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DEPARTAMENTO DE BIOLOGIA ANIMAL

FORENSIC ENTOMOLOGY: DNA BARCODING FOR COLEOPTERA IDENTIFICATION

Susana Fernandes Torres Lopes

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Dissertação orientada por:

Professora Doutora Deodália Dias

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Nota prévia

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Resumo

A Entomologia Forense é a ciência que recorre ao estudo de insectos e outros artrópodes e os aplica em investigações do foro legal. Para além de contribuírem para o processo de decomposição do corpo, os insectos podem ainda fornecer informações importantes em investigações criminais. A aplicação mais frequente da Entomologia Medicolegal é a determinação do tempo decorrido desde a morte do indivíduo (intervalo *postmortem* ou IPM), contudo, existem outras informações importantes como a ocorrência de deslocação do corpo, situações de negligência e associação do corpo com drogas ou toxinas.

Apesar da fauna associada ao cadáver incluir uma grande diversidade de artrópodes, Diptera e Coleoptera são as duas principais ordens de insectos que fornecem a maioria da informação em investigações forenses. As espécies pertencentes à ordem Diptera são as primeiras a colonizar o cadáver, sendo que a ordem Coleoptera tem o seu pico, em número e diversidade, durante os estádios mais avançados de decomposição. As espécies de insectos encontradas, assim como o seu padrão de colonização estão dependentes de inúmeros factores, sendo a região geográfica onde se encontram um dos mais importantes. Desta forma, para que seja possível uma aplicação sustentável da Entomologia Forense em investigações do foro legal, é extremamente importante a elaboração de bases de dados específicas para cada região geográfica. A identificação de espécies de insectos encontradas em cadáveres é a etapa mais crucial neste tipo de investigação. Este processo baseia-se na observação directa de caracteres morfológicos e uso de chaves dicotómicas. Contudo, trata-se de uma abordagem demorada e que muitas vezes não permite a identificação de espécimes em estádio imaturo. De modo a ultrapassar estas limitações foi proposta uma identificação molecular que se baseia na sequenciação de uma pequena região do genoma do espécime alvo, denominada por *DNA barcode*, e que posteriormente é comparada com sequências de referência que se encontram em bases de dados *online*, pelo que esta ferramenta é designada por “DNA barcoding”. O marcador molecular de eleição, usado no *DNA barcoding* é o gene mitocondrial Citocromo *c* oxidase subunidade I (COI).

Contudo, a utilização de um único marcador para a identificação de espécies é controversa, pelo que se tem vindo a apostar cada vez mais numa abordagem que envolve outros genes. Uma outra região genómica que tem vindo a ser utilizada com sucesso, incluindo estudos com Coleópteros, é o Citocromo b (Cytb).

Desta forma, o presente estudo surge com o principal objectivo de proceder à identificação molecular de espécies com interesse forense, pertencentes à ordem Coleoptera, recolhidas na região de Aveiro, Portugal. Os indivíduos colectados foram identificados morfologicamente procedendo-se, de seguida, à extracção do DNA e posterior sequenciação dos marcadores moleculares COI e Cytb. Deste modo, pretendeu-se testar a eficácia do gene Cytb como marcador molecular e simultaneamente comparar a sua eficiência relativamente ao marcador COI.

Neste trabalho foram colectados 35 espécimes de Coleópteros, pertencentes às famílias Staphylinidae, Silphidae, Histeridae, Chrysomelidae, Curculionidae, Nitidulidae, Scarabaeidae e Carabidae. Destes 35 espécimes foram obtidas 30 sequências com 658 pb para o gene COI e 31 sequências com 433 pb para o gene Cytb. Embora a amplificação tenha ocorrido sem grandes complicações para ambos os genes, quando o gene Cytb foi analisado, foi possível verificar que 17 das 31 sequências eram idênticas, mesmo entre indivíduos pertencentes a famílias distintas. Este resultado veio demonstrar que esta região parece ser bastante conservada entre as diferentes espécies, impossibilitando a sua diferenciação. Assim sendo, esta região do Cytb mostrou não ser um bom marcador molecular para ser utilizado no *DNA barcoding* destas espécies. Todas as sequências obtidas foram comparadas com sequências de referência disponíveis nas bases de dados *online* GenBank e BOLD, de forma a corroborar a identificação morfológica. Porém, para a grande maioria dos indivíduos não foi possível a confirmação ao nível da espécie, uma vez que o *DNA barcoding* é uma abordagem relativamente recente e consequentemente ainda existe um grande número de espécies que não se encontra nestas bases de dados, impossibilitando uma comparação. As sequências obtidas neste trabalho irão contribuir para a implementação de uma Base de Dados Nacional, permitindo aumentar o conhecimento de espécies de Coleópteros com interesse forense, em Portugal, e que no futuro poderão ser utilizadas como evidências entomológicas em

investigações do foro legal. As sequências serão também submetidas nas bases de dados GenBank e BOLD.

As variações intra e interespecíficas foram calculadas relativamente aos dados do COI, usando apenas sequências de indivíduos identificados até ao nível da espécie. Neste tipo de análise é importante ter em conta que o valor de variação intraespecífica deverá ser inferior a 3%, enquanto a variação interespecífica deverá ser superior a este limite, de forma a permitir a diferenciação das espécies. Outro critério está relacionado com a variação interespecífica, que deverá ser 10 vezes superior à média da variação intraespecífica. Contudo, ao analisar os resultados, foi possível observar que os valores de divergência intraespecífica para duas das espécies eram muito superiores ao esperado. Desta forma, foi necessário voltar a analisar a matriz inicial e tentar perceber quais os indivíduos que apresentavam um maior número de diferenças. Estas diferenças poderão estar relacionadas com o facto de os indivíduos terem sido morfologicamente mal identificados, podendo estar a considerar-se indivíduos de espécies diferentes como pertencentes à mesma espécie, obtendo-se assim valores errados. Outro critério usado na delimitação de espécies é a análise filogenética, onde cada espécie deverá surgir como um grupo monofilético. É importante ter em conta que geralmente a sequência *barcode* não apresenta um sinal filogenético suficientemente forte para determinar relações evolutivas, pelo que a análise destes dados deve ser realizada com precaução. A análise filogenética foi feita para ambos os genes através das metodologias Máxima Verosimilhança, Neighbour-Joining e Máxima Parcimónia. Relativamente à árvore obtida com os dados do COI, foi possível verificar que algumas espécies não agrupavam da forma esperada, o que nos leva a considerar, mais uma vez, que devido ao elevado grau de dificuldade, a identificação morfológica de alguns espécimes terá sido incorrecta. Em relação ao Cytb, visto que foram obtidas 17 sequências idênticas entre si, verifica-se que este gene não tem resolução para estimar relações filogenéticas nas espécies estudadas. Assim, este trabalho vem demonstrar que, na ordem Coleoptera, o COI é um marcador mais adequado do que o Cytb para ser usado no *DNA barcoding*.

Palavras-chave: Entomologia Forense; Coleoptera; DNA barcoding; COI; Cytb.

Abstract

Besides the contribution to the body decomposition process, insects are entomological evidences that can provide important information in criminal investigations. In order to study these events, it is essential to accomplish an accurate identification of species founded on corpses.

The main goal of this study was to perform a molecular identification of species with forensic interest, belonging to the order Coleoptera. With this purpose, two mitochondrial markers, COI and Cytb, were used. A total of 35 specimens belonging to the families Staphylinidae, Silphidae, Histeridae, Chrysomelidae, Curculionidae, Nitidulidae, Scarabaeidae and Carabidae were collected. Of the 35 Coleoptera specimens, there were obtained 30 sequences with 658 bp for COI gene and 31 sequences with 433 bp for Cytb gene. When Cytb sequences were analysed it was noticed that 17 of the 31 sequences were identical. This region appears to be highly conserved among different species, showing that this fragment is not a good molecular marker to be used for DNA barcoding of these Coleoptera species. Obtained sequences were compared with reference sequences in GenBank and BOLD databases. The majority of studied specimens did not have a sequence to be compared with, showing a lack of reference sequences for a great number of species.

Intra and interspecific variation was determined for COI gene, using only specimens identified to species level. In this analysis it was realized that intraspecific divergence values for some species were much higher than expected (>3%). Therefore, the original dataset was reanalyzed to see which individuals presented greatest differences. Phylogenetic analysis was performed for both genes. Regarding the tree based on COI gene data, some species were not grouped as expected, leading us to consider that these specimens have been misidentified morphologically. About Cytb gene, since 17 obtained sequences were identical, phylogenetic analysis for this gene is not suitable. Comparing these two genes, results showed that COI gene is a most suitable marker to be used in DNA barcoding for the order Coleoptera.

Key words: Forensic Entomology; Coleoptera; DNA barcoding; COI; Cytb.

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List of Abbreviations

BLAST - Basic Local Alignment Search Tool

BLASTn - Nucleotide Basic Local Alignment Search Tool

BOLD - Barcode of Life Data System

BOLD-IDS - Identification System of BOLD

bp - Base Pair

BSA – Bovine serum albumine

CBOL - Consortium for the Barcode of Life

cDNA - complementary DNA

COI - Cytochrome *c* Oxidase Subunit I

Cytb - Cytochrome b

DNA - Deoxyribonucleic Acid

dNTP - Deoxyribonucleotide Triphosphate

ML - Maximum Likelihood

MP - Maximum Parsimony

mtDNA - Mitochondrial DNA

NCBI - National Center for Biotechnology Information

NJ - Neighbor-Joining

NUMT - Nuclear Mitochondrial Pseudogene

PCR - Polymerase Chain Reaction

PMI - *Postmortem* Interval

RNA - Ribonucleic Acid

rRNA - Ribosomal RNA

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State of the Art

I. State of the Art

1.1. Forensic Entomology

"Forensic" can be defined as the use of scientific techniques to solve legal issues and "Entomology" as the study of insects. Thus, "Forensic Entomology" is a branch of forensic sciences which applies the study of insects and other arthropods with forensic interest in legal investigations (Jobling & Gill, 2004; Amendt *et al*., 2007).

1.1.1. Retrospective

Although Forensic Entomology has only begun to play an important role in criminal investigations over the past few decades, the potential of using insects to solve criminal cases has been known for centuries (Oliveira-Costa & Mello-Patiu, 2004).

The first documented case was written in 1235 by Sung Tz'u, a Chinese lawyer and criminalist, in a Legal Medicine book entitled "The Washing Away of Wrongs" (Benecke, 2001). In the 13th century, Sung Tz'u investigated a murder in a local village, in which a farmer had been stabbed in a rice field with a sharp weapon. The criminalist believed that a farming tool was the murder weapon, so he called all the farm workers and told them to place their working tools on the ground. He noticed that numerous blowflies were attracted by one of the sickles, probably due to the presence of invisible traces of blood. Confronted by this evidence, the stickle owner confessed to be the murderer (Amendt *et al*., 2004; Gupta & Setia, 2004).

During the Middle Ages, many sculptures, painters and poets have observed the decomposition process of human bodies, paying particular attention to the role of maggots. The realistic and detailed illustrations of corpses infested with maggots were usual (Benecke, 2001; Amendt *et al*., 2004). There are documents dating from 15th and 16th century that illustrate the importance of insects in decomposition, showing the pattern of body mass reduction and the skeletonization process by insect activity (Gupta & Setia, 2004).

In 1831, during the mass exhumations in France, the medical doctor Orfila noticed that insects and their larvae played an important role in body decomposition. That was an important step to understand the correlation between maggots and corpses decomposition (Benecke, 2001).

The first application in Europe is attributed to the French doctor Bergeret (in 1855), when the skeletonised remains of a child were found behind a chimney, by a workman, during a house redecoration. Bergeret studied the insect fauna discovered on these remains and made an estimation of the *postmortem* interval (PMI), i.e. time since death occurred, proving that the current occupants of the house could not have been the murderers since they had moved recently (Benecke, 2001; Gupta & Setia, 2004).

Later, in 1894, Mégnin published his most important book "*La Faune des Cadavres: Application de l'entomologie à la Medicine Légale*". In this book, he describes the morphological features of different insect families that helped in their identification (Benecke, 2001; Gupta & Setia, 2004). He was the first to describe the relation between the chronological sequence of the decomposition process and insect colonization. He recognized eight invasion waves of arthropods related to the different stages of human decomposition on freely bodies, and two waves on buried bodies. This knowledge about insect succession became the basis for forensic entomologists to estimate the PMI (Campobasso *et al*., 2001; Gennard, 2007).

Despite Mégnin studies, Forensic Entomology was neglected for many years, not only due to the lack of specialized entomologists in cadaveric fauna, but mainly due to the distance between entomologists and medicolegal specialists (Pujol-Luz *et al*., 2008).

Only in the second half of the 20th century the interest in Forensic Entomology emerged again. In Europe, the medical Doctor Marcel Leclercq and the Professor of biology Pekka Nuorteva were the first ones to employ Forensic Entomology for the determination of the PMI (Benecke, 2001; Amendt *et al*., 2004). Nowadays, Forensic Entomology is recognized in many countries as an important forensic tool (Amendt *et al*., 2004).

1.1.2. Main Areas

Forensic Entomology is the broad field where the study of insects and legal system interact. Insects can be used as evidence to solve crimes and many times are in the centre of legal disputes for causing damages (Pujol-Luz *et al*., 2008; Hall & Huntington, 2010).

Lord and Steveson (1986) divided this scientific field into three main areas: urban, stored products and medicolegal.

Urban Entomology deals with arthropods that affect humans and their environment, usually involving infestation of houses and other facilities by termites and cockroaches (Pujol-Luz *et al*., 2008; Hall & Huntington, 2010).

Stored products Entomology is related with cases regarding infestation of stored products or food contamination. Some examples are parts of insects in cereals, caterpillar in cans of vegetables and fly maggots in sandwiches from fast-food restaurants (Hall & Huntington, 2010). Arthropods can cause direct damage to food resources and can also be accidentally ingested. This can lead to toxic or allergic reactions and other health disorders. They also may defecate or regurgitate fluids, contaminating food with potential pathogens, which will be ingested by humans or other animals (Durden & Mullen, 2002).

Lastly, Medicolegal Entomology, also known as Medicocriminal Entomology, involves the study of necrophagous insects that colonize human corps to help to resolve crimes, most often crimes of violence like homicide and suicide (Pujol-Luz *et al*., 2008).

Although less known, Wildlife Forensic Entomology is another area that should be taken into account. It is a more recent area proposed by Anderson (1999) that focuses on surveillance and protection of wild animals in captivity from neglect situations or physical abuse and on the resolution of questions about illegal hunting of protected species (Anderson, 1999; Hall & Huntington, 2010).

