C_SA_NEL_01 (F)	Nelspruit	South Africa	JN792738	JN792816	JN792658
	C_SA_NEL_02 (F)	Nelspruit	South Africa	JN792739	JN792817	JN792659
	C_THA_01 (F)	Chiang Mai	Thailand	JN792740	JN792818	JN792660
	C_THA_02 (F)	Chiang Mai	Thailand	JN792741	JN792819	JN792661
	C_THA_03 (F)	Chiang Mai	Thailand	JN792742	JN792820	JN792662
	C_THA_04 (F)	Chiang Mai	Thailand			JN792663
	C_USA_01 (F)	Merced	USA	JN792743	JN792821	JN792664
	C_USA_02 (F)	Merced	USA	JN792744	JN792822	JN792665
	C_ZIM_01 (F)	Matobos	Zimbabwe			JN792666
	C_ZIM_02 (F)	Matobos	Zimbabwe	JN792745	JN792823	JN792667
	S_AUS_01 (M)	Seaford	Australia	JN792746	JN792824	JN792668
	S_CAN_01 (F)	Windsor	Canada	JN792747	JN792825	JN792669
	S_CAN_02 (F)	Windsor	Canada	JN792748	JN792826	JN792670
	S_FRC_01 (F)	Montferrier-Sur-Lez	France	JN792749	JN792827	JN792671
	S_FRC_02 (F)	Montferrier-Sur-Lez	France	JN792750	JN792828	JN792672
	S_FRC_03 (F)	Montferrier-Sur-Lez	France	JN792751	JN792829	JN792673
	S_GER_01 (F)	Kempen	Germany	JN792752		JN792674
	S_GER_02 (F)	Kempen	Germany		JN792830	JN792675
	S_GRC_01 (F)	Crete	Greece	JN792753		JN792676
	S_GRC_02 (F)	Crete	Greece			JN792677
	S_JPN_01* (F)	Osaka	Japan	JN792754	JN792831	JN792678
	S_JPN_02* (F)	Osaka	Japan	JN792755	JN792832	JN792679
	S_JPN_03* (F)	Iwate	Japan	JN792756	JN792833	JN792680
	S_JPN_04* (F)	Iwate	Japan	JN792757	JN792834	JN792681
	S_NAM_01 (F)	Possession Island	Namibia	JN792758	JN792835	JN792682
	S_NAM_02 (F)	Possession Island	Namibia	JN792759	JN792836	JN792683
	S_SA_CT_01* (F)	Cape Town	South Africa	JN792760	JN792837	JN792684
	S_SA_CT_02 (F)	Cape Town	South Africa	JN792761	JN792838	JN792685
Lucilia sericata	S_SA_CT_03* (M)	Cape Town	South Africa	JN792762	JN792839	JN792686
	S_SA_CT_04* (F)	Cape Town	South Africa	JN792763	JN792840	JN792687
	S_SA_CT_05 (F)	Cape Town	South Africa	JN792764	JN792841	JN792688
	S_SA_CT_06* (F)	Cape Town	South Africa	JN792765	JN792842	JN792689
	S_SA_CT_07* (F)	Cape Town	South Africa	JN792766	JN792843	JN792690
	S_SA_CT_08* (F)	Cape Town	South Africa	JN792767	JN792844	JN792691
	S_SA_GHT_01 (F)	Grahamstown	South Africa	JN792768	JN792845	JN792692
	S_SA_GHT_02 (F)	Grahamstown	South Africa	JN792769	JN792846	JN792693
	S_SA_PTA_01 (M)	Pretoria	South Africa	JN792770	JN792847	JN792694
	S_SA_PTA_02 (F)	Pretoria	South Africa	JN792771	JN792848	JN792695
	S_SA_PTA_03 (F)	Pretoria	South Africa	JN792772	JN792849	JN792696
	S_SA_PTA_04 (M)	Pretoria	South Africa	JN792773	JN792850	JN792697
	S_SA_WLK_01 (F)	Welkom	South Africa	JN792774	JN792851	JN792698
	S_SA_WLK_02 (F)	Welkom	South Africa	JN792775	JN792852	JN792699
	S_SA_WTB_01 (F)	Witbank	South Africa	JN792776	JN792853	JN792700
	S_SA_WTB_02 (F)	Witbank	South Africa	JN792777	JN792854	JN792701
	S_SWZ_01 (M)	Lausanne	Switzerland			JN792702
	S_USA_01 (F)	Michigan	USA	JN792778	JN792855	JN792703
	S_USA_02 (M)	Michigan	USA	JN792779	JN792856	JN792704

