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The toad fly *Lucilia bufonivora*: its evolutionary status and molecular identification

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Abstract. The blow fly genus *Lucilia* is composed largely of saprophages and facultative myasis agents, including the economically important species *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae) and *Lucilia sericata* (Meigen). Only one species is generally recognized as an obligate agent of myiasis, *Lucilia bufonivora* Moniez, and this is an obligate parasite of toads. *Lucilia silvarum* (Meigen), a sister species, behaves mainly as a carrion breeder; however, it has also been reported as a facultative parasite of amphibians. Morphologically, these species are almost identical, and historically this has led to misidentification, taxonomic ambiguity and a paucity of studies of *L. bufonivora*. In this study, dipterous larvae were analysed from toad myiasis cases from the U.K., The Netherlands and Switzerland, together with adult specimens of fly species implicated in amphibian parasitism: *L. bufonivora*, *L. silvarum* and *Lucilia elongata* Shannon (from North America). Partial sequences of two genes, *cox1* and *ef1α*, were amplified. Seven additional blow fly species were analysed as outgroups. Bayesian inference trees of *cox1*, *ef1α* and a combined-gene dataset were constructed. All larvae isolated from toads were identified as *L. bufonivora* and no specimens of *L. silvarum* were implicated in amphibian myiasis. This study confirms *L. silvarum* and *L. bufonivora* as distinct sister species and provides unambiguous molecular identification of *L. bufonivora*.

Key words. *Bufo bufo*, Calliphoridae, cytochrome *c* oxidase subunit 1, elongation factor 1 alpha, myiasis, obligate parasitism.

Introduction

The cosmopolitan genus of calliphorid blow flies, *Lucilia*, is composed largely of saprophages and facultative agents of myiasis, the latter showing species-specific differences in their propensity to infest living hosts. Of most economic importance within the genus are *Lucilia cuprina* (Wiedemann) and *Lucilia sericata* (Meigen), which are primary agents of sheep myiasis in many areas of the world. Only one species is believed to be an obligate agent of myiasis, *Lucilia bufonivora* Moniez, which has a high host specificity for anurans. Eggs are laid on the living host and, after hatching, the first-stage larvae migrate to the nasal cavities, where larval development takes place (Fig. 1), usually resulting in the death of the amphibian host (Zumpt, 1965). *Lucilia bufonivora* has been reported as the cause of myiasis in a range of amphibian hosts; however, most reports relate to infestations of the common toad, *Bufo bufo* (Anura: Bufonidae) (Weddeling & Kordges, 2008; van Diepenbeek & Huijbregts, 2011; Martín *et al.*, 2012). This blow fly is widely distributed in Europe (Rognes, 1991; Verves & Khrokalo, 2010) and Asia (Fan *et al.*, 1997), and adult specimens of *L. bufonivora* have recently been reported in North America and Canada (Tantawi & Whitworth, 2014).

Lucilia silvarum (Meigen) is another widely distributed blow fly species in the Palaearctic (Schumann, 1986) and the Nearctic (Hall, 1965). It lives mainly as a carrion breeder in the

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Fig. 1. Common toad (*Bufo bufo*) with nasal myiasis due to *Lucilia bufonivora*, Bridgnorth, Shropshire, U.K.; posterior ends of live third instar larvae are visible within the enlarged wounds at the site of each nostril (photograph courtesy of Dr A. Breed, Animal and Plant Health Agency, Defra, U.K.). [Colour figure can be viewed at wileyonlinelibrary.com].

Palaearctic (Zumpt, 1956). However, there are several reports of *L. silvarum* being involved in amphibian myiasis in North America (Hall, 1948; Bolek & Coggins, 2002; Bolek & Janovy, 2004; Eaton *et al.*, 2008). Therefore, it is usually considered a facultative rather than an obligate parasite (Nuorteva, 1963). There is no reliable evidence of the involvement of this species in amphibian myiasis in Europe.

Although most cases of toad myiasis by *L. bufonivora* have been reported to occur in the nasal cavities of their host (van Diepenbeek & Huijbregts, 2011; Martín *et al.*, 2012), toad myiases due to *L. silvarum* have been reported to occur in the back, neck, legs and parotid glands of the host; there are no reports of *L. silvarum* developing in the nasal cavities (Bolek & Coggins, 2002; Bolek & Janovy, 2004). Despite this apparent behavioural difference, the adults of these two closely related blow fly species are almost identical on the basis of morphology, and reliable identification requires examination of the male genitalia or the female ovipositor. Morphological identification and differentiation of the larval stages is even more problematic, and Zumpt (1965) argued that in Europe most records of toad myiasis thought to have been caused by *L. silvarum* should probably be attributed to *L. bufonivora*.

