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DNA BARCODING OF TWO FORENSICALLY IMPORTANT FLESHFLY SPECIES (DIPTERA: SARCOPHAGIDAE) FROM SPAIN AND NOTES ON BARCODING SUCCESS WITHIN GENUS *SARCOPHAGA* MEIGEN, 1826

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Resumen: Se presenta la secuenciación del gen de la citocromo oxidasa I (COI) de poblaciones españolas de *Sarcophaga tibialis* (Macquart, 1850) y *Sarcophaga cultellata* Pandellé, 1896, dos especies con interés forense, que fueron previamente identificadas empleando otros criterios. *S. tibialis* está relacionada con *Sarcophaga dux* Thomson, 1869 dentro del subgénero *Liosarcophaga,* mientras que *S. cultellata* está relacionada con *Sarcophaga crassipalpis* Macquart, 1839 dentro del subgénero *Liopygia.* Los resultados permiten identificar estados preimaginales, adultos y restos de ambas especies procedentes de casos forenses.

El análisis de nuestros resultados moleculares en relación a los datos de 61 especies del género *Sarcophaga* extraídos de las bases de datos GenBank y BOLD, mostró una correspondencia aceptable entre la identificación

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previa y la derivada del estudio molecular. La mayor correspondencia (99% de éxito) se obtuvo con umbrales elevados del parámetro Kp2 (10,55%), mientras que con umbrales inferiores (5,02%) la correspondencia descendió al 94%. Aunque se trata de un análisis preliminar, se advierte que la división actual de *Sarcophaga* en subgéneros tiene que ser revisada, ya que algunos de ellos parecen ser polifiléticos.

Palabras clave: Citocromo oxidasa I, ciencia forense, entomología forense, *Sarcophaga cultellata*, *Sarcophaga tibialis*, identificación específica.

Abstract: COI barcoding sequence of two species of forensic interest, *Sarcophaga tibialis* (Macquart, 1850) and *Sarcophaga cultellata* Pandellé, 1896, from Spain has been determined. Both species were successfully identified under different criteria. S. *tibialis* was related to *Sarcophaga dux* Thomson, 1869, within the subgenus *Liosarcophaga*, whereas *S. cultellata* was related to *Sarcophaga crassipalpis* Macquart, 1839, within the subgenus *Liopygia*. This study provides a good tool to identify preimaginal stages, male and female adults, and the remnants of these two species for forensic purposes.

A first analysis of 61 species of the genus *Sarcophaga* with all available information obtained from GenBank and BOLD databases, showed that success in correct identification raised up to 94 and 99% under different threshold values of the Kp2 parameter. This analysis also showed that current division of *Sarcophaga* into subgenera is worth to be revised as many of these subgenera might be polyphyletic.

Key words: Cytochrome oxidase I, forensic science, forensic entomology, *Sarcophaga cultellata*, *Sarcophaga tibialis*, species identification.

1. INTRODUCTION

Forensic entomology deals with entomological evidence that is relevant in legal cases, particularly those related to corpses. Proper identification of evidence is crucial as a misidentification may lead to inaccurate and erroneous conclusions of potentially dramatic consequences. Identification is usually made on the basis of morphological characters observed on adults and compiled in identification keys. However, morphological characters are sometimes difficult to be observed or do not provide a good discrimination among related taxa (Smith, 1986, Gennard, 2007, Wells & Stevens, 2010).

To the difficulty of identifying entomological evidence it should be added that when dealing with a cadaver, usually preimaginal insect stages are involved making the species identification more difficult due to the lack of adequate keys based on larval characters; in some instances these preimaginal stages are yet unknown (Greenberg & Kunich, 2002, Gennard, 2007, Byrd & Castner, 2010). A major advance for solving this problem

has been the publication of identification keys based on larval morphology (Velásquez *et al.,* 2010), although these are only valid for few species.

To solve this problem some molecular techniques have been developed to provide an alternative to morphological identification. DNA barcoding, as proposed by Hebert *et al.* (2003), uses DNA to identify unknown sample in terms of a known classification (Kress *et al.,* 2005). Dipterists were among the first systematists to extensively use DNA sequences for species identification and delimitation, as the order Diptera contains a large number of economically and forensically important species, of which many are difficult to identify using traditional methods (Meier & Zhang, 2009).