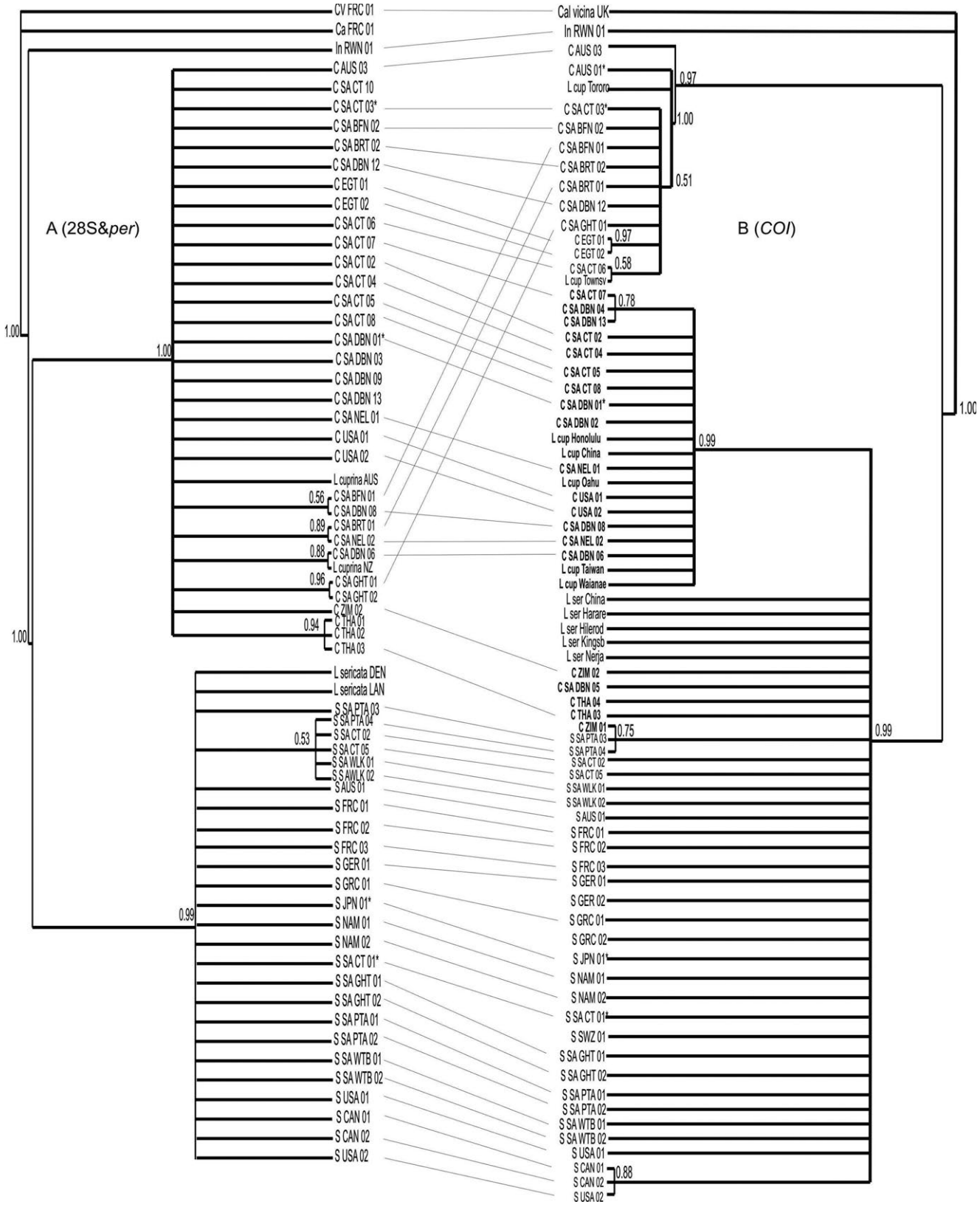

Fig. 2. Bayesian Inference trees constructed from nuclear genes (*28S* and *per*) (A) and mitochondrial genes (*COI*) (B) data. Posterior probabilities are indicated on nodes. C – *cuprina*, S – *sericata*, CV – *Calliphora vicina*, In – *Lucilia infernalis*, CA - *Lucilia caesar*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, GRC – Greece, JPN – Japan, NAM – Namibia, EGT – Egypt, RWN – Rwanda, SWZ – Switzerland, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

Fig. 3. NeighborNet network diagram constructed from *28S* & *per* data. C – *cuprina*, S – *sericata*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, JPN – Japan, NAM – Namibia, EGT – Egypt, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

within the *L. sericata* clade (Fig. 2B) also appear within the *L. sericata* cluster (Fig. 4). The network diagram of the total evidence concatenated data sets (Fig. 5) shows a clear split between the *L. sericata* and *L. cuprina* clusters, and the *L. cuprina* samples split into two clusters which are linked by more pathways to each other than to the *L. sericata* cluster.

DISCUSSION

A number of studies have been conducted on *L. sericata* and *L. cuprina*, looking at morphological identification, the possibility that they are interbreeding and whether *L. cuprina* should be classified as two subspecies or two independent species (Ullyett, 1945; Waterhouse & Paramonov, 1950; Norris, 1990; Holloway, 1991a, b; Stevens & Wall, 1996; Stevens et al., 2002; Stevens, 2003; Wallman et al., 2005; Wells et al., 2007; Harvey et al., 2008; Tourle et al., 2009; DeBry et al., 2010). This study focuses on these two species in South Africa, but also examines specimens from across the globe to place the South African situation into a global context. This study used two nuclear and one mitochondrial gene where most previous studies have either used only one mitochondrial gene or a combination of mitochondrial genes and one nuclear gene (Table 1). Stevens & Wall (1996) used RAPDs, which encompasses multi-locus nuclear genotype data, but without targeting explicit genes (Table 1).

Individually and together, the nuclear *28S* and *per* genes show *L. sericata* and *L. cuprina* to be two monophyletic clades (Fig. 2A) with very strong posterior probability support (0.99 and 1.00 respectively). However, the mitochondrial *COI* gene suggests that *L. cuprina* is paraphyletic with respect to *L. sericata* (Fig. 2B). There is a monophyletic clade of *L. cuprina* specimens that have *L. sericata*-like mtDNA, which has been seen in previous studies (Table 1). This monophyletic clade of *L. cuprina* with *L. sericata*-like mtDNA has been suggested to represent an ancient hybridization event (Stevens & Wall, 1996; Stevens et al., 2002; Tourle et al., 2009). The *L. sericata* mtDNA appears to have been fixed in this lineage of *L. cuprina* and not lost through lineage sorting.

However, there are also five specimens with the morphology of *L. cuprina* and mtDNA of *L. sericata* that are not representative of the ancient, introgressed clade (Figs

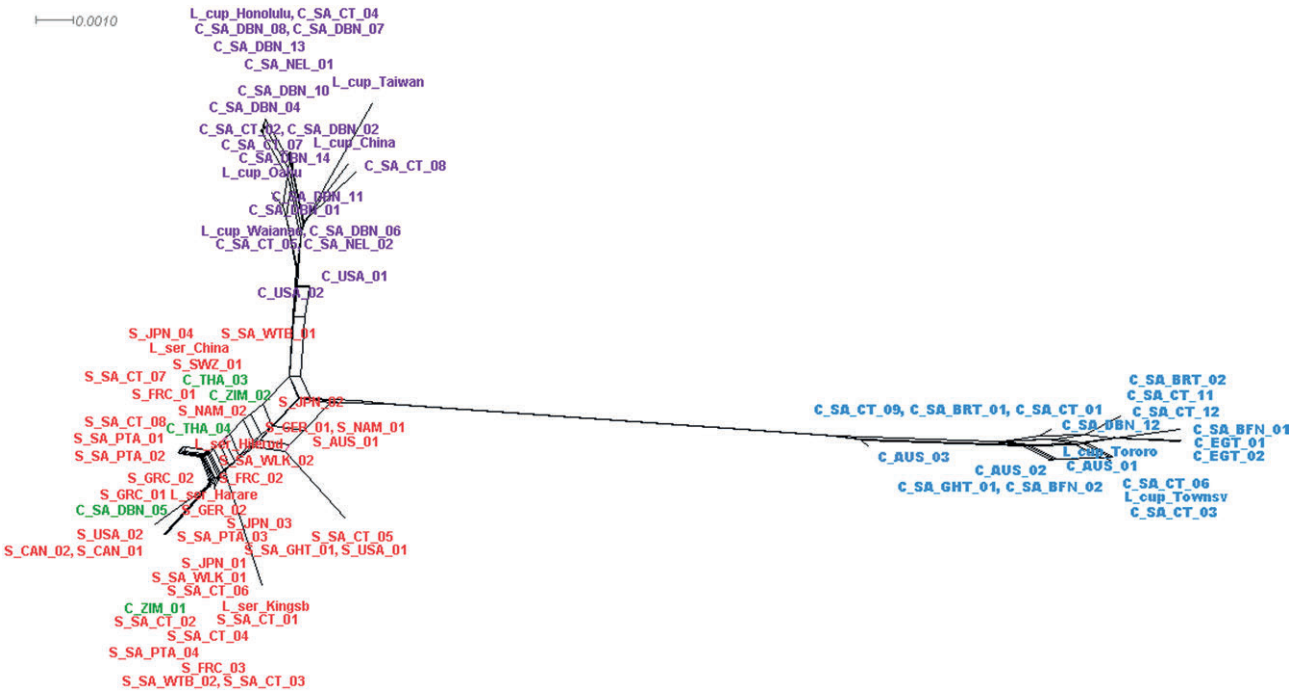

Fig. 4. NeighborNet network diagram constructed from *COI* data. C – *cuprina*, S – *sericata*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, JPN – Japan, NAM – Namibia, EGT – Egypt, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

2B and 4), implying novel mismatches of nuclear and mitochondrial genomes. Nuclear genes were not amplified for three of these specimens, but the other two, from Zimbabwe and Thailand, have (different) *28S* and *per* genotypes typical of *L. cuprina*, which suggests modern hybridization. This has not been seen in any previous studies on *L. sericata* / *L. cuprina* (Table 1) and provides the first direct genetic evidence of modern-day natural interbreeding between these species.

Ancient hybrids and introgression

The specimens that form the monophyletic clade of *L. cuprina* with *L. sericata*-like mtDNA originate from Durban, Nelspruit and Cape Town in South Africa, and from Merced in California in the continental USA, Hawaii, China, and Taiwan (Tables 2 and 3). It was once suggested that this lineage was restricted to the Hawaiian Islands (Stevens & Wall, 1996; Stevens et al., 2002), but since then the lineage has been found in North America, Africa, and Asia. It would be difficult to determine where it originated because it is so widespread. There does not appear to be any geographical coherence within the two *L. cuprina* clades (Fig. 2B). It was suggested that the two named subspecies of *L. cuprina* – *L. c. cuprina* and *L. c. dorsalis* – could be distinguished using *COI* sequences because both subspecies formed monophyletic clades (DeBry et al., 2010), with *L. c. cuprina* forming a monophyletic clade that was sister to the *L. sericata* clade, thus suggesting that all *L. cuprina* with *L. sericata*-like mtDNA are *L. c. cuprina*. Sequences from South Africa (Tourle et al., 2009) that were included in this analysis (DeBry et al., 2010) all grouped with the putative clade of *L. c. cuprina*, although African *L. cuprina* are considered to be *L. cuprina dorsalis* (Waterhouse & Paramonov, 1950). Perhaps *L. c. cuprina* has been introduced into South Africa like some other synanthropic blow flies (Williams & Villet, 2006), but the problem remains of distinguishing them morphologically, an issue that was addressed by Tourle et al. (2009), who found the "hybrid" clade to have a morphological index that was more *cuprina*-like than "pure" *cuprina* specimens.

Four cases of mtDNA introgression without detectable nuclear introgression, as seen in this study, were reported for *Protocalliphora* blowflies (Whitworth et al., 2007). Interspecific mitochondrial introgression linked to selective sweeps induced by nuclear-cytoplasmic incompatibility due to *Wolbachia* infections has been described in various insects (Ballard, 2000) as an explanation for how mtDNA introgression without nuclear introgression is possible. Cytoplasmic incompatibility is a process where, if uninfected females mate with infected males, some or all of their eggs will die. But if an infected female mates with either an infected or uninfected male, her eggs remain viable but all will be infected with *Wolbachia*. So infected females outcompete uninfected ones and the overall population of *Wolbachia*-infected flies (and therefore *Wolbachia*) increases (Zimmer, 2001). Thus the mitochondria of infected individuals have a greater chance than uninfected individuals of being passed on because mitochondria are passed down the female line, leading to fixed introgression. *Wolbachia* infection in the blowfly *Protocalliphora sialia* (Baudry et al., 2003) and infections of three different strains of *Wolbachia* in *Protocalliphora* in North America (Whitworth et al., 2007)

