UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Molecular Analysis of Necrophagous Diptera of the Iberian Peninsula

Análisis Molecular de Dípteros Necrófagos de la Península Ibérica

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2018

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Tesis Doctoral

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2018

Escuela Internacional de Doctorado
Universidad de Murcia

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Support projects

El trabajo presentado en esta tesis se ha realizado en el Área de Biología Animal del Departamento de Zoología y Antropología Física de la Universidad de Murcia bajo la dirección de los Doctores José Galián Albaladejo y Elena Romera Lozano, como parte de los proyectos "Análisis molecular de dípteros necrófagos de la península Ibérica" (CGL2011-25298) financiado la Secretaría de Estado de Investigación, Desarrollo e Innovación y "Animal Phylogeny and Evolution" (19908-GERM-15) financiado por la Fundación Séneca en la convocatoria de Grupos de Excelencia Regional.

El autor ha disfrutado de un contrato asociado a un proyecto CDTI "Selección y Evaluación de Semioquímicos para la Atracción de Dípteros" (IDI-20160234). Durante el desarrollo del mismo ha realizado una estancia de tres meses en el Departamento de Biología Animal de la Facultad de Ciencias de la Universidad de Lisboa, (Portugal), bajo la supervisión de la Doctora María Teresa Rebelo, y con el apoyo económico del programa Erasmus + Prácticas de la Universidad de Murcia.

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RESUMEN

Resumen

Los artrópodos son el phylum más diverso, amplio y extenso del reino animal. Están presentes en casi todos los hábitats de la tierra (agua salada, fresca y profunda, parásitos, suelo, tierra, aire, etc.) y están compuestos por alrededor de diez millones de especies estimadas. La relación entre los humanos y los artrópodos no se limita a la alimentación basada en crustáceos o, en algunas culturas, en otros artrópodos terrestres. Desde la antigüedad, los humanos han usado diferentes productos producidos por artrópodos como miel, seda, tintes, toxinas, etc. Además, diferentes especies polinizan un alto porcentaje de plantas que son la base de la dieta humana, mientras que otros se consideran dañinos. Todos estos usos han llevado a la implementación del conocimiento de muchas especies a lo largo de la historia, ya sea para mejorar su productividad, su uso en medicina o para usarlas como controladores de plagas. Debido a la biología de algunos órdenes de insectos, algunas especies pueden estar presentes en diferentes escenarios delictivos. Por lo tanto, su estudio desde un punto de vista judicial fue un paso natural.

Entomología forense

La entomología forense se define como la ciencia que estudia los insectos (u otros artrópodos) presentes en la escena del crimen de una investigación médico-criminal. El primer informe que puede ajustarse a esta definición se remonta al siglo XIII en China, donde el abogado Sun Tźu usó datos entomológicos para resolver un asesinato por primera vez. El arma homicida fue una hoz, por lo que las hoces de los sospechosos fueron expuestas a una serie de moscas adultas (Fig. 1). Estas moscas fueron atraídas por la hoz que tenía rastros de sangre. Durante los siglos siguientes, esta ciencia evoluciona junto con la biología eliminando el concepto de que las larvas aparecen por generación espontánea. En el siglo XIX se produce la primera determinación de la edad de un cadáver. El francés Dr. Bergeret analizó un cadáver de un bebé encontrado detrás de la chimenea. Sus resultados fecharon que el cadáver estaría muerto dos años, liberando a los actuales inquilinos de la propiedad de la culpa. Más tarde, Pierre Mégnin sistematizó estos estudios. Desde entonces, los estudios en este campo se han convertido en algo regular.

Aunque la definición anterior evoca el escenario de muerte violenta, debido a la imagen errónea de los entomólogos forenses (y científicos forenses en general) que aparece en algunas series de televisión o películas, los insectos son útiles en diversas situaciones. En ese sentido, podemos diferenciar (1) entomología urbana, (2) entomología de productos almacenados y (3) entomología medicolegal. La entomología urbana estudia los insectos que afectan a los humanos y su entorno, por ejemplo, las plagas. La entomología de los productos almacenados se ocupa de una posible contaminación de alimentos o bebidas por insectos. La entomología medicolegal estudia los artrópodos que se encuentran en un cadáver alimentándose de él, como evidencia legal. Esta última especialidad es la más famosa para la opinión pública y hay diferentes maneras en que estos estudios podrían ayudar a la resolución de un delito.

Cuando las larvas de díptero se alimentan de un cadáver, absorben las sustancias presentes en el cadáver debido a la bioacumulación. Estos procesos han sido estudiados en los últimos años por trabajos de entomotoxicología. Aunque esta ciencia todavía tiene algunas limitaciones, los resultados han demostrado ser útiles en la detección de drogas en los cadáveres. Esto es algo a tener en cuenta, porque estas sustancias cambian la tasa de crecimiento de los insectos, alterando la interpretación de las investigaciones. Sin embargo, el uso más popular de los dípteros en la entomología medicolegal es el cálculo del *intervalo post mortem* (PMI). De hecho, el momento de la muerte solía calcularse a través de algunas características físicas del cadáver debido a los cambios post mortem, como *algor mortis*, *livor mortis* y *rigor mortis*, pero estos cambios solo son utilizables durante las primeras 72 horas. Después de eso, la técnica más precisa es el cálculo de la edad del cadáver según el tamaño de las muestras de dípteros presentes en la escena, identificando correctamente la especie y conociendo los factores abióticos del medio ambiente. En realidad, esta técnica calcula el momento en que el cadáver es colonizado por los dípteros, por lo que es importante tener en cuenta los factores abióticos. Durante el proceso de descomposición, los dípteros y otros insectos van al cadáver para desarrollar su ciclo de vida (Fig. 2).

El cadáver pasa por un proceso en serie de descomposición. Entre estas fases, el cadáver sufre variaciones físicas y químicas. Durante el proceso de descomposición, el cadáver produce diferentes tipos de gases y líquidos que atraen a algunas especies de insectos, especialmente dípteros y coleópteros. Las fases son (Fig. 3):

1) Fresco. Este es el momento inicial de la muerte. Aparecen los primeros cantos de hinchazón. Aparentemente, no hay olor asociado a esta fase.

2) Hinchazón (fase enfisematosa). El cuerpo comienza a hincharse por el efecto de las bacterias anaeróbicas. Aparecen las manchas violeta y verde. Las larvas comienzan a ser evidentes.

3) Descomposición activa. El cadáver comienza a desinflarse por la acción de las larvas de dípteros. Se observa una gran pérdida de masa corporal. El olor es muy intenso debido a la salida de fluidos corporales.

4) Descomposición avanzada. El cadáver pierde la humedad y todo el tejido muscular. En esta fase, aparecen las larvas de coleópteros y las larvas de dípteros comienzan a migrar.

5) Restos. Solo quedan los huesos, la piel y el cartílago deshidratado. El olor es menos evidente.

Todas las especies tienen un ciclo de vida específico. Este ciclo está formado por cuatro fases: huevo, larva, pupa y adulto. Además, la fase larvaria está compuesta por tres estadios: larva I, larva II y larva III (Fig. 2). La duración del ciclo depende de factores como la temperatura y la humedad. En ese sentido, se han realizado trabajos sobre el ciclo de vida de varias especies con interés forense. Los isomegalendiagramas desarrollados en estos estudios (Fig. 4) relacionaban el tamaño de las larvas con los factores abióticos. Serían utilizados en casos forenses para calcular el PMI mínimo, después de la identificación de la especie.

Identificación de especies

El paso delicado en estos estudios es la identificación precisa de la especie. Debido a la tasa de crecimiento específica de las especies, la identificación errónea de una especie produciría resultados erróneos en el PMI. Por lo tanto, estas evidencias no podrían ser

utilizadas en procedimientos legales. La identificación de la especie generalmente se realiza a través de claves morfológicas, lo que popularmente se conoce como taxonomía clásica. Las claves morfológicas han evolucionado a partir de claves hechas con dibujo a mano a claves hechas con fotografía de alta resolución y técnicas de microscopía. Sin embargo, estas claves se han diseñado para funcionar con muestras bien conservadas. En el campo de la entomología forense, las muestras suelen estar rotas, sucias y no siempre son capaces de llegar a la edad adulta en el laboratorio. Además, los métodos morfológicos son limitados debido a taxones morfológicamente crípticos, plasticidad fenotípica (un genotipo que produce más de un fenotipo), claves limitadas a estados de vida particulares y la experiencia requerida para su uso. Esos problemas han producido el uso creciente de técnicas moleculares en este campo.

Técnicas moleculares

Las técnicas moleculares presentan algunas ventajas para trabajar sin muestras bien conservadas. El ADN puede usarse en cada etapa de larva, adultos o huevos. Incluso las pupas vacías se han utilizado en la identificación molecular, produciendo resultados positivos. Además, algunas especies estrechamente relacionadas son demasiado difíciles de identificar morfológicamente, incluso por expertos, cuando las muestras no se encuentran en buen estado de conservación. Las técnicas moleculares han demostrado ser útiles para diferenciar especies con interés forense. Sin embargo, hay casos en los que esta diferenciación aún no está clara, según el estudio. La identificación de especies a través de métodos moleculares está condicionada por la comparación con otras secuencias bien identificadas. Las bases de datos públicas deben mantenerse y actualizarse con secuencias sin errores de secuencia o identificación errónea para crear conjuntos de secuencias con buena representatividad por especie. Por esa razón, no se recomiendan las técnicas moleculares para investigar nuevas especies, especialmente con un solo locus. De hecho, al comienzo de la popularización de estas técnicas, algunos taxónomos tomaron esta herramienta como un problema, ya que los límites propuestos en la divergencia interespecífica no se cumplieron entre algunas especies cercanas. Sin embargo, se crearon varios proyectos para comenzar a acumular información genética.

En el Nacional Center of Biological Information (NCBI), el proyecto GenBank está funcionando desde 1992. Desarrollaron la herramienta de búsqueda llamada BLAST. Esta herramienta compara una secuencia con la base de datos y muestra las similitudes más altas con otras secuencias. Entre los diferentes parámetros dados por la herramienta, los dos más importantes son el E value (cuanto más pequeño es, más similitud hay entre las secuencias) y el Grade (cuanto más alto es, más similar hay entre las secuencias). El problema de esta herramienta es que siempre produce un resultado de similitud, pero la secuencia más similar podría no ser una identificación precisa. De la misma manera, la técnica llamada "ADN barcoding" comienza a usarse rutinariamente. Esta técnica consiste en la secuenciación de un fragmento estándar del gen *citocromo oxidasa subunidad 1* y su uso para la identificación de especies (figura 5). La alta efectividad de esta técnica propició el desarrollo de la base de datos del sistema Barcode of Life Data (BOLD). Esta base de datos se centra en la técnica de código de barras de ADN y utiliza la herramienta Biological Index Number (BIN) para identificar las secuencias. Esta herramienta puede asignar un BIN específico de especie a una secuencia para reconocerlo. Además, si las secuencias no fueran lo suficientemente similares a cualquier BIN, se reconocerían como "Singleton". Eso significaría que la secuencia putativa sería la primera incluida en la base de datos de esa especie, aumentando la diversidad de la base de datos. Esa es la ventaja de esta herramienta.

ADN mitocondrial

El genoma mitocondrial consiste en una molécula circular que contiene 37 genes (Fig. 6): 13 genes que codifican proteínas, 22 ARN de transferencia y 2 ARN ribosómicos. Tiene un porcentaje muy bajo de ADN no codificante o ADN repetitivo. Su tasa de evolución es más alta que el ADN nuclear, por lo tanto, es una herramienta útil para inferir las filogenias. Además, cada célula tiene miles de mitocondrias, por lo que los resultados en las prótesis de extracción suelen ser más altos que el ADN nuclear. El ADN mitocondrial es completamente heredado por la madre, por lo que no se produce recombinación de ADN. Por lo tanto, todos los cambios en el ADN mitocondrial se deben a mutaciones. Aunque se han descrito algunos casos de heteroplasmia (diferentes poblaciones mitocondriales dentro de un individuo), se consideraron casos

aislados. Todas estas características hacen que el ADN mitocondrial sea una herramienta útil para la diferenciación especies y las relaciones entre ellas, ya que necesita menos tiempo para mostrar las diferencias evolutivas.

ADN nuclear

El ADN nuclear, al contrario que el ADN mitocondrial, recibe herencia materna y paterna. Debido a eso, se ha utilizado en las ciencias forenses para investigar las relaciones de paternidad. Además, el ADN nuclear presenta áreas de velocidad de evolución rápida. Es el caso del ADN ribosomal, compuesto por tres genes y dos áreas no codificantes. Estas secuencias se han usado en filogenias y en estudios de diferenciación de especies que producen buenos resultados, incluso siendo el *internal transcribed spacer 2* propuesto como marcador de código de barras de ADN para algún grupo de organismos.

Filogeografía

La información genética se puede utilizar para estudiar las relaciones filogeográficas entre especies o dentro de las especies. La búsqueda de haplotipos (un conjunto de un polimorfismo genético ubicado en un solo cromosoma / gen) relacionado con subespecies o ubicaciones geográficas ayuda a describir la historia y / o los movimientos de una especie. Algunos trabajos han sido desarrollados estudiando los haplotipos geográficos que han resultado en subespecies de algunos insectos. En Diptera, se han desarrollado algunos estudios de extensas áreas geográficas, llegando a diferenciar áreas geográficas basadas en haplotipos. El uso de esta técnica enfocada en entomología forense sería el reconocimiento de un lugar de ubicación en donde se encontraron pruebas entomológicas.

Un uso más general para la información genética es la reconstrucción de estados genéticos ancestrales. La historia biogeográfica de una especie o un grupo de especies podría estudiarse con esta técnica. La información sobre ubicaciones combinada con esta técnica muestra la permanencia de un grupo en un área geográfica. La relación entre esto y la historia geológica o la influencia de los humanos nos ayuda a conocer los antecedentes de una especie para comprender mejor su situación real.

Morfometría geométrica

La morfometría geométrica es una técnica de análisis cuantitativo de formas en espacios de dos o tres dimensiones. Se basa en la visualización de los cambios de forma medidos con puntos de referencia llamados *landmarks* (figura 7). Estos puntos corresponden a rasgos particulares presentes en todos los especímenes y que siguen el mismo orden en todos ellos. Este tipo de análisis no tiene en cuenta el tamaño, posición u orientación, por lo que solo se analiza la forma. Estos análisis de la variación morfológica se utilizan para responder a una gama cada vez más amplia de preguntas sobre la evolución y el desarrollo de los organismos. Los resultados de estos análisis permiten a los investigadores visualizar los cambios de forma a través de programas estadísticos, más fácil que en una tabla de resultados. Se puede hacer mostrando el desplazamiento y la dirección de cada landmark o mostrando la deformación de una superficie hecha con una combinación de landmarks. Además, cuando los cambios de formas no son lo suficientemente claros como para ser detectados con métodos previos, podemos realizar algunos análisis algebraicos. El Análisis de Componentes Principales (PCA) se usa para determinar la variable o variables en las cuales ocurrieron los cambios y buscar la explicación biológica de la misma. El Análisis de Varianza Canónica (CVA) se usa para explorar las diferencias entre grupos. Estos análisis se han utilizado para diferenciar especies o grupos de especies basados en la forma de las alas. Esta es la estructura más utilizada debido a su forma bidimensional y su fácil aislamiento, pero se pueden usar otras estructuras con buenos resultados. En diferentes tipos de dípteros, esta técnica se ha utilizado produciendo una buena separación de especies y poblaciones.

Objetivos

Objetivos generales

El objetivo principal de esta tesis es aplicar la identificación de barcoding de ADN y otras técnicas a las familias de dípteros necrófagos recogidos en un estudio intensivo de la Península Ibérica. Este enfoque permitirá: 1) confirmar la presencia de las especies encontradas en trabajos previos identificados usando caracteres morfológicos; 2) agregar datos para la taxonomía del ADN (análisis filogenéticos) de

estas especies distribuidas cosmopolitas; 3) evaluar genes mitocondriales y nucleares (COI, 16S e ITS2) como una herramienta para su uso en la identificación de especies para detectar taxones crípticos; 4) analizar los haplotipos y los estados ancestrales de especies diseminadas en la Península Ibérica para obtener datos filogeográficos; 5) analizar mediante técnicas geométricas morfométricas las estructuras anatómicas de algunas especies en las que las diferencias morfológicas y moleculares no son lo suficientemente claras.

Capítulo I. Identificación molecular de especies de moscas de interés forense en España utilizando COI barcoding.

El objetivo de este capítulo es utilizar barcoding de ADN para identificar Diptera con interés forense en España. Estos análisis corroboran la identificación basada en la morfología utilizada para la identificación preliminar, así como los diferentes métodos de delimitación de especies. Para esto, los objetivos específicos son:

- Generar datos de la secuencia COI de especies de moscas forenses relevantes, recolectadas en la Península Ibérica durante las cuatro estaciones.

- Definir el número de unidades taxonómicas operacionales moleculares (MOTU) de moscas asociadas a cadáveres, incluidas aquellas especies con diferenciación difícil.

- Caracterizar la diversidad molecular y el conocimiento de la fauna de dípteros necrófagos de la Península Ibérica, proporcionando información para la identificación de especies en entomología forense.

Capitulo II. Identificación basada en ADN y taxonómica de Sarcophagidae (Diptera) de importancia forense en el sudeste de España.

El objetivo de este capítulo es identificar con técnicas moleculares especímenes de la familia Sarcophagidae recolectados en casos forenses ocurridos en Alicante (Comunidad Valenciana, sureste de España) y comparar estas muestras con otras recolectadas en esta zona y provincias aledañas con trampas cebadas. Los objetivos específicos son:

- Aplicar técnicas de código de barras de ADN a esta familia de dípteror.

- Comparar las diferencias relacionadas con las especies presentes en los cuerpos humanos y las capturadas en la naturaleza en la misma área geográfica.

- Analizar con técnicas filogeográficas a las especies más extendidas que buscan haplotipos locales.

- Verificar si esta información puede ser útil en el cálculo del PMI en comparación con la realizada con especies pertenecientes a otras familias encontradas en estos casos forenses.

Los datos moleculares generados en este documento aumentarán los conocimientos disponibles sobre la familia Sarcophagidae en España y proporcionarán información valiosa esencial para la identificación de especies en el futuro a través de técnicas de códigos de barras.

Capítulo III. Diversidad de la especie de mosca de importancia forense Calliphora vicina, (Diptera: Calliphoridae) en la Península Ibérica utilizando secuencias COI, 16S e ITS2.

El objetivo general del capítulo es generar datos que podrían ser útiles para determinar la ubicación original de un cadáver que se hubiera movido después del crimen. Los objetivos de este capítulo son:

- Generar secuencias de COI, 16S e ITS2 de muestras de *C. vicina* de España y Portugal, útiles en la identificación de especies.

- Analizar la biodiversidad de estos genes para determinar haplotipos locales

- Analizar estados ancestrales de estos genes para determinar la colonización plausible de la Península Ibérica por esta especie.

Capítulo IV. Especies del género Lucilia (Diptera: Calliphoridae) con interés forense en el norte de España: un enfoque molecular y morfogeométrico.

El objetivo general de este capítulo es verificar diferentes métodos para delimitar estas especies estrechamente relacionadas. Los objetivos específicos de este trabajo son:

- Generar secuencias del gen *citocromo oxidasa subunidad 1* (COI) y del *Internal transcribed spacer 2* (ITS2) de *L. ampullacea*, *L. caesar* y *L. illustris* recolectadas en el norte de España.

- Realizar análisis de identificación molecular y técnicas filogeográficas.

- Analizar alas y cabezas de estas especies con técnicas morfométricas geométricas para reconocer con precisión la especie y buscar diferencias entre las poblaciones.

Capítulo I

Identificación molecular de especies de moscas de importancia forense en España utilizando COI barcoding

La identificación de especies con códigos de barras de ADN ha demostrado su eficacia en diferentes organismos y, en particular, se ha convertido en una herramienta rutinaria y precisa en entomología forense para estudiar especies de dípteros necrófagos. En este estudio, analizamos 215 especímenes pertenecientes a 42 especies de 17 géneros, de nueve diferentes familias Diptera. Las moscas fueron recolectadas en 39 localidades españolas de la Península Ibérica muestreadas durante tres años en las cuatro estaciones. La variación intraespecífica varió de 0 a 2.46%, mientras que la variación interespecífica varió de 3.07 a 14.59%, midiendo 651 pb del gen de la subunidad uno del gen *citocromo oxidasa subunidad 1* (COI). El análisis de Neighbour-Joining se llevó a cabo para investigar las capacidades de identificación molecular de la región de barcoding, recuperando casi todas las especies como distintos grupos monofiléticos. Las agrupaciones de especies fueron generalmente consistentes con la identificación morfológica y molecular. Este trabajo muestra que el barcoding de COI es un marcador apropiado para la identificación inequívoca de dípteros de importancia forense en España.

Capítulo II

Identificación basada en ADN y taxonómica de Sarcophagidae (Diptera) de importancia forense en el sudeste de España.

El estudio de los dípteros en el lugar de la muerte proporciona información esencial para la interpretación de evidencias y, por lo tanto, para la resolución del caso. Las herramientas moleculares se han incorporado a las tareas de identificación a un nivel específico en casos de especies crípticas o en la conservación deficiente de especímenes. Estos métodos también son útiles debido a la falta de personal calificado en taxonomía y sistemática de insectos en la policía científica. Además, una reconstrucción filogeográfica dentro de una especie podría ayudar a diferenciar los haplotipos de un área geográfica, contribuyendo a la clarificación en casos de reubicación del cadáver. La identificación taxonómica de las especies de Sarcophagidae a menudo es difícil usando caracteres morfológicos, especialmente las hembras. Este hecho aumenta la duda de usarlos para identificar el mínimo intervalo post mortem (PMI). En este trabajo, comparamos los sarcófagos hallados en cadáveres humanos en casos forenses reales en Alicante (sureste de España) con especímenes recolectados en trampas con cebo en la misma área y provincias circundantes. Se recolectó un total de 189 especímenes, 72 de casos forenses reales y 117 de trampas con cebo. La identificación morfológica de las muestras se realizó con claves taxonómicas hasta nivel familiar. La identificación molecular se realizó por secuenciación del gen mitocondrial COI y análisis de secuencias mediante los métodos ABGD, GMYC y BIN. Para la construcción de redes filogeográficas, se utilizó el algoritmo Median Joining del programa PopART. Se identificaron ocho especies de la familia Sarcophagidae. Cinco secuencias, a priori asignadas a *Sarcophaga argyrostoma*, producen controversia entre los tres métodos de delimitación de especies utilizados. Cuatro especies no se encontraron en los casos forenses. Además, tres de estas especies se encontraron en un solo lugar cada una. Las especies más ampliamente recolectadas fueron *S. argyrostoma* y *S. tibialis*. Las redes de haplotipos de estas especies no revelaron una clara distribución geográfica de los haplotipos. Las muestras de *S. argyrostoma* de

Alcoy fueron claramente aisladas. Estos resultados demuestran que este método es útil para la identificación de muestras de Sarcophagidae en investigaciones forenses.

Capítulo III

Diversidad de especies de mosca de importancia forense *Calliphora vicina*, (Diptera: Calliphoridae) en la Península Ibérica utilizando secuencias COI, 16S e ITS2.

El estudio de los dípteros en la escena de un crimen proporciona información esencial para la interpretación de las evidencias y, por lo tanto, para su resolución. Su uso más conocido es el cálculo del PMI (intervalo post mortem), pero la entomología forense tiene otras aplicaciones: casos de abandono y / o abandono de menores y ancianos, confirmando la presencia de sustancias químicas, e incluso confirmando la reubicación de un cadáver. La reconstrucción filogeográfica podría ayudar a diferenciar los haplotipos de una especie específica de un área geográfica, contribuyendo a la clarificación de la posible transferencia del cadáver. Además, la reconstrucción de estados ancestrales ayuda a comprender el estado real de la especie y su historia biogeográfica. En este estudio, se utilizaron 464 especímenes de *C. vicina* recolectados en España y Portugal entre 2012 y 2015. Se identificaron con claves morfológicas y se analizaron por técnicas moleculares secuenciando los genes COI, 16S e ITS2. Para ello, se construyeron tres matrices en GENEIOUS 7.1.3 y se utilizó la herramienta BLAST. Para la construcción de redes filogeográficas, se utilizó el algoritmo Median Joining del programa PopART. La reconstrucción de estados ancestrales se realizó con el programa RASP. Las técnicas moleculares han corroborado las identificaciones hechas en aspectos morfológicos. Las redes filogeográficas muestran que no existe una estructura geográfica con haplotipos compartidos entre casi todas las poblaciones. Los análisis RASP mostraron una alta tasa de movimiento entre poblaciones con colonizaciones que cruzan la Península Ibérica, posiblemente relacionadas con la actividad humana.

Capítulo IV

Especies del género *Lucilia* (Diptera: Calliphoridae) con interés forense en el norte de España: un enfoque molecular y morfogeométrico.

La correcta identificación de las especies es un paso esencial en los estudios de entomología forense, ya que la identificación incorrecta puede conducir a errores en el cálculo del intervalo post mortem (PMI). Esta tarea es más complicada cuando se analizan especies estrechamente relacionadas con caracteres morfológicos difíciles de observar en especímenes dañados o mal conservados. Este estudio ha sido desarrollado con tres especies *Lucilia ampullacea*, *Lucilia caesar* y *Lucila illustris* (Diptera, Calliphoridade) empleando análisis morfológicos, moleculares y morfogenométricos. En este estudio, hemos realizado un análisis filogenético y morfogeométrico de las muestras recolectadas entre 2012 y 2014 en el norte de España. Hemos amplificado y secuenciado el fragmento mitocondrial citocromo oxidasa subunidad I (COI) y el fragmento nuclear internal transcribed spacers 2 (ITS2). Han sido analizados filogenéticamente por métodos de distancia con Neighbor-Joining y filogeográficamente a través de redes de haplotipos. Hemos definido 18 landmarks en las alas y 18 en la cabeza. En ambas estructuras, comparamos las diferencias usando el análisis de componentes principales (PCA) y el análisis de variables canónicas (CVA). Los análisis filogenéticos y filogeográficos han mostrado una clara diferencia entre *L. ampullacea* y las otras dos especies, pero no había una distancia clara entre *L. caesar* y *L. illustris*. La distancia intra e interespecífica ha demostrado la falta de diferencias genéticas entre estas dos especies. El análisis morfogeométrico de las alas y la cabeza corroboraron la separación de *L. ampullacea*. La separación de *L. caesar* y *L. illustris* por el análisis de CVA mostró dos grupos bien diferenciados con una pequeña zona de separación en ambas estructuras. Por lo tanto, estas herramientas podrían ser la clave para diferenciar estas especies de interés forense. Además, al agrupar las muestras por ubicación, los diferentes grupos estaban bien definidos. La procedencia de una muestra podría investigarse de esta manera.

Conclusiones

Las conclusiones generales de esta tesis son:

1. Las técnicas de barcoding y los métodos de delimitación de especies son herramientas importantes en entomología forense, identificando muestras dañadas / mal conservadas.

2. Las técnicas moleculares ayudan a caracterizar la diversidad de especies, de un conjunto de secuencias, de las moscas de la península Ibérica de importancia forense.

3. Las técnicas moleculares han demostrado ser útiles para identificar muestras de casos forenses, incluso cuando las muestras estaban en mal estado de conservación.

4. La identificación a través del barcoding nos permitió cambiar el nombre de algunas muestras que se asignaron a la familia de dípteros incorrecta en un caso forense.

5. El análisis filogeográfico mostró que las muestras de Alcoy de casos forenses conllevan haplotipos locales.

6. No existe una estructura biogeográfica para *C. vicina* para los genes COI, 16S e ITS2. Esto confirma que las barreras geográficas no son suficientes para detener el flujo de génico.

7. La colonización de la península Ibérica por *C. vicina* no presenta un patrón geográfico para las secuencias COI y 16S. El análisis mostró colonizaciones seriadas múltiples posiblemente relacionadas con la actividad humana.

8. Las especies hermanas *L. ampullacea*, *L. caesar* y *L. illustris* se han diferenciado por varios métodos.

9. Nuestros resultados confirman la conclusión de Boehme et al, (2012) que dice que un fragmento más corto elimina las zonas homólogas, mejorando la identificación molecular.

10. El análisis de haplotipos ayuda a visualizar la diferenciación de las especies. Esto nos permitió renombrar seis secuencias erróneamente identificadas por morfología.

11. Las técnicas de morfometría geométrica nos permiten identificar a las especies usando ambas estructuras, alas y cabezas, con casi la misma precisión.

12. Los resultados muestran que las técnicas de morfometría geométrica se pueden utilizar para diferenciar el origen de las muestras con las dos estructuras analizadas, especialmente si consideramos separadamente muestras orientales y occidentales. Esto puede usarse en casos forenses en los que se haya producido la reubicación del cadáver.

13. El conjunto de landmarks diseñados en este trabajo para cabezas ha demostrado la misma efectividad que aquellos diseñados para alas.

14. Un total de 1398 nuevas secuencias se han producido en esta tesis: 669 de COI, 206 de 16S y 523 de ITS2. Con las secuencias COI se ha creado el proyecto "Barcoding of Iberian Diptera" en BOLD. Este proyecto será útil en futuros trabajos.

15. Toda la información producida en esta tesis aumenta el conocimiento sobre los dípteros con interés forense en la península Ibérica.

INTRODUCTION

Introduction

The arthropods are the more diverse, large and widespread phylum of the animal kingdom. They are presents in almost all habitats on earth (salty, fresh and deep waters, parasite, soil, underground, air, etc.) and they are composed of around ten million estimated species (Byrd & Castner, 2010). The relationship between humans and arthropods is not restricted to the feeding based on crustacean or, in some cultures, other terrestrial arthropods (DeFoliart, 1989). Since ancient times, the humans have used different products produced by arthropods as honey, silk, dyes, toxins, etc. (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2010; Hamamura, 1959; Pemberton, 1999; Quirce, Cuevas, Olaguibel, & Tabar, 1994). Besides, different species pollinize a high percentage of plants that are the base of human diet (Westphal, Steffan-Dewenter, & Tscharntke, 2003) while others are considered harmful (White, 1976). All these uses have led to the implementation of the knowledge of many species through history, either to improve their productivity, their use in medicine or to use them as pest controllers (Jonsson, Wratten, Landis, & Gurr, 2008). Due to the biology of some orders of insects, some species are able to be present is different crime scenarios. Therefore, their study from a judicial point of view was a natural step forward.


宋提刑洗寃集錄卷之二

（五）疑難雜說下

有檢驗被殺屍在路傍，始疑盜者殺之，及點檢沿身衣物俱在，遍身鏃刀砍傷十餘處。檢官曰：「盜只欲人死取財，今物在傷多，非寃讎而何！」遂屏左右，呼其妻問曰：「汝夫自來與甚人有寃讎最深？」應曰：「夫自來與人無寃讎，只近日有某甲來做債，不得，曾有對期之言。然非寃讎深者。」檢官默識其居，逕多差人分頭告示：側近居民各家所有鏃刀盡底將來，只今呈驗。如有隱藏，必是殺人賊，當行根勘。俄而，居民貨到鏃刀七八十張。令布列地上。時方盛暑，內鏃刀一張，蠅子飛集。檢官指此鏃刀問：「爲誰者？」忽有一人承當，乃是做債對期之人。就擒訊問，猶不伏。檢官指刀令自看，衆人鏃刀無蠅子，令汝殺人，血腥氣猶在，蠅子集聚，豈可隱耶？左右攘觀者失聲碳服，而殺人者叩首服罪。昔有深池中溺死人，經久，事屬大家因檢事發。體究官見皮肉盡無，惟惻髏骨

Figure 1. Chapter 5 of Sun Tźu's Chinese book on Forensic Medicine deals with a case stabbing solved by use of insects. From Benecke (2001)

Forensic entomology

Forensic entomology is defined as the science that study the insects (or other arthropods) present in the crime scene of a medicocriminal investigation (Amendt, Krettek, & Zehner, 2004; Byrd & Castner, 2010). The first report that can fit this definition goes back to 13th century in China, where the lawyer Sun Tźu used entomological data to solve a murder for the first time (Benecke, 2001). The murder weapon

was a sickle, so the sickles of the suspects were exposed to a series of adult flies (Fig. 1). These flies were attracted by the sickle that had traces of blood. During the following centuries, this science evolves together with the biology eliminating the topic that larvae appear by spontaneous generation (González-Zymla & Herbert, 2014). In the 19th century the first determination of the age of a corpse occurs. The French Dr. Bergeret analysed a corpse of a baby found behind the chimney. His results dated the corpse to be two years dead, freeing the current tenants of the property from the guilt. Later, Pierre Mégnin systematized these studies (Benecke, 2001). Since then, the studies in this field has become something regular.

Although the previous definition evokes the scenario of violent death, due to the erroneous image of forensic entomologists (and forensic scientists in general) that appears in some television series or movies, insects are useful in various situations. In that sense, we can differentiate (1) urban entomology, (2) entomology of stored products and (3) medicolegal entomology (Byrd & Castner, 2010). Urban entomology studies the insects that affect humans and their environment, for instance, plagues. The entomology of stored products deals with a possible contamination of food or drinks by insects. Medicolegal entomology studies the arthropods found in a corpse feeding from it, as a legal evidence. This last speciality is the most famous for public opinion and there are different ways in which these studies could help to the resolution of a crime.

When the dipteran larvae feed of a corpse, they absorb the substances presents in the corpse due to the bioaccumulation. These processes have been studied in the last years by works of entomotoxicology (Amendt et al., 2004; Gosselin et al., 2011; Introna, Campobasso, & Goff, 2001; O'Brien & Turner, 2004; Sadler, Richardson, Haigh, Bruce, & Pounder, 1995). Although this science still having some limitations, the results have demonstrated to be useful in the detection of drugs in the corpses. This is something to have into account, because these substances change the growth rate of insects, altering the interpretation of the investigations. However, the most popular use of the Diptera in medicolegal entomology is the calculation of *post mortem interval* (PMI). In fact, the moment of the death used to be calculated throughout some physical characteristics of the corpse due to the post mortem changes, like *algor*

mortis, *livor mortis* and *rigor mortis* (Amendt et al., 2004), but this changes are only usable during the first 72 hours (Amendt et al., 2004; Henßge & Madea, 2004). After that, the more accurate technique is the calculation of the age of the corpse age based on the size of the specimens of Diptera presents in the scene, identifying correctly the species and knowing the abiotic factors of the environment (Amendt, Richards, Campobasso, Zehner, & Hall, 2011). Actually, this technique calculates the moment in which the corpse is colonized by the Diptera, because of that the abiotic factors are important to be taking into account. During the decomposition process, the Diptera and other insects go to the corpse to develop their life cycle (Fig. 2).

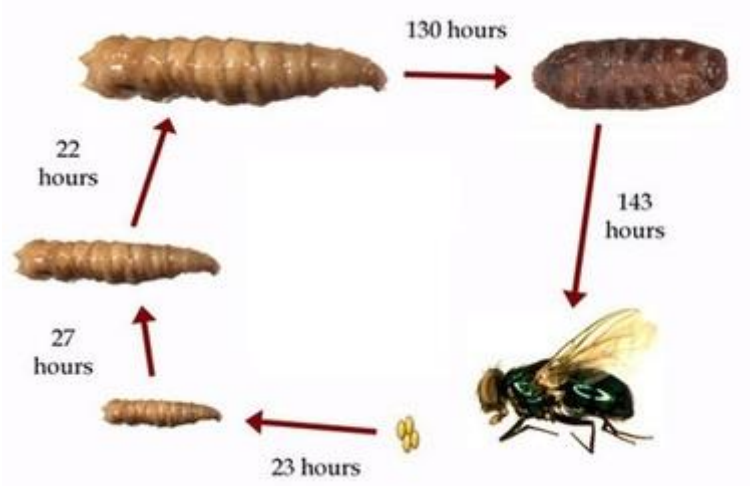

Figure 2. The typical blow fly life cycle. Times given in the chart are for 21 degrees (by The Cleveland Natural History Museum, 2011)

The corpse goes through a serial process of decomposing. Among these phases, the corpse undergoes physical and chemical variations. During the decomposing process, the corpse produces different types of gasses and liquids that attract some species of insects, especially Diptera and Coleoptera (Anderson & VanLaerhoven, 1996). The phases are (Fig. 3):

1) **Fresh**. This is the initial time of death. The first sings of swelling appear. Apparently, no smell is associated to this phase.
2) **Bloat (emphysematous phase).** The body begins to swell by the effect of the anaerobic bacteria. The purple and green spots appear. The larvae begin to be evident.

3) **Active decomposition.** The corpse begins to deflate by the action of the Diptera larvae. A large loss of body mass is observed. The smell is very intense due to the outflow of body fluids.
4) **Advanced decomposition.** The corpse loss the humidity and all the muscular tissue. In this phase, the larvae of Coleoptera appear and the Diptera larvae begin to migrate.
5) **Remains.** Only bones, skin and dehydrated cartilage remains. The smell is less evident.

Figure 3. Pig carcass in the different stages of decomposition. From left to right: Fresh, Bloat, Active Decomposition, Advanced Decomposition and Remains. From www.academia.dk

All the species have a specific life cycle. This cycle is formed by four phases: egg, larvae, pupae and adult. Besides, the larvae phase is composed of three stadiums: larvae I, larvae II and larvae III (Fig. 2). The length of the cycle depends on factors such as temperature and humidity (Amendt et al., 2011; Oliveira-Costa & de Mello-Patiu, 2015). In that sense, works on the life cycle of several species with forensic interest have been made (Martínez-Sánchez, Magaña, Toniolo, Gobbi, & Rojo, 2015; Reibe, Doetinchem, & Madea, 2010; Richards, Crous, & Villet, 2009; Wang et al., 2018; Wang et al., 2016; Yang et al., 2015). The isomegalen diagrams developed in these studies (Fig. 4) related the size of the larvae with the abiotic factors. They would be used in forensic cases to calculate the minimum PMI, after the identification of the species (Pedreño Sala, 2014).

Species identification

The essential step in these studies is the accurate identification of the species (Farinha et al., 2014). Due to the species-specific growing rate, miss-identification of the species would produce erroneous results in the PMI. Therefore, these evidences could not be used in legal proceedings. The species identification usually is made throughout

morphological keys, what is popularly known as classical taxonomy. The morphological keys have evolved from keys made with hand drawing (Barrientos, 2004; Velásquez, Magaña, Martínez-Sánchez, & Rojo, 2010) to keys made with high resolution photography and microscopy techniques (Pinto e Vairo, Moura, & de Mello-Patiu, 2015; Szpila, Pape, & Rusinek, 2008; Szpila, 2012; Szpila, Richet, & Pape, 2015; Szpila & Villet, 2011; Ubero-Pascal et al., 2015). However, these keys have been designed to work with well-preserved samples. In the forensic entomology field, samples use to be broken, dirty and not always are able to reach the adulthood in laboratory (Harvey, Dadour, & Gaudieri, 2003; Zehner et al., 2004). Besides, the morphological methods are limited because of morphologically cryptic taxa, phenotypic plasticity (one genotype producing more than one phenotype), keys limited to particular life states and the experience required for their use (Hebert, Cywinska, Ball, & Jeremy, 2003; Waugh, 2007). Those issues have produced the increasing use of molecular techniques in this field.

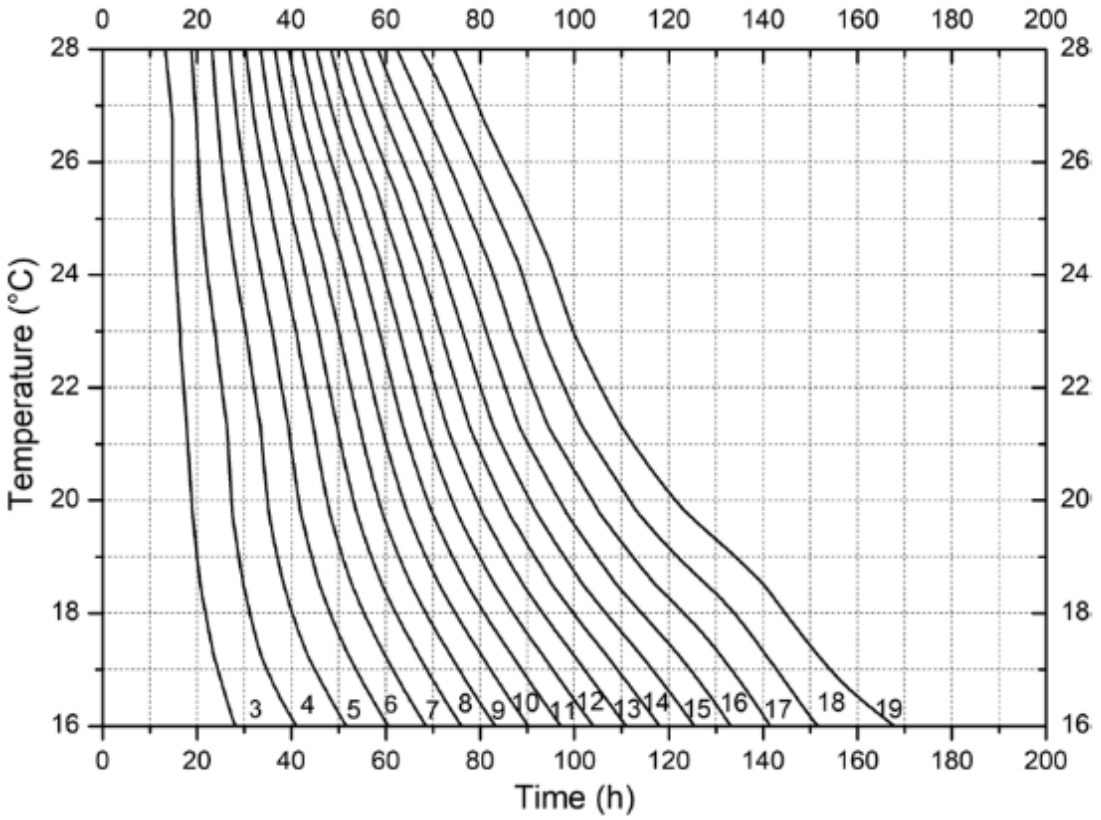

Figure 4. Isomegalen diagram of Aldrichina graham larvae from hatching to peak feeding state. Time is plotted against temperature where each line represents developmental larval length in mm (3-19). From Wang et al. (2018)

Molecular techniques

Molecular techniques present some advantages to work with no well-preserved samples. The DNA is able to be used in each larval stage, adults or eggs (Amendt et al., 2004; Meiklejohn, Wallman, & Dowton, 2012). Even the empty puparia has been used in molecular identification (Mazzanti, Alessandrini, Tagliabracci, Wells, & Campobasso, 2010), producing positive results. Besides, some closely related species are so difficult to identified morphologically, even by experts, when samples are not in good state of preservation (Amendt et al., 2011; Sperling et al., 1994). Molecular techniques have demonstrated to be useful to differentiate species with forensic interest (Aly, 2014; Boehme, Amendt, & Zehner, 2012; Jordaens, Sonet, Richet, et al., 2013; Kim et al., 2014; Koroiva, de Souza, Roque, & Pepinelli, 2018; Li et al., 2010; Tan, Rizman-Idid, Mohd-Aris, Kurahashi, & Mohamed, 2010). However, there are cases in which this differentiation is still not clear, depending of the study (Boehme et al., 2012; Picard, Wells, Ullyot, & Rognes, 2018; Sonet, Jordaens, Braet, & Desmyter, 2012).

