DNA barcoding of forensically important flies in the Western Cape, South Africa

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DNA barcoding of forensically important flies in the Western Cape, South Africa

Authors:

Tenielle Cooke, Kyle Kulenkampff, Marise Heyns and Laura Jane Heathfield

Affiliation for all authors:

Division of Forensic Medicine and Toxicology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Anzio Road, Observatory, Cape Town, South Africa.

Corresponding Author:

Laura Jane Heathfield

Physical address: Division of Forensic Medicine and Toxicology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Anzio Road, Observatory, South Africa

Postal address: Division of Forensic Medicine and Toxicology, P.O. Box 13914, Mowbray, 7705, South Africa

E-mail: laura.heathfield@uct.ac.za

Telephone: +27 21 406 6569

Email addresses of other authors:

Tenielle Cooke: teniellecooke91@gmail.com

Kyle Kulenkampff: KLNKYL004@myuct.ac.za

Marise Heyns: marise.heyns@uct.ac.za
Physical address: Division of Forensic Medicine and Toxicology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Anzio Road, Observatory, South Africa

Postal address: Division of Forensic Medicine and Toxicology, P.O. Box 13914, Mowbray, 7705, South Africa
Abstract

Forensic entomology aids the determination of post mortem interval based on arthropods associated with a deceased body. This relies on the accurate identification of insects that visit the body, particularly first colonisers such as Calliphoridae (Diptera). Traditional species identification though morphological keys can be challenging as immature or closely related specimens can look similar. Some of these challenges have been overcome through ‘DNA barcoding’, which involves the sequencing of informative regions within a species’ DNA and comparison to a database of reference sequences. However, reference DNA sequences of blow fly species in South Africa is currently limited. In this study, adult blow flies representing four species common to the Western Cape, South Africa (*Chrysomya chloropyga*, *Chrysomya albiceps*, *Chrysomya marginalis*, *Lucilia sericata*) were examined using morphological keys and DNA barcoding of two regions: COI and ITS2. These DNA sequences were then used as references for the successful identification of seven unknown immature specimens. Intraspecific divergence showed a maximum of 0.36% and 2.25% for COI and ITS2, respectively; interspecific divergence showed a minimum of 6.14% and 64.6% for COI and ITS2, respectively. According to these results, COI and ITS2 have sufficient discriminatory power for species level identification for the four species studied.

Keywords: post mortem interval; species identification; DNA barcoding; blow fly; medico-legal investigation
1. Introduction

The use of insect evidence to estimate the post mortem interval (PMI) is valuable in forensic investigations (Catts and Goff 1992, Amendt et al. 2004). The PMI is calculated based on succession patterns of insects and the life cycle stage of blow flies collected at the scene (Byrd and Castner 2000, Amendt et al. 2007). Insects within the taxon family Calliphoridae (blow flies) are first colonisers of the deceased and consequently offer the most information regarding PMI. Calliphoridae comprise over one thousand species, each with differing life cycle patterns. Since the life cycle is central to PMI determination, correct species identification is critical (Catts and Goff 1992). Traditionally, species identification involves the use of a morphological key that assigned species based on distinguishing characteristics (Amendt et al. 2011). This method requires personnel with specialised taxonomic knowledge especially in closely related or recently divergent species, where differences between species are subtle (Wallman 2001).

A lack of distinguishable external features in blow flies during immature stages (especially first and second instar stages), results in incomplete and sometime non-existent morphological keys for these initial developmental stages thereby making species-level identification fairly difficult (Byrd and Castner 2000). Therefore, larvae need to be reared to adulthood for definite identification, although, these efforts are time consuming (Amendt et al. 2000) and may fail (Amendt et al. 2004). In addition, specimens that are damaged, acquired using incorrect storage or handling methods, may not be identifiable due to the destruction of distinctive characteristics (Rolo 2010, GilArriortua et al. 2014).
For the past decade, attempts to circumvent the shortfalls of morphological identification have focused on using molecular techniques which take advantage of diversity in different species’ DNA (Hebert et al. 2003a). More recently, DNA barcoding has become a common means of species identification for forensically important insects whereby specifically chosen target regions (barcodes) in the DNA are amplified and sequenced, followed by comparison to reference sequences of known species in a database (Nelson et al. 2007, Meiklejohn et al. 2013, Bhaskaran and Sebastian 2015).

Mitochondrial DNA has been the preference for molecular analysis due to its comparably higher mutation rate than nuclear DNA (Saccone et al. 1999). Focus has been centred on a 658 bp region of a mitochondrial gene that encodes the cytochrome oxidase subunit I (COI). Hebert et al. (2003a), suggested this region as a universal genetic marker to classify and identify all of animal life. DNA barcoding projects have since escalated and COI is currently the standard marker for animal identification (Wells and Stevens 2008; Hebert et al. 2003b). Several studies however, have found limitations in the discriminating power of the COI barcode in closely related species of blow fly, and another region, the second internal transcribed spacer (ITS2), has been assessed as an alternative or supplementary barcode (Song et al. 2008, Nelson et al. 2008).

Since the initiation of DNA barcoding, a substantial amount of barcode sequences have accumulated in databases such as Barcoding of Life Data System (BOLD) and GenBank®. While some geographical regions are well represented, data pertaining to species common in the
Western Cape, South Africa, is limited. The paucity of reference data unfortunately restricts the use of DNA barcoding in the local forensic setting. The presence of geographical differences within and between species requires reference data that is both comprehensive and representative of species from various regions (Wells and Stevens 2008). This pilot study aimed to generate reference data for two DNA barcodes (COI and ITS2) for four blow fly species common to the Western Cape area of South Africa that are of forensic significance, and subsequently assess its intra- and inter-specificity to identify immature specimens which were not morphologically distinguishable.

2. Materials and Methods

2.1 Specimens

Ten adult specimens from *Lucilia sericata* (voucher number (vn) #16.04.01.004), *Chrysomya albiceps* (vn#16.04.01.005), *Chrysomya marginalis* (vn #16.04.01.009) and *Chrysomya chloropyga* (vn #16.04.01.027) were collected at the Medical Research Council Primate Unit premises in Delft, Cape Town from a Cape Flats Dune Strand-veld (CFDS) biome with GPS coordinates 33° 59' 27" S, 18° 39' 04"E. Collection was done on 16 April 2015 (autumn season) at approximately midday. The specimens were placed directly into 70% ethanol in accordance with previously published methods (Harvey et al. 2003). Each specimen was identified using a stereomicroscope (Carl Zeiss, Oberkochen, Germany) and morphological keys adapted from Zumpt (Zumpt n.d.). Images of all flies were captured, and the sample specimens were submitted to the Diptera collection of University of Cape Town, with the voucher numbers as indicated. Unrelated larvae of unknown species to the researcher, but known to an
independent researcher, were sampled at the third instar stage. This was carried out to test the
reliability of barcoding on immature specimens.

2.2 DNA extraction, amplification and sequencing

Genomic DNA was extracted from whole flies and immature specimens using the ZR Insect
extraction kit (Zymo Research, CA, USA) according to the manufacturer’s instructions. The COI
barcode was first amplified using Folmer primers LCO1490 and HCO2198 (Folmer et al. 1994).
Upon analysis, two species (Ch. chloropyga and Ch. albiceps) failed to amplify; thus an
additional set of primers was used, LepF1 and LepR1 for these two species (Hebert et al. 2004).
The ITS2 region was amplified using previously published primers ITS2_F and ITS2_R (Song et al.
2008). Each 25µL reaction contained 0.3µM of each primer, 1X HiFi Hotstart Ready Mix (KAPA
Biosystems, South Africa) and all reactions contained a minimum of 10ng of template DNA. PCR
was carried out on the T100 thermal cycler (BioRad, Berkeley, CA, USA) at the following cycling
conditions: initial denaturation, 95°C for three minutes, followed by 25 cycles of denaturation
at 98°C for 20 seconds, annealing at 55°C for 15 seconds, elongation at 72°C for 30 seconds.
Final extension was carried out at 72°C for five minutes. For samples which were low in yield
and where 10ng of template DNA was not available, a secondary PCR was performed. This
entailed the use of the PCR product from the first PCR as the template for a second PCR,
keeping the other setup and cycling conditions as described above.

PCR amplicons were then purified with Exonuclease 1 (New England Biolabs, Ipswich, MS, USA)
and recombinant shrimp alkaline phosphatase (rSAP) (New England Biolabs, Ipswich, MS, USA).
Sanger sequencing was performed in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA,) according to manufacturer’s instructions. Labelled products were cleaned using ZR-96 DNA Sequencing Clean-up Kit (Zymo Research, California) and separated using the ABI 3500xl analyser (Applied Biosystems, CA, USA).

2.3 Analysis

Forward and reverse sequences were analysed using GeneMapper v4 and visualised and using ChromasLite (Technelysium 2007) and BioEdit 7.2.5 (Hall 1999). Multiple sequence alignments were performed for each marker using MUSCLE as given in MEGA version 6 (Tamura et al. 2013). Phylogenetic analyses were carried out in MEGA 6 using Maximum Likelihood (Kimura 1980) based on the Kimura 2-parameter model, and to assess the reliability of phylogenetic tree construction bootstrapping was performed with 1000 replicates. *Stomoxys calcitrans* (Diptera, Muscidae) was used as an out group.

Intraspecific and interspecific differences as well as pairwise distances were calculated using Arlequin version 3.5. Consensus sequences were compiled from adult flies using Seaview, and these were used as reference sequences for species identification of immatures. The Basic Local Alignment Search Tool (BLAST) was used for alignment of sequences from immature specimens to the reference data. BOLD (Ratnasingham and Hebert 2007) was used a secondary identification for COI sequences.
3. Results

Reference sequences from both DNA barcode regions were successfully generated from all specimens in this study. These sequences were submitted to BOLD (process ID: BFRSA001-18 to BFRSA040-18) as well as to GenBank® (accession numbers: MH716055-MH716094 and MH765355-MH765394).

The molecular phylogeny of *Ch. chloropyga*, *Ch. albiceps*, *Ch. marginalis* and *L. sericata* was inferred by using the Maximum Likelihood method based on COI (Figure 1) and ITS2 (Figure 2). For each species node, phylogenetic support was high, with *Ch. chloropyga* having the lowest nodal support (85%). All the nodal support values were above the threshold value of 75% for maximum likelihood.

*Intra and interspecific analysis of four species studied*

Intraspecific analysis was performed for each species based on COI and ITS2 sequences. ITS2 in *Ch. chloropyga* appeared to be the most variable, with ten different haplotypes and a total of 19 polymorphic sites. For *Ch. marginalis*, ITS2 displayed homogeneity across all ten specimens (Table 1).

To demonstrate intra- and interspecific divergence, distance matrices (Table 2) shows nucleotide divergence values within and between species for COI. Intraspecific diversity ranged from 0% to 0.313% across all four species. Interspecific divergence for species used in this study varied from 6.1% to 10.7%. The smallest divergence values corresponded to the species
Ch. chloropyga and Ch. marginalis. The highest divergence value was seen between L. sericata and Ch. marginalis.

Table 2 also shows nucleotide divergence values within and between species for ITS2. The lowest intraspecific variation was observed in Ch. marginalis which showed no variation. Maximum percentage variation was 0.873% and 0.181% for Ch. albiceps, L. sericata respectively. Ch. chloropyga showed the highest intraspecific diversity (2.251%). Interspecific divergence of ITS2 for species used in this study varied from 53.3% - 64.6%. The smallest divergence values corresponded to the species Ch. albiceps and Ch. marginalis. The highest divergence value was seen between L. sericata and Ch. marginalis.

Identification of immature specimen

Adult specimens were used as references for identification of immature specimens. Table 3 reports the similarity scores reported after alignment. The majority of sequences matched with 100% similarity. Furthermore, pairwise difference between unknown and reference sequences were computed and are shown in Table 3. COI sequences from immature specimen were also submitted to BOLD to assess its ability to identify sequences. Table 3 shows the BOLD identification and the top result and the reported percentage similarity.