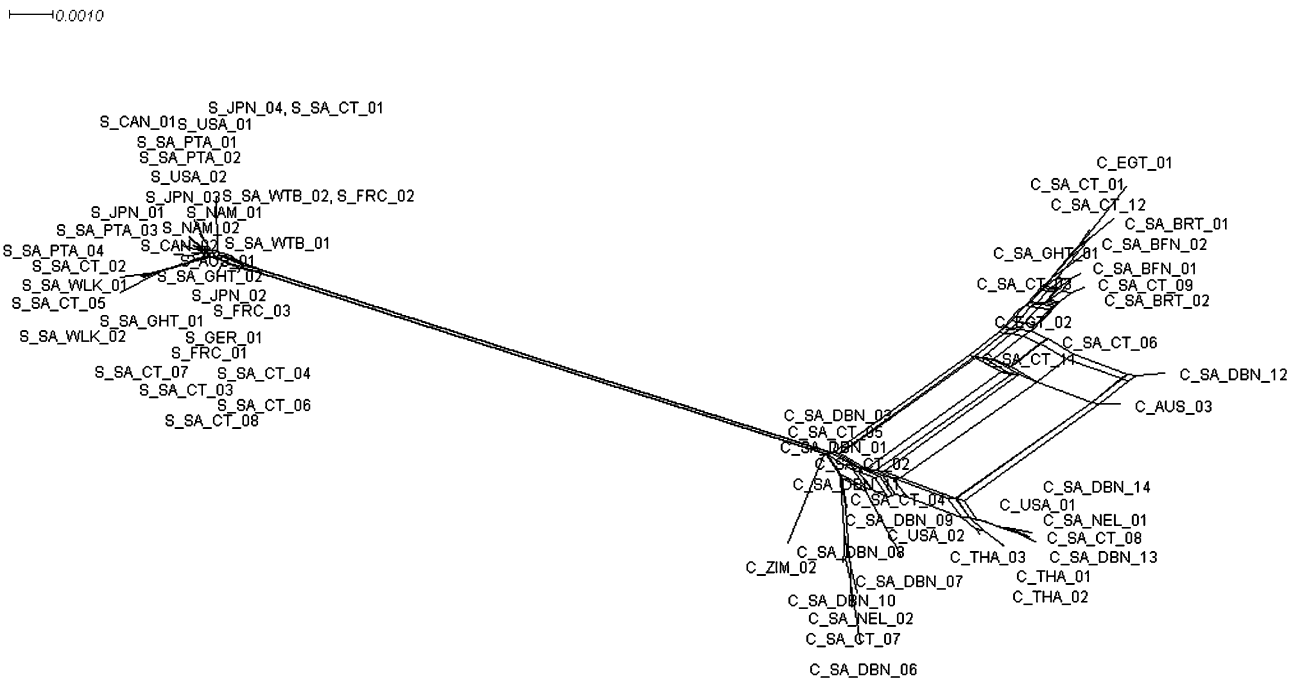

Fig. 5. NeighborNet network diagram constructed from *28S* & *per* & *COI* concatenated data. C – *cuprina*, S – *sericata*, AUS – Australia, CAN – Canada, FRC – France, GER – Germany, JPN – Japan, NAM – Namibia, EGT – Egypt, SA – South Africa, THA – Thailand, USA – United States of America, ZIM – Zimbabwe, CT – Cape Town, BFN – Bloemfontein, BRT – Britstown, DBN – Durban, GHT – Grahamstown, NEL – Nelspruit, PTA – Pretoria, WLK – Welkom, WTB – Witbank.

have been reported. All of these infections resulted in mtDNA introgression without any detectable nuclear introgression. Further studies are recommended to determine if *Lucilia* blowflies are affected by *Wolbachia* infections as an explanation for the pattern seen in this study. However, such infections can die out over time, so that the only evidence of them may be cytoplasmic introgression (Zimmer, 2001).

The combined *28S* and *per* data show a very clear split between the *L. sericata* and *L. cuprina* samples (Fig. 3). The splits show very little internal incompatibility. The mtDNA (*COI*) shows a much higher degree of incompatibility between the splits (Fig. 4) which represents incompatible signals (Huson & Bryant, 2006). There are three important splits that group *L. sericata* together and two *L. cuprina* splits. This grouping is consistent with the Bayesian Inference tree (Fig. 2B). The concatenated total data set (*28S*, *per* and *COI*) (Fig. 5) shows a high level of incompatibility between the *L. cuprina* samples and a high degree of compatibility between the *L. sericata* samples. The *L. cuprina* samples show a number of splits and this incompatibility is probably as a result of the *L. sericata*-like mitochondrial DNA which results in the two clusters of *L. cuprina*.

Modern hybrids

The genetic component of an organism's morphology is determined by its nuclear DNA. One would expect recombination of the nuclear DNA if interbreeding occurs, resulting in morphology that is either intermediate (for multi-locus traits) or a mosaic of the two parental phenotypes (for single-locus traits). However, if one species' alleles are consistently dominant over the other, then despite recombination, the dominant phenotype will prevail (Lewin, 1997). Thus, although the putative modern hybrids had *sericata*-like mtDNA indicating hybridisation, they were still *L. cuprina*-like in morphology, suggesting that *L. cuprina*'s alleles for morphology are dominant over those of *L sericata*. In crossing experiments carried out in a laboratory, it was suggested that the femur colour of *L. cuprina* and the abdomen colour of *L. sericata* were dominant characteristics, giving the hybrids a combination of the two species' morphologies (Ullyett, 1945). However, this study used only two characters (femur and abdomen colour) which Ullyett (1945) described as not being "scientific criteria" because there are gradations in both characters depending on both the age and condition of the specimens and the observers' opinion and thus they could not be considered reliable criteria for identification.

Even when hybridization occurred in *Hyalomma* (Acari: Ixodidae), no intermediate morphologies were observed and the morphology of one parent appeared to be inherited over that of the other (Rees et al., 2003). Funk & Omland (2003) suggest that most hybrid species originate via asymmetrical hybridization and would be mitochondrially monophyletic. This might explain what we see in this study regarding the ancient hybridization "hybrid" group, but not the modern hybrids (which are derived from several sources). mtDNA may be more susceptible to introgression than nuclear loci (Machado & Hey, 2003). One is therefore less likely to have consistent gene trees for mtDNA and they may even suggest a different phylogeny. This gives support to the well-established idea that more than just one nuclear or

mitochondrial gene needs to be used when trying to determine species and gene trees (Funk & Omland, 2003; Machado & Hey, 2003; Hurst & Jiggins, 2005).

DNA-based identification

The use of *COI* sequences to correctly identify the two presumed subspecies of *L. cuprina* seems unlikely to succeed due to the presence of *L. cuprina* flies that group within the *L. sericata* clade (Fig. 2B). The phylogenetic positioning of these flies indicates their relationship relative to other specimens, but does not necessarily give an identification that agrees with their morphology. This problem is even more acute for modern hybrids. It also raises the issue of using *COI* as the universal "barcoding" gene and whether it is suitable, especially for insects (Rubinoff et al., 2006; Roe & Sperling, 2007; Whitworth et al., 2007; Jordaens et al., 2012; Sonet et al., 2012). The idea of using part of *COI* as a universal diagnostic gene is to allow the identification of unknown specimens when comparing them to identified species' sequences (Roe & Sperling, 2007). However, using *COI* alone could result in incorrect identifications, as seen in this study, as numerous insect species have undergone hybridisation and may carry mtDNA of another species (Zimmer, 2001; Baudry et al., 2003; Whitworth et al., 2007). The sequences of unidentified specimens may align with species with which they share mtDNA, but which are in fact a different species based on nuclear DNA or morphology. Although a study on blowflies in Australia suggested that using *COI* for identification is tenable, the authors also raised the issue of misidentifications when hybridisation was involved and suggested the use of a nuclear gene for confirmation (Nelson et al., 2007). A study of 1333 mitochondrial sequences (minimum of 300 bp) for 449 species of flies concluded that using *COI* alone for identification had a less than 70% success rate at identifying the species correctly (Meier et al., 2006).

The results show that in some cases both nuclear and mitochondrial genes are needed for reliable species identification and hybrid detection. It is well known that the use of just one gene can generally be taxonomically misleading as can be seen in the *L. sericata* / *L. cuprina* situation (Wallman et al., 2005; Harvey et al., 2008; Tourle et al., 2009; DeBry et al., 2010), especially if modern hybridisation is occurring at any appreciable rate. By using nuclear genes in conjunction with mitochondrial genes, a potentially misleading situation can be avoided (Rubinoff et al., 2006; Nelson et al., 2007; Roe & Sperling, 2007; Williams et al., 2008; Tantawi et al., 2010).

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