The species identification through molecular methods is conditionate by the comparison with other well identified sequences. The public databases must be maintained and updated with sequences without sequencing errors or misidentification in order to create pools of sequences with good representativeness per species (Sonet et al., 2013; Wells & Stevens, 2008). For that reason, molecular techniques are not recommended to investigate new species, especially with a single locus (DeSalle, 2006; DeSalle, Egan, & Siddall, 2005). In fact, at the beginning of the popularization of these techniques, some taxonomist took this tool as a problem (Blaxter, 2003; Janzen, 2004; Prendini, 2005; Taylor & Harris, 2012), since the proposed limits in interspecific divergence were not met between nearby species (Meyer & Paulay, 2005). However, several projects were created to start accumulating genetic information.

In the National Center of Biological Information (NCBI), the GenBank project is working since 1992. They developed the searching tool called BLAST (Altschul et al., 1997). This tool compares a sequence with the database and shows the highest similarities with other sequences. Among the different parameters given for the tool, the most important two are the E-value (the smaller it is, the more similarity there is between

sequences) and the Grade (the higher it is, the more similar there is between sequences). The problem of this tool is that always produce a similarity result, but the more similar sequence might not be an accurate identification. In the same way, the technique called "DNA barcoding" starts to be routinely used (Hebert, Ratnasingham, & Jeremy, 2003). This technique consists in sequencing of a standard fragment of the *cytochrome oxidase subunit one* gene and use it for species identification (Fig. 5). The high effectiveness of this technique propitiated the development of the Barcode of Life Data (BOLD) System database (Ratnasingham & Hebert, 2007). This database is focused in the DNA barcoding technique and used the Biological Index Number (BIN) tool to identify the sequences (Ratnasingham & Hebert, 2013). This tool is able to assign a species-specific BIN to a sequence to recognise it. Besides, if the sequences were not similar enough to any BIN, it would be recognized as "Singleton". That would mean that the putative sequence would be the first one included in the database of that species, increasing the diversity of the database. That is the advantage of this tool.

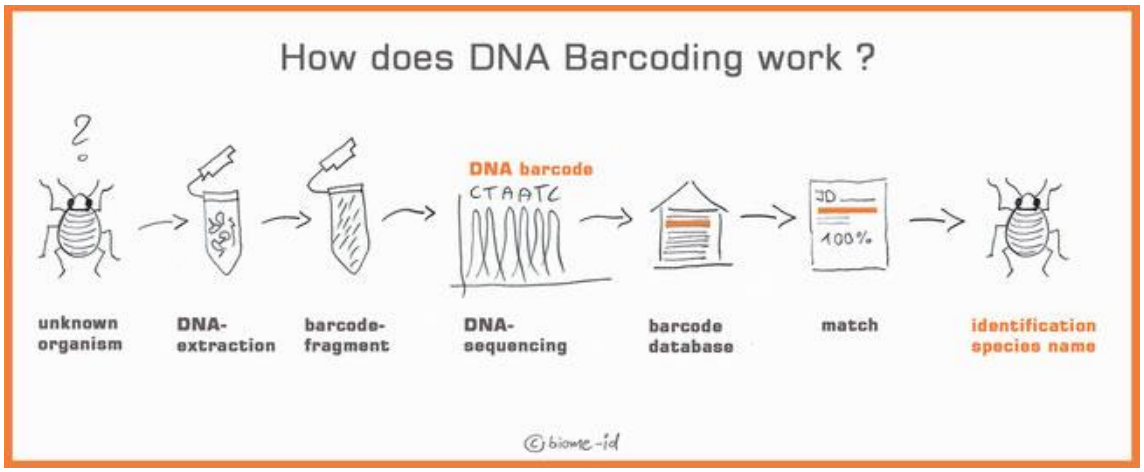

Figure 5. Schematic use of the DNA barcoding technique. From www.biome-id.com

Mitochondrial DNA

The mitochondrial genome consist of a circular molecule that contains 37 genes (Fig. 6): 13 protein coding genes, 22 transfer RNAs and 2 ribosomal RNAs (Boore, 1999). It has a very low percentage of non-coding DNA or repetitive DNA. Its evolutionary rate is higher than the nuclear DNA, therefore is a useful tool to infer phylogenies (Piganeau, Gardner, & Eyre-Walker, 2004; Simon et al., 1994; Zhao et al., 2013). Besides, each cell has thousands of mitochondria, so the results in the extraction prosses use to be

higher than the nuclear DNA. The mitochondrial DNA is completely inherited through the mother, so no recombination of DNA is produced. So, all the changes in the mitochondrial DNA is due to mutations (Avise et al., 1987). Although some cases of heteroplasmy (different mitochondrial populations within one individual) has been described, it were consider isolated cases (Galtier, Nabholz, Glémin, & Hurst, 2009). All these characteristics makes the mitochondrial DNA a useful tool to differentiated species and the relationships among them, since it needs less time to show evolutionary differences (Hewitt, 2004).

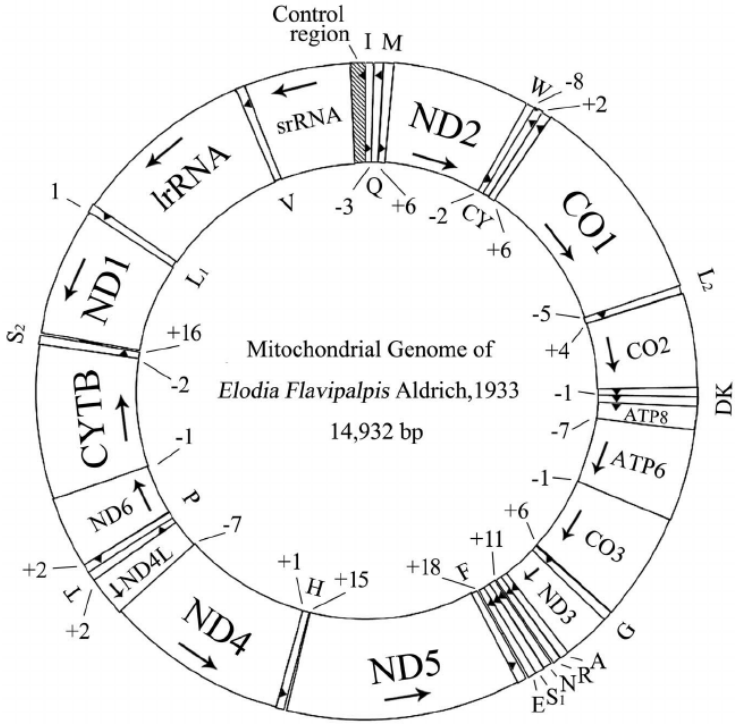

Figure 6. Structure of the mitochondrial DNA molecule. From Zhao et al, (2013)

Nuclear DNA

Nuclear DNA, in different way from mitochondrial DNA, receives maternal and paternal inheritance. Due to that, it has been used in forensic sciences to investigate relationships of paternity (Jeffreys, Brookfield, & Semeonoff, 1985; Jobling, Pandya, & Tyler-Smith, 1997). In addition, nuclear DNA presents areas of rapidly evolving rate. It is the case of ribosomal DNA, composed of three genes and two non-coding areas. These sequences have been used in phylogenies and in studies of species differentiation producing good results, being the *internal transcribed spacer 2* even

proposed as DNA barcode marker for some group of organisms (Brower & DeSalle, 1994; Nelson, Wallman, & Dowton, 2008; Schoch et al., 2012).

Phylogeography

The genetic information can be used to study the phylogeographic relationships among species or within species (Lambertini et al., 2006; Roderick, 1996). The searching of haplotypes (a set a genetic polymorphism located on a single chromosome/gen) related with subspecies or geographic locations helps to describe the history and/or the movements of a species. Some works have been developed studying the geographic haplotypes that have resulted in subspecies of some insects (Hurtado-Burillo, May-Itzá, Quezada-Eúan, De la Rúa, & Ruiz, 2016; López-López, Hudson, & Galián, 2016). In Diptera, some studies of extensive geographical areas have been developed (Izumitani, Kusaka, Koshikawa, Toda, & Katoh, 2016; Pfeiler et al., 2013; Ruiz-Arce, Owen, Thomas, Barr, & McPheron, 2015), reaching to differentiate geographical areas based on haplotypes. The use of this technique focused in forensic entomology would be the recognition of a location place in where a entomological evidence were found (Alacs, Georges, FitzSimmons, & Robertson, 2009).

A more general use for the genetic information is the reconstruction of ancestral genetical states (Pagel, 1999). The biogeographic history of one species or a group of species could be studied with this technique (Blaimer et al., 2015; Bourguignon et al., 2016; Katoh, Izumitani, Yamashita, & Watada, 2016; Zaspel, Weller, Wardwell, Zahiri, & Wahlberg, 2014). The information about locations combined with this technique shows the permanence of a group in a geographical area. The relationship between this and the geological history or the influence of humans help us to know the background of a species to better understand its actual situation of it (Seabra et al., 2015).

Geometric morphometrics

Geometric morphometrics is a technique of quantitative analysis of shapes in two or three dimensional spaces (Bookstein, 1997). It is based in the visualization of shape changes measured with reference points called *landmarks* (Fig. 7). This points correspond to a particular traits present in all specimens and that follow the same

order in all of it (Zelditch, Swiderski, & Sheets, 2012). This type of analysis does not take into account the size, position or orientation, so only the shape is analysed (Dryden & Mardia, 2016). These analyses of morphological variation are used to answer an increasingly wide range of questions about the evolution and development of organisms (Klingenberg, 2010, 2013). The results of these analyses allows to the investigators to visualize the shape changes through statistical programs (Klingenberg, 2011) easier than in a table of results. It can be done showing the displacement and direction of each landmark or showing the deformation of a surface made with combination of landmarks (Klingenberg, 2011, 2013). Besides, when the shapes changes are not clear enough to be detected with previous methods, we can perform some algebraical analyses. The Principal Components Analysis (PCA) is used to determine the variable or variables in which the changes were happening and search the biological explanation of it. The Canonical Variate Analysis (CVA) is used to explore differences among groups (Zelditch et al., 2012). These analyses have been used to differentiate species or groups of species based in the shape of the wings (Aytekin, Terzo, Rasmont, & Çağatay, 2007; Cheverud, 2017; Roggero & Passerin d'Entrèves, 2005; Su, Guan, Wang, & Yang, 2015). This is the more used structure due to its two dimensional form and its easy isolation, but other structures can be used with good results (de Freitas & Morales, 2009; Gamboa & Arrivillaga, 2010). In different types of Diptera, this technique have been used producing good separation of species and populations (Espra et al., 2015; Grzywacz, Ogiela, & Tofilski, 2017).

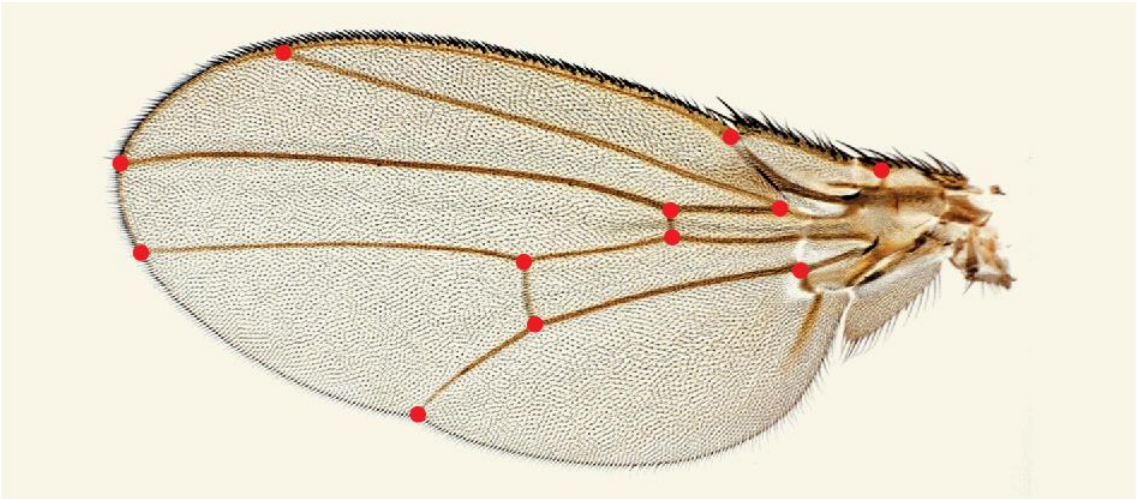

Figure 7. Example of landmarks position. From Cheverud (2017)

OBJETIVES

Objectives

Main objective and outlines of the thesis

The Main objective of this thesis is to apply DNA barcoding identification and other techniques to the families of necrophagous Diptera collected in an intensive survey of the Iberian Peninsula. This approach will allow: 1) to confirm the presence of the species found in previous works identified using morphological characters; 2) to add data for DNA taxonomy (phylogenetic analyses) of these cosmopolitan distributed species; 3) to evaluate mitochondrial and nuclear genes (COI, 16S and ITS2) as a tool for their use in identification of species to detect cryptic taxa; 4) to analyse the haplotypes and the ancestral states of widespread species in the Iberian Peninsula for obtaining phylogeographic data; 5) to analyse through geometric morphometric techniques anatomical structures of some species in which morphological and molecular differences are not clear enough.

Chapter I. Molecular identification of forensically important fly species in Spain using COI barcodes

The objective of this chapter is to use DNA barcoding to identify Diptera with forensic interest in Spain. These analyses will corroborate the identification based on morphology used for preliminary identification, as well as the different species delimitation methods. For this, the specific objectives are:

- To generate COI sequence data from forensically relevant fly species, collected in the Iberian Peninsula during the four seasons.
- To define the number of molecular operational taxonomic units (MOTUs) from carrion-associated flies including those species with difficult differentiation.
- To characterize the molecular diversity and knowledge of the necrophagous Dipteran fauna of the Iberian Peninsula, providing information for species identification in forensic entomology.

Chapter II. DNA-based and Taxonomic identification of forensically important Sarcophagidae (Diptera) in Southeastern Spain.

The objective of this chapter is to identify with molecular techniques specimens of the family Sarcophagidae collected in forensic cases occurred in Alicante (Comunidad Valenciana, southeastern Spain) and to compare these samples with others collected in this area and surrounding provinces with baited traps. Specific objectives are:

- To apply DNA barcoding techniques to this Diptera family.
- To compare the differences related to the species present in human bodies and those captured in nature in the same geographical area.
- To analyze with phylogeographic techniques the more widespread species searching for local haplotypes.
- To verify if this information can be useful in the calculation of the PMI compared to that made with species belonging to other families found in these forensic cases.

The molecular data generated in this paper will increase the available knowledge on the family Sarcophagidae in Spain and will provide essential valuable information for species identification in the future through barcoding techniques.

Chapter III. Diversity of the forensically important fly species *Calliphora vicina*, (Diptera: Calliphoridae) in the Iberian Peninsula using COI, 16S and ITS2 sequences.

The general goal of the chapter is to generate data that could be useful to determine the original location of a corpse that would had been moved after the crime. The objectives of this chapter are:

- To generate sequences of COI, 16S and ITS2 of *C. vicina* samples from Spain and Portugal, useful in species identification.
- To analyse the biodiversity of these genes to determinate local haplotypes
- To analyse ancestral states of these genes to determinate the plausible colonization of the Iberian Peninsula by this species.

Chapter IV. Species of the genus *Lucilia* (Diptera: Calliphoridae) with forensic interest in northern Spain: a molecular and morphogeometrical approach.

The general goal of this chapter is to check different methods to delimitate these closely related species. The specific objectives of this work are:

- To generate sequences of *cytochrome oxidase subunit one* (COI) and *Internal transcribed spacer 2* (ITS2) of *L. ampullacea*, *L. caesar* and *L. illustris* collected in northern Spain.
- To carry out molecular identification analyses and phylogeographic techniques.
- To analyse wings and heads of these species with geometric morphometric techniques to accurately recognize the species and searching for differences among populations.

CHAPTER I

Chapter I

Molecular identification of forensically important fly species in Spain using COI barcodes

Abstract

Species identification with DNA barcodes has been proven effective on different organisms and, particularly, has become a routinely and accurate tool in forensic entomology to study necrophagous Diptera species. In this study, we analysed 215 specimens belonging to 42 species of 17 genera, from nine different Diptera families. Flies were collected in 39 Spanish localities of the Iberian Peninsula sampled across three years in the four seasons. Intraspecific variation ranged from 0 to 2.46% whereas interspecific variation fluctuated from 3.07 to 14.59%, measuring 651 pb of the cytochrome oxidase subunit one (COI) gene. Neighbour-Joining analysis was carried out to investigate the molecular identification capabilities of the barcoding region, recovering almost all species as distinct monophyletic groups. The species groupings were generally consistent with the morphological and molecular identification. This work shows that the COI barcode is an appropriate marker for unambiguous identification of forensically important Diptera in Spain.

Key words: Cytochrome oxidase subunit one (COI), forensic entomology, Iberian Peninsula, necrophagous Diptera, species identification.

Introduction

Accurate identification of necrophagous Diptera found on corpses, together with the recording of abiotic factors (such as environmental temperature) and the knowledge of species-specific developmental rates, may lead to consistent estimation of the corpse's age and, thus, to specify the minimum post-mortem interval (PMI) (Amendt, Richards, Campobasso, Zehner, & Hall, 2011).

In decaying bodies, the minimum PMI can be calculated according to the identification and analysis of the community of Diptera present on the corpse. However, proper

species identification of flies is often difficult, as some forensically important species can be barely distinguished based on morphology, especially in the juvenile stages (Nelson, Wallman, & Dowton, 2007). Moreover, rearing the individuals collected in the crime scene to the adult stage is not always possible, and well preserved specimens are often not available (Harvey, Dadour, & Gaudieri, 2003; Zehner et al., 2004).

Molecular techniques are increasingly useful for dealing with those identification difficulties. They have the advantage of being able to make use of old or damaged samples (Wells & Stevens, 2008), immature life stages (Meiklejohn et al., 2012), or empty puparia (Mazzanti et al., 2010). They are also able to distinguishing between morphologically similar species (Meiklejohn, Wallman, & Dowton, 2011). Moreover, in practice, a DNA-based strategy for quick and accurate species identification is desirable, as not all the staff who work in police scientific units have been formally trained in the morphological identification of Diptera. In addition, the acquisition of general knowledge in molecular techniques and the use of databases are easier than acquiring sufficient experience as a taxonomist in a particular group of organisms. However, the molecular barcoding identification involves the comparison of sequences obtained from the samples with well-identified sequences available in public databases (Sonet et al., 2013). Therefore, the work of the molecular taxonomists is essential to provide the databases of pool of sequences with enough representativeness, without misidentification or sequencing errors (Wells & Stevens, 2008). If the biological groups are not well represented in molecular databases or the sequences that they contain are not well identified, molecular identification for forensic purposes, such as the PMI calculation would not have the desirable accuracy.

In recent years, many research has been carried out worldwide to contribute to the accurate identification of dipteran species of forensic interest. Studies has been developed in Asia (Kim et al., 2014; Park et al., 2009; Saigusa, Takamiya, & Aoki, 2005; Sharma, Singh, & Sharma, 2015; Tan et al., 2009, 2010), America (Koroiva, de Souza, Roque, & Pepinelli, 2018; Nakano & Honda, 2015; Oliveira et al., 2016; Yusseff-Vanegas & Agnarsson, 2017), Europe (Caine et al., 2009; Olekšáková, Žurovcová, Klimešová, Barták, & Šuláková, 2018; Oliveira, Farinha, Rebelo, & Dias, 2011) even Africa and Oceania (Harvey, Mansell, Villet, & Dadour, 2003). In these type of studies different

genes were analysed, either nuclear (Nelson, Wallman, & Dowton, 2008), mitochondrial (Boehme et al., 2012; Lessard, Wallman, & Dowton, 2009), or a combinations of both (Phayuhasena, Colgan, Kuvangkadilok, Pramual, & Baimai, 2010). The use of mitochondrial DNA, widely considered a valuable tool for this kind of analyses (Hebert, Cywinska, et al., 2003; Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Hebert, Ratnasingham, et al., 2003), is justified due to its easy isolation even from small or degraded samples (Waugh, 2007), and the amount of information that it provides for population genetics and evolutionary studies (Aly, 2014). DNA barcoding is based on sequencing of a standardized small fragment of the *cytochrome oxidase subunit one* (COI), known as the "DNA barcode", widely represented in public databases, as GenBank and BOLD (Barcode of Life Data), and already used to identify fly species (Nelson et al., 2007). In that sense the BIN (Barcode Index Number) tool was developed in the BOLD database (Ratnasingham & Hebert, 2013), and it has been proven useful for barcoding studies (Borges et al., 2016; Pohjoismäki, Kahanpää, & Mutanen, 2016). The BIN system assigns sequences to a sequence cluster that is then assigned a unique BIN identifier. This tool can be decisive in cases such as (1) to rename species of cryptic morphology; (2) to help to reliably assign an unknown sample to a species present in BOLD; and (3) to estimate the diversity of species in groups with an unknown taxonomic framework (Hausmann et al., 2013).

More than 200 species of sarcosaprophagous Diptera have been collected from the Iberian Peninsula in works that use animals as bait (Baz, Botías, Martín-Vega, Cifrián, & Díaz-Aranda, 2015; Farinha et al., 2014; Gaminde, 2015; Martín-Vega & Baz, 2013; Prado e Castro, Sousa, Arnaldos, Gaspar, & García, 2011; Rolo et al., 2013; Zabala, Díaz, & Saloña-Bordas, 2014; Zajac et al., 2016). A total of 44 species have been reported from human remains (Arnaldos, Romera, García, & Luna, 2001; Arnaldos, García, Romera, Presa, & Luna, 2005; Arnaldos, Luna, Presa, López-Gallego, & García, 2006; Arnaldos, Prado e Castro, Presa, Castro, & García, 2006; Arnaldos, Romera, Luna, Presa, & García, 2004; Arnaldos, Sánchez, Álvarez, & García, 2004; Arnaldos, Romera, García, & Luna, 2001; Cainé et al., 2006; Cainé et al., 2009; Couri, de Souza, Cunha, Pinheiro, & Cunha, 2008; GilArriortua, Saloña-Bordas, Cainé, Pinheiro, & de Pancorbo, 2013; González Medina et al., 2011; Mora & Perez, 1990; Prado e Castro et al., 2011; Prado e

Castro, Arnaldos, & García, 2010; Romera, Arnaldos, García, & González-Mora, 2003). This indicates that almost 25% of the species colonize human corpses in this area (Arnaldos et al., 2006). The scarceness of molecular data of necrophagous Diptera in Spain (Arnaldos, Ruiz, García, González-Mora, & Serrano, 2015) and the lack of broad sampling schemes makes it necessary to address this study, on the boundary between basic knowledge and its application to forensic investigations.

The aims of this work are (1) to generate COI sequence data from forensically relevant fly species, collected in the Iberian Peninsula during the four seasons, (2) to define the number of molecular operational taxonomic units (MOTUs) from carrion-associated flies including those species with difficult differentiation and (3) to characterize the molecular diversity and knowledge of the necrophagous Dipteran fauna of the Iberian Peninsula, providing information for species identification in forensic entomology. These analyses will corroborate the identification based on morphology used for preliminary identification, as well as the different species delimitation methods.

Material and methods

Fly collection and morphological identification

The 215 specimens analysed in this study were collected at 39 localities placing traps every 200 km in average, covering 37 provinces and all the Spanish Autonomous communities (Fig. 1.1). Sampling was carried out in all season of the year since 2012 until 2014, to capture the largest number of carrion-associated flies species from all Spain. Larvae and adults were wild-caught with bottle traps (Allemand & Aberlenc, 1991) baited with pig blood and liver. Traps were placed in peri-urban areas during 3-5 days. In each place, three traps were placed: two of them hanging to 1.5 meters and one semi-buried on the field. The captured adults were washed in 70% alcohol to remove dirtiness and bait. Then, samples were preserved in absolute ethanol. Some of the larvae were reared to adulthood using pig liver as substrate, to be used in other experiments. Adults were then identified based on external morphology to species, genera or family level and classified in morphospecies (Barrientos, 2004; Barros de Carvalho & de Mello-Patiu, 2008; Barták, Preisler, Kubík, Šuláková, & Sloup, 2016;

Marshall, Whitworth, & Roscoe, 2011; Pinto e Vairo et al., 2015; Szpila, 2012; Whitworth, 2006; Yang, Kurahashi, & Shiao, 2014). The level of identification depends on the samples preservation state. A subsample of each sampling site was selected in order to maximize the forensically important fly species diversity through space and time. Approximately half of the selected specimens were broken or dirty by the remnants of blood, in the same way that they would found in a forensic case.

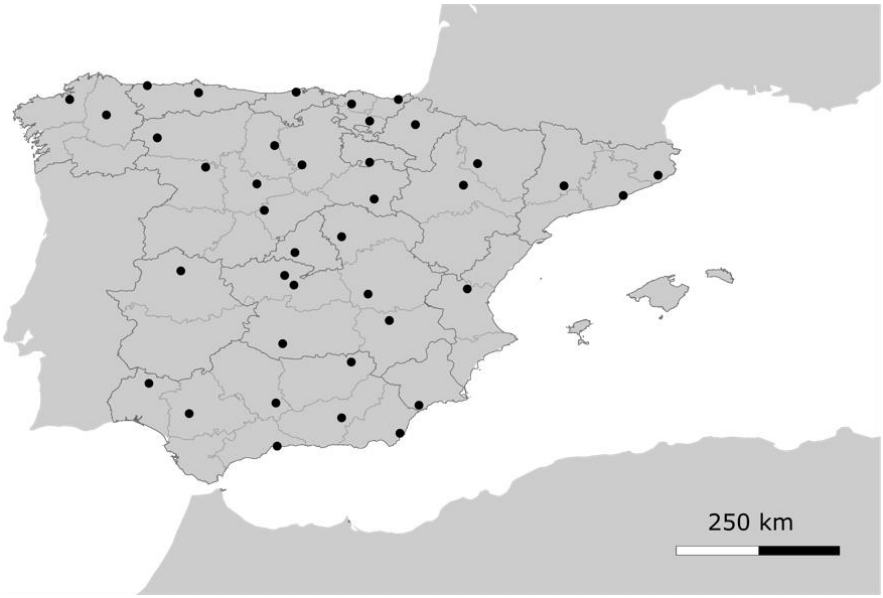

Figure 1.1. Chosen sampling localities in Spain.

DNA barcoding analysis

Genomic DNA was extracted from one or two legs and thorax muscles for genera of the families Calliphoridae and Sarcophagidae, or the whole specimen for genera of the remaining families due to their small size. It was used the Glass Fiber Plate DNA Extraction protocol of the CCDB (Ivanova, Dewaard, & Hebert, 2006). All extractions were diluted in 60 µL of ddH_2O prewarmed to 56°C.

Amplification of the COI barcoding region was performed using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') described by Folmer (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). Amplification was performed in a thermocycler 2720 Thermal cycler (Applied Biosystems, Foster City, U.S.A.). A KAPABIOSYSTEMS PCR kit (Wilmington, U.S.A.) including all components necessary for the PCR cocktail except

water, primers and the target DNA was used. The components of this cocktail were buffer (10x), dNTPs and Taq polymerase. The total reaction volume was 12.5 µl containing 1.5 µl of sample DNA for each reaction. The PCR program consists of an initial activation of 95 °C for 3 min, followed by 35 cycles of 60 sec at 94 °C, 60 sec at 45 °C, and 90 sec at 72 °C, and then a final extension of 5 min at 72 °C. After amplification, 2 µl of each PCR product was visualized by electrophoresis gel on a 2% agarose stained with RED SAFE (iNtRON Boitechnology, Seongnam, South Korea) dyed fluorescent product and visualized under ultraviolet light.

Samples were sequenced in Macrogen (Amsterdam, The Netherlands) using an ABI Prism 3730XL. Sequences were edited in MEGA7 (Kumar, Stecher, & Tamura, 2016). The obtained sequences have been deposited in GenBank (Accession numbers provided in Supplementary Table 1.1 (Appendix 1.1)) and uploaded in the project "Biodiversity of Diptera" within BOLD system.

We used the R package Spider version 1.3–0 (Brown et al., 2012) in R statistical software to check the absence of ambiguous codons, to calculate the base composition and the basic statistics.

Morphological identifications (IDs) were compared with molecular IDs using different approaches. First, the COI sequences were compared with Dipteran sequences on the NCBI using the BLAST tool (Altschul et al., 1997). Sequences with the lowest E-Value and higher grade of representative taxa were considered as BLAST_ID.

To estimate the number of molecular operational taxonomic units (MOTUs) from carrion-associated flies, three commonly used species delimitation methods were employed: Barcode Index Number (BINs) (Ratnasingham & Hebert, 2013), Automatic Barcode Gap Discovery (ABGD) (Puillandre, Lambert, Brouillet, & Achaz, 2012) and generalized mixed Yule-coalescent (GMYC) (Monaghan et al., 2009; Pons et al., 2006). BIN and ABGD were distance-based methods while GMYC is a coalescence tree-based approach. The congruence between the three methods can be a measure of the robustness of the resulting MOTUs due to their differing analytical approaches (Carstens, Pelletier, Reid, & Satler, 2013). Each MOTU was categorized into one of three categories, in order to examine the congruence of putative species across the different

delimitation methods: i) Match, when the particular MOTU was recognized by all three methods, ii) Partial match when not all the methods recognized this MOTU and iii) No match, when no congruence was obtained across methods.

BOLD generates automatically the Barcode Index Number (BINs) for every sequence using the "Refined Single Linkage (RESL) Analysis". This analysis consists in a three steps algorithm that cluster all the COI available sequences, using a fixed 2.2% threshold followed by Markov clustering, to improve the accuracy of the obtained clusters, and finally chooses the clustering result with the highest Silhouette score. BOLD consider a Discordant BIN when the information submitted for the sequences included conflict in some taxonomic level. When the information submitted and the BOLD information match, the BIN is considered as Concordant. At last, when BOLD assign a new BIN for a sequence that not match whit any existing BIN, it is considered Singleton. When discordant BINs were obtained the samples were revised for checking misidentification or contamination issues, to confirm the BINs results. This is the only method that provides us a putative identification of the species.

ABGD divides the data into groups based on the barcode gap and reanalyses the group obtained recursively using Markov clustering conducting a second round of splitting. ABGD analysis was carried out online (www.abi.snv.jussieu.fr/public/abgd/) with the default settings, by K2P and *p*-distance model with relative gap width (X = 1.5). The third method, the GMYC analysis required an ultrametric tree, therefore, sequences were collapsed to haplotypes with the web FaBox (http://users-birc.au.dk/biopv/php/fabox/). The best fitting model of evolution (GTR+I+G) was estimated with jModelTest2 (Darriba, Taboada, Doallo, & Posada, 2015). Ultrametric tree was obtained with BEAST v2.4.8 (Bouckaert et al., 2014) using a constant population size tree prior and strict clock, as both are considered more conservative option for generating the tree (Monaghan et al., 2009). MCMC chains were run for 20 million generations. Resulting trees were pooled removing 25 % of samples as initial burn-in, and consensus trees were obtained in LogCombiner 1.8.2 and TreeAnnotator 1.8.2 (Drummond & Rambaut, 2007). The GMYC analysis was done using the SPLITS v1.0-19 package (Ezard, Fujisawa, & Barraclough, 2009) implemented in R statistical software (R Development Core Team, 2012) with the single threshold option

(Michonneau, 2015). These methods provide us a list of MOTUs but no identification itself because do not compare the sequences with any database.

The sequence divergence was calculated using de "Barcode Gap Analysis (BGA)" tool on BOLD (Puillandre et al., 2012). The BGA relates the mean and maximum intraspecific variation and minimum genetic distance to the nearest-neighbor species. The distance among sequences of species belonging to the same family was calculated using TaxonDNA (SpeciesIdentifier 1.7.8-sy1) (Meier, Shiyang, Vaidya, & Ng, 2006).

Species whose BOLD identification resulted in discordant BINs, were analysed in detail using available sequences from BOLD (Appendix 1.2 (Supplementary Table 1.2) and Appendix 1.3 (Supplementary Table 1.3)) if them did not produce any discordance. A median Joining network was realized with PopArt program (Bandelt, Forster, & Röhl, 1999) to analyse the phylogeographic structure of the samples.

Sequences were aligned using MUSCLE (Edgar, 2004) and a Neighbor-Joining (NJ) tree was performed with sequences produced in this work with BOLD's tool, using the Kimura-2-Parameter (K2P) distance algorithm. Although this algorithm is being subject of controversy, in our conditions it is expected to produce results as valid as *p*-distances (Srivathsan & Meier, 2012). Two specimens of the family Anisopodidae were used as external group.

Results

A total of 651 nucleotides of the barcoding region were obtained for 215 specimens of 25 dipteran morphospecies of forensically important Brachycera of Spain plus a species of Nematocera (Anisopodidae, *Sylvicola fenestralis*), that are collected in this study too but do not have forensic interest. Specimens belonging to the families Calliphoridae, Sarcophagidae, Muscidae, Fanniidae, Drosophilidae, Anthomyiidae, Ulidiidae, Heleomyzidae and Phoridae could be identified, despite the difficulties of the conservation state of some samples. Specimens of family Calliphoridae were identified up to species level using morphology keys, except for one species that was identified up to family level. In the family Sarcophagidae the specimens of *Sarcophaga (Liosarcophaga) caerulescens*, *Sarcophaga (Hellicophagella) melanura*, *Sarcophaga*

(Liopygia) crassipalpis, *Sarcophaga (Liopygia) argyrostoma* and *Sarcophaga (Bercaea) africa* were identified up to species level with morphology keys. The remaining samples were identified based on external morphology up to family level, except for *Musca domestica*. Species, location, date of collection, accession number of NCBI and BIN from BOLD database are shown in Supplementary Table 1.1 (Appendix 1.1).

Collection in each season generally provided different outcomes. The specimens of family Sarcophagidae were collected mostly in summer (between June and September), except four specimens collected in May from south and centre of Spain. These species are presents in every geographical zone, although in the south we only collected *Sarcophaga lehmanni* and *S. africa*. Fanniidae, Musciadae, Ulidiidae and specimens of genus *Chrysomya*, *Pollenia* and *Lucilia* (Calliphoridae) were also collected in warm seasons. Drosophilidae, Heleomyzidae and Anthomyiidae were collected in cold seasons, mostly in southern areas. In the same way, specimens of the genus *Calliphora* were also collected in cold seasons, but *Calliphora vicina* was even collected in July and September in places that were still cold in those months. This species is present in almost all sampling places, as well as *Chrysomya albiceps* and *Lucilia sericata*, being these three species the most widely represented. The other species of the genus *Lucilia* are concentrated in the north area.

BLAST ID. Molecular identification with the BLAST tool corroborates the identification based on morphological characters. The species of Calliphoridae that could not be identified at species level was assigned to *Pollenia rudis* and *Pollenia* sp. Another eleven putative species were recognized for the family Sarcophagidae, seven species for the family Muscidae, three for Fanniidae, two for Drosophilidae and two more Anthomyiidae. For Heleomyzidae, Phoridae and Ulidiidae only one species was identified for each family.

The base composition of the matrix generated was: A=0.301, C=0.160, G=0.159 and T=0.379. The amino acid translation of the sequences showed no frameshift mutations, stop codons or unusually divergent amino acid profiles in the alignment. All the sequences of our dataset meet BOLD quality requirements, absence of stop codons and sequence length. The distance analysis showed an intraspecific distance between 0

– 2.46%. The interspecific distance range was between 3.07 – 14.59%. This shows a gap between the intraspecific and interspecific distances. The TaxonDNA stablished the intraspecific cutoff in 1.53%. Despite that distances were calculated giving the same name to MOTUs in where several morphospecies merge ("*Lucilia caesar/illustris*" group and "*Sarcophaga variegata/lehmanni*" group) some taxa (*Hydrotaea armipes*, "*Lucilia caesar/illustris*" group, *L. sericata* and *C. vicina* with 2.46%) have higher intraspecific distance. This is due to the fact that most species have a very low intraspecific distance, displacing the cutoff value.

DNA barcode-based identification

Of the species diversity collected in this work, 36 species (85.71%) have been reported in previous studies from the Iberian Peninsula in which traps baited with different tissues of vertebrates species were used (Baz et al., 2015; Farinha et al., 2014; Gaminde, 2015; Martín-Vega & Baz, 2013; Prado e Castro et al., 2011; Rolo et al., 2013; Zabala et al., 2014; Zajac et al., 2016). Half of them (18 species; 42.85%) have been reported on samples collected on human corpses (Arnaldos et al., 2001; Arnaldos et al., 2004; Arnaldos et al., 2005; Arnaldos et al., 2006; Cainé et al., 2006; Caine et al., 2009; Couri et al., 2008; GilArriortua et al., 2013; González Medina et al., 2011; Mora & Perez, 1990; Prado e Castro et al., 2010; Prado e Castro et al., 2011; Romera et al., 2003).

The three methods of species delimitation recovered almost identical results. The comparison was based on the BIN identification. They match in 44 MOTUs (97.78%) and only one partial match (2.22%) among them. There was no no-matches. ABGD and BINs recovered 45 MOTUs while GMYC recovered 46 clusters. The maximum number of specimens belonging to one MOTU was 38 (*C. albiceps*), while the median was five. There were 12 MOTUs only represented by one sequence (Table 1.1). If we take into account the preliminary identification, 84.4% (38 MOTUs) of them match with the MOTUs provided by the species delimitation methods. In three cases (6.67%) the preliminary identification produces a split: *L. sericata*, *Delia platura* and *C. vicina* (this last only for GMYC method). Two pairs of species merged together in same MOTUs each (8.93%).

Table 1.1. Correspondence between preliminary identification (morphology or BLAST) with BOLD Identification of forensically important fly species. Number of specimens for each species and Barcode Index Number (BINs) assigned by BOLD are given. Samples that have finally change the first identification are shaded.

Preliminary identification (Morphosp. or BLAST-ID)	Nº of samples	BINs	BOLD Identification
Calliphora vicina Robineau-Desvoidy, 1830	32	AAB6579	***Calliphora vicina***
Calliphora vomitoria (Linnaeus, 1758)	4	AAA8931	***Calliphora vomitoria***
Chrysomya albiceps (Wiedemann, 1819)	38	ABX6432	***Chrysomya albiceps***
Lucilia sericata (Meigen 1826)	34	AAA6618	***Lucilia sericata***
Lucilia sericata	1	ADF1790	***Lucilia* sp.**
Lucilia ampullacea Villenueve, 1922	4	AAC3450	***Lucilia ampullacea***
Lucilia caesar (Linnaeus, 1758)	12	AAA7470	***Lucilia caesar***
Luclia illustris (Meigen, 1826)	1	AAA7470	***Lucilia caesar***
Pollenia rudis (Fabricius, 1794)	1	AAI2766	***Pollenia griseotomentosa* (Jacentkovský, 1944)**
Pollenia rudis	1	ABY0051	***Pollenia* sp. Robineau-Desvoidy, 1830**
Pollenia sp.	1	AAH3035	***Pollenia rudis***
Hemilucilia semidiaphana (Rondani, 1850)	1	AAG2452	**Anthomyiidae sp.**
Delia platura (Meigen, 1826)	4	AAG2511	***Delia platura***
Delia platura	1	ADE9643	***Delia* sp.**
Sarcophaga (Liosarcophaga) marshalli (Parker, 1923)	8	ACB4485	***Sarcophaga marshalli***
Sarcophaga (Liosarcophaga) tibialis Macquart, 1851	2	ACB6283	***Sarcophaga tibialis***
Sarcophaga (Liosarcophaga) caerulescens (Zetterstedt, 1838)	4	ABZ2577	***Sarcophaga caerulescens***

Sarcophaga (Pandelleisca) similis Meade, 1876	2	ACB4532	***Sarcophaga similis***
Sarcophaga (Hellicophagella) melanura Meigen, 1826	1	ACB5109	***Sarcophaga melanura***
Sarcophaga (Liosarcophaga) teretirostris (Pandellé, 1896)	2	ACB4317	***Sarcophaga teretirostris***
Sarcophaga (Parasarcophaga) albiceps (Meigen, 1826)	1	AAE9461	***Sarcophaga albiceps***
Sarcophaga (Sarcophaga) lehmanni Mueller, 1922	3	AAZ0264	***Sarcophaga lehmanni***
Sarcophaga (Sarcophaga) variegata (Scopoli, 1963)	2	AAZ0264	***Sarcophaga variegata***
Sarcophaga (Liopygia) crassipalpis Macquart, 1839	4	AAC1709	***Sarcophaga crassipalpis***
Sarcophaga (Liopygia) cultellata Pandellé, 1896	1	ADF1212	***Sarcophaga cultellata***
Sarcophaga (Liopygia) argyrostoma (Robineau-Desvoidy, 1830)	4	AAI0975	***Sarcophaga argyrostoma***
Sarcophaga (Bercaea) africa (Weidemann, 1824)	3	AAC1710	***Sarcophaga africa***
Sarcophaga (Heteronychia) filia Rondani, 1860	2	ACB5207	***Sarcophaga filia***
Sarcophaga (Heteronychia) pandellei (Rohdenfort, 1837)	1	ADE9531	***Sarcophaga pandellei***
Sarcophaga (Hellicophagella) hirticrus Pandellé, 1896	2	ACB4527	***Sarcophaga hirticrus***
Fannia canicularis (Linnaeus, 1761)	4	AAF7101	***Fannia canicularis***
Fannia prisca Stein, 1918	1	ACY6258	***Fannia prisca***
Fannia monilis (Haliday, 1838)	2	AAD9983	***Fannia monilis***
Musca domestica Linnaeus, 1758	10	AAA6020	***Musca domestica***
Muscina prolapsa (Harris, 1780)	2	AAI3240	***Muscina prolapsa***
Muscina stabulans (Fallén, 1817)	1	AAM4634	***Muscina stabulans***
Ophyra leucostoma (Weidemann, 1817)	2	ABW3765	***Hydrotaea ignava* (Harris, 1780)**

Hydrotaea dentipes (Fabricius, 1805)	1	AAX2553	***Hydrotaea cyrtoneurina* (Zetterstendt, 1845)**
Hydrotaea armipes (Fallén, 1825)	4	AAG6908	***Hydrotaea armipes***
Helina evecta (Harris, 1780)	1	AAG1711	***Helina depuncta* (Fallén, 1825)**
Phaonia subventa (Harris, 1780)	3	AAG7029	***Phaonia subventa***
Drosophila simulans Sturtevant, 1919	2	AAE8098	***Drosophila simulans***
Drosophila funebris (Fabricius, 1787)	1	AAY0369	***Drosophila funebris***
Tephrochlamys rufiventris (Meigen, 1830)	2	ABX7666	***Tephrochlamys rufiventris***
Physiphora clausa Macquart, 1843	2	ABA5170	***Physiphora alceae* (Preyssler, 1791)**
Megaselia scalaris Loew, 1866	1	ADA4621	***Megaselia scalaris***
Sylvicola fenestralis (Scopoli, 1763)	2	AAG1996	***Sylvicola fenestralis***

ABGD analysis recovered 45 MOTUs. Almost all of them agree with the species recovered with NCBI identification (BLAST-ID). Species *L. sericata* and *D. platura,* identified by BLAST-ID, were grouped in two MOTUs. On the other hand, two pair of sister species identified by BLAST-ID (*Lucilia illustris-Lucilia caesar*; and *Sarcophaga variegata-Sarcophaga lehmanni*) were recovered in the same MOTU respectively.

BIN results grouped the sequences in the same MOTUs as ABGD. The RESL analysis recovered 17 concordant BINs, 23 discordant BINs and four singletons. The morphospecies *C. vicina* was recovered in only one BIN, not in agreement with the GMYC method. As with ABGD, couples of sister species *L. illustris-L. caesar* and *S. variegata-S. lehmanni* were recovered in the same BINs each (AAA7470 and AAZ0264 respectively). The morphospecies *D. platura* and *L. sericata* were recovered in two BINS each, as happened with ABGD and GMYC methods. The sequences of these species that form a MOTU in themselves were recovered as singletons BINs (ADF1790 and ADE9643). Seven of the discordant BINs contain mostly sequences that do not match

the BLAST-ID and four others could only be identified up to genus level (Table 1.4). GMYC analysis resulted in 46 MOTUs, what mostly agree with distance-based delimitation methods, however the morphospecies *C. vicina* was splitted in two MOTUs. Considering the results of the BINs with respect to the preliminary identification and the MOTUs generated by ABGD and GMYC, 17 MOTUs match with the BIN provided by BOLD, four MOTUs do not match with any BIN (so they were recognized as singletons) and 23 were discordant BINs, two of them include species that merge (*L. illustris-L. caesar* and *S. variegata-S. lehmanni*). The remaining 21, split preliminary groups in two or more clusters, some of them due to errors in our identification (shaded in Table 1.1).

The NJ tree (Fig. 1.2) resolved generally well-grouped species clades. The sister species *L. illustris* and *L. caesar* were grouped mixed in the same cluster, while *S. variegata* and *S. lehmanni* were grouped in two sister clusters very close to each other (0.61% of interspecific distance). Within *L. sericata* cluster, the sequence recovered as different MOTU by the three species delimitation methods (accession number KX161609) is located as a sister branch of the remaining sequences of the species. The same structure was observed in the cluster of *D. platura*. The sequences identified as genus *Pollenia* were grouped in three clusters that agrees with MOTUs delimitation. The rest of species were ordered in monophyletic groups coinciding with the recovered MOTUs, although not always coinciding with the identification of BOLD database, as we discuss below.

The network analysis of sister species *L. illustris-L. caesar* and *S. variegata-S. lehmanni*, including the sequences available in BOLD, showed the same pattern. In the BIN AAA7470, sequences of both *L. illustris-L. caesar* were present (275 of *L. caesar* and 126 of *L. illustris*). The haplotype network of both species resulted in two groups of haplotypes each, separated by three mutational steps that roughly corresponds to the expected species in both cases. However, there were some individuals placed in the opposite place according its identification between the two groups.

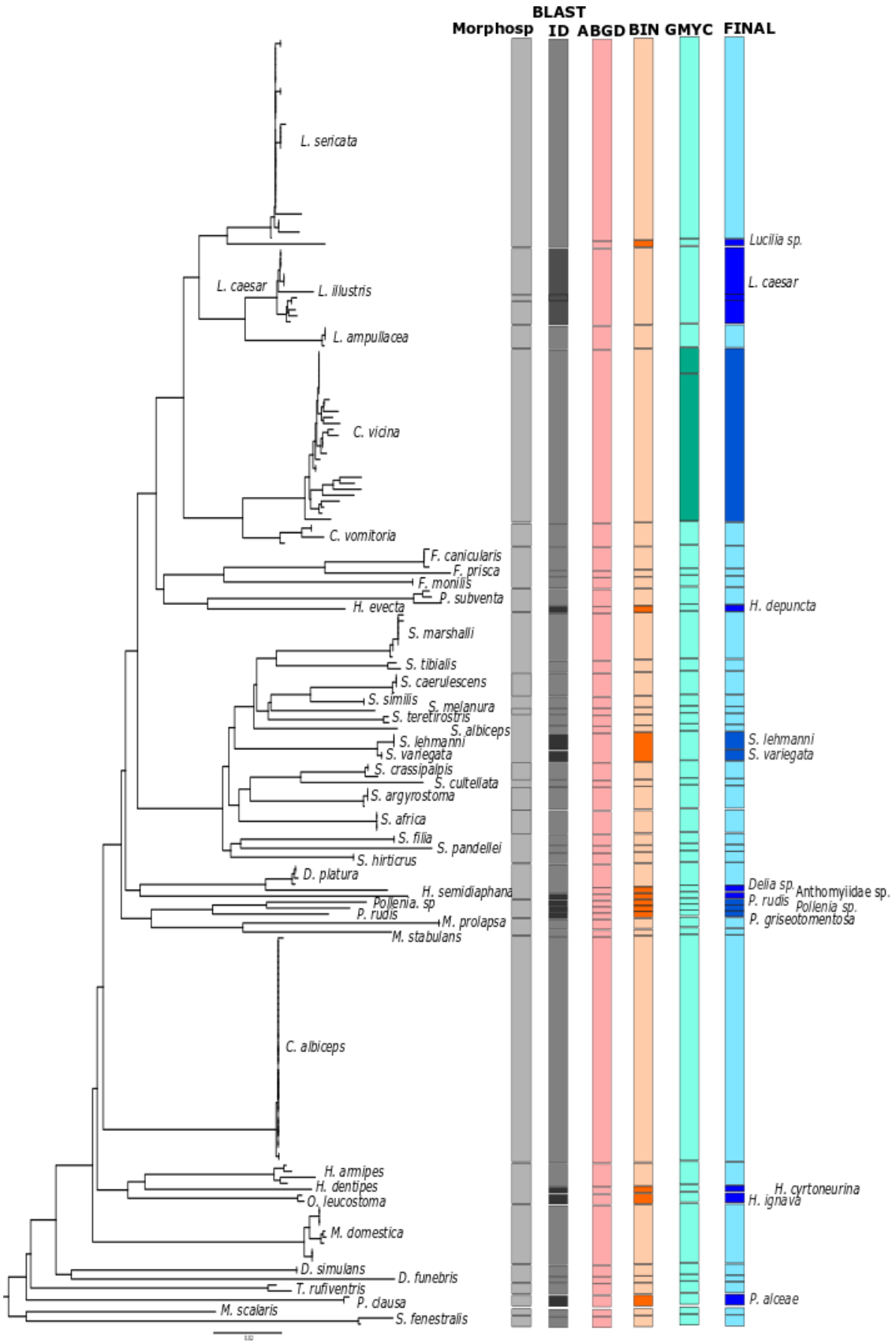

Figure 1.2. NJ tree of forensically important fly species. Bars show different ID and species delimitation methods. Discrepancies among them are showed shaded. Identification changes are shown in right margin.

Therefore, these sequences could have been miss-identified. All our sequences were located in the *L. caesar* group (Fig. 1.3). There was no geographic structure in the haplotype network.

Likewise, two groups of haplotypes separated by three mutational steps, define *S. variegata-S. lehmanni* species. Only two individuals identified as *S. lehmanni* were located in the *S. variegata* group. Our sequences were located in the two groups matching with the preliminary BLAST-ID. Both haplogroups were from individuals from western Europe, showing no geographic structure, although a certain geographic structure was observed within each haplogroup.

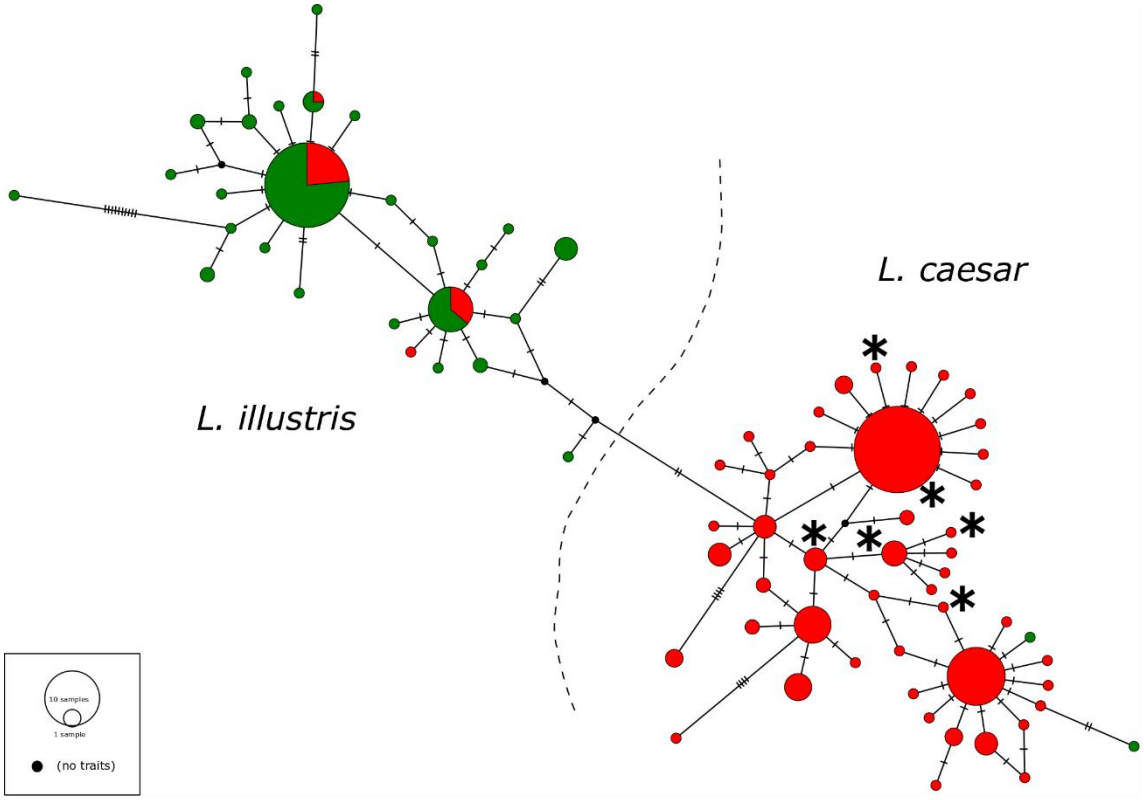

Figure 1.3. Haplotype network of L. caesar (red) and L. illustris (green). Asterisk indicates the haplotypes recovered in our sampling.

The geographical distribution of *S. lehmanni* presents a haplotype from the centre of Europe (Italy and Denmark), and two other haplotypes: one from France and other shared between France and our sequences from Spain (west Europe). For *S. variegata* the analysis shows several haplotypes from Germany, some of them shared with nearby countries and the unique sequence from Croatia. Belgium and France present their own haplotypes (Fig. 1.4).

Discussion

The present work extends the known distribution of forensically important Diptera in the Iberian Peninsula. For instance, for *S. africa* that has been collected previously in Málaga (Andalucía) (Velásquez, Gobbi, Martínez-Sánchez, & Rojo, 2015), in this work we add samples from Llagostera (Gerona) and Ponferrada (León), where it has not been reported so far. In the same way, we add samples of *S. argyrostoma* from Llagostera (Gerona), Coca (Segovia) and Almazán (Soria) to the known data (Yelitza Velásquez et al., 2015). Samples from Gerona and Toledo of *S. tibialis* and from Huesca and León of *S. teretirostris* were added too. Although our samples of *L. caesar* are from the north area, previously it has been reported in Murcia and Alicante (Saloña, Moneo, & Díaz, 2009; Velásquez et al., 2015). The distribution of the more widespread species (*C. vicina*, *L. sericata* and *C. albiceps*) agrees with previous works (Baz, Cifrián, Díaz-Aranda, & Martín-Vega, 2007; García-Rojo, 2004; GilArriortua et al., 2013; González Medina, González Herrera, Martínez Téllez, Archilla Peña, & Jiménez Ríos, 2011; Martínez-Sánchez, Smith, Rojo, Marcos-García, & Wall, 2007; Saloña et al., 2009; Velásquez et al., 2015) that demonstrated that they are present in all geographic areas. Our work helps to complete this with samples from some provinces absent so far. Other species present insufficient sequences to compare with other works.

All the methods for species delimitation show a good congruence among them, resulting in the same assignment for 97.78% of MOTUs. Only the cluster corresponding to *C. vicina* recovered by BIN and ABGD was splitted in two by the GMYC method. This could have happened because the GMYC method typically generates oversplit, producing more MOTUs than the other methods (Kekkonen & Hebert, 2014; Talavera, Dincă, & Vila, 2013).

There were several cases where species delimitation methods revealed cryptic diversity or misidentification of the sampling. Species identified as single morphospecies resulted in various MOTUs with the species delimitation methods. For example, sequences preliminary identified as *L. sericata* were grouped in two MOTUs by the three methods. In the NJ tree, both MOTUS were recovered as sister clusters. This agrees with previous studies that showed certain difficulties for the

molecular identification of this species (Debry, Timm, Dahlem, & Stamper, 2010; Harvey, Gaudieri, Villet, & Dadour, 2008). In the same way, sequence of *D. platura* were splitted into two MOTUs (Frey, Frey, & Baur, 2004). In both cases, this could be the result of a high intraspecific diversity resulting in two MOTUs or cryptic diversity not detected in the preliminary identification. Likewise, sequences of the genus *Pollenia* were split in three different MOTUs, that contrast with other works (Nelson et al., 2012; Rolo et al., 2013). Two of them were finally identified as *P. rudis* and *Pollenia griseotomentosa* when compared with BOLD databases. The remaining sequence assigned to another MOTU was named as *Pollenia* sp. These and other sequences of this work are influenced by the problems of using a single sequence per species (Wells, Wall, & Stevens, 2007). Further sampling to increase the size of the sample in a wide geographic range (Wells & Williams, 2007) will be necessary to detect COI paraphyly. The analysis of additional genes, distribution and natural history data are needed in order to explore this cryptic diversity in future works. In the same way, the sequences that produced the rest of discordant BINs were compared with the BOLD data, with molecular data of other related works and, whenever possible, with morphological data. We had into account geographical information (e. g, www.gbif.org) and possible synonyms (Barták et al., 2016; Frantisek, Rozkosny, Barták, & Vanhara, 2016; Galinskaya, Suvorov, Okun, & Shatalkin, 2014; Grzywacz, 2013; Grzywacz, Wallman, & Piwczyński, 2017; Jewiss-Gaines, Marshall, & Whitworth, 2012; Kim et al., 2014; Kutty, Pape, Pont, Wiegmann, & Meier, 2008; Marinho et al., 2012; Moon, 2002; Yang et al., 2014). All these discordant BINs were due to correctable errors: synonym names/erroneous information in BOLD, morphological misidentification due to the preservation conditions of samples or BINs shared by species with too low molecular distance. Therefore, the names of some of our sequences were modified accordingly, becoming them as concordant (match). After this process, almost all samples were identified, even those that were in poor state of conservation or broken. The only exceptions were the samples whose morphological identification was inaccurate and whose molecular identification was inconsistent (*Lucilia* sp., *Delia* sp., *Pollenia* sp. and Anthomyiidae sp.), which represents only the 1.86% of samples.

The sister species *L. illustris* and *L. caesar* were assigned to the same MOTU by the tree methods. This may be due to an error in the identification of *L. illustris*, which would be consistent with the actual distribution of both species, being *L. illustris* absent in the Iberian Peninsula to some authors (Velásquez, Gobbi, Martínez-Sánchez, & Rojo, 2015). The key taxonomic characters to differentiate between these sister-species (i.e. the shape of the hypopygium for males, the shape of the tergit VI for females, the numbers of setae on the arista or the colour of the legs) (Rognes, 1991; Szpila & Villet, 2011) are missing or difficult to observe in not well-preserved specimens, so molecular identification could help for these forensically important sister species. There have been previous studies showing low genetic divergences and shared haplotypes in mitochondrial and nuclear markers questioning the validity *L. caesar* and *L. illustris* (Reibe, Schmitz, & Madea, 2009; Sonet, Jordaens, Braet, & Desmyter, 2012; Williams, Lamb, & Villet, 2016). This is supported by the RESL analysis that recovered both taxa in the same BIN (AAA7470). However, other studies have found genetic differences between both species, supporting the validity of both species (Boehme et al., 2012; GilArriortua, Saloña-Bordas, Cainé, Pinheiro, & de Pancorbo, 2015; Picard et al., 2018). Despite the low interspecific distance between both species (0.01036%), our COI network analysis showed low percentage of individual misplacement (0.06% within *L. illustris* and 0.005% within *L. caesar*) what could be explained by misidentification or introgressive hybridization. An integrative taxonomy study is needed to clarify the status of both species. There is no geographical structure, samples from Europe, Asia and America share haplotypes. Therefore, although this MOTU is shared by sequences of both species, the haplotypes analysis can help to clarify the identification. It would be necessary to get more morphological information. All our sequences will be named as *L. caesar*.

Similar results were obtained with the sister species *S. variegata* and *S. lehmanni*, a pair of sister species that also are difficult to differentiate morphologically (Szpila, Mądra, Jarmusz, & Matuszewski, 2015; Szpila, Richet, & Pape, 2015) and that show low interspecific distance (0.61%). Both species were recover in the same MOTU in all the species delimitation methods. The BIN AAZ0264 included sequences of both species (76 sequences of *S. variegate* and seven of *S. lehmanni*). Also, these species are part of

the same clade in different phylogenetic studies (Buenaventura & Pape, 2017; Jordaens, Sonet, Richet, et al., 2013). The molecular distance between them falls below the limit suggested by several authors (Hebert et al., 2003; Hebert et al., 2004; Meiklejohn et al., 2011). However, the network analysis shows two distinct haplotypes groups between both species separated by three mutational steps. Only two sequences (0.02%) identified as *S. lehmanni* from France (GBPD12557 and GBDP12559, # in Fig. 1.4) were misplaced in the *S. variegata* group, what could be the result of a misidentification. Moreover, a phylogeographic structure was recovered in the haplotype network when both groups of haplotypes were considered, recovering samples of the same geographic origin for both species in different haplotype groups. Those results are congruent with a recent speciation event for both species as previous studies has pointed out (Buenaventura & Pape, 2017; Jordaens, Sonet, Richet, et al., 2013; Zhang, Buenaventura, Pape, & Zhang, 2016).

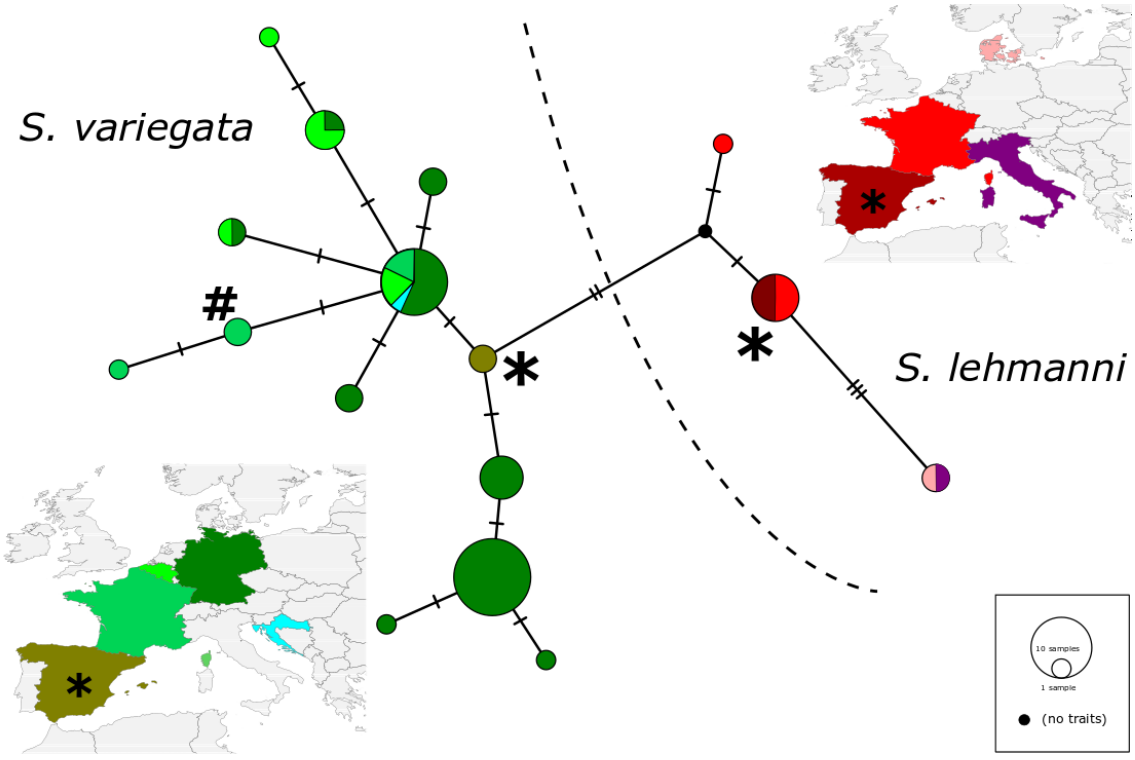

Figure 1.4. Haplotype network of S. variegata (greenish colours) and S. lehmanni (reddish colours). Asterisk indicates the haplotypes recovered in our sampling, Sequences located in an unexpected place according to its identification in database are indicated by hashtag. The haplotype colour indicates country of origin of the sequence.

Our results show that DNA barcoding and the species delimitation methods are important tools in forensic entomology, identifying damaged/poorly-preserved samples and characterizing the diversity of Iberian forensically important fly species. This work provides 215 new sequences to the BOLD and NCBI databases. These data will provide better identification in future works carried out in the Iberian Peninsula.

Appendices

Appendix 1.1. Supplementary Table 1.1: Locality, GenBank reference, BIN assigned by BOLD and final identification of all new sequences of the present study.

FAMILY	Morphospecies	BLAST-ID	NCBI ACCESION No.	LOCATION	Collection Date	BOLD BIN	FINAL IDENTIFICATION
Calliphoridae	***Calliphora vicina***	***Calliphora vicina***	KX161581	Almazán (Soria)	12/3/14		***Calliphora vicina***
			KX161582	Almazán (Soria)	12/3/14		
			KX161588	Almazán (Soria)	4/11/13		
			KX161580	Barañain (Navarra)	12/3/14		
			KX161567	Dos Hermanas (Sevilla)	3/3/13		
			KX161570	Guadix (Granada)	2/2/13		
			KX161565	Huesca (Huesca)	22/3/13	AAB6579	
			KX161572	Jabugo (Huelva)	3/3/13		
			KX161568	La Roda (Albacete)	3/2/13		
			KX161590	La Roda (Albacete)	9/9/12		
			KX161573	Lerma (Burgos)	20/2/14		
			KX161583	Lubiano (Álava)	3/11/13		
			KX161574	Lugo (Lugo)	14/3/14		
			KX161591	Navia (Asturias)	14/5/12		

			KX161577	Osorno (Palencia)	15/2/13		
			KX161586	Osorno (Palencia)	6/7/12		
			KX161569	Oviedo (Asturias)	12/3/14		
			KX161578	Oviedo (Asturias)	13/3/14		
			KX161571	Plasencia (Cáceres)	3/3/13		
			KX161589	Ponferrada (León)	19/9/12		
			KX161575	Rincón de la Victoria (Málaga)	21/2/14		
			KX161584	San Sebastián (Guipúzcoa)	12/3/14		
			KX161587	San Sebastián (Guipúzcoa)	3/11/13		
			KX161579	Santander (Cantabria)	20/2/14		
			KX161576	Toledo (Toledo)	16/2/13		
			KX161585	Villanueva de Cameros (La Rioja)	12/3/14		
			KX161564	Villar del Arzobispo (Valencia)	23/3/13		
			KX161563	Zaragoza (Zaragoza)	1/2/13		
			KX161566	Zaragoza (Zaragoza)	1/2/13		
	Calliphora vomitoria	***Calliphora vomitoria***	KX161559	Almazán (Soria)	3/11/13		***Calliphora vomitoria***
			KX161561	Betanzos (A Coruña)	2/3/13	AAA8931	
			KX161560	Villanueva de Cameros (La Rioja)	3/11/13		
			KX161562	Villanueva de Cameros (La Rioja)	12/3/14		

	Lucilia sericata	***Lucilia sericata***	KX161614	Almazán (Soria)	23/6/12		***Lucilia sericata***
			KX161632	Almazán (Soria)	23/6/12		
			KX161643	Baena (Córdoba)	7/7/12		
			KX161623	Badalona (Barcelona)	8/6/12		
			KX161631	Badalona (Barcelona)	21/7/12		
			KX161633	Barañain (Navarra)	7/9/12		
			KX161638	Barañain (Navarra)	23/6/12		
			KX161626	Benavente (Zamora)	19/9/12		
			KX161627	Benavente (Zamora)	19/9/12		
			KX161610	Cabezón de Pisuerga (Valladolid)	7/7/12		
			KX161637	Cabezón de Pisuerga (Valladolid)	6/7/12		
			KX161628	Coca (Segovia)	6/7/12		
			KX161640	Coca (Segovia)	6/7/12		
			KX161616	Huesca (Huesca)	21/7/12		
			KX161617	Huesca (Huesca)	21/7/12		
			KX161620	Huesca (Huesca)	21/7/12		
			KX161641	Huesca (Huesca)	21/7/12		
			KX161635	La Roda (Albacete)	24/6/12		
			KX161639	La Roda (Albacete)	24/6/12		
			KX161625	Lerma (Burgos)	6/7/12	AAA6618	

			KX161634	Llagostera (Gerona)	8/6/12		
			KX161636	Llagostera (Gerona)	21/7/12		
			KX161612	Ponferrada (León)	14/5/12		
			KX161613	Ponferrada (León)	19/9/12		
			KX161619	Puertollano (Ciudad Real)	28/4/12		
			KX161629	Puertollano (Ciudad Real)	28/4/12		
			KX161618	Rincón de la Victoria (Málaga)	28/4/12		
			KX161630	Rincón de la Victoria (Málaga)	21/2/14		
			KX161615	Tarancón (Cuenca)	24/6/12		
			KX161622	Tarancón (Cuenca)	24/6/12		
			KX161611	Torrijos (Toledo)	27/4/12		
			KX161624	Torrijos (Toledo)	27/4/12		
			KX161621	Zaragoza (Zaragoza)	8/6/12		
			KX161642	Zaragoza (Zaragoza)	8/6/12		
			KX161609	Lerma (Burgos)	6/7/12	ADF1790	***Lucilia* sp.**
	Lucilia ampullacea	***Lucilia ampullacea***	KX161594	Betanzos (A Coruña)	19/9/12		***Lucilia ampullacea***
			KX161595	Navia (Asturias)	19/9/12	AAC3450	
			KX161592	San Sebastián (Guipúzcoa)	7/9/12		
			KX161593	San Sebastián (Guipúzcoa)	23/6/12		

Lucilia caesar	***Lucilia caesar***	KX161600	Arrigorriaga (Vizcaya)	7/9/12		***Lucilia caesar***
		KX161608	Arrigorriaga (Vizcaya)	23/6/12		
		KX161596	Betanzos (A Coruña)	19/9/12		
		KX161603	Betanzos (A Coruña)	14/5/12		
		KX161601	Lugo (Lugo)	14/5/12		
		KX161604	Oviedo (Asturias)	19/9/12		
		KX161605	Oviedo (Asturias)	19/9/12	AAA7470	
		KX161598	Ponferrada (León)	14/5/12		
		KX161597	Santander (Cantabria)	6/7/12		
		KX161606	Santander (Cantabria)	6/7/12		
		KX161602	Villanueva de Cameros (La Rioja)	7/9/12		
		KX161607	Villanueva de Cameros (La Rioja)	7/9/12		
Lucilia illustris	***Lucilia illustris***	KX161599	Betanzos (A Coruña)	19/9/12		
Chrysomya albiceps	***Chrysomya albiceps***	KX161533	Baena (Córdoba)	7/7/12		***Chrysomya albiceps***
		KX161535	Baena (Córdoba)	7/7/12		
		KX161542	Baena (Córdoba)	7/7/12	ABX6432	
		KX161527	Benavente (Zamora)	19/9/12		
		KX161530	Benavente (Zamora)	19/9/12		
		KX161531	Benavente (Zamora)	19/9/12		

			KX161548	Benavente (Zamora)	19/9/12			
			KX161539	Brunete (Madrid)	6/7/12			
			KX161544	Brunete (Madrid)	6/7/12			
			KX161553	Cabezón de Pisuerga (Valladolid)	6/7/12			
			KX161528	Coca (Segovia)	6/7/12			
			KX161537	Coca (Segovia)	6/7/12			
			KX161543	Coca (Segovia)	6/7/12			
			KX161558	Coca (Segovia)	6/7/12			
			KX161526	La Roda (Albacete)	24/6/12			
			KX161554	La Roda (Albacete)	24/6/12			
			KX161549	Lerma (Burgos)	6/7/12			
			KX161557	Llagostera (Gerona)	21/7/12			
			KX161534	Lubiano (Álava)	7/9/12			
			KX161536	Osorno (Palencia)	6/7/12			
			KX161522	Ponferrada (León)	19/9/12			
			KX161529	Ponferrada (León)	19/9/12			
			KX161545	Ponferrada (León)	14/5/12			
			KX161546	Ponferrada (León)	19/9/12			
			KX161523	San Sebastián (Guipúzcoa)	7/9/12			
			KX161532	San Sebastián (Guipúzcoa)	7/9/12			

			KX161556	San Sebastián (Guipúzcoa)	7/9/12		
			KX161538	Santander (Cantabria)	6/7/12		
			KX161550	Santander (Cantabria)	6/7/12		
			KX161552	Santander (Cantabria)	6/7/12		
			KX161555	Santander (Cantabria)	6/7/12		
			KX161551	Tàrrega (Lérida)	21/7/12		
			KX161521	Valdenoches (Guadalajara)	7/9/12		
			KX161524	Valdenoches (Guadalajara)	7/9/12		
			KX161540	Valdenoches (Guadalajara)	7/9/12		
			KX161547	Valdenoches (Guadalajara)	7/9/12		
			KX161541	Villanueva de Cameros (La Rioja)	23/6/12		
			KX161525	Zaragoza (Zaragoza)	8/6/12		
	Anthomyiidae	***Hemilucilia semidiaphana***	KX161644	Carboneras (Almería)	2/2/13	-	**Anthomyiidae sp.**
	Calliphoridae	***Pollenia rudis***	KX161505	Barañain (Navarra)	7/9/12	AAI2766	***Pollenia griseotomentosa***
			KX161506	La Roda (Albacete)	8/9/12	ABY0051	***Pollenia* sp.**
		***Pollenia* sp.**	KX161507	Barañain (Navarra)	23/6/12	AAH3035	***Pollenia rudis***
Sarcophagidae	***Sarcophaga africa***	***Sarcophaga africa***	KX161469	Llagostera (Gerona)	21/7/12		***Sarcophaga africa***
			KX161466	Ponferrada (León)	19/9/12	AAC1710	
			KX161467	Rincón de la Victoria (Málaga)	28/4/12		

		KX161468	Rincón de la Victoria (Málaga)	28/4/12		
Sarcophaga melanura	***Sarcophaga melanura***	KX161497	Lerma (Burgos)	6/7/12	ACB5109	***Sarcophaga melanura***
Sarcophaga caerulescens	***Sarcophaga caerulescens***	KX161503 KX161502 KX161504 KX161501	Arrigorriaga (Vizcaya) Betanzos (A Coruña) Betanzos (A Coruña) San Sebastián (Guipúzcoa)	7/9/12 19/9/12 19/9/12 7/9/12	ABZ2577	***Sarcophaga caerulescens***
Sarcophaga argyrostoma	***Sarcophaga argyrostoma***	KX161473 KX161472 KX161470 KX161471	Almazán (Soria) Coca (Segovia) Llagostera (Gerona) Zaragoza (Zaragoza)	23/6/12 6/7/12 8/6/12 8/6/12	AAI0975	***Sarcophaga argyrostoma***
Sarcophaga crassipalpis	***Sarcophaga crassipalpis***	KX161475 KX161476 KX161477	Coca (Segovia) Lerma (Burgos) Tàrrega (Lérida)	6/7/12 6/7/12 21/7/12	AAC1709	***Sarcophaga crassipalpis***
Sarcophagidae	***Sarcophaga pandellei***	KX161450	Cabezón de Pisuerga (Valladolid)	6/7/12	ADE9531	***Sarcophaga pandellei***
	Sarcophaga filia	KX161464 KX161465	Tàrrega (Lérida) Zaragoza (Zaragoza)	21/7/12 8/6/12	ACB5207	***Sarcophaga filia***

Sarcophaga variegata	KX161478 KX161479	Betanzos (A Coruña) San Sebastián (Guipúzcoa)	19/9/12 7/9/12	AAZ0264	***Sarcophaga variegata***
Sarcophaga lehmanni	KX161481 KX161480 KX161482	Barañain (Navarra) La Roda (Albacete) La Roda (Albacete)	23/6/12 24/6/12 24/6/12	AAZ0264	***Sarcophaga lehmanni***
Sarcophaga tibialis	KX161494 KX161493	Llagostera (Gerona) Torrijos (Toledo)	21/7/12 27/4/12	ACB6283	***Sarcophaga tibialis***
Sarcophaga marshalli	KX161486 KX161488 KX161491 KX161489 KX161485 KX161487 KX161492 KX161490	Badalona (Barcelona) Badalona (Barcelona) Barañain (Navarra) Cabezón de Pisuerga (Valladolid) Huesca (Huesca) Huesca (Huesca) Lerma (Burgos) Tàrrega (Lérida)	8/6/12 21/7/12 7/9/12 6/7/12 21/7/12 21/7/12 6/7/12 8/6/12	ACB4485	***Sarcophaga marshalli***
Sarcophaga teretirostris	KX161496 KX161495	Huesca (Huesca) Ponferrada (León)	8/6/12 19/9/12	ACB4317	***Sarcophaga teretirostris***

		Sarcophaga albiceps	KX161498	Badalona (Barcelona)	8/6/12	AAE9461	***Sarcophaga albiceps***
		Sarcophaga similis	KX161499 KX161500	Lubiano (Álava) Lubiano (Álava)	23/6/12 23/6/12	ACB4532	***Sarcophaga similis***
		Sarcophaga cultellata	KX161474	Torrijos (Toledo)	27/4/12	ADF1212	***Sarcophaga cultellata***
		Sarcophaga hirticrus	KX161483 KX161484	Huesca (Huesca) Llagostera (Gerona)	8/6/12 8/6/12	ACB4527	***Sarcophaga hirticrus***
Muscidae	***Musca domestica***	***Musca domestica***	KX161459 KX161460 KX161455 KX161463 KX161456 KX161454 KX161458 KX161457 KX161461 KX161462	Almazán (Soria) Almazán (Soria) Baena (Córdoba) Benavente (Zamora) Brunete (Madrid) Huesca (Huesca) Lerma (Burgos) Tàrrega (Lérida) Valdenoches (Guadalajara) Valdenoches (Guadalajara)	23/6/12 23/6/12 7/7/12 19/9/12 6/7/12 21/7/12 6/7/12 21/7/12 7/9/12 7/9/12	AAA6020	***Musca domestica***
	Muscidae	***Muscina stabulans***	KX161453	Lubiano (Álava)	7/9/12	AAM4634	***Muscina stabulans***
		Muscina prolapsa	KX161448 KX161449	Osorno (Palencia) San Sebastián (Guipúzcoa)	6/7/12 7/9/12	AAI3240	***Muscina prolapsa***

		Hydrotaea armipes	KX161513 KX161512 KX161510 KX161511	Arrigorriaga (Vizcaya) Cabezón de Pisuerga (Valladolid) San Sebastián (Guipúzcoa) Santander (Cantabria)	23/6/12 6/7/12 7/9/12 6/7/12	AAG6908	***Hydrotaea armipes***
		Hydrotaea dentipes	KX161514	Santander (Cantabria)	6/7/12	AAX2553	***Hydrotaea cyrtoneurina***
		Ophyra leucostoma	KX161509 KX161508	Cabezón de Pisuerga (Valladolid) Llagostera (Gerona)	6/7/12 8/6/12	ABW3765	***Hydrotaea ignava***
		Phaonia subventa	KX161445 KX161446 KX161447	Oviedo (Asturias) Betanzos (A Coruña) Betanzos (A Coruña)	19/9/12 19/9/12 19/9/12	AAG7029	***Phaonia subventa***
		Helina evecta	KX161520	Barañain (Navarra)	7/9/12	AAG1711	***Helina depuncta***
Fanniidae	**Fanniidae**	***Fannia monilis***	KX161451 KX161452	Arrigorriaga (Vizcaya) Barañain (Navarra)	23/6/12 23/6/12	AAD9983	***Fannia monilis***
		Fannia canicularis	KX161441 KX161442 KX161443 KX161444	Tàrrega (Lérida) Oviedo (Asturias) Llagostera (Gerona) Lerma (Burgos)	8/6/12 19/9/12 21/7/12 6/7/12	AAF7101	***Fannia canicularis***
		Fannia prisca	KX161440	Lubiano (Álava)	23/6/12	ACY6258	***Fannia prisca***
Drosophilidae	**Drosophilidae**	***Drosophila simulans***	KX161438	Águilas (Murcia)	3/2/13	AAE8098	***Drosophila simulans***

			KX161439	Águilas (Murcia)	3/2/13		
		Drosophila funebris	KX161432	La Roda (Albacete)	24/6/12	AAY0369	***Drosophila funebris***
Anthomyiidae	**Anthomyiidae**	***Delia platura***	KX161517	Arroyo del Ojanco (Jaén)	2/2/13	AAG2511	***Delia platura***
			KX161518	Arroyo del Ojanco (Jaén)	2/2/13		
			KX161516	Carboneras (Almería)	2/2/13		
			KX161519	La Roda (Albacete)	24/6/12		
			KX161515	Osorno (Palencia)	6/7/12	ADE9643	***Delia* sp.**
Ulidiidae	**Ulidiidae**	***Physiphora clausa***	KX161433	Coca (Segovia)	6/7/12	ABA5170	***Physiphora alceae***
			KX161434	Huesca (Huesca)	21/7/12		
Heleomyzidae	**Heleomyzidae**	***Tephrochlamys rufiventris***	KX161435	Guadix (Granada)	2/2/13	ABX7666	***Tephrochlamys rufiventris***
			KX161436	Guadix (Granada)	2/2/13		
Phoridae	**Phoridae**	***Megaselia scalaris***	KX161437	Badalona (Barcelona)	22/3/13	ADA4621	***Megaselia scalaris***
Anisopodidae	**Anisopodidae**	***Sylvicola fenestralis***	KX161430	Navia (Asturias)	2/3/13	AAG1996	***Sylvicola fenestralis***
			KX161431	Santander (Cantabria)	15/2/13		

Appendix 1.2. Supplementary Table 1.2: Code assigned by BOLD, location of the samples and recognized species used in phylogeographic analysis of L. caesar and L. illustris.