Owing to their morphological similarity, the taxonomic status of *L. bufonivora* and *L. silvarum* has been debated over many decades; indeed, Townsend (1919) proposed a new genus, *Bufolucilia*, which included *L. bufonivora* as the type species, along with *L. silvarum*. Subsequently, Hall (1948) included *Lucilia elongata* Shannon in this genus, which has also been reported as a facultative amphibian parasite in North America (James & Maslin, 1947; Bolek & Janovy, 2004). More recently, the genus *Bufolucilia* was dismissed as a synonym of *Lucilia* by Rognes (1991), although it continues to be recognized as a genus or subgenus by a number authors (e.g. Kraus, 2007; Verves & Khrokalo, 2010; Draber-Mońko, 2013). However, although several studies provide strong support for the grouping of *L. bufonivora* and *L. silvarum* as closely related sister species (e.g. Stevens & Wall, 1996a; McDonagh & Stevens, 2011), recognition of genus *Bufolucilia* would leave other *Lucilia* species in a heterogeneous and paraphyletic group, as observed with some other proposed (but poorly supported) genera, e.g. *Phaenicia* (Stevens & Wall, 1996a).

Here, we utilize sequence data from the mitochondrial protein-coding gene cytochrome *c* oxidase subunit I (*cox1*) and the nuclear gene elongation factor 1 alpha (*ef1α*) to facilitate unambiguous identification of *L. bufonivora* larvae infesting live toads, and we identify the causal agent of obligate amphibian myiasis. Additionally, we confirm the hypothesis that *L. bufonivora* and *L. silvarum* are distinct sister species, and we discuss the evolutionary relationships between the closely related taxa associated with amphibian myiasis.

Materials and methods

Adult and larval specimens

Larval specimens putatively identified as *L. bufonivora* were sampled from 16 separate toad myiasis cases from six different locations in Britain (eight cases), four locations in The Netherlands (seven cases) and one site in Switzerland (one case) (Table 1, Fig. S1). Four adult specimens of *L. bufonivora* were also analysed: two from southern Germany and two collected with the aid of baited traps in The Netherlands (Table 2, Fig. S1). Five adult specimens of *L. silvarum* were analysed, including three from the U.K., one from the U.S.A. and one from The Netherlands. A specimen of *L. elongata* from Alberta, Canada, was also added to facilitate further exploration of the evolutionary relationships across the broader group of fly species reported as amphibian parasites.

For comparative purposes, adult specimens of seven other *Lucilia* species were also analysed (Table 2, Fig. S1). Specimens were collected in the U.K. and The Netherlands using liver-baited traps and identified using keys by van Emden (1954). Additionally, two new specimens of adult *Lucilia mexicana* Macquart from Chapingo, Mexico, were analysed (Table 2). Sequence data for specimens of *L. silvarum*, *L. sericata*, *L. cuprina* and *Lucilia illustris* (Meigen) and *Lucilia ampullacea* Villeneuve were obtained from EMBL/GenBank and also included in the analysis. Three adult samples of *Calliphora vicina* (Diptera: Calliphoridae) collected in the U.K. and Switzerland were included as outgroup taxa. All specimens were stored in 100% ethanol at 4 °C prior to analysis.

DNA extractions and polymerase chain reaction procedures

Thoracic muscle of adult specimens was used for DNA extraction to avoid contamination with ingested protein, eggs or parasites. To avoid potential contamination from larval gut contents, the anterior and posterior ends of larvae were used for DNA extraction from LII and LIII life stages, whereas whole specimens were used if samples were LI; live larvae were maintained on damp filter paper for 3–6 h prior to storage in ethanol to allow them to evacuate their gut contents. DNA extractions were carried out using a QIAGEN DNeasy® Blood

Table 1. Larval *Lucilia* specimens studied, including the location of collection, name of sample used for phylogenetic analysis and accession numbers for EMBL/GenBank DNA sequences for both *cox1* and *ef1α*.