A wide variety of molecular markers has been used in Diptera (Wells & Stevens 2008), but the best taxon coverage is available for the mitochondrial COI gene, which is the standard for DNA barcoding (Meier *et al.,* 2009). Despite opinions, such as that of Will & Rubinoff (2004) disagreeing with using this analytical method, and some failures in preliminary studies on Dipteran taxa (Meier *et al.,* 2006), recent research has shown that DNA barcoding is an effective tool to correctly assign DNA sequences to described species (e.g. Vincent *et al.,* 2000, Wells *et al.,* 2001, Harvey 2003, Zehner, 2004, Ames *et al.,* 2006a, b, Smith *et al.,* 2006, 2007, Nelson *et al.,* 2007, Meier & Zhang, 2009, Cywinska *et al.*, 2010, Guo *et al.,* 2010b, Tan, 2010, Alfred, 2011, Dalton & Kotze, 2011, Jinbo *et al.,* 2011, Meiklejohn *et al.,* 2012). Following Dawnay *et al.* (2007), COI gene has sufficiently discrimination and consistently identifies species where authenticated reference sequence data exist.

Sarcophagidae is a worldwide distributed dipteran family which includes common members of the sarcosaprophagous fauna, as many of their species develop in excrement, carrion and other media related to forensic issues (Smith, 1986, Povolný & Verves, 1997). These flies can be found associated with carcasses throughout both the early and late stages of decomposition (Byrd & Castner, 2010) and therefore have characteristics that make them ideal forensic indicators. However, their utility is severely hampered due to their complex taxonomic framework, the difficulties met in species identification and the lack of enough trained taxonomists (i.e. the taxonomic impediment) (Wells *et al.,* 2001). In fact, Sarcophagidae flies are notoriously difficult to identify because of their highly similar morphological appearance and that task often requires the study of male genitalia; for many sarcophagid species only adult males can be certainly identified (Guo *et al.,* 2011). However, female genitalia do not include valuable taxonomic characters and thus females are frequently identified in relation to co-occurring males (Prado e Castro *et al.,* 2010).

To date, there are not many DNA barcoding studies related to sarcophagid flies (e.g. Draber-Monko *et al.,* 2009, Alfred, 2011, Meiklejohn *et al.,* 2011, Stamper *et al.,* 2013) although several studies have dealt with DNA based identification (e.g. Kiyoshi *et al.,* 2005, Cainé *et al.,* 2009, Hall *et al.*, 2009, Guo *et al.,* 2010a, b, Tan *et al.,* 2010) or phylogenetic relationships

among lineages of this family based on molecular data (Wells *et al.,* 2001, Zehner *et al.,* 2004, Bajpai & Tewari, 2010, Kutty *et al.,* 2010).

The knowledge of local fauna is very useful in forensic investigations because data from other regions, which may have both different environmental and fauna characteristics, may not provide a sufficient degree of accuracy (Arnaldos *et al.,* 2004). Although the faunistic knowledge is increasing in the Iberian Peninsula (e.g. Arnaldos *et al.,* 2001, Arnaldos *et al.,* 2004, Arnaldos *et al.,* 2005, Prado e Castro, 2009, Prado e Castro *et al.,* 2010, Prado e Castro *et al.,* 2011a,b) molecular data available with respect to the sarcophagid flies are very limited. In fact there are no records for analysing sequences of the COI gene of flesh flies from Spain. In the Iberian Peninsula sarcophagid species are commonly related to corpses (Castillo Miralbés, 2002, Romera *et al.,* 2003, Cainé *et al.,* 2009, Prado e Castro *et al.,* 2010). Some of their species have been referred from human cadavers (Velásquez *et al.,* 2010) and are taxa of forensic interest. Among them there are *Sarcophaga tibialis* (Macquart, 1851) and *S. cultellata* (Pandellé, 1896). Males of both species are easily recognizable on the basis of their genitalia characters (see below Figures 1 and 2).

Sarcophaga tibialis is widely distributed in the Palaearctic, Afrotropical, Oriental, Australasian and Oceanian regions (Pape, 1996, Richet *et al.,* 2011) while *S. cultellata* is mainly restricted to Mediterranean areas from France, Italy and Spain (Pape, 1996, Richet *et al.,* 2011). The biology of *Sarcophaga cultellata* is almost unknown (Romera *et al.,* 2003, Arnaldos *et al.*, 2013), *Sarcophaga tibialis* tends towards synanthropy (Povolný & Verves, 1997), its larvae develop in carcasses and faeces (Aspoas 1991) and cause traumatic dermal myiasis (Zumpt, 1965). This species has potentially significance in forensic investigation and as vector of disease (Zumpt & Patterson, 1952). It has already been sequenced (Zehner *et al.,* 2004) but

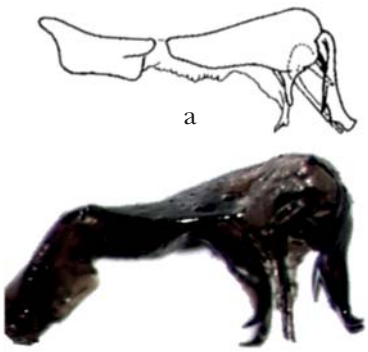


Figura 1. Genitalia de los ejemplares macho de Sarcophaga tibialis utilizados en este trabajo; a: distifalo según Peris *et al.* (1999).

Figure 1. Male genitalia of Sarcophaga tibialis from a specimen used in this study; a: distiphallus from Peris *et al.* (1999).

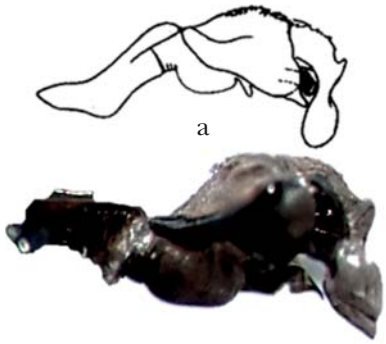


Figura 2. Genitalia de los ejemplares macho de Sarcophaga cultellata utilizados en este trabajo; a: distifalo según Peris *et al.* (1999).
Figure 2. Male genitalia of Sarcophaga cultellata from a specimen used in this study; a: distiphallus from Peris *et al.* (1999).

the fragment analyzed does not correspond to the part of the COI gene usually considered for DNA barcoding.

Here we present barcoding data of *S. cultellata* and *S. tibialis*, as part of a study aimed to develop a library of barcodes of sarcophagid flies inhabiting Southeast Spain, which can be used for forensic purposes. DNA barcodes of the two species are provided to enable accurate identification of their larval stages, as well as the females. These data are useful as the major limitation to species identification is the lack of authenticated reference DNA sequence data (Dawnay *et al.*, 2007).

2. MATERIALS AND METHODS

Specimens were obtained from laboratory breeding colonies maintained at a temperature of 25 ºC and relative humidity of 50%. These colonies were established from wild specimens of *S. cultellata* captured in Sierra Espuña (Murcia, SE Spain) and *S. tibialis* captured in the University Campus of Murcia (SE Spain). Samples were frozen and conserved in absolute ethanol. DNA data of related taxa *Sarcophaga crassipalpis* Macquart, 1839, *S. ruficornis* (Fabricius, 1794), *S. argyrostoma* (Robineau-Desvoidy, 1830), *S. princeps* Wiedemann, 1830, *S. portschinskyi* (Rohdendorf, 1937), *S. dux* Thomson, 1869 and *S. misera* Walker, 1849 (= *S. orchidea* Böttcher, 1913), and of other members of this genus were obtained from BOLD and Genbank databases and used to interpreting barcode results of the studied species. Accession numbers of these sequences are indicated besides the species name in Figure 3.

Figura 3. Árbol de consenso del 50% derivado del análisis de Neighbor-Joining (NJ) aplicado a las secuencias del gen COI en el género *Sarcophaga*.