BOLD code	Location	Species
CNIVD022-14	Canada, Yokun, Ivvavik Nat. Park	*L. illustris*
DWF001-13	Portugal	*L. caesar*
DWF045-13	Portugal	*L. caesar*
DWF046-13	Portugal	*L. caesar*
DWF047-13	Portugal	*L. caesar*
DWF048-13	Portugal	*L. caesar*
DWF049-13	Portugal	*L. caesar*
DWF050-13	Portugal	*L. caesar*
DWF051-13	Portugal	*L. caesar*
DWF052-13	Portugal	*L. caesar*
DWF053-13	Portugal	*L. caesar*
DWF054-13	Portugal	*L. caesar*
DWF055-13	Portugal	*L. caesar*
DWF056-13	Portugal	*L. caesar*
DWF057-13	Portugal	*L. caesar*
DWF058-13	Portugal	*L. caesar*
DWF087-13	Portugal	*L. caesar*
GBDP0976-06	UK, South Kensington, London	*L. caesar*
GBDP0977-06	UK, South Kensington, London	*L. caesar*
GBDP0978-06	UK, South Kensington, London	*L. caesar*
GBDP0979-06	UK, South Kensington, London	*L. caesar*
GBDP0980-06	UK, South Kensington, London	*L. caesar*
GBDP0986-06	UK, South Kensington, London	*L. illustris*
GBDP0987-06	UK, South Kensington, London	*L. illustris*
GBDP0988-06	UK, South Kensington, London	*L. illustris*
GBDP0989-06	UK, South Kensington, London	*L. illustris*
GBDP0990-06	UK, South Kensington, London	*L. illustris*
GBDP0991-06	UK, South Kensington, London	*L. illustris*
GBDP0992-06	UK, South Kensington, London	*L. illustris*
GBDP0993-06	UK, South Kensington, London	*L. illustris*
GBDP0994-06	UK, South Kensington, London	*L. illustris*
GBDP12943-13	UUEE, Winona, MN	*L. illustris*
GBDP12944-13	UUEE, Trail, ND	*L. illustris*
GBDP12945-13	UUEE, Onondaga, NY	*L. illustris*
GBDP15923-15	Canada	*L. illustris*
GBDP15924-15	Canada	*L. illustris*
GBDP16417-15	France	*L. caesar*
GBDP16418-15	France	*L. caesar*
GBDP16529-15	UUEE	*L. illustris*
GBDP16530-15	Japan	*L. illustris*
GBDP16531-15	Japan	*L. illustris*
GBDP2286-06	India	*L. illustris*
GBDP4485-08	France	*L. illustris*
GBDP4655-08	-	*L. illustris*
GBDP5800-09	-	*L. illustris*
GBDP7655-09	UUEE	*L. illustris*

GBDP7668-09	UUEE	*L. illustris*
GBDP7669-09	UUEE	*L. illustris*
GBDP7670-09	UUEE	*L. illustris*
GBDP7671-09	UUEE	*L. illustris*
GBDP7899-09	Korea	*L. illustris*
GBDP7900-09	Korea	*L. illustris*
GBDP7901-09	Korea	*L. illustris*
GBDP7902-09	Korea	*L. illustris*
GBDP7903-09	Korea	*L. illustris*
GBDP7904-09	Korea	*L. illustris*
GBDP7905-09	Korea	*L. illustris*
GBDP7906-09	Korea	*L. illustris*
GBDP7907-09	Korea	*L. illustris*
GBDP9379-11	Germany	*L. illustris*
GBDP9380-11	Germany	*L. illustris*
GBDP9381-11	Germany	*L. illustris*
GBDP9382-11	Germany	*L. illustris*
GBDP9383-11	Germany	*L. caesar*
GBDP9384-11	Germany	*L. caesar*
GBDP9385-11	Germany	*L. caesar*
GBDP9386-11	Germany	*L. caesar*
GBMIN26196-13	UUEE	*L. illustris*
GBMIN26197-13	UUEE	*L. illustris*
GBMIN26198-13	UUEE	*L. illustris*
GBMIN26199-13	UUEE	*L. illustris*
GBMIN26200-13	UUEE	*L. illustris*
GBMIN26201-13	UUEE	*L. illustris*
GBMIN26202-13	UUEE	*L. illustris*
GBMIN26237-13	UUEE	*L. illustris*
GBMIN26238-13	UUEE	*L. illustris*
GBMIN26239-13	UUEE	*L. illustris*
GBMIN26240-13	UUEE	*L. illustris*
GBMIN26241-13	UUEE	*L. illustris*
GBMIN53629-17	Italy	*L. caesar*
GBMIN53634-17	Italy	*L. caesar*
GBMIN53638-17	Italy	*L. caesar*
MHFLY030-06	Canada, Manitoba, Churchill	*L. caesar*
LUCIL003-12	Belgium	*L. caesar*
LUCIL004-12	Germany	*L. caesar*
LUCIL005-12	Germany	*L. caesar*
LUCIL006-12	Germany	*L. caesar*
LUCIL007-12	Germany	*L. caesar*
LUCIL008-12	Germany	*L. caesar*
LUCIL009-12	Germany	*L. caesar*
LUCIL010-12	Belgium	*L. caesar*
LUCIL011-12	Germany	*L. caesar*
LUCIL012-12	Germany	*L. caesar*
LUCIL014-12	France	*L. caesar*
LUCIL015-12	France	*L. caesar*
LUCIL016-12	France	*L. caesar*

LUCIL017-12	UK	*L. caesar*
LUCIL018-12	UK	*L. caesar*
LUCIL019-12	UK	*L. caesar*
LUCIL020-12	UK	*L. caesar*
LUCIL021-12	UK	*L. caesar*
LUCIL022-12	UK	*L. caesar*
LUCIL023-12	UK	*L. caesar*
LUCIL024-12	UK	*L. caesar*
LUCIL025-12	UK	*L. caesar*
LUCIL026-12	UK	*L. caesar*
LUCIL027-12	UK	*L. caesar*
LUCIL028-12	UK	*L. caesar*
LUCIL029-12	UK	*L. caesar*
LUCIL030-12	Poland	*L. caesar*
LUCIL031-12	Poland	*L. caesar*
LUCIL032-12	Poland	*L. caesar*
LUCIL033-12	Poland	*L. caesar*
LUCIL034-12	Poland	*L. caesar*
LUCIL035-12	Poland	*L. caesar*
LUCIL036-12	Poland	*L. caesar*
LUCIL038-12	Belgium	*L. illustris*
LUCIL039-12	Belgium	*L. illustris*
LUCIL040-12	Belgium	*L. illustris*
LUCIL041-12	Belgium	*L. illustris*
LUCIL042-12	Belgium	*L. illustris*
LUCIL043-12	Germany	*L. illustris*
LUCIL044-12	Belgium	*L. illustris*
LUCIL045-12	Belgium	*L. illustris*
LUCIL046-12	Belgium	*L. illustris*
LUCIL047-12	Belgium	*L. illustris*
LUCIL048-12	Belgium	*L. illustris*
LUCIL049-12	Belgium	*L. illustris*
LUCIL051-12	Belgium	*L. illustris*
LUCIL052-12	Belgium	*L. illustris*
LUCIL053-12	Belgium	*L. illustris*
LUCIL054-12	Belgium	*L. illustris*
LUCIL055-12	Germany	*L. illustris*
LUCIL056-12	France	*L. illustris*
LUCIL057-12	France	*L. illustris*
LUCIL058-12	France	*L. illustris*
LUCIL059-12	UK	*L. illustris*
LUCIL060-12	UK	*L. illustris*
LUCIL078-12	Belgium	*L. caesar*
LUCIL079-12	Belgium	*L. caesar*
LUCIL080-12	Belgium	*L. caesar*
LUCIL081-12	Belgium	*L. caesar*
LUCIL082-12	Belgium	*L. caesar*
LUCIL083-12	Belgium	*L. caesar*
LUCIL084-12	Belgium	*L. caesar*
LUCIL085-12	Belgium	*L. caesar*

LUCIL086-12	Belgium	*L. caesar*
LUCIL087-12	Belgium	*L. caesar*
LUCIL088-12	Belgium	*L. caesar*
LUCIL089-12	Belgium	*L. caesar*
LUCIL090-12	Belgium	*L. caesar*
LUCIL091-12	Belgium	*L. caesar*
LUCIL092-12	Belgium	*L. caesar*
LUCIL093-12	Belgium	*L. caesar*
LUCIL094-12	Belgium	*L. caesar*
LUCIL095-12	Belgium	*L. caesar*
LUCIL096-12	Belgium	*L. caesar*
LUCIL097-12	Belgium	*L. caesar*
LUCIL098-12	Belgium	*L. caesar*
LUCIL099-12	Belgium	*L. caesar*
LUCIL100-12	Belgium	*L. caesar*
LUCIL101-12	Belgium	*L. caesar*
LUCIL102-12	UK	*L. caesar*
LUCIL103-12	UK	*L. caesar*
LUCIL104-12	UK	*L. caesar*
LUCIL105-12	UK	*L. caesar*
LUCIL106-12	UK	*L. caesar*
LUCIL107-12	UK	*L. caesar*
BBDEC349-09	Canada, Newfoundland y Labrador, Gros Morne NP	*L. illustris*
BBDEE003-10	Canada, New Brunswick, Kouchicouguac NP	*L. illustris*
GBDP0705-06	UK, Bristol	*L. caesar*
GBDP0758-06	UK, Somerset	*L. illustris*
GBDP15207-14	Germany	*L. caesar*
GBDP15208-14	Germany	*L. caesar*
GBDP15624-14	-	*L. caesar*
GBDP16425-15	Spain	*L. caesar*
GBDP16453-15	Spain	*L. caesar*
GBDP16455-15	Spain	*L. caesar*
GBDP16458-15	Spain	*L. caesar*
GBDP16462-15	Spain	*L. caesar*
GBDP16483-15	Spain	*L. caesar*
GBDP16486-15	Spain	*L. caesar*
GBDP16496-15	Spain	*L. caesar*
GBDP16504-15	Spain	*L. caesar*
GBDP16510-15	Spain	*L. caesar*
GBDP16532-15	Spain	*L. illustris*
GBDP16533-15	Spain	*L. illustris*
GBDP16534-15	Spain	*L. illustris*
GBDP16535-15	Spain	*L. illustris*
GBDP16536-15	Spain	*L. illustris*
GBDP16537-15	Spain	*L. illustris*
GBDP16538-15	Spain	*L. illustris*
GBDP16539-15	Spain	*L. illustris*
GBDP16540-15	Spain	*L. illustris*
GBDP16541-15	Spain	*L. illustris*
GBDP16542-15	Spain	*L. illustris*

GBDP16543-15	Spain	*L. illustris*
GBDP16544-15	Spain	*L. illustris*
GBDP16545-15	Spain	*L. illustris*
GBDP16546-15	Spain	*L. illustris*
GBDP16547-15	Spain	*L. illustris*
GBDP16548-15	Spain	*L. illustris*
GBDP16549-15	Spain	*L. illustris*
GBDP16550-15	Spain	*L. illustris*
GBDP16551-15	Spain	*L. illustris*
GBDP16552-15	Spain	*L. illustris*
GBDP16553-15	Spain	*L. illustris*
GBDP16554-15	Spain	*L. illustris*
GBDP16555-15	Spain	*L. illustris*
GBDP16556-15	Spain	*L. illustris*
GBDP16557-15	Spain	*L. illustris*
GBDP16558-15	Spain	*L. illustris*
GBDP18472-15	Spain	*L. caesar*
GBDP18473-15	Spain	*L. caesar*
GBDP18474-15	Spain	*L. caesar*
GBDP18476-15	Spain	*L. caesar*
GBDP18477-15	Spain	*L. caesar*
GBDP18478-15	Spain	*L. caesar*
GBDP18479-15	Spain	*L. caesar*
GBDP18480-15	Spain	*L. caesar*
GBDP18481-15	Spain	*L. caesar*
GBDP18482-15	Spain	*L. caesar*
GBDP18483-15	Spain	*L. caesar*
GBDP18484-15	Spain	*L. caesar*
GBDP18485-15	Spain	*L. caesar*
GBDP18486-15	Spain	*L. caesar*
GBDP18487-15	Spain	*L. caesar*
GBDP18488-15	Spain	*L. caesar*
GBDP18489-15	Spain	*L. caesar*
GBDP18490-15	Spain	*L. caesar*
GBDP18491-15	Spain	*L. caesar*
GBDP18492-15	Spain	*L. caesar*
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GBDP18495-15	Spain	*L. caesar*
GBDP18496-15	Spain	*L. caesar*
GBDP18497-15	Spain	*L. caesar*
GBDP18498-15	Spain	*L. caesar*
GBDP18499-15	Spain	*L. caesar*
GBDP18500-15	Spain	*L. caesar*
GBDP18501-15	Spain	*L. caesar*
GBDP18502-15	Spain	*L. caesar*
GBDP18503-15	Spain	*L. caesar*
GBDP18504-15	Spain	*L. caesar*
GBDP18505-15	Spain	*L. caesar*
GBDP18506-15	Spain	*L. caesar*

GBDP18507-15	Spain	*L. caesar*
GBDP18508-15	Spain	*L. caesar*
GBDP18509-15	Spain	*L. caesar*
GBDP18511-15	Spain	*L. illustris*
GBDP2531-06	UK	*L. caesar*
GBDP7908-09	Korea	*L. caesar*
GBDP7909-09	Korea	*L. caesar*
GBDP7910-09	Korea	*L. caesar*
GBDP7911-09	Korea	*L. caesar*
GBDP9040-10	-	*L. illustris*
GBDP9041-10	-	*L. illustris*
GBMIN53635-17	China, Jilin, Baekdu Mountain	*L. caesar*
GBMIN53636-17	China, Jilin, Baekdu Mountain	*L. caesar*
GBMIN53639-17	China, Jilin, Baekdu Mountain	*L. caesar*
GBMIN53729-17	China, Changchun, Jilin	*L. illustris*
GBMIN53730-17	China, Suihua, Heilongjian	*L. illustris*
GBMIN53731-17	China, Jilin, Baekdu Mountain	*L. illustris*
MHFLI237-06	Canada, Manitoba, Churchill	*L. illustris*
MHFLY031-06	Canada, Manitoba, Churchill	*L. illustris*
GBDP13550-13	-	*L. caesar*
GBDP15209-14	Germany	*L. caesar*
GBDP16419-15	Spain	*L. caesar*
GBDP16420-15	Spain	*L. caesar*
GBDP16421-15	Spain	*L. caesar*
GBDP16422-15	Spain	*L. caesar*
GBDP16423-15	Spain	*L. caesar*
GBDP16424-15	Spain	*L. caesar*
GBDP16426-15	Spain	*L. caesar*
GBDP16427-15	Spain	*L. caesar*
GBDP16428-15	Spain	*L. caesar*
GBDP16430-15	Spain	*L. caesar*
GBDP16432-15	Spain	*L. caesar*
GBDP16433-15	Spain	*L. caesar*
GBDP16434-15	Spain	*L. caesar*
GBDP16435-15	Spain	*L. caesar*
GBDP16437-15	Spain	*L. caesar*
GBDP16439-15	Spain	*L. caesar*
GBDP16440-15	Spain	*L. caesar*
GBDP16441-15	Spain	*L. caesar*
GBDP16442-15	Spain	*L. caesar*
GBDP16444-15	Spain	*L. caesar*
GBDP16445-15	Spain	*L. caesar*
GBDP16446-15	Spain	*L. caesar*
GBDP16447-15	Spain	*L. caesar*
GBDP16449-15	Spain	*L. caesar*
GBDP16451-15	Spain	*L. caesar*
GBDP16457-15	Spain	*L. caesar*
GBDP16460-15	Spain	*L. caesar*
GBDP16464-15	Spain	*L. caesar*
GBDP16466-15	Spain	*L. caesar*

GBDP16468-15	Spain	*L. caesar*
GBDP16470-15	Spain	*L. caesar*
GBDP16472-15	Spain	*L. caesar*
GBDP16473-15	Spain	*L. caesar*
GBDP16475-15	Spain	*L. caesar*
GBDP16477-15	Spain	*L. caesar*
GBDP16479-15	Spain	*L. caesar*
GBDP16480-15	Spain	*L. caesar*
GBDP16481-15	Spain	*L. caesar*
GBDP16485-15	Spain	*L. caesar*
GBDP16487-15	Spain	*L. caesar*
GBDP16488-15	Spain	*L. caesar*
GBDP16489-15	Spain	*L. caesar*
GBDP16490-15	Spain	*L. caesar*
GBDP16491-15	Spain	*L. caesar*
GBDP16492-15	Spain	*L. caesar*
GBDP16493-15	Spain	*L. caesar*
GBDP16494-15	Spain	*L. caesar*
GBDP16495-15	Spain	*L. caesar*
GBDP16497-15	Spain	*L. caesar*
GBDP16498-15	Spain	*L. caesar*
GBDP16499-15	Spain	*L. caesar*
GBDP16500-15	Spain	*L. caesar*
GBDP16501-15	Spain	*L. caesar*
GBDP16502-15	Spain	*L. caesar*
GBDP16506-15	Spain	*L. caesar*
GBDP16508-15	Spain	*L. caesar*
GBDP16512-15	Spain	*L. caesar*
GBDP16514-15	Spain	*L. caesar*
GBDP16516-15	Spain	*L. caesar*
GBDP16518-15	Spain	*L. caesar*
GBDP16520-15	Spain	*L. caesar*
GBDP16522-15	Spain	*L. caesar*
GBDP16524-15	Spain	*L. caesar*
GBMIN53623-17	China	*L. caesar*
GBMIN53624-17	China	*L. caesar*
GBMIN53625-17	China	*L. caesar*
GBMIN53626-17	China	*L. caesar*
GBMIN53627-17	Spain	*L. caesar*
GBMIN53628-17	Spain	*L. caesar*
GBMIN53630-17	Spain	*L. caesar*
GBMIN53631-17	Spain	*L. caesar*
GBMIN53632-17	Spain	*L. caesar*
GBMIN53633-17	Spain	*L. caesar*
GBMIN53637-17	Spain	*L. caesar*
BBDCP288-10	Canada, Alberta, Elk Island NP	*L. illustris*
BBDCP289-10	Canada, British Columbia, Pacific Rim NP	*L. illustris*
BBDCP290-10	Canada, Sascatchewan, Prince Albert NP	*L. illustris*
BBDCP291-10	Canada, Sascatchewan, Prince Albert NP	*L. illustris*
BBDCP297-10	Canada, Sascatchewan, Prince Albert NP	*L. illustris*

BBDCP299-10	Canada, British Columbia, Kootenay NP	*L. illustris*
BBDEC133-09	Canada, Newfoundland y Labrador, Gros Morne NP	*L. illustris*
BBDEC134-09	Canada, Newfoundland y Labrador, Terra Nova NP	*L. illustris*
BBDEC135-09	Canada, Newfoundland y Labrador, Gros Morne NP	*L. illustris*
BBDEC136-09	Canada, Nova Scotia, Cape Breton Highlands NP	*L. illustris*
BBDEC137-09	Canada, Nova Scotia, Cape Breton Highlands NP	*L. illustris*
BBDEC138-09	Canada, Newfoundland y Labrador, Gros Morne NP	*L. illustris*
BBDED712-10	Canada, New Brunswick, Kouchiboguac NP	*L. illustris*
BBDED748-10	Canada, Newfoundland y Labrador, Gros Morne NP	*L. illustris*
BBDED906-10	Canada, New Brunswick, Kouchiboguac NP	*L. illustris*
BBDED925-10	Canada, New Brunswick, Kouchiboguac NP	*L. illustris*
BBDEE783-10	Canada, Newfoundland y Labrador, Terra Nova NP	*L. illustris*
BBDEE977-10	Canada, New Brunswick, Kouchiboguac NP	*L. illustris*
CDFD005-12	Portugal, Lisbon	*L. caesar*
GBDP13549-13	-	*L. illustris*
GBMIN53722-17	China	*L. illustris*
GBMIN53723-17	China	*L. illustris*
GBMIN53724-17	China	*L. illustris*
GBMIN53725-17	China	*L. illustris*
GBMIN53726-17	China	*L. illustris*
GBMIN53727-17	China	*L. illustris*
GBMIN53728-17	China	*L. illustris*
JSYKA259-10	Canada, Yukon Territory, Firth River	*L. illustris*
JSYKA261-10	Canada, Yukon Territory, Firth River	*L. illustris*
TTDFW075-08	Canada Manitoba, Riding Mountain NP	*L. illustris*
TTDFW718-08	Canada, Ontario, Pukaskwa NP	*L. illustris*
UAMIC893-13	EEUU, Alaska	*L. illustris*
UAMIC900-13	EEUU, Alaska	*L. illustris*

Appendix 1.3. Supplementary Table 1.3: Code assigned by BOLD, location of the samples and recognized species used in phylogeographic analysis of S. lehmanni and S. variegata.

BOLD code	**Location**	**Species**
GBDP12450-12	France	*S. lehmanni*
GBDP12518-12	France	*S. variegata*
GBDP12532-12	Belgium	*S. variegata*
GBDP12533-12	Belgium	*S. variegata*
GBDP12537-12	France	*S. lehmanni*
GBDP12540-12	France	*S. variegata*
GBDP12542-12	France	*S. lehmanni*
GBDP12543-12	France	*S. variegata*
GBDP12544-12	Belgium	*S. variegata*
GBDP12545-12	Belgium	*S. variegata*
GBDP12547-12	Belgium	*S. variegata*
GBDP12549-12	Belgium	*S. variegata*
GBDP12551-12	Belgium	*S. variegata*
GBDP12557-12	France	*S. lehmanni*

GBDP12558-12	France	*S. lehmanni*
GBDP12559-12	France	*S. lehmanni*
GBDP13675-13	UUEE	*S. lehmanni*
GBDP13967-13	Denmark, Lejre	*S. lehmanni*
GBMIN60307-17	Italy	*S. lehmanni*
GBMIN60457-17	Croatia	*S. variegata*
GMGMA303-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMA380-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB022-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB025-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB028-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB029-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB040-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB044-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB045-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB059-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMB589-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMC057-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMC339-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMD207-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMD217-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMD228-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMH1280-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMH1315-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMH1324-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMI106-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMJ233-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK044-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK050-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK051-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK053-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK061-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK062-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK063-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK271-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK289-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK291-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK305-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMK314-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML068-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML085-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML092-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML1389-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML207-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML218-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML237-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML241-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML257-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML273-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML276-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*

GMGML277-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGML500-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMM217-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMM242-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMM251-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMM274-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMN1595-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMN205-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMN213-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGMN302-14	Germany, Renania-Palatinado, Ahrweiler	*S. variegata*
GMGRB1542-13	Germany, Bavaria, Neiderbeyrn	*S. variegata*
GMGRF6346-13	Germany, Bavaria, Neiderbeyrn	*S. variegata*
GMGRF6351-13	Germany, Bavaria, Neiderbeyrn	*S. variegata*
GMGRG4863-13	Germany, Bavaria, Neiderbeyrn	*S. variegata*

CHAPTER II

Chapter II

DNA-based and Taxonomic identification of forensically important Sarcophagidae (Diptera) in Southeastern Spain.

Abstract

The study of dipterans at the scene of a death provides essential information for the interpretation of evidences and therefore, for the case resolution. Molecular tools have been incorporated into identification tasks at a specific level in cases of cryptic species or in poor conservation of specimens. These methods are also useful because of a lack of qualified staff in taxonomy and systematics of insects in the scientific police. In addition, a phylogeographic reconstruction within a species could help to differentiate the haplotypes from a geographic area, contributing to the clarification in cases of relocation of the corpse. The taxonomic identification of Sarcophagidae species is often difficult using morphological characters, especially the females. This fact increases the doubt to use them to identify minimum post mortem interval (PMI). In this paper, we compare Sarcophagids found in human corpses in real forensic cases in Alicante (southeast of Spain) with specimens collected in baited traps in the same area and surrounding provinces. A total of 189 specimens were collected, 72 from real forensic cases and 117 from baited traps. The morphological identification of samples was made with taxonomic keys until family level. The molecular identification was made sequencing the COI mitochondrial gene and analysing sequences through ABGD, GMYC and BIN methods. For the construction of phylogeographic networks, the Median Joining algorithm of the PopART program was used. Eight species of the family Sarcophagidae were identified. Five sequences, *a priori* assigned to *Sarcophaga argyrostoma*, produce controversy among the three methods of species delimitation used. Four species were not found in the forensic cases. Besides, three of these species was found in a single location each. The most widely collected species were *S. argyrostoma* and *S. tibialis*. The haplotype networks of these species did not reveal a clear geographic distribution of haplotypes. The *S. argyrostoma* samples from Alcoy

were clearly isolated. These results demonstrate that this method is useful for the identification of Sarcophagidae samples in forensic investigations.

Key words

DNA Barcodes, Sarcophagidae, human corpses, forensic entomology, species identification.

Introduction

The identification of specimens of Diptera is an essential step in forensic entomology investigation. The knowledge of the species and the abiotic factors allow investigators calculating the minimum Post Mortem Interval (PMI) (Amendt, Richards, Campobasso, Zehner, & Hall, 2011). Due to the species-specific developmental rates, this tool would not be useful if the identification of the specimens were not accurate.

The family Sarcophagidae (commonly named “flesh flies”), especially female specimens, represents a complex challenge for forensic entomologist due to their morphological similarity (Jordaens, Sonet, Richet, et al., 2013). In males, morphological identification is based on genitalia. However, these structures are often difficult to see because of the inappropriate preservation of the specimens collected in forensic cases (Harvey et al., 2003; Zehner et al., 2004). Besides, the available morphological keys (Richet, Blackith, & Pape, 2011; Szpila, Richet, et al., 2015) and some not still published (Szpila et al., in preparation, Keys for Eurpean Sarcophagidae) do not include all species of Diptera with forensic interest detected in previous reports in southeastern Spain (Baz et al., 2015; Farinha et al., 2014; Romera et al., 2003). These issues hinders its use in the calculation of PMI (Jordaens, Sonet, Richet, et al., 2013; Tan et al., 2010).

In the Iberian Peninsula 32 species belonging to the family Sarcophagidae have been reported in previous papers (Arnaldos, Romera, Luna, Presa, & García, 2004; Martín-Vega & Baz, 2013; Prado e Castro, García, Arnaldos, & González-Mora, 2010; Prado e Castro, Arnaldos, Sousa, & García, 2011; Romera et al., 2003). Only eight of them have been collected on human corpses, therefore as happened in other families, only a few species of this family seem to participate in human corpse decomposition, (Arnaldos, Prado e Castro, Presa, Castro, & García, 2006). Recognizing and identifying these species accurately is essential to include them in forensic investigations.

Molecular techniques are able to identify inadequately preserved or damaged samples (Wells & Stevens, 2008) and can distinguish between closely related species with very similar morphology, whether they are adults or immature life stages (Meiklejohn et al., 2011, 2012). Although so many genes can be used for the identification (Bourke et al., 2013; Vences, Thomas, Meijden, Chiari, & Vieites, 2005; Zaidi, Wei, Shi, & Chen, 2011; Zajac et al., 2016) the *cytochrome oxidase* subunit 1 (COI) is the most widely considered gene for this task (Aly, 2014; Boehme, Amendt, & Zehner, 2012; Farinha et al., 2014; Jordaens, Sonet, Braet, et al., 2013; Meier, Shiyang, Vaidya, & Ng, 2006; Nelson, Wallman, & Dowton, 2007; Pohjoismäki, Kahanpää, & Mutanen, 2016; Zhang, Buenaventura, Pape, & Zhang, 2016). The use of this gene has been developed as "DNA barcode" marker, so it has a large number of sequences in public databases such as GenBank and BOLD (Barcode of Life Data). In this last, the tool BIN (Biological Index Number) has been made available. This analysis assigns sequences to clusters associated with the BIN identifier of a particular species (Ratnasingham & Hebert, 2013). The results of the BIN analysis help 1) to evaluate the diversity of a group of unknown species, 2) to separate species with cryptic morphology and 3) to identify species with sequences present in BOLD (Hausmann et al., 2013). The utility of this tool has been proven in previous works of different phyla (Borges et al., 2016; Hausmann et al., 2013; Kekkonen & Hebert, 2014; Morinière et al., 2014; Pohjoismäki et al., 2016). The barcoding analysis can help Scientific Police to identify samples without sending it to an entomologist, since the main part of the staff who works in those departments have none or scarce taxonomic training. Besides, the phylogeographic analysis has demonstrated utility to differentiate populations relatively close in species of several taxa (López-López et al., 2016; Pfeiler et al., 2013). This could be useful in forensic cases with displacement or dislocation of corpses.

The aim of this work is to identify with molecular techniques specimens of the family Sarcophagidae collected in forensic cases occurred in Alicante (Comunidad Valenciana, southeastern of Spain) and to compare these samples with others collected in this area and surrounding provinces with baited traps. This will allow us to know whether there are differences related to the species present in human bodies and those captured in nature in the same geographical area. Once identified, it will be verified if this

information can be useful in the calculation of the PMI compared to that made with species belonging to other families found in these forensic cases. The molecular data generated in this paper will increase the available knowledge on the family Sarcophagidae in Spain and will provide essential valuable information in the future for species identification through barcoding techniques.

Methods

Fly Specimens

Sampling method in forensic cases

Nine corpses were found in Alicante province (Spain) by Scientific Police between 2009 and 2010 having Sarcophagidae samples on them (Fig. 2.1). Eight of the bodies were found indoors and one outdoors. Samples were picked up by Alicante Scientific Police Brigade, after the judicial commission gave permission. The specimens were taken from different parts of the body (nostrils, eyes, wounds, etc.) in the crime scene in all stages of development (eggs, larvae, pupae, puparium, adults) (Amendt et al., 2007), checking the surroundings of the body as well. During the autopsy, clothes and shroud were examined searching for more samples. Adults were preserved in absolute ethanol, and larvae were reared in cages in laboratory under controlled environmental conditions of temperature and relative humidity to allow them to reach adulthood. Some of the larvae were preserved in absolute ethanol. The police protocol, in Spain, oblige that samples has to be sent to Toxicology National Institute. Authors were authorized to study a representative number of individuals.

Sampling method by bottle traps

Species analyzed in this study were collected in 11 localities (sampled four times, one per season). Nine of this sampling points are located surrounding the province of Alicante, while two of them are within this province (Fig. 2.1), in order to find a representative selection of carrion-associated Sarcophagidae of this area. Three bottle traps (Allemand & Aberlenc, 1991) baited with pig liver and pig blood were placed in each locality and stayed there between 3 and 6 days. Sampling were carried out from 2012 to 2014. Larvae were reared to adulthood in cages with liver and adults were preserved in absolute ethanol. All samples were morphologically identified (Barrientos, 2004; Richet et al., 2011; Szpila, Richet, et al., 2015) as far as possible prior to DNA

extraction. All the information about the samples collected with both methods is in Table 2.1.

Table 2.1: Species collected, number of forensic case, location name, place of the corpse/trap, number of specimens and date of collection.

Species	Case	Localities		Places	Samples	Data
Sarcophaga argyrostoma	1	3	Alcoy (Alicante)	Indoor	5	July
	3	1	Alicante	Indoor	4	April
	4	1	Alicante	Outdoor	9	May
	8	2	San Vicente (Alicante)	Indoor	7	July
	9	6	Denia (Alicante)	Indoor	1	July
	28	1	Alicante	Indoor	13	June
	32	4	Villajoyosa (Alicante)	Indoor	5	August
	38	5	Torrevieja (Alicante)	Indoor	14	August
	-	9	Rambla Campus (Murcia)	Outdoor	21	January, June, July, August, October
	-	10	Granja Veterinaria (Murcia)	Outdoor	2	December
	-	11	Águilas (Murcia)	Outdoor	5	September
	-	12	Carboneras (Almería)	Outdoor	1	September
	-	14	Arroyo del Ojanco (Jaén)	Outdoor	2	September
	-	13	Guadix (Granada)	Outdoor	1	December
	-	7	Denia (Alicante)	Outdoor	2	June
	-	8	Elda (Alicante)	Outdoor	2	July
	-	16	Villar del Arzobispo (Valencia)	Outdoor	5	June, July
					99	
Sarcophaga tibialis	-	9	Rambla Campus (Murcia)	Outdoor	4	February
	-	7	Denia (Alicante)	Outdoor	22	June, August
	-	8	Elda (Alicante)	Outdoor	9	July, October
	-	16	Villar del Arzobispo (Valencia)	Outdoor	13	June, August, December
	-	17	Catarroja (Valencia)	Outdoor	3	July
	-	14	Arroyo del Ojanco (Jaén)	Outdoor	5	June, October, December
	-	15	La Roda (Albacete)	Outdoor	2	July
					58	
Sarcophaga cultellata	-	13	Guadix (Granada)	Outdoor	**13**	Feb, March, July, Oct, Sep, Dec
Sarcophaga dux	32	4	Villajoyosa (Alicante)	Indoor	**8**	August
Sarcophaga jacobsoni	-	11	Águilas (Murcia)	Outdoor	**3**	September
Sarcophaga africa	1	3	Alcoy (Alicante)	Indoor	**2**	July
Sarcophaga crassipalpis	1	3	Alcoy (Alicante)	Indoor	**1**	July
Wohlfahrtia magnifica	-	15	La Roda (Albacete)	Outdoor	**2**	July
Calliphora vicina	6	3	Alcoy (Alicante)	Indoor	**3**	June

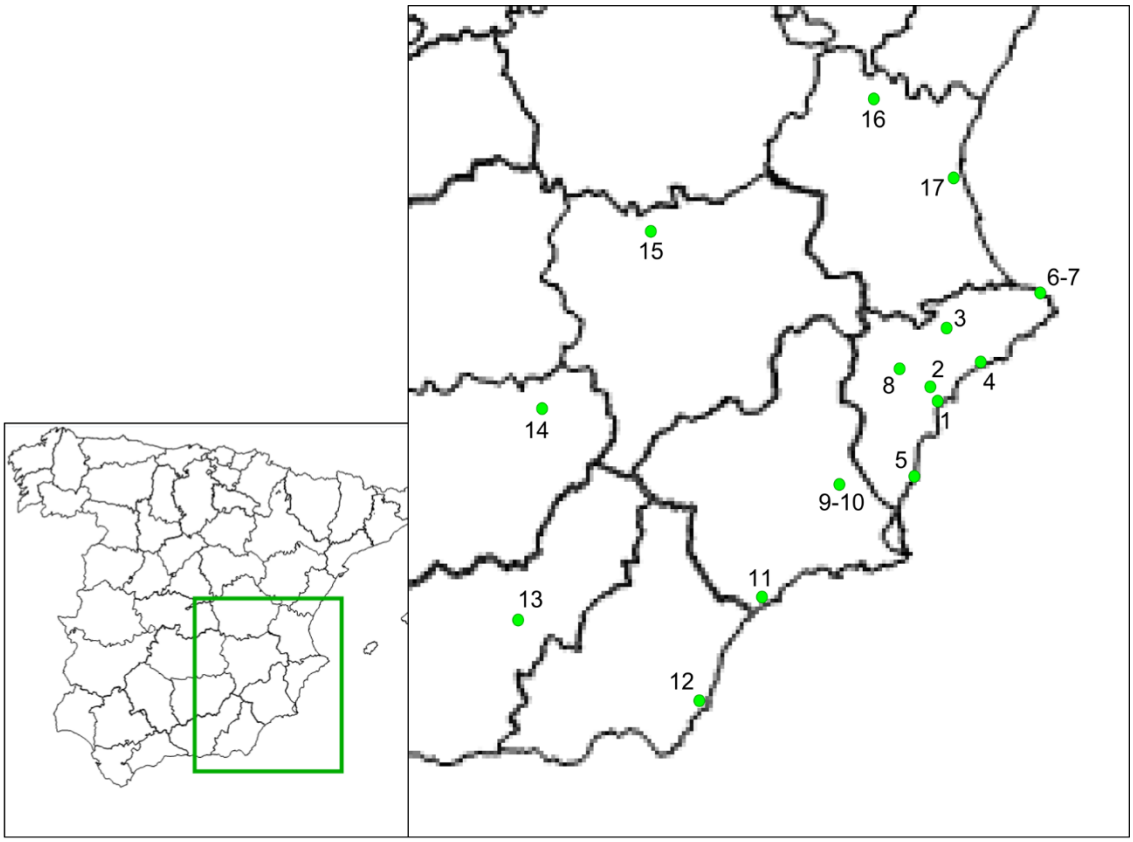


Figure 2.1: Location of sampling points.

DNA extraction and PCR amplification

For the genomic DNA extraction, we used one or two legs and thorax muscles for adult specimens. For the larvae, a portion of the middle area of the body was used. The extraction was carried out using the Glass Fiber Plate DNA Extraction protocol of the CCDB (Ivanova et al., 2006). After extraction, all samples were diluted in 60 µL of ddH_2O prewarmed to 56°C.

We performed the amplification of the COI gene, barcoding region, using the primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') described by Folmer (Folmer et al., 1994). It was performed in a thermocycler 2720 Thermal cycler Applied Biosystems (Applied Biosystems, Foster City, U.S.A.). A Kapabiosystems PCR kit (Wilmington, U.S.A.) was used. This kit includes all components necessary for the PCR cocktail except primers, target DNA and water. The components of this cocktail are Buffer (10x), dNTPs and Taq polymerase. The total reaction volume was 12.5 µl containing 2 µl of sample DNA for each reaction. The PCR program consists in: initial activation of 95 °C for 3 min, 35

cycles of 60 sec at 94 °C, 60 sec at 45 °C, and 90 sec at 72 °C, and final extension of 5 min at 72 °C. Finally, 2 µl of each PCR product was subjected to electrophoresis on a 2% agarose gel stained with RED SAFE (iNtRON Boitechnology, Seongnam, South Korea) dye fluorescent product. The result was visualized under ultraviolet light.

DNA sequencing and COI barcode analysis

Samples were sent to Macrogen (Amsterdam, The Netherlands) to be sequenced. Sequences were edited and aligned, using MUSCLE (Edgar, 2004), with MEGA7 (Kumar et al., 2016). The sequences have been uploaded in the project "Biodiversity of Diptera" within BOLD system. We used the BGA (Barcode Gap Analysis) tool from BOLD to calculate the sequence divergence. A Neighbor-Joining (NJ) tree was carried out with BOLD tool using the Kimura-2-Parameter (K2P) distance algorithm. We used the BLAST tool (Altschul et al., 1997) to compare all sequences with the NCBI database. Searches that obtained lowest E-value and higher Grade were consider as an initial molecular identification (BLAST_ID).

Three species delimitation methods were used to analyze the number of molecular operational taxonomic units (MOTUs): generalized mixed Yule-coalescent (GMYC) (Monaghan et al., 2009; Pons et al., 2006), automatic barcode gap discovery (ABGD) (Puillandre et al., 2012) and barcode index number (BINs). GMYC is a coalescence tree-based approach. ABGD and BIN are both methods based on distance. Each method generates a putative group of MOTUs. To check the congruence among them, the resulting MOTUs were categorized into one of three categories: 1) Match (MOTU recognized by all methods), 2) Partial Match (one of the methods not recognized the MOTU) and 3) No Match (none of the methods recognized the MOTU).

The GMYC analysis required to collapse the sequences into haplotypes, using the web tool FaBox (http://users-birc.au.dk/biopv/php/fabox/), to develop an ultrametric tree. The evolution model for these data was calculated with jModelTest2 (Darriba et al., 2015), the result was "GTR+I+G". Tree was obtained with BEAST v2.4.8 (Bouckaert et al., 2014), with constant population size tree prior and strict clock as more conservative options (Monaghan et al., 2009). MCMC were run for 20 million generation. First 25% of resulting trees were removed as burn-in. The consensus tree was obtained using LogCombiner 1.8.2 and TreeAnnotator 1.8.2 (Drummond &

Rambaut, 2007). The GMYC analysis was made with R statistical software (R Development Core Team, 2012), using the SPLITS v1.0-19 package (Ezard et al., 2009) choosing the single threshold option (Michonneau, 2015). The ABGD analysis forms groups based on the barcode gap. The groups thus formed were reanalyzed recursively using Markov clustering, so divisions in secondary groups can occur. ABGD analysis was performed online (www.abi.snv.jussieu.fr/public/abgd/) using default settings (K2P and p-distance model with relative gap width X=1.5). The BIN analysis was made automatically by the BOLD database. All sequences were analysed with the algorithm "Refined Single Linkage Analysis" (RESL). This analysis cluster the sequences with COI sequences available in the database using a 2.2% threshold to compare with. A secondary Markov clustering delimit more accuracy the previous results. Finally, the system chooses the result with highest Silhouette score (Rousseeuw, 1987). The resulting BINs can be defined in three groups: 1) Singleton, when the sequence does not match with any existing BIN, 2) Concordant BIN, when the information of the sequence match with that of the BIN, and 3) Discordant BIN, when the information of the sequence does not match with that of the BIN at genus/species level.

To study the phylogeographic relationship among populations of the most widespread species in this area, the PopArt program (Bandelt et al., 1999) was used. We developed the haplotypes network using the Median Joining algorithm.

Results

A total of 189 specimens were analyzed, 72 of them were collected in forensic cases and 117 from baited tramps. The obtained sequences have a length of 644 pb in all cases and meet the BOLD requirements to be included in it. All sequences belong to the Sarcophagidae family, except three belonging to the species *Calliphora vicina* Robineau-Desvoidy 1830 from the forensic case number 6. In this work eight Sarcophagidae species were found, seven of the genus *Sarcophaga* and one of the genus *Wohlfarthia*. These species were impossible to identified with morphological keys because of the bad preservation state of the samples. For this reason, all of them had to be identified only by molecular techniques.

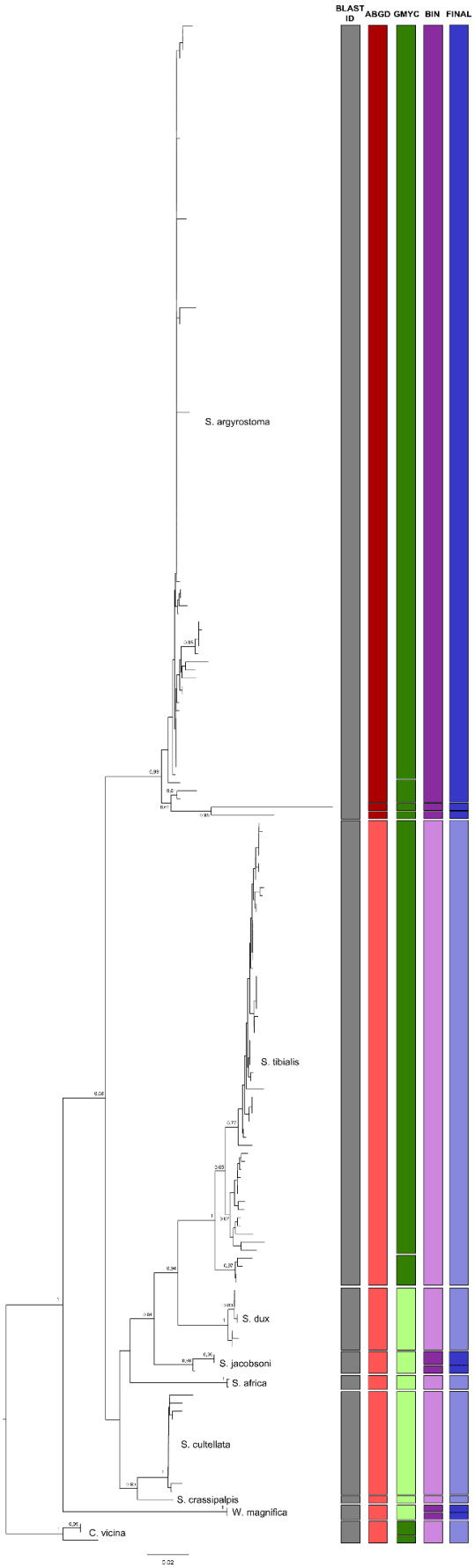


Figure 2.2. NJ tree of forensically important Sarcophagidae species. Bars show different ID and species delimitation methods. Discrepancies among them are showed shaded.

Molecular identification with BLAST tool allow us to realize the identification and recognized the eight species as *putative* species. The matrix built with these sequences meet the quality requirements demanded by BOLD, sequence length and absence of stop codons. The distance analysis shows an intraspecific distance of 0 - 10.67% and an interspecific distance of 3.18 - 13.06%. The distances did not show a clear gap. *S. argyrostoma* presents an intraspecific distance (10.67%) higher than the distance to the nearest neighbour (6.36%).

