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Ecology and Conservation of *Cephalota* (*Taenidia*) *Deserticoloides* (Codina, 1931) (*Coleoptera, Cicindelidae*)

Ecología y Conservación de *Cephalota* (*Taenidia*) *Deserticoloides* (Codina, 1931) (*Coleoptera, Cicindelidae*)

D. José Herrera Russert

2018

UNIVERSIDAD DE MURCIA

Ecología y conservación de *Cephalota (Taenidia) deserticoloides* (Codina, 1931) (Coleoptera, Cicindelidae)

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José Herrera Russert

2018

Escuela Internacional de Doctorado
Universidad de Murcia

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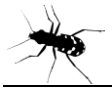
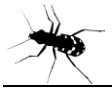
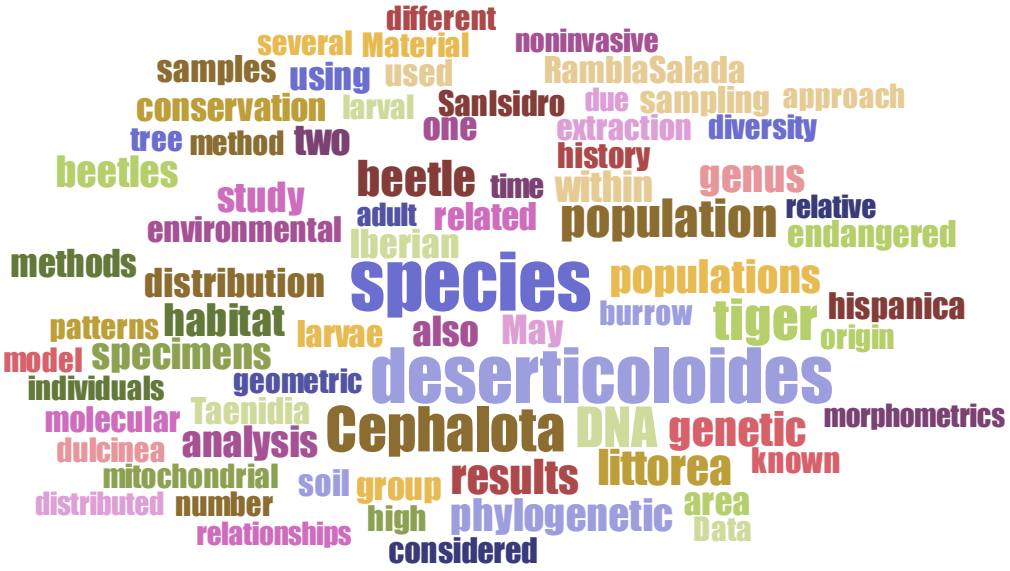


Este trabajo se ha realizado en el Área de Biología Animal del Departamento de Zoología y Antropología Física de la Universidad de Murcia, bajo la financiación obtenida en la convocatoria de Grupos de Excelencia Regional otorgada por la Fundación Séneca de la Región de Murcia al grupo de investigación "Filogenia y Evolución Animal" (19908/GERM/15), cuyo I.P. es el Dr. José Galián Albaladejo, director de esta tesis.

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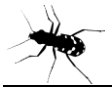

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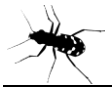

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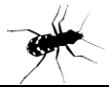

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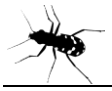

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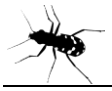

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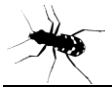
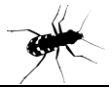




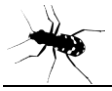
Abstract

The use of molecular techniques has become essential as a means to study biological diversity. This need is especially pronounced in endangered organisms, where a detailed assessment of their genetic diversity, ecology, and populational status are required in order to design more effective management measures that may ensure their survival. Additionally, knowledge of the phylogenetic relationships among organisms is essential in order to establish a taxonomic classification coherent with their evolutionary history. Recently, environmental DNA has become an important source of information on biodiversity that does not require an invasive disturbance of the taxa brought under analysis.

The tiger beetle genus *Cephalota*, including the subgenera *Cephalota* and *Taenidia*, is native to an area encompassing the Mediterranean coast and much of Central Asia, and includes some endangered endemics restricted to reduced areas in the Iberian Peninsula. The genesis of this genus has previously been related to the closure of the Tethys Ocean and the formation of the Mediterranean Sea.

Cephalota deserticoloides is an endemic species, with a distribution restricted to a few sites in south eastern Spain, where it occupies only the patchy arid saline-steppe type habitat. Due to this high level of patchiness and to its ecological specialization, its current populations are assumed to be local and isolated. Although regarded as vulnerable, very little is known about its actual population dynamics and degree of endangerment. A capability to assess its presence and identity is paramount to its management, but made more complicated by the co-occurrence of its larvae with those of the very similar species *C. littorea*.

The main objective of this thesis is to offer an approach to the knowledge of the evolution of *C. deserticoloides*, starting with its origin within the Palearctic genus *Cephalota*, and then to focus on the internal dynamics and the relationship between its populations.



In the first chapter, a phylogenetic tree of eleven *Cephalota* species is inferred. The observed phylogenetic relationships between these species challenges the hypotheses previously formulated about the evolution of this group. Additionally, the results do not support the established systematics of this genus, suggesting that the subgenus *Taenidia* is not monophyletic. The origin of *Cephalota* is dated back to 13.5 million years ago, once the Mediterranean Sea was already formed. Hypotheses concerning the changes on the suitable habitat for this halophile group caused by fluctuating levels of the Mediterranean Sea are proposed.

In the second chapter, we use a twofold approach to assess the population dynamics and origin of *C. deserticoloides*, combining genetic-based phylogeography with a geometric morphometrics approach. The results attest that *C. deserticoloides* is divided into a small group of isolated populations with little contact with each other. These results improve our understanding of the species and its ecological dynamics, which will enable an improved management and protection of the Iberian saline steppe and of *C. deserticoloides*.

In the third chapter, we make use of environmental DNA to conduct a non-invasive sampling of tiger beetle larvae. A novel protocol is developed to discriminate between *C. deserticoloides* and *C. littorea* soil-bound genetic material from larval burrows. The observed results confirm the ability of this method to separate both species and give rise to new hypotheses regarding relative abundance and niche partitioning between them.

Finally, in the fourth chapter, mark-recapture estimates of total population size are presented for one population of *Cephalota deserticoloides*. The observations gathered indicate that *C. deserticoloides* makes a narrow use of the habitat available, with activity peaks that are separated from co-occurrent species. The area under consideration supports a relatively dense tiger beetle population, numerically comparable to those of other endangered cicindelids. These results will help assess

the conservation state of *C. deserticoloides* and set the stage for more long-term efforts to analyse its population viability for protective measures.

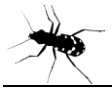
Resumen

El uso de técnicas moleculares se ha convertido en una herramienta esencial para estudiar la biodiversidad. Es especialmente importante en relación con organismos amenazados, en los que es preciso evaluar su diversidad genética, ecología y estado poblacional para asegurar su supervivencia. Además, conocer las relaciones filogenéticas entre los organismos es esencial para establecer una clasificación coherente con su historia evolutiva. Recientemente, el ADN ambiental se ha convertido en una fuente importante de información sobre la biodiversidad que no requiere la perturbación de los taxones analizados.

El género de escarabajos tigre *Cephalota*, que está compuesto por los subgéneros *Cephalota* y *Taenidia*, se distribuye desde el Mediterráneo hasta Asia central, e incluye algunos endemismos amenazados restringidos a áreas reducidas de la península Ibérica. El origen de este género ha sido relacionado con el cierre del Mar de Tetis y la formación del Mediterráneo.

Cephalota deserticoloides es una especie endémica con distribución restringida a escasas localidades en el sureste ibérico, donde ocupa parches de estepa árida salina. Dado su alto nivel de fragmentación y su especialización ecológica, se asume que sus poblaciones están aisladas. Aunque considerada vulnerable, se conoce poco sobre su dinámica real y su nivel de riesgo. La capacidad de detectar su presencia e identidad es primordial para su manejo, pero es fácil confundir sus larvas con las de la especie coexistente *C. littorea*.

El principal objetivo de esta tesis es ofrecer una aproximación al conocimiento sobre la evolución de *C. deserticoloides*, comenzando con su origen en el seno del género



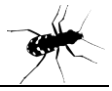
paleártico *Cephalota*, y centrándose después en su dinámica interna y la relación entre sus poblaciones.

En el primer capítulo, se infiere un árbol filogenético de once especies de *Cephalota*. Las relaciones filogenéticas observadas entre las especies contradicen las hipótesis previamente formuladas acerca de la evolución de este grupo. Además, los resultados no apoyan la sistemática establecida para este género, sugiriendo que el subgénero *Taenidia* no es monofilético. El origen de *Cephalota* se remonta a hace 13.5 millones de años, una vez que el Mediterráneo ya estaba formado. Se proponen hipótesis alternativas relacionadas con los cambios en el hábitat adecuado para este grupo halófilo, causados por el nivel fluctuante del Mediterráneo.

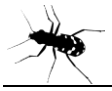
En el segundo capítulo, se usan dos enfoques para evaluar la dinámica poblacional y origen de *C. deserticoloides*, combinando el análisis filogeográfico con el de morfometría geométrica. Los resultados indican que *C. deserticoloides* se compone de un pequeño grupo de poblaciones con escaso contacto entre ellas. Estos resultados mejoran nuestro entendimiento de la especie y su dinámica ecológica, y permitirán mejorar el manejo y protección tanto de la estepa salina como de *C. deserticoloides*.

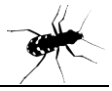
En el tercer capítulo, se usa ADN ambiental para muestrear de forma no invasiva larvas de cicindélidos. Se desarrolla un protocolo novedoso para diferenciar entre *C. deserticoloides* y *C. littorea* tomando muestras de suelo de las galerías larvarias. Los resultados confirman la validez de este método para separar ambas especies y generan nuevas hipótesis acerca de la abundancia y repartición del nicho entre ellas.

Finalmente, en el cuarto capítulo se estima el tamaño poblacional de una población de *C. deserticoloides* mediante métodos de marcaje y recaptura. Las observaciones indican que *C. deserticoloides* ocupa un nicho reducido, separado de especies coexistentes. El área considerada soporta una densidad alta de estos escarabajos tigre, comparable a las de otras especies amenazadas. Estos resultados ayudarán a

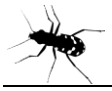


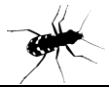
evaluar el estado de conservación de *C. deserticoloides* y a sentar las bases para medidas de protección a largo plazo.





Introduction

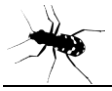




Tiger beetles as an object of study

Tiger beetles are a group of adephagan beetles (Coleoptera, Carabidae) with a widespread distribution across all major landmasses except for Antarctica. Across their range, tiger beetles occupy a very wide range of habitats, although individual species are often specialized on a narrow spectrum of habitat types or conditions. Tiger beetles are also conspicuous, medium-sized and visually attractive insects which often display a very active and easily observable behaviour. These characteristics have made them a rather well-known medium-sized family of beetles among scientists and amateur naturalists alike (Pearson and Vogler, 2001). Tiger beetles have been variably considered either a distinct subfamily within the wider Carabidae (ground beetles), or a family on their own (Cassola, 2001; López-López and Vogler, 2017), the Cicindelidae. While most studies tend to lean in favor of the subfamily hypothesis (Maddison et al., 2009, 1999; McKenna et al., 2015; Shull et al., 2001), some recent analyses hint at their status as a separate family from Carabidae (Bocak et al., 2014; Linard et al., 2018; López-López and Vogler, 2017; Zhang et al., 2018).

Conservation programs focused on tiger beetles (Cornelisse et al., 2013; Dang and Aitken, 2014) show a different and often more urgent set of challenges in comparison with more popular groups such as vertebrate animals or vascular plants, which makes them an interesting asset in conservation, especially given their vulnerability to specific types of environmental change across temperate regions (Kirby, 1992). Some of the characteristics that make them so appealing in conservation progams are their often highly specialized habitat or even microhabitat, further complicated by the existence of differentiated life stages with different demands. For example, plant cover and landscape makeup may be important to the mobile adult stage, while soil composition, stability and drainage are of most importance to the ground-dwelling larvae. Prey type, size and pattern of occurrence are also different for the adult, an active hunter, and for the immobile larva. The relative shortness of the life cycle also



means that these insects will not be able to avoid seasonal adverse conditions as easily, while smaller size and more limited dispersal ability (or behavioral tendency to disperse) renders tiger beetle populations more prone to isolation and fragmentation. Maintenance of habitat quality, continuity, heterogeneity and connectedness are thus fundamental traits in the approach to tiger beetle conservation (Stewart et al., 2007).

These factors all contribute to making tiger beetles ideal for the role of environmental indicators, as well as very interesting for their potential as model insects in fields ranging from population ecology to genetic studies and molecular analyses.

Tiger beetles lead a mainly predatory life style, although adults are also known to be opportunistic scavengers (Jaskuła, 2013). The adult tiger beetle is a very mobile insect that actively hunts down its prey by running (Kamoun and Hogenhout, 1996) thanks to its thin, long legs and an often adequate flight capability. Its protruding eyes enable it to easily spot other animals in its surroundings, while its sickle-shaped mandibles are ideally suited to efficiently dispatch its mostly invertebrate prey (Figure 1).

Adult tiger beetles usually occupy flat, open terrain that suits their hunting and evasion capabilities, and they also display thermoregulatory behavior in their diel activity and through activities such as stilting and basking (Figure 2). The adult phase is usually short-lived, surviving only for one season during which reproduction occurs, although some species are known to winter in the adult form (Gwiazdowski et al., 2011; Pearson and Vogler, 2001; Serrano, 1990).

Figure 1. *Cephalota (Cephalota) hispanica* pair feeding on a mealworm (*Tenebrio molitor*) (Herrera-Russert, 2016).

Figure 2. *Cephalota (Taenidia) deserticoloides* exhibiting thermoregulatory behaviour. (Herrera-Russert, 2016).

Eggs are laid in moist substrate and usually hatch within days or weeks. The larval phase is the longest, taking anywhere from a few months to several years, and progresses through three instars, of which the third by far takes up the most time (Figure 3).

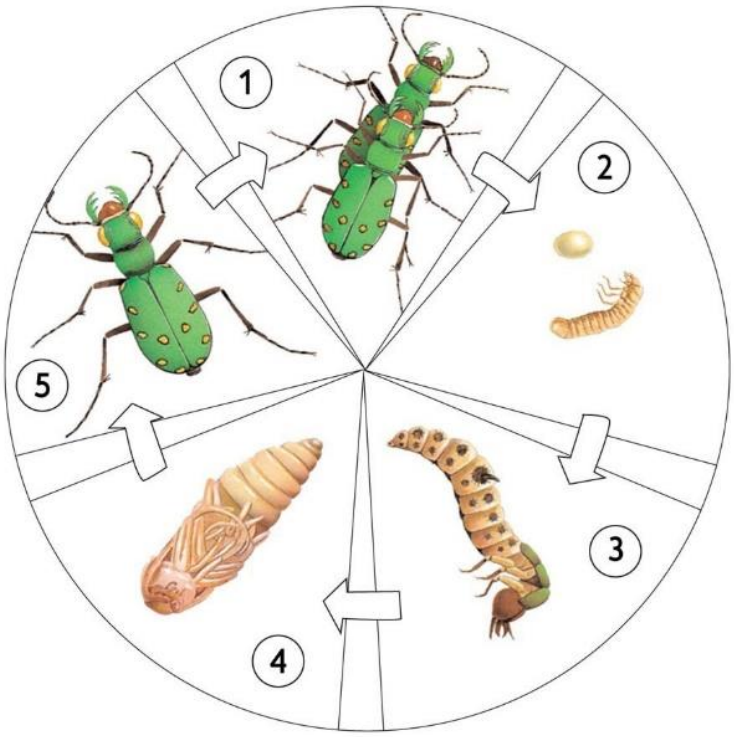


Figure 3. Life cycle of a tiger beetle. 1: adults mating, 2: egg and first instar larva, 3: larva, 4: pupa, 5: adult. Picture by 'Q-Files, the Great Illustrated Encyclopedia' (https://www.q-files.com/life/insects/beetles).