4. Discussion

The determination of the post mortem interval (PMI) is often an integral part of forensic investigations. After 72 hours, conventional methods of PMI determination become less
reliable and forensic entomology assumes a more prominent role. Due to the limitations of morphological identification, molecular methods such as DNA barcoding have become increasingly popular for the forensic identification of insects. This study generated DNA barcode reference data for four common species in the Western Cape, South Africa, and then assessed whether these barcodes could provide enough discriminatory value to identify immature species. This is important because if the discriminatory value of the investigated region and generated barcodes are not specific enough to generate monophyletic groups for each of the observed species then it will fail as a means of identification. This includes instances where the taxonomy or lineage sorting of the observed species is incomplete or if the traditional identification was done incorrectly (Meyer and Paulay 2005).

The phylogenetic analysis of both **COI** (Figure 1) and **ITS2** (Figure 2) by means of Maximum likelihood trees show each species as a monophyletic group indicating that both barcodes share the ability to distinguish the four species in this study. High support values at species nodes of both trees, demonstrate the suitability of **COI** and **ITS2** for species determination. Nodal support for **COI** (>99%) was higher than that of **ITS2** (>85%). The lower nodal support values for **ITS2** may be due to the higher amounts of intraspecific variation. This suggests that **ITS2** may have better discrimination power than **COI** for the studied species. This is in line with other studies that have found success in using **ITS2** (Song et al. 2008, Nelson et al. 2008). However, it also shows that additional markers should be used in conjunction with **ITS2** for proper species identification.
Threshold values within the context of percentage diversity between sequences constitute another criterion for DNA barcoding. These are based on the observation that nucleotide divergence between insect species, in most cases, exceed 3% or that there is a 10x or greater difference in nucleotide distance among species than within species (Hebert et al. 2003a). Referred to as the barcoding gap, this was calculated using mean interspecific distances. However, Meier et al. (2008) showed that this exaggerated the barcoding gap and proposed the use of the smallest interspecific distance value, instead of using the mean interspecific distances. In this study, COI showed that the lowest interspecific divergence returned for the species analyses was higher than 6%, as well as, meeting the 10x criterion. This demonstrates the discriminatory power of COI. However, according to Hebert et al. (Hebert et al. 2003a), a criterion for the selection of a universal barcode is ease of amplification. In this study, the ITS2 sequence was easier to amplify than COI due to the failure of universal primers to amplify COI in two of four species. A similar study performed on mites, found amplification of COI problematic and suggested the utilisation of ITS2 over COI for convenience (Yang et al. 2011). Though the use of universal primers was a key aim for DNA barcoding, the use of primers that only amplify certain species may also add another layer of discrimination.

The ITS2 marker showed a large amount of interspecific diversity (>50%) between the four species analysed in this study, exceeding 3% and meeting the 10x criterion, indicating that ITS2 may have greater discriminatory power than COI. This is corroborated by a study done by GilArriortua et al. (2014) which noted that ITS2 had greater discriminatory power that of the traditional COI-5P and a another 3P COI marker combined. This may make ITS2 valuable for
delimiting closely related species as a result of having more informative sites. One exception
was *Ch. marginalis* which displayed a very low degree of intraspecific variability in ITS2, while
demonstrating highest genetic variation for COI compared to other species. In Table 1, ITS2
presents with what appears to be complete homogeneity for *Ch. marginalis* which is indicative
of minimum variability in this region.

Based on the low intraspecific variation and moderate interspecific variation for most species
studied, COI appears to be more suited to general species identification than ITS2, which was
also seen by Yao *et al.*, (2010) who suggested that ITS2 be used as complementary to COI for
animal identifications (Yao et al. 2010). However, in the scope of this study, both markers were
capable of delineating the aforementioned species.

Sequences from adult specimens were used as reference material for immature specimens.
Table 3 shows the similarity scores, for each marker. Species were assigned to immature
specimens based on high (>98%) sequence similarity. All specimens were correctly identified.
Sample 6 was identified as *Ch. albiceps* although initial morphological identification reported it
to be *Ch. rufifacies*, sister species of *Ch. albiceps*. Genbank® and BOLD were used to confirm the
identity of this sample, which both reported it to be *Ch. albiceps*. Due to the likeness of these
and other sister species, errors like this can be introduced. Since the development stages within
the life cycle of blow flies differ, PMI estimations inferred by forensic entomology will be
incorrect if based on incorrect data.
Immature specimens were also identified using BOLD, which displays the 99 best matches and details species-level identification for sequences that show less than 1% sequence divergence (Ratnasingham and Hebert 2007). Most of the larval specimens matched with 100% percent similarity to their own species from other parts of South Africa as well as other countries. This indicated that COI, in the studied species, did not have the discriminatory capacity to distinguish within species from different haplogroups, beyond the species level. The database was able to confirm identity to species level for Ch. chloropyga, Ch. albiceps and Ch. marginalis. Interestingly, L. sericata could only be identified to genus level and was reported as being 100% to both L. sericata and L. cuprina as well as having a 99.5% similarity score to the hybrid species L. cuprina x sericata. These two species have been reported as being almost morphologically indistinguishable (Holloway 1991). However, this may be due to incorrect taxonomy of the reference sequences.

According to Wells et al. (Wells et al. 2007), COI alone is insufficient to distinguish between these two species due to some cases showing L. cuprina haplotypes being more similar to those of L. sericata than other L. cuprina. This means that within phylogenetic trees, these two species would not exhibit a pattern of monophyly. However, when using nuclear DNA data, such as ITS2, monophyly of the species can be seen (Tourle et al. 2009), demonstrating that the incorporation of a secondary marker would be useful for closely related species. More recently Williams and Villet (2014) developed a key to distinguish L. sericata and L. cuprina as well as their naturally occurring hybrids. In this circumstance, morphological data appeared to be
superior to molecular analysis; therefore combining both disciplines could strengthen the final
collections as suggested by Chan et al. (2014).

Conclusion
The ability of the COI and ITS2 regions was assessed as a marker for species identification for
blow flies common to the Western Cape of South Africa. The results showed that COI and ITS2
had sufficient discriminatory value to allow for species level identification for the studied
species and provided suitable reference data for the successful identification of immature
specimens. ITS2 had higher levels of interspecific diversity than COI and thus supplements COI
data especially in closely related species. Additionally, ambiguous results reported on COI
sequences by databases can be refined by the analysis of supplementary regions. If DNA
barcoding is incorporated into forensic practice in the Western Cape, South Africa, we
recommend that it includes both DNA barcode regions, and combines the molecular results
with the traditional morphological analyses. Future work should focus on the inclusion of
additional species and increased sample size to provide a more holistic overview of these
methods, as well increase statistical power of these results. Overall, this pilot study has
contributed towards the lack of reference DNA barcode data in the Western Cape, South Africa,
and demonstrates the suitability of both COI and ITS2 for species level identification based on
DNA barcoding methods.

Acknowledgements
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Table 1: The intraspecific analyses performed on each species for COI and ITS2 sequences. Table displays the species, number of specimen per species (N), molecular marker, the number of haplotypes observed, the nucleotide diversity, the mean number of pairwise differences and the number of polymorphic sites.

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Molecular marker</th>
<th>Number of haplotypes observed</th>
<th>Nucleotide diversity</th>
<th>Mean number of pairwise differences</th>
<th>Number of polymorphic sites</th>
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<tr>
<td>Ch. chloropyga</td>
<td>10</td>
<td>COI barcode</td>
<td>4</td>
<td>0.001±0.001</td>
<td>0.800±0.628</td>
<td>4</td>
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<td></td>
<td></td>
<td>ITS2</td>
<td>10</td>
<td>0.022±0.013</td>
<td>6.711±3.485</td>
<td>19</td>
</tr>
<tr>
<td>Ch. albiceps</td>
<td>10</td>
<td>COI barcode</td>
<td>1</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td>ITS2</td>
<td>6</td>
<td>0.008±0.005</td>
<td>2.622±1.528</td>
<td>5</td>
</tr>
<tr>
<td>Ch. marginalis</td>
<td>10</td>
<td>COI barcode</td>
<td>4</td>
<td>0.003±0.002</td>
<td>2.222±1.336</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITS2</td>
<td>1</td>
<td>0.000±0.000</td>
<td>0.000±0.000</td>
<td>0</td>
</tr>
<tr>
<td>L. sericata</td>
<td>10</td>
<td>COI barcode</td>
<td>2</td>
<td>0.000±0.000</td>
<td>0.200±0.263</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITS2</td>
<td>4</td>
<td>0.001±0.001</td>
<td>0.600±0.519</td>
<td>3</td>
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</table>
Table 2: Percentage of divergence values between *L. sericata*, *Ch. albiceps*, *Ch. marginalis* and *Ch. chloropyga* at the *COI* (below the diagonal) and *ITS2* (above the diagonal) regions. Bolded values indicate intraspecific distances for COI/ITS2

<table>
<thead>
<tr>
<th></th>
<th><em>Ch. chloropyga</em></th>
<th><em>Ch. albiceps</em></th>
<th><em>Ch. marginalis</em></th>
<th><em>L. sericata</em></th>
</tr>
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<tr>
<td><em>Ch. chloropyga</em></td>
<td>0.131/2.251</td>
<td>54.611</td>
<td>59.765</td>
<td>63.093</td>
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<tr>
<td><em>Ch. albiceps</em></td>
<td>6.802</td>
<td>0.000/0.827</td>
<td>53.259</td>
<td>63.093</td>
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<tr>
<td><em>Ch. marginalis</em></td>
<td>6.135</td>
<td>6.260</td>
<td>0.363/0.000</td>
<td>64.628</td>
</tr>
<tr>
<td><em>L. sericata</em></td>
<td>10.757</td>
<td>10.423</td>
<td>9.577</td>
<td>0.032/0.181</td>
</tr>
</tbody>
</table>
Table 3: Identification of immature specimen, percentage of sequence similarity to specimens used in this study. Also indicated are identifications reported by BOLD and corresponding similarity

<table>
<thead>
<tr>
<th>Unknown specimen</th>
<th>Most similar to</th>
<th>COI %</th>
<th>ITS2 %</th>
<th>BOLD identification</th>
<th>BOLD reported % similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ch. albiceps</td>
<td>100%</td>
<td>100%</td>
<td>Ch. albiceps</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2 L. sericata</td>
<td>100%</td>
<td>99%</td>
<td>L. sericata</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. cuprina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Ch. chloropyga</td>
<td>99%</td>
<td>99%</td>
<td>Ch. chloropyga</td>
<td>99.5%</td>
<td></td>
</tr>
<tr>
<td>4 L. sericata</td>
<td>100%</td>
<td>99%</td>
<td>L. sericata</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. cuprina</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>L. cuprina x L. sericata</td>
<td>98.8%</td>
<td></td>
</tr>
<tr>
<td>5 Ch. albiceps</td>
<td>98%</td>
<td>100%</td>
<td>Ch. albiceps</td>
<td>99.83%</td>
<td></td>
</tr>
<tr>
<td>6 Ch. albiceps</td>
<td>99%</td>
<td>100%</td>
<td>Ch. albiceps</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>7 Ch. albiceps</td>
<td>99%</td>
<td>100%</td>
<td>Ch. albiceps</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
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Figure 1: Maximum likelihood tree for 40 COI sequences from four blow fly species and one out group, Stomoxys calcitrans. Values on tree branches refer to bootstrap values shown as a percentage of 1000 replicates and indicate support for nodes.

Figure 2: Maximum likelihood tree for 40 ITS2 sequences from four blow fly species and one out group, Stomoxys calcitrans. Values on tree branches refer to bootstrap values shown as a percentage of 1000 replicates and indicate support for nodes.