1.2. Medical Entomology

1.2.1. Initial *Postmortem* Changes

After death, a body undergoes different stages of decomposition. Decomposition process is responsible for returning the organic matter of a body to the ecosystem. This process consists on the metabolic transformation of organic matter into simple organic and inorganic compounds (Souza *et al*., 2008). During this process, the body undergoes several changes caused by cells autolysis (promoted by the chemical breakdown), tissue autolysis (due to the released enzymes) and external processes, where fungi, bacteria and arthropods are the main responsible (Campobasso *et al*., 2001; Amendt *et al*., 2004).

During the first 72 hours after death, the forensic pathologist has the function of analyse the interior and exterior of the cadaver to be able to estimate the time since death (PMI), as well as the manner and the cause of death, through the observation of body changes (Henβge & Madea, 2004; Schoenly *et al*., 2006). Several changes of the body may occur in the first 72 hours after dead.

One of the earliest changes of the body is the red discoloration of the skin (*livor mortis* or lividity). Upon death, circulation stops and blood begins to settle to the lowest parts of the body by the action of gravidity. Skin becomes red in those areas, but pale in the rest of the body (Goff, 2010). Body temperature also decreases (*algor mortis*) because body ceases the regulation of its internal temperature and begins to approximate ambient temperature. The size of the individual and the exposure to sunlight or heat are factors that can influence this stage. For a more accurate measurement, liver temperature is usually used (Amendt *et al*., 2004; Goff, 2010).

Another change is the stiffening of muscle fibbers due to the breakdown of glycogen and accumulation of lactic acid in muscles (*rigor mortis*) (Amendt *et al*., 2004). The duration of *rigor mortis* depends on the metabolic state of the body. If the individual has been involved in intense exercise immediately before death, *rigor mortis* will develop faster. It also depends on the body temperature: lower temperatures tend to accelerate the beginning of *rigor mortis* and to prolong its duration, while in warmer temperatures the opposite occurs (Henβge & Madea, 2004; Goff, 2010).

Skin slippage also occurs, resulting in the separation of epidermis from dermis, due to the production of hydrolytic enzymes. Consequently, skin can be easily removed as well as nails and hair. A large quantity of putrefaction gases causes a physical distortion of the cadaver. One of the formed gases is hydrogen sulphide (H_2S), which reacts with haemoglobin forming sulfhemoglobin, a green pigment that can be seen in blood vessels and in other areas of the body (Amendt *et al*., 2004; Goff, 2010).

All these changes occur within the first 72-96 hours after death. Beyond this period there is less medical information and no reliable estimation of PMI interval is possible (Amendt *et al*., 2004). Therefore, insects found on the corpse can provide an important source of information.

1.2.2. Relationship between Insects and Corpses

Arthropods are by far the largest and most important biological group in our planet (Benecke, 2001). Insects, the major group of arthropods, can travel considerable distances in search of food or a suitable place for laying their eggs (Castner, 2010). There are about one million species described, living in a wide variety of habitats, including crime scenes (vertebrate corpses provide an excellent food source) (Benecke, 2001; Amendt *et al*., 2004). However, not all insects found near a body are feeding on it or have oviposited there. Therefore, it is important to distinguish which insects can be used as forensic evidences (Benecke, 1998; Campobasso *et al*., 2001; Amendt *et al*., 2011).

Hundreds of arthropods species are attracted to corpses, mostly flies and beetles, but also mites, isopods, opiliones and nematodes. Some insects are attracted to a body shortly after death and soon begin their activities (Benecke, 2001; Goff, 2010). These species can feed, live and/or lay their eggs on the cadaver, depending on their biological preferences and on the state of decomposition of the body (Benecke, 2001).

Based on their food preferences and behaviour, carrion insects can be grouped into four ecological categories: necrophagous, predators or parasites, omnivorous, adventitious and accidental species.

Necrophagous species feed only on decomposing tissues of the body. They are the first to arrive and constitute the most important category to estimate the PMI (Wolff *et al*., 2001; Goff, 2010). Diptera species are dominant in this group, followed by Coleoptera (Campobasso *et al*., 2001).

Predators or parasites of necrophagous species feed on other insects (especially on Diptera larvae and pupae) or arthropods and are the second most important category. This group includes essentially Coleoptera and some schizophagous species that become predators during the last stages of their development (Amendt *et al*., 2004; Gennard, 2007; Amendt *et al*., 2011).

Omnivorous species such as wasps, ants and some beetles are not obligate necrophages and can feed both on the cadaver and associated fauna (Oliveira & Vasconcelos, 2010; Amendt *et al*., 2011). Large populations of these species may delay the rate of decomposition since they feed on necrophagous species (Campobasso *et al*., 2001).

It is possible to find other species, such as adventitious species, which use the corpse as an extension of their own habitat or as a refuge, and accidental species, which have no real relationship with the corpse (Goff, 2010; Oliveira & Vasconcelos, 2010).

1.2.3. Insects with Forensic Importance

Although the fauna associated with a corpse includes a wide variety of arthropod taxa such as Hymenoptera, Lepidoptera and Acari, Diptera (flies) and Coleoptera (beetles) are the two main insect orders that provide the majority of information in forensic investigations, probably due to their activity and frequency on human remains (Campobasso *et al*., 2001; Castner, 2010; Schilthuizen *et al*., 2011; Horenstein *et al*., 2012).

Diptera species are the first to colonize the corpse. They are attracted in large numbers by the smell of inner viscera and can locate an odour source with high precision and deposit their eggs on the body only a few minutes after death.

Oviposition first occurs at wounds or natural orifices of the body, namely eyes, nasal openings, mouth and ears (Campobasso *et al*., 2001; Amendt *et al*., 2011; Zhuang *et al*., 2011). Female insects do not oviposit in dehydrated tissues because their eggs and pupae need protection, good source of food and humidity for their development (Schroeder *et al*., 2002). Later, the larvae will hatch from the eggs and feed on death tissues (Amendt *et al*., 2011). The families Calliphoridae, Sarcophagidae and Muscidae (Diptera) are the most important colonizers of corpses (Kulshrestha & Satpathy, 2001).

The majority of research has focused on Diptera species, being Coleoptera much less popular, probably due to the fact that Diptera are the firsts to colonize a body, and therefore gives a more accurate estimate of PMI (Cai *et al*., 2011; Schilthuizen *et al*., 2011; Zhuang *et al*., 2011).

Although the order Coleoptera (Fig. 1) is much more diverse than Diptera, both taxonomically and ecologically, little knowledge is available about this group (Kulshrestha & Satpathy, 2001; Schilthuizen *et al*., 2011). The order Coleoptera is the largest group within all insects and it is believed that over 6 000 000 species have been described (Kulshrestha & Satpathy, 2001). Coleoptera species have their peak in number and diversity during advanced and dry stages of decomposition (Wolff *et al*., 2001; Almeida & Mise, 2009). In cases where mummification occurs, beetles contribute to the decay of a corpse, since they are detritivores. Therefore, when badly decomposed or skeletonised corpses are found, they are the main entomological evidence for the investigation (Kulshrestha & Satpathy, 2001; Shroeder *et al*., 2002; Zhuang *et al*., 2011).

Some of the most important families with forensic interest are Staphylinidae, Silphidae, Histeridae Dermestidae, Nitidulidae, Cleridae, Carabidae and Scarabaeidae (Kulshrestha & Satpathy, 2001; Almeida & Mise, 2009; Anton *et al*., 2011). Silphids are the firsts to arrive, followed by staphylinids and histerids. As decomposition progresses, dermestids, nitidulids and clerids are attracted to the corpse (Ozdemir & Sert, 2009; Schilthuizen *et al*., 2011).

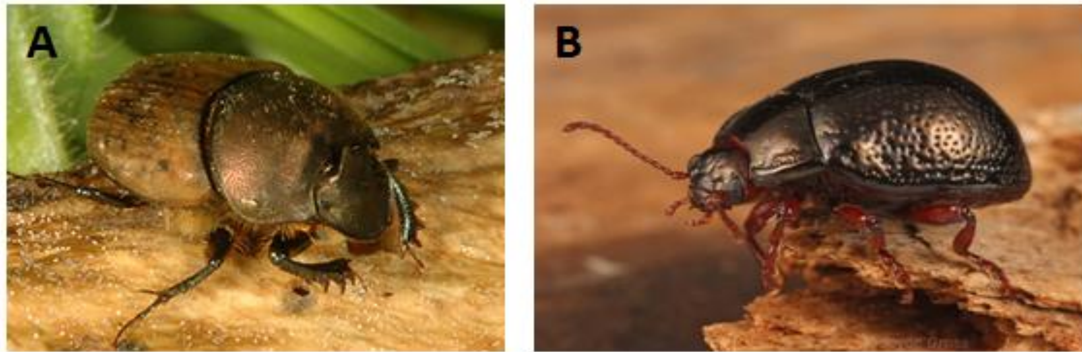

Fig. 1. Coleoptera species. **A:** *Ontophagus coenobita* (Photo: Angela Schwarz); **B:** *Chrysolina bankii* (Photo: Joyce Gross).

1.2.4. Role of Arthropods in Decomposition Process

Although being a continuous process, corpse decomposition can be divided into five stages to facilitate their study and analysis. These stages are related to the eight waves or arthropod colonization proposed by Mégnin (1984), since each stage is attractive to a different group of insects, creating a succession pattern (Campobasso *et al*., 2001; Gennard, 2007; Horenstein *et al*., 2012). The stages of *postmortem* changes are known as fresh, bloated, active decay, post decay and skeletozination (Ozdemir & Sert, 2009).

Fresh stage starts in the moment of death and ends at the first signs of bloating. There are no visible changes besides a slight muscular rigidity. Internal bacteria activity begins, producing odours that attract the first insects. Insect invasion begins on this stage, being Diptera species the first to arrive (Souza *et al*., 2008; Goff, 2010; Kreitlow, 2010).

Bloated stage is the easiest phase to distinguish. The abdominal region begins to swell due to the gases generated through the metabolism of anaerobic bacteria and eventually the whole body becomes inflated. It is in this stage that oviposition can be well noted, particularly in natural openings of the body. The odour of gases may be responsible for the increasing of the number of species attracted by the corpse during this stage (Gennard, 2007; Souza *et al*., 2008).

Active Decay stage is recognized by the rupture of skin and rapid mass loss, resulting from putrefactive bloating and maggot activity (Carter *et al*., 2007). Skin can

be easily removed from the body. In this stage, decomposition gases escape and strong odours of decomposition are present (Carter *et al*., 2007; Gennard, 2007; Goff, 2010). Diptera larvae are predominant, attracting predators as Coleoptera (Kreitlow, 2010).

In **Post Decay stage** most of flesh is removed from the corpse, remaining only skin, cartilage and bones. The biggest indicator of this stage is an increase of beetles (Coleoptera) and a reduction of flies (Diptera) on the body. Diptera larvae migrate to the soil to pupate (Carter *et al*., 2007; Gennard, 2007).

Skeletonization is reached when skin dries completely and only bones and hair remain. There are no obvious groups of insects during this stage. This final phase is not always reached, because it needs specific conditions of temperature and humidity to arise (Arnaldos *et al*., 2004; Goff, 2010; Kreitlow, 2010).

The duration of each stage and the rate of decay are dependent of intrinsic factors such as age, constitution of the body and cause of death, but also extrinsic factors like geographical region, temperature, seasonality, wind, rainfall and humidity. Temperature is the most important factor, being directly proportional to decomposition rate (Ozdemir & Sert, 2009; Horenstein *et al*., 2012).

It is well noticed that some species are directly attracted by the corpse while others are attracted by the presence of different insects that they use as a food resource (Anderson, 2010). Insects succession and their times of arrival are also affected by geographic and seasonal variations, being temperature a key factor, affecting their growth and development rate (Arnaldos *et al*., 2004; Oliveira-Costa & Melo-Patiu, 2004; Souza *et al*., 2008; Horenstein *et al*., 2012).

1.3. Applications

1.3.1. Estimating the *Postmortem* Interval

As mentioned above, beyond 72 hours after death it is only possible to estimate PMI through the study of insect species found in the corpse (Amendt *et al*., 2004; Amendt *et al*., 2011). In fact, the major contribution of forensic entomology is PMI estimation (Catts, 1992; Amendt *et al*., 2007).

An accurate PMI estimation is essential in any homicide investigation to identify the victim, reconstruct events and death circumstances, establish the credibility of statements made by witnesses, narrow the field of suspects or even to indentify the real criminal (Amendt *et al*., 2007; Wells & Lamotte, 2010; Aments *et al*., 2011). Even when the cause of death is natural, it is important to estimate the time of death for legal matters such as inheritance and insurance (Wells & Lamotte, 2010).

Estimating PMI involves the setting of minimal and maximal probable time interval between death and discovery of the corpse (Campobasso & Introna, 2001). The exact time of death cannot be determined, but it is possible to do an estimative through the period of insect activity. The estimation of PMI based on entomological evidences is also referred as the period of insect activity or the time of colonization (Kreitlow, 2010). Hence, when insect specimens are found associated with a body, samples should be collected and processed as evidence, like any other biological sample (Campobasso & Introna, 2001; Amendt *et al*., 2007).

There are essentially two main approaches to determine PMI, briefly described afterwards.

During short periods, PMI determination is based on the life cycle of insects associated with the corpse or, more precisely, on a direct age estimation of immature insects (Amendt *et al*., 2007; Gennard, 2007). This approach infers a minimum PMI, which corresponds to the time when insects first colonized the body and not the actual time of death (Amendt *et al*., 2011). The maximum PMI cannot be estimated by this approach because even though flies may be attracted immediately to fresh remains, it is impossible to know when the females laid their eggs (Catts, 1992; Wells & Lamotte, 2010). For measure insect development it is required that immature insects are still present on the body (Higley & Haskell, 2010).

After an accurate identification of the species found on a corpse, the next step is to associate this data to the temperatures to which the larvae were exposed, since insects are poikilotherms (cold-blooded) and use the environmental temperature as a source of heat to their development (Oliveira-Costa & Melo-Patiu, 2004; Gennard, 2007; Amendt *et al*., 2011).

The most sophisticated technique used to estimate the rate of development of insects is named "accumulated degrees hours" or "accumulated degree days", and it is based on the sum of temperature (in ºC) multiplied by time (Amendt *et al*., 2007). This model has a lower development threshold, i.e., the temperature value below which insect development stops (diapausa), and an upper development threshold, i.e., the temperature value above which the development slows down and can be lethal at some point. Between these two points there is a linear relationship between temperature and development rate (Fig. 2) (Amendt *et al*., 2007; Gennard, 2007).

It is required a specific number of accumulated degree hours to each stage complete their development (Amendt *et al*., 2011).

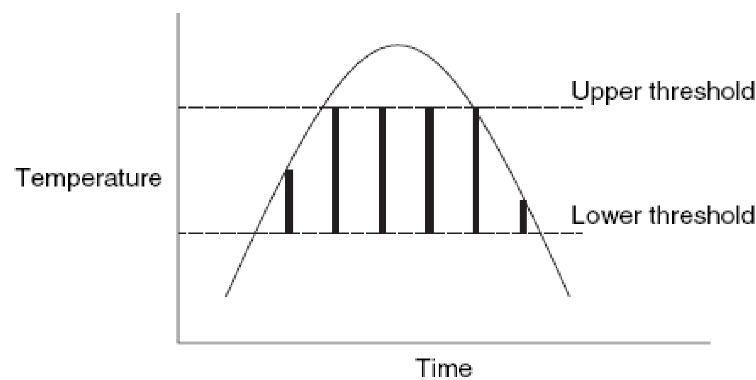

Fig. 2. Insect growth in relation to upper and lower thresholds (outlined area is the area of growth) (Gennard, 2007).

The presence of a large number of larvae at the same location is an important factor that must be taken into account. The metabolic heat generated by a mass of maggots can be significantly higher than the ambient temperature, which is going to affect their development and consequently PMI estimation (Amendt *et al*., 2007; Wells & Lamotte, 2010).

Diptera specimens are good indicators of PMI over short periods of time due to their fast development. However, when they are no longer developing on carrion, Coleoptera species are also useful (Catts, 1992).

In cases of long *postmortem* periods, the first colonizers of the body have already left, so PMI estimation must be based on the succession pattern of arthropods found on a cadaver, although the two methods should be complementary (Amendt *et al.*, 2007; Kreitlow, 2010).

The succession pattern on corpses can be divided into different waves that correspond to different stages of decay (Amendt *et al.*, 2004). Each of these stages is attractive to a different group of insects. This produces a faunal succession on corpses that can be used to determine PMI together with the knowledge of growth rates under specific environmental conditions (Benecke, 1998). This process can be used to estimate both minimum and maximum PMI. Succession data have been used to calculate a PMI up to 52 days and can be applied to a much longer time interval (Amendt *et al.*, 2004; Wells & Lamotte, 2010).

1.3.2. Forensic Implications of Myiasis

The study of insects can also give information about cases of neglect or abuse (Benecke & Lessig, 2001). Some insects are attracted to ammonia odour, resulting from urine or faecal contamination. Patients in hospitals, elderly people or babies, who have not been assisted in their personal hygiene, occasionally suffer of an infection called myiasis (Hall & Huntington, 2010; Gennard, 2007).

Myiasis (from the Greek myia = fly) can be defined as the invasion of an organ or tissue of live humans or vertebrate animals by dipterous larvae, which feed on living or necrotic tissues, like wounds, ulcers or natural openings of the body (Catts & Mullen, 2002; Service, 2004). It can be classified by the level of dependency of the parasite to the host. There are three main categories: accidental, obligatory and facultative. Facultative parasites, such as blowflies (Calliphoridae) and fleshflies (Sarcophagidae) are the main group involved in this type of cases (Amendt *et al.*, 2011). If a person was already infested when death occurred, insects present at that time could cause an overestimation of PMI (Wells & Lamotte, 2010). Entomological evidence recovered from the body can prove that negligence happened earlier than actual death (Benecke & Lessig, 2001).

1.3.3. Entomotoxicology: Insects as Toxicological Evidences

Entomotoxicology is a recent branch of Forensic Entomology that deals with the use of toxicological analysis of carrion feeding insects in order to identify drugs and toxins present in a death body (Introna *et al*., 2001; Carvalho, 2010). It can be applied in cases involving possible sudden death without obvious causes, criminal abuse by use of drugs, poisoning and suicide (Introna *et al*., 2001; Wolff *et al*., 2001; Gennard, 2007; Goff & Lord, 2010).

Insects, mainly larvae, which feed on a corpse, ingest and incorporate chemical metabolites of toxins into their own tissues (Wolff *et al*., 2001; Goff & Lord, 2010). It is possible to detect drugs and other chemical substances in insects and their chitinized remnants, recovered from badly decomposed bodies. These entomological techniques are important when the body is in an advanced stage of decomposition or when blood, urine or internal organs are not available or not suitable for analyses (Campobasso & Introna, 2001; Carvalho, 2010).

In recent years, some studies have been developed in order to understand the effects of drugs and toxins on carrion feeding insects (Introna *et al*., 2001; Goff & Lord, 2010). Studies showed that some drugs can delay insects colonization and also interfere with their rate of development and behaviour, which may affect an accurate PMI estimation. The decomposition process can be accelerated or decelerated depending on the substance and its concentration (Wells & Lamotte, 2010; Amendt *et al*., 2011).

1.3.4. Gene Expression Analysis

Gene expression studies, a more recent application of Forensic Entomology, can help to determine the age of developing individuals (Tarone *et al*., 2007; Zehner *et al*., 2009). There are development stages, such as eggs and pupae that exist over a period of time without any morphological changes. Nevertheless, metamorphosis is characterized by several intrinsic processes that are regulated by several genes.

Therefore, a differential gene expression could be used to determine the age, since gene expression pattern is specific for each development stage.

For this type of analysis is necessary to determine mRNA quantification of specific genes through RNA extraction, followed by a reverse transcription into cDNA and quantification of this cDNA through real-time PCR (Tarone *et al*., 2007; Zehner *et al*., 2009; Amendt *et al*., 2011). Although these studies are very expensive, they are extremely important to achieve an accurate PMI estimation, since a more precise age of insects is given.

1.3.5. Additional Applications

Besides these applications, it is possible to obtain additional information through the study of insects found in a crime scene. For example, if a suspect have been bitten by an insect, it is possible to link him to the crime scene through DNA analysis from the blood ingested by the insect (DiZinno *et al*., 2002; Pujol-Luz *et al*., 2008). In some cases, it may be possible to prove that a body was moved after death from one area to another and it may provide information about the location of death itself, if insects, which live in a restricted geographical region, are found on a corpse in a different area (Wolff *et al*., 2001; Gennard, 2007). Besides that, the presence of live insects or their larvae in an empty house may indicate that the body has been removed after death (Benecke, 1998; Campobasso & Introna, 2001). Forensic Entomology can, as well, give information about the time of decapitation, dismemberment, *postmortem* artefacts and specific sites of trauma on the body because insects will lay their eggs in these places (Campobasso & Introna, 2001).

Thus, a good knowledge of general entomology is essential for the accurate interpretation of insect evidence.

Knowing the distribution, biology and behaviour of the insect species found in a place where a body has been discovered can be determinant for many types of forensic investigations by providing information on when, where and how the crime occurred (Amendt *et al*., 2007).

There are many crucial considerations and limitations in making such estimations (Higley & Haskell, 2010). Colonization pattern is different depending whether the body was buried (it will influence the time required for insects to reach the body and the species composition on the corpse), left on the soil surface or in the water (faunal succession will be different from that seen on soil surface) (Anderson, 2010; Amendt *et al*., 2004). Factors such as physical barriers (for example, presence of clothing), chemical barriers, climate factors, season, weather and location can influence the type and species of insects present, their development and consequently PMI estimation (Catts, 1992; Anderson, 2010; Goff, 2010).

Since there are so many parameters that can affect species colonization, it is extremely important to develop a geographical database of species with forensic interest to apply in criminal investigations (Hall & Huntington, 2010; Cainé *et al*., 2009). Due to ethical and logistical constrains of using human cadavers for this type of research studies, usually animal carcasses are used (Oliveira & Vasconcelos, 2010). The majority of these studies use domestic pig (*Sus scrofa*) as a model due to its high homology with human in internal anatomy, fat distribution, size of chest cavity, skin, intestinal flora due to omnivorous diet; consequently, the decomposition rate is similar of a human body with the same weight (Campobasso & Introna, 2001; Wolff *et al*., 2001; Schoenly *et al*., 2006; Anton *et al*., 2011; Horenstein *et al*., 2012). There are also other studies that test bait traps to capture species with forensic interest since they are a cheaper and easier alternative (Leccese, 2004; Hwang & Turner, 2005; Brundage *et al*., 2011; Farinha *et al*., unpublished data).

In Portugal, forensic entomology studies concerning insect succession and species identification are also emerging (Gusmão, 2008; Marques, 2008; Prado e Castro & García, 2009, 2010; Prado e Castro *et al*., 2010a, 2010b, , 2010c, 2011, 2012) as well as studies employing DNA methodology to achieve species identification (Cainé *et al*., 2006, 2009; Oliveira *et al*., 2011; Ferreira *et al*., 2011).

1.4. DNA Analysis

1.4.1. Species Identification

An accurate identification of insect species is the first and most crucial step in Forensic Entomology. Some species can seem similar but they may have considerably different growth rates, behaviours and habitat preferences (Zehner *et al*., 2004; Wells & Stevens, 2008). A misidentification may result in the application of an incorrect development data and consequently, the estimated PMI would be wrong (Benecke & Wells, 2001; Harvey *et al*., 2003).

Insects identification is typically based on morphological characters. This methodology often requires entomological experts, with specialized taxonomic knowledge and appropriate morphological keys, which are often incomplete or only effective for a particular life stage or gender, when species identification is mainly based on male genitalia (Harvey *et al*., 2003; Zehner *et al*., 2004; Valentini *et al*., 2008; Raupach *et al*., 2010). The proper use of morphological keys may also be affected by geographic variations or by the loss of some morphological characters, like colour patterns, due to preservation process (Wells & Stevens, 2008; Buhay, 2009). Moreover, for most immature stages, identification based on morphological characters is difficult and sometimes impossible due to similarities among species (Cainé *et al*., 2006; Raupach *et al*., 2010). For this reason, experts often prefer rearing the immature stages up to eclosion of the adult fly, and then apply identification keys to adult specimens. However, this approach is time consuming, delaying a criminal investigation, and is not always possible because the larval evidence can be already dead (Harvey *et al*., 2003; Amendt *et al*., 2011; Mazzanti *et al*., 2010). Besides that, the use of keys often demands such a high level of expertise that misdiagnosis are common (Hebert *et al*., 2003).

In order to overcome these difficulties, a molecular approach has been proposed. Molecular identification is based on species specific nucleotide sequences of certain genes (Cainé *et al*., 2006; Raupach *et al*., 2010; Amendt *et al*., 2011).

The development of Polymerase Chain Reaction (PCR) made a great contribution to the study of specimens at the molecular level, since through this

method it is possible to amplify *in vitro* a particular genomic sequence, allowing small amounts of DNA analysis (Gennard, 2007). This is a very fast and simple method when compared with morphological procedures. This approach also has the advantage of being applicable to any life stage of insects and even to damaged specimens, like puparium remains (Cainé *et al*., 2006; Mazzanti *et al*., 2010).

1.4.2. DNA Barcoding

Herbert *et al*. (2003) proposed a molecular identification system known as DNA barcoding, which provides a fast and accurate identification by using short standardized gene regions (DNA barcodes) of the target specimen (Hebert & Gregory, 2005; Hajibabaei *et al*., 2007). These sequences can be viewed as genetic labels that are present in every cell and each species will have a unique DNA "barcode" (Hebert *et al*., 2003; Frézal & Lebois, 2008).

There is an international organization named Consortium for the Bacode Life (CBOL) that coordinates the DNA barcodes collection with the aim of building an international reference database for species molecular identification (Dawnay *et al*., 2007; Virgilio *et al*., 2010).

DNA barcoding has two main goals. The first is to use the barcode sequence to identify specimens (distinguish between known species) and the second one is to discover new species (species delimitation or description) (DeSalle *et al*., 2005; Frézal & Leblois, 2008).

After suitable laboratory procedures, obtained sequences are compared to reference sequences of known species, already deposited on an online database (Hebert & Gregory, 2005). For these purposes, two databases are commonly used: GenBank (www.ncbi.nlm.nih.gov/genbank/) and BOLD (www.barcodinglife.com/).

Hebert *et al.* (2003) proposed the use of a divergence threshold to delimit species, showing that the genetic distance between species almost always exceeds 3%. Another criterion is that the standard divergence threshold value should be ten times higher than the mean of intraspecific variation (Frézal & Leblois, 2008). However, the use of a divergence threshold to distinguish between intra and interspecific variations

should be done carefully, since these thresholds can suffer from statistical problems, compromising species identification (Valentini *et al*., 2008).

Phylogenetic reconstruction could also be used as a complementary analysis in species identification (Dawnay *et al*., 2007). Usually, DNA barcodes do not have sufficient phylogenetic signal to resolve evolutionary relationships, especially at deeper levels, so barcode-based trees should not be interpreted as phylogenetic trees (Hajibabaei *et al*., 2007). Nevertheless, they can be used to reveal the specimens closest relatives and narrow the choice of possible species (Benecke & Wells, 2001).

Since DNA barcoding is a relatively recent approach, there is a large number of undescribed species, which may constrain the representation of the overwhelming insect diversity on DNA barcode databases (Frézal & Leblois, 2008; Virgilio *et al*., 2010). Nevertheless, although DNA barcoding does not allow a complete taxonomic resolution, several studies demonstrate that the success in species identification exceeds 95% of the cases. Even when it fails it will reduce the option to a small number of congeneric taxa (Hebert & Gregory, 2005; Waugh, 2007).

Therefore, several studies showed that DNA barcoding is a reliable, cost effective and easy molecular identification tool for Diptera and Coleoptera species identification. This approach would be beneficial in the application of forensic insect evidences, since it allows an accurate identification in all life stages, which are often impossible to identify based on morphological characteristics (Nelson *et al*., 2007; Frézal & Leblois, 2008; Virgilio *et al*., 2010). DNA barcoding does not replace morphological taxonomists, but build alliances between molecular and morphological areas. In fact, DNA barcoding requires fully described voucher species to match the sequence of an unknown specimen to a reference sequence on a database (Hebert & Gregory, 2005; Dawnay *et al*., 2007; Waugh, 2007).

1.4.3. Nuclear vs Mitochondrial DNA

Molecular identification is based on the analysis of DNA sequences of the target specimen. Insects, as eukaryotic organisms, contain two distinct genomes, the nuclear and the mitochondrial genomes (Budowle *et al*., 2003).

The nucleus of eukaryotic cells contains a double-stranded molecule of DNA, called nuclear DNA (nDNA) (Gennard, 2007; Lodish *et al*., 2008). Eukaryotic organisms also have DNA in mitochondria, named mitochondrial DNA (mtDNA), which is separated and distinct from the nuclear genome of the cell (Boore, 1999, Gennard, 2007). Insect's mitochondria contain their own double-stranded circular genome, containing around 16,000 base pairs (bp) (Hu *et al*., 2009). Eukaryotic cells contain hundreds to thousands of mitochondria and each mitochondrion contains several copies of mtDNA. This property makes it easier to obtain mtDNA, especially in cases where the amount of DNA is very limited or degraded (Isenberg & Moore, 1999; Budowle *et al*., 2003). The number of mitochondria varies with the nature of the cell. The size of mtDNA also varies considerably between species. Despite this, the gene content of mtDNA is extremely conserved. With few exceptions, it encodes 37 genes, including 13 protein-coding subunits, 2 rRNAs of the mitochondrial ribosome and 22 tRNAs (Boore, 1999; Hwang & Kim, 1999).

Usually, the gene map of *Drosophila yakuba* mtDNA (Fig. 3) is used as a model, since gene arrangements of many insect species are identical to those (Boore, 1999).

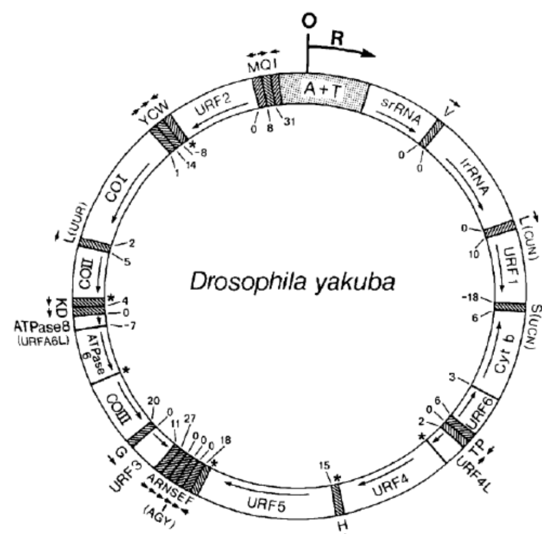

Fig. 3. Gene map of *Drosophila yakuba* (Diptera) mtDNA molecule (Clary & Wolstenholme, 1985).

Mitochondrial genome contains a large non-coding region, which is involved in initiation and regulation of mitochondrial transcription and replication, known as control region or D-loop. This region is the main responsible for the differences in size of mtDNA between species (Isenberg & Moore, 1999; Hwang & Kim, 1999; Hu *et al*., 2009).

Mitochondrial DNA is maternally inherited, through cytoplasm (Lodish *et al*., 2008). Thus, the mtDNA of siblings and all maternal relatives is identical. The haploid and monoclonal nature of mtDNA simplifies the interpretation of DNA sequencing results because only one sequence can be detected (Benecke & Wells, 2001; Budowle *et al*., 2003). On the other hand, does not allow the discrimination between individuals of the same maternal lineage (Isenberg & Moore, 1999).

The mutation rate of mtDNA is several times higher than the nuclear genes probably due the low fidelity of mtDNA polymerase, inefficient repair mechanisms or the presence of free radicals formed during the phosphorylation process. The most frequently varying part of the mtDNA is the control region that appears to evolve at rates 5-10 times higher than nuclear genes (Hwang & Kim, 1999; Budowle *et al*., 2003; Witas & Zawicki, 2004).

The characteristics described above have made mtDNA a valuable tool in insect genomic studies. These includes a small genome with simple structure and organization, easy to isolate due their high copy number, lack of introns, haploidy, maternal inheritance, lack of recombination (which means that all differences result only from fixed changes), high mutational rates in different regions of the molecule and the availability of robust primers to amplify a target region. These features make it easy to obtain mtDNA sequences from unstudied insect species (Zhang & Hewitt, 1996; Branicki *et al*., 2003; Witas & Zawacki, 2004; Dawnay *et al*., 2007; Wells & Stevens, 2008).

1.4.4. Molecular Markers

Several molecular markers may be used for identification purposes, such as nuclear and mitochondrial DNA sequences.

To select a molecular marker it is important to take into account certain factors: the chosen segment of DNA must be relatively easy to isolate, easy to amplify, should have few insertions and deletions to facilitate sequence alignment and must have a mutation rate rapid enough to allow interspecific variation, allowing species discrimination, but sufficiently slow to minimize intraspecific variation (Blaxter, 2004; Hajibabaei *et al*., 2007; Waugh, 2007).

1.4.4.1. *Cytochrome* c *oxidase I*

Cytochrome *c* oxidase is a large transmembrane complex of proteins found in mitochondrion that acts as an oxidizing enzyme in the respiratory chain, which receives electrons from cytochrome complex and transfers them to oxygen molecules (Gennard, 2007; Waugh, 2007). This protein complex comprises four subunits encoded by the nuclear genome and three mitochondrially encoded, known as subunits I, II and III (Frati *et al*., 1997). Subunit I is the largest of the three mitochondrially encoded cytochrome oxidase subunits and was originally chosen to be used in genetic analysis due its great resolving-power for species discrimination, being the most conserved among the three subunits (Lunt *et al*., 1996; Hwang & Kim, 1999; Gennard, 2007; Frézal & Leblois, 2008).

DNA barcode itself consists of a 658 bp region of the cytochrome *c* oxidase I (COI), corresponding to nucleotide positions 1490 to 2198 of the *Drosophila yakuba* mitochondrial genome (Nelson *et al*., 2008). This mitochondrial gene has some characteristics which make it particularly suitable to be used as molecular marker. The universal primers for this gene are very robust, which enables the routine amplification of this specific segment (Hebert *et al*., 2003). Its size and structure are highly conserved across all aerobiotic organisms. On the other hand, the evolution of this gene is rapid enough to allow the discrimination of species and insertions/ deletions are rare (Lunt *et al*., 1996; Waugh, 2007).

Several studies demonstrate that COI gene has successfully been used for species identification of a large range of animal taxa (Harvey *et al*., 2003; Danway *et al*., 2007; Hajibabaei *et al*., 2007; Frézal & Lebois, 2008; Raupach *et al*., 2010).

However, a lack of reference sequences in online databases prevents a match for a large number of species, limiting the use of COI. In addition, COI based identifications sometimes fail to distinguish closely related species, due to shared barcode sequence (Nelson *et al*., 2007). For example, this gene does not allow the distinction between sister species of the genus *Lucilia* (Raupach *et al*., 2010). In these cases, additional analysis of other COI segments, or even other gene, would be required to provide extra information in cases of uncertain species identification (Raupach *et al*., 2010; Oliveira *et al*., 2011). The idea of a multi-locus DNA barcoding approach has been increasingly applied in order to enlarge the power of sequence assignments (DeSalle *et al*., 2005; Frézal & Lebois, 2008).

The occurrence of non-funcional copies of mitochondrial sequences (nuclear mitochondrial pseudogenes – NUMTs) is an important factor that should be taken into account when COI gene is used as a molecular marker. NUMTs are copies of mitochondrial DNA sequences that have been translocated into the nuclear genome and could lead to some ambiguity in species identification since they could mimic mitochondrial copies of COI gene (Zhang & Hewitt, 1996; Frèzal & Leblois, 2008; Song *et al*., 2008). When NUMTs have been also amplified, it is possible to see more than one band in PCR amplification or double peaks in chromatograms. Due to the random nature of nucleotide substitutions, NUMTs can also be detected when unexpected insertions/deletions, frameshifts or stop codons occur (Zhang & Hewitt, 1996; Grosso *et al*., 2006). A fine phylogenetic analysis can also be useful in NUMTs recognition, since their molecular evolution is much slower than the real mtDNA copy (Grosso *et al*., 2006).

1.4.4.2. *Cytochrome b*

Another region of mtDNA that has been used as a molecular marker to species identification and to establish phylogenetic links is a fragment of the gene cytochrome b (Cytb) (Branicki *et al*., 2003; Prusak & Grzybowski, 2004; Hajibabaei *et al*., 2007).

Cytb is a transmembrane protein of the mitochondrial oxidative phosphorylation system responsible for the electron transport and thus the production of ATP, being only encoded by the mitochondrial genome. It is the central redox catalytic subunit of the ubiquinol cytochrome *c* reductase, an enzyme that is present in the respiratory chain of mitochondria. All eukaryotic organisms require this class of redox enzymes, and consequently Cytb, for energy conservation (Esposito *et al*., 1993; Prusak & Grzybowski, 2004).

This gene has been applied specially in studies with vertebrates, where the analysis proved to be a very sensitive, reliability and powerful technique, using primers given by Parson and his collaborators (Parson *et al*., 2000; Branicki *et al*., 2003). However, the use of Cytb gene as molecular marker has been applied to several species of animals, including Coleoptera (Barraclough *et al*., 1999; Kergoat *et al*., 2004; Balke *et al*., 2005; Pons, 2006; Sembène, 2006; Sembène *et al*., 2010; Ndiaye *et al*., 2011).

The typical variation on this region of mtDNA can be insufficient to enable unequivocal differentiation of species belonging to the same genus (Branicki *et al*., 2003). Although a few regions of Cytb sequences tend to be more conserved, other regions exhibit considerable variability and thus are valuable for determining the phylogenetic distance among species (Esposito *et al*., 1993).

1.5. Thesis Aims

In Portugal, the interest in Medicolegal Entomology is increasing, being a large majority of the studies focused on insect composition and succession patterns (Gusmão, 2008; Marques, 2008; Prado e Castro & García, 2009, 2010; Prado e Castro *et al*., 2010a, 2010b, 2010c, 2011, 2012). However, these studies are based on the morphological identification of insects, which is time consuming and often inconclusive. In order to overcome these limitations, molecular approaches have emerged, but previous studies of insect species with forensic interest in Portugal only focused on Diptera identification (Cainé, 2006, 2009; Rolo, 2010; Ferreira, 2011).

The present study represents the first molecular approach to identify Coleoptera species with forensic interest, in Portugal.

Therefore, the main goals of this study are:

1. Molecular identification of insect species belonging to the Order Coleoptera using two mitochondrial markers (COI and Cytb);
2. Test the suitability of a fragment of Cytb gene for Coleoptera identification;
3. Compare the effectiveness of COI and Cytb genes for Coleoptera DNA barcoding;
4. Contribute to increase the National Database of the Forensic Entomology Project.

Material and Methods

II. Material and Methods

2.1. Sampling

Coleoptera specimens used in this study were collected in Aveiro, Portugal, using traps baited with pork liver (300-500 grams) and then stored in 96% ethanol. Morphological identification was performed using dichotomous keys (see References). Once identified, each specimen was separated individually, labelled, stored in absolute ethanol and maintained at 4 ºC.

In order to allow further confirmation of identification, photographs were taken, since in the case of small specimens, whole individual should be used in DNA extraction.

2.2. DNA Extraction, Amplification and Sequencing

DNA was extracted from 35 adult specimens using DNeasy® Blood and Tissue (Qiagen, Germany) extraction kit, following the manufacturer's protocol, with an overnight incubation step. For large specimens, DNA was obtained by removing 2-3 legs from the same side of the body and the remaining specimen was preserved for morphological re-examination, if necessary. For small specimens the whole individual was used. For maximum DNA yield, two independent elutions were performed, using 50 µL of Elution Buffer for the first one and 30 µL for the second. DNA stock was stored at 4 ºC.

After DNA extraction, Polymerase Chain Reactions (PCRs) were performed in order to amplify the two genomic regions of interest, COI and Cytb. The specific primers used to amplify each genomic region are summarized in Table 1.

Table 1. Genes and primers used in this study.

Genes	Primers	Direction	Sequences	References
COI	LCO1490	F	5’ GGT CAA CAA ATC ATA AAG ATA TTG G 3’	Hebert *et al.*, 2003
	HCO2198	R	5’ TAA ACT TCA GGG TGA CCA AAA AAT CA 3’	
Cytb	CB1	F	5’ TAT GTA CTA CCA TGA GGA CAA ATA TC 3’	Sembène, 2006
	CB2	R	5’ ATT ACA CCT CCT AAT TTA TTA GGA AT 3’	

Table 2 summarizes PCR reagents and their final concentration used in the amplification process, for a final volume of 25µl.

Table 2. Final concentration of reagents used in PCR amplification for COI and Cytb genes.

	Genes	
Reagents	**COI**	**Cytb**
Buffer	1x	1x
dNTPs (mM)	0,1	0,1
MgCl2 (mM)	2	1,5
Primer F (pmol/µl)	0,2	0,18
Primer R (pmol/µl)	0,2	0,18
BSA (µg/µl)	0,16	-
Taq Polymerase (U)	0,25	0,3
DNA (µl)	3	2

In order to confirm the absence of contamination, a negative control was added in each PCR reaction. A positive control was also included to guarantee that PCR reactions were achieved.

COI amplification consisted in an initial denaturation step at 94 ºC for 1 minute, followed by 5 cycles of 94 ºC for 30 seconds, 45 ºC for 1 minute, 72 ºC for 1 minute, then 35 cycles of 94 ºC for 1 minute, 50 ºC for 1 minute and 30 seconds 72 ºC for 1 minute, and a final extension step of 72 ºC for 5 minutes. For Cytb amplification there was performed an initial denaturation at 92 ºC for 3 minutes followed by 35 cycles of 92 ºC for 1 minute, 59 ºC for 1 minute and 30 seconds, 72 ºC for 1 minute and a final extension step at 72 ºC for 10 minutes.

PCR fragments were detected by 1% agarose gel electrophoresis, stained with RedSafe (iNtRON Biotechnology, Korea), along with a molecular weight marker HyperLadder^{TM} IV (Bioline, UK), at 100V for 20 minutes, and then visualized under ultraviolet light in a trans-illuminator and analysed with appropriate software.

The amplified products were stored at 4 ºC until purification process.

PCR products were purified using the commercial SureClean kit (Bioline, UK) according to manufacturer's instructions. Sequencing reactions were carried out by a specialized company (Macrogen Inc., Amsterdam/Korea). DNA fragments were sequenced in both directions using the same primers used in amplification step.

2.3. Data Analysis

Obtained sequences were analyzed and edited in Sequencer® v4.0.5 (Gene Codes Corporation, USA) and BioEdit Sequence Alignment Editor v7.0.5.3 (Hall, 1999). After all corrections, obtained sequences were aligned with ClustalX v2.0.12 (Thompson *et al*., 1997).

In order to confirm species identification, obtained sequences were compared with reference sequences deposited in the online databases GenBank (produced and maintained by the National Center for Biotechnology Information - NCBI) and BOLD (Barcode of Life Data Systems).

With the obtained sequences three matrices were prepared: one for each gene individually and one matrix with both genes concatenated. Matrices were converted into a .NEXUS format using the software Concatenator v1.1.0 (Pina-Martins & Paulo, 2008) in order to allow subsequent analysis. Matrices concatenation was also performed using the software Concatenator v1.1.0.

With the purpose of studying intra and interspecifc divergence of COI, uncorrected *p*-distances were determined in PAUP* v4.0.d99 (Swofford, 2002).

Sequences available in GenBank from *Sarcophaga autralis* and *Lucilia sericata* (two Diptera species) were selected to be used as outgroup in phylogenetic analyses. Table 3 shows the accession numbers for each gene and species.

Table 3. Code and accession numbers of the specimens used as outgroup.

Gene	Code	Species	Accession number
COI	Saust	*Sarcophaga australis*	HQ978732
	Lseri	*Lucilia sericata*	GQ254431
Cytb	Saust	*Sarcophaga australis*	DQ400489
	Lseri	*Lucilia sericata*	GQ409427

Phylogenetic analyses were performed in MEGA v5.05 (Tamura *et al*., 2011) using Maximum Likelihood (ML), Neighbour-Joining (NJ) and Maximum Parsimony (MP) methods for all matrices. Phylogenetic analysis by ML method was based on the Tamura-Nei model (Tamura & Nei, 1993). For NJ method, the evolutionary distances were computed using *p*-distance method (Nei & Kumar, 2000). MP analysis was obtained using the Close-Neighbour-Interchange (CNI) algorithm (Nei & Kumer, 2000). In all methods, 1,000 bootstrap replicates were used to calculate support for nodes.

Results and Discussion

III. Results and Discussion

3.1. Laboratorial Constraints

3.1.1. DNA Extraction

All specimens were collected in Aveiro, Portugal. Initially, it was also intended to analyse Coleoptera specimens that were collected in two previous studies (Gusmão, 2008; Marques, 2008), since they have not been genetically analysed. However, some laboratorial constrains occurred during DNA extraction preventing their use.

At first, commercial E.Z.N.A.® Insect DNA Isolation Kit (Omega Bio-Tek, USA) was used to perform DNA extraction, since it had been used in previous studies for Diptera extraction (Rolo, 2010; Ferreira, 2011). After the PCR procedure, since no amplification bands were obtained, with the exception of positive control (Diptera), DNA from these samples was quantified in order to determine its quantity and quality. Results showed that the amount of DNA present in each sample was insufficient to perform PCR amplification.

Therefore, a new commercial kit was tested to perform DNA extraction. The commercial DNeasy ® Blood and Tissue Kit (Qiagen, Germany) was chosen, since it is commonly used for DNA extraction of Coleoptera specimens (Caterino & Tishechkin, 2006; Kölsch & Pedersen, 2008; Seago *et al.*, 2011; Schilthuizen *et al.*, 2011). However, results were identical to the ones obtained with the previous commercial kit.

Later, it was noticed that this fact was related with an inappropriate form of preserving Coleoptera specimens in traps. In the referred previous studies, Coleoptera specimens were collected using *pitfall* traps containing ethylene glycol as preserving liquid (Marques, 2008; Gusmão, 2008). This liquid is used because it has low toxicity, no odour, evaporates slowly, kill the animals very quickly and conserve the specimens during approximately two weeks. Nevertheless, this particular liquid, is the same liquid that is used in cars to avoid water to freeze, and contains several substances that may damage DNA, leading to problems in DNA extraction and PCR amplification.

As a result, those stored specimens could not be analysed in this study. It is important to refer that when insects were collected, in 2008, it was not intended to perform molecular analyses. Therefore, new specimens were collected without the preserving liquid, using only water and one drop of detergent to decrease the surface tension of water to prevent animals from escaping. In this way, DNA extraction was no longer compromised.

3.1.2. PCR Amplification

Initially, Cytb amplification conditions included the primer pair proposed by Parson *et al*. (2000). However, when PCR products were run in agarose gel electrophoresis, there were no amplification bands, but only some smear. Since it was not possible to achieve a correct amplification of the target DNA fragment, a new primer pair, specific for Coleoptera, was chosen from a previous work (Sembène, 2006). This new primer pair allowed Cytb amplification without major problems.

Universal primers used for COI amplification are very robust, allowing that the majority of COI amplifications occurred without major problems. Nevertheless, it was not possible to amplify this specific segment for three Coleoptera specimens, which were successfully amplified for Cytb gene. Several PCR reactions were tested, using different concentrations of reagents and different PCR thermal cycles, without any successful result. One possible explanation for this may be the fact that the target sequence has suffered point mutations, compromising the annealing of the primers to that region and consequently COI amplification.

Amplification of another region of COI gene was also tested in order to increase the size of this segment and consequently raise the amount of genetic information. Primers used for amplification were F1 and R2 proposed by Malgorn & Coquoz (1999). However, unspecific DNA amplification was noticed. Several optimization reactions were then performed, but no good results were achieved and this region was not used in this study. This result may be related with the fact that primers used for amplification of this fragment are not universal, and they were originally designed for Diptera species.

Besides amplification problems, other specimens could not be used in this study due to sequencing problems. Table 8 (Appendix I) shows all the results for DNA extraction and amplification for each specimen and gene.

3.2. Coleoptera Composition

A total of 35 adult Coleoptera specimens, belonging to 8 families were studied. Due to the difficulties to achieve a correct identification based on morphologic criteria, it was not possible to identify all specimens to species level, and therefore, some specimens were grouped in morphospecies, i.e., morphologically identical specimens.

Table 4 represents all the specimens analysed in this study.

Table 4. Specimens collected in this study: family, genus, species; number of specimens of each species - N (Total); and number of analysed specimens – N (Analysed).

Family	Genus	Species	N (Total)	N (Analysed)
Staphylinidae	Creophilus	*Creophilus maxillosus*	16	3
	Ocypus	*Ocypus aethiops*	4	4
	?	Morphospecies G	1	1
	?	Morphospecies H	1	1
Silphidae	?	Morphospecies A	1	1
Histeridae	Saprinus	*Saprinus semistriatus*	3	3
Chrysomelidae	Chrysolina	*Chrysolina bankii*	7	7
		Chrysolina diluta	3	3
	?	Morphospecies F	5	5
	?	Morphospecies B	1	1
Curculionidae	?	Morphospecies I	1	1
Nitidulidae	?	Morphospecies C	1	1
Scarabaeidae	Onthophagus	*Onthophagus coenobita*	1	1
Carabidae	Licinus	*Licinus punctatulus granulatus*	1	1
	Notiophilus	*Notiophilus biguttatus*	1	1
	Harpalus	Morphospecies D	1	1

The majority of collected species were common with previous studies (Miralbes, 2002; Arnaldos *et al*., 2004; Matuszewski *et al*., 2008; Almeida & Mise, 2009; Ozdemir & Sert, 2009; Matuszewski *et al*., 2010; Anton *et al*., 2011; Prado e Castro, 2011; Horenstein *et al*., 2012). Concerning the abundance, Staphylinidae is the most abundant family. *Creophilus maxillosus* (Linnaeus, 1758) is the most abundant species within this family (Table 4), being present in almost every study of insect composition in carrion (Matuszewski *et al*., 2008; Almeida & Mise, 2009; Ozdemir & Sert, 2009; Matuszewski *et al*., 2010; Anton *et al*., 2011; Horenstein *et al*., 2012). Both *Creophilus maxillosus* and *Ocypus aethiops* (Waltl, 1835) appeared in a previous study concerning carrion beetles of forensic interest in Portugal (Prado e Castro, 2011). *Saprinus semistriatus* (Scriba, 1790) and *Onthophagus coenobita* (Herbst, 1783) were also found in studies of insect succession conducted by Matuszewski and his collaborators in Central Europe (Matuszewski *et al*., 2008, 2010). Although *Licinus punctatulus granulatus* (Dejean, 1826) and *Notiophilus biguttatus* (Fabricius, 1779) were not found in current literature, both species belong to the family Carabidae, which is known for having forensic interest (Arnaldos *et al*., 2004; Almeida & Mise, 2009; Anton *et al*., 2011; Prado e Castro, 2011).

All the families found are well known for being related with body decomposition process, with the exception of Chrysomelidae and Curculionidae. Anton *et al*. (2011) conducted a study on a pig carrion, in Thuringia (Germany), where they referred families Chrysomelidae and Curculionidae as accidental. However, several species belonging to the family Chrysomelidae including *Chrysolina bankii* (Fabricius, 1775) and *Chrysolina diluta* (Germar, 1824) and one specimen belonging to Curculionidae were collected in this experimental study. Besides our study, different species belonging to these same families were also found in Portugal (Prado e Castro, 2011) and Spain (Miralbes, 2002) studies. Therefore, it is important to carry out further studies, concerning these two families, since they could have some forensic role that is still unknown.

It is important to refer that the comparison of our collected species with previous studies must be done carefully, since beetle colonization may vary according to geographical areas (Kulshestha & Satpathy, 2001).

Moreover, in this study, specimens were collected using bait traps, which are a cheaper and easier alternative to capture insects, allowing us to overcome ethical and logistical constrains of using animal carcasses (Leccese, 2004; Hwang & Turner, 2005; Brundage *et al*., 2011). However, the use of baits also has some limitations since it does not allow studying the different stages of carrion decomposition, and consequently the insect succession pattern associated with each stage. Additionally, since decomposition time is too short, the abundance and richness of Coleoptera species is much smaller. Another fact that must be taken into account is that this study occurred only in autumn months and Prado e Castro (2011) noticed that Coleoptera communities changed greatly in each season.

Further studies should be made in order to increase the abundance and species richness and consequently allow deeper analysis.

3.3. Molecular Identification

Of the 35 Coleoptera specimens, 30 sequences with 658 bp for COI gene and 31 sequences with 433 bp for Cytb gene were obtained (Appendix I, Table 8). The alignment was straightforward and no insertions or deletions have been found. Well defined peaks in chromatograms and the absence of stop codons indicated that amplification of pseudogenes did not occur. Nevertheless, when Cytb sequences were analyzed in BioEdit, it was verified that 17 of the 31 obtained sequences were identical, even among individuals from different families. This result leads us to believe that this mitochondrial fragment is not a good molecular marker to be used for DNA barcoding of these species, since Cytb appears to be highly conserved among different species, preventing their identification and differentiation.

Obtained sequences were compared with reference sequences of known species contained in online databases (GenBank and BOLD). These databases allow species identification when our sequence matches the available reference sequence with an identity value greater than 97%, given that intraspecific genetic distance should not exceed 3% (Hebert *et al*., 2003).

For Cytb, it was only used BLASTn tool of GenBank database, since BOLD does not have records for this gene. None specimen was successfully identified at species level, given that identity values were all very low (83 - 88%) (see Appendix II, Table 9).

Regarding COI gene, sequences were blasted using not only BLASTn (GenBank) but also BOLD-IDS (BOLD) tool. More species were identified at species level using COI than Cytb gene. This can be easily explained since COI is the most widely used molecular marker for DNA barcoding. Thus, it is predictable that more COI sequences are available in these databases. BOLD database showed higher values of specimen similarity than GenBank, where, in the majority of cases, identity values were too low to permit identification at species level (Appendix II, Table 10).

According to BOLD database, both *Creophilus maxillosus* (Cmax1, Cmax2 and Cmax3) and *Notiophillus biguttatus* (Cnot) showed a high identity value (above 99%), allowing the corroboration of morphological identification.

Nevertheless, morphological and molecular identification was not always congruent. For example, OcSp1 and OcSp2 specimens, morphologically identified as *Ocypus aethiops,* matched with a reference sequence identified as *Ocypus opthalmicus* with identity values of 97.07% and 96.79%, respectively. It is important to refer that there are no reference sequences identified as *Ocypus aethiops* in these databases, and consequently, the morphological identification may be correct. Another case occurred with Csp7 specimen, identified as *Crysolina bankii*, that matches with a reference sequence identified as *Apteropeda orbiculata*.

In some cases, our sequence matched, with high identity values, to others reference sequences identified only at order, family or genus level. For instance, C*hrysolina bankii* (Cban1, Cban2, Cban3, Csp1 and Csp5) and *Chrysolina diluta* (Cdil1, Cdil2 and Cdil 3) matched with reference sequences identified to family level (Chrysomelidae).

Regarding Char specimen, morphological identification to species level was not possible to achieve. However, molecular identification allows the confirmation of identification at genus level (*Harpalus* sp.), with high identity values (97 – 98.62%), in both databases.

Other morphospecies also showed high identity values. Nevertheless, since morphological identification was not achieved in these cases, it was not possible to confirm molecular identification.

In order to understand if the match between our sequences and reference sequences did not occur because these species do not exist in these databases, a search by species name was made. In addition to *Creophilus maxillosus* and *Notiophillus biguttatus,* it was possible to find some records in GenBank database for *Chrysolina diluta*, *Chrysolina bankii*, *Onthophagus coenobita* and *Licinus punctatulus granulatus*. One possible reason for the match between these species and our species did not occur may be due to the fact that a different region of COI gene has been amplified for those species. Another explanation could be that the identification of our specimen or the reference specimen may be wrong. Moreover, it has already been noticed that geographic distance between species could enhance genetic differences (Ball *et al*., 2005). However, even taking into account geographical differences, genetic distances should not be so high, preventing the match. Nonetheless, further studies are needed to evaluate the level of COI diversity among species with large geographic distributions. In fact, the goal of DNA barcode databases is to include sequences of specimens from a wide geographic range to cover COI diversity of each species in the world.

It was also noticed, that even though Coleoptera is the largest group within all insects (Kulshrestha & Satpathy, 2001), little knowledge is available about this group, leading to a lack of reference sequences for a great number of species in online databases. Besides that, due to difficulties in morphological identification, many of the reference sequences are only identified to family or genus level, preventing the identification of some specimens to species level.

Obtained sequences will be submitted both to Genbank and BOLD Systems online databases.

3.4. Intra and Interspecific variation

In order to study intra and interspecific divergence, a distance matrix was calculated in PAUP*, using uncorrected *p*-distances. Since several of the obtained sequences for Cytb were identical, even between different families, this analysis was only performed for COI gene. It is important to remember that according to Hebert *et al*. (2003), intraspecific variation should not exceed 3%, while interspecific variation needs to exceed that threshold value to allow species differentiation. Another criterion is related with interspecific variation, whose value should be ten times higher than the mean value of intraspecific variation (Frézal & Leblois, 2008).

The analysis was performed using all the specimens identified to species level for COI gene. With the purpose of increasing the number of specimens, an online search by species name was made. One barcode sequence, belonging to a specimen identified as *Notiophilus biguttatus* (accession number: FN868620), was found in GenBank and added to the original matrix. Regarding BOLD database, no public records were available.

Percentages of nucleotide divergence values and standard deviation for uncorrected *p*-distances are presented in Table 5. In some cases, only one specimen of each species (*Saprinus semistriatus*, *Onthophagus coenobita* and *Licinus punctatulus granulatus*) was present in the matrix. In these cases, it was not possible to determine intraspecific variation.

In this analysis, intraspecific divergence values for *Creophilus maxillosus*, *Chrysolina diluta* and *Notiophilus biguttatus* were below the 3% threshold. However, it was possible to verify that intraspecific variation for *Ocypus aethiops* and *Chrysolina bankii* were much higher than expected (24.417 ± 12.057 and 13.475 ± 2.034, respectively). In fact, intraspecific divergence value concerning *Ocypus aethiops* was, in some cases, higher than interspecific values. With such high values it is almost impossible that all these specimens belong to the same species.

Table 5. Summary of intra and interspecific percentages of uncorrected p-distances obtained for COI data of eight species. Standard deviation values are shown in brackets.

	Creophilus maxillosus	*Ocypus aethiops*	*Saprinus semistriatus*	*Chrysolina bankii*	*Chrysolina diluta*	*Onthophagus coenobita*	*Licinus punctatulus granulatus*	*Notiophilus biguttatus*
Creophilus maxillosus	0.405 (±0.134)	23.387 (±3.019)	18.997 (±0.088)	21.935 (±0.089)	19.706 (±0.057)	15.350 (±0.088)	16.413 (±0.088)	18.921 (±0.065)
Ocypus aethiops		24.417 (±12.057)	26.950 (±6.282)	27.845 (±1.818)	25.498 (±2.834)	24.063 (±5.446)	25.127 (±5.902)	25.557 (±3.525)
Saprinus semistriatus			-	21.277 (±0.549)	20.618 (±0.051)	19.301 (±0.000)	21.429 (±0.000)	19.529 (±0.076)
Chrysolina bankii				13.475 (±2.034)	15.746 (±0.377)	19.985 (±0.052)	21.884 (±0.321)	20.846 (±0.346)
Chrysolina diluta					0.912 (±0.456)	17.427 (±0.203)	20.263 (±0.101)	17.452 (±0.073)
Onthophagus coenobita						-	17.325 (±0.000)	18.389 (±0.000)
Licinus punctatulus granulatus							-	15.122 (±0.076)
Notiophilus biguttatus								0.152 (±0.000)

One possible reason for this discrepancy may be related with the fact that identification of Coleoptera specimens based on morphologic characters is extremely difficult. This can lead to a misidentification, and consequently to some incongruence between morphological and molecular data. In order to confirm morphological identification, some of the most problematic specimens were sent to the group of the entomological expert Dr. Stuart Hine, of the Natural History Museum of London. Unfortunately, it was not possible yet to include these data in the analyses, due to the difficulty in identifying those samples.

Therefore, it was necessary to look back at the original matrix and try to understand within each specimens were the greatest differences and remove these individuals from analysis. A new matrix was made, in which specimens OcAet (identified as *Ocypus aethiops*) and Csp7 (identified as *Chrysolina bankii*) were excluded from the analysis, for presenting a great number of genetic differences.

Table 6 shows the average of nucleotide divergence percentage and standard deviation for uncorrected *p*-distances within and between species.

Table 6. Summary of intra and interspecific percentages of uncorrected *p*-distances obtained for eight species of the reanalyzed COI dataset. Standard deviation values are shown in brackets.

	Creophilus maxillosus	*Ocypus aethiops*	*Saprinus semistriatus*	*Chrysolina bankii*	*Chrysolina diluta*	*Onthophagus coenobita*	*Licinus punctatulus granulatus*	*Notiophilus biguttatus*
Creophilus maxillosus	0.405 (±0.134)	17.351 (±0.047)	18.997 (±0.088)	22.047 (±0.079)	19.706 (±0.057)	15.350 (±0.088)	16.413 (±0.088)	19.921 (±0.065)
Ocypus aethiops		0.304 (±0.000)	20.669 (±0.152)	22.933 (±0.035)	19.833 (±0.034)	18.617 (±0.076)	19.225 (±0.076)	19.985 (±0.098)
Saprinus semistriatus			-	21.003 (±0.583)	20.618 (±0.051)	19.301 (±0.000)	21.429 (±0.000)	19.529 (±0.076)
Chrysolina bankii				9.878 (±2.305)	15.065 (±0.095)	20.000 (±0.061)	21.854 (±0.392)	20.653 (±0.389)
Chrysolina diluta					0.912 (±0.456)	17.427 (±0.203)	20.263 (±0.101)	17.452 (±0.073)
Onthophagus coenobita						-	17.325 (±0.000)	18.389 (±0.000)
Licinus punctatulus granulatus							-	15.122 (±0.076)
Notiophilus biguttatus								0.152 (±0.000)

With this new dataset, it was possible to verify that intraspecific divergence value for *Ocypus aethiops* do not exceed the 3% threshold (0.304 ± 0.000), which is now in accordance with the value proposed in Hebert *et al*. (2003). Therefore, it was possible to confirm that OcAet was the specimen responsible for the high intraspecific value. The exclusion of this specimen also allowed the decrease of interspecific divergence values.

Regarding *Chrysolina bankii*, the intraspecific value decreased but remains higher than expected (9.878 ± 2.305). Looking back at the original matrix, it was noticed that specimens belonging to *Chrysolina bankii* formed two groups with distinct sequences, one containing specimens Cban1, Cban2 and Cban3 and the other one the specimens Csp1 and Csp5. In order to confirm this suspicion, both groups were separated into a new matrix. With this new analysis, it was possible to observe that intraspecific values were below the 3% threshold in both cases (Table 7). This leads us to consider that these two groups may correspond to different species, but they probably belong to the same family (see Appendix II, Table 10).

Table 7. Summary of intra and interspecific percentages of uncorrected *p*-distances obtained for eight species of the reanalyzed COI dataset. Standard deviation values are shown in brackets. A – Cban1, Cban2 and Cban3 specimens; B – Csp1 and Csp5 specimens.

	Creophilus maxillosus	***Ocypus aethiops***	***Saprinus semistriatus***	***Chrysolina bankii (A)***	***Chrysolina bankii (B)***	***Chrysolina diluta***	***Onthophagus coenobita***	***Licinus punctatulus granulatus***	***Notiophilus biguttatus***
Creophilus maxillosus	0.405 (±0.134)	17.351 (±0.467)	18.997 (±0.088)	22.273 (±0.037)	21.707 (±0.047)	19.706 (±0.057)	15.349 (±0.088)	16.413 (±0.088)	19.921 (±0.065)
Ocypus aethiops		0.304 (±0.000)	20.669 (±0.152)	22.974 (±0.047)	22.872 (±0.044)	19.833 (±0.034)	18.617 (±0.076)	19.225 (±0.076)	19.985 (±0.098)
Saprinus semistriatus			-	20.061 (±0.152)	22.416 (±0.076)	20.618 (±0.051)	19.301 (±0.000)	21.429 (±0.000)	19.529 (±0.076)
Chrysolina bankii (A)				1.925 (±0.963)	15.476 (±0.073)	15.198 (±0.136)	19.909 (±0.000)	22.492 (±0.000)	19.732 (±0.164)
Chrysolina bankii (B)					0.152 (±0.000)	14.868 (±0.073)	20.137 (±0.076)	20.897 (±0.076)	22.036 (±0.062)
Chrysolina diluta						0.912 (±0.456)	17.427 (±0.203)	20.263 (±0.101)	17.452 (±0.073)
Onthophagus coenobita							-	17.325 (±0.000)	18.389 (±0.000)
Licinus punctatulus granulatus								-	15.122 (±0.076)
Notiophilus biguttatus									0.152 (±0.000)

High interspecific divergence values allow the differentiation between all presented species, with values above the 3% threshold, and ten times higher than the average values of intraspecific divergence. Interspecific variation ranged from a minimum of 14.868% (±0.073), to a maximum of 22.974% (±0.047). The minimum value was found between congeneric species *Chrysolina bankii* (B) and *Chrysolina diluta*. *Chrysolina bankii* (A) and *Ocypus aethiops,* the species with the maximum higher interspecific divergence value are from different infraorders, being genetically more distant.

It is more frequent to perform this analysis with species belonging to the same genus or family. In this case, individuals belong to very distinctive families, which explain the large differences found. Moreover, the number of analysed specimens was not ideal, preventing a more robust analysis.

As a final point, it is important to remember that it is necessary to use the threshold values carefully. Some authors defend that these thresholds can suffer from statistical problems and can compromise species identification (Valentini *et al*., 2008).

Closely related species may present very similar nucleotide sequences, leading to a misleading species determination. For example, the 3% threshold is not sufficient to distinguish between sister species from genus *Lucilia* (Boehme *et al*., 2011). Therefore, group-specific thresholds should be established. However, it will take a long time to establish new parameters, since DNA barcoding is a relatively recent approach and there is a lack of knowledge for a great number of species.

3.5. Phylogenetic Analysis

The aim of DNA Barcode Project is not to resolve phylogenetic relationships. In fact, in most of the cases, DNA barcodes do not have sufficient phylogenetic signal to resolve evolutionary relationships (Hajibabaei *et al*., 2007). Nevertheless, phylogenetic analysis should be done in order to distinguish monophyletic groups and consequently permit species delineation (Nelson *et al*., 2007).

Sometimes, an oversimplified or inappropriate method may fail to distinguish monophyletic groups, compromising all the analysis. Therefore, it is advisable to make a comparison of tree-building methods in order to achieve best results (Nelson *et al*., 2007).

Thus, three methodologies were chosen for phylogenetic reconstruction: Maximum Likelihood (ML), Neighbour-joining (NJ) and Maximum Parsimony (MP). ML tree was chosen to explain obtained data for both genes and concatenated data.

3.5.1. *Cytochrome* c *oxidase I*

ML, NJ and MP analysis based on COI did not converge into straightforward topologies, since phylograms showed few differences at some levels. In these cases, bootstrap values were too small (<50%), preventing a robust analysis. ML tree and bootstrap values for ML, NJ and MP analysis are shown in Fig. 4.

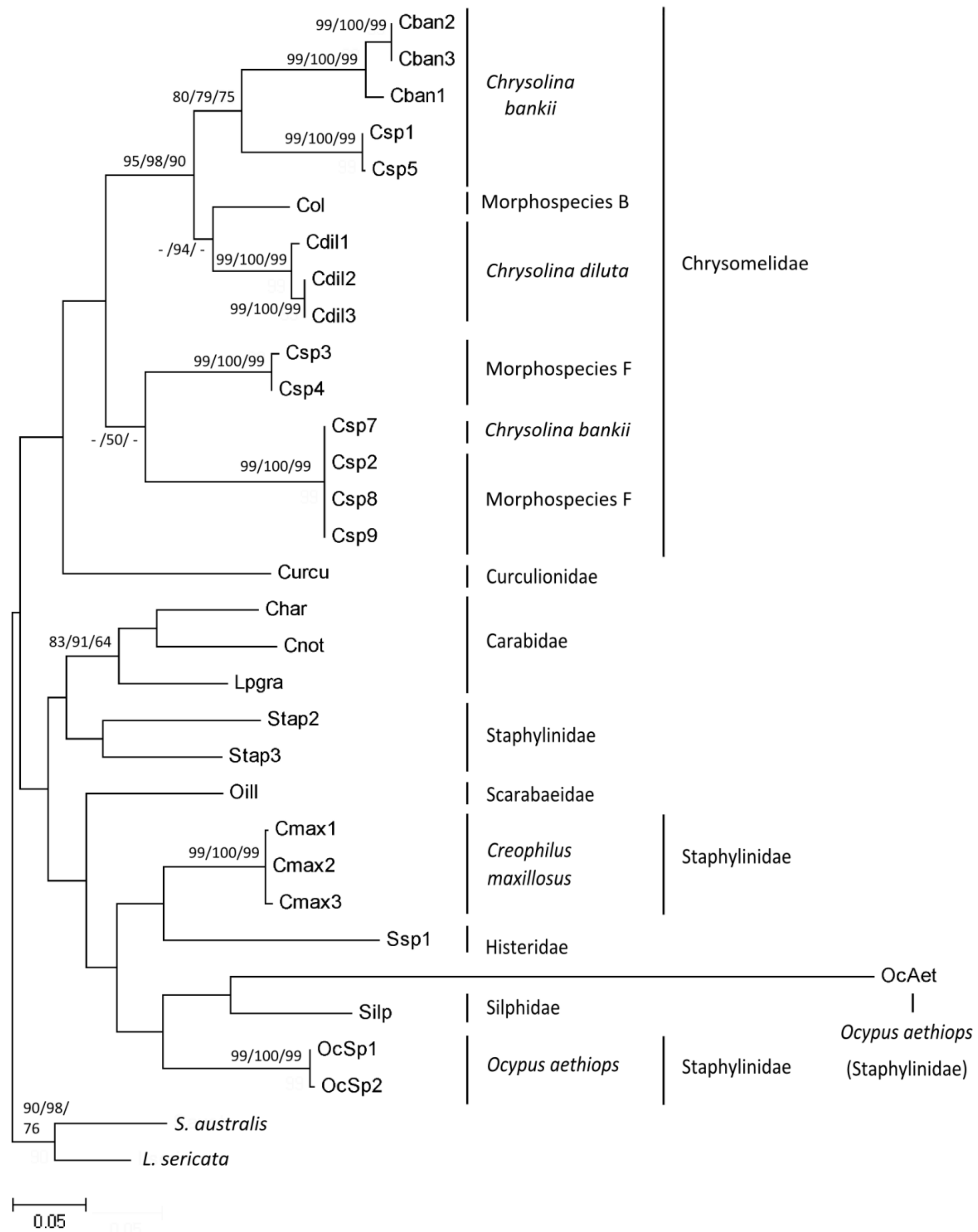

Fig. 4. Maximum likelihood phylogram (1,000 bootstrap replicates) based on 32 sequences of Cytochrome *c* oxidase I (COI) gene from 8 species, 7 morphospecies and 2 outgroups (*Sarcophaga australis* and *Lucilia sericata* - Diptera). Bootstrap values (above 50%) obtained for the analysis of ML, NJ and MP are shown next to the tree nodes.

It is possible to observe that different species/morphospecies belonging to the family Chrysomelidae are all grouped together. However, the specimen Csp7, originally identified as *Chrysolina bankii,* appears associated with morphospecies F and not with the rest of the specimens belonging to the same species. It is important to remember that in genetic distances analysis, it was also noticed some incongruence in intraspecific variation within individuals belonging to *Chrysolina bankii* species, and that this individual was removed from the analysis for showing a great number of genetic differences. In addition, when this sequence was blasted in GenBank database, the result was different from the other specimens belonging to same species. Thus, phylogenetic analysis corroborates previous results, leading us to consider that this specimen has been misidentified morphologically, and should be included in morphospecies F. Furthermore, within *Chrysolina bankii* species, it was possible to identify two distinct groups. These groups are the same ones previously recognized in genetic distances analysis, showing once more that may be two different species identified as *Chrysolina bankii*. This is corroborated by the really high bootstrap values associated with this separation.

Concerning morphospecies F there are also two distinct groups, possibly belonging to different species. With respect to morphospecies B it was observed that it is, in fact, different from morphospecies F, and it is possible that this specimen belongs to genus *Chrysolina*, since bootstrap value is very high for all the analyses performed.

Another specimen which also presented problems in genetic distances analysis was the individual OcAet, since it showed high genetic differences compared to the other specimens (OcSp1 and OcSp2) belonging to the same species (*Ocypus aethiops*). With phylogenetic analysis it was noticed that OcSp1 and Ocsp2 grouped together, but not OcAet, which supports previous results. Moreover, when this sequence was blasted in Genbank database, the match for this sequence was different from the other specimens (OcSp1 and OcSp2). Once again, this may be due to a morphological misidentification. The individuals OcAet and Csp7 are two of the problematic specimens that were sent to Dr. Stuart Hine, an entomological expert, to confirm morphological identification. However, it was not possible yet to confirm the results due to difficulties associated with morphological identification process.

Specimens belonging to the family Carabidae (Lpgra, Char and Cnot) cluster together, as expected, with good bootstrap values.

Stap2, Stap3 and specimens belonging to *Creophilus maxillosus* (Cmax1, Cmax2 and Cmax3) and *Ocypus aethiops* (OcSp1, OcSp2) all belong to the family Staphylinidae, but they not appear together in the tree. However, bootstrap values were below 50%. Nevertheless, *Creophilus maxillosus* specimens are all grouped together, with a high bootstrap value.

3.5.2. *Cytochrome b*

Despite minor differences, all phylogenetic analyses performed for Cytb, resulted in trees with similar topologies. The exceptions presented bootstrap values below 50%. ML tree and bootstrap support values for ML, NJ and MP analysis are shown in Fig. 5.

As mentioned above, 17 of the 31 obtained sequences were identical, even between specimens belonging to different families. This demonstrates that this region of Cytb gene is not a good molecular marker for the analysis of these specimens, since it appears to be very conserved among species, preventing their distinction. The only specimens that grouped as expected were the individuals belonging to *Chrysolina bankii* (Cban1, Cban2 and Cban3; Csp1 and Csp5), forming to distinct groups as seen before (Table 7). The individuals belonging to *Creophilus maxillosus* appear in different groups: Cmax1 and Cmax2 in one group and Cmax3 in another group, along with Ssp1 (Histeridae) and Cdil2 (Chrysomelidae).

Thereby, phylogenetic analysis of this gene is not appropriate to be used for these species, since is not possible to infer about phylogenetic relationships.

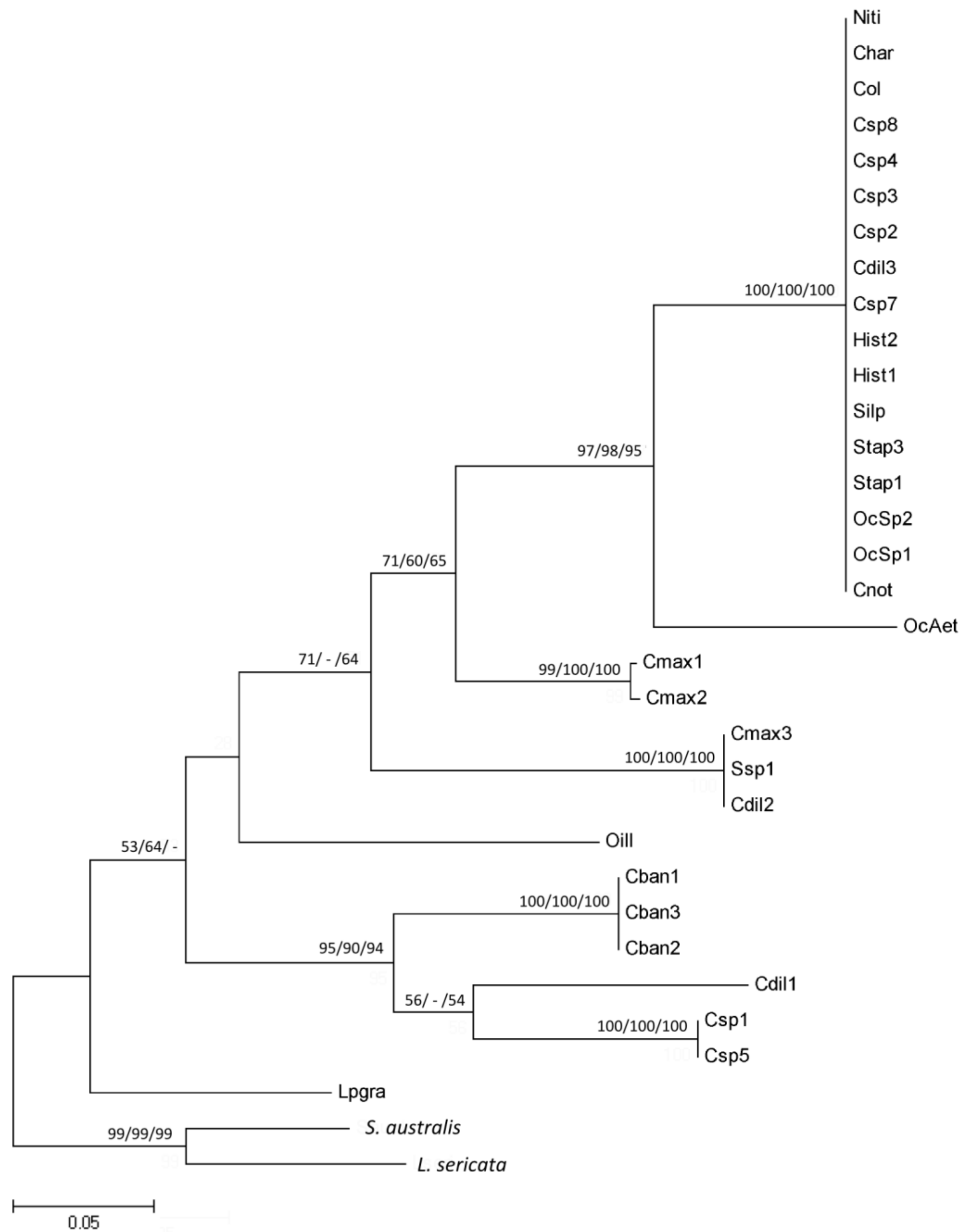

Fig. 5. Maximum likelihood phylogram (with 1,000 replicates) based on 33 sequences of Cytochrome b (Cytb) gene from 8 species, 6 morphospecies and 2 outgroups (*Sarcophaga australis* and *Lucilia sericata* - Diptera). Bootstrap values (above 50%) obtained for the analysis of ML, NJ and MP are shown next to the tree nodes.

3.5.3. *Concatenated data*

Despite knowing that results would be biased due to Cytb data, it was also constructed a concatenated tree, assembling information from both genes (COI and Cytb).

Since tree topologies obtained for the different analysis were very different, it was decided to present the tree obtained through ML method (Fig. 6), and show the remaining results in Appendix III (Fig. 7 and 8).

Regarding ML tree, is possible to observe that morphospecies F appear associated, as well as specimens belonging to *Chrysolina bankii,* with the exception of Csp7. As seen before in COI analysis, within morphospecies F and *Chrysolina bankii*, there are two distinct groups that may belong to different species. Specimens belonging to *Chrysolina diluta* species are also appear associated, but without a high bootstrap value. Unlike the analysis for COI data, family Chrysomelidae is not grouped together. Once again, the specimen OcAet does not appear associated with the rest of the specimens belonging to the same species, *Ocypus aethiops*. Char and Cnot belonging to the family Carabidae appears together in the same group with high bootstrap value, but the specimen Lpgra that belongs to the same family, has a basal position.

As a final point, it is important to mention that these specimens belong to very distinct families and that the number of individuals is particularly small for this sort of analysis. In fact, in some cases only one specimen exists per family. Further studies are needed to increase the number of specimens belonging to each species.

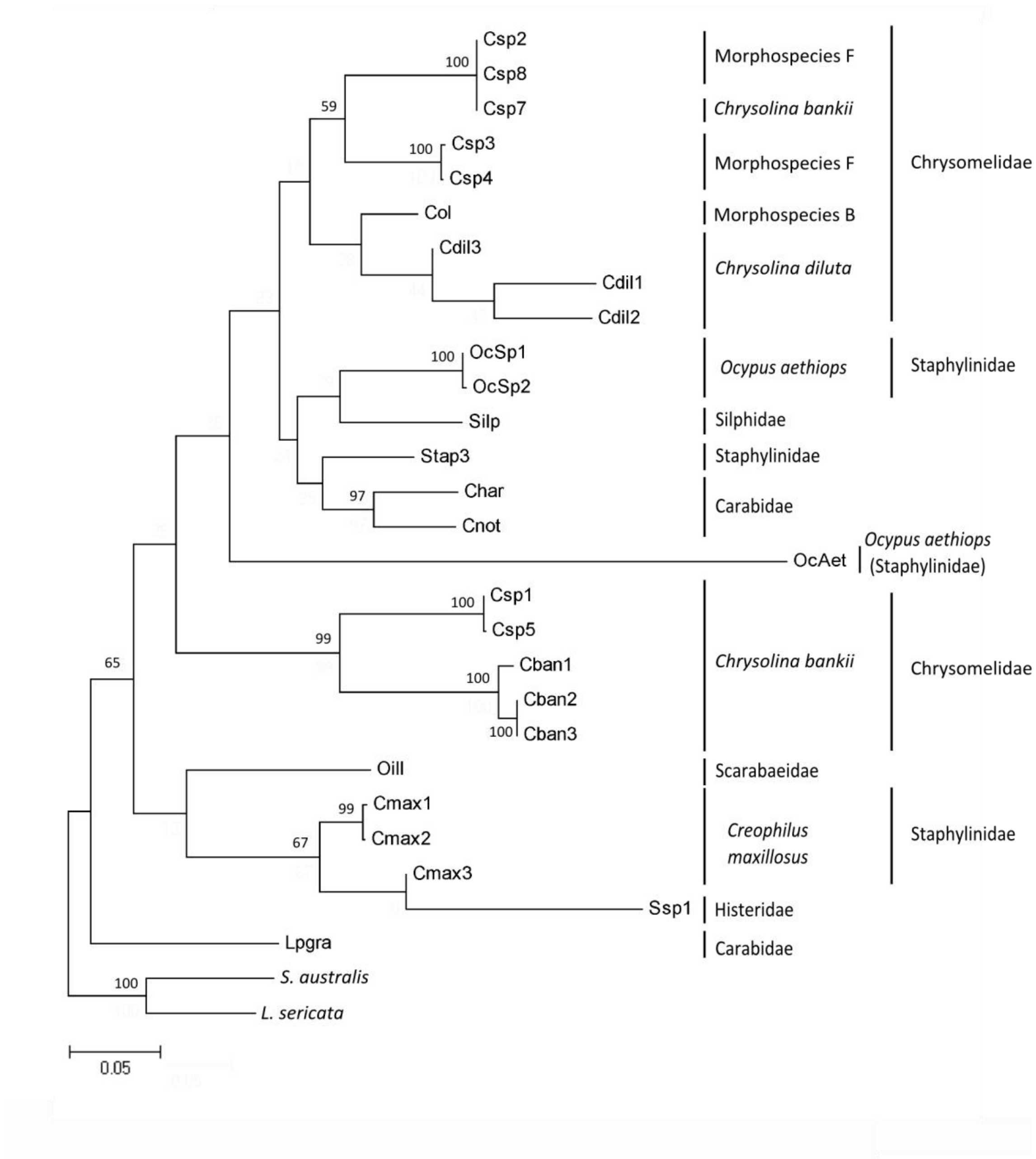

Fig. 6. Maximum likelihood consensus phylogram (1,000 bootstrap replicates) based on 29 sequences from 8 species, 6 morphospecies and 2 outgroups (*Sarcophaga australis* and *Lucilia sericata* - Diptera). Bootstrap values (above 50%) obtained for the analysis of ML, NJ and MP are shown next to the tree nodes.

Final Considerations

IV. Final Considerations

It is well known that it is extremely important to develop a molecular database of species with forensic interest, in order to apply in criminal investigations. This database should include geographic data, since many factors can affect species composition and succession pattern. In Portugal, studies concerning insect succession and identification of species with forensic interest have been made, using both morphological and molecular approaches. Nevertheless, the majority of studies focused on Diptera. As a result, little knowledge is available about Coleoptera species, mainly at molecular level. Nevertheless, such studies are extremely important, since Coleoptera specimens are the main entomological evidence in cases where badly decomposed or skeletonised corpses are found.

Difficulties in achieving a correct morphological identification were noticed in this study, preventing the identification of some specimens to species level. As a result, some specimens were grouped in morphospecies, based on morphological characters. In the future it is intended to continue with identifications in order to obtain complete information about each specimen. This shows once again the great importance of the molecular approach since it is a reliable, accurate and faster method of insect specimens identification.

Successful amplification of COI comes to confirm that it is a robust molecular marker to be used in Coleoptera identification. On the other hand, although Cytb amplification has occurred with success, when obtained sequences were analysed it was noticed that 17 of the 31 sequences were identical, proving that this region is highly conserved among these species, and therefore Cytb is not a good molecular marker to be used with this aim. Thereby, results showed that COI gene is a most suitable marker to be used in DNA barcoding, when compared with Cytb.

It was possible to observe a lack of reference sequences in online databases for a great number of species for both genes, mainly due to a poor knowledge of species. This fact prevented a match with the obtained sequences, and consequently the corroboration of morphological identification.

In some cases, it was noticed some incongruence between morphological and molecular data, due to an uncertainty in morphological identification. In the future it is intended to confirm species identification to include these data in the analysis.

After confirmation of morphological identification, sequences will be submitted to GenBank and BOLD online databases. Molecular identification was performed for the first time for several Coleoptera species, and obtained sequences will contribute to increase the number of available sequences for comparison, contributing to future species determination through DNA barcoding methodology. In addition, the submission of obtained sequences will contribute to the implementation of a National Database, increasing the amount of entomological information available for some important Coleoptera species in Portugal.

The present study was the first molecular approach to identify Coleoptera specimens in Portugal. New studies are required to increase the abundance and richness of Coleoptera species, and new molecular markers should be tested. It is important to invest both in morphological and molecular identification, since countless Coleoptera species remain unexplored. For a more robust research these studies should be carried out using an animal carcass, preferably pig carrion, due to its high homology with human. Those experiments should be settled in different areas of the country to evaluate geographical and climatic differences.

It would also be important to perform laboratory studies concerning insect development rate in order to make an association between the insect stage and PMI estimation. In the future, these data may contribute to increase the number of entomological evidences used in forensic investigations, in Portugal.

Finally, in addition to the main objectives, this thesis was also important to acquire competences in laboratory practice and in data analysis of obtained results.

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Appendices

Appendix I

Table 8. Species identification, success of extraction and amplification for COI and Cytb.