Figure 3. 50% of the majority consensus tree resulting from the NJ analysis of COI sequence of taxa of the genus *Sarcophaga*.

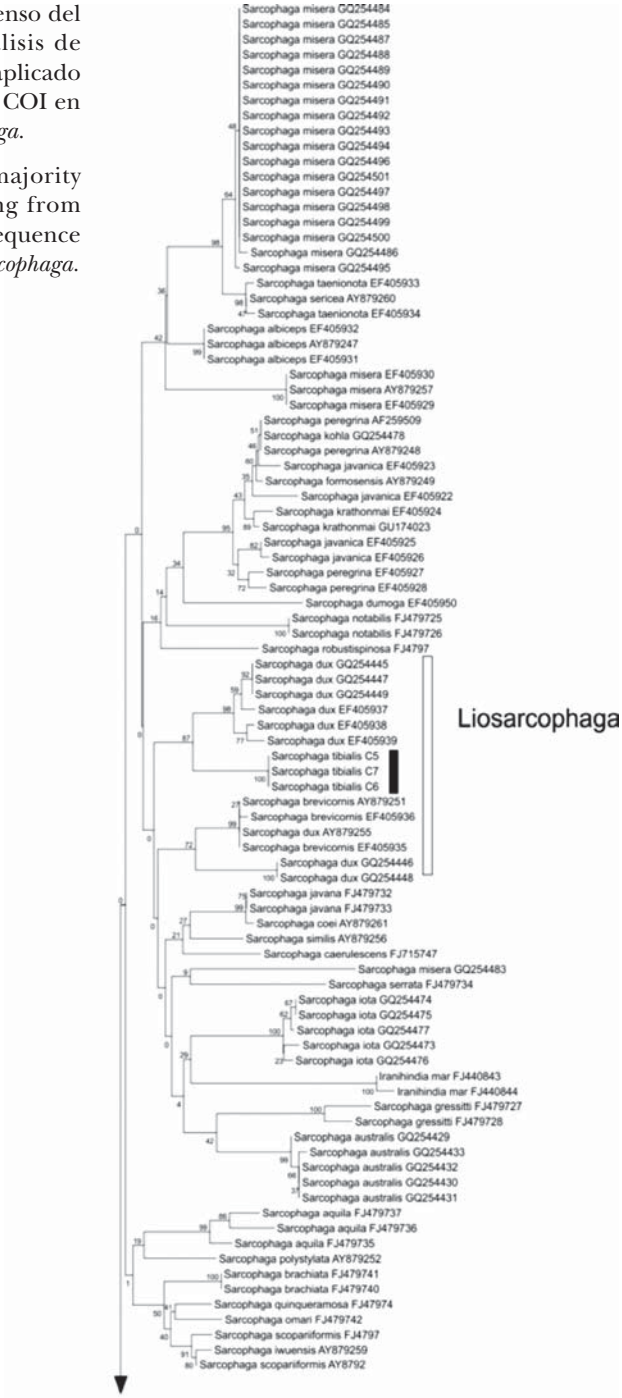
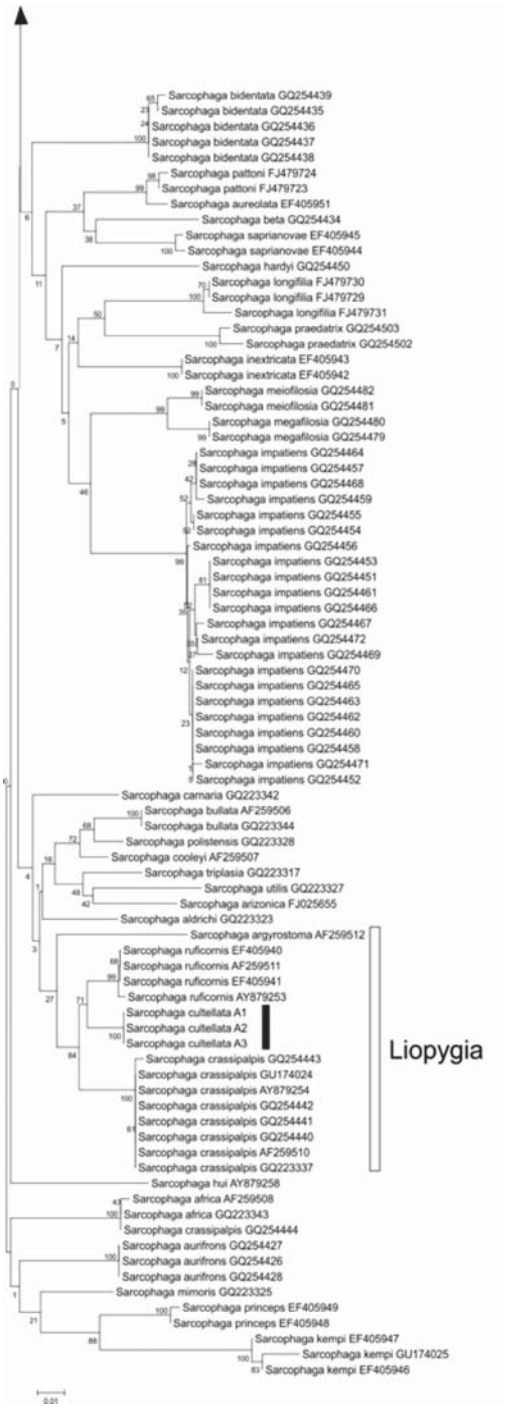


Figura 3. (continuación) Árbol de consenso del 50% derivado del análisis de Neighbor-Joining (NJ) aplicado a las secuencias del gen COI en el género *Sarcophaga.*

Figure 3. (continued) 50% of the majority consensus tree resulting from the NJ analysis of COI sequence of taxa of the genus *Sarcophaga.*



Genomic DNA from 4 adult specimens of these two species was extracted using the thorax and legs tissues with Invisorb spin tissue mini kit (Berlin, Germany).

A barcoding COI region was amplified with primers LCO1490 (5′- GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAA ATCA-3′) (Folmer *et al.,* 1994) using standard PCR conditions as described in Ruiz, Serrano (2006): 40 cycles at 94ºC for 1 min, annealing at 47 ºC for 1min and extension of 72ºC for 2 min. An amplicon of about 650 bp was obtained and purified with isopropanol and 5M ammonium acetate. Sequencing was performed in both directions using standard protocol for the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Sequences were edited and unambiguously aligned using the software MEGA v4.0 (Tamura *et al.,* 2007). A first dataset was built up using all sequences of the genus *Sarcophaga* available from GenBank and BOLD databases (the FULL dataset). As sequences from GenBank are known to include misidentified species (e.g. Harris, 2003, Hebert, 2003, Vilgalys, 2003), a second data set was built up using only BOLD sequences (BOLD dataset). We also considered species with at least 2 or 3 specimens and reanalyzed the data using a subset of original datasets (FULL2, FULL3, BOLD2 and BOLD3) (Table I).

The proportion of correct matches followed the three distance-based identification criteria described by Meier *et al.* (2004). These were the Best Match (BM), Best Close Match (BCM) and All Species Barcodes (ASB). The distance below which 95% of all intraspecific distances are found was used as cut-off. These parameters were calculated using TaxonDNA v1.7 (Meier *et al.,* 2004).

A NJ tree was calculated with MEGA 4.0 (2000 bootstrap) using the model of Kimura 2-parameter (K2P) that has become the metric most widely used in barcoding studies (CBOL, http://www.barcoding.si.edu/protocols.html). A bayesian analysis was carried out to infer the phylogenetic relationships between taxa with MrBayes v.3.1.(Ronquist & Huelsenbeck, 2003). Searches were performed with 6,000,000 generations, sampling trees every 100 generations under the GTR + I + Γ model. Likelihood values were observed with Tracer v1.4 (Rambaut & Drummond, 2005), discarding all the trees before stability in likelihood values as a «burnin». Stationarity was also reassessed using the convergence diagnostic: the average standard deviation of split frequencies and the potential scale reduction factor (PSRF).

Voucher specimens are kept at Forensic Entomology Laboratory, Zoology and Physical Antropology Department from the University of Murcia.