DNA barcode-based identification

The three methods of species identification produced very similar results. All MOTUs coincided in at least two of the methods, and we did not get any no-match. The MOTUs match among them in four cases (44.4%). In the rest of the cases (66.6%), one of the methods produced different number of MOTUs (splitting one MOTU in several), so they are considered partial-match. The MOTU with the largest number of specimens was *Sarcophaga argyrostoma*, and *Sarcophaga crassipalpis* got the lowest number (only one specimen). The number of specimens for each species and its origin, number of the forensic case when necessary and data and location of the corpses/tramps are shown in Table 2. All the species reported in this work represents 24.2% of Sarcophagidae species with forensic interest present in Spain. However, *Sarcophaga jacobsoni* and *W. magnifica* has not yet been reported on human corpses and *Sarcophaga dux* has not yet been reported on animal corpses.

GMYC analysis produced 14 MOTUs. Six of them match with the BLAST_ID done before. The remaining eight are the product of the split of these groups in two (*S. tibialis* and *C. vicina*) or four MOTUs (*S. argyrostoma*). This produces three of the five partial-match results among methods.

ABGD method recovered 11 MOTUs, coinciding with the identifications of the BLAST_ID. Only differ in two sequences *a priori* identified as *S. argyrostoma* that produced a single MOTU each. This agrees with the result of the GMYC analysis for these sequences.

BIN analysis grouped the sequences in 13 BINs. It mostly matches with the groups formed by the ABGD analysis differing only in the grouping of *S. jacobsoni* and *W. magnifica* sequences, that are splitted in two BINs considered singleton each. The

identification of the rest of the BINs matches with the BLAST_ID, even the *C. vicina* sequences. The two sequences *a priori* named *S. argyrostoma,* that form an individual MOTU each in the previous analyses form a Singleton BIN each also in this analysis. This is the unique congruence among the three methods that not agree with the BLAST_ID. The BIN Discordance reports five Singleton BINs, corresponding to the two problematic sequences of *S. argyrostoma* (ADL6991 and ADL6992), the two sequences of *W. magnifica* (ADL6346) and one of the *S. jacobsoni* (ADL5678). The other two sequences of *S. jacobsoni* are grouped in the same BIN (ADL5679), so that, the system did not recognize them as Singleton but it behaves in the same way. The rest of the BINs were Concordant. The discordances that some of them could present were due to rectifiable errors.

As far as the NJ tree produced by BOLD is concerned, the sequences of *C. vicina* were used as external outgroup (Fig. 2.2). The different species delimitation methods are represented in the right margin of the tree. The *W. magnifica* samples are in an external branch to the genus *Sarcophaga* group. Generally, all MOTUs forms a monophyletic branch. The group of *S. argyrostoma* presents two long branches that correspond to the sequences that formed individuals MOTUs/BINs. In the cluster of species such a *S. argyrostoma*, *S. tibialis* and *S. jacbsoni*, some sequences form little branches inside the same cluster, that are recognized as other putative MOTUs by GMYC or BIN analyses, producing Partial Match as mentioned above.

The distribution of the species thus recognized showed four species that are only present in one sampling locality (Fig. 2.3). Two species, *S. africa* and *S. crassipalpis*, are only present together in one locality in this work. *S. argyrostoma* and *S. tibialis* are widespread in the whole area, however *S. tibialis* is not collected in forensic cases and is present in the northern localities, while *S. argyrostoma* is in southern localities. There are four localities in which the two species were present together, producing a kind of border. *S. argyrostoma* is the only Sarcophagidae species which has been collected in both forensic cases and baited traps. The species *S. dux*, *S. africa* and *S. crassipalpis* were only present in forensic cases, while *S. tibialis*, *S. cultellata*, *S. jacobsoni* and *W. magnifica* were only found in baited traps.

The network analysis of the most widely distributed species (*S. tibialis* and *S. argyrostoma*) does not show a clear phylogeographic structure. In the case of *S.*

tibialis, 35 haplotypes were observed (Fig. 2.4). All sequences are spread in haplotypes formed by one to six sequences. Only one haplotype, represented by one sequence each, is shared between Denia (Alicante) and Rambla Campus (Murcia). In the case of *S. argyrostoma*, the shape of the network is different, but it did not produce a clear phylogeographic structure either (Fig. 2.5).

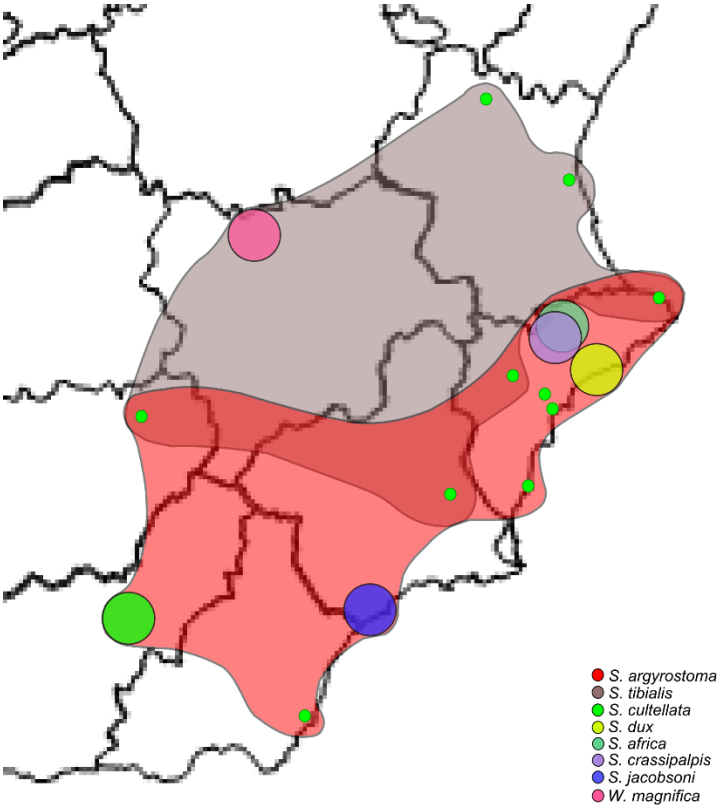


Figure 2.3. Species distribution in the studied area.

A total of 17 haplotypes with only one sequence each are produced, three with two and one with three sequences from the same locality, one haplotype with three sequences from two localities and one with seven sequences from three localities. Finally, one highly abundant and widespread haplotype is shared by 58 sequences from all localities except one, Alcoy (Alicante). Samples from this locality are grouped in three closely related haplotypes separated from the rest by five mutational steps.

Discussion

The identification of specimens of the family Sarcophagidae is known to be a difficult task due to the morphological similarities among the species (Alfred, 2011; Jordaens, Sonet, Richet, et al., 2013; Sukontason, Bunchu, Chaiwong, Moophayak, & Sukontason, 2010; Sukontason, Sanit, Klong-Klaew, Tomberlin, & Sukontason, 2014; Zehner et al.,

2004; Zhang, Zhang, Pape, Gu, & Wu, 2013; Zhang et al., 2016). In the case of our samples, even three *C. vicina* larvae specimens were initially identified as Sarcophagidae. The preservation process is essential, producing errors in subsequent analysis if it was not properly done (Day & Wallman, 2008). The preservation state of these samples made impossible to distinguish the species by morphological key, although some keys allows to distinguish adults (Pinto e Vairo et al., 2015) or larvae (Szpila, Richet, et al., 2015), so that the molecular identification is badly needed.

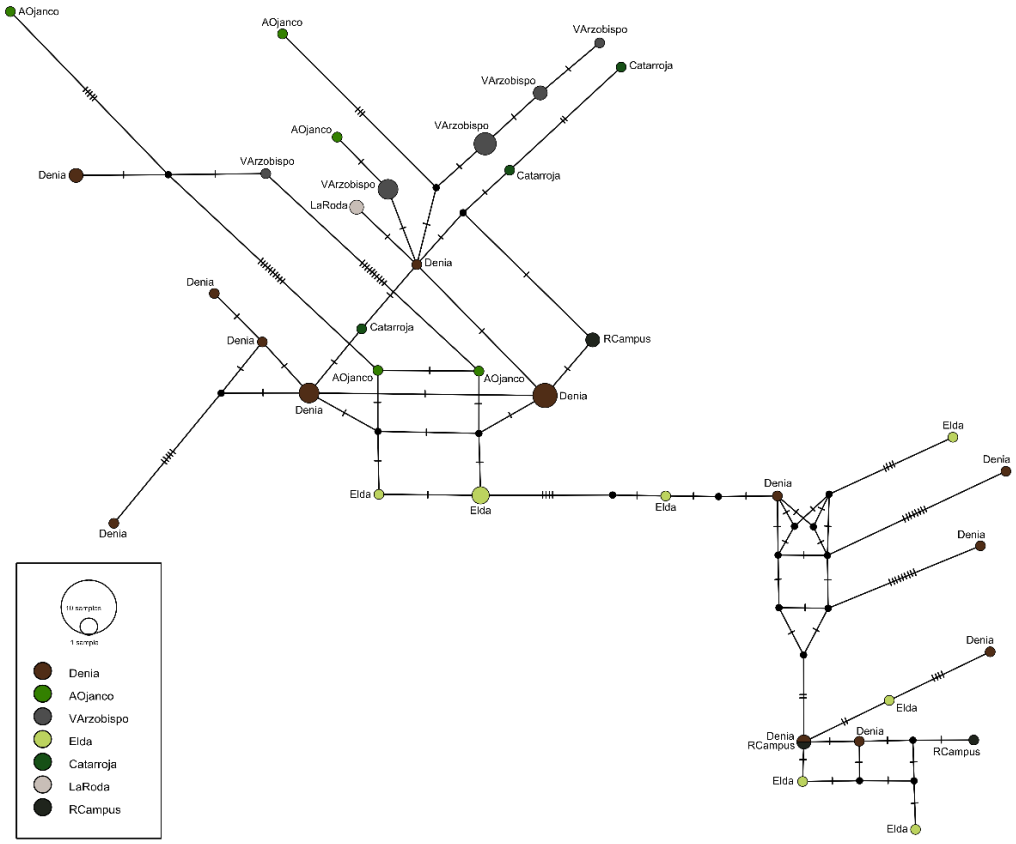


Figure 2.4. Haplotype network of S. tibialis.

The utility of the molecular methods has been proven in previous works (Alfred, 2011; Buenaventura & Pape, 2017; Jordaens, Sonet, Richet, et al., 2013; Kim et al., 2014; Meiklejohn et al., 2012; Piwczyński, Szpila, Grzywacz, & Pape, 2014; Tan et al., 2010; Whitmore, Pape, & Cerretti, 2013; Zehner et al., 2004). The distance analysis presents two species with higher intraspecific than interspecific distance. These two species, *S. argyrostoma* and *S. tibialis*, are the most widely distributed. Their distances, 10.67 and 4.65 respectively, overlap the minimum intraspecific distance (3.18%). The reason of this could be due to the names assigned to some sequences. The sequences which

produce the incongruences among methods could be the result of an erroneous molecular identification due to limited information in the databases (Wells & Stevens, 2008). However, the identification of the sequences with BLAST_ID and especially BINs tools provided good results (E-Value, Grade and Concordance respectively). Even if we change the names of the sequences of *S. argyrostoma* that produce controversy in the three species delimitation methods by "*Sarcophaga sp.*", the overlap continues to occur (data not shown). This agrees with authors that suggest that the thresholds routinely used are not always effective (Hebert, Cywinska, et al., 2003; Hebert et al., 2004; Meiklejohn et al., 2011).

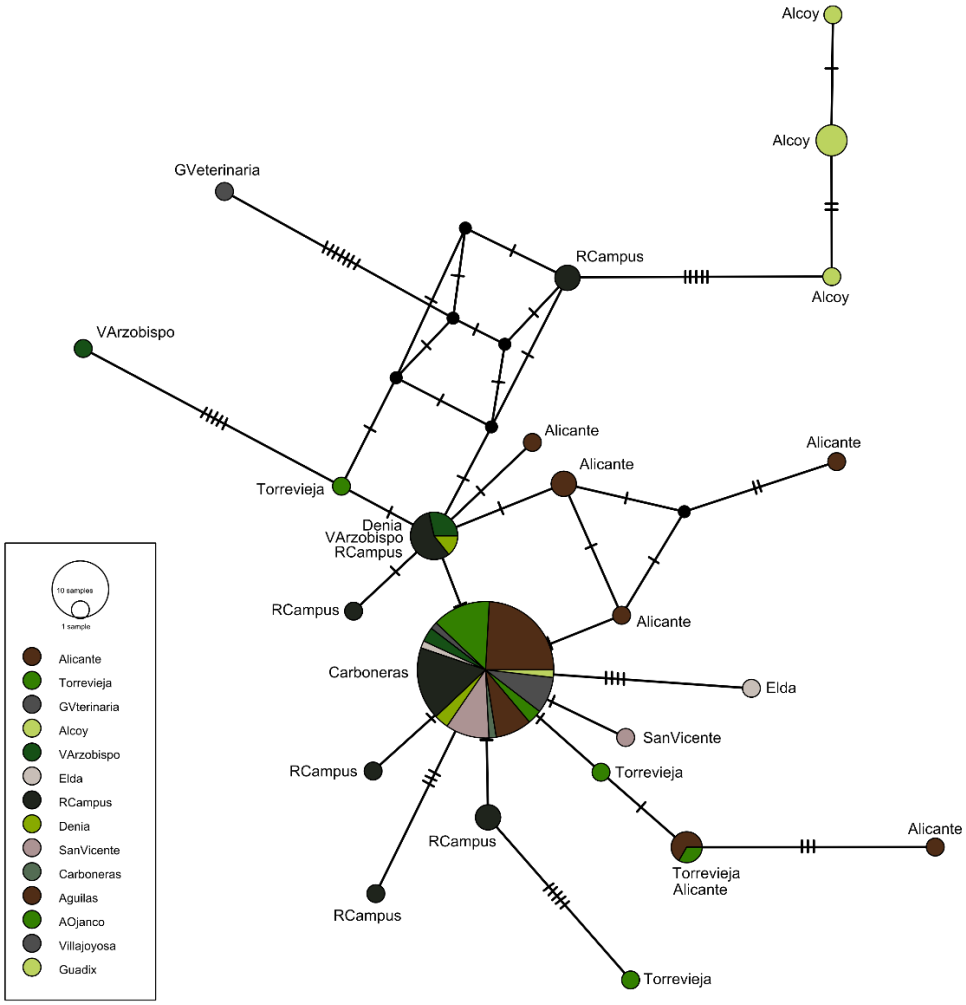


Figure 2.5. Haplotype network of S. argyrostoma.

GMYC method produces some MOTUs in these species that could be the result of oversplit (Kekkonen & Hebert, 2014; Talavera et al., 2013), but this result together with the distance result are indicators of the presence of different haplotypes. The

results of the Discordant BINs and the fact that there are other BINs for that species (*S. argyrostoma*, *S. jacobsoni* and *W. magnifica*) indicate that these identifications are not accurate enough. Therefore, these sequences would be named as "*Sarcophaga* sp." expecting for more information.

All the species reported in this work has been reported before in previous papers (Arnaldos, Ruiz, García, González-Mora, & Serrano, 2015; Baz et al., 2015; Carles-Tolrá, 2002; Martín-Vega & Baz, 2013; Velásquez, Magaña, Martínez-Sánchez, & Rojo, 2010; Velásquez, Gobbi, Martínez-Sánchez, & Rojo, 2015). However, in Spain we only have clear evidences of the presence of *S. argyrostoma* and *S. crassipalpis* in human corpses (Velásquez et al., 2010; Velásquez et al., 2015). Our samples agree with this and demonstrate the presence of other two species in human corpses in Spain: *S. dux* and *S. africa*. The *S. argyrostoma* specimens were collected outdoors and indoors from human corpses in spring season. That contrasts with the results of Baz et al. (2015) that only collected this species outdoors using tramps baited with squid (Baz et al., 2015). The type of bait could be the key of this difference. However, in summer season indoor and outdoor samples of this species were collected in both works. In the same way, they collected *S. africa* outdoors while our samples were collected indoors in the same season. In both works the *S. crassipalpis* were collected in July indoors. Samples of *S. tibialis* and *S. jacobsoni* match in the data and places of collection in both studies, but they found *S. tibialis* indoors in June and October. These data indicate that location and type of bait influence the flies attraction but the species with forensic interest are able to colonize decaying matter wherever it was place.

Although there are more Partial-Match than Match, the analysis showed high level of congruence among methods. Some of the Partial-Match are consequence of the overspliting commented above (Kekkonen & Hebert, 2014; Talavera et al., 2013). Other Partial-Match are consequence of the lack of information of some species in the databases, and few sequences can produce these incongruences. The two sequences of *S. argyrostoma* that produces Discordant BINs will be named "*Sarcophaga* sp." in the BOLD database waiting for more information that clarify this situation. For *S. jacobsoni* and *W. magnifica* no other BINs corresponding to these species are presents in the BOLD database, so these are the first sequences of these species uploaded to this database. But more information is needed to corroborate the BINs grouping of

both species. These are examples of the difficulties that limited information can produce (Wells & Stevens, 2008).

The distribution in the NJ tree is congruent with the information published in previous papers (Arnaldos et al., 2015; Buenaventura & Pape, 2017; Jordaens, Sonet, Richet, et al., 2013), producing a clear group with the species of the genus *Liosarcophaga* (*S. dux*, *S. tibialis* and *S. jacobsoni*) as a sister group of *S. africa*. The species of the subgenus *Liopygia* (*S. crassipalpis*, *S. cultellata* and *S. argyrostoma*) are related as it was shown in previous works, but the basal relationship among them is not as clear as in the *Liosarcophaga* group. The most distantly related Sarcophagidae is *W. magnifica* (Paramacronychiidae), as it was expected (Piwczyński et al., 2014). The distribution of *S. dux* and *S. crassipalpis* reported by Meiklejohn et al. (2011) is not congruent with our results since some of their sequences are located as sister species in that work. Finally, the authors, that defined these sequences as *outliers*, suggest that some divergences indicate that these sequences correspond to different species (Meiklejohn et al., 2011).

The distribution of these species in this area shows two species widely distributed. *S. argyrostoma* have been collected in the southern area, but this does not mean that this is the only area with presence of this species. In other works, this species have been collected in central Spain (Baz et al., 2015; Martín-Vega & Baz, 2013), north Spain and north Portugal (Prado e Castro, García, & Arnaldos, 2010). In the same way, *S. tibialis* was collected in northern Spain and also in the Balearic, Canary and Madeira Islands (Carles-Tolrá, 2002). The species collected only in one place in this work were found in more localities in previous works (Carles-Tolrá, 2002; Martín-Vega & Baz, 2013), but in a relatively small sampling area. Therefore, this could indicate that these species (*S. cultellata*, *S. jacobsoni*, *S. dux*, *S. africa*, *S. crassipalpis* and *W. magnifica*) tend to concentrate on limited populations.

The phylogeographic analysis has been useful in previous studies with Diptera (Marquez, Cummings, & Krafsur, 2007; Pfeiler et al., 2013; Ruiz-Arce et al., 2015). The distance among the sample points of our study are shorter than that of other studies, what seems to diminish the capacity of discrimination of the COI. Only the sequences of *S. argyrostoma* from Alcoy (Fig. 2.4) can be differentiated of the rest of populations

with this marker. Other markers should be investigated in future works to increase the capacity for discrimination.

Our results show the usefulness of the molecular techniques to identify samples of forensic cases, even when the samples were in poor preservation state. This has allowed us to rename some specimens that were assigned to the wrong dipteran family. This work provides 72 sequences of Sarcophagidae collected in forensic cases and other 117 of specimens collected with baited tramps with forensic interest. The data generated in this work improve the state of the molecular information of Sarcophagidae in the Iberian Peninsula, what will be of great help in future studies.

CHAPTER III

Chapter III

Diversity of the forensically important fly species *Calliphora vicina*, (Diptera: Calliphoridae) in the Iberian Peninsula using COI, 16S and ITS2 sequences.

Abstract

The study of Diptera in the scene of a crime provides essential information for the interpretation of evidence and, therefore, resolution of it. Its best known use is the calculation of the PMI (post mortem interval), but the forensic entomology has other applications: cases of neglect and/or abandonment of minors and the elderly, confirming the presence of chemical substances, and even confirming the relocation of a corpse. Phylogeographic reconstruction could help to differentiate haplotypes of a specific species from a geographical area, contributing to the clarification of the possible transfer of the corpse. Besides, reconstruction of ancestral states helps to understand the actual status of the species and its biogeographic history. In this study, 464 specimens of *C. vicina* collected in Spain and Portugal between 2012 and 2015 were used. They were identified with morphological keys and analysed by molecular techniques sequencing the COI, 16S and ITS2 genes. For it, three matrices were built in GENEIOUS 7.1.3 and the BLAST tool was used. For the construction of phylogeographic networks, the Median Joining algorithm of the PopART program was used. Reconstruction of ancestral states was realized with RASP program. Molecular techniques have corroborated the identifications made on morphological aspects. The phylogeographic networks show that there is no geographical structure with haplotypes shared among almost all populations. RASP analyses showed a high rate of movement among populations with colonizations crossing the Iberian Peninsula, possibly related to human activity.

Key words: *Calliphora vicina*, Iberian Peninsula, phylogeographic network, ancestral states.

Introduction

Study of the Diptera found in a crime scene can provide important information for resolution of cases. Identifying species correctly, knowing the species-specific developmental rate and the abiotic factors, the minimum post mortem interval (PMI) can be calculated (Amendt et al., 2011). Besides, they can be used in cases of neglect or abandonment of elderly / children / animals, entomotoxicology or relocation of corpses (Amendt et al., 2011; Introna, Campobasso, & Goff, 2001; Picard & Wells, 2010; Wells & Stevens, 2008).

One of the more widely studied species is *Calliphora vicina* Rovineau-Desvoidy, 1830 (Diptera: Calliphoridae). This species is distributed worldwide (Baz et al., 2015; Bonacci et al., 2009; Harvey et al., 2008; Marshall et al., 2011; Park et al., 2009; Williams & Villet, 2006). Generally, it is easy to differentiate this species from the closest ones with morphological keys (Akbarzadeh, Wallman, Sulakova, & Szpila, 2015) and its developmental rate of growing is well known (Donovan, Hall, Turner, & Moncrieff, 2006). Due to this, this species has been studied in all aspects of forensic entomology (Brown, Thorne, & Harvey, 2015; Matuszewski, Szafałowicz, & Jarmusz, 2013; O'Brien & Turner, 2004; Sadler, Richardson, Haigh, Bruce, & Pounder, 1995) and it has been reported in several forensic cases (Arnaldos, García, Romera, Presa, & Luna, 2005; Arnaldos, Sánchez, Álvarez, & García, 2004; Bonacci et al., 2009; Introna, Campobasso, & di Fazio, 1998).

However, in some cases the preservation state of the samples makes impossible to identify the specimens (Harvey, Dadour, & Gaudieri, 2003; Nelson, Wallman, & Dowton, 2007; Zehner et al., 2004), so the molecular techniques have become an important tool in this type of investigations. These techniques are able to identify specimens in any developmental state (Mazzanti et al., 2010; Meiklejohn et al., 2012), even with samples were damaged (Wells & Stevens, 2008). In this type of studies, the *cytochrome oxidase subunit one* (COI) gene has been widespread used (Aly, 2014; Boehme, Amendt, & Zehner, 2012; Desmyter & Gosselin, 2009; Nelson et al., 2007), while *16S ARN ribosomal* (16S) and *internal transcribed spacer 2* (ITS2) genes have been less studied or used for secondary analysis (Jordaens et al., 2013; Li et al., 2010; Nelson et al., 2007; Piwczyński, Szpila, Grzywacz, & Pape, 2014; Zaidi, Wei, Shi, & Chen,

2011), producing good results in the identification. These three genes are well represented in databases as National Center for Biotechnology Information (NCBI) and, for COI, Barcode of Life Data (BOLD).

In the same way, phylogeographic studies have been developed with different species of Diptera (Izumitani et al., 2016; Marquez et al., 2007; Pfeiler et al., 2013; Ruiz-Arce et al., 2015) and other insects (Hurtado-Burillo, May-Itzá, Quezada-Eúan, de la Rúa, & Ruiz, 2016; López-López et al., 2016). In these studies, relatively close locations could be differentiated by analysing the haplotypes of different genes. This type of analysis would allow the best reconstruction of the scene of a crime in which a corpse would have been relocated.

This information, combined with geographical and/or morphological information, has been used to investigate evolutionary and biogeographic history, and ancestral state reconstruction on different insect species (Blaimer et al., 2015; Bourguignon et al., 2016; Hedtke, Patiny, & Danforth, 2013; Zaspel et al., 2014). In Diptera, several studies were developed using different types of biogeographical approaches (Petersen, Meier, Kutty, & Wiegmann, 2007; Wagner & Müller, 2002; Yassin et al., 2008). Other studies used genetic information exclusively (Izumitani et al., 2016; Katoh et al., 2016) producing good results in the reconstruction of evolutionary history of the species. This type of analysis would allow us to know the background of the species, which is useful to understand the current status of the species and its populations.

The aims of this study are; (1) to generate sequences of COI, 16S and ITS2 of *C. vicina* samples from Spain and Portugal; (2) to analyse the biodiversity of these genes to determinate local haplotypes; and (3) to analyse ancestral states of these genes to determinate the plausible colonization of the Iberian Peninsula by this species. The general goal of the work is to generate data that could be useful to determine the original location of a corpse that would had been moved after the crime. No data of these subjects were found in the Iberian Peninsula, which makes this study necessary.

Material and Methods

Fly collection and morphological identification

Specimens were collected in 25 localities from north and south of the Iberian Peninsula, covering the Cantabric area and occidental south part from Spain, and north and south part from Portugal (Fig. 3.1). The sampling was carried out in autumn in the Portuguese areas and across all year in the Spanish areas, between 2012 and 2015. Sampling was made using bottle traps (Allemand & Aberlenc, 1991). The traps were baited with pig liver and blood, and they were placed in peri-urban areas of each collection locality for 3 – 6 days. Due to the dirtiness that were on the specimens collected, they were washed in 70% alcohol to remove it before preserving them in absolute alcohol. The specimens were morphologically identified using keys until species level (Akbarzadeh et al., 2015; Szpila, 2012).

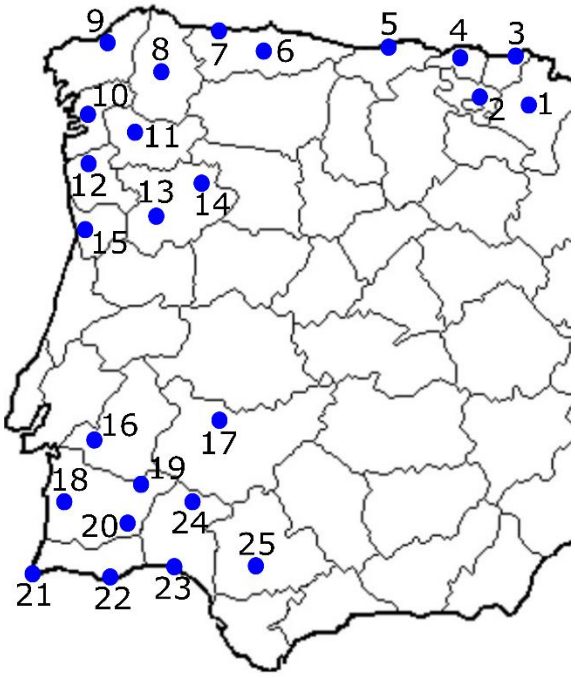


Figure 3.1: Sampling localities from Spain and Portugal. The localities are: (1) Barañain, (2), Lubiano, (3) San Sebastián, (4) Arrigorriaga, (5) Santander, (6) Oviedo, (7) Navia, (8) Lugo, (9) Betanzos, (10) Pontevedra, (11) Ourense, (12) Fontoura, (13) Vila Real, (14) Bragança, (15) Porto, (16) Montemor-o-Novo, (17) Mérida, (18) São Luis, (19) Moura, (20) Mértola, (21) Sagres, (22) Faro, (23) Punta Umbría, (24) Jabugo and (25) Dos Hermanas.

DNA extraction and PCR amplification

The extraction of DNA was made using two legs and thoracic muscle of each specimen. Glass Fiber Plate DNA extraction protocol of the CCDB was used (Ivanova et al., 2006). The extractions were diluted in 60 µl of prewarmed (56°C) ddH_2O.

Amplification of the genes was performed using a thermocycler 2720 Thermal Cycler (Applied Biosystems, Foster City, U.S.A.). For COI mitochondrial gen was amplified using LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers (Folmer et al., 1994). PCR for COI was performed using a program with an initial activation of 95°C for 3 min, followed by 35 cycles of 60 sec at 94°C, 60 sec at 45°C and 90 sec at 72°C, and a final extension of 5 min at 72°C. 16S mitochondrial gen was amplified using 16S-F (5'-CCGAGTATTTTGACTGTGC-3') and 16S-R (5'-TAATCCAACATCGAGGTCGCAA-3') primers (Zerm et al., 2007). PCR program for 16S gen had an initial activation of 94°C for 5 min, followed by 40 cycles of 30 sec at 94°C, 30 sec at 50°C and 1 min at 72°C, and a final extension of 10 min at 72°C. ITS2 nuclear gen was amplified using 5.8sF (5'-GTGAATTCTGTGAACTGCAGGACACATGAAC-3') and 28Sr (5'-ATGCTTAAATTTAGGGGGTA-3') primers (Porter & Collins, 1991). PCR for ITS2 gen consisted in an initial activation of 94°C for 3 min, followed by 37 cycles of 30 sec at 94°C, 30 sec at 54°C and 30 sec at 72°C, and a final extension of 72°C for 10 min. We prepared the PCR-cocktails with a KAPABIOSYSTEMS PCR kit (Wilmington, U.S.A.) for performed the PCRs. The kit includes buffer (10x), dNTPs and Taq polymerase. Target DNA, primers and water were included by us. For the reaction, 12.5 µL of volume were obtained, 1.5 µL of which were DNA. 2 µL of every PCR product were visualized on a 2% agarose gel stained with RED SAFE (iNtRON Biotechnology, Seongnam, South Korea) by electrophoresis and results were visualized under ultraviolet light.

Sequencing of genes and molecular analyses

Samples were sent to Macrogen (Amsterdam, The Netherlands) to be sequenced with an ABI Prism 3730XL. We used the GENEIOUS 7.1.3 program (Kearse et al., 2012) to edit manually the possible reading mistakes on the sequences. The resulting sequences were aligned using the MUSCLE algorithm (Edgar, 2004) to obtain three different

matrix. The morphological identification of the samples was confirmed using the BLAST tool (Altschul et al., 1997).

The haplotypes analyses were developed using the PopArt program (Bandelt et al., 1999). The Median-Joining algorithm was used for developing the haplotypes. The samples were organized by the localities in order to know if the haplotypes could differentiate the origin of a sample. To reconstruct the ancestral areas of distribution, the program RASP has been used (Yu, Harris, Blair, & He, 2015). This method infers possible ranges of ancestral species (populations in our case) at each node. We used the Bayesian Binary MCMC algorithm (BBM) to construct the tree, using the default variables and grouping the samples in seven biogeographical areas (Fig. 3.2). The Neighbor-Joining (NJ) trees used in these analyses were inferred in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) using the Kimura-2-Parameter (K2P) algorithm.

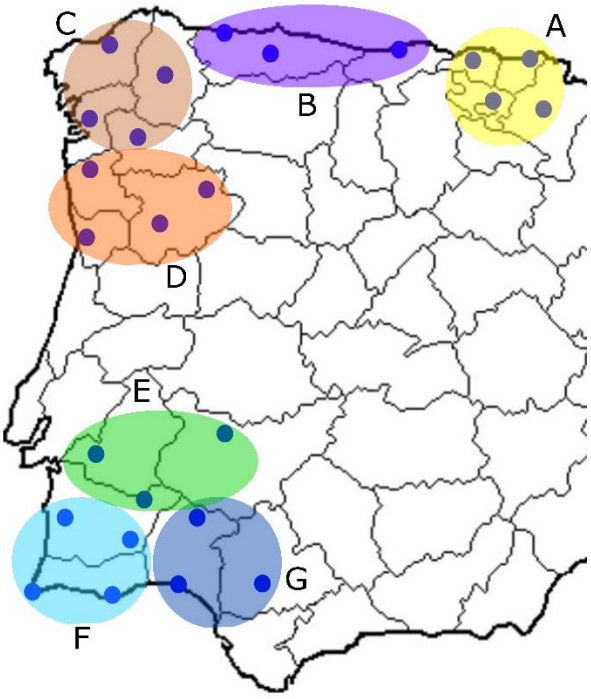


Figure 3.2: Geographical areas chosen for the RASP analyses. Names and localities of each areas are: "A" Basque Country area (Barañain, Lubiano, San Sebastián and Arrigorriaga), "B" Asturias-Cantabria area (Santander, Oviedo and Navia), "C" Galicia area (Lugo, Betanzos, Pontevedra and Ourense), "D" North Portugal area (Fontoura, Bragança, Vila Real and Porto), "E" Extremadura area (Mérida, Montemor-o-Novo and Moura), "F" South Portugal area (São Luis, Mértola, Sagres and Faro) and "G" Andalucía area (Jabugo, Punta Umbría and Dos Hermanas).

Results

A total of 464 samples of *C. vicina* were selected. During the amplification and sequencing processes, not all the samples could be used for the three genes due to their state of preservation and deterioration. The number of sequences obtained for each gene from each locality is indicated in Table 3.1. All the specimens were molecularly identified with BLAST to confirm the morphological identification. The three genes produced positive results. The size of the matrices constructed were: 408 bp for COI matrix, 251 bp for 16S matrix and 356 bp for ITS2 matrix.

Table 3.1: Sequences used to build the matrix of the three genes, total of sequences for each matrix and localities that where the sequences come from.

	Sequences number		
Locality	**COI**	**16S**	**ITS2**
Barañain	1	1	18
San Sebastián	6	11	19
Lubiano	4	3	21
Arrigorriaga	5	5	19
Santander	-	1	7
Oviedo	3	13	19
Navia	4	17	20
Lugo	-	5	12
Betanzos	19	4	20
Ourense	20	12	19
Pontevedra	15	8	20
Dos Hermanas	10	11	19
Punta Umbría	14	12	19
Jabugo	12	7	19
Mérida	2	4	19
Faro	20	20	19
Sagres	-	13	20
Mértola	2	5	20
São Luis	10	14	21
Moura	14	9	20
Motemor-o-Novo	1	17	19
Porto	3	8	18
Bragança	3	1	19
Vila Real	6	5	20
Fontoura	13	-	18
Total	**187**	**206**	**464**

The haplotype network developed for the ITS2 did not show any geographical distribution of haplotypes (Fig. 3.3). One widespread haplotype was represented ("Haplotype 1" in Fig. 3.3) including samples from all the localities. Other haplotype

shared among nine localities from north and south of Iberian Peninsula was located at only one mutational step from it. Others minor haplotypes are represented close to the Haplotype 1 including one or two localities.

In the case of 16S gene, one widespread haplotype was represented too (Fig. 3.4, "Haplotype 1"). One haplotype with samples from three localities and other with samples from five localities were represented next it, but they did not show a local haplotype structure. Seven haplotypes with 1-5 samples from Dos Hermanas and Punta Umbría (southeast of Spain) seems to be related among them and with the Haplotype 1. Generally, no geographical structure was shown for this gen.

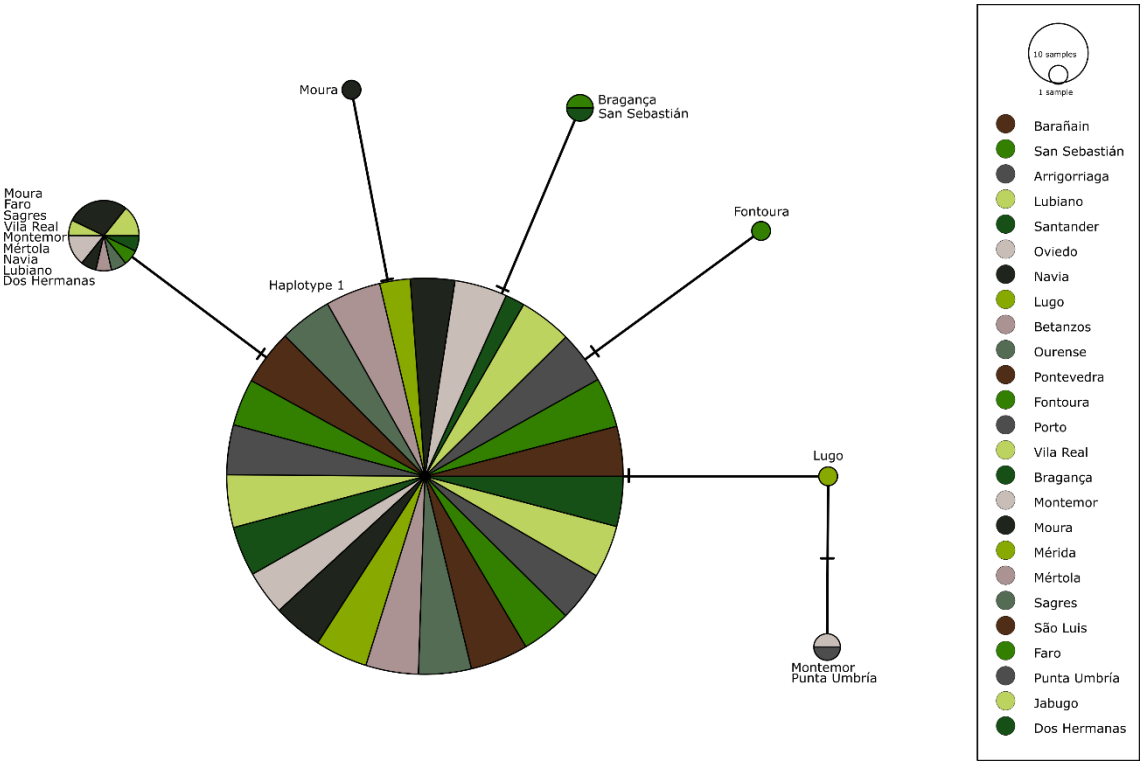


Figure 3.3: Haplotype network build with PopArt for the ITS2 gen.

The COI gene produced the most complex haplotype network (Fig. 3.5), but no geographical structure was observed. In this case two widespread haplotypes were represented. In the Haplotype 1, a total of 19 localities were included while in the Haplotype 2 samples from 13 localities were included. Between these two widespread haplotypes, 11 localities are shared. The sequences from Lubiano were not included in any one of these haplotypes, but they did not form a haplotype by them self.

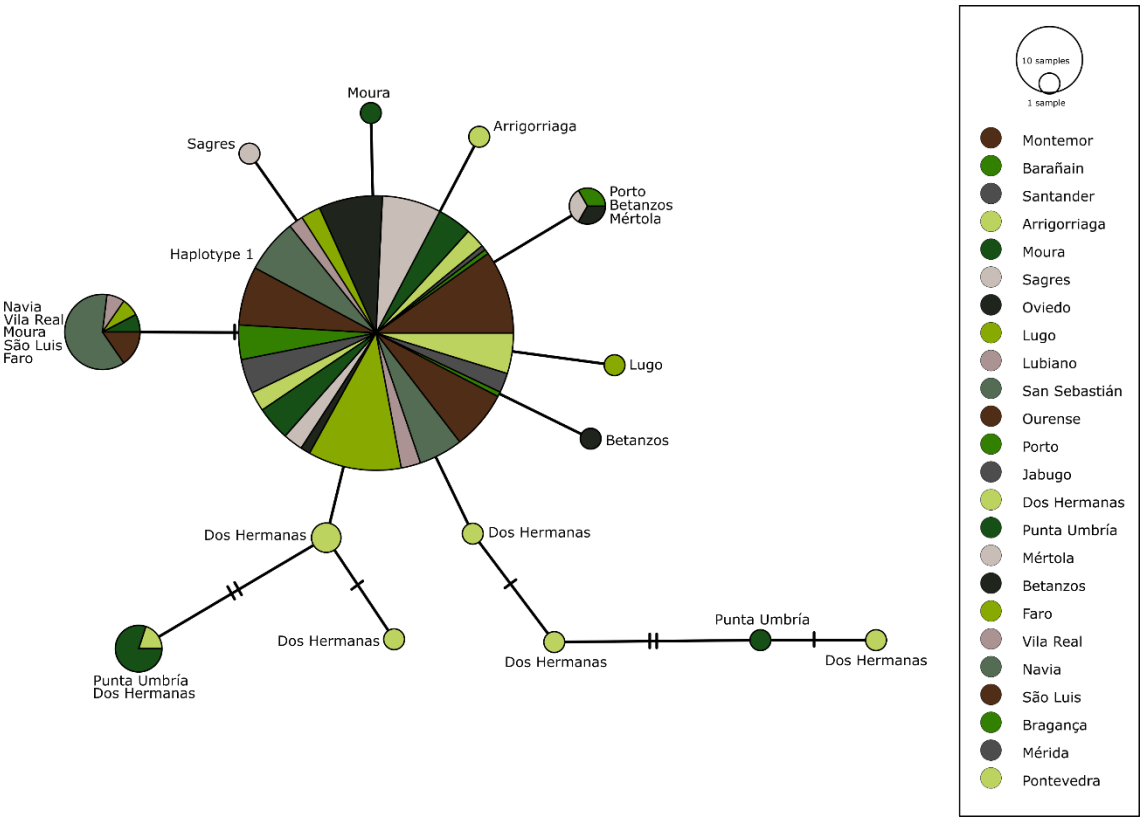


Figure 3.4: Haplotype network build with PopArt for the 16S gen.

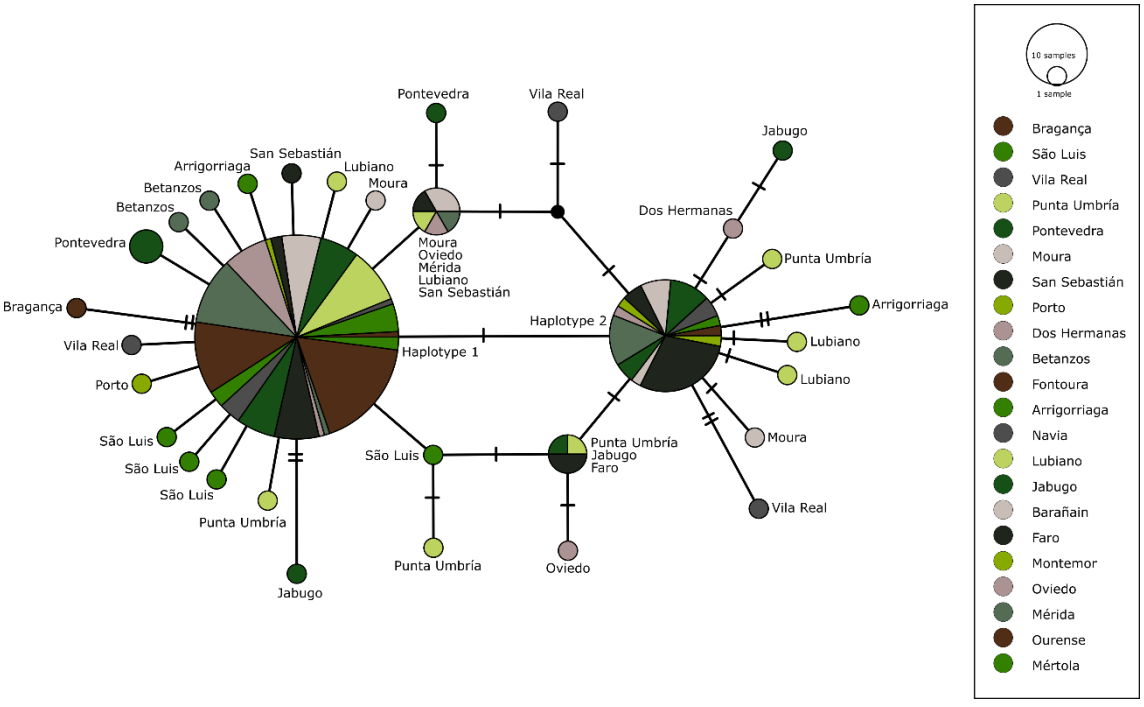


Figure 3.5: Haplotype network build with PopArt for the COI gen.

Three sequences from Pontevedra form a haplotype, but the other sequences from this locality are included in the widespread haplotypes commented above. The rest of

the haplotypes were formed for only one sequence or by sequences no geographical related.

The RASP analyses showed a high degree of movements between different geographical areas in the Iberian Peninsula (Fig. 3.6, Fig. 3.7 and Fig. 3.8). Along the trees, the ancestral nodes tend to conserve the geographic area, but multiple and frequent changes, corresponding to long-distance colonizations, can be observed. These colonizations usually follow a north-south direction, or vice versa.

Discussion

In this study, specimens of *C. vicina* species were collected in localities of north and south of Spain and Portugal, increasing the number of localities in that this species were collected in previous works (Arnaldos et al., 2005; Baz et al., 2015; Carles-Tolrá, 2002; Castillo Miralbes, 2002; González Medina et al., 2011; Martínez-Sánchez, Rojo, & Marcos-García, 2000; Moneo Pellitero & Saloña-Bordas, 2007; Peralta Álvarez, GilArriortua, & Saloña Bordas, 2013; Prado e Castro, Sousa, Arnaldos, Gaspar, & García, 2011; Prado e Castro, Serrano, Martins da Silva, & García, 2012; Velásquez, Gobbi, Martínez-Sánchez, & Rojo, 2015). The morphological identification was effective even with dirty samples, confirming de effectiveness of these morphological keys (Akbarzadeh et al., 2015; Szpila, 2012). Morphological identification has been confirmed by molecular identification, showing the potential utility to recognize this species in cases of deteriorated samples, in agreement with previous works (Boehme et al., 2012; Nelson et al., 2012; Reibe, Schmitz, & Madea, 2009; Rolo et al., 2013).

Previous phylogeographic studies have shown positive results delimiting local haplotypes. The study of López-López et al. (2016) showed good congruence between the haplotypes and the origin of the samples of *Pseudotetracha* tiger beetles. The dispersion capacity of these Coleoptera through the seasonal lakes derived in specific haplotypes of each area. In Hurtado-Burillo et al. (2016), phylogeographic techniques were useful to distinguish among Mesoamerican *Scaptotrigona* species (stingless bees) and the mitochondrial markers (COI and 16S) were able to reveal the geographic structure of some species.

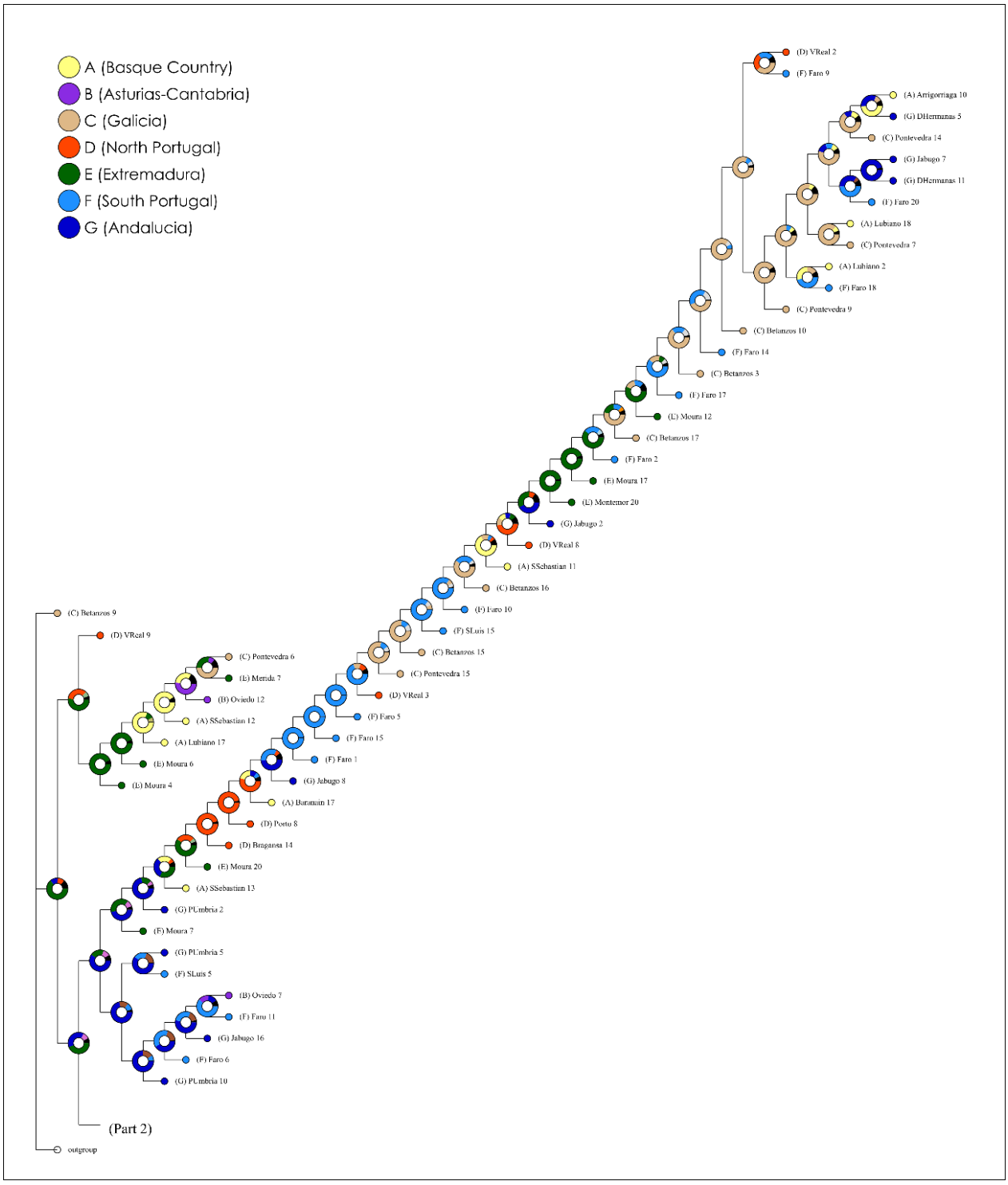


Figure 3.6: Ancestral states RASP tree for COI gene (part 1). Nodes color correspond to the probability that the antecesor came from any location.

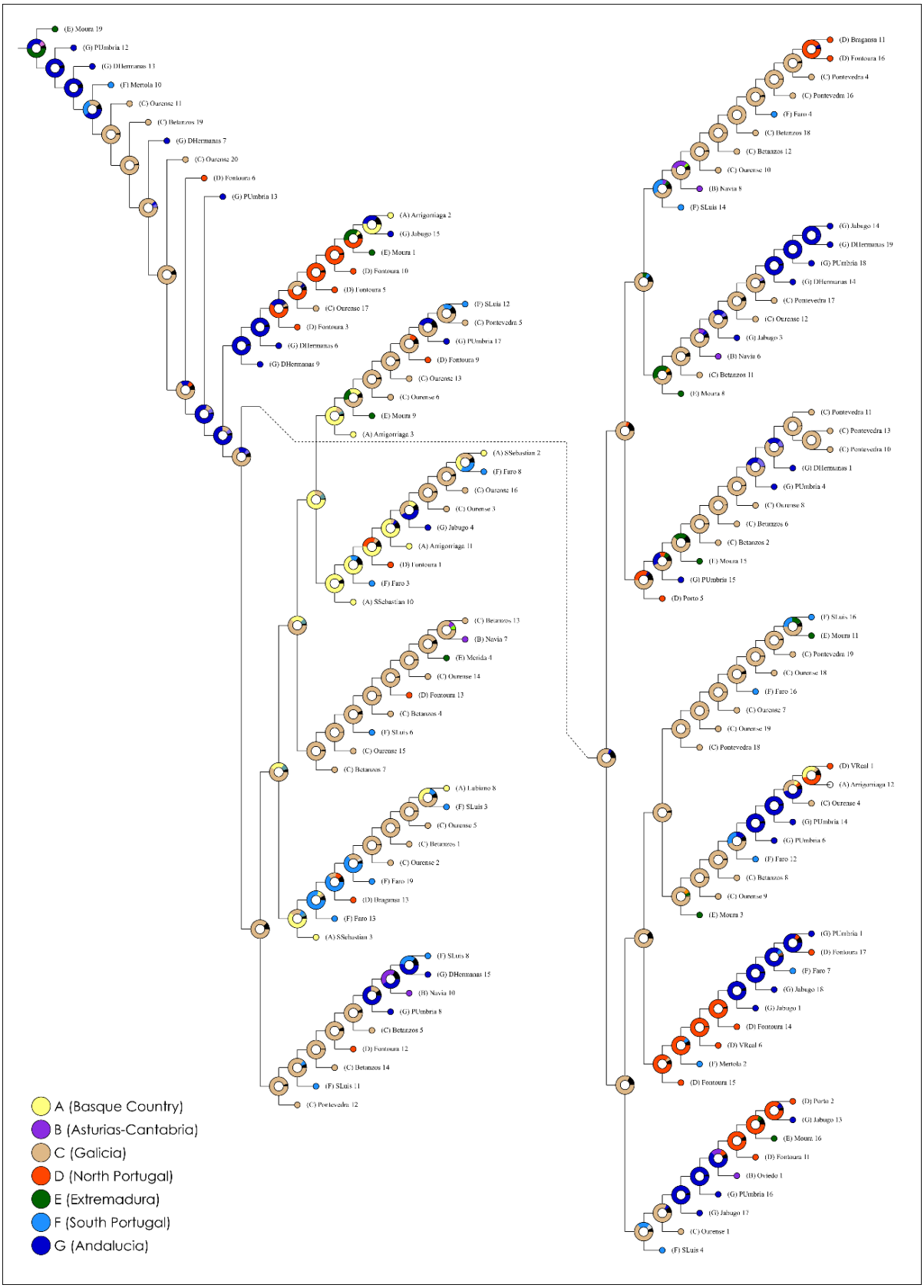


Figure 3.7: Ancestral states RASP tree for COI gene (part 2). Nodes color correspond to the probability that the antecesor came from any location.

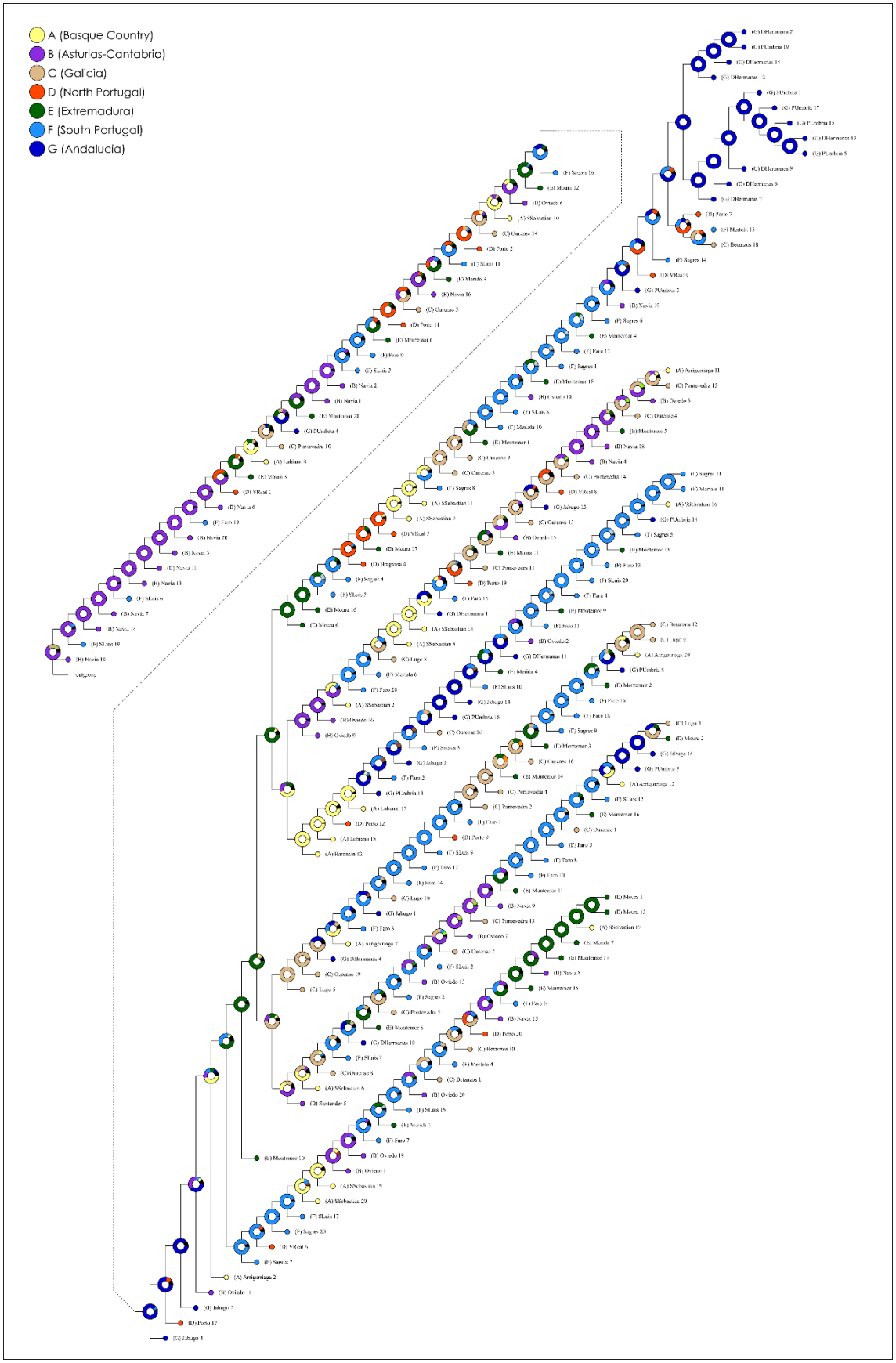


Figure 3.8: Ancestral states RASP tree for 16S gene. Nodes color correspond to the probability that the antecesor came from any location.

The nuclear marker ITS2 had been compared with the COI mitochondrial gene (Song, Shao, Atwell, Barker, & Vankan, 2011) to distinguish between two species of *Ixodes*, producing good results differentiating some geographical areas. In Diptera, mitochondrial and nuclear genes have been used with this technique, but no information about the ITS2 gene of *C. vicina* species has been found (Izumitani et al., 2016; Marquez et al., 2007; Pfeiler et al., 2013; Ruiz-Arce et al., 2015).

The area of study of some of these studies were much more higher than ours (Izumitani et al., 2016; Marquez et al., 2007), but they reach to distinguish local haplotypes and reconstruct the evolutionary history of its species of study. The other studies were able to differentiate local haplotypes of closest regions (Pfeiler et al., 2013; Ruiz-Arce et al., 2015). Only one study with samples of Diptera from Iberian Peninsula have been found (Seabra et al., 2015). Our results do not show geographical structure of the populations, in disagreement with studies that found it for samples with similar distances among them (López-López et al., 2016; Pfeiler et al., 2013). Increasing the number of specimens studied could produce better results, but similar amount of samples produce enough differences in other studies (Marquez et al., 2007; Ruiz-Arce et al., 2015). So that, this situation would be better explained by a very fast/recent spatial and demographic expansion (Seabra et al., 2015). Besides, the potential geological barriers present in south (Vale do Guadiana, Sierra de Aracena, etc.) and north (Cordillera Cantábrica, Macizo Galaico, Serra da Estrela, etc.) of the Iberian Peninsula do not seem to be enough to not allow the genetic flow among the populations. This agrees with studies of widespread species in which the authors did not found geographical structure (Karsten, van Vuuren, Barnaud, & Terblanche, 2013; van Gremberghe et al., 2011). In our case, *C. vicina* is a very anthropized species usually associated to farm animals (Carles-Tolrá, 2002; Martínez-Sánchez et al., 2000). So, it is probably that the dispersal process has been associated to human movements or, even, to the national and international trade of different types of farm animals, in the same way as Seabra et al. (2015).

These results can also be observed in the output of the RASP analyses, where the populations of *C. vicina* are shown to stay in determinated geographical areas for certain periods of time, but a high degree of movement is also observed. Some of

these movements or colonizations are even between localities separated by a long distance, such as southern Portugal and the Basque Country. This reinforces the hypothesis of that the geographical barriers found in the Iberian Peninsula have no effect impeding the translocation of *C. vicina* between localities. Most of the colonizations follow a latitudinal gradient, suggesting that the temperature changes might be the factor that drives the movement of *C. vicina*. Thus, a colder than normal winter may result in the northern populations traveling to the south, or warmer summers would push the southern populations northwards. In that case, we must not underestimate the putative role of the current global change in the future evolution and distribution of the economically and forensically important species.

CHAPTER IV

Chapter IV

Species of the genus *Lucilia* (Diptera: Calliphoridae) with forensic interest in northern Spain: a molecular and morphogeometrical approach.

Abstract

The correct identification of species is an essential step in studies of forensic entomology, since incorrect identification can lead to errors in the calculation of Post Mortem Interval (PMI). This task is more complicated when closely related species are analysed with morphological characters difficult to observe in damaged or poorly conserved specimens. This study has been developed, with three species *Lucilia ampullacea*, *Lucilia caesar* and *Lucila illustris* (Diptera, Calliphoridade) employing morphological, molecular and morphogeometrical analyses. In this study, we have realized phylogenetic and morphogeometric analysis of the samples collected between 2012 and 2014 in northern Spain. We have amplified and sequenced the mitochondrial fragment cytochrome oxidase I (COI) and the nuclear fragment internal transcribed spacers 2 (ITS2). They have been analysed phylogenetically by distance methods with Neighbor-Joining and phylogeographically through haplotype networks. We have defined 18 landmarks on the fly wings and 18 on the head. In both structures, we compared the differences using principal component analysis (PCA) and canonical variable analysis (CVA). The phylogenetic and phylogeographic analyses have shown a clear difference between *L. ampulacea* and the other two species, but there was no clear distance between *L. caesar* and *L. illustris*. Intra and interspecific distance has shown the lack of genetic differences between these two species. The morphogeometric analysis of the wings and the head corroborated the separation of *L. ampullacea*. The separation of *L. caesar* and *L. illustris* by the CVA analysis showed two well-differentiated groups with a small gap zone in both structures. Therefore, these tools could be the key to differentiate these species of forensic interest. Besides, grouping the samples by location different groups were well defined. The provenance of a sample could be investigated in this way.

Key words: Species identification, forensic entomology, morphometric geometry, genus *Lucilia*, molecular techniques.

Introduction

In forensic entomology the necrophagous Diptera are used to calculate the minimum post mortem interval (PMI). This is an estimation of the corpse's age based on the developmental stage of the larvae growing in it. To carry out the calculation of the PMI is necessary to know some abiotic factors and the identification of the species must be accurate (Aly, 2014; Amendt et al., 2011; Sharma et al., 2015; Zajac et al., 2016). If the identification of the species would not be accurate, the generated data could not be used in a forensic investigation.

There are cases in which the identification results a hard task, even for expert taxonomists. Differentiate morphologically among some related species is often difficult in juvenile stages (larvae or egg) (Nelson, Wallman, & Dowton, 2007). Besides, the samples collected in a crime scene are not always well preserved (Harvey, Dadour, & Gaudieri, 2003; Zehner et al., 2004). Rearing the samples to the adult stage is not possible in all cases either.

Lucilia (Diptera: Calliphoridae) is one of the more widespread genus of flies and it has been described worldwide (Stevens & Wall, 1996). Species of this genus have medical and veterinary importance since they produce myasis (Picard et al., 2018). The importance of this genus in forensic entomology lies in the species that are used in the PMI calculation and those that have the potential for it (Day & Wallman, 2008; Debry, Timm, Dahlem, & Stamper, 2010; GilArriortua, Saloña-Bordas, Cainé, Pinheiro, & de Pancorbo, 2015; Klong-Klaew et al., 2012; Szpila, Pape, & Rusinek, 2008; Tourle, Downie, & Villet, 2009; Wells, Wall, & Stevens, 2007; Wilson, Nigam, Jung, Knight, & Pritchard, 2016). However, some close species are difficult to use in these analysis because of the morphological similarity and the problems commented above. This is the case of *Lucilia ampullacea* Villenueve, 1922, *Lucilia caesar* (Linaeus, 1758) and *Lucilia illustris* (Meigen, 1826). These are considered sister species (Akbarzadeh, Wallman, Sulakova, & Szpila, 2015; Boehme, Amendt, & Zehner, 2012; Harvey, Gaudieri, Villet, & Dadour, 2008; Malgorn & Coquoz, 1999) that share distribution

areas, although *L. illustris* is considered less anthropized than *L. caesar* and *L. ampullacea* (Martínez-Sánchez, Rognes, Rojo, & Marcos-García, 2001; Martínez-Sánchez, Rojo, & Marcos-García, 2000; Rognes, 1991; Saloña, Moneo, & Díaz, 2009). Moreover, the differences among them in morphological keys are based in structures easily confused in samples not well preserved (presence/absence of coxopleural streak, shape of ovipositor VIth tergite (female) or shape of surstyli (male)) (Akbarzadeh et al., 2015; Szpila, 2012).

Molecular techniques has been proven to be a very useful tool for species identification (Kim et al., 2014; Park et al., 2009; Pettersson et al., 2013; Saigusa et al., 2005). These techniques are able to be used with samples not well preserved, broken samples and specimens at any state of development (Mazzanti et al., 2010; Meiklejohn et al., 2011, 2012; Wells & Stevens, 2008). Thus, forensic entomology studies has been developed with molecular techniques worldwide in last years (Harvey, Mansell, Villet, & Dadour, 2003; Olekšáková, Žurovcová, Klimešová, Barták, & Šuláková, 2018; Sharma et al., 2015; Tan et al., 2009; Yusseff-Vanegas & Agnarsson, 2017), with nuclear (Nelson, Wallman, & Dowton, 2008) and mitochondrial genes (Koroiva et al., 2018). However, there are cases in which these tools are not enough accurate because of the threshold of the species delimitation (Hebert et al., 2004; Hebert, Ratnasingham, et al., 2003; Meiklejohn et al., 2011) and the molecular distance among very closely related species (Sonet et al., 2012). Therefore, other techniques should be used to complete studies in which these types of problems occur.

Geometric morphometrics techniques consist of quantitative analyses based on the visualization of the shape changes, in two or three dimensional spaces, comparing distances among discrete anatomical points designated by landmarks (Bookstein, 1997; Klingenberg, 2013; Zelditch, Swiderski, Sheets, & Fink, 2004). These landmarks can be analysed by Principal components analysis (PCA), to determine variables, or Canonical variation analysis (CVA), to explore differences among groups (Zelditch et al., 2004). Although the structures more widely used for geometric morphometric studies are the wings (Aytekin et al., 2007; Roggero & Passerin d'Entrèves, 2005; Su et al., 2015), other structures has been used producing positive species differentiation (de Freitas & Morales, 2009; Gamboa & Arrivillaga, 2010). In previous studies, these

techniques have been proven to be useful tools to analyse dipteran groups (Brown, 1980; Espra et al., 2015; Grzywacz, Ogiela, & Tofilski, 2017; Hall, MacLeod, & Wardhana, 2014). No information about this group of *Lucilia* is available.

The aims of this work are (1) to generate sequences of *cytochrome oxidase subunit one* (COI) and *Internal transcribed spacer 2* (ITS2) of *L. ampullacea*, *L. caesar* and *L. illustris* collected in northern Spain, (2) to carry out several molecular analyses and (3) to analyse several structures with geometric morphometric techniques. The general goal is to check different ways to delimitate these related species. Not much information has been gathered about it in Europe (Boehme et al., 2012; Picard et al., 2018) and none at all in the Iberian Peninsula, due to which this study is badly needed.

Material and Methods

Fly collection and morphological identification

Samples were collected in eight localities across the Cantabrian area between 2012 and 2014 (Fig. 4.1), covering seven Autonomous Communities of northern Spain. The sampling was carried out in all year season, except winter, using bottle taps (Allemand & Aberlenc, 1991) baited with pig liver and blood.

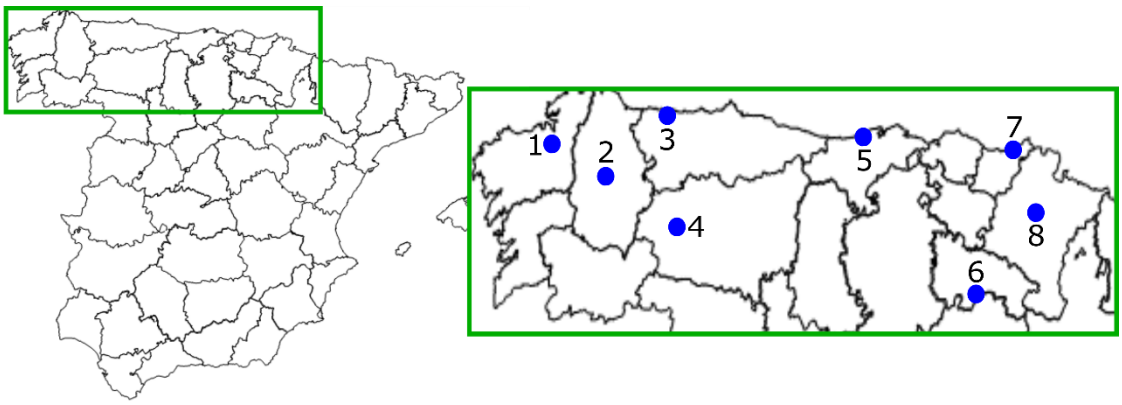


Figure 4.1: Studied area and location of the sampling points. The locations are: 1 Betanzos (Galicia), 2 Lugo (Galicia), 3 Navia (Asturias), 4 Ponferrada (Castilla y León), 5 Santander (Cantabria), 6 Villanueva de Cameros (La Rioja), 7 San Sebastián (Euskadi) and 8 Barañain (Navarra).

Traps were placed in peri-urban areas for 3 days. Adults captured were washed in 70% alcohol to remove dirtiness before preserving them in 100% alcohol. Specimens were identified using morphological keys (Akbarzadeh et al., 2015; Szpila, 2012). However,

some samples had difficulties to identified because the rests of bait. These identifications were considered *putative* until confirmation by molecular identification.

DNA extraction and PCR amplification

One or two legs and thorax muscle were used to extract genomic DNA using the Glass Fiber Plate DNA extraction protocol of the CCDB (Ivanova et al., 2006). All extractions were diluted in 60 µL of ddH_2O prewarmed to 56°C.

Amplification of the COI gene was made using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') of the barcoding region described by Folmer (Folmer et al., 1994). ITS2 gene was amplified using those described by Porter and Collins (Porter & Collins, 1991): 5.8sF (5'-GTGAATTCTGTGAACTGCAGGACACATGAAC-3') and 28Sr (5'-ATGCTTAAATTTAGGGGGTA-3'). Amplification was performed in a thermocycler 2720 Thermal Cycler (Applied Biosystems, Foster City, U.S.A.). The PCR was carried out preparing PCR-cocktails with a KAPABIOSYSTEMS PCR kit (Wilmington, U.S.A.), that included all components needed for it (buffer (10x), dNTPs and Taq polymerase) except water, primers and the target DNA. The final reaction volume was 12.5 µL of which 1.5 µL were DNA. The PCR program consisted in an initial activation of 95°C for 3 min, followed by 35 cycles of 60 sec at 94°C, 60 sec at 45°C and 90 sec at 72°C, and then a final extension of 5 min at 72°C. After amplification, 2 µL of every PCR product were visualized by electrophoresis on a 2% agarose gel stained with RED SAFE (iNtRON Biotechnology, Seongnam, South Korea) dye fluorescent product and visualized under ultraviolet light.

Sequencing of COI and ITS2 genes and molecular analyses

Samples were sequenced in Macrogen (Amsterdam, The Netherlands) using an ABI Prism 3730XL. The program GENEIOUS 7.1.3 (Kearse et al., 2012) was used to edit manually the sequences. Sequences were compared with those included in the NCBI (National Center of Biotechnology Information) database using the BLAST tool (Altschul et al., 1997). Comparisons that obtained the lowest E-Value and higher Grade were considered as putative identifications. The alignments of matrix of both genes were done with MUSCLE (Edgar, 2004). Neighbor-Joining (NJ) trees were done with MEGA X

(Kumar et al., 2018) using Kimura-2-Parameter (K2P) distance algorithm (Kimura, 1980).

Molecular distances of both matrix were calculated with TaxonDNA (SpeciesIdentifier 1.7.8-sy1) (Meier et al., 2006). Haplotype networks were developed with PopArt program (Bandelt et al., 1999) using the Median-Joining algorithm, in order to know if the species could be identified due to its separation in different groups of haplotypes.

Digitization of landmarks of the wings and head. Multivariate analysis

Wings were removed from the thorax of 89 specimens. When the specimens were in bad preservation conditions, both wings were collected to choose the one that was least degraded. The wings were extended between two crystal slides with a drop of water. Then, the slides were inside a heater for 24 hours to eliminate bubbles (Espra et al., 2015; Hall et al., 2014). For the head samples, some of them were not in enough good conditions to be used. Besides, due to the sexual dimorphism and the low number of males, we used 49 female heads that were available. These samples were used without previous treatment, with the frontal part of the head directed to the camera (de Freitas & Morales, 2009; Gamboa & Arrivillaga, 2010).

All samples were studied using a stereo microscope STEMI-200-C (Fisher Scientific, Madrid, Spain) calibrate with SPOT 4.6 program (Taubman, Leffler, & Silicon, 2001). The chosen landmarks were digitalized using the Tps package (Rohlf, 2010). In case of wing samples, the 18 selected landmarks are based on those of the work of Espra (Espra et al., 2015), that are also focused in the *Lucilia* genus (Fig. 4.2, table 4.1). In the case of head samples, no information about morphometric geometric techniques in dipteran head was found. Therefore, we based our 18 landmarks in the work of de Freitas (de Freitas & Morales, 2009), that are focused in Neuroptera insects but some structures are usable in the same way (Fig. 4.3, table 4.2). To determinate if the landmarks are enough accurate for the analysis, we performed the sumperimposition using the R geomorph package in R statistical software (Adams & Otárola-Castillo, 2013).

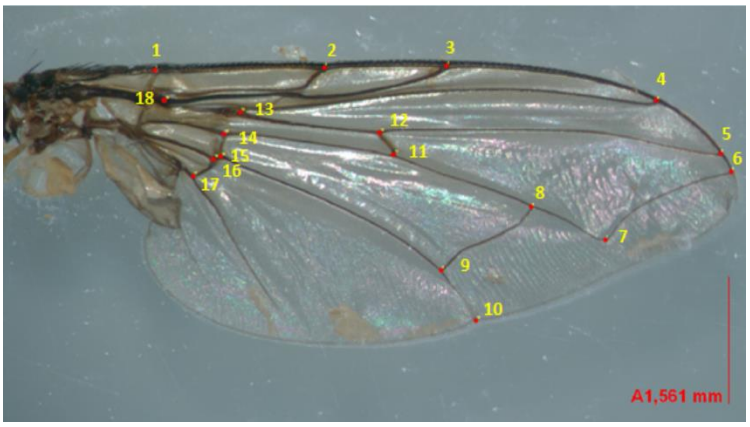


Figure 4.2: Landmark positions on a L. caesar wing.

Table 4.1: Description of the landmarks used on the wings.

Landmarks	Description of the landmarks
1	Humeral cross-vein
2	Subcostal vein
3	Anterior branch of radius R_2 vein
4	R_{2+3} vein, the distal end of Radius
5	R_{4-5} vein, the distal end of Radius
6	Branching endpoint of media vein
7	Curve point of CVA_1 vein
8	Intersection between Media vein and bm-cu vein
9	Intersection between bm-cu vein and CuA_2 vein
10	Branching point of CuA_2 vein
11	Intersection between Median vein and r-m vein
12	Intersection between r-m vein and R_{4+5} vein
13	Branching point of vein Rs vein
14	Intersection between bm-cu and Median vein
15	Intersection between CuA_2 vein and bm-cu vein
16	Intersection between CuA_2 and bm vein
17	Intersection between A_1+ CuA_2 vein and CuA_2 vein
18	Intersection of Rs vein and R_1 vein

To examine the wing and head shapes variation among specimens, species and population, the data files were analysed with the statistical software MorphoJ (Klingenberg, 2011). The arrangement of specimens in morpho-space or the main features of shape variation in a sample, was analysed with PCA analysis. Ordination analysis of separation of specified groups (species, populations, etc.) was evaluated using CVA analysis.

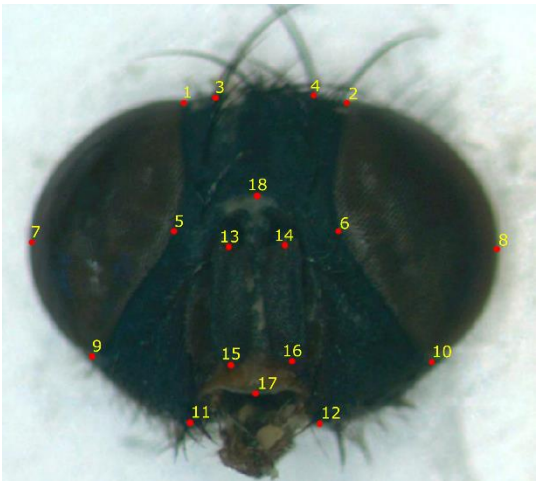


Figure 4.3: Landmark positions on a L. caesar head.

Table 4.2: Description of the landmarks used on the heads.

Landmarks	Description of the landmarks
1	Right eye upper margin
2	Left eye upper margin
3	Upper final of right orbital bristles line
4	Upper final of left orbital bristles line
5	Interior angle of right eye
6	Interior angle of left eye
7	Right eye lateral margin
8	Left eye lateral margin
9	Right eye lower margin
10	Left eye lower margin
11	Lower right margin of the mouth
12	Lower left margin of the mouth
13	Flagellum base of the right antennae
14	Flagellum apex of the right antennae
15	Flagellum base of the left antennae
16	Flagellum apex of the left antennae
17	Lower margin of the clypeus
18	Upper margin of the frontal suture

Results

A total of 89 specimens were selected: 63 of *L. caesar*, 13 of *L. illustris* and 13 of *L. ampullacea*. They were previously identified with morphological keys. These quantities are the result of re-identification after comparing to the molecular information. Therefore, six specimens previously identified as *L. illustris* were finally identified as *L.*

caesar. In addition to these samples, four sequences of *L. illustris* and four of *L. caesar* from Boehme *et al*., work (2012) had been included to compared our results with theirs (Boehme et al., 2012). The preservation state of the samples made impossible to use every specimen in all analyses. Therefore, we developed different data matrix to ITS2, COI and COI plus the sequences from Boehme *et al*. (2012), besides the data matrix of the geometric morphometic analyses. The samples used in each analysis are indicated in table 4.3.

Table 4.3: Geographical location and sex of the specimens. Analyses in which each one is included is indicated by ●.

		Molecular techniques			Geometric Morph.	
Species	**Location Point (Sex)**	**COI**	**COI Boehme**	**ITSII**	**Wing**	**Head**
Lucilia caesar	Barañain (F)	●		●	●	●
	Betanzos (F)	●	●	●	●	●
	Betanzos (F)	●	●	●	●	
	Santander (F)	●	●	●	●	●
	V. de Cameros (F)	●	●	●	●	●
	Betanzos (F)	●	●	●	●	
	Santander (F)	●	●	●		
	Santander (F)			●	●	●
	Betanzos (F)	●	●	●	●	●
	V. de Cameros (F)	●	●	●	●	●
	V. de Cameros (F)	●	●	●	●	●
	Betanzos (F)	●	●	●	●	●
	Betanzos (F)	●	●	●	●	●
	V. de Cameros (F)	●	●	●	●	●
	Barañain (F)	●	●	●	●	
	San Sebastián (M)	●	●	●	●	●
	Lugo (F)	●	●	●	●	
	V. de Cameros (F)	●	●	●	●	●
	Barañain (F)	●	●	●	●	●
	Barañain (F)	●		●	●	●
	Barañain (F)	●	●	●		
	Santander (M)	●	●	●	●	
	Navia (F)	●	●	●		
	Lugo (F)			●	●	●
	Navia (M)			●	●	●
	Betanzos (F)			●		
	Barañain (F)			●	●	

	Betanzos (F)			●		
	Santander (F)			●	●	●
	Lugo (F)			●	●	
	Betanzos (F)			●		
	Barañain (F)			●	●	●
	Santander (F)			●		
	Lugo (F)			●		
	Lugo (F)			●	●	●
	V. de Cameros (F)	●	●		●	●
	Barañain (F)	●	●		●	●
	Santander (F)	●	●	●	●	●
	Barañain (F)	●	●		●	●
	Betanzos (F)	●	●		●	
	Lugo (F)	●	●		●	
	Ponferrada (F)	●	●		●	●
	Lugo (F)	●	●			
	Santander (F)	●	●		●	
	Betanzos (F)	●	●		●	●
	V. de Cameros (M)	●	●		●	●
	Betanzos (F)	●	●		●	●
	Navia (F)	●	●		●	●
	Navia (M)	●	●			
	Santander (F)	●	●			
	Barañain (F)	●		●		
	Santander (M)	●	●			
	V. de Cameros (F)	●	●	●	●	
	San Sebastián (F)	●		●	●	●
	Barañain (F)	●	●	●		
	V. de Cameros (F)	●	●	●	●	
	San Sebastián (F)	●	●	●	●	●
	Santander (M)			●	●	●
	Betanzos (F)	●	●		●	
	San Sebastián (F)	●	●		●	●
	Betanzos (F)	●	●	●	●	●
	Barañain (F)				●	●
	Barañain (F)				●	
JN257231	Germany (Boehme)		●			
JN257232	Germany (Boehme)		●			
JN257233	Germany (Boehme)		●			
JN257234	Germany (Boehme)		●			
Lucilia illustris	Lugo (F)	●		●		
	Santander (F)	●	●	●	●	●
	Lugo (F)	●	●	●	●	●

	Betanzos (F)	●	●		●	
	Betanzos (F)	●	●		●	
	Lugo (F)	●	●			
	Lugo (F)		●			
	Santander (F)	●	●			
	Betanzos (F)	●	●			
	Betanzos (F)	●	●		●	●
	San Sebastián (F)				●	●
	Lugo (F)	●	●	●	●	●
	Santander (F)				●	●
JN257235	Germany (Boehme)		●			
JN257236	Germany (Boehme)		●			
JN257237	Germany (Boehme)		●			
JN257238	Germany (Boehme)		●			
Lucilia ampullacea	San Sebastián (F)	●	●	●	●	
	San Sebastián (F)	●	●	●	●	
	San Sebastián (F)	●	●	●	●	●
	San Sebastián (F)	●	●	●	●	●
	San Sebastián (F)	●	●	●	●	●
	San Sebastián (F)	●	●	●	●	●
	San Sebastián (F)	●	●	●	●	●
	San Sebastián (F)	●	●	●	●	●
	San Sebastián (F)	●	●	●	●	
	Barañain (F)	●	●	●	●	●
	Barañain (F)	●	●	●	●	
	San Sebastián (F)		●		●	●
	Barañain (F)				●	
TOTAL		69	73	59	72	49

The distance analysis did not show clear gap among the species. The matrix developed with COI gen produced the same inter- and intra-specific distances: 0 – 9.5%. In the case of the ITS2 gen, the results were almost the same. Intra-specific distance between 0 – 26% and inter-specific distance between 0 – 25.5%. This indicates that, in both genes were sequences with erratic distances, since sequences equally to those of other specie until sequences extremely different to those of the same species.

Almost all the morphological identifications were corroborated with the molecular identification of the BLAST tool. Only six specimens were reidentified after the molecular analysis, as we said above.

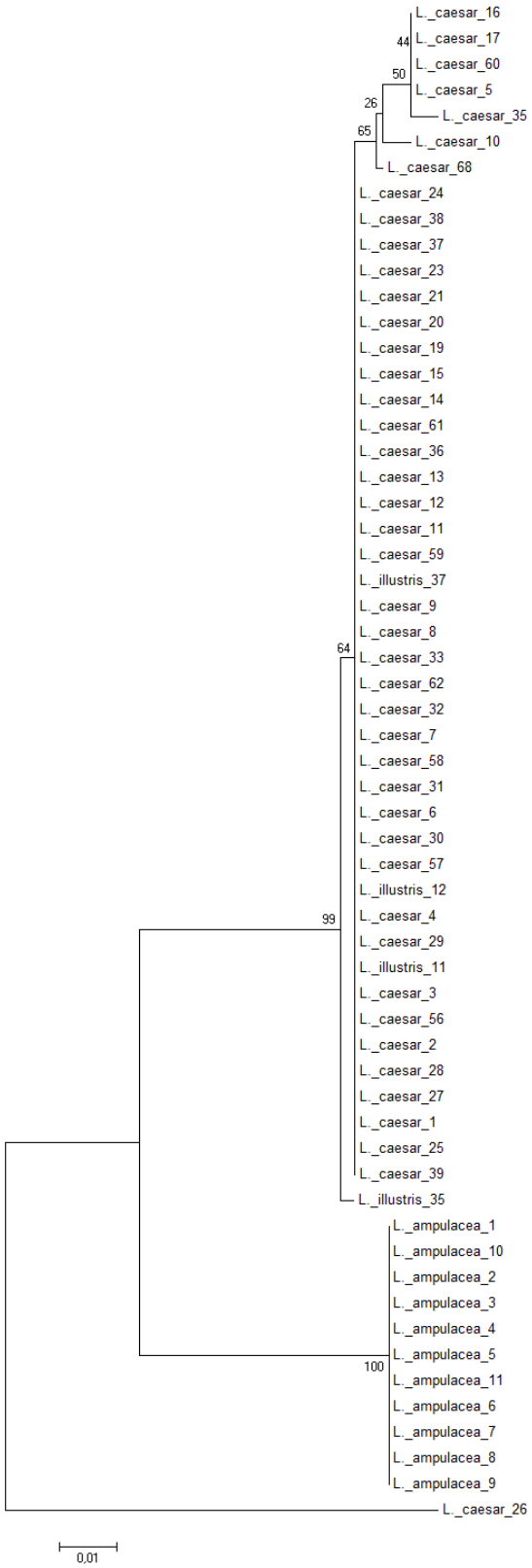


Figure 4.4. NJ tree of ITS2 gene.

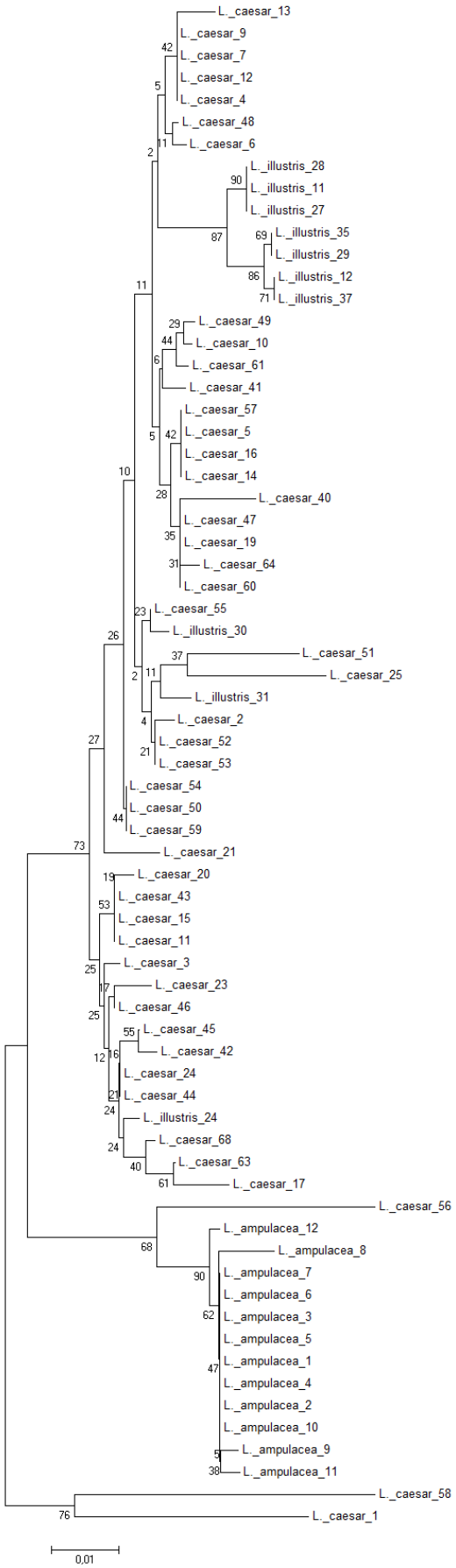


Figure 4.5: NJ tree of the COI gene.

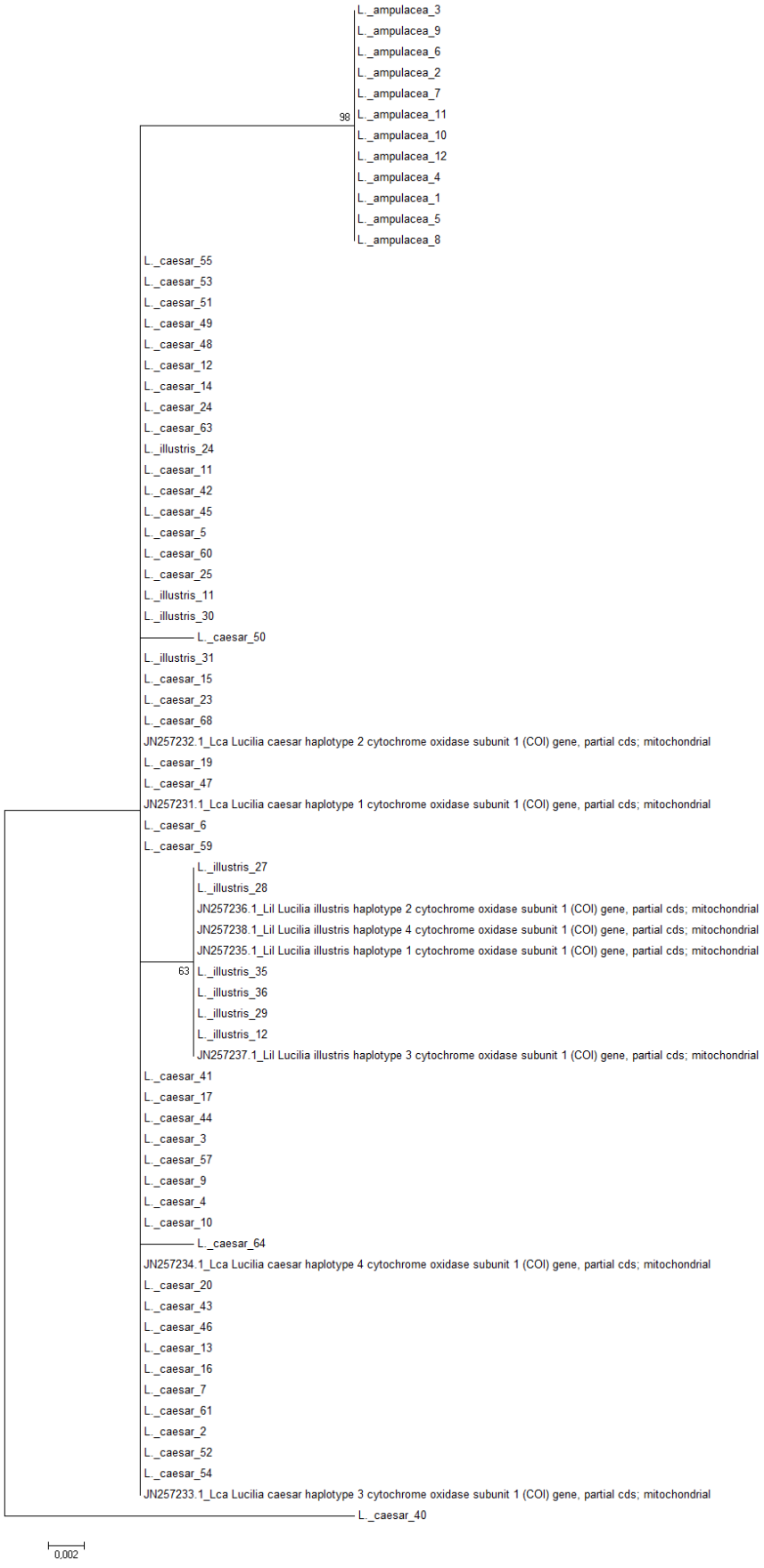


Figure 4.6: NJ tree of COI fragment coinciding with the sequences from Boehme et al., (2012).

The NJ trees developed for the different matrix showed similar results. ITS2 tree grouped the *L. ampullacea* sequences in a monophyletic group (Fig. 4.4). The rest of sequences are mixed in the same group, not producing clear separation between *L. caesar* and *L. illustris*. The support values of both groups of sequences were 99 and 100% respectively. For COI gene, the NJ tree recovered two groups of sequences either (Fig. 4.5). One of them, formed by the sequences of *L. ampullacea*, as in previous analysis. In the other group, sequences of *L. caesar* and *L. illustris* were mixed, but not in the same way that in the previous tree. Seven of the *L. illustris* sequences were grouped in a branch inside this group. Only sequences from San Sebastian were not present in this branch. The rest of sequences of this species were mixed with the *L. caesar* sequences. NJ tree of the matrix coinciding with the fragment of Boehme *et al*. (2012) produced similar results to the previous matrix of COI (Fig. 4.6). Group of *L. ampullacea* was formed in the same way than previous trees. The rest of sequences were mixed in the other branch, as in the COI tree. One branch inside this group was formed by six sequences of *L. illustris* and those of the same species from the work of Boehme *et al*. (2012). Another four *L. illustris* sequences were mixed with the sequences of *L. caesar* from this work and Boehme *et al*. (2012) work.

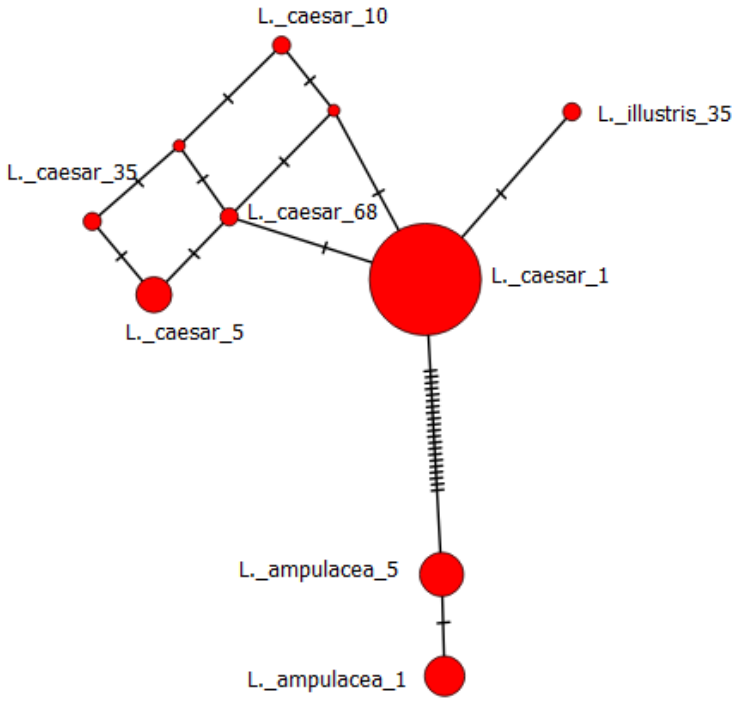


Figure 4.7: Haplotype network of ITS2 gene.

Phylogeographic analysis produced three different haplotypic networks. The ITS2 network (Fig. 4.7) produced two groups of haplotypes separated by 21 mutational steps: one group with two haplotypes for *L. ampullacea* and other group with six haplotypes for *L. caesar* and *L. illustris*, coinciding with the branches distribution of the NJ result for this gene. In the COI gene network (Fig. 4.8), the haplotypes do not form clear groups. Five haplotypes of *L. ampullacea* were grouped together. The group of *L. illustris* was formed by another five haplotypes. The rest of haplotypes correspond to *L. caesar*, that did not for a distinctive group. However, in this last group, three sequences of *L. illustris* were included, as it was observed in the NJ analysis.

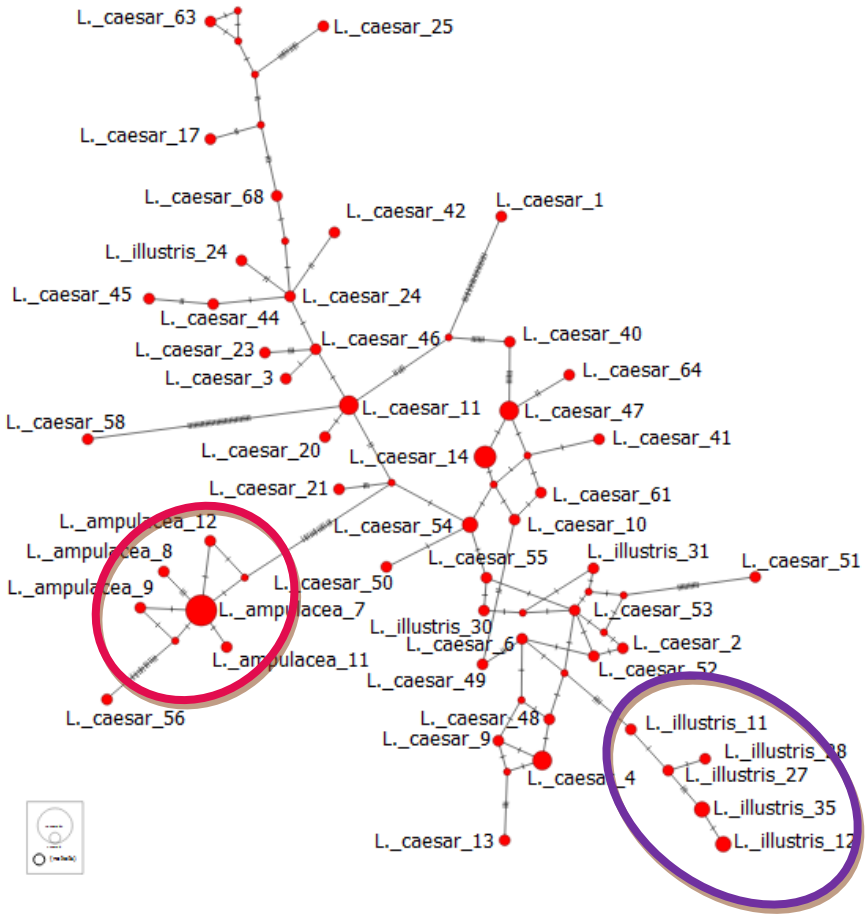


Figure 4.8: Haplotype network of COI gene.

The groups of haplotypes formed for the matrix of COI plus the sequences from Boehme *et al*. (2012) (Fig. 4.9) showed a clearer clustering than the previous network. Three groups of haplotypes were formed. One for *L. ampullacea* with five haplotypes separated by 12 mutational steps. Another group for *L. illustris* with five haplotypes separated by four mutational steps, where the sequences from Boehme *et al*. (2012) of

this species were included. The last group was formed by 14 haplotypes for *L. caesar* in the middle of the other two groups. In that, three sequences of *L. illustris* were included as well.

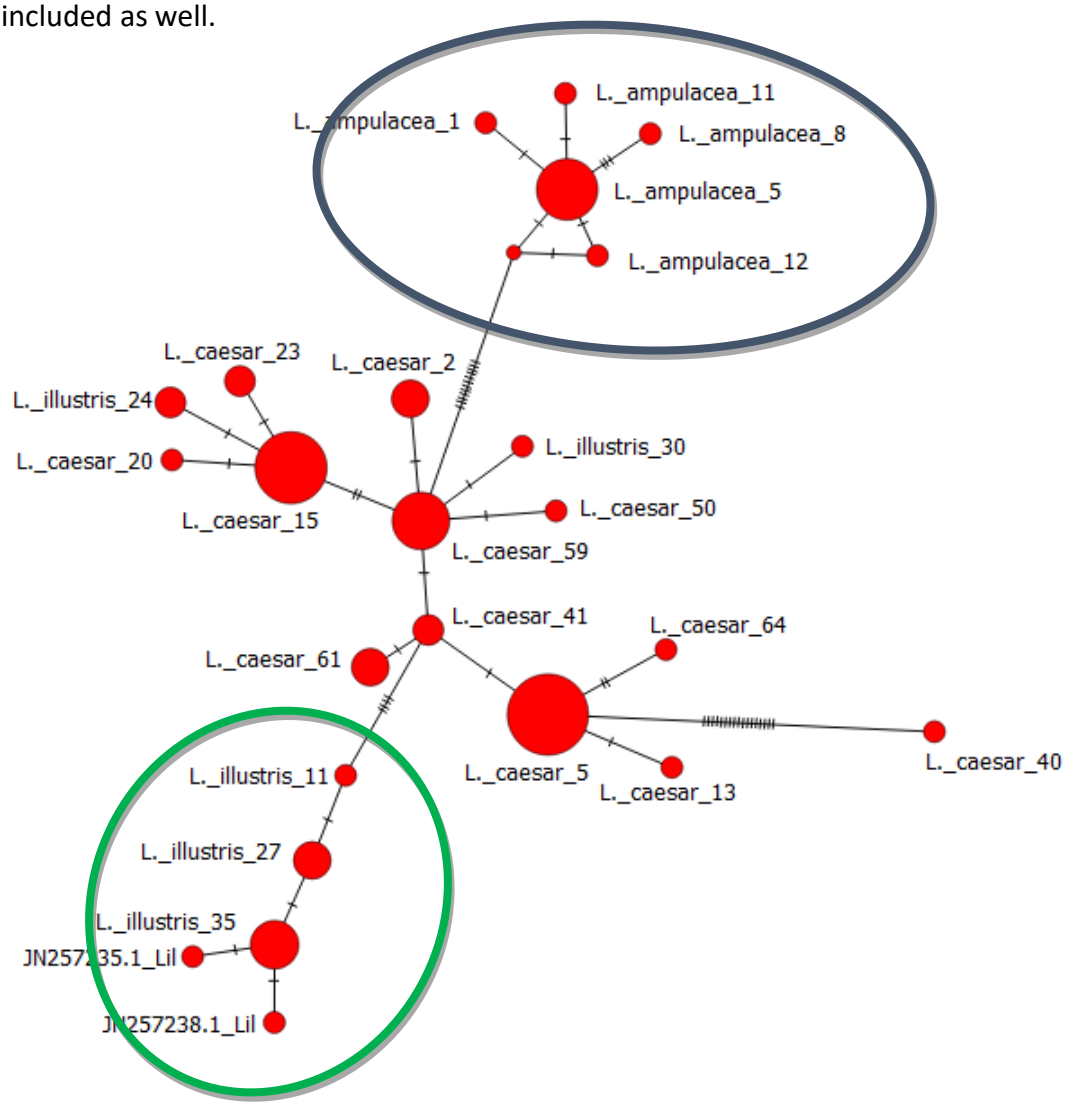


Figure 4.9: Haplotype network of COI gene coinciding with the sequences from Boehme et al., (2012).

The PCA analysis made with the wings samples showed that the landmarks with the highest differences in order of variation from the target were: 2, 3, 4, 5, 6, 7 and 9. Transformation grid and wireframe of the landmarks is shown in Appendix 4.1. The projection of the landmark configuration geometries for the datasets into the subspace formed by the first two PC axes is shown in Appendix 4.2. In those results we could not observe real differences that can be assigned to the diversity of species or to the locality. Using the CVA with localities as classifier variables for groups, the landmark that showed main differences were: 1, 2, 3, 8, 10 and 15 (Appendix 4.3). This result produced enough differences among localities, especially between western

localities (Santander, Betanzos, Lugo and Ponferrada) and eastern localities (San Sebastián, Barañain and Villanueva de Cameros), with only a little overlap between the ellipses of San Sebastian and Betanzos (Fig. 4.10).

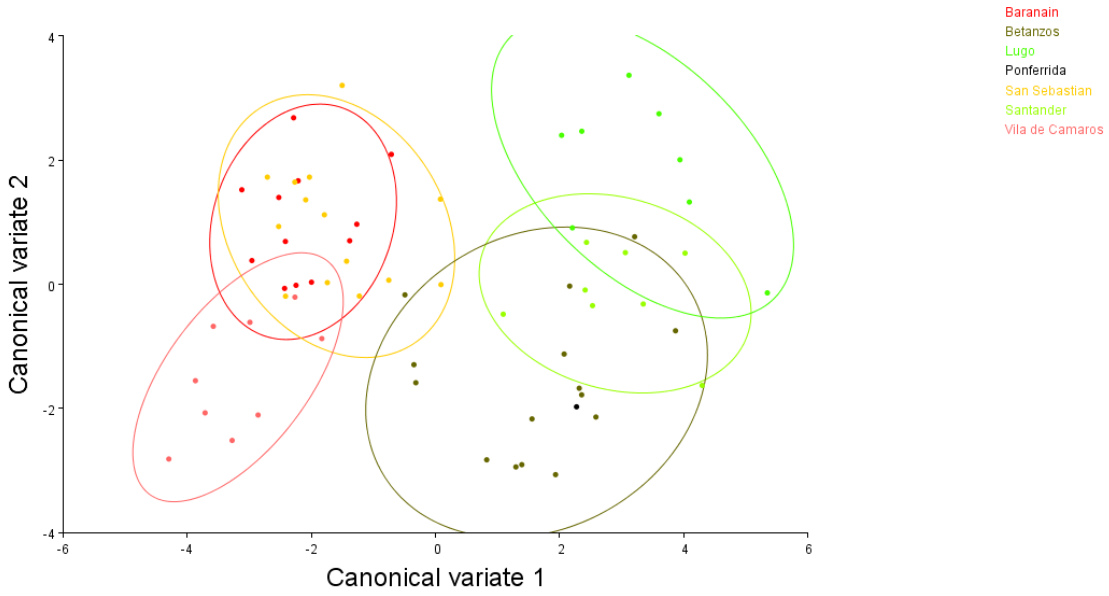


Figure 4.10: Distribution of CVA of wings with localities as grouping variable.

Due to this result, both areas were reanalysed separately. The landmarks transformation is shown in Appendix 4.4.

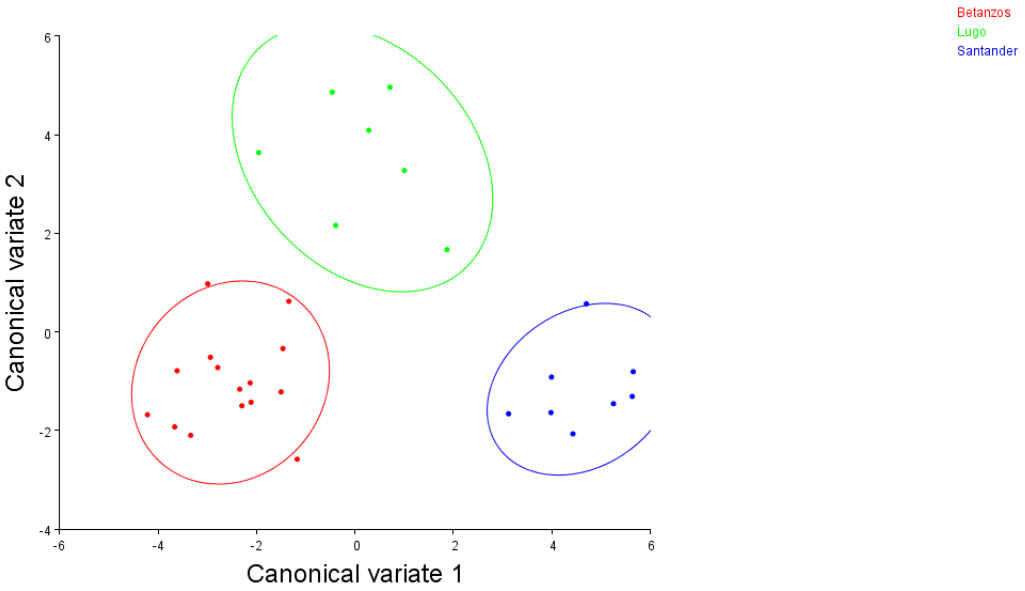


Figure 4.11: Distribution of CVA of wings with western localities as grouping variable

Both result showed good differentiation of each population in each geographical area (eastern or western). No overlap was shown in both cases (Fig. 4.11 and Fig. 4.12).

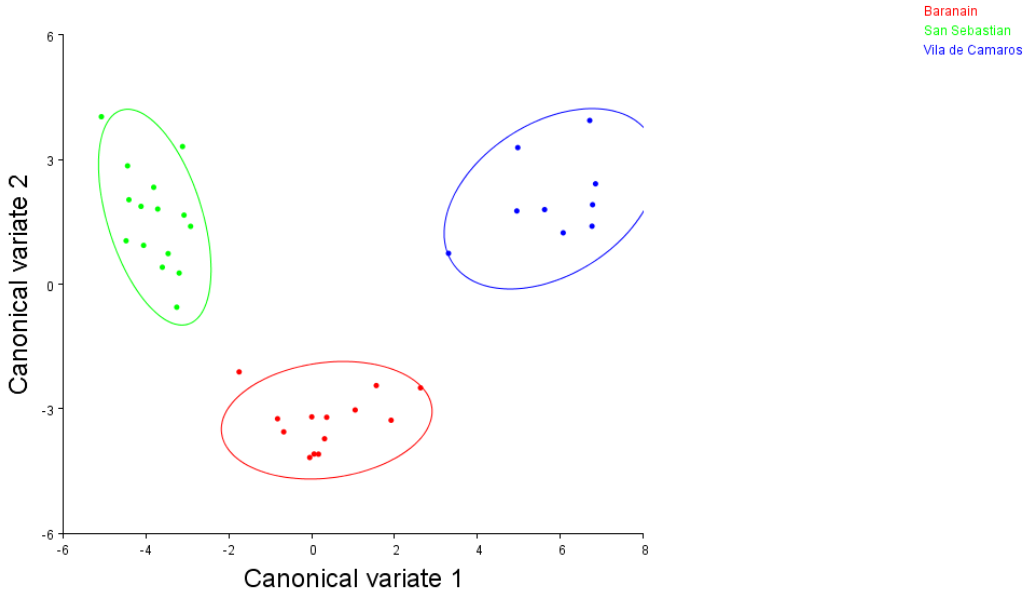


Figure 4.12: Distribution of CVA of wings with eastern localities as grouping variable.

The CVA analysis shown the main differences in landmarks 3, 5, 6, 7 and 10 using the species as variable for groups formation (Appendix 4.5). The representation of these results shows three groups of species (Fig. 4.13). The species more accurately represented is *L. ampullacea*, *L. caesar* and *L. illustris* species form both groups well defined but with an overlap zone, which evidences the morphological closeness between these two species.

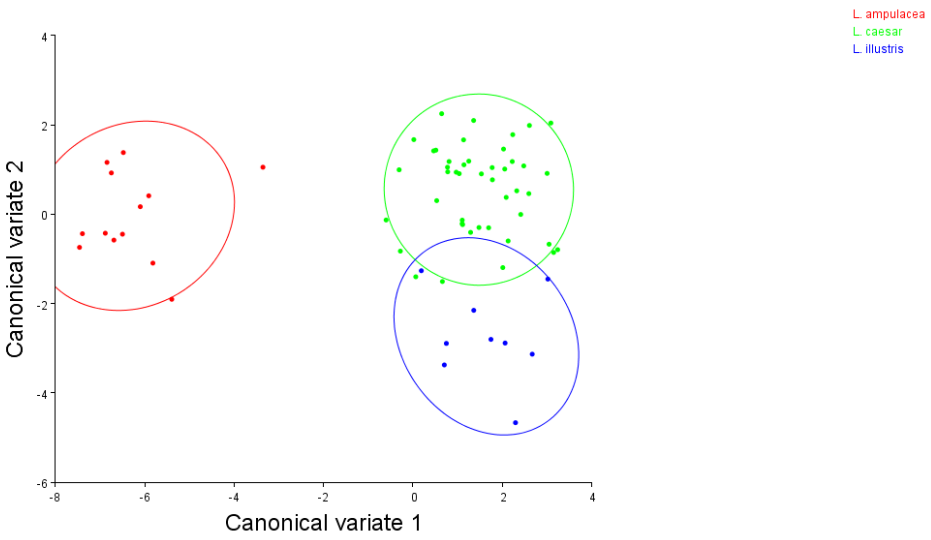


Figure 4.13: Distribution of CVA of wings with species as grouping variable.

The samples of fly heads were analysed with the PCA detecting the main differentiation in landmarks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 16 and 17 (transformation grid and wireframe of landmarks is shown in Appendix 4.6). As occurred with the wings samples, no difference among localities or species (Appendix 4.7) were observed with this analysis. With the CVA analysis using the localities as classifier variables, the main differences were observed in landmarks 1, 2, 5, 6, 7, 8, 9, 10, 14, 16 and 17 (transformation grid and wireframe of landmarks is shown in Appendix 4.8). This result produces certain separation among the localities, but not so clear as the wings results (Fig. 4.14).

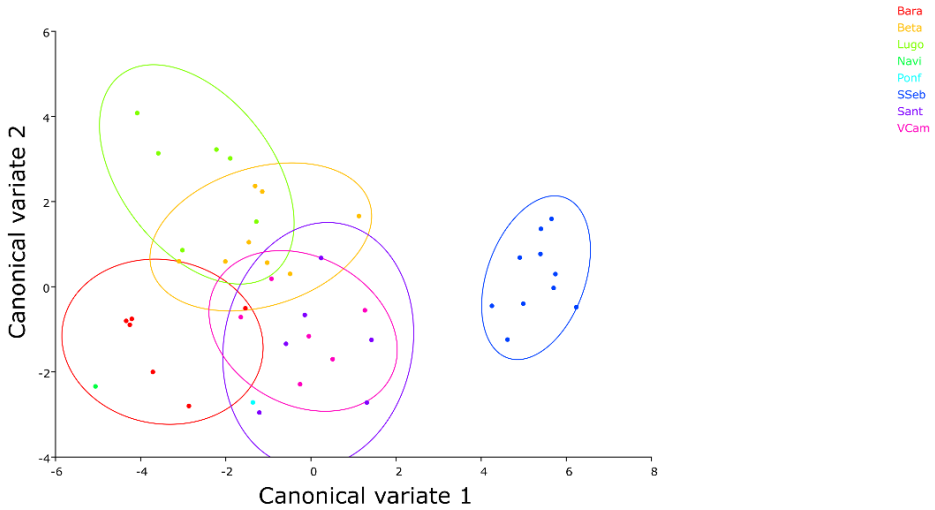


Figure 4.14: Distribution of CVA of heads with localities as grouping variable.

In this case the samples form Santander are totally overlapped with the samples from Villanueva de Cameros. This would reorganize these samples in the eastern group with respect to the wings results. Besides, the one sample from Navia and the one sample of Ponferrada were plot in the area of the eastern samples. Due to these results and following the geographical analyses developed for the wings samples, the eastern and the western samples were analysed separately. But in this case, the samples from Santander were included in the eastern area due to their ubication in the previous analysis. The transformation grids and wireframes are shown in Appendix 4.9. In western samples, the CVA analysis produced well discrimination in almost all localities (Fig. 4.15). Samples from Betanzos and Lugo form both groups separated and without overlap. The one sequence from Ponferrada was separated from the other species while the one sequence form Navia was included in the group of samples from Lugo. In

Eastern samples, the CVA analysis produced well separation of the four localities. No overlap was observed among them (Fig. 4.16).

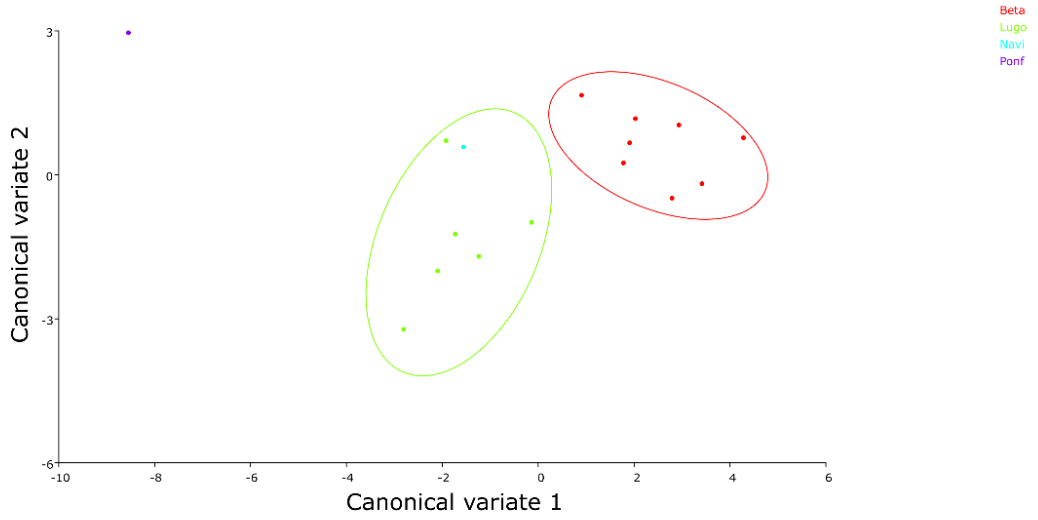


Figure 4.15: Distribution of CVA of heads with western localities as grouping variable.

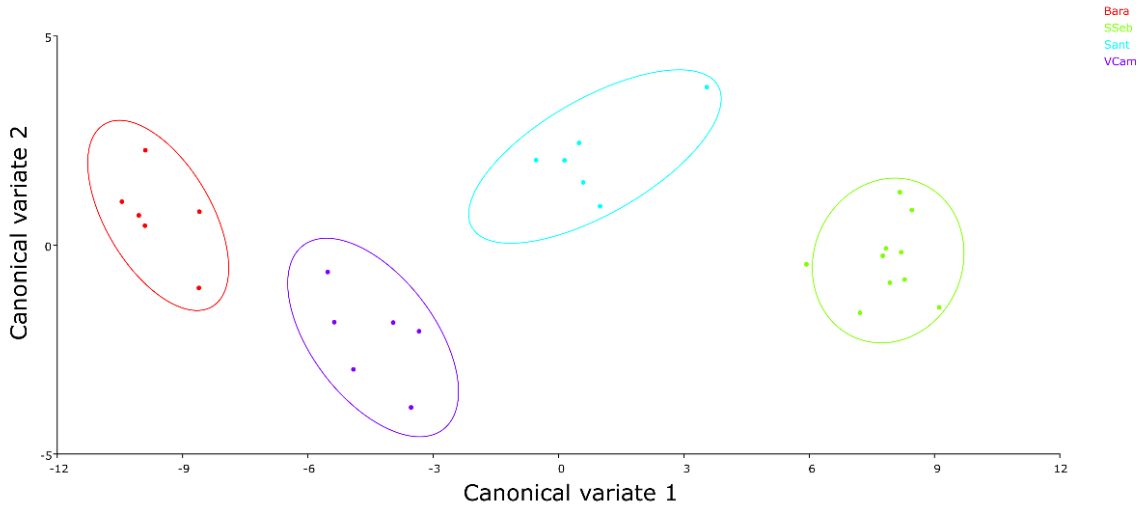


Figure 4.16: Distribution of CVA of heads with eastern localities as grouping variable.

The landmarks that produces the main differences in the CVA with species as variable for groups formation were: 1, 2, 5, 6, 7, 8, 9, 10, 14, 16 and 17 (Appendix 4.10). The results shown three species grouped, as happened the analysis of the wings (Fig. 4.17). The structure observed was the same, *L. ampullacea* well separated while *L. caesar*

and *L. illustris* were closer to each other with an overlap zone. In this case, the overlap zone was higher than the observed in the wings analysis.

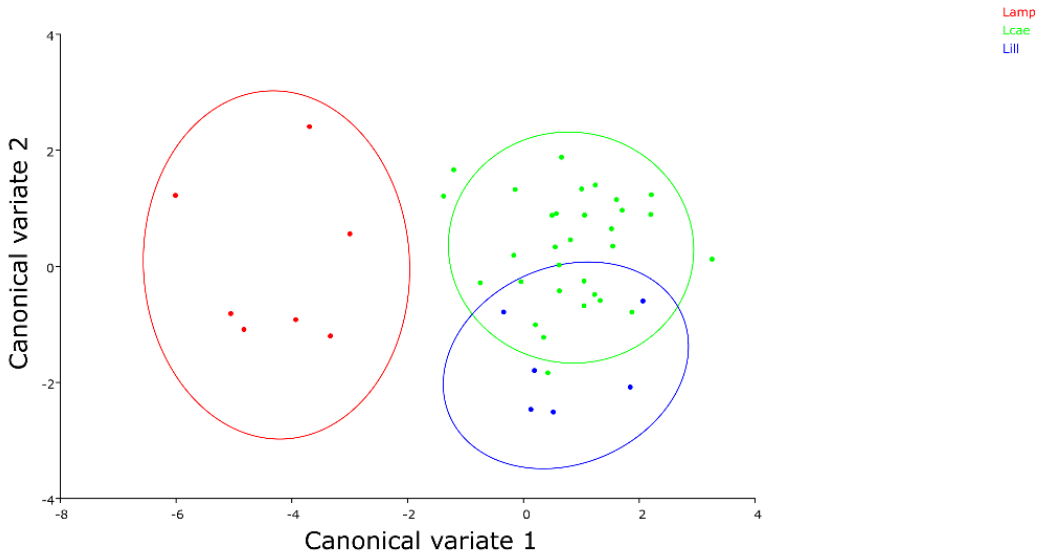


Figure 4.17. Distribution of CVA of heads with species as grouping variable.

Discussion

The present work adds valuable information about the identification of *L. ampullacea*, *L. caesar* and *L. illustris* in northern Spain. The results of the identification using morphological keys has demonstrated that is not always effective due to the observational difficulties of some structures in not well preserved samples (Akbarzadeh et al., 2015; Arnaldos et al., 2015; GilArriortua et al., 2013). In agreement with this, in this work up to six samples have been renamed based in molecular information. The final quantity of samples of *L. caesar* was higher than that of *L. illustris*. Having into account the peri-urban location of the traps, this agrees with the assumption of *L. caesar* being considered more anthropized than *L. illustris* (Martínez-Sánchez et al., 2001; Martínez-Sánchez et al., 2000; Saloña, Moneo, & Díaz, 2009). However, in this work we find that *L. ampullacea* samples are less anthropized than *L. caesar*, in disagreement with previous works that said that both species were equally anthropized. The distribution of the three species in this work was not equal, being *L. ampullacea* concentrated in the eastern area, in disagreement with previous works that collected this species in other areas of Iberian Peninsula (Carles-Tolrá, 2002;

Martínez-Sánchez, Rognes, Rojo, & Marcos-García, 1998) and in other localities of northern Spain (Prado e Castro, García, Arnaldos, et al., 2010).

The genetic distances were higher than previous works (Boehme et al., 2012; Tourle et al., 2009). Even the intraspecific distance were almost equally than the interspecific, in disagreement with previous studies (Sonet et al., 2012). This could be the result of some erroneous identifications, an unusually large intraspecific diversity for these species in this geographical zone or the putative presence of hybrid specimens.

Although the ITS2 gene was used as helpful tool to differentiate species in previous works with other Diptera groups (Nelson et al., 2008; Thanwisai, Kuvangkadilok, & Baimai, 2006; Zaidi et al., 2011), in our case it did not produce good separation of the species. NJ tree and haplotypic network (Fig. 4 and 7) produce two groups of sequences, the *L. ampullacea* species group and *L. caesar/illustris* group. This is a similar grouping to that produced by the COI in BOLD database with the BIN tool, in which *L. ampullacea* has its own BIN (AAC3450) while *L. caesar* and *L. illustris* share it (AAA7470). This corroborates that the ITS2 gene is able to distinguish among species, as long as they do not have a genetic distance smaller than what is typically used as a threshold (Hebert, Cywinska, et al., 2003; Meiklejohn et al., 2011).

The COI gene analysis produced better results than the previous analysis. NJ tree produced almost the same groups that the ITS2 analysis. But in the COI case, some of the sequences of *L. illustris* form an individual branch inside the *L. caesar* branch (Fig. 4.5), in the same way than previous works (Sonet et al., 2012). The haplotype network analysis produced a large number of haplotypes in which the three species can be mostly differentiated (Fig. 4.8). However, some sequences of *L. illustris* were still mix with haplotypes of *L. caesar*, in agreement with the structure observed in Sonet et al. (2012). These results suggest that the haplotypes analysis with this gene is useful to differentiate these species.

The matrix built with the sequence coinciding with the length of sequences from Boehme *et al*. (2012) produced similar results than the previous analysis. In NJ tree (Fig. 4.6) the samples were located in the same manner than previous tree. Most of the *L. illustris* samples were located in a single branch coinciding whit the sequences of

this species from Boehme et al. (2012) work. In the branch of *L. caesar* the sequences of this species from Boehme et al. (2012) were presents too. In disagreement with this study, some sequences of *L. illustris* were included also in this branch. In the haplotype network (Fig. 4.9) the groups of species were well defined, and the number of haplotypes were much less than in the previous analysis. This corroborates that a shorter fragment of these gene is more accurate to determine species due to the elimination of high variable zones of the gene (Boehme et al., 2012; Guo et al., 2012).

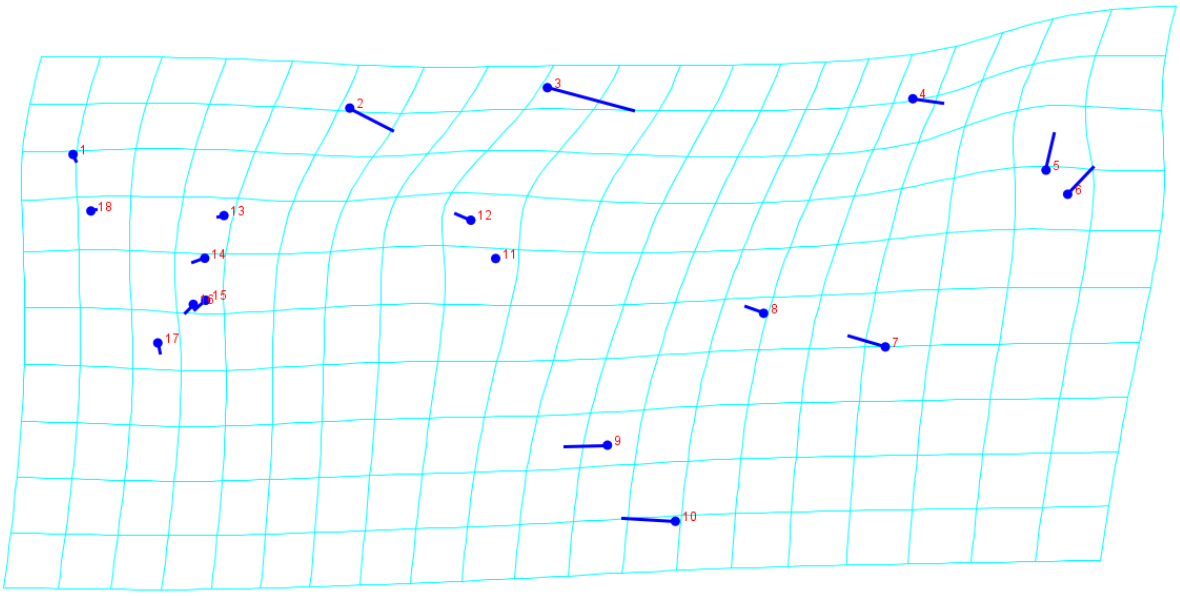
The results of geometric morphometric analysis have shown utility to differentiate localities and species. As happened in previous works with different organisms (Espra et al., 2015; Hurtado-Burillo, Jara, et al., 2016; Loh, David, Debat, & Bitner-Mathé, 2008; Padilla-Gil & García-López, 2016; Roggero & Passerin d'Entrèves, 2005), the localities can be differentiated using the selected landmarks of both structures, head and wing. Although we made different decisions with the samples of the frontier locality between eastern and western (Santander), the closer localities were able to be distinguish with enough accuracy. These types of results demonstrate that these analyses can be used to distinguish the provenance of the samples. This is the first study in the Iberian Peninsula in which both structures (wing and head) have been compared in this family. As regards to the differentiation of species, these species have not been previously studied in this way. Previous studies in Diptera (Calle, Quiñones, Erazo, & Jaramillo, 2008; Changbunjong, Sumruayphol, Weluwanarak, Ruangsittichai, & Dujardin, 2016; Grzywacz, Ogiela, et al., 2017; Vásquez & Liria, 2012) and other types of organisms (Gamboa & Arrivillaga, 2010; Hernández, 2015; Martí, Rissech, Juan, & Turbón, 2013) have demonstrated the utility of these analyses for distinguishing species. The better differentiation of *L. ampullacea* agrees with the molecular data of this family (Boehme et al., 2012; Stevens & Wall, 1996). In the same way, the more difficult differentiation between *L. caesar* and *L. illustris* agrees with studies that said that these are two species too close to be distinguished in some cases (Sonet et al., 2012) with molecular data. However, there is an overlap area in analyses of both structures (Fig. 4.13 and 4.17), in agreement with other studies in which the target species were considered well identified despite the overlap zone (Vásquez &

Liria, 2012). Therefore, these analyses are useful to distinguish among these species with almost the same accuracy between the two studied structures.

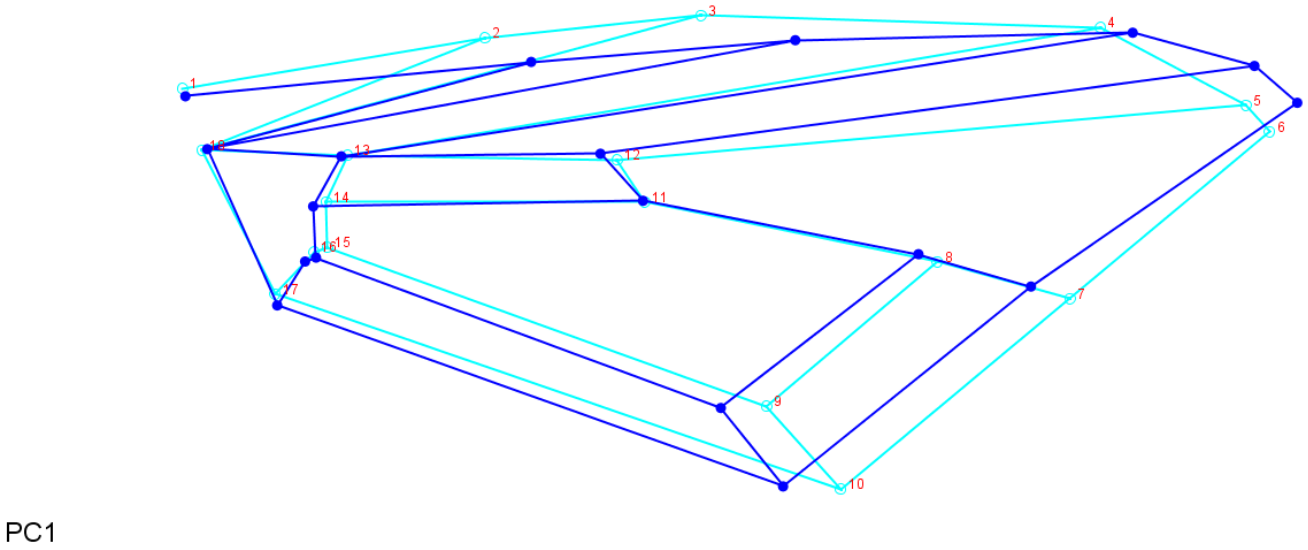
The data produced in this (multidisciplinary) study shows that there are different types of analysis useful to distinguish these species. In a forensic investigation, this is relevant because of the developmental or conservation state of the samples. This study shows that the DNA information can be used to differentiate the species with good accuracy, especially if the haplotypes are represented in a network. If the DNA information were not available, geometric morphometric information can be used in the same way with accurate results. Besides, this information can be used for determining the source locality of samples, which could be useful in cases of relocation of corpses. This study produces useful information for the differentiation of species of the genus *Lucilia* with forensic interest, besides a collection of landmarks for fly heads, useful in future works for differentiating species or populations.

Appendices

Appendix 4.1.

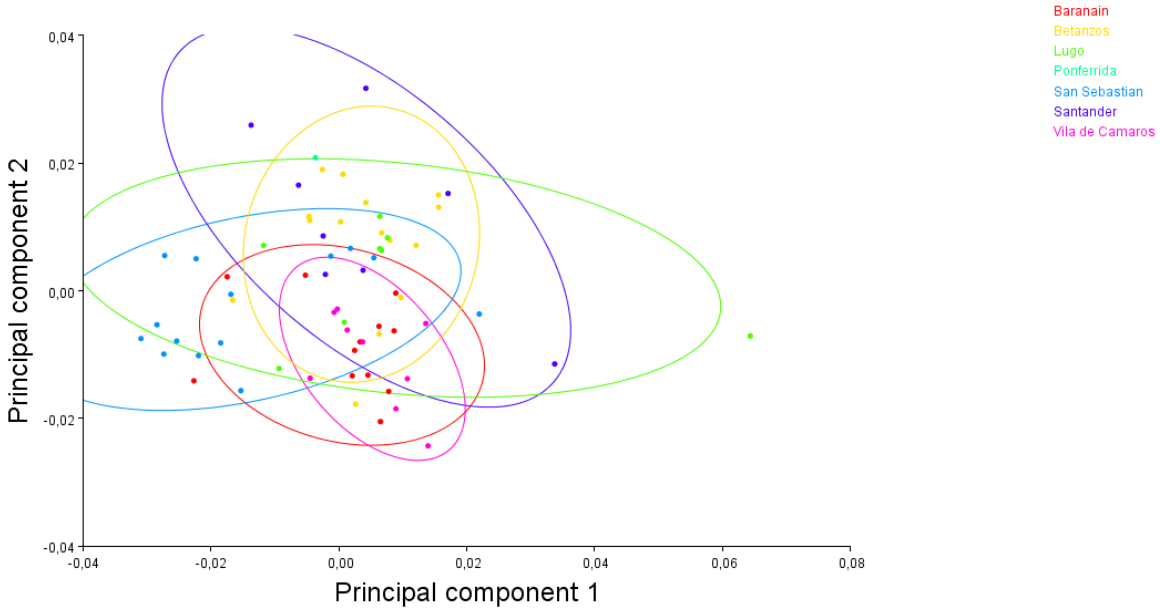


(a) Figure 4.18: Distribution of PCA with transformation grid for wings analysis.

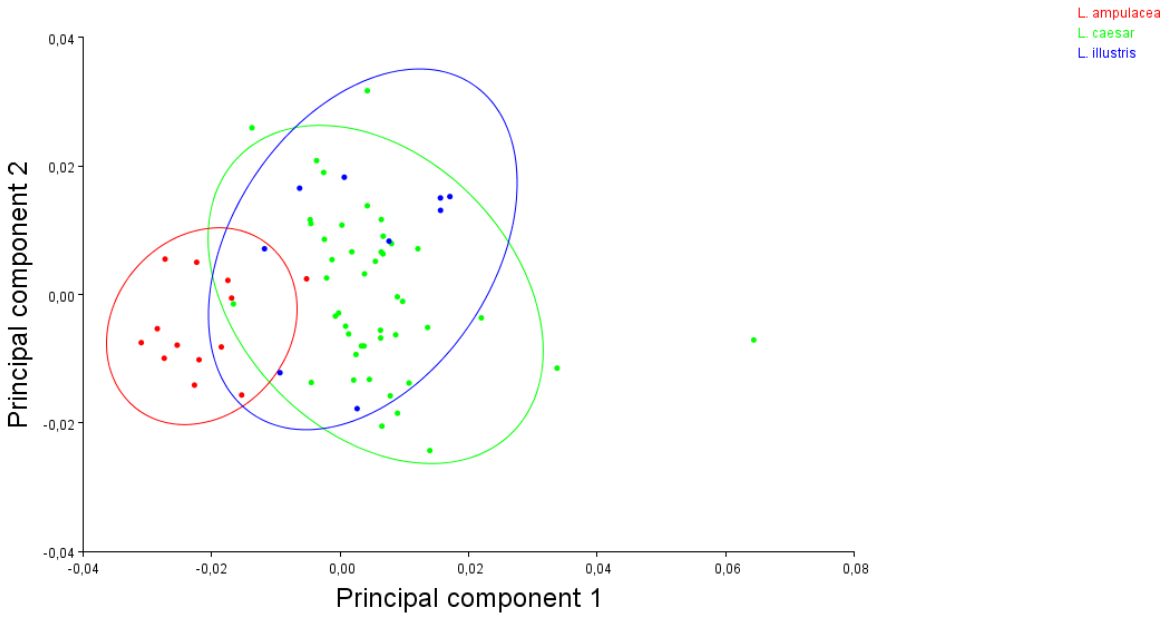


(b) Figure 4.19: Distribution of PCA with wireframe for wings analysis.

Appendix 4.2.

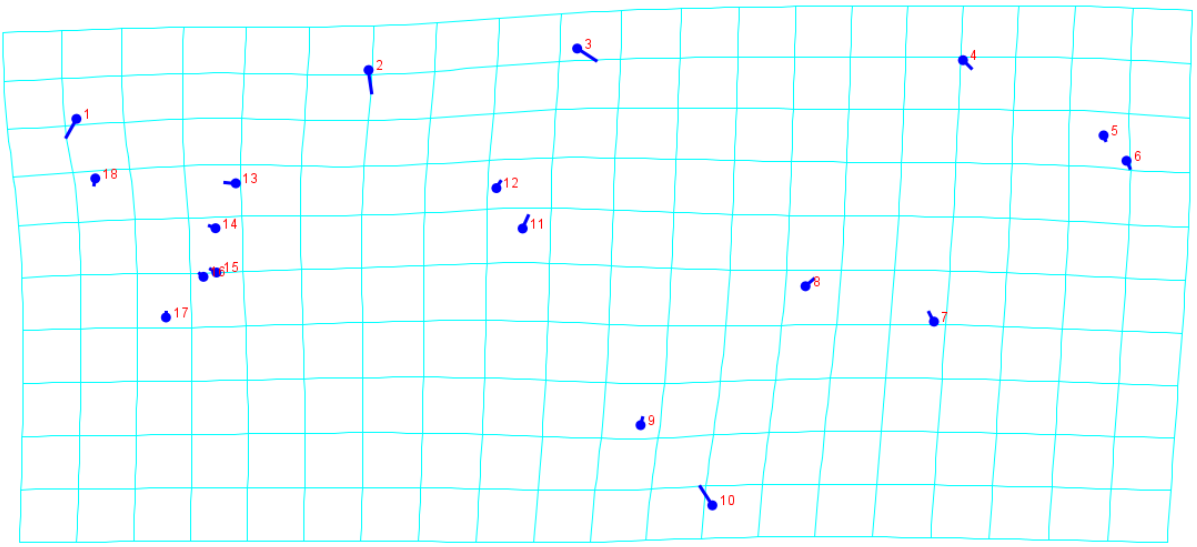


(a) Figure 4.20: Distribution of PCA with locality ellipses



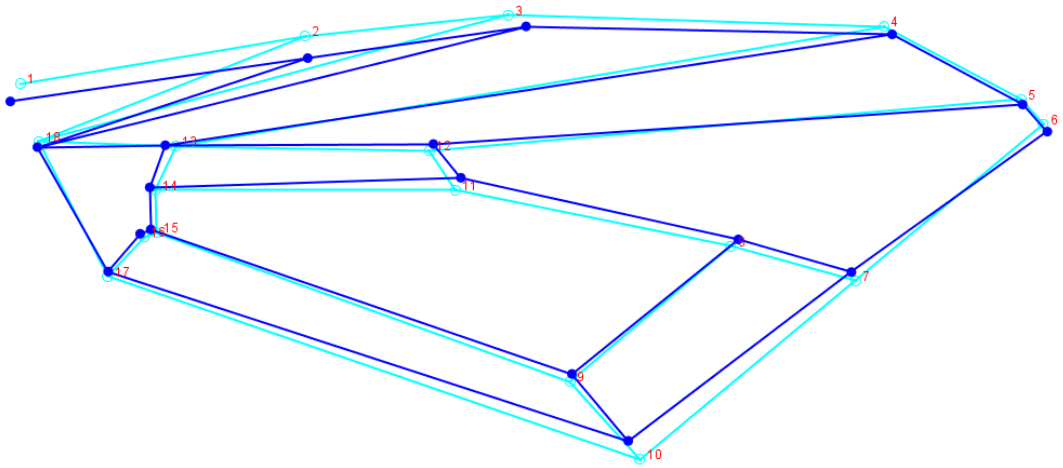
(b) Figure 4.21: Distribution of PCA with species ellipses

Appendix 4.3.



CV1

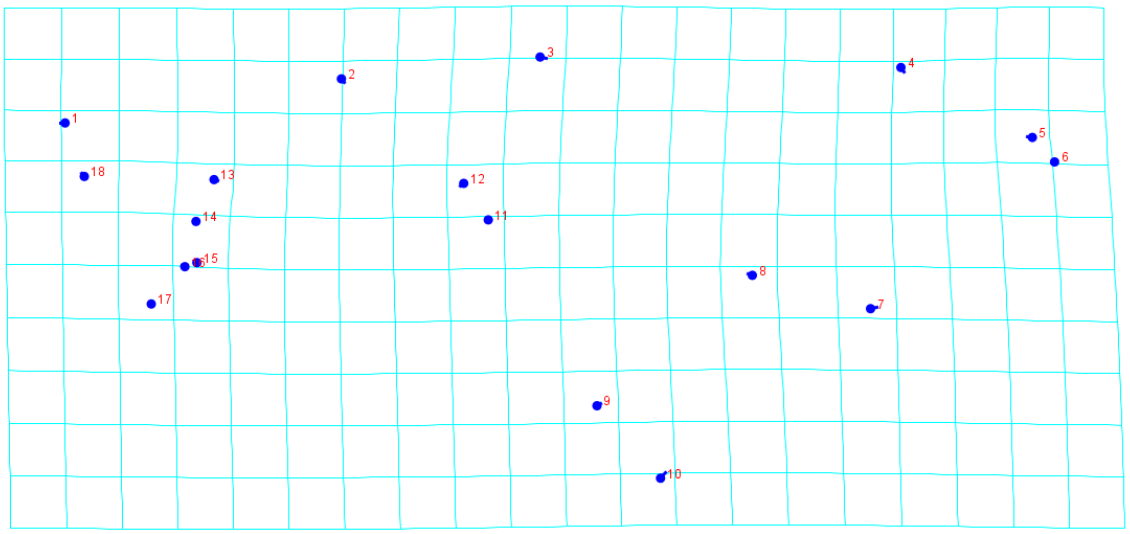
(a) Figure 4.22: Distribution of CVA with transformation grid for localities



CV1

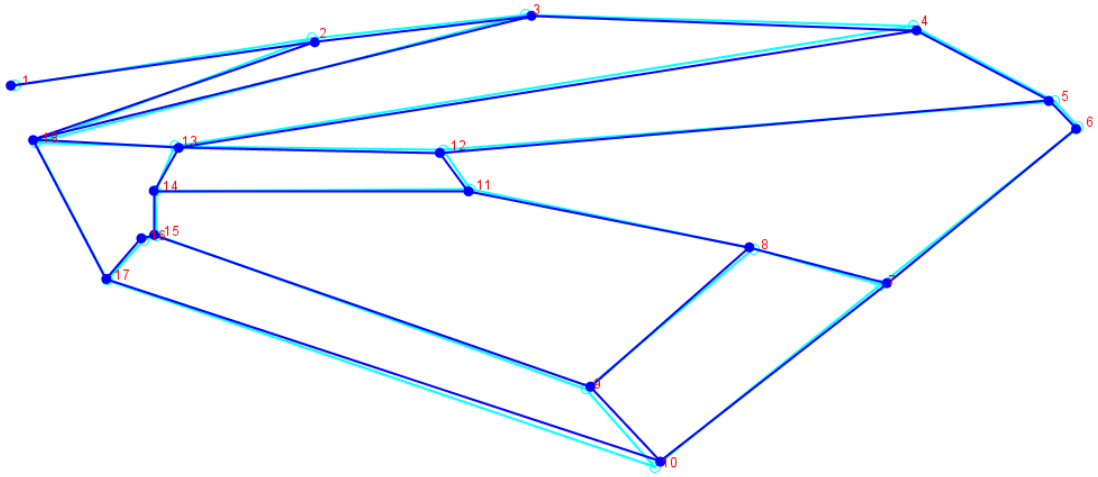
(b) Figure 4.23: Distribution of CVA with wireframe for localities

Appendix 4.4.



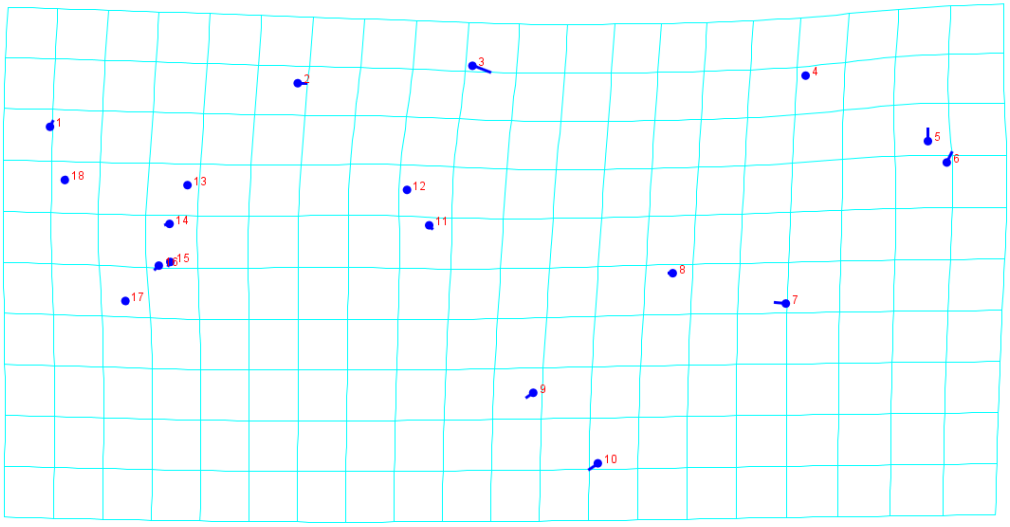
CV1

(a) Figure 4.24: Distribution of CVA with transformation grid for the western localities

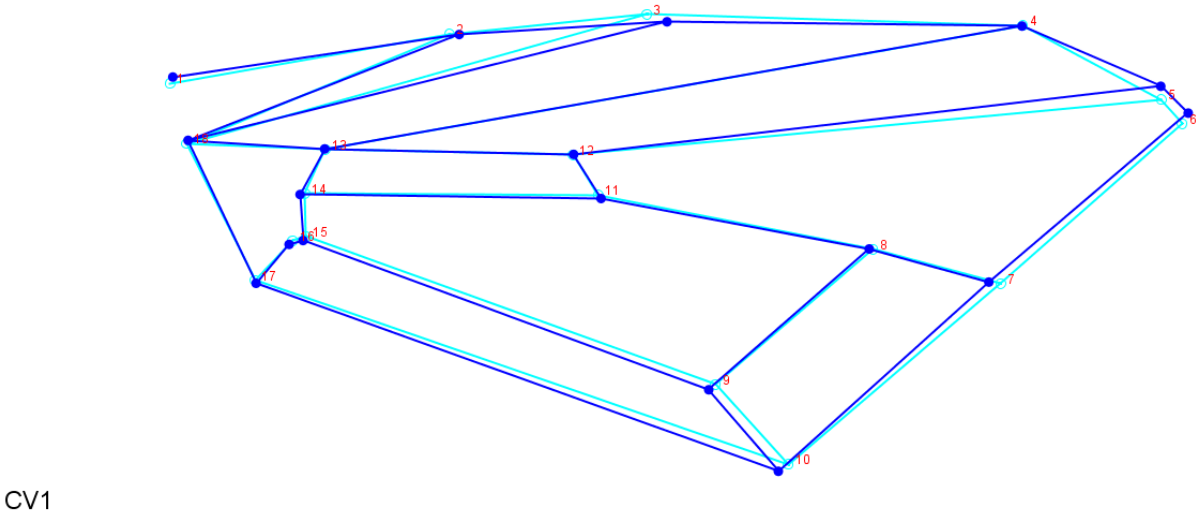


CV1

(b) Figure 4.25: Distribution of CVA with wireframe for the western localities

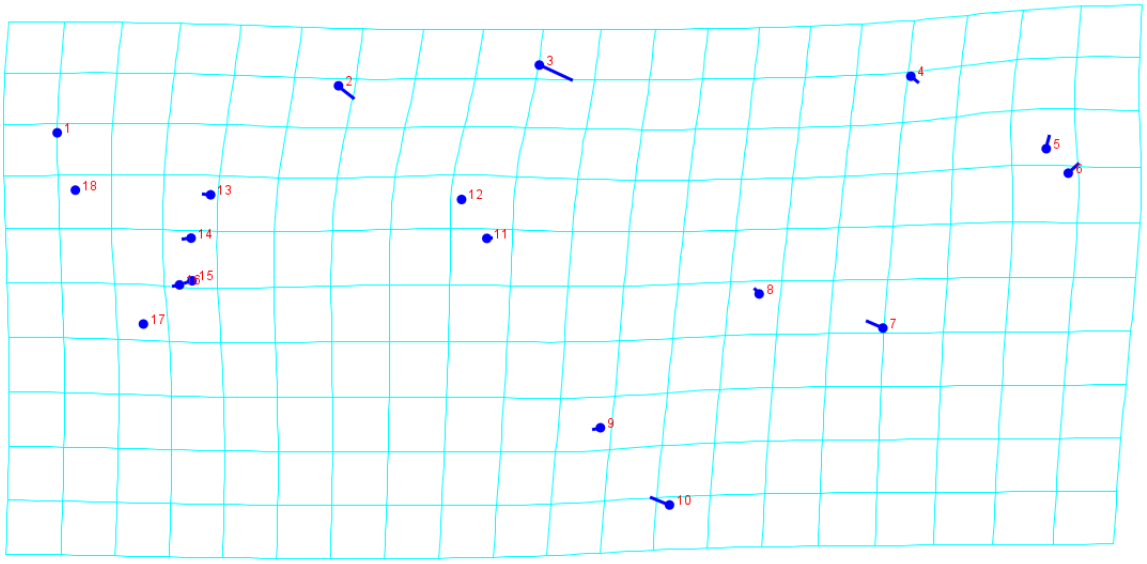


(c) Figure 4.26: Distribution of CVA with transformation grid for eastern localities



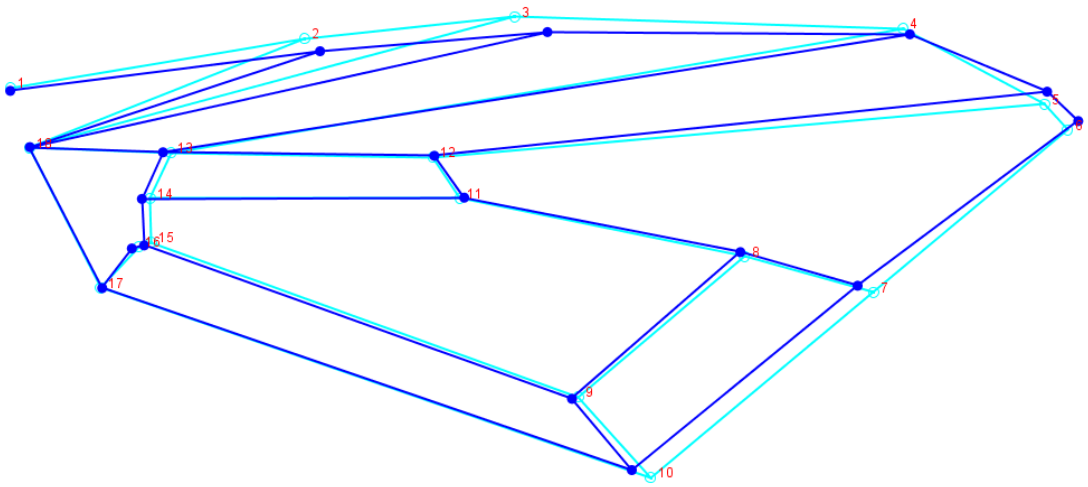
(d) Figure 4.27: Distribution of CVA with wireframe for eastern localities

Appendix 4.5.



CV1

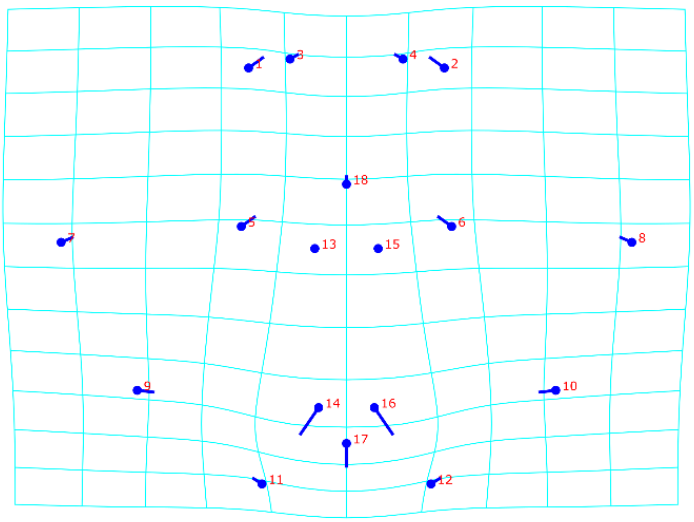
(a) Figure 4.28: Distribution CVA with transformation grid for species



CV1

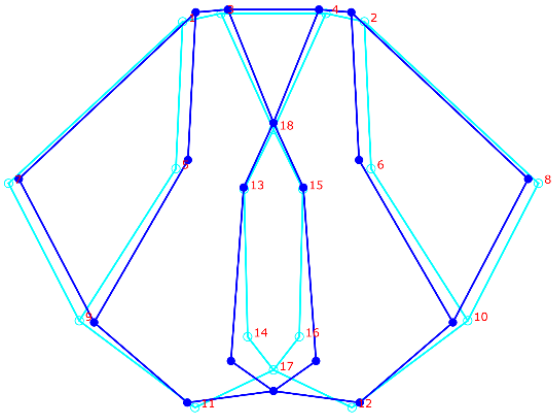
(b) Figure 4.29: Distribution CVA with wireframe for species

Appendix 4.6.



CV1

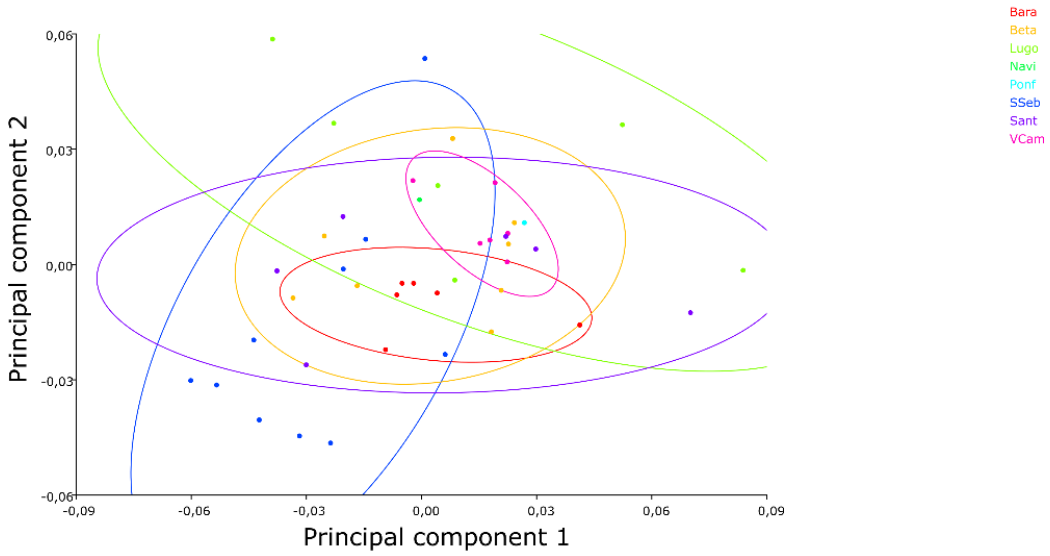
(a) Figure 4.30: Distribution of PCA with transformation grid for heads analysis



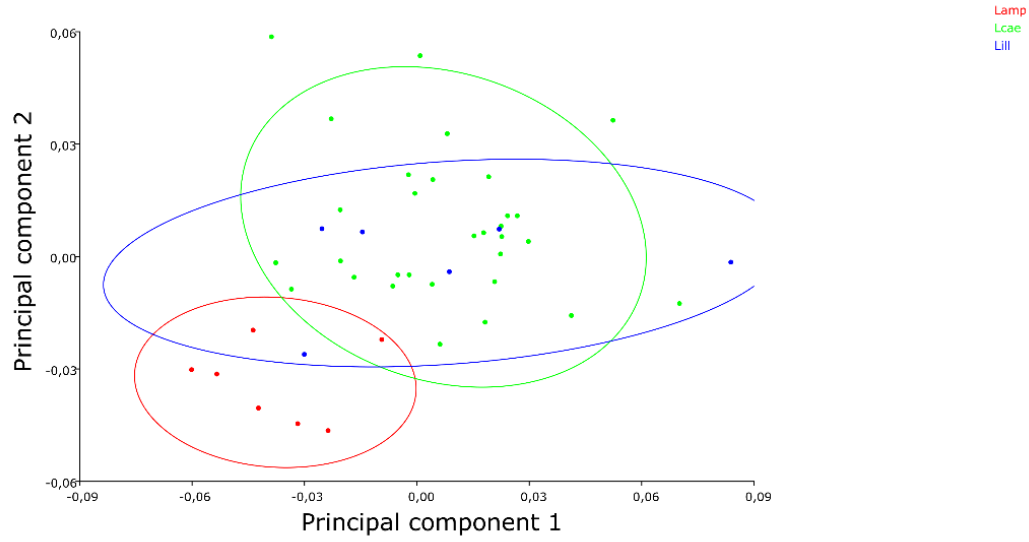
CV1

(b) Figure 4.31: Distribution of PCA with wireframe for heads analysis

Appendix 4.7.

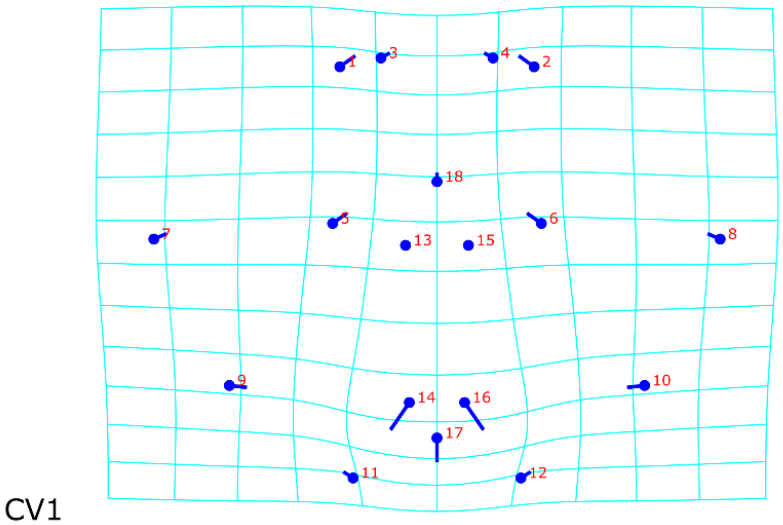


(a) Figure 4.32: Distribution of PCA of heads with locality ellipses

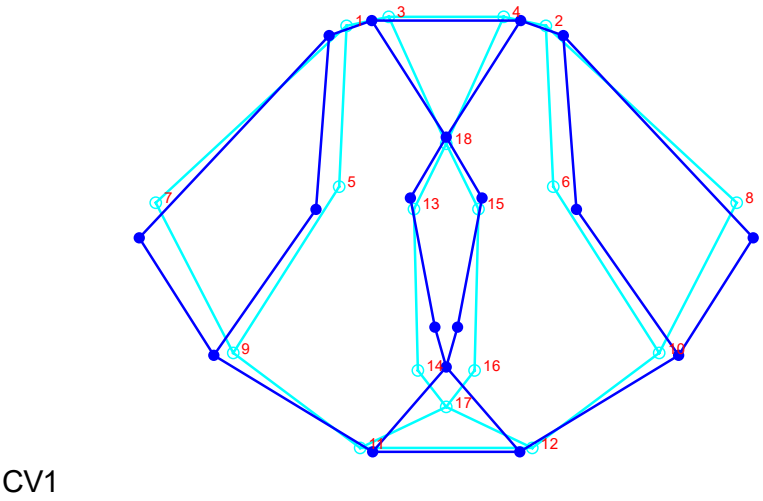


(b) Figure 4.33: Distribution of PCA of heads with species ellipses

Appendix 4.8.

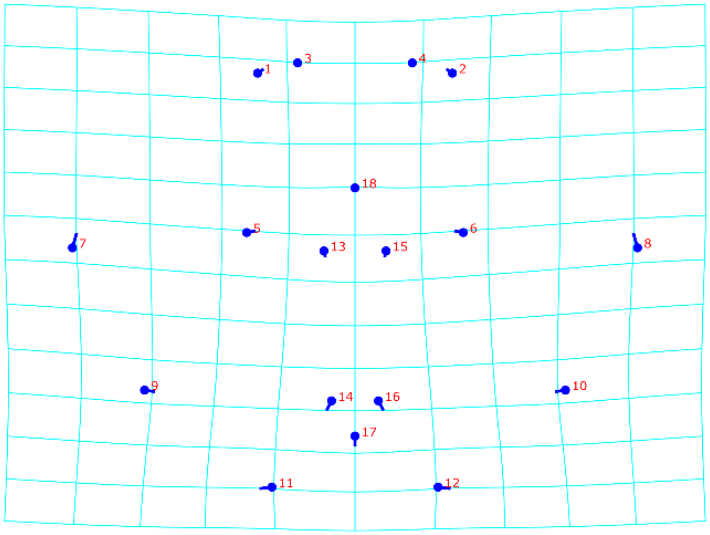


(a) ***Figure 4.34: Distribution of CVA with transformation grid for head analysis.***



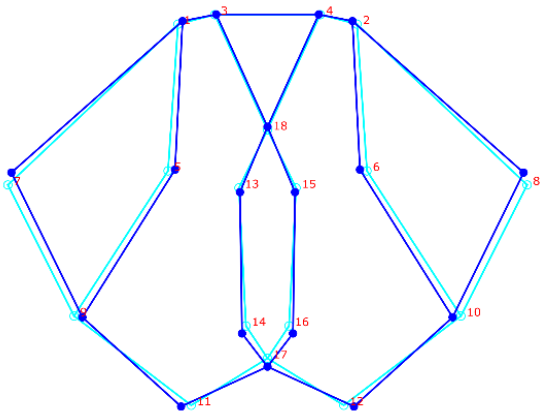
(b) ***Figure 4.35: Distribution of CVA with wireframe for head analysis***

Appendix 4.9.



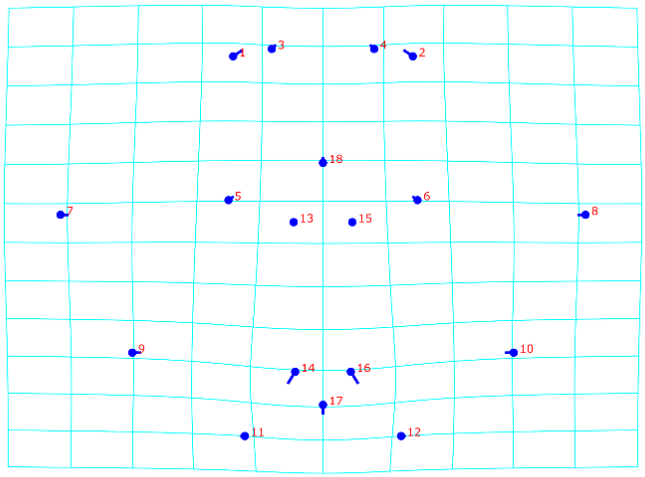
CV1

(a) Figure 4.36: Distribution of CVA of heads with transformation grid for the western localities



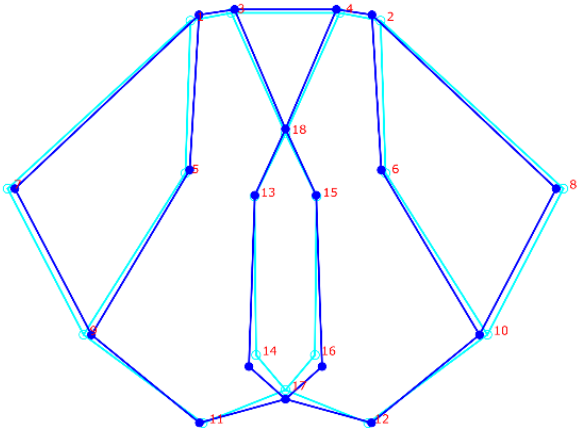
CV1

(b) Figure 4.37: Distribution of CVA of heads with wireframe for the western localities



CV1

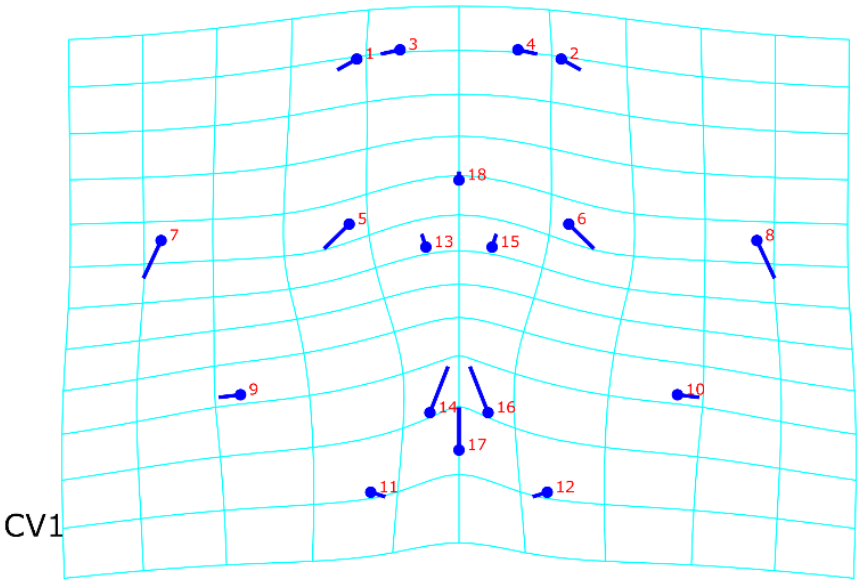
(c) Figure: 4.38: Distribution of CVA of heads with transformation grid for the eastern localities



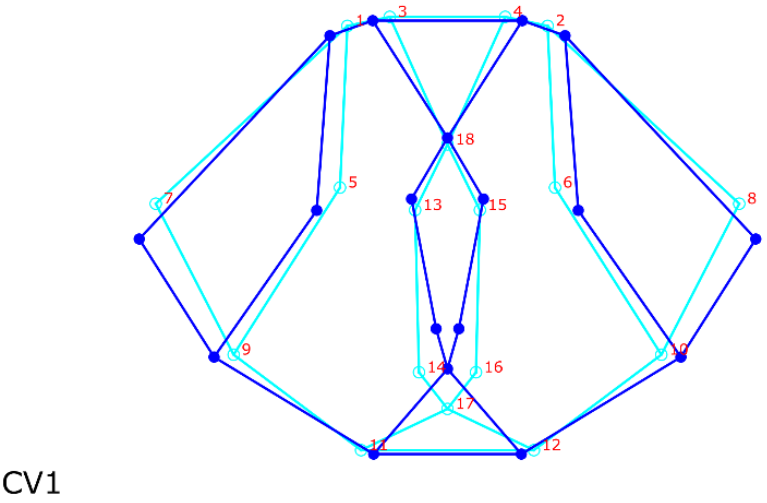
CV1

(d) Figure 4.39: Distribution of CVA of heads with wireframe for the eastern localities

Appendix 4.10.



(a) ***Figure 4.40: Distribution for CVA with transformation gride for species***



(b) ***Figure 4.41: Distribution for CVA with wireframe for species***

CONCLUSIONS

Conclusions

The general conclusions of this thesis are:

1. DNA barcoding and the species delimitation methods are important tools in forensic entomology, identifying damaged/poorly-preserved samples.
2. Molecular techniques help to characterize the diversity of forensically important Iberian fly species in a pool of sequences.
3. Molecular techniques have demonstrated to be useful to identify samples of forensic cases, even when the samples were in poor preservation state.
4. The identification through DNA barcoding allowed us to rename some specimens that were assigned to the wrong dipteran family in a forensic case.
5. The phylogeographical analysis showed that samples from Alcoy of forensic cases carry local haplotypes.
6. There is no biogeographical structure for *C. vicina* for the COI, 16S and ITS2 genes. This confirms that the geographical barriers are not enough to hold back gene flow.
7. The colonization of the Iberian Peninsula by *C. vicina* does not present a geographical pattern for COI and 16S sequences. The analysis showed multiple serial colonizations possibly related to human activity.
8. The sister species *L. ampullacea*, *L. caesar* and *L. illustris* have been differentiated by several methods.
9. Our results confirm the conclusion of Boehme et al, (2012) that said that a shorter fragment removes homologous zones, improving the molecular identification.
10. The haplotype analysis helps to visualize the species differentiation. This allows us to rename six sequences erroneously identified by morphology.
11. The geometric morphometric techniques allow us to identify the species using both structures, wings and heads, with almost the same accuracy.
12. The results show that the geometric morphometric techniques can be used to differentiate the origin of samples with the two structures tested, especially if we consider separately eastern and western samples. This can be used in forensic cases in which relocation of the corpse may have occurred.

13. The set of landmarks designed in this work for heads have demonstrated the same effectiveness than those designed for wings.
14. A total of 1398 new sequences have been produced in this thesis: 669 of COI, 206 of 16S and 523 of ITS2. With the COI sequences the project "Barcoding of Iberian Diptera" have been created in BOLD. This project will be helpful in future works.
15. All the information produced in this thesis increase the knowledge about the Diptera with forensic interest in the Iberian Peninsula.

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