Code	Family	Genus	Species	COI	Cytb
Cmax1	Staphylinidae	Creophilus	*Creophilus maxillosus*	✓	✓
Cmax2			*Creophilus maxillosus*	✓	✓
Cmax3			*Creophilus maxillosus*	✓	✓
OcAet		Ocypus	*Ocypus aethiops*	✓	✓
OcSp1			*Ocypus aethiops*	✓	✓
OcSp2			*Ocypus aethiops*	✓	✓
Stap1			*Ocypus aethiops*	×	✓
Stap2		?	Morphospecies G	✓	×
Stap3		?	Morphospecies H	✓	✓
Silp	Silphidae	?	Morphospecies A	✓	✓
Ssp1	Histiridae	Saprinus	*Saprinus semistriatus*	✓	✓
Hist1			*Saprinus semistriatus*	×	✓
Hist2			*Saprinus semistriatus*	×	✓
Cban1	Chrysomelidae	Chrysolina	*Chrysolina bankii*	✓	✓
Cban2			*Chrysolina bankii*	✓	✓
Cban3			*Chrysolina bankii*	✓	✓
Csp1			*Chrysolina bankii*	✓	✓
Cps5			*Chrysolina bankii*	✓	✓
Cps6			*Chrysolina bankii*	×	×
Cps7			*Chrysolina bankii*	✓	✓
Cdil1			*Chrysolina diluta*	✓	✓
Cdil2			*Chrysolina diluta*	✓	✓
Cdil3			*Chrysolina diluta*	✓	✓
Csp2		?	Morphospecies F	✓	✓
Csp3		?	Morphospecies F	✓	✓
Csp4		?	Morphospecies F	✓	✓
Csp8		?	Morphospecies F	✓	✓
Csp9		?	Morphospecies F	✓	×
Col		?	Morphospecies B	✓	✓
Curcu	Curculionidae	?	Morphospecies I	✓	×
Niti	Nitidulidae	?	Morphospecies C	×	✓
Oill	Scarabaeidae	Onthophagus	*Onthophagus coenobita*	✓	✓
Lpgra	Carabidae	Licinus	*Licinus punctatulus granulatus*	✓	✓
Char		Harpalus	Morphospecies D	✓	✓
Cnot		Notiophilus	*Notiophilus biguttatus*	✓	✓

Appendix II

Table 9. Molecular Identification of insect specimens obtained for Cytb in GenBank database.

	GenBank	
Samples	**Species**	**%**
Cmax1	*Amphichroum* sp.	86
Cmax2	*Tachinus luridus*	85
Cmax3	*Blackburnia constricta*	84
OcAet	*Dropephylla cacti*	85
OcSp1	*Tachinus luridus*	86
OcSp2	*Tachinus luridus*	86
Stap1	*Tachinus luridus*	86
Stap3	*Tachinus luridus*	86
Silp	*Tachinus luridus*	86
Ssp1	*Blackburnia constricta*	84
Hist1	*Tachinus luridus*	86
Hist2	*Tachinus luridus*	86
Cban1	*Pachyta bicuneata*	84
Cban2	*Pachyta bicuneata*	84
Cban3	*Pachyta bicuneata*	84
Csp1	*Tegeticula treculeanella*	85
Cps5	*Sphaerius* sp.	84
	Galerucella grisescens	84
Cps7	*Tachinus luridus*	86
Cdil1	*Trechus navaricus*	84
Cdil2	*Blackburnia constricta*	84
Cdil3	*Tachinus luridus*	86
Csp2	*Tachinus luridus*	86
Csp3	*Tachinus luridus*	86
Csp4	*Tachinus luridus*	86
Csp8	*Tachinus luridus*	86
Col	*Tachinus luridus*	86
Niti	*Tachinus luridus*	86
Oill	*Sphaerocanthon hanskii*	87
	Helictopleurus fulgens	87
Lpgra	*Cychrus caraboides*	88
	Blackburnia depressa	88
Char	*Tachinus luridus*	86
Cnot	*Tachinus luridus*	86

Table 10. Molecular Identification of insect specimens obtained for COI in BOLD and GenBank databases.

	BOLD		GenBank	
Sample	**Species**	**%**	**Species**	**%**
Cmax1	*Creophilus maxillosus*	99,85	*Atheta myrmecobia*	86
Cmax2	*Creophilus maxillosus*	100	*Atheta myrmecobia*	86
Cmax3	*Creophilus maxillosus*	99,54	*Stethusa spuriella*	86
			Atheta myrmecobia	86
OcAet	*Nehemitropia lividipennis*	94,59	*Wolbachia*	80
OcSp1	*Ocypus opthalmicus*	97,07	*Ocypus olens*	88
OcSp2	*Ocypus opthalmicus*	96,79	*Ocypus olens*	88
Stap2	*Anotilus nitidulus*	99,85	*Smidtia fumiferanae*	86
Stap3	*Tachinus marginellus*	93,58	*Bembidion erasum*	88
Silp	*Thanatophilus sinuatus*	99,69	*Oxypoda praecox*	85
Ssp1	*Saprinus semistriatus*	87,61	*Staphylinidae* sp.	83
Cban1	Chrysomelidae	99,69	*Chrysolina* sp.	85
Cban2	Chrysomelidae	99,85	*Chrysolina* sp.	85
Cban3	Chrysomelidae	99,85	*Chrysolina* sp.	85
Csp1	Chrysomelidae	99,69	*Zygogramma bicolorata*	85
Cps5	Chrysomelidae	99,54	*Zygogramma bicolorata*	85
	Chrysolina haemoptera	87,4		
Cps7	*Apteropeda orbiculata*	97,09	*Drosophila bifasciata*	83
Cdil1	Chrysomelidae	99,23	*Chrysolina* sp.	87
Cdil2	Chrysomelidae	98,31	*Chrysolina* sp.	87
Cdil3	Chrysomelidae	98,31	*Chrysolina* sp.	87
Csp2	*Apteropeda orbiculata*	97,09	*Drosophila bifasciata*	83
Csp3	Chrysomelidae	94,32	Coleoptera sp.	87
Csp4	Chrysomelidae	94,78	Coleoptera sp.	87
Csp8	*Apteropeda orbiculata*	97,09	*Drosophila bifasciata*	83
Csp9	*Apteropeda orbiculata*	98,09	*Drosophila bifasciata*	83
Col	Chrysomelidae	99,69	*Chrysolina haemoptera*	99
	Chrysolina haemoptera	98,62		
	Chrysolina haemoptera	97,29		
Curcu	*Trachyphloeus asperatus*	86,24	*Lachnopus seini*	86
Oill	Coleoptera	99,5	*Onthophagus nuchicornis*	92
Lpgra	*Licinus depressus*	95,41	*Agriotes aequalis*	89
Char	*Harpalus amputatus*	98,62	*Harpalus aeneus*	98
	Harpalus affinis	98,58	*Harpalus affinis*	98
	Harpalus distinguendus	98,51	*Harpalus rubripes*	97
Cnot	*Notiophilus biguttatus*	99,85	*Notiophilus biguttatus*	99

Appendix III

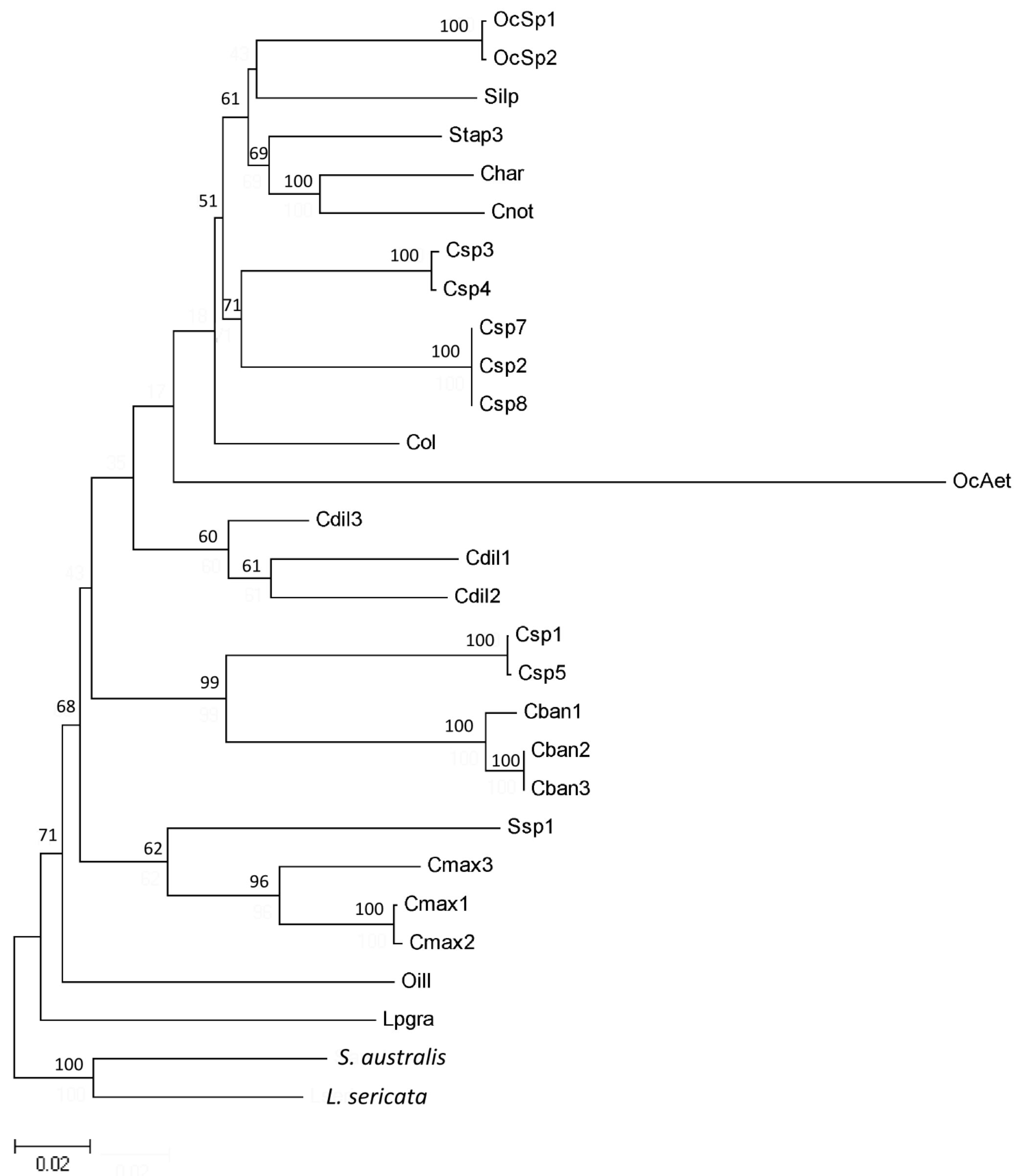

Fig. 7. Neighbour-joining consensus phylogram (1,000 bootstrap replicates) based on 29 sequences from 8 species, 6 morphospecies and 2 outgroups (*Sarcophaga australis* and *Lucilia sericata* - Diptera). Bootstrap values (above 50%) obtained for the analysis of ML, NJ and MP are shown next to the tree nodes.

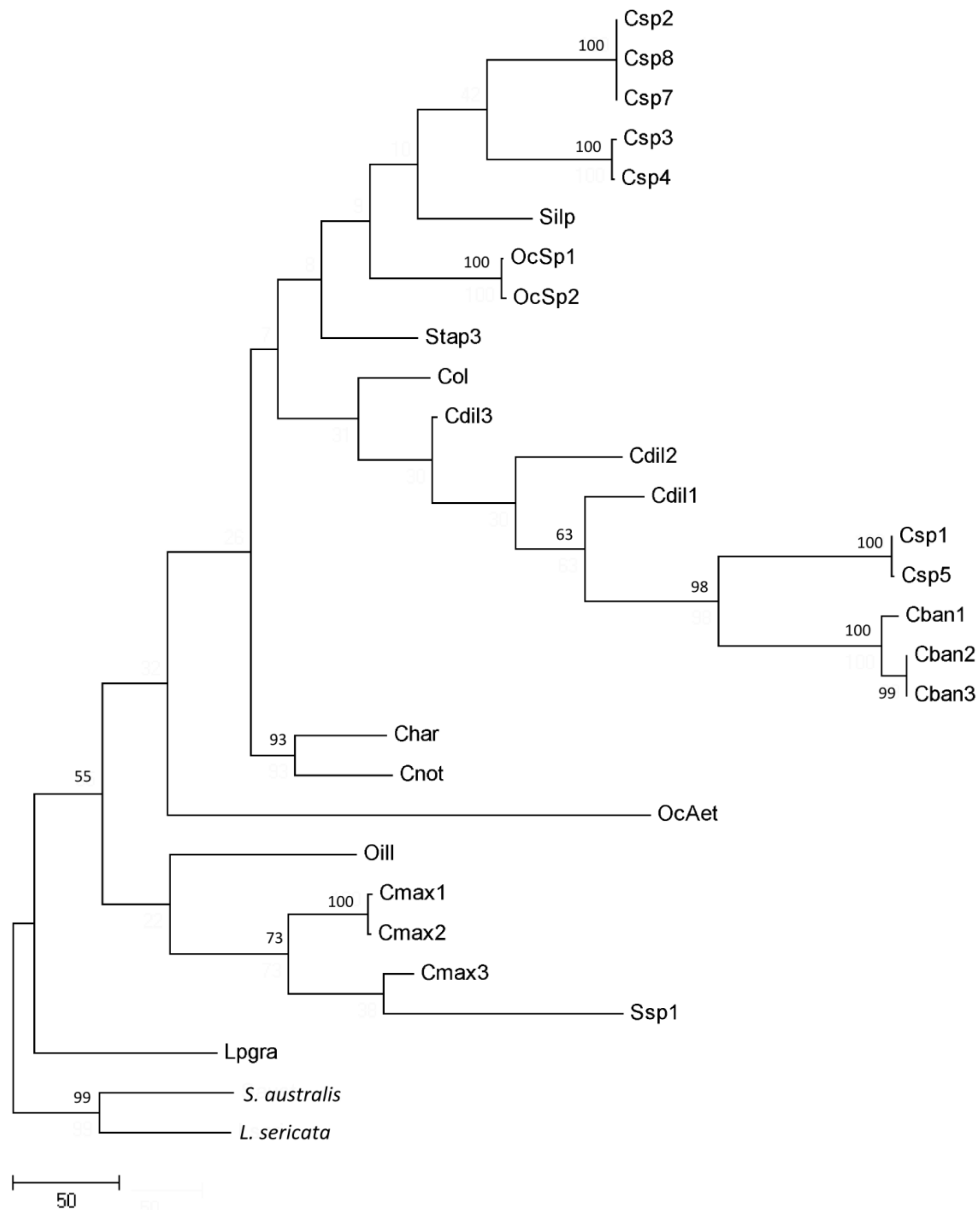

Fig. 8. Maximum Parsimony consensus phylogram (1,000 bootstrap replicates) based on 29 sequences from 8 species, 6 morphospecies and 2 outgroups (*Sarcophaga australis* and *Lucilia sericata* - Diptera). Bootstrap values (above 50%) obtained for the analysis of ML, NJ and MP are shown next to the tree nodes.