Infestation ID	Larvae analysed	Country/region of origin	Code	*cox1*	*ef1α*
BB016-2	1	Haaksbergen, The Netherlands	L. bufo (NLD1)	FR719161	FR719238
BB016-3	1	Haaksbergen, The Netherlands	L. bufo (NLD2)	FR719161	FR719238
BB016-1	1	Zelhem, The Netherlands	L. bufo (NLD3)	FR719161	FR719238
BB016-4	1	Haaksbergen, The Netherlands	L. bufo (NLD4)	FR719161	FR719238
BBSP1	1	Haaksbergen, The Netherlands	L. bufo (NLD5)	FR719161	FR719238
Friesl-1	1	Friesland, The Netherlands	L. bufo (NLD6)	FR719161	FR719238
Rott-1	1	Rotterdam, The Netherlands	L. bufo (NLD7)	FR719161	FR719238
Oss-Ch-1	1	Ossingen, Switzerland	L. bufo (CHE)	FR719161	FR719238
WV15 6QR-1	1	Bridgnorth, Shropshire, U.K.	L. bufo (GBR1)	FR719161	FR719238
WV15 6QR-2	1	Bridgnorth, Shropshire, U.K.	L. bufo (GBR2)	FR719161	FR719238
XT767-16	1	Loughborough, UK	L. bufo (GBR3)	FR719161	FR719238
XT931-16	1	Bridgnorth, Shropshire, U.K.	L. bufo (GBR4)	FR719161	FR719238
Holk-1	2	Holkam, U.K.	L. bufo (GBR5), L. bufo (GBR6)	FR719161	FR719238
Shrew-446	2	Shrewsbury, U.K.	L. bufo (GBR7)	FR719161	+LT900481
			L. bufo (GBR8)	FR719161	FR719238
Nott-1	2	Nottingham, U.K.	L. bufo (GBR9), L. bufo (GBR10)	FR719161	FR719238
Suff-1	2	Suffolk, U.K.	L. bufo (Suff1), L. bufo (Suff2)∗	FR719161	FR719238

∗See McDonagh & Stevens (2011)
+ = new sequence.

and Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions.

DNA was extracted as total nucleic acid and subjected to polymerase chain reaction (PCR) to amplify the cytochrome oxidase I (*cox1*) region of the mitochondrial protein-coding gene and the EF1–EF4 region of the nuclear protein-coding gene elongation factor 1 alpha (*ef1α*). Universal insect primers previously published (Table 3) were used. The PCR protocol published by Folmer *et al.* (1994) was modified to amplify *cox1* and *ef1α* (EF1–EF4 region) with the following cycling conditions: 94 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C (*cox1*) or 48 °C (EF1–EF4) for 30 s, 72 °C for 1 min and a final step of 72 °C for 1 min. A negative control (no template DNA) was included in each set of PCR amplifications. PCR products were separated by gel electrophoresis, and bands were visualized by ethidium bromide staining. Targeted bands of *cox1* were cut out and purified using a QIAquick® Gel Extraction Kit (Qiagen GmbH, Germany). Successful EF1–EF4 products were purified using 0.5 μL of exonuclease I and 0.5 μL of Antarctic phosphatase per 20 μL of PCR product. A total of 658 bp of the *cox1* region were amplified in a single fragment with primers HCO2198 and LCO1490. A fragment of 638 bp of the *ef1α* region was amplified with primers EF1 and EF4. Purified PCR products were sequenced using commercial sequencing facilities: EUROFINS® (*ef1α*) and GENEWIZ® (*cox1*).

Sequence alignment

The quality of the sequences was checked and edited manually for both forward and reverse fragments; sequences were then assembled into a single consensus sequence using BIOEDIT software (Hall, 1999). Each consensus sequence was checked against previously published sequences in EMBL/GenBank using BLAST. Multiple sequence alignment was carried out using BIOEDIT implementing the CLUSTALW algorithm.

Phylogenetic analysis

The best-fitting nucleotide substitution model for each dataset was selected using JMODELTEST (Posada, 2008) (TreNef + I was selected for the EF1–EF4 dataset; TIM3 + I + G was selected for *cox1*). Prior to Bayesian inference analyses, the best-fitting model selected for each gene was implemented by changing the default settings (*nst*, *rates*, *ngammacat*, *statefreqpr*, *revmat*, *shapepr* and *pinvarpr*) in the software MRBAYES v3.2.6 (Huelsenbeck & Ronquist, 2001). Phylogenetic analysis was then carried out implementing a Markov chain Monte Carlo method starting from two independent analyses simultaneously, each with three heated chains and one cold chain; they were run for 10 000 generations, sampling every 10 generations. Analyses were stopped when the critical value for the topological convergence diagnostic fell below the default threshold (0.01). A fraction (0.25) of the sampled values was discarded (*burninfrac* = 0.25) when the convergence diagnostics were calculated. Substitution model parameters (*sump*) and branch lengths (*sumt*) were summarized; tree topology was then calculated with the remaining data by constructing a majority-rule consensus tree.

A combined-gene analysis was also carried out with a partitioned dataset; model parameters for each gene were implemented separately (unlinked), allowing each gene to evolve under different rates. An incongruence length difference (ILD) test was run in PAUP*4.0a152 to test phylogenetic congruence and to quantify the differences in topology between the single-gene trees. Analysis was conducted on a partitioned dataset with the combined dataset (*ef1α* and *cox1*).

Table 2. Adult *Lucilia* specimens studied, including the location of collection, name of sample used for phylogenetic reconstruction, and accession numbers for GenBank DNA sequences for both *cox1* and *ef1α*. [Correction added on 13 August 2018, after first online publication: table caption has been updated.]

Species	ID	Country/region of origin	Code	*cox1*	*ef1α*
Lucilia bufonivora	DM	Baden-Württemberg, Germany	L. bufo (DEU1)	FR719161	FR719238
L. bufonivora	DM	Baden-Württemberg, Germany	L. bufo (DEU2)	FR719161	FR719238
L. bufonivora	GAR	Olst, The Netherlands	L. bufo (Olst)	FR719161	FR719238
L. bufonivora	GAR	Winssen, The Netherlands	L. bufo (WN)	FR719161	FR719238
Lucilia elongata	AT	Canada	L. elongata (CAN)	KM858341*	+LT965032
Lucilia silvarum	GAR	Bristol, U.K.	L. silv (GBR1)	KJ394947	FR719260
L. silvarum	GAR	Bristol, U.K.	L. silv (GBR2)	KJ394947	FR719260
L. silvarum	GAR	Bristol, U.K.	L. silv (GBR4)	KJ394947	FR719260
L. silvarum	RLW	San Francisco, U.S.A.	L. silv (USA)	FR719259*	FR719259*
L. silvarum	RLW	Sacramento, U.S.A.	Lsilv SacrUSA-2	+LT963484	+LT965034
L. silvarum	GAR	Olst, The Netherlands	Lsilv (NLD-1)	+LT963483	FR719253
Lucilia richardsi	GAR	Bristol, U.K.	L. rich (1)	FR872384	FR719253
L. richardsi	GAR	Bristol, U.K.	L. rich (2)	KJ394940	FR719253
Lucilia sericata	GAR	Bristol, U.K.	L. sericata (UK)	AJ417714	+LT965035
L. sericata	JRS	Los Angeles, U.S.A.	L. sericata (USA)	AJ417715*	FR719257*
Lucilia cuprina	RLW	Perth, Australia	L. cuprina (AUS)	AJ417707*	FR719245*
L. cuprina	AH/ DMB	Dorie, South Island, New Zealand	L. cuprina (NZ)	AJ417706*	FR719244*
Lucilia caesar	GAR	Bristol, U.K.	L. cae (Bristol-1)	+LT900367	+LT900482
Lucilia illustris	RLW	Somerset, U.K.	L. illus	FR872384*	FR719253*
Lucilia ampullacea	GAR	Bristol, U.K.	L. amp (Bristol-2)	+LT963485	+LT965033
L. ampullacea	RLW	Somerset, U.K.	L. amp	FR719236*	EU925394*
Lucilia mexicana	FAV	Chapingo, Mexico	L. mex (MEX1)	+LT900368	+LT900483
L. mexicana	FAV	Chapingo, Mexico	L. mex (MEX2)	+LT900368	+LT900483
Calliphora vicina^	GAR	Switzerland (laboratory reared)	C. vic (CHE)	KJ635728#	FR719219
C. vicina	GAR	Bristol, U.K.	C. vic (1)	KJ635728	FR719219
C. vicina	GAR	Bristol, U.K.	C. vic (2)	KJ635728	FR719219

Adult specimen identification: GAR, Gerardo Arias-Robledo (Bristol, U.K.); JRS, Jamie Stevens (Exeter, U.K.); RLW, Richard Wall (Bristol, U.K.); FAV, Francisco Arias-Velazquez (Chapingo, Mexico); DM, Dietrich Mebs (Frankfurt, Germany); AH, Allen Heath (AgResearch, New Zealand); DMB, Dallas Bishop (AgResearch, New Zealand); AT, Angela Telfer (Guelph, Canada).
+ = new sequence; * = sequence data from EMBL/GenBank; ^ = unidentified specimens provided by G. Guex (Zurich) and identified at University of Exeter by GAR; # = identity based on 540 bp of sequence data.

Table 3. Amplification and internal sequencing primers used to amplify the two genes studied, including the source of published primers.

Gene	Primer	Sequence	Source
ef1α	EF1	ACAGCGACGGTTTGTCTCATGTC	McDonagh *et al.* (2009)
	EF4	CCTGGTTCAAGGGATGGAA	McDonagh *et al.* (2009)
cox1	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* (1994)
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* (1994)

Results

Molecular identification of Lucilia bufonivora

All 20 larval specimens from the 16 infestations studied (Table 1) gave nuclear and mitochondrial sequence data consistent with BLAST searches for *L. bufonivora*. Additionally, molecular data reaffirmed the identity of adult fly samples identified as *L. bufonivora* on the basis of morphology. All *L. bufonivora* samples were grouped together in a single unstructured clade in all phylogenies (Fig. 2, Fig. 3).

Single-gene phylogenies

In both single-gene phylogenies, all amphibian parasite taxa grouped together. In the *ef1α*-based phylogeny, amphibian parasite taxa formed a monophyletic clade (Fig. 2A); in the *cox1*-based phylogeny, *L. bufonivora* and *L. elongata* formed a monophyletic clade, and *L. silvarum* was paraphyletic and incorporated *Lucilia richardsi* Collin (Fig. 2B). Within the amphibian parasite group (in each single-gene phylogeny) all *L. bufonivora* specimens analysed were classified together in a well-supported monophyletic clade (Fig. 2A,B) with minimal intraspecific variation (only one English specimen, L. bufo (GBR7), showed minor variation). However, analysis of *ef1α*-sequence data did not show clear distinction of *L. elongata* (a North American species) from *L. silvarum* (Fig. 2A), although within this grouping both U.S.A. samples of *L. silvarum* (Sacramento and San Francisco) were placed together with strong support. In the *cox1* phylogeny (Fig. 2B) *L. silvarum* samples from the U.S.A. also grouped together with strong support, but were placed apart from European

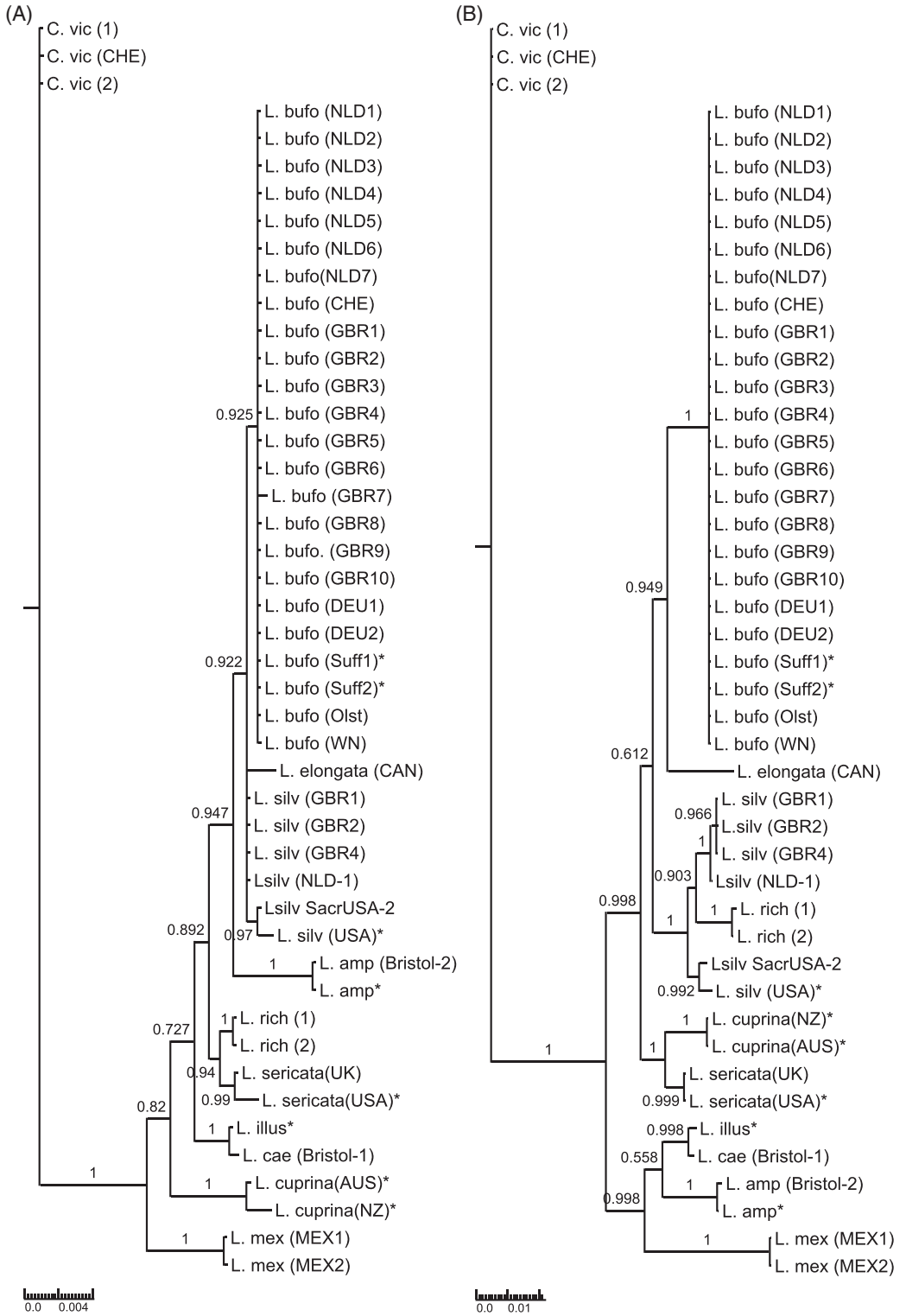

Fig. 2. Bayesian inference trees constructed from (A) the EF1–EF4 region of the nuclear gene *ef1α* and (B) the mitochondrial gene *cox1*. Posterior probability values are labelled on each node. AUS, Australia; CAN, Canada; CHE, Switzerland, DEU, Germany; GBR or UK, United Kingdom; NLD, The Netherlands; NZL, New Zealand; Suff, Suffolk (U.K.); US or USA, United States of America; WN, Winssen (The Netherlands); Olst, Olst (The Netherlands). * = sequence data from EMBL/GenBank. L. bufo = *Lucilia bufonivora*, L. silv and Lsilv = *Lucilia silvarum*, L. rich = *Lucilia richardsi*, L. illus = *Lucilia illustris*, L. cae = *Lucilia caesar*, L. amp = *Lucilia ampullacea*, L. mex = *Lucilia mexicana*, C. vic = *Calliphora vicina*.

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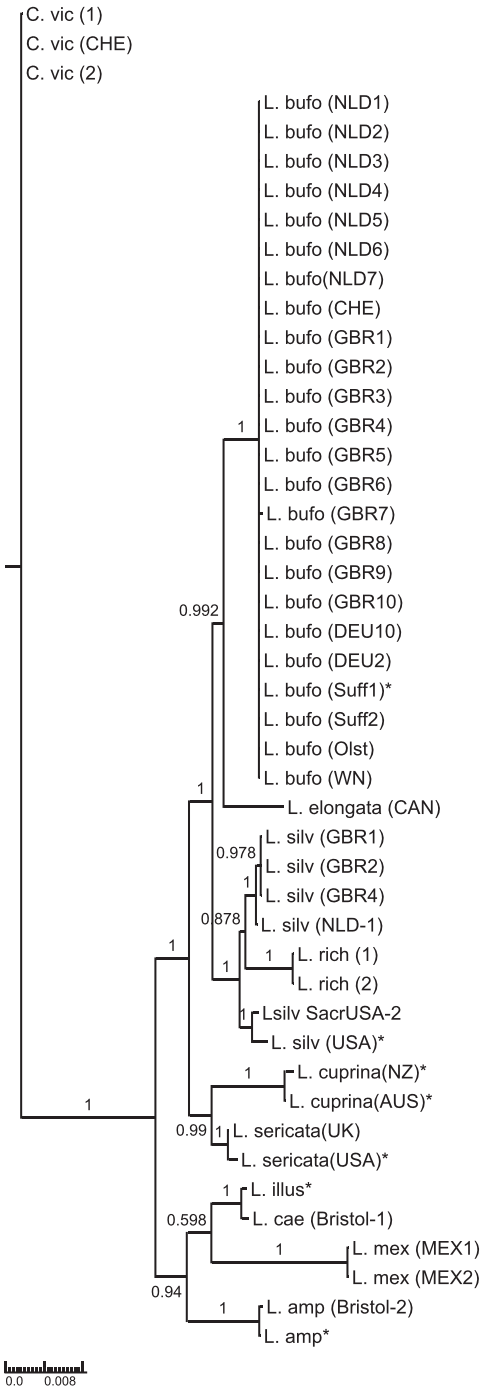

Fig. 3. Bayesian inference tree constructed from a partitioned dataset of the combined genes *ef1α* and *cox1*. Posterior probability values are labelled on each node. AUS, Australia; CAN, Canada; CHE, Switzerland; DEU, Germany; GBR or UK, United Kingdom; NLD, The Netherlands; NZL, New Zealand; Suff, Suffolk (U.K.); US or USA, United States of America; WN, Winssen (The Netherlands); Olst, Olst (The Netherlands). * = sequence data from EMBL/GenBank. L. bufo = *Lucilia bufonivora*, L. silv and Lsilv = *Lucilia silvarum*, L. rich = *Lucilia richardsi*, L. illus = *Lucilia illustris*, L. cae = *Lucilia caesar*, L. amp = *Lucilia ampullacea*, L. mex = *Lucilia mexicana*, C. vic = *Calliphora vicina*.

L. silvarum, suggesting relatively high intraspecific variation in *L. silvarum*.

The placement of other *Lucilia* spp. relative to the amphibian parasite taxa was essentially as described previously (McDonagh & Stevens, 2011). All sequences of *C. vicina* analysed grouped together in the same outgroup clade.

Combined-gene phylogeny

The ILD test detected incongruence between the two genes used in this study ($P = 0.01$); nonetheless, Bayesian inference analysis of a combined partitioned dataset produced a phylogeny with generally strong posterior probabilities (Fig. 3). All *L. bufonivora* samples were grouped in a single clade as a sister species to *L. elongata*. As observed in the *cox1* tree, a monophyletic European *L. silvarum* group (GBR + NDL) was recovered, with *L. richardsi* grouped as its sister taxon (Fig. 3); again, both U.S.A. specimens of *L. silvarum* were placed outside of this group as sister taxa with high support values. Both sheep blow fly species, *L. sericata* and *L. cuprina*, were recovered as a monophyletic group with strong support. The closely related species *L. illustris* and *Lucilia caesar* (Linnaeus) were recovered as sister species; however, this combined-gene analysis placed *L. mexicana* more closely related to the *L. caesar* group than the *L. ampullacea* clade. Subfamily relationships of Luciliinae were recovered with strong posterior probability (p. p. = 1.0), grouping all *C. vicina* samples as an outgroup and differentiating subfamily Calliphorinae from Luciliinae with strong support (Fig. 3).

Discussion

Using mitochondrial data (*cox1*) McDonagh & Stevens (2011) differentiated *L. bufonivora* from *L. silvarum* and placed them as separate sister species. However, in the same study, both species were placed in the same clade using *ef1α* and *28S rRNA* as phylogenetic markers, the latter failing to classify them as distinct species. In this study, the EF1–EF4 region of the protein-coding nuclear gene *ef1α* showed just a single nucleotide difference between the sequence data of *L. silvarum* and *L. bufonivora*; however, Bayesian inference analysis showed clear groupings, identifying them as distinct sister species. Addition of data from the North American amphibian parasite *L. elongata*, another putatively closely related taxon, allowed an even clearer understanding of the evolutionary relationships between *L. silvarum* and *L. bufonivora*, resulting in the differentiation of them as distinct sister species. The *ef1α* tree supported the suggestion that *L. bufonivora* has diverged relatively recently from its sister taxon *L. silvarum* (Stevens & Wall, 1996a). The *cox1*-based phylogeny showed clear relationships and distinction between *L. bufonivora* and *L. silvarum*, a finding reiterated in the combined-gene tree. It is probable that in the combined-gene tree a stronger signal in the mtDNA data (*cox1*) is driving this clear distinction and is dominating the weaker phylogenetic signal of the nuclear data (*ef1α*). The low signal present in the *ef1α* sequence data accords with the relatively slow rate of evolution reported previously in this nuclear gene

(McDonagh & Stevens, 2011) compared with that reported in the majority of insect mitochondrial genes (McDonagh *et al.*, 2016). Indeed, *cox1* has been widely used in blow fly systematics (Otranto & Stevens, 2002; Stevens *et al.*, 2002; Wells *et al.*, 2002), and owing to generally higher rates of sequence change in mtDNA it is expected to reach reciprocal monophyly before nuclear genes (Funk & Omland, 2003; Dowton, 2004; Lin & Danforth, 2004). As such, mitochondrial sequence data (e.g. *cox1*) are useful for inferring the relationships of recently diverged species (Stevens & Wall, 1997; Shao *et al.*, 2001), and our results reaffirm this, suggesting that *L. bufonivora* is clearly a separate but closely related species to *L. silvarum*. Taken together, such findings call into question the utility of apparently slowly evolving genes such as *ef1α* for evolutionary analysis of relatively recently diverged Diptera. As such, future studies of this group may be advised to consider alternative nuclear genetic markers evolving at a rate better suited to the question(s) being asked. For example, Williams & Villet (2013) showed the *period* gene and a nuclear rRNA locus to be well suited to elucidating the extent of hybridization between two closely related *Lucilia* species (*L. cuprina* and *L. sericata*); moreover, their use of two nuclear loci overcame some of the problems of species determination and accurate phylogenetic reconstruction associated with ancient mitochondrial introgression and potentially recent hybridization events that have unquestionably disrupted mtDNA-based blow fly phylogenies (Stevens & Wall, 1996b; Stevens *et al.*, 2002). In short, blow fly phylogenetic analyses do need to employ nuclear markers, but it is apparent that *ef1α* may not be the ideal locus for elucidating relationships between closely related blow fly taxa.

Molecular analysis of different populations of *L. bufonivora* from across Europe detected no intraspecific differences in mitochondrial sequence data, and the nuclear gene *ef1α* also exhibited only minimal intraspecific sequence variation (Fig. 2A). However, in *L. silvarum*, marked intraspecific variation in both nuclear and mitochondrial sequence data was observed between European and North American populations of this fly; recent phylogenetic analysis of populations of this species from the U.S.A. and Germany also showed a high degree of intraspecific difference (Williams *et al.*, 2016). In the current study, intraspecific variation was also observed between European samples, with U.K. *L. silvarum* differing from a Dutch specimen of the same species. By contrast, a lack of significant variation in both nuclear and mitochondrial genes in the different European populations of *L. bufonivora* analysed suggests that it may be a recently diverged species that has accumulated less molecular variation. Further studies would be of value, particularly to explore the differences between European and North American populations of *L. bufonivora* (e.g. Tantawi & Whitworth, 2014).

Even when both species have been reported as amphibian parasites (Baumgartner, 1988), *L. bufonivora* has never been observed breeding in carrion. By contrast, its sister species *L. silvarum* is reported mainly as a common carrion-breeding species in Europe (Rognes, 1991), with no confirmed records of parasitism in amphibians due to it in this region (van Diepenbeek & Huijbregts, 2011; Fremdt *et al.*, 2012). In North America, however, there have been several reports of amphibian myiasis cases apparently involving *L. silvarum* (Bolek & Coggins, 2002; Bolek & Janovy, 2004; Eaton *et al.*, 2008). The phylogeny constructed from the combined dataset characterized *L. silvarum* from the U.S.A. as more closely related to *L. bufonivora* than to *L. silvarum* from Europe. This finding is congruent with the reported amphibian parasitic behaviour of North American *L. silvarum*, and reiterates the significance of the relatively high intraspecific variation present between European and North American populations of *L. silvarum*, which in turn reflects the fact that very different larval feeding strategies can be exhibited even between closely related blow fly taxa (Stevens, 2003; Stevens & Wallman, 2006).

Using the nuclear marker *ef1α*, amphibian parasitism in *Lucilia* appears as a monophyletic trait with the inclusion of *L. bufonivora*, *L. silvarum* and *L. elongata*. However, in the combined-gene and *cox1* trees this group becomes paraphyletic due to the inclusion of the European species *L. richardsi*. It is important to mention that the biology of *L. elongata* has been poorly studied, and this species has never been reported as a carrion breeder (James & Maslin, 1947; Bolek & Janovy, 2004), possibly behaving only as an obligate parasite of anurans in North America. Thus, *L. elongata* and *L. bufonivora* may be the only two species that exhibit this obligate parasitism behaviour among the genus *Lucilia*. Interestingly, they are placed together as monophyletic sister taxa in both the *cox1* and combined-gene trees.

Lucilia bufonivora is considered a rare species in England; there are few reports of confirmed toad myiasis cases where it is involved (McDonagh & Stevens, 2011), and adult flies of this species are rarely caught using carrion-baited traps (Arias-Robledo, unpublished data). This may illustrate the highly specific nature of the cues emanating from a living amphibian host that are required to attract *L. bufonivora*, or simply may reflect its restricted distribution and low abundance in the field. In this study, the molecular identification of larval samples extracted from toad myiasis cases as *L. bufonivora* reaffirmed the presence of this obligate parasite in Britain (Fig. 3). A study in Germany suggests that this species is highly variable in its local abundance (Weddeling & Kordges, 2008).

Based on mitochondrial sequence data, European specimens of *L. silvarum* were more closely related to *L. richardsi* than to *L. bufonivora*. However, the *ef1a*-based phylogeny placed *L. richardsi* as a sister species of *L. sericata* outside of the amphibian parasite group of flies, as observed in previous phylogenetic analyses (McDonagh & Stevens, 2011). Although *L. sericata* and *L. silvarum* have been reported as facultative parasites of sheep and amphibians respectively (Macleod, 1937; Hall, 1948), there are no records of *L. richardsi* being involved in either sheep or toad myiasis. However, Nuorteva (1959) reported that three males of *L. richardsi* were reared from a single case of wound myiasis in a bird (a nightjar). The high similarity of *L. richardsi* with *L. sericata* based on nuclear DNA and with *L. silvarum* based on mtDNA might be attributed to introgressive hybridization; however, more detailed studies are required to confirm this. The occurrence of hybridization has important implications for speciation, and this phenomenon has been reported several times as occurring within the genus *Lucilia*, as is the case of the hybridization between the closely related species *L. sericata* and *L. cuprina* (Stevens & Wall, 1996b; Williams & Villet, 2013). Similarly, *L. illustris* and *L. caesar*

present very low genetic distances, and they could not be reliably identified using mitochondrial markers, which might result from hybridization or incomplete lineage sorting (Sonet *et al.*, 2012).

In conclusion, it has been suggested that the myiasis habit may have arisen in multiple independent evolutionary events within the subfamily Luciliinae (Stevens, 2003). The results presented here support this and suggest that the *obligate* parasitic habit in the genus *Lucilia* possibly diverged from *L. silvarum*. Further studies that include more specimens of *L. elongata* from different geographical regions are required to explore its molecular identity and to resolve its evolutionary relationships within the broader amphibian parasite group of blow fly species.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Distribution map of Lucilia bufonivora samples analysed (larvae and adults).

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