Table I. Rates of successful species identification in the genus *Sarcophaga* using all COI sequences from GenBank and BOLD (FULL), from solely BOLD (BOLD), and from BOLD database with at least 2 or 3 conspecific per species (FULL2, FULL3, BOLD2, BOLD3) under different identification criteria: Best Match (BM), Best Close Match (BCM), and All Species Barcodes (ASB). Cut-off threshold was set to 10.55% (see text). N, number of specimens; sp, number of species.

Tabla I. Niveles de éxito en la identificación en el género *Sarcophaga* utilizando todas las secuencias COI de GenBank y BOLD (FULL), únicamente BOLD (BOLD) y BOLD con al menos 2 o 3 coespecíficos por e-specie (FULL2, FULL3, BOLD2 BOLD3) bajo criterios de identificación diferentes: correspondencia correcta (BM), correspondencia más aproximada (BCM) y considerando todos los datos de barcoding (ASB). El umbral de corte se estableció en 10,55 (ver texto). N, número de ejemplares; sp, número de especies.

DATABASE	N SP	CRITERION	CORRECT ID. %	AMBIGUOUS ID. %	INCORRECT ID. %	WITHOUT ANY MATCH THR.
	184	BM	76.6	5.43	17.9	
FULL	61	BCM	76.6	5.43	17.9	0.54
		ASB	10.86	84.78	3.8	0.54
	153	BM	93.54	1.29	5.16	
FULL2	32	BCM	93.54	1.29	4.51	0.64
		ASB	14.19	83.22	1.93	0.64
	126	BM	94.44	1.58	3.96	
FULL3	19	BCM	94.44	1.58	3.17	0.79
		ASB	17.46	80.95	0.79	0.79
	85	BM	95.29	0	4.7	
BOLD	16	BCM	95.29	0	4.7	0
		ASB	17.6	82.35	0	0
	82	BM	98.78	0	1.2	
BOLD2	13	BCM	98.78	0	1.2	0
		ASB	18.29	81.7	0	0
	48	BM	98.66	0	1.33	
BOLD3	10	BCM	98.66	0	1.33	0
		ASB	20.00	80.00	0	1.4

3. RESULTS

Morphological identification of studied taxa

The structure and form of male terminalia is important for both taxonomical and phylogenetical purposes (Povolný & Verves, 1997). In our case, the sequences proceed from vouchers whose male genitalia match those described in Peris *et al.* (1999), Lehrer (2006) and Richet *et al.* (2011) (Figures 1 and 2), so we are confident of having correctly identified these vouchers.

Barcoding analysis

A fragment of 658 bp of the COI barcoding region was obtained from three specimens of each species. This sequence lack insertions, deletions or codon stops, and revealed 53 positions that are phylogenetically informative. No intraspecific variation was found, a result that should be corroborated with the study of specimens from more localities. The sequence of both species has been submitted to GenBank, with accession numbers JX987058 (*S. tibialis*) and JX987057 (*S. cultellata*).

Interspecific K2P distance between S. *tibialis* and *S. cultellata* was 5.04%.

Both NJ and Bayesian analyses of COI sequences of the genus *Sarcophaga* showed the same main relationships between taxa and thus only the NJ cladogram is shown (Figure 3). Clades including the two species investigated had high bootstrap support (100%) (Figure 3). *Sarcophaga tibialis* was related to *S. dux*, and *S. cultellata* to *S.ruficornis* and *S. crassipalpis*. Both species were successfully identified under Best Match (BM), Best Close Match (BCM) and All Species Barcode (ASB) criteria.

A first analysis with all available information of the genus *Sarcophaga* obtained from GenBank and BOLD databases (FULL dataset) comprised 184 specimens representing 61 species. Of these, 47% have more than one individual per species. Intraspecific distance was usually lower than 1% but in some cases raised up to 10%; interspecific distance varied between 8% and 17%. The cut-off value that comprises the 95% of the intraspecific distances was 10.55%. When using this threshold the correct identification under the ASB criterion was 10.86%, and ambiguous 85%. Under the BM criterion, the correct identification was 76.6% while ambiguous identification corresponded to 5.4% and the remaining specimens (17.9%) were incorrectly identified (Table I). The total overlap between intraspecific and interspecific distances was 10.7% (from 0 to 10.7%, 82% of the pairwise comparisons. This overlap was still high, 4.12% (from 6.42 to 10.55%, 74% of the pairwise comparisons), when the 5% of extreme intra and interspecific divergences was removed. These values are in accordance with previous barcoding Dipteran studies (Meier *et al.*, 2006), in which it resulted in a success rate in species identification lower than 70%.

When considering only BOLD database (85 sequences representing 16 species with 82% of them with a valid conspecific) the proportion of correct identifications increased to 84.9% using BM and BCM, and to 17.6% using ASB (Table I).

If only sequences with at least 2 conspecific are considered (FULL2 and BOLD2) the proportion of correct identification by BM and BCM criteria increased substantially to 94 and 99% respectively (although the ASB criterion remained low: 14 and 18% respectively; Table I). The proportion of correct identification when considering at least 3 conspecific (FULL3 and BOLD3) remained almost the same (94 and 99%) using BM and BCM although it increased using ASB (17 and 20% respectively). Additionally, when the 5% extreme distances were removed the overlap remained the same in FULL2 and 3 (4.1%), or slightly decreased to 3.9% in BOLD 2 and 3.

4. DISCUSSION

The main goal of the genetic species identification is to match the sequence of the evidence item to an authenticated reference DNA sequence. In our case, the two investigated species, *Sarcophaga tibialis* and *S. cultellata* were shown to be unequivocally identified by the COI barcoding sequence with regard to related taxa, as *S. tibialis* was included in one of the clades of the possibly polyphyletic subgenus *Liosarcophaga*, whereas *S. cultellata* was included within the clade corresponding to the subgenus *Liopygia* (Figure 3). Analyses of samples of these two species from other localities are needed to determine whether there exists intraspecific variation.

Among the genus *Sarcophaga* barcoding of species has an identification success up to 99% using BM and BCM criteria, that is, when misidentifications from GenBank data and the absence of conspecific sequences are considered. This resolution is comparable to that found in other barcoding studies (Nelson *et al.,* 2007, Meiklejohn *et al.,* 2011), and in studies based on other fragments of COI (Tan *et al.,* 2010). GenBank sequences are known to include misidentified sequences (e.g. Harris, 2003, Hebert *et al.,* 2003, Vilgalys, 2003), what explains that identification success increases to 95% when these wrong sequences are removed (BOLD, Table I).

Additionally, species represented by a single DNA barcode (53% in FULL and 18% in BOLD) substantially affect the results of BM and BCM criteria. The presence of single sequences may generate incorrect identification because there are no other conspecific reference sequences in the dataset to which they can be matched (Ross *et al.,* 2008). This caution was not taken into account by Meier *et al.* (2006), who underestimated the proportion of correct species identification for Diptera (<70%) (Ross *et al.,*

2008, Virgilio *et al.,* 2010). The lower identification success of the ASB criterion is clearly related to a more stringent «decision rules» (Virgilio *et al.,* 2010).

A broad overlap was observed between intra- and interspecific distances, as noted in previous studies on *Sarcophaga* (Meier *et al.*, 2006) and instances of similar results have been reported in other insect orders (Virgilio *et al.,* 2010). These findings show that the extent of barcoding overlap does not necessarily predict the identification success (Ross *et al.,* 2008, Virgilio *et al.,* 2010).

Although the amplicon size and sample size were both small, these data provide an opportunity to evaluate the value of COI for basic biological studies of both sarcophagid species. These data (COI gene) can be used as a supplemental means of morphological method in identification of sarcophagid species, as the technology is easier to perform and saves more time for forensic scientist (Guo *et al.,* 2011).

In addition to the increase of biological knowledge of these species, our contribution provides new data to identify populations from Iberian Peninsula increasing the current knowledge worldwide (Piwcznsky, 2014). This will enable a correct identification even when inadequate morphological information (larvae, females and fragments of specimens) is the only available evidence.

5. ACKNOWLEDGEMENTS

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