Larvae are morphologically adapted to a vertical life style in the burrow they construct in the substrate, which the seldom if ever abandon (Figure 4).

Figure 4. Captive *Cicindela* larva lunging at prey (https://cicindela.wordpress.com/2010/06/30/gotcha/)

They ambush small invertebrates at the entrance, which they then drag and feed upon inside the burrow. When larvae molt, pupate or enter diapause, they will seal the entrance of their burrow with soil for variable periods of time, and each time a molt is completed the diameter of the burrow is enlarged, so that developmental state can often be noticed by noting the width of the entrance (Pearson and Vogler, 2001).

Tiger beetles have been recorded to suffer high mortality rates, especially as larvae, from causes such as starvation, catastrophic events such as flooding (Figure 5), to which some species are well adapted (Zerm et al., 2004), and a not negligible rate of parasitoidism by other insects such as ichneumonoid wasps and bombyliid flies. Adults are more occasionally fed upon by insects such as robber flies (Figure 6), birds and lizards (Knisley and Juliano, 1988).

Figure 5. *Pseudotetracha sp.* running form a sudden water flood. (A. Lopez-López, 2012).

Figure 6. Robber fly feeding on *Cephalota (Taenidia) circumdata* (R. Batlle).

The genus *Cephalota*

The genus *Cephalota*, to which some 21 species have been ascribed worldwide (Lorenz, 2005; Putchkov and Matalin, 2017), is a halobiontic group that purportedly already existed 35 million years ago populating coastal areas of the Tethys Ocean (Hieke, 1983). The genus shows a Mediterranean and Central-Asian distribution pattern, that possibly took its current shape with the formation of the Mediterranean Sea. There are currently six species of *Cephalota* living in the Iberian Peninsula, with *C. maura*, *C. circumdata* and *C. littorea* (Figure 7) being part of a wider Mediterranean distribution while *C. hispanica*, *C. dulcinea* and *C. deserticoloides* are local Iberian endemics (Rodríguez-Flores et al., 2016). All of these species have a patchy distribution, occupying coastal salt marsh or salt steppe habitats, as well as interior salt flats and salty endorheic lagoon complexes (Jaskuła and Rewicz, 2015; Vives and Vives, 1978).

Figure 7. *Cephalota (Taenidia) littorea*. This species often co-occurs with *C. deserticoloides*. Note hairless frons, widening of elytra and maculae that do not reach scutellum, as well as overall bronze colouring. (Herrera-Russert, 2017).

The genus *Cephalota* was described by Dokhtouroff (1883). Later, Rivalier (1950) established the subgenus *Taenidia* for most of the species, with the exception of *C. hispanica*, *C. turcica*, *C. pseudodeserticola* and *C. luctuosa*, which fall under subgenus *Cephalota s. str.* On the other hand, *C. maura* (Figure 8) is at present included in the subgenus *Cassolalia* (Lorenz, 2005) or considered as the only species of an independent genus altogether (Putchkov and Matalin, 2017).

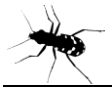
Figure 8. *Cephalota (Cassolaia) maura* (Herrera-Russert, 2017).

Cephalota deserticoloides

C. deserticoloides is currently considered a member of the subgenus *Taenidia* (Lorenz, 2005; Rivalier, 1950), represented in the Iberian Peninsula by three other species (Serrano, 2013): *C. circumdata* and *C. littorea*, of Circum-Mediterranean distribution, and *C. dulcinea*, an Iberian endemic of inland lagoons (Rodríguez-Flores et al., 2016; Serrano, 2013). *C. deserticoloides*, however, has a much more restricted range and shows a special affinity towards arid areas, especially in its larval phase, occupying only a few sites in south-eastern Iberia (Diogo et al., 1999; Lencina and Serrano, 2011; Rosas et al., 1992). It is considered a vicariant taxon whose closest relative could be *Cephalota deserticola* (Faldermann, 1836), which is found in central Asia (Diogo et al., 1999; Domingo et al., 2007). Recent results obtained by means of molecular phylogeny methods indicate that *C. deserticoloides* belongs to a kinship that is clearly separated from the grouping of its Iberian congeners (Diogo et al., 1999; Herrera-Russert, 2015; López-López and Galián, 2010).

C. deserticoloides is a cicindelid of a body length ranging between 9 and 11 mm. Dorsally, it shows a brownish-copper background colouring and ivory elytral maculae (Figure 9). The head is covered with a snowy pubescence, and has large jaws and very pronounced eyes. It presents parallel elytra with an ivory-coloured external marginal band resulting from the merging of the maculae, which runs the entire elytral length from the humerus to the apex. This patch presents three extensions on the elytral disc in the form of bands, one subhumeral, another median and the third reaching the apex of the elytral suture. Ventrally, *C. deserticoloides* is characterized by a dark purple metalic glean, in stark contrast to the bottle green of the other Iberian *Taenidia*. Finally, it has long legs adapted for quick motion and very conspicuously coated with a white pubescence (Lencina and Serrano, 2011).

Figure 9: *Cephalota (Taenidia) deserticoloides* from Rambla Salada (Herrera-Russert, 2017).



It is a halophile species known only as an exclusive specialist of a habitat typical of southeastern Iberia, the arid salt steppe (Ortiz et al., 1987). This habitat is considered to be of special interest, since it only occurs over small and fragmented extensions, normally forming part of endorrheic and salt-water systems of very high ecological interest, classified as natural spaces and Special Protection Areas for Birds: SPAs (http://www.murcianatural.carm.es/; http://www.magrama.gob.es/).

This habitat can be described as an interior saline plain that, although influenced by groundwater, remains dry throughout the year and has a vegetation coverage of less than 50% of its surface, consisting mainly of halophilic genera such as *Sarcocornia*, *Suaeda*, *Halocnemun*, *Frankenia*. Especially noteworthy are the several species of *Limonium* (Esteve et al., 1995).

Of the eight locations where the species has been recorded historically, the presence of *Cephalota deserticoloides* has only been confirmed in four of them in the most recent surveys. Two of them are located in the Region of Murcia, in the natural spaces of the Guadalentín salt marshes and in the cryptic wetlands of Ajauque and Rambla Salada (where it coexists with *C. littorea*). The other two populations are located in the province of Alicante, on a small plot adjacent to the town of San Isidro that lacks any protection status and in the Vinalopó River in Elche (López-López, personal communication) (Figure 10).

Figure 10. Rambla Salada (Murcia, Spain). Typical habitat of *C. deserticoloides* (Alejandro López-López).

Within this very specific habitat, *C. deserticoloides* develops its activity as a diurnal predator of other invertebrates. The adult phase is active only during the summer season and can be observed running, flying, and actively pursuing its prey on the flat extensions of open ground (Figure 11). In contrast, larvae occupy deep vertical galleries in the same substrate, from which they passively await and ambush their prey. These characteristics are generalizable to the natural history of most cicindelids (Pearson and Vogler, 2001).

Figure 11. The tiger beetle *Cicindela hybrida* hunting an ant (*Formica sp*). (https://www.naturbildarchiv-guenter.de/en/photo-reports/perfect-adaption-cicindela-hybrida/)

Conceptual and methodological aspects

Phylogenetic analysis

Knowledge of the phylogenetic relationships of organisms (Figure 12) is essential for the establishment of a taxonomic classification coherent with evolutionary history (Tautz et al., 2003; Vogler and Monaghan, 2007). It is also essential to draw up conservation strategies, identifying operational taxonomic units susceptible to being subject to special protection measures. Phylogenetic information is also crucial for systematics, since taxonomic categories should not be arbitrary abstractions but real entities that express the unique and distinctive historical perspective of each group of organisms.

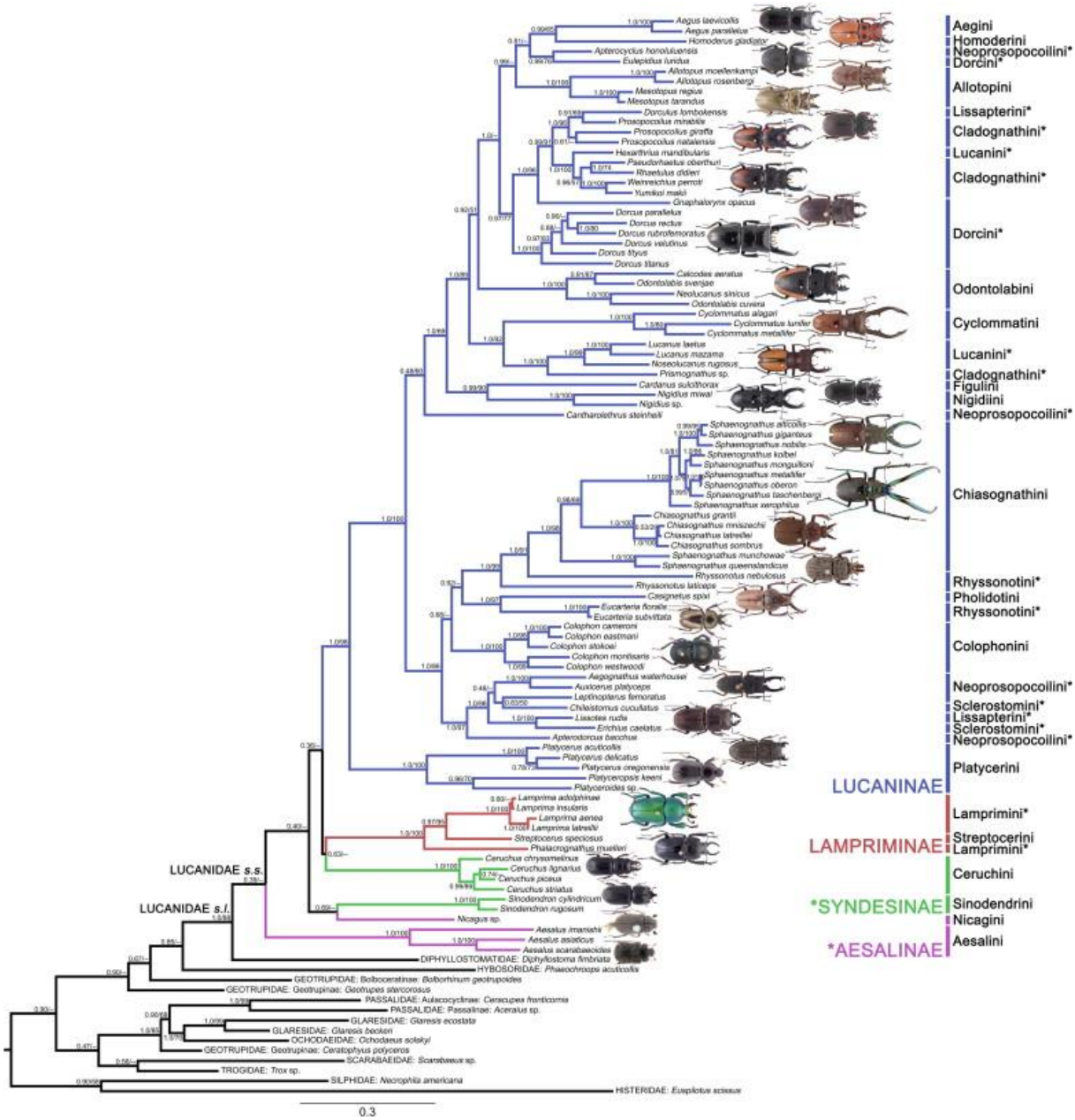
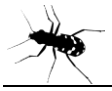


Figure 12. Bayesian topology showing the relationships within the family Lucanidae and outgroups (Kim and Farrell, 2015).

Phylogenetic inference aims to reconstruct the evolutionary relationships among organisms, among populations of a species, among species of a genus, among genera, etc. The approach of analysing the topology of a tree and dating its nodes let us to estimate times of divergence among taxa, in order to infer patterns and processes that have shaped the evolutionary history of a group.

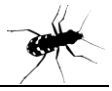


For a long part of its history, phylogenetic reconstruction, originally proposed by Willi Hennig (1950), has depended exclusively on morphological, ecological, ethological or similar characters. Nevertheless, the recent development of DNA-based methods and molecular phylogeny have provided a large number of markers to improve the knowledge of the phylogenetic relationships between organisms (Rodrigo et al., 2008). These markers are getting increasingly cheaper and more accessible every day, and provide more reliable results than traditional characters would. In addition, when combined with these traditional characters, molecular data are richer and produce a deeper insight on the evolutionary history of the analysed taxa.

Nowadays, phylogenetic inference plays a fundamental role in any study dealing with the evolution of a group of organisms. In the words of Yang and Rannala (2012): "Nowadays, every biologist needs to know something about phylogenetic inference".

The simplest molecular phylogenetic methods are those based on the genetic distances between the analyzed sequences, properly modified by nucleotide substitution models to correct the deviation between the number of observed differences and the actual number of mutations. Within these methods, one of the most popular is the Neighbor-Joining (Saitou and Nei, 1987), which is still widely used for some objectives such as DNA barcoding and evolutionary genomic analysis of particular genes.

The Maximum Likelihood algorithms (Felsenstein, 1981) search for the most probable tree according to the data. Maximum Likelihood allows for greater statistical flexibility by allowing evolutionary rates to vary by branch. These methods can be used to test alternative hypotheses, such as the search for the most suitable nucleotide substitution model (Goldman, 1993) or molecular clock model (Wilke et al., 2009).



One way to test the reliability of these methods is the bootstrap test (Felsenstein, 1985; Felsenstein and Kishino, 1993). This method repeats the analysis in multiple replicates of the original matrix, in each of which the characters have been randomly shuffled. The fidelity of a particular node is given by the percentage of these trees in which the original node can be found.

A more advanced way of applying Maximum Likelihood for inferring phylogenies is Bayesian Inference (Mau and Newton, 1997; Rannala and Yang, 1996; Yang and Rannala, 1997), which applies the Bayes Theorem, through an algorithm that uses Markov chain Monte Carlo to calculate the tree probability according to the data matrix. Finally, the posterior probability of each node is calculated on the most probable tree. It is a very expensive method in computer processing, but feasible for modern computers.

Phylogeography: haplotype networks

Phylogenetic trees adequately represent the relationships among species or entities of a higher taxonomic level, whose divergences are clear and correspond to speciation events. However, when establishing relationships between populations or other intraspecific entities, trees may not faithfully represent all the possible events that have contributed to the underlying diversity and distribution.

For these cases, phylogeographic networks are used (Posada and Crandall, 2001), consisting of a graph in which the different haplotypes, both those sampled in the studied population and those inferred, are linked by multiple branches and separated by the number of mutational steps that separate them (Templeton et al., 1992) (Figure 13).

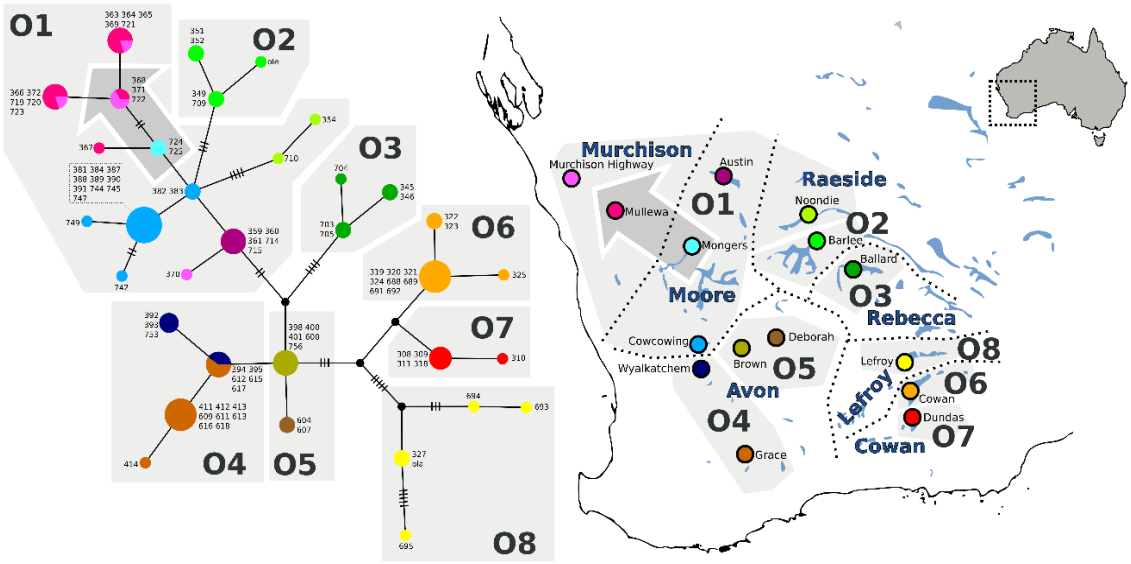


Figure 13: Phylogeographic network (López-López et al., 2016), showing the relationship between haplotypes and geographic distribution in the *Pseudotetracha oleadorsa* species complex from Western Australia.

In general, phylogeography integrates the phylogenetic aspects, which now take the form of a haplotype network and a geographical structure (Avise, 2000). Phylogeography focuses on representing and understanding the patterns and processes of separation of lineages at the intra-specific level, highlighting their geographical distribution as well as their characteristics and genetic relationships.

The most frequent outcome in phylogeographic investigations reveals that the genetic variation pattern shows a certain geographic structure that it is not only explained by the geography itself, but also by the historical relationship of populations and their interaction with geological or paleoecological events.

A haplotype is a unique nucleotide sequence. Individuals with identical haplotypes will be more closely related to each other. Haplotypes are one of the most common tools in phylogeography, usually displayed as a network (Gehring et al., 2012). In these networks, haplotypes are usually shown as disks, where the partition by colors usually corresponds to different localities. The haplotypes are connected by lines that represent the inferred genetic distance, with intermediate nodes representing inferred mutational steps that have not been found in the sampled population.

Haplotype networks provide a means to examine the history of genetic exchange between populations, with the potential to discern biogeographic patterns of genetic variation, which can ultimately be traced back to the genetic flow originated by common ancestors (Schaal et al., 1998). The combination and interpretation of both methods (phylogeny and phylogeography) is the most advisable approach to infer the population history of a species or a group of closely related species (López-López et al., 2016, 2015; Mardulyn, 2012; Posada and Crandall, 2001).

Mitochondrial markers

In order to carry out phylogenetic and phylogeographic studies, DNA sequences can be used to provide enough information on the relationship between organisms. The assumed hypothesis is that the most related organisms are those that have the most similar sequences, while the most distant ones have greater differences. It is also possible to calculate the time of divergence by correlating the changes in the sequences with the mutational rates (molecular clock). Nevertheless, it must be remembered that there is no universal molecular clock, as each gene and organism have a particular nucleotide substitution rate.

Mitochondrial DNA is the genetic material of mitochondria, the organelles that generate energy for the cell through a process called oxidative phosphorylation, which uses oxygen and simple sugars to create adenosine triphosphate (ATP), the main energy source of cells (Nass and Nass, 1963).

The animal mtDNA is a small circular double-stranded molecule (15-20 kb) (Figure 14). It includes 37 genes, of which 22 genes code for tRNA, 2 genes code for rRNA and 13 genes code for proteins that participate in oxidative phosphorylation (Figure 14).

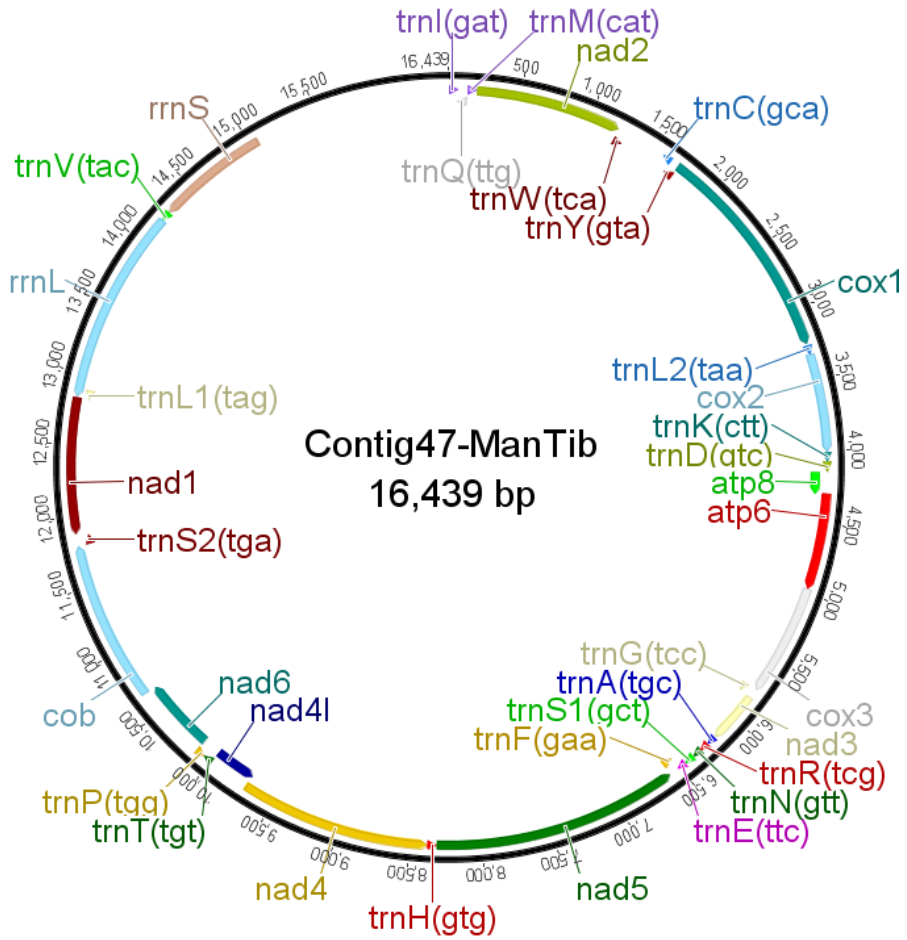
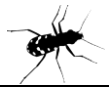


Figure 14: Mitochondrial genome of the tiger beetle *Manticora tibialis* (López-López and Vogler, 2017), with labels indicating the position of the genes.

Mitochondrial DNA (mtDNA) has great potential for inferring phylogenies and evolutionary relationships at low taxonomic level (species and genera) due to its high variability compared to nuclear DNA (Avise, 2000). Mitochondrial DNA has several additional characteristics that makes it more appropriate than nuclear DNA for phylogenetic analysis.

First, the study of mtDNA is easier than that of other fragments, since the size of the molecule is smaller than nuclear DNA. In addition, due to the large number of copies present in each cell, its extraction yields better results. It also has a very high percentage of coding DNA (absence of introns) and an apparent lack of repetitive DNA and other artefacts that would otherwise disturb phylogenetic estimations. Additionally, recombination does not take place in mtDNA, since it is only



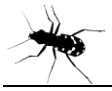
maternally inherited. This implies that the only changes that may have occurred in a mitochondrial DNA lineage are due exclusively to mutations (Avise et al., 1987). Although some cases of heteroplasmy (different mitochondrial populations in an individual) and other artefacts have been described (Galtier et al., 2009), they represent exceptional events.

One of the most widely used fragments for this kind of analysis, due to its particular high variation rate (Pons et al., 2010), is the subunit 1 of the cytochrome c oxidase (*cox1*). This fragment has been successfully used in numerous phylogenetic and phylogeographic works, many of them studying the order Coleoptera (Martínez-Navarro et al., 2004; Maus et al., 2001) and specifically in cicindelids (García-Reina et al., 2014; López-López et al., 2015).

DNA barcoding

DNA barcoding (Hebert et al., 2003) is a taxonomic method that uses a short genetic marker in the DNA of an organism to identify the taxon to which it belongs. Its goal is not to determine proximity or kinship patterns, but to identify the sample against a preestablished genetic database (such as BOLD or GenBank). One of the applications of this approach is to ascribe larval stages to species in which these stages are morphologically difficult to identify, or simply when the larvae have not been described (Ahrens et al., 2007).

The most common fragment used for barcoding animals is a mtDNA segment of ~600bp included in the *cox1* gene. Due to its characteristics, this fragment has been sequenced in numerous studies, giving rise to a massive worldwide database that includes more than 4.7 million of barcodes.



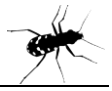
Environmental DNA

The assessment of species distribution, presence and ecological patterns is often a critical first step for biological studies within many disciplines, such as biogeography, conservation biology and ecology. However, many species are difficult to detect due to their scarcity, reclusive life style or simply due to extended periods of dormancy or during certain developmental stages. In these cases, a promising but still novel approach takes advantage of the persistence of DNA in the environment to detect the presence of a species. Short mitochondrial markers have been used for this approach due to their easier amplification and their resolution to identify species. The extraction of DNA from soil has been used routinely to identify the microbial or animal biodiversity in soil samples (Andújar et al., 2015; Venter, 2004), while more recently this approach has been applied to wider settings such as the analysis of DNA in water samples to detect the presence and identity of fish and frogs (Ficetola et al., 2008).

Indeed, the concept and application of environmental DNA (eDNA) is a young and expanding field that will provide otherwise difficult to achieve mechanistic insights into ecological and evolutionary processes. Foremost among these is the ability to explore ecosystem-level processes and patterns that would otherwise be out of reach (Bohmann et al., 2014).

Geometric morphometrics

Visualization of shape change remains an important tool for understanding morphological variation, while at the same time allowing us to address an increasingly wide range of questions about the evolution and development of organisms (Klingenberg, 2010). Shape is defined technically as all the geometrical features of an object once we have excepted its size, position and orientation (Dryden and Mardia, 1998).



Geometric morphometrics is a quantitative method which could be defined as the study of shape in a two or three-dimensional space (Bookstein, 1996). The typical approach in geometric morphometrics places its emphasis on the visualization of shape variation based on selected landmarks, or discrete anatomical points of reference, that are asigned to the same view of the same biological structure across many individual specimens. These points must be defined carefuly so that they show true homology in their correspondence to the position of a particular feature in an object. For example, maxima are generally bad reference points for a landmark-based geometric morphometric approach, as they need not (and often do not) correlate with a true structural homology. In such cases, other approaches such as semilandmark sliding or Fourier analysis are preferable (Zelditch et al., 2012).

The great advantage offered by geometric morphometrics is that data can be directly visualized with statistics programs (Klingenberg, 2013; Rohlf and Marcus, 1993) allowing to infer the nature and pattern of morphological change.

This technique has often been used to study differences the wing shape across various orders of insects, most notably the Hymenoptera, Diptera and Lepidoptera (Roggero and Passerin d'Entrèves, 2005; Su et al., 2015). Within the tiger beetles, it should be noted that a classical morphometric study in the genus *Cicindela* was carried out by Franzen (2007) on several populations of tiger beetles of the *Cicindela campestris* group from southern Turkey and Lebanon. This work investigated the morphometric ratios, male genitalia, and color patterns of several taxa from this species complex (Figure 15).

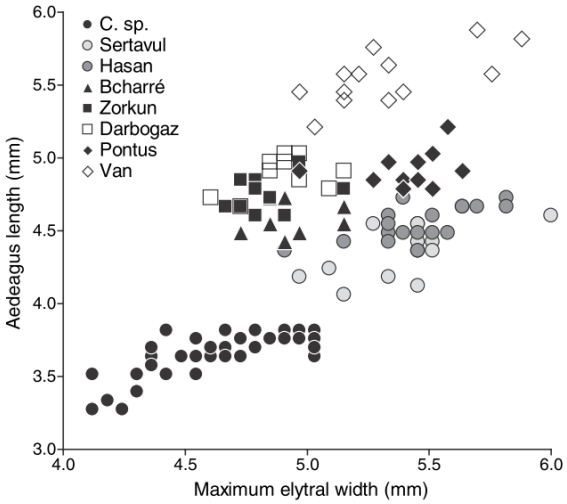
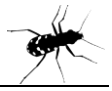


Figure 15: Partial results of the analyses carried out by Franzen et al. (2007), indicating the relation of aedeagus length and elytral width within specimens of the *Cicindela campestris* species complex.

Population ecology: mark-recapture

One of the most fundamental issues in population ecology is the quantification of the number of individuals in a population. In the frequent cases where a census is not possible, it is necessary to implement estimation methods. Of these, mark and recapture obtains numerical estimates of population parameters such as total number, survival probabilities and probability of recapture (Pradel, 1996; Sandercock, 2003). Part of the population is captured, marked and returned to freedom. On subsequent occasions, the capture and marking is repeated, taking note of the captured individuals that have already been marked on previous occasions. Different types of models can be applied to the mark and recapture data matrix, consisting of individualized records of presence/absence in each capture event.

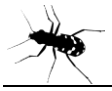
The simplest and oldest model is the Lincoln-Petersen estimator (White, 1996) that only uses two capture occasions. More complex and recent models, referring to open or closed populations, such as those of Jolly Seber (Schwarz, 2001) or Cormack Jolly Seber (CJS) (Schwarz and Stobo, 1999), establish different types of restrictions on capture parameters and recapture probability between samplings. The CJS model, more widely used for practical reasons, is a restricted model that imposes a

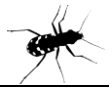


single value for Φ (fi) and for g throughout the study, these being probability of survival and recapture from one event to the next. These restrictions are often acceptable when contrasted with the limitations and scope of a mark-recapture approach, especially given the benefit they bring by reducing model complexity and parameterization (Amstrup et al., 2010).

In all these models, the following premises must be met (Amstrup et al., 2010):

1. The population remains constant in composition and size.
2. The animals do not lose their marks, and they remain recognizable.
3. All marks are correctly registered.
4. The animals act independently of each other.





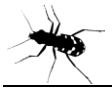
Aims and hypotheses

The main objective of this thesis is to offer a stepwise approach to the knowledge of the evolution of *C. deserticoloides*, starting with its origin within the Palearctic genus *Cephalota*, then to focus on the internal dynamics and the relationship between its populations.

For this purpose, the relationships and differences between representative species of the genus *Cephalota* will be studied at a phylogenetic level, while populations of *C. deserticoloides* will receive further attention at the genetic, morphometric and phylogeographic levels. The data provided by a mark-recapture study on one of its four current populations will in turn clarify the real conservation situation faced by the species by providing the first quantitative population estimates for this species.

The hypotheses that will be tested in this thesis are the following:

1. The species of the genus *Cephalota* are genetically related to each other according to a geographic pattern, whereby neighbouring or overlapping species are more closely related.
2. Within the genus *Cephalota*, there also exists a pattern of vicariance specifically pertaining *C. deserticoloides*.
3. The extant populations of *C. deserticoloides* are differentiated genetically and morphologically. Interpopulation differentiation is strong enough to gain further insights into the species' history and current distribution patterns.
4. Species identification of the larval stage of tiger beetles can be achieved by using exclusively environmental DNA from the soil around or inside the larval burrow. The information obtained in this way can be used to further assess the biology and conservation efforts required for the vulnerable Iberian endemic species *Cephalota deserticoloides*.



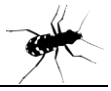
5. The entire population size of *C. deserticoloides* falls within the range of those of other vulnerable and endangered tiger beetle species.
6. *C. deserticoloides* is not evenly distributed with other tiger beetle species across the habitat where it occurs, but shows mutual segregation with these species at a temporal and spatial scale.

The ultimate objective of this study is to offer a precedent that will allow to i) better focus the future conservation strategies for this species through a reassessment of its status and the feasibility of the population viability analysis, and ii) have a more in-depth knowledge of its ecology. For the latter purpose, the objective of this thesis is to develop a method to differentiate larval galleries of *C. deserticoloides* from those of other cicindelids with which they coexist, through a non-invasive procedure based on genetic analyses of soil-bound larval biological remains.

The specific objectives of this thesis are the following:

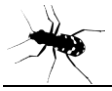
Chapter 1. Molecular phylogeny and origin of the halophile tiger beetle genus *Cephalota* (Coleoptera: Cicindelidae): The aim of this chapter is to analyse the mitochondrial *cox1* gene to shed some light on the degree of genetic relatedness among several species of the genus *Cephalota*, and to test whether geographically close or even overlapping species are also phylogenetically related to each other.

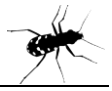
Chapter 2: Geometric morphometrics and molecular characterization of the endangered endemism *Cephalota (Taenidia) deserticoloides:* The main aim of this chapter is to study the extent to which the known populations of *C. deserticoloides* are differentiated based on a fragment of the subunit 1 of the mitochondrial cytochrome c oxidase and on geometric morphometrics. This will allow us to establish the degree of inter and intrapopulation diversity and genetic flow among them, infer the history of the species and attempt to establish the processes that explain its current distribution.



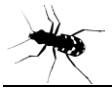
Chapter 3: Non-invasive identification of endangered tiger beetle species (Coleoptera: Cicindelidae) from soil samples: The aim of this chapter is to achieve identification of the larva by using exclusively environmental DNA from the soil around or inside the larval burrow to confirm species identity. This will provide a useful tool in the population assessment and conservation efforts without affecting living beetles of the vulnerable Iberian endemic species *C. deserticoloides*.

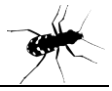
Chapter 4: Mark-recapture population estimates of *Cephalota deserticoloides:* The aim of this chapter is to provide some rough estimates of the entire population size of *C. deserticoloides* that may set the stage for an as yet impossible assessment of its precise status and long-term tendencies through techniques such as PVA. In addition, we also expect to gain insights into the possible mutual segregation of several coexisting tiger beetle species at a temporal and at a spatial scale.





Chapter 1: Molecular phylogeny and origin of the halophile tiger beetle genus *Cephalota* (Coleoptera: Cicindelidae)





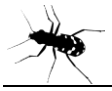
Abstract

The halophile tiger beetle genus *Cephalota* is distributed from the Mediterranean Sea to Central Asia, and includes some endangered endemisms restricted to reduced areas in the Iberian Peninsula. It is currently considered to include the subgenera *Cephalota* and *Taenidia*. The genesis of this genus has been related to the closure of the Tethys Ocean and the formation of the Mediterranean Sea. In this work, a phylogenetic tree of eleven *Cephalota* species is inferred. The observed phylogenetic relationships between these species challenges the hypotheses previously formulated about the evolution of this group. Additionally, the results do not support the established systematics of this genus, suggesting that the subgenus *Taenidia* is not monophyletic. The origin of *Cephalota* is dated back to 13.5 million years ago, once the Mediterranean Sea was already formed, rejecting the hypotheses previously proposed about the origins of this genus. Alternative hypotheses concerning the changes on the suitable habitat for this halophile group caused by fluctuating levels of the Mediterranean Sea are proposed.

Introduction

The systematics of a taxonomic group should reflect the phylogenetic relationships among its members (Andújar et al., 2014; Tautz et al., 2003; Vogler and Monaghan, 2007). Phylogenetic inference based on molecular data has become an essential tool for the knowledge of the evolutionary history of taxonomic groups, and for inferring the processes that contributed to its current distribution and diversity. This information should be contrasted and examined in the light of other data in order to provide a reliable overview of the evolution of a taxonomic group, according to the principles of the integrative taxonomy (Dayrat, 2005; Padial et al., 2010).

Tiger beetles are a widely distributed family that includes over 2600 species (Pearson and Cassola, 2005, 1992; Pearson and Vogler, 2001), spanning a high variety of

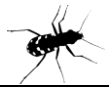


habitats. Tiger beetles have received more attention than other Adephaga in fields such as natural history, population dynamics, communities, patterns of diversity, and the taxonomy of certain groups (Cardoso and Vogler, 2005; Knisley and Schultz, 1997). This amount of data, together with their high habitat specialization, strengthens the case for their use as biological indicators (Pearson and Vogler, 2001; Rodríguez et al., 1998).

The genus *Cephalota* includes 21 species within the Palearctic region (Putchkov and Matalin, 2017). It is a halophile group that was likely already found populating the coasts of the Tethys Ocean 35 million years ago (Hieke, 1983). This genus is distributed throughout the Mediterranean coast and deep into central Asia, with the species *C. littorea* and *C. vonderdeckeni* reaching as far south as the Sudan region and Somalia (Gebert, 1999; Werner, 2000). In this context, *C. deserticoloides*, an Iberian endemic, has been postulated to be a vicariant taxon whose closest relative could be *C. deserticola*, a Eurasian species (Diogo et al., 1999). Several species, such as *C. deserticoloides* and *C. dulcinea*, have extremely restricted distributional patterns (López et al., 2006; Rodríguez-Flores et al., 2016), which makes them interesting endemic insect species for wildlife conservation plans and has already warranted the inclusion of *C. deserticoloides* as an endangered species in the Spanish Red Book (Lencina and Serrano, 2011).

Most *Cephalota* tiger beetles are placed within the subgenus *Taenidia*, with the exceptions of *C. hispanica*, *C. turcica*, *C. pseudodeserticola* and *C. luctuosa*, which are considered to be *Cephalota s. str.* Alternatively, *C. maura*, was originally placed in its own subgenus, *Cassolaia*, and has been either considered a suspect member *Cephalota* (Lorenz, 2005; Rivalier, 1950) or as a separate genus (Putchkov and Matalin, 2017).

Mitochondrial DNA is suitable for phylogenetic and phylogeographic analysis for several reasons (Avise, 2000). It is easy to extract in sufficient quantity and its rate of mutation is high (Hewitt, 2004), which allows for the resolution of taxonomical relationships at a closer time scale than those resolved by most nuclear DNA.



Additionally, the maternal inheritance of mitochondrial DNA prevents the effects derived from meiotic recombination. One of the most widely used fragments for this kind of analyses in insects, because of its high degree of variation (Pons et al., 2010), is the subunit 1 of the cytochrome c oxidase (*cox1*), which has been successfully employed in several phylogenetic and phylogeographic works with Coleoptera (Martínez-Navarro et al., 2004; Maus et al., 2001), and specifically with Cicindelidae (García-Reina et al., 2014; López-López et al., 2015).

The aim of this paper is to analyse the mitochondrial *cox1* gene to shed some light on the degree of genetic relatedness among several species of the genus *Cephalota*, and to test whether geographically close or even overlapping species are also phylogenetically related to each other.

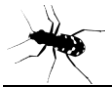
Material and methods

Specimens

Specimens belonging to eleven different species were used in this study (Table 1).

Table 1. List of specimens used in this work. The outgroups are marked with an asterisk.

Species	**N**	**Locality**	**Material**
Cephalota atrata	2	Russia, lake Elton	Dried specimens
Cephalota besseri	3	Ukraine	Dried specimens
Cephalota chiloleuca	3	Russia, lake Elton, Omsk, Buryatia	Dried specimens
Cephalota circumdata	9	Spain, Murcia	GenBank sequences
Cephalota deserticoloides	4	Spain, Murcia	Fresh specimens
Cephalota dulcinea	5	Spain, Toledo	GenBank sequences
Cephalota elegans	6	Russia, lake Elton	Fresh specimens (larvae)
Cephalota hispanica	4	Spain, Cádiz	Fresh specimens
Cephalota littorea	3	Spain, Cádiz, Murcia	Fresh specimens
Cephalota zarudniana	1	Iran	Dried specimens
Cicindela campestris *	1	-	GenBank sequence
Ellipsoptera sperata *	1	-	GenBank sequence
Eugrapha minuta *	1	-	GenBank sequence



Sequence obtention

DNA was extracted from the specimens, previously fixed in absolute alcohol, using the Invisorb® Spin Tissue Mini Kit®. For the dried specimens an additional method using Chelex® was tested. Before amplifying the gene fragment of interest, the yield of each extraction was checked with a Thermo Fisher Scientific® Nanodrop® 1000. A region of ~800 bp from the mitochondrial cytochrome c oxidase subunit 1 was amplified using the primers Jerry (CAACATTTATTTTGATTTTTTGG) and Pat (TCCAATGCACTAATCTGCCATATTA) (Simon et al., 1994). The PCR consisted of an initial activation at 94°C for 5 min; 40 amplification cycles consisting of 94 °C for 30 s (denaturalization), 50 °C for 30s (annealing), and 72 °C for 1 min (extension); and a final extension of 72°C for 10 min.

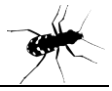
The success of the PCR was ascertained in a 1.5% agarose gel, and the PCR products were sequenced in Macrogen (Amsterdam, Netherlands).

Phylogenetic analysis

The sequences were aligned in GENEIOUS R7 (Biomatters, available at http://www.geneious.com), using the MUSCLE algorithm, and manually edited to correct possible sequencing errors and to delete low-resolution terminal segments.

Additional sequences from GenBank corresponding to *C. circumdata* and *C. dulcinea* were added. Sequences of *Cicindela campestris*, *Ellipsoptera sperata* and *Eugrapha minuta* were also included at this stage as outgroups.

The Bayesian Inference analyses were performed in MRBAYES v3.1.2 (Ronquist and Huelsenbeck, 2003), running for 1000000 generations and sampling a tree for every 1000 generations, using the most appropriate model according to jMODELTEST v2.1.7 (Darriba et al., 2012).



Four separate Bayesian Inference analyses were also carried out in BEAST 1.8.3 (Drummond et al., 2012), in which two different clock models and two tree priors were combined. The analyses were run in the CIPRES Science Gateway (Miller et al., 2010). The best combination was selected according to the Bayes factors calculated in TRACER 1.6 (available from http://beast.bio.ed.ac.uk). The molecular clock was based on the rates obtained by Andújar et al. (2012) for the *cox1* fragment. The analyses ran for 50 million generations and the consensus tree for the best clock and tree prior was built using TREEANNOTATOR (distributed with BEAST).

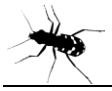
Results

DNA extraction, sequences and models

The extraction kit proved to be the better of the two methods of DNA extraction on account of higher yields of DNA concentration in a greater number of samples. Thus, only the DNA obtained with this method was used for further amplification and sequencing.

The resulting fragment had a length of 571 bp. The most appropriate model of nucleotide substitution was the General Time Reversible, corrected with a gamma distribution and the proportion of invariant sites (GTR+I+Γ). This resulted in the phylogenetic tree depicted in Figure 1.

For the BEAST analyses, the strict clock performed better than the log-normal relaxed clock, an expected result assuming the species considered have all evolved at similar rates. The Yule prior proved the better option compared to the Coalescent model. The resulting tree is depicted in Figure 2.



Phylogenetic results

The phylogenetic trees separate the *Cephalota* species considered in this study, with one exception (Figures 1 and 2). They show that *Cephalota* taxa make up a separate clade from the outgroup genera *Ellipsoptera*, *Eugrapha* and *Cicindela*. Within *Cephalota*, there is one clade with the samples corresponding to *C. atrata*, *C. chiloleuca*, *C. circumdata*, *C. elegans*, *C. littorea* and *C. zarudniana*; and a second clade grouping *C. deserticoloides*, *C. hispanica*, *C. dulcinea* and *C. besseri* in tree from BEAST (Figure 2). These last species appear as an unresolved polytomy with the exception of the closely related *C. hispanica* and *C. dulcinea* in the tree from MRBAYES (Figure 1).

Within the first clade, relationships between species hold in both trees with the exception of *C. littorea*. *C. zarudniana* is most basal, while the pairs formed first by *C. atrata* and *C. chiloleuca* and then by *C. circumdata* and *C. elegans* branch out within the clade. *C. littorea* alternates between forming a sister species to the basal *C. zarudniana* in the BEAST tree and being sister to the solid *C. circumdata* + *C. elegans* clade. *C. atrata* and *C. chiloleuca* are closely related in both trees.

Within the second clade resolved in BEAST, *C. deserticoloides* is most basal, with *C. besseri* as a sister species to the tight *C. dulcinea* + *C. hispanica* clade. This close proximity between *C. dulcinea* and *C. hispanica* still holds in the MRBAYES tree and, while not surprising from a geographic, ecological and morphological point of view, directly contravenes the current taxonomical status of *C. hispanica* at the subgeneric level.

According to the chronogram (Figure 2), the origin of the genus *Cephalota* goes back to 13.5 million years ago (with a 95% confidence interval between 8.1–27 million years ago). Each species (including the pair *C. atrata* + *C. chiloleuca* as a single taxa) is estimated to have originated less than 1 Mya, except *C. besseri* (1.7 Mya).

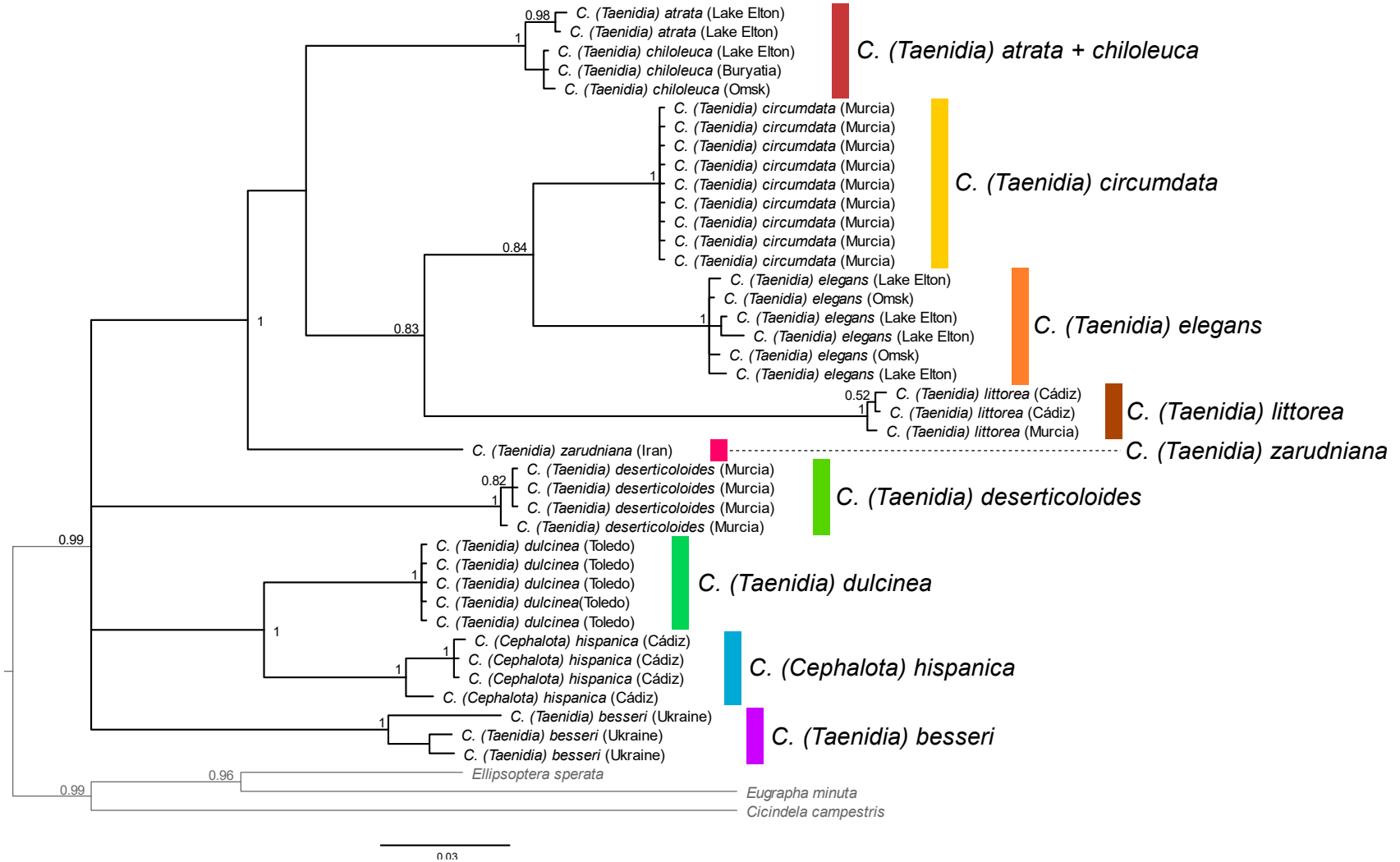


Figure 1: Bayesian inference tree based on the *cox1* alignment. Numbers in the nodes correspond to the posterior probability (upper number). Branch lengths are proportional to the phylogenetic distance between samples. The taxonomic identity and the locality of each sample are provided as terminal tips. The outgroups are greyed for clarity.

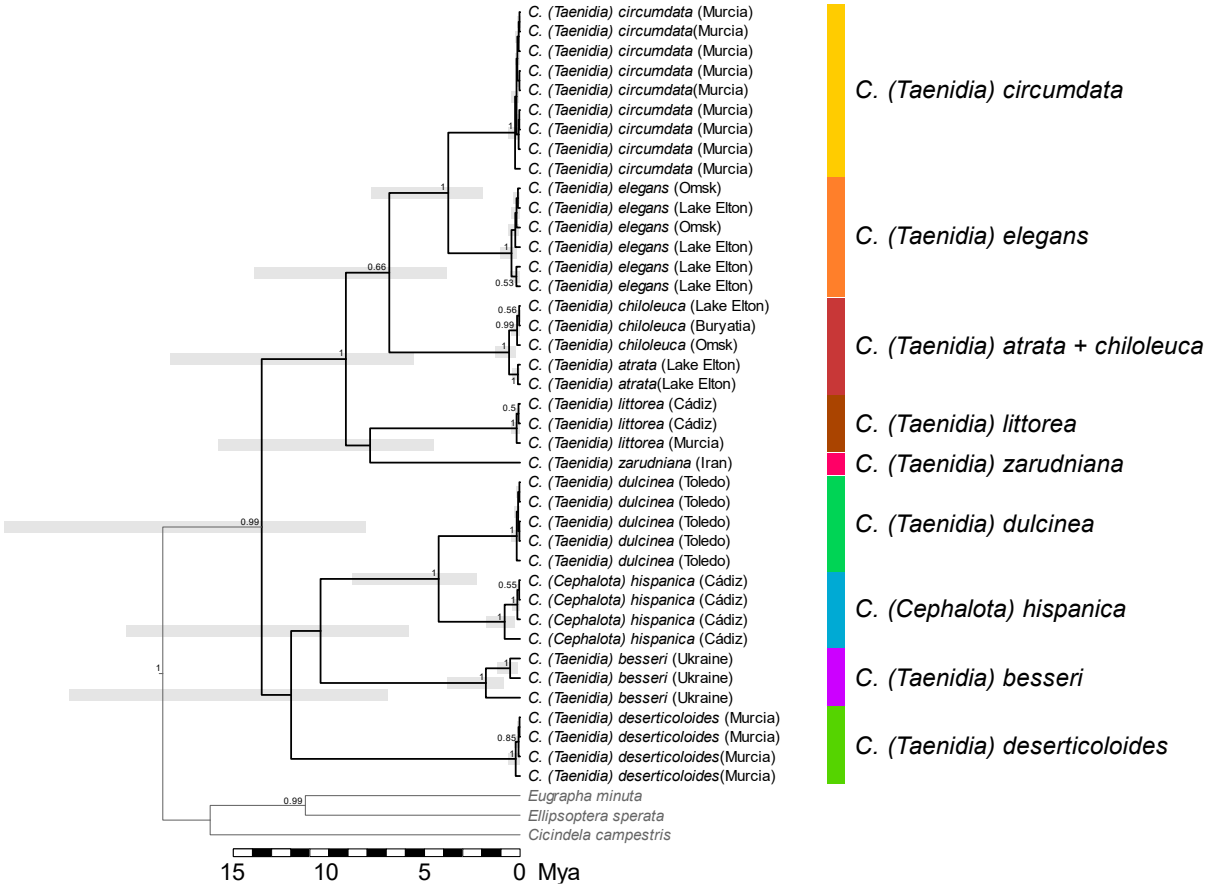
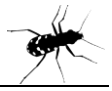


Figure 2: Chronogram of the *cox1* fragment reconstructed in BEAST using the rates by Andújar et al. (2012). A timescale is provided below (time in million years before present). Ninety-five per cent confidence intervals for the ages of clades are indicated with grey bars. The taxonomic identity and the locality of each sample are provided as terminal tips. Numbers in the nodes represent the posterior probability. The outgroups are greyed for clarity.

Discussion

The main aim of this chapter was to assess the phylogenetic relationships between some representative species of *Cephalota*, and to test if the geographical distribution, especially in those species that overlap, is related to their phylogenetic proximity.

According to our results, it seems that relatedness between species holds a weak overall correlation, if any, with geographical distribution. There are two notable exceptions to this, those being the pairs *C. atrata* + *C. chiloleuca* and *C. dulcinea* + *C. hispanica*, which are composed of geographically very close or even overlapping



species that, in addition, bear strong resemblance to each other in morphological terms (López et al., 2006). In fact, *C. atrata* and *C. chiloleuca* should be considered as a single species (nominally *C. atrata*), based on their phylogenetic placement and the reduced genetic differences between them (Table 2).

Table 2: Maximum genetic distances within species (Max intra) and minimum genetic distances to other species (Min inter). *C. atrata* and *C. chiloleuca* are shown both as separate species and as a single taxon.

Species	**Max intra**	**Min inter**
C. atrata	0.0018	0.0088
C. chiloleuca	0.0018	0.0088
C. atrata+chiloleuca	0.0124	0.0935
C. circumdata	0.0000	0.0612
C. elegans	0.0106	0.0612
C. littorea	0.0035	0.1046
C. zarudniana	NA	0.0949
C. deserticoloides	0.0018	0.1112
C. dulcinea	0.0000	0.0647
C. hispanica	0.0178	0.0647
C. besseri	0.0363	0.1044

Another result with taxonomic implications is the placement of *C. hispanica*, considered as a member of the subgenus *Cephalota s. str.*, within the species classified in the subgenus *Taenidia*. This indicates that either *C. hispanica* should be reclassified as a member of the subgenus *Taenidia*, or that the validity of this subgenus should be revised. Nonetheless, data from more species should be gathered and analyzed before properly proposing any taxonomic change.

The chronogram obtained in BEAST places back the origin of the genus *Cephalota* to 13.5 million years ago (Figure 2), once the Mediterranean Sea was already formed. This clashes with the proposed hypothesis that linked the origin and diversification of *Cephalota* with the evolution of this inner sea (Gebert, 1991). The *Cephalota* ancestor gave rise to two lineages, each occupying the Mediterranean and the Central Asia areas (Figure 3).

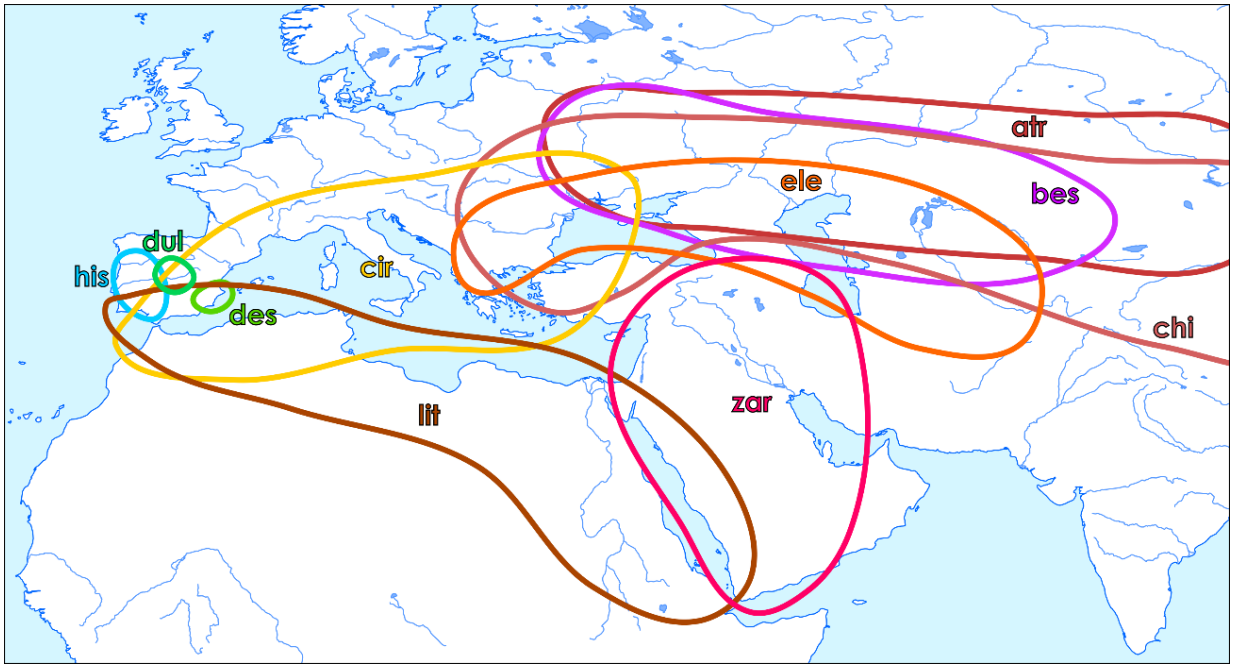


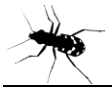
Figure 3: Approximate distribution of the species analyzed in this work. atr: *C. atrata*, bes: *C. besseri*, chi: *C. chiloleuca*, cir: *C. circumdata*, des: *C. deserticoloides*, dul: *C. dulcinea*, ele: *C. elegans*, his: *C. hispanica*, lit: *C. littorea*, zar: *C. zarudniana*. Colors correspond to those used in the phylogenetic trees.

The first lineage is divided in two clades, one distributed along the southern shores of the Mediterranean Sea (*C. littorea* and *C. zarudniana*), and other along the northern shores of the Mediterranean and the Central Asian steppes (*C. circumdata*, *C. elegans*, and *C. atrata*). It is possible that the origin of these two lineages may be linked to a vicariance event, where an original population became split by the sea, which led to speciation by geographical barriers. Lately, the divergences within each clade may be related to dispersal event in a longitudinal axis. Molecular information from other *Cephalota* species, *C. tibialis* and *C. arabiana*, not included in this work, may show that they are possibly related to *C. littorea* (Gebert, 1991). This information will complete our understanding about the evolutionary history of this group. Additionally, information from *C. littorea* collected across its complete distribution area, will clarify the taxonomic status of its subspecies, which have sometimes been proposed as separate species (Gebert, 1991).

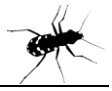
Noteworthy, the divergence between the north Mediterranean *C. circumdata* and the Ponto-Caspian *C. elegans* is concurrent with the Messinian crisis (Andeweg, 2002; Manzi et al., 2013), which suggests that the separation between these species may be related to this event that drastically changed the environmental conditions in the Mediterranean basin.

The second lineage is formed by species with a restricted distribution in the Iberian Peninsula (*C. dulcinea*, *C. hispanica* and *C. deserticoloides*). This suggests the role of the Iberian Peninsula as a center of speciation for this group of *Cephalota*. Within this lineage it is also included the Central Asian *C. besseri*, but additional molecular information from other related species is necessary in order to assess the evolution of these taxa, and how the Iberian species are related to this Central Asian taxon. Especially, molecular data from *C. deserticola* are needed in order to test the hypothesis that links this species to *C. deserticoloides*, a critically endangered endemism from southeast Spain (Diogo et al., 1999). According to our results, *C. deserticoloides* originated from a lineage that split from the other *Cephalota* quite early, 12 million years ago. This highlights the phylogenetic singularity of this species, which reinforces the necessity of conservation measures to protect this ecologically important taxon from the threats that challenge its future.

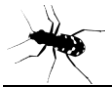
It appears that the evolutionary history of *Cephalota* is made up by dispersal events of ancestral populations across the Mediterranean basin, followed by episodes of habitat fragmentation, isolation and speciation. This dynamics can be easily reconciled with the ebb and flow of suitable habitats for this halophile group caused by fluctuating levels and profiles of the Mediterranean Sea. Such is the case of currently vulnerable species like *C. deserticoloides*, and of individual populations of *C. hispanica*, *C. dulcinea* and *C. circumdata* (López et al., 2006). Conversely, the periods during which these habitats increased their extension may be the cause of the wide distribution range of other taxa, such as *C. littorea*, *C. besseri* or *C. atrata* + *chiloleuca*. Whereas the existence of salty place geographically well connected has probably



favoured dispersal of *Cephalota* taxa (i.e. during the Messinian salinity crisis 5-5.8 Mya, Ben-Avraham (2018)) the opening of the Gibraltar strait and tectonic movements have caused notable isolation among salt places, thus favouring speciation.



Chapter 2: Geometric morphometrics and molecular characterization of the endangered endemism *Cephalota (Taenidia) deserticoloides*



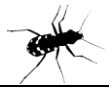
Abstract

The halophile tiger beetle *Cephalota deserticoloides* is an endemic species, with a distribution restricted to a few sites in south eastern Spain, where it occupies only the patchy arid saline steppe type habitat. Due to this high level of patchiness and ecological specialization, its current populations are assumed to be local and possibly isolated. Here, we use a twofold approach to assess the population dynamics and origin of the species, combining genetic-based phylogeography with a geometric morphometric approach. The results reinforce our understanding of *C. deserticoloides* as a small group of isolated populations with little current contact with each other. Additionally, we gain some insights into the possible origins and ancient dispersal of the species. These results improve our understanding of the species and, as a result, of the ecological dynamics of the area it inhabits, which will enable an improved management and protection of the Iberian saline steppe and of *C. deserticoloides*.

Introduction

Tiger beetles are a widely distributed family composed by more than 2600 species, spanning over a wide range of habitats (Pearson and Cassola, 1992, 1992; Pearson and Vogler, 2001). Tiger beetles have received more attention than many other insect taxa in fields such as natural history, population dynamics, communities and patterns of diversity and the taxonomy of certain groups within the family (Cardoso and Vogler, 2005; Knisley and Schultz, 1997; Vogler and DeSalle, 1993). This amount of data, together with their high specialization for their habitats, strengthens the case for their use as biological indicators (Pearson and Vogler, 2001; Rodríguez et al., 1998).

The genus *Cephalota* if formed by 25 halophile species (Wiesner, 1992; Puchkov and Matalin, 2017) , whose origin and diversification has been related to the expansions

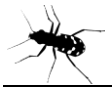


and disappearances of suitable habitat following the evolution of the Mediterranean Sea (Chapter 1). This genus is distributed through the Mediterranean coast and deep into central Asia, with the species *C. littorea* and *C. vonderdeckeni* reaching as far south as the Sudan and Somalia (Gebert, 1999; Werner, 2000).

Within the genus *Cephalota*, *C. deserticoloides* is currently placed within the subgenus *Taenidia* (Lorenz, 2005; Rivalier, 1950), which also encompasses other three species in Iberia (Serrano, 2013): *C. circumdata* and *C. littorea*, both found along the Mediterranean coast, and *C. dulcinea*, an Iberian inland endemism (López et al., 2006; Rodríguez-Flores et al., 2016). *C. deserticoloides*, however, is more restricted and shows a pronounced affinity towards arid salt steppes, having been found only at some isolated localities in the provinces of Murcia and Alicante (Diogo et al., 1999). The species is believed to be a vicariant taxon whose closest relative could be *C. deserticola*, a Eurasian species (Diogo et al., 1999).

Cephalota deserticoloides (Codina, 1931) stands out from among the 23 Iberian tiger beetle species (Serrano, 2013) because of this extremely restricted geographic distribution, which has contributed to its inclusion as a vulnerable species in the *Spanish Red Book of Endangered Invertebrates* (Lencina and Serrano, 2011). Its unique salt steppe habitat has historically been heavily disturbed by drainage and desalination, transformed into agricultural land and, more recently, into rubbish dumps, illegal buildings and industrial complexes. The remaining populations are confined to a few small and fragmented patches of habitat (Diogo et al., 1999).

The genetics of *C. deserticoloides* have been studied by Diogo et al. (1999), who analyzed an 1896 bp fragment from three different individuals captured at different localities. These samples showed very low intraspecific genetic diversity. However, the results obtained by López-López and Galián (2010) hinted at that this diversity could be greater than that previously estimated.



Landmark-based geometric morphometrics are a powerful shape-analysis tool for taxonomists and anatomists (Alibert et al., 2001; Gumiel et al., 2003; Rohlf, 1990). Insect wings are an ideal biological structure for this kind of analysis (Pavlinov, 2001); however, in beetles there are comparatively few studies of this kind covering hind wing analysis, with most studies focused on Polyphaga (Bai et al., 2011). Geometric morphometrics methods will be used here to test if there are significant differences in hindwing morphology between the studied populations, which will allow us to further refine our view of the population structure of *C. deserticoloides*.

The main aim of this paper is to study the extent to which the known populations of *C. deserticoloides* are differentiated based on a fragment of the subunit 1 of the mitochondrial cytochrome c oxidase and on geometric morphometrics. This will allow us 1) to establish the degree of inter and intrapopulation diversity, 2) to better know the history of the species and 3) to attempt to establish the processes that explain its current distribution.

Material and methods

Specimens

Most of the specimens were collected over the course of several visits to different localities between late May and early July 2015, to assess the presence of populations of *C. deserticoloides* (Figure 1). Sites were selected in accordance with the existence of historical records reported by Lencina and Serrano (2011) and visiting other candidate localities areas that seemed to be apparently suitable.

A total of 31 specimens were gathered from three sites (Table 1): i) Alhama and ii) San Isidro de Albatera, both located along a 100 km stretch formed by the Segura valley and the river Guadalentín; and iii) Rambla Salada, located in the crypto-wetland system of Ajauque and Rambla Salada in the Abanilla-Fortuna basin (Esteve et al., 1995).

Table 1: Specimens captured during this study. Three additional samples of *C. deserticoloides* were included, as well as other *Cephalota sp.*, all of them from the GenBank database.

Species	**Site**		**Date**	**N**
C. deserticoloides	Alhama	37°51'25.24"N,1°22'42.18"W	06/22/2015	9
C. deserticoloides	Rambla Salada	38°7'22.09"N,1°6'39.07"W	06/17/2015	13
C. deserticoloides	San Isidro	38°9'57.71"N,0°50'27.93"W	06/15/2015	9

Sequence obtention

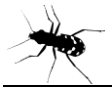
DNA was extracted from the specimens, previously fixed in absolute alcohol, using two different methods: Invisorb® Spin Tissue Mini Kit® and Chelex®. Before amplifying the gene fragment of interest, the yield of each extraction was checked with a Thermo Fisher Scientific® Nanodrop® 1000. A region of ~800 bp from the mitochondrial cytochrome c oxidase subunit 1 was amplified using the primers Jerry and Pat (Simon et al., 1994). The PCR consisted of an initial activation at 94 °C for 5 min; 40 amplification cycles consisting of 94 °C for 30 s (denaturalization), 50 °C for 30s (annealing), and 72 °C for 1 min (extension); and a final extension of 72 °C for 10 min.

The success of the PCR was ascertained in a 1.5% agarose gel, and the products were sequenced in Macrogen (Amsterdam, Netherlands).

Phylogeographic analysis

The sequences were aligned in GENEIOUS R7 (Biomatters, available at http://www.geneious.com), using the MUSCLE algorithm, and manually edited to correct possible sequencing errors and to delete low resolution terminal segments.

Additional sequences already available on GenBank, belonging to 3 specimens of *C. deserticoloides* from San Isidro (access codes: KJ395000, KJ394999 and KJ394998) originally obtained by López-López and Galián (2010), were added to this matrix.



The haplotype network for the *C. deserticoloides* matrix was constructed in PopART (available at http://popart.otago.ac.nz) using the Median Joining method (Bandelt et al., 1999).

Geometric morphometrics analysis

Both hind wings were dissected from 19 of the specimens preserved in absolute ethanol and mounted on glass slides. Some of the specimens were briefly submerged in propylene glycol to aid in the unfolding of the wings and avoid damage while being handled. The wings were then photographed using a Zeiss Stemi 2000-C microscope and SPOT 5.0 advanced software (http://www.spotimaging.com/software/spot-advanced/). Photographs were first imported into tpsUTIL1.67 (available at http://life.bio.sunysb.edu/morph/). Two dimensional Cartesian coordinates of 19 landmarks from the hind wings were then digitized using tpsDIG2.22 (available at http://life.bio.sunysb.edu/morph/). GLS Procrustes superimposition (Alibert et al., 2001; Bookstein, 1996; Rohlf and Marcus, 1993; Rohlf, 1990), generation of Covariance Matrix, PCA and Canonical Variates Analysis were performed using MORPHOJ (Klingenberg, 2011).

Results

DNA extraction, amplification and sequencing

The extraction kit proved to be the better of the two methods of DNA extraction on account of higher yields of DNA concentration in a greater number of samples. Thus, only the DNA obtained with this method was used for further amplification and sequencing. The resulting fragment had a length of 613 bp.

Phylogeographic results

The haplotype network (Fig. 1) shows an abundant central haplotype, formed by specimens from Alhama and Rambla Salada, from which other haplotypes radiate in a star-shape pattern. These haplotypes that form these derived branches are separated from the central haplotype by 1-3 mutational steps, and are restricted to single localities.

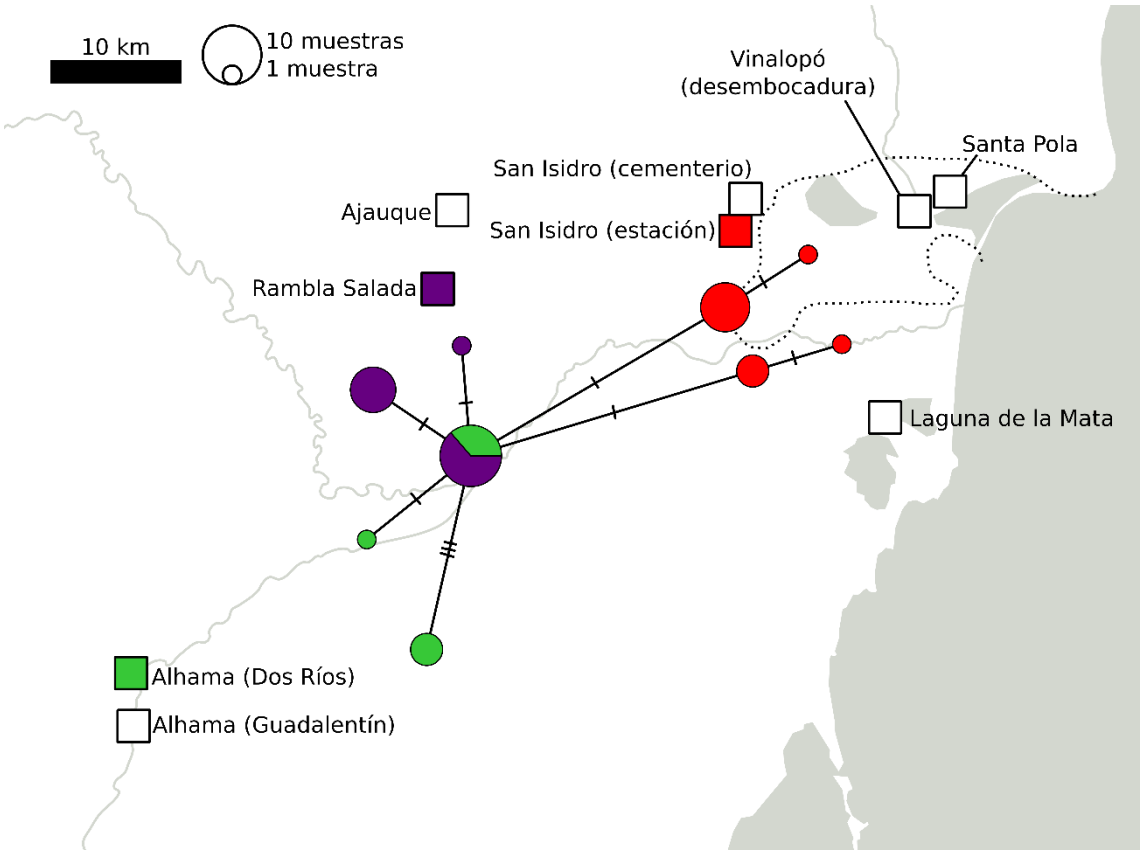


Figure 1. Map showing the locations sampled of *C. deserticoloides*. A colored square indicates locations where the specimens were sampled and a white square those where no specimens were found. The dotted line outlines the approximate coastline of the lagoon of Elche. The haplotype network of our *C. deserticoloides* samples is superimposed to the map. Each circle represents an individual haplotype, joined to the adjoining haplotype by crossed lines indicating the mutational steps separating them. A higher diversity can be observed in San Isidro.

Geometric morphometric results

Nineteen landmark coordinates were digitized for each wing (Figure 2). The plot of the first two relative warps (Figure 3) shows the positions scored by each specimen in that shape space, as well as the shape change explained by each axis. Singular values explained by the first two relative warps for consensus were 24.505% and 20.513% respectively (total percentage of 45.018%).

Results of the CVA analysis are shown in Figure 4. CVA, a method that maximizes the among group variation relative to the pooled within-group variation, separated more clearly the samples according to their site of origin.

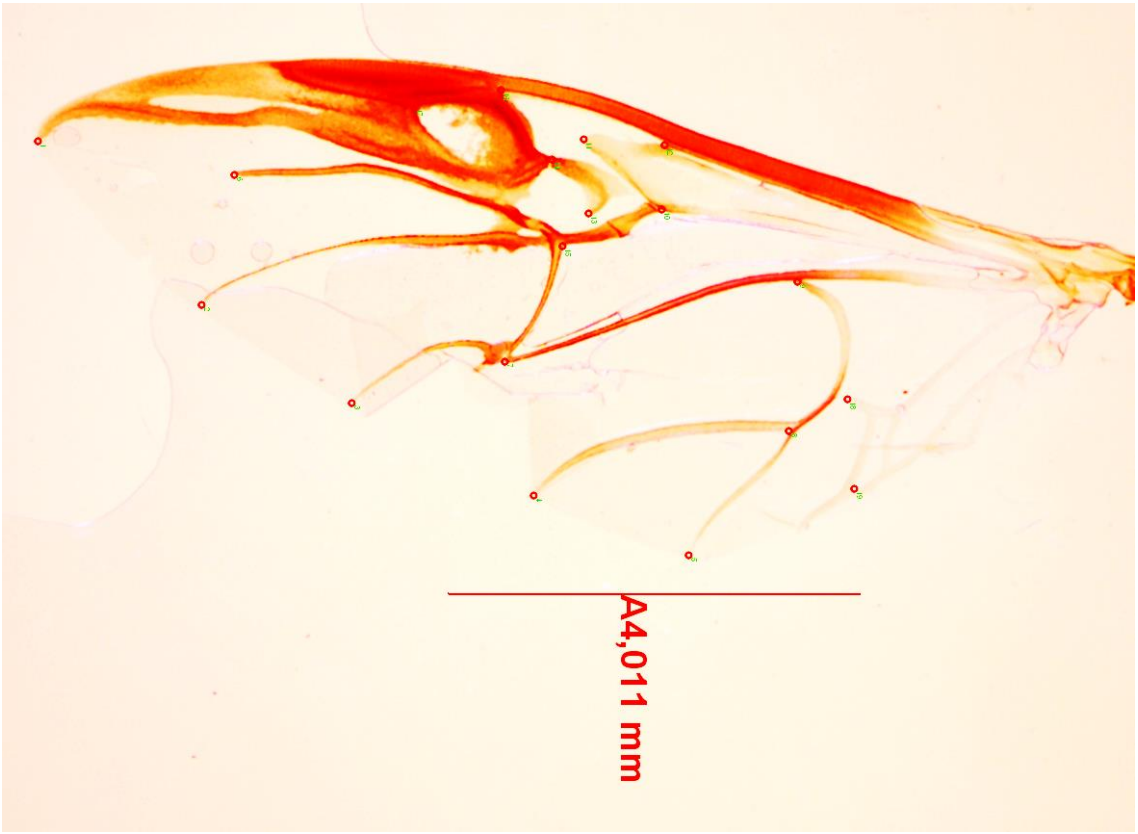


Figure 2: Location of the 19 landmarks on the tiger beetle hindwing.

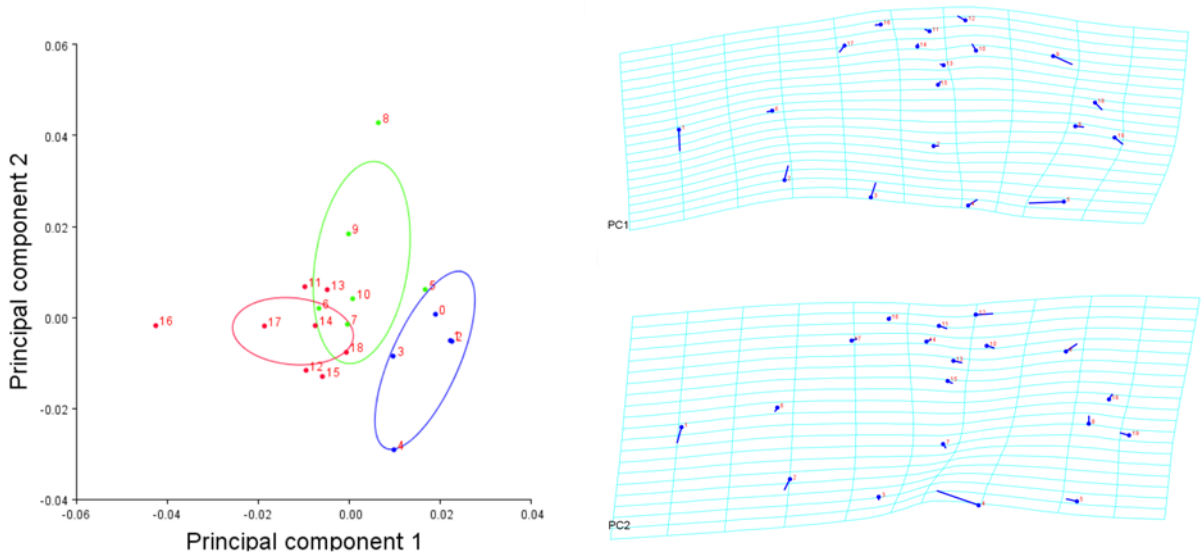


Figure 3: Relative positions of the *C. deserticoloides* specimens in the shape space defined by the first two relative warps, together with deformation grids with the variation explained by each component. Red points correspond to Rambla Salada, green ones to San Isidro and blue ones to Alhama.

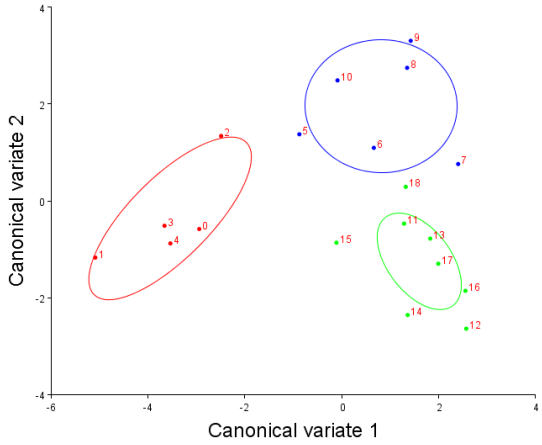
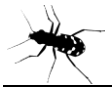


Figure 4: Relative positions of the *C. deserticoloides* specimens according to the Canonical Variates Analysis. Red points correspond to Rambla Salada, green ones to San Isidro and blue ones to Alhama.

Discussion

History of *C. deserticoloides*

The geographic distribution of the haplotypes, and their positions in the star-shaped phylogeographic network, suggest that the central haplotype may represent an



ancestral population that lived in Rambla Salada, Alhama, or a nearby locality. At least two additional haplotypes appear to have evolved independently in each of these two sites. The specimens from the most distant locality, San Isidro, would have been originated from two independent eastward colonizations.

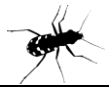
In this scenario, San Isidro is the only locality whose haplotypes are derived, which would signify a greater diversity and uniqueness when compared to the other two locations, less diverse and holding shared haplotypes what means a faster evolution in a large area with favourable conditions .

Genetic diversity of *C. deserticoloides*

C. deserticoloides is a valuable endemism due to its high degree of genetic distance from its closest Iberian relatives, set at values of 6.6 to 9.6% by Diogo et al. (1999). The phylogeny of the genus *Cephalota* (Chapter 1) also clearly reflects the relatively distant relationship of *C. deserticoloides* to the more closely related group formed by the other Iberian *Taenidia spp*.

The practically non-existent within-species differentiation found by Diogo et al. (1999) is in overall accordance with the usual patterns of local genetic differentiation of holarctic Cicindelini, as exemplified by more thorough studies with species such as *Cicindela dorsalis*, *C. puritana* (Vogler and DeSalle, 1993) and *C. hybrida* (Cardoso and Vogler, 2005). However, we have now found that *C. deserticoloides* exhibits a higher level of differentiation than previously thought.

Our results show that there is a certain degree of within-population genetic differentiation, with peak genetic distance values of 0.67% in San Isidro and 0.66% in Rambla Salada and Alhama (Table 2). In contrast, the ten specimens of the much more widespread *C. littorea* analyzed during this study showed no differences, and were also identical to another *C. littorea* sequence from a relatively distant location,



the Rasall salt marshes (GenBank access code KC963763), located South to the Mar Menor Lagoon.

Table 2: Maximum genetic distance (Max intra) found within each population of *C. deserticoloides*, and minimum genetic distances between the three populations (Min inter).

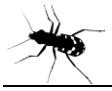
	Max intra	**Min inter**	
		Alhama	**San Isidro**
Rambla Salada	0.0033	0.0066	0.005
Alhama	0.0067	-	0.0083
San Isidro	0.0067	-	-

Additionally, the three sampled populations appear to be well separated from each other by wing shape, according to our relative warps analysis. The CVA analysis suggests that wing shape could also be a good predictor to assign each specimen to its population. These differences may be yet another piece of evidence of a high degree of differentiation between fragmented and isolated populations. However, the variance explained by the relative warps is low, and further studies with more specimens and different structures should be conducted to validate these results.

C. deserticoloides is more highly specialized in arid environments than its closest relatives, and also has a much more restricted range (Gebert, 1991) and a lower local abundance (Diogo et al., 1999). These factors may have contributed to the isolation of populations and consequently, to the local genetic and morphological differentiation suggested by our results.

Future expectations

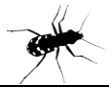
During this study we visited several sites east of San Isidro where *C. deserticoloides* has been collected before (Lencina and Serrano, 2011), but found no sign of *C. deserticoloides* despite our repeated sampling effort. Only a few *C. circumdata* individuals were observed in the Santa Pola locality.



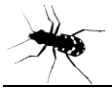
These sites, together with San Isidro, are all relatively close to each other in an area occupied by greater extensions of suitable habitats that have been subject to an intense and long-standing instability owing to the proximity of the sea, well documented in the historical records (Figure 1) (Saumell and Pérez, 1978; Tent-Manclús et al., 2014).

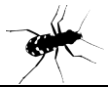
The haplotypes of the putative *C. deserticoloides* from this area are expected to be related to those found in San Isidro. Further studies based on beetles from more localities in the province of Alicante are needed to explain the genetic diversity patterns, in comparison to the results found in the localities of the Region of Murcia (Rambla Salada and Alhama). However this research line seems to be quite difficult given the serious threat as a result of the intense natural and human modification processes to which the Albufera de Elche and its adjacent environments have been subjected (Giménez-Font, 2008; Tent-Manclús et al., 2014).

Due to its relative richness and uniqueness of haplotypes, San Isidro has an especially high value compared to the other two locations as far as the conservation of genetic diversity is concerned. At the same time, the expanse of suitable habitat is very low and under multiple threats by the building of new roads, railways and the urban growth of the town of San Isidro, which, unlike Rambla Salada or Alhama, is not a protected natural space (Pavón-García, 2009) and therefore urgent conservation regulations are badly needed.



Chapter 3: Non-invasive identification of endangered tiger beetle species (Coleoptera: Cicindelidae) from soil samples





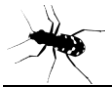
Abstract

Recently, environmental DNA has become an essential source of information about biodiversity that does not require an invasive disturbance of the analyzed taxa. The halophile tiger beetle *Cephalota deserticoloides* is an ecologically and geographically restricted species, with only a few known populations. A capability to assess its presence and identity is paramount to its management, but is confounded by the very similar co-occurrent larvae of *C. littorea*. In this work, we make use of environmental DNA to conduct a low-effort, non-invasive sampling of tiger beetle larvae. A novel protocol is developed to discriminate between *C. deserticoloides* and *C. littorea* soil-bound genetic material. Our approach focused on the identity of separate larval tiger beetle burrows. The observed results confirm the ability of this method to separate both species and give rise to new hypotheses regarding relative abundance and niche partitioning between these two tiger beetles.

Introduction

DNA data are nowadays a fundamental part of many biological and ecological studies. DNA analysis may be necessary, for instance, to reliably identify cryptic species or simply to be able to tell apart different genotypes within a single species in contexts ranging from forensic entomology to conservation biology (Lefort et al., 2015). In many cases, especially when vulnerable or endangered biological populations are concerned, there is a strong interest in obtaining DNA samples with as little harm to individual specimens as possible. Although this approach has been taken when sampling bigger vertebrates (González and Duarte, 2007) and plants (Fahner et al., 2016; Taberlet et al., 2012), the non-invasive sampling of small, physically delicate but also possibly threatened arthropods has been less explored, mainly in fresh water systems (Krehenwinkel et al., 2018; Thomsen et al., 2012).

Specifically, when approaching the problem of sampling DNA without harming the animal source, two concepts should be considered: non-invasive and non-disruptive



sampling (Lefort et al., 2015). Non-invasive sampling refers to preserving the physical integrity of involved specimens, while the term non-disruptive emphasizes the effects of the sampling method not only on physical integrity, but also on the fitness and behavior of the organism from which the sample is obtained.

It is within this context of non-invasive and non-disruptive DNA sampling that we consider environmental DNA (eDNA) as a way of gathering this genetic information. Environmental DNA can be defined as the genetic material obtained directly from environmental samples (soil, sediment, water, etc) without any obvious signs of biological source material. The possibility of obtaining this kind of material has been enabled by the availability of ever-improving DNA extraction and sequencing technologies, and it has even been suggested as the cornerstone of most future genetic studies in field ecology and conservation (Thomsen and Willerslev, 2015).

Tiger beetles are a well-known group of insects (Pearson and Vogler, 2001), and most of the currently recognized species in the Palaearctic and, more specifically, in the Iberian Peninsula, may be readily identified directly on the field in their adult phase, most often without the need to capture them (personal observation). However, tiger beetle larvae pose much more of a challenge to identify at the species level (Putchkov and Arndt, 1997), as larval morphology is highly conservative. In this context, DNA analysis is most often needed to confirm species identity where more than one species is known to coexist (López-López and Galián, 2010).

Due to tiger beetle larval habits, living in narrow soil burrows, obtaining genetic material directly from the animal involves significant disruption and most often the death of the animal. Thus, the use of a non-invasive method for obtaining DNA is particularly needed in the case of vulnerable species with limited range, like the critically endangered endemism *Cephalota (Taenidia) deserticoloides* (Diogo et al., 1999), are particularly interesting for using non-invasive method of DNA isolation. This Iberian endemism inhabits hypersaline environments where it coexists with

other tiger beetle species such as the common and non-endangered *C. littorea* in Rambla Salada. This fact does not allow to identify the larva inhabiting a particular burrow according to characteristics such as burrow diameter or detritus surrounding the lining of the burrow.

It is in this context that we attempted to find a non-invasive and non-disruptive alternative for obtaining DNA through the use of environmental DNA, in order to discern what larval burrows correspond to *C. deserticoloides* and how they are spatially distributed.

DNA from saliva may be present in the lining of the burrow (Figure 1), as larvae clean the entrance continuously (Pearson, 1988).

Figure 1. Opening of a *Cicindela* burrow

Thus, the aim of this paper is to achieve identification of the larva by using exclusively environmental DNA from the soil around or inside the larval burrow, to confirm species identity. This will provide a useful tool in the population assessment and conservation efforts of the vulnerable Iberian endemic species *Cephalota deserticoloides*.

Material and methods

A suitable habitat was visited in the Ajauque-Rambla Salada natural space (Figure 2), where the related species *C. deserticoloides* and *C. littorea* are known to co-occur. Fourty larval burrows well-separated from each other (as nearby larvae would probably be siblings) were sampled for soil DNA within this area in March 2017. Samples were stored in the buffer provided by the PowerSoil extraction kit from Qiagen.

Figure 2. Rambla Salada (Murcia) (Alejandro López-López).

For each individual burrow, two methods of extraction were attempted. In the first method, a cotton bud (Figure 3, left) was used to scoop soil from the inner lining of the burrow. A second method involved scrapping the soil from the outer limits of the hole with a scalpel (Figure 3, right).

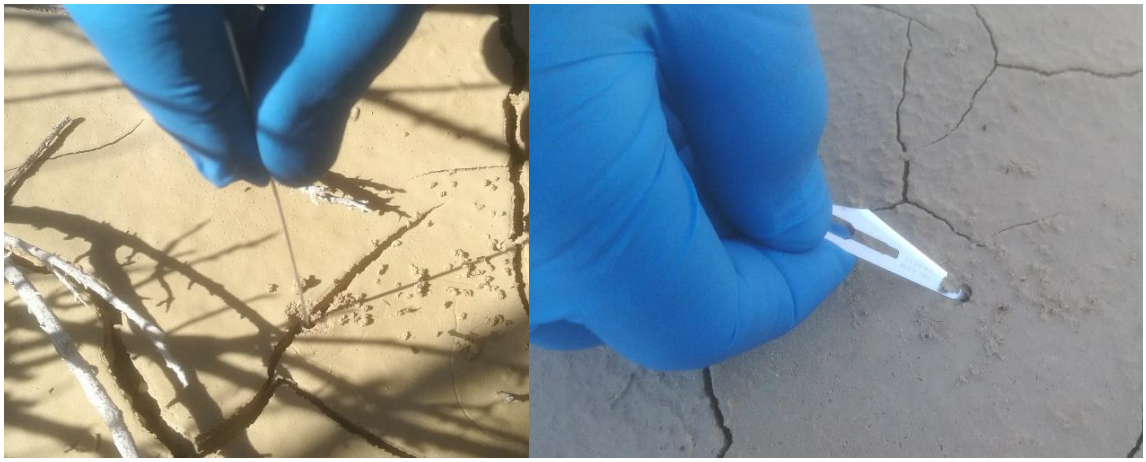


Figure 3. Collecting environmental DNA from tiger beetle burrows in Rambla Salada, Murcia, using a cotton bud (left) and a scalpel (right).

Soil DNA was extracted using the aforementioned extraction kit. Two pairs of primers were developed to amplify a short region of the coxI mitochondrial DNA exclusively for each of the species (Table 1).

Table 1. List of primers pairs designed for this study

Name	Species	Direction	Sequence
278F-des	*C. deserticoloides*	F	GTGGACTAACGGGGGTTGTT
576R-des	*C. deserticoloides*	R	TCATGCCACATATGCATCAGGA
278F-lit	*C. littorea*	F	GTGGATTGACCGGAGTTGTT
576R-lit	*C. littorea*	R	TCATGATACGTAGGCGTCGGGG

The discriminating ability of both primer pairs was validated both on *C. littorea* and *C. deserticoloides* DNA previously extracted from tissue samples of known origin (those from Chapter 1 and Chapter 2). Primer pairs were then used on the environmental DNA samples.

The PCR experiments were run in duplicate with both extraction methods (cotton bud and scalpel). The PCR program, especially designed for samples that are difficult to amplify (López-López et al., 2015), consisted on the following cycles:

- Initial denaturalization: 94 °C 5 min
- 5 cycles:
    - Denaturalization: 94 °C 30 sec
    - Annealing: 45 °C 30 sec
    - Elongation: 72 °C 1 min

- 5 cycles:
    - Denaturalization: 94 °C 30 sec
    - Annealing: 47 °C 30 sec
    - Elongation: 72 °C 1 min
- 30 cycles:
    - Denaturalization: 94 °C 30 sec
    - Annealing: 50 °C 30 sec
    - Elongation: 72 °C 1 min
- Final elongation: 72 °C 10 min

Results and discussion

Validation of the primers confirmed the utility of both primer pairs to exclusively amplify DNA of the respective species (Figure 4). When the primers were used with the DNA isolated from the forty soil samples, 39 amplified with the *C. deserticoloides* exclusive primers and only one with the *C. littorea* primers. No sample amplify with both set of primers, corroborating their specificity.

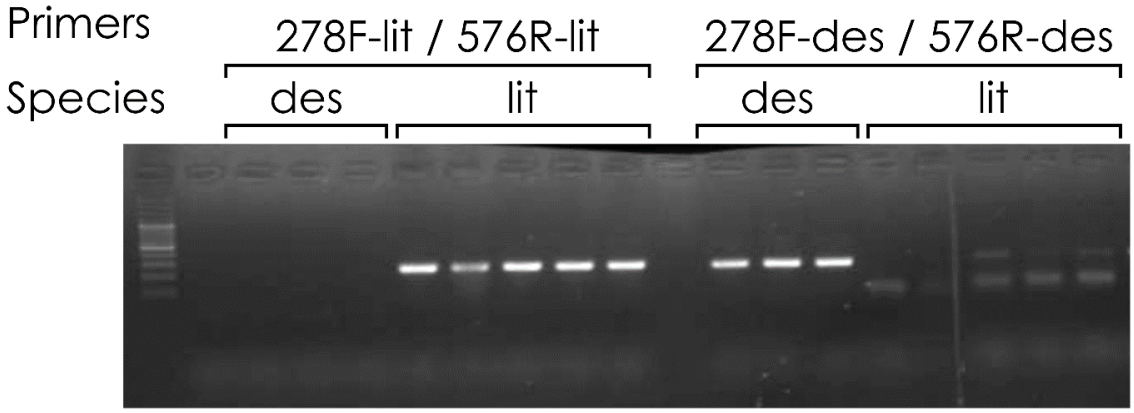


Figure 4: Results of the validation of the primers pairs with known samples. The same set of eight samples (3 *C. deserticoloides*, des; and 5 *C. littorea*, lit) are tested with the primers exclusive for *C. littorea* (left) and the primers exclusive for *C. deserticoloides* (right).

The results of this work support that DNA extraction from soil can be a valid alternative to invasive and disruptive collection of larvae for their identification. In all cases a clear read was achieved so that each sample could be ascribed to one species or the other.

Even though both species, *C. deserticoloides* and *C. littorea*, are known to inhabit the sampled area and are not rare as adults during their peak times of activity. However, only one sample turned out to be *C. littorea* while the rest were *C. deserticoloides*.

Adult tiger beetles show asynchronous diel activity during favourable seasons when living in sympatry (Zerm and Adis, 2001), probably to avoid interspecific competition. It seems that the same pattern arises with arvae, as the number of *C. deserticoloides* burrows was notably higher than that of *C. littorea* .

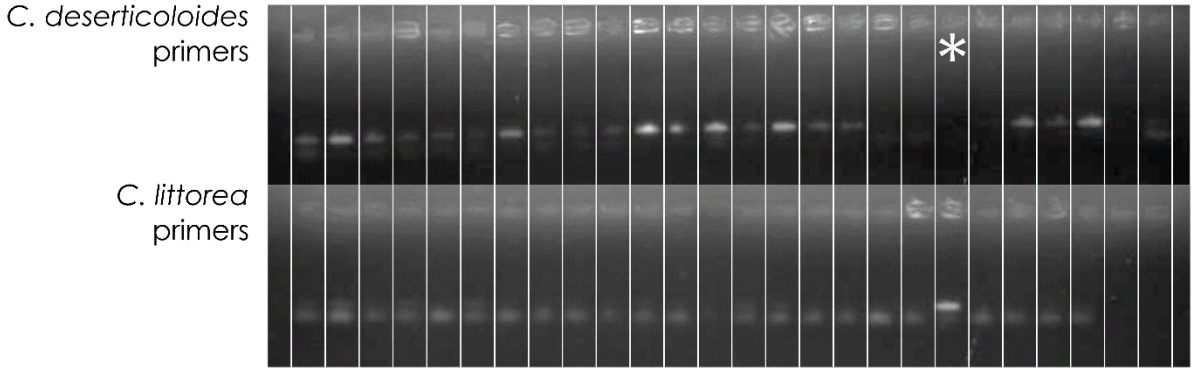
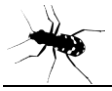


Figure 5: Agarose gel showing the PCR products for several samples (one for each column) obtained with the scrapping method. Above: PCR carried out with the primers exclusive to *C. deserticoloides* (278F-des and 576R-des). Below: PCR carried out with the primers exclusive to *C. littorea* (278F-lit and 576R-lit). The white asterisk (*) in the upper row marks the only sample that could be ascribed to *C. littorea*.

Tiger beetle larvae will plug their burrow while molting, pupating or while digesting large prey, and some species such as *Grammognatha euphratica* have even been observed to do this on an almost daily basis if well-fed (personal observation). In view of the observed local abundance of *C. littorea* adults in the same area further down the season, their larvae may have been inactive or already pupating by the time sampling was conducted, so that despite a wide sampling effort almost only *C. deserticoloides* was sampled.

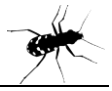
As for the results themselves and their implications for future studies, it seems that this methodology will be useful to quantify larval abundance, survival rates and microhabitat selection, which are interesting data to be considered in future



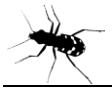
conservation studies. The two main restrictions to this kind of research are solved, those being the undesired death of a sufficient number of individuals to make the sampling statistically relevant and the ability to readily discriminate between two very similar species whose possibly significantly differing ecological preferences are of great interest in conservation. In the case of our study, the 40 sampled larvae were not disturbed, where they would have otherwise been collected and sacrificed, which is especially relevant when facing the study of vulnerable or endangered species with limited numbers of individuals. It should also be noted that extracting (Brust et al., 2010) such a number or larvae is a very uncertain and time-consuming process, while collecting soil from the surface of a tiger beetle burrow represents less effort and difficulty.

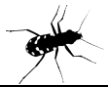
In this study it was also noted that the soil collected from the burrow surface yielded much better results than that from the inner lining of the hole this could be explained by the behaviour of the larva. While one might expect that the surface would be more exposed to the effects of weather and outside contamination, it is for these same reasons that the larva will most likely be spending most of its effort to rebuild and maintain its burrow at the entrance, meaning it may leave behind more recent and more abundant saliva with genetic material as it does so. In addition, tiger beetle larvae are also known to spend a long time at the very entrance of their burrow ambushing their prey (Pearson, 1988).

This technique could easily be applied to other endangered tiger beetle species that have been managed and monitored currently, such as *Cicindela ohlone* (Cornelisse et al., 2013; Knisley and Arnold, 2013) or *Ellipsoptera lepida* (Dang and Aitken, 2014).



Chapter 4: Mark-recapture population estimates of *Cephalota deserticoloides*



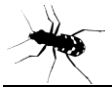


Abstract

The tiger beetle *Cephalota deserticoloides* is a species endemic to a few localised sites in southeastern Iberia, where it is a specialised inhabitant of the arid saline steppe type habitat. Although regarded as vulnerable, very little is known about the actual population dynamics and degree of endangerment the species actually faces. In this work, mark-recapture estimates of total population size are presented for one of the main known populations of *Cephalota deserticoloides*. Additionally, some further remarks on seasonality and co-occurring tiger beetle species are made. The observations gathered indicate that *C. deserticoloides* makes a narrow use of the habitat available, with activity peaks that are earlier and spatially separated from those of *C. littorea* and *Myriochila melancholica*. At its peak, the area under consideration holds a relatively dense tiger beetle population, which is numerically comparable to those of other endangered cicindelids. These results will help assess the conservation state of *C. deserticoloides* and set the stage for more long-term efforts to analyze population viability and the priority of *C. deserticoloides* and its habitat as targets for protective measures.

Introduction

Tiger beetles are a widely distributed family (Pearson, 1988; Pearson and Cassola, 1992) that includes over 2600 species (Pearson and Cassola, 2005), spanning a high variety of habitats. Tiger beetles have received more attention than other insect taxa in fields such as natural history, population dynamics, communities and patterns of diversity and the taxonomy of certain groups within the family (Cardoso and Vogler, 2005; Knisley and Schultz, 1997). This amount of data, together with their high specialization for their habitats, strengthens the case for their use as biological indicators (Pearson and Vogler, 2001; Rodríguez et al., 1998).

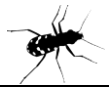


The genus *Cephalota*, to which 21 species have been ascribed worldwide (Wiesner, 1992), is a halophile group that was likely already found populating the coasts of the Tethys Sea 35 million years ago (Hieke, 1983). This genus is distributed throughout the Mediterranean coast and deep into central Asia, with the species *C. littorea* and *C. vonderdeckeni* reaching as far south as the Sudan and Somalia (Gebert, 1999; Werner, 2000). The current distribution patterns of most extant species were in all likelihood influenced by the formation of the Mediterranean Sea. In this context, *C. deserticoloides*, an Iberian endemic, has been postulated to be a vicariant taxon whose closest relative could be *C. deserticola*, a Eurasian species (Diogo et al., 1999). Several species, such as *C. deserticoloides* and *C. dulcinea*, have extremely restricted distributional patterns (López et al., 2006; Rodríguez-Flores et al., 2016), which makes them interesting endemic insect species for wildlife conservation plans and has already warranted the inclusion of *C. deserticoloides* in the in the Spanish Red Book of Endangered Invertebrates (Lencina and Serrano, 2011).

Its unique salt steppe habitat has historically been heavily disturbed by drainage and desalination, and transformed into agricultural land and, more recently, into rubbish dumps, illegal buildings and industrial complexes. The remaining populations are confined to a small and fragmented patches of habitat (Diogo et al., 1999).

In spite of this dire situation, no serious characterization of population size and seasonal fluctuation has been performed up to this date. Such efforts have however been made with Iberian species relevant to our case such as *C. littorea* and *C. hispanica* using mark-recapture methodologies (Serrano, 1990) in their Portuguese populations, and less labor intensive methods like index counting have been employed to obtain similar estimates for North American species of conservationist interest, such as *Cicindela dorsalis*, *C. albissima* and *C. puritana* (Gowan and Knisley, 2014; Knisley, 2009).

C. deserticoloides is a very mobile beetle that occurs at great local densities with peak activity during the warmest hours of the day from late May to late October.



Previous field work showed that the beetle is most abundant from early June to mid-July. Because of these characteristics, manual sampling with the help of a butterfly net was not feasible, and methods such as index counting or distance sampling (Buckland et al., 2015) were initially considered but discarded for fear of repeated counts of a single individual. Many tiger beetle species are generally known to occur in more or less discrete clusters of individuals (Simon-Reising et al., 1996), and this is indeed the case of *Myriochila melancholica* in our study area and of *C. deserticoloides* and *C. littorea* to a lesser extent (personal observation). Analysing clusters of beetles instead of individual captures could be an interesting direction for future work, but the spatial scope of the sampling would need to be enlarged, which necessarily entails a greater sampling effort.

The Rambla Salada natural space, one of the three known locations where *C. deserticoloides* is known to exist, is, for the most of its extension, a narrow creek with well-delimited vegetation zones. *C. deserticoloides* inhabits the low, flat, and yearlong moist *Sarcocornia*-dominated strip between the very near approaches of the creek and the *Limonium* and *Tamarix*-dominated arid saline steppe that starts abruptly with a suficient ground-level increase. This strip of rather well defined habitat is not usually wider than 10 m on each side of the creek, often being much smaller and even disrupted due the effects of the growth of dense *Phragmites australis* and *Arundo donax* formations, which have been brought about by a relatively recent decrease in salinity levels (Esteve et al., 1995).

The aim of this paper is to provide some rough estimates of the entire population size of *C. deserticoloides* that may set the stage for a yet impossible assessment of its precise status and long-term tendencies through techniques such as PVA. In addition, we also expect to gain insights into the possible mutual segregation of several coexisting tiger beetle species at a temporal and at a spatial scale.

Material and methods

Mark-recapture study

Field work was performed between early June and early August 2017, which is the time frame of most intense adult activity. Sampling was conducted every two days across this period under the assumption of an open population (Schwarz, 2001).

Twelve sets of two pitfall traps each were deployed in late May across a 150 meter-long path of very favorable *C. deserticoloides* habitat. The need to capture the tiger beetles along a narrow corridor of habitat was addressed by arranging the traps in pairs, each connected by an aluminum barrier perpendicular to the stream (Hansen and New, 2005) (Figure 1). An inverted plastic cup was placed into each trap to retain the living insects (Taboada et al., 2012), and traps were covered with plastic plates when not in use.

Figure 1: Individual barrier pitfall trap deployed in Rambla Salada.

The work routine involved an early visit between 8:00 and 9:00, before any significant adult beetle activity, during which each trap was disclosed and inverted cups put in place. The traps were then revisited at 18:00 to 20:30, during which time

all captured tiger beetles were recorded, additionaly marking and releasing living *C. deserticoloide*s. Traps were then dismounted and covered until further use.

The tiger beetles were marked on the prothorax with a customized numbering system using La Pajarita tempera paint. A double code system was partially used (Hagler and Jackson, 2001), painting a colored mark on the elytral tips of each individual to ensure no marks were lost or repeated.

Data analysis

Analyses were started by fitting a fully time dependent model using POPAN (Schwarz and Arnason, 1996), which implements a CJS type model. The program RELEASE (White and Burnham, 1999) was used to estimate goodness of fit of this starting model.

The full time dependent model was deemed appropriate and population estimates were extracted from it. These N estimates were converted to population density and applied to all habitat across the area of study (Figure 2), encompassed by the 1200 square meter area of Los Baños, where population was estimated, and an equivalent surface 2.4 km upstream. Total adult numbers during the peak time of activity (first week of July) were estimated in this way.

Figure 2: Area considered in this study. Mark recapture analysis was conducted in yellow area (Los Baños), while orange area (Los Periquitos) was visually inspected during the whole study. Pink areas in between are equivalent patches of habitat where tiger beetle activity is assumed to be equivalent to what was seen directly.

Results

A total of 123 *C. deserticoloides* specimens were sampled. *C. littorea* was also trapped on occasion, while the presence of nearby population clusters of *Myriochile melancholica* was also noted. However, these three species appeared to spatially be well segregated, and despite their immediate presence, an overwhelming majority of the tiger beetles captured were *C. deserticoloides* due to our placement selection.

Goodness of fit analysis was deemed sufficient according to TEST 2 and TEST 3 provided by program RELEASE, and hence the full time dependent model was sufficient for our population analysis.

The results for the full time dependent model are detailed in Table 1.

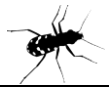


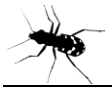
Table 1: Results of the full time dependent model.

Real Function Parameters of {phi(1)p(t)pent(t)}				
	95% Confidence Interval			
Parameter	**Estimate**	**Standard Error**	**Lower**	**Upper**
1:Phi	0.8744866	0.2463660	0.0788426	0.9982399
2:Phi	1.0000000	0.4794077E-006	0.9999991	1.0000009
3:Phi	0.4301325	0.2086868	0.1245536	0.8001731
4:Phi	0.1739117	0.0790335	0.0668331	0.3822705
5:p	1.0000000	0.0021983	0.3752888E-296	1.0000000
6:p	0.1524823	0.0402260	0.0890447	0.2487722
7:p	0.1894477	0.0478076	0.1126636	0.3008218
8:p	0.2470761	0.1281612	0.0783797	0.5587360
9:p	1.0000000	0.0011678	0.6524601E-296	1.0000000
10:pent	0.8092046	0.0503494	0.6911857	0.8893414
11:pent	0.1498760E-007	0.0000000	0.1498760E-007	0.1498760E-007
12:pent	0.3116955E-006	0.3463488E-003	0.3073218E-310	1.0000000
13:pent	0.0383703	0.0263497	0.0097467	0.1392343
14:N	229.62218	38.209387	176.94727	333.72965

Population estimates for the studied area of Los Baños showed a density of 0.2 adults per square meter. Male and female individuals appeared to be equivalent in activity, distribution and tendency to be trapped. Expanding this population density for the whole area that was visually inspected in Los Baños gives 2140 individuals. Expanding this estimation to the area of equivalent habitat encompassed in the 3 km considered in this study gives another 2020 individuals for the area inspected at the upstream end (Los Periquitos) and another 7273 individuals in the area in between, where appropriate habitat was indirectly selected using Google Maps and calculating areas with SigPac (http://sigpac.mapama.gob.es/fega/visor/). Total number of adults in the area under consideration was estimated to be 11400 in the first half of June.

Discussion

Three tiger beetle species were encountered: *C. deserticoloides*, *C. littorea* and *M. melancholica*. The study areas of Los Baños and Los Periquitos exhibited visually

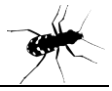


similar densities of adult tiger beetle activity. Almost no spatial and temporal overlap was seen between *C. littorea*, *M. melancholica* and *C. deserticoloides*. The two former species occupy much more wet or even flooded areas and are more sparsely distributed, although individual patches are very densely occupied. *C. deserticoloides* occupies humid but not damp soil, and is more extensively distributed along the stream. *C. deserticoloides* shows a peak of abundance in late May and early June, while *C. littorea* and *M. melancholica* only really become numerically relevant in early to mide July. It was possible to observe specific aspects of the natural history of *C. deserticoloides* on the field. Specifically, frequent predation upon *Cataglyphis* ants and more sporadic feeding on *Porcellio ornatus* woodlice was observed.

Estimated population totals fall within the range suggested for other endangered tiger beetle species such as *C. dorsalis* and *C. puritana*. For instance, *C. puritana* is known to be constituted by a metapopulation of 6 patches with 1100 to 9200 individuals per patch, while *C. dorsalis* is known from several tens of locations populated by 7000 to 12000 adults (Knisley and Schultz, 1997). Considering that *C. deserticoloides* has only been collected from 3 locations in recent times, despite intensive sampling efforts, one of which was covered in this study and was estimated at 11400 adult individuals, and that *C. puritana* was listed as endangered in 1996 under UICN criteria, a reevaluation of the conservation status of this Iberian endemic may be desirable.

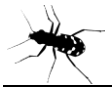
Although the complete area of distribution of *C. deserticoloides* was not covered in this study, the numbers obtained suggest that this species is far from population levels of hundreds of thousands or millions of adults, the number suggested by (Willey and Perkins, 2007) as typical of a fully viable tiger beetle population.

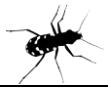
In addition to these numeric considerations, the historical fall in salinity and fluctuation of water line levels have brought about both extensive invasion by *Phragmites australis* and drying up of previously good habitat in different areas. Both



of these phenomena may be currently increasing the fragmentation of the three known populations, and may even have an effect within the area studied.

In view of these results, further management of *C. deserticoloides* may benefit from a more extensive mark-recapture effort to assess individual mobility and establish whether there are discrete boundaries between populations as those described in other species. Although genetic distance is another approach to this problem, mark-recapture may offer a better view of the effect of barriers such as vegetation change and salinity variations that have only taken effect recently. Although the study was conducted in the Ajauque-Rambla Salada SPA, a protected natural space, the other two locations where *C. deserticoloides* may be found enjoy no such status. Should it turn out that those populations have similar or lower numbers, a case could be made to protect those spaces as well to prevent the eventual extinction of *C. deserticoloides*.





Final remarks and conclusions

The results of this thesis suggest that *Cephalota deserticoloides* is a species whose conservation should be considered of high interest, due to i) its evolutionary singularity in a genetic and geographic context within the genus *Cephalota*, and ii) its reduced distribution area and small population size, albeit showing locally high densities.

The genus *Cephalota* has experienced at least two expansion events through the Mediterranean basin and Central Eurasia, producing two distinct lineages. In both lineages, pairs of vicariant species can be found, like *C. hispanica* and *C. besseri*, or *C. circumadata* and *C. chiloleuca*. On the other hand, sister species like the Iberian endemisms *C. hispanica* and *C. dulcinea* derive from most recent and geographically located divergence events.

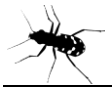
According to our results, the separation between the subgenera *Taenidia* and *Cephalota s. str.* should be revised, as *C. hispanica* is shown within the subgenus *Taenidia*.

The phylogenetic analysis also show that *C. deserticoloides* has a singular origin compared to the other Iberian *Cephalota*, deriving from an ancient and less diversified branch.

Despite the intensive and recurrent sampling carried out during this thesis, including sites with potential habitat for *C. deserticoloides* and localities with historical records, no population beside the three considered (Rambla Salada, Alhama and San Isidro) has been found[1].

The well-structured phylogeographic network reveals that these three populations are isolated. Remarkably, the San Isidro population showed both the highest degree

[1] An additional population has been rediscovered during the edition of this document (López-López, pers. com.).



of separation from the other localities and the highest genetic variability and singularity. It also seems to be the most vulnerable, with the lowest density and threatened habitat. This locality is unprotected, and is being reduced by urban expansion and the construction of road infrastructure and railways. On the other hand, the Alhama and Rambla Salada populations are included in protected areas, have a wider extension and show a higher density of individuals.

The mark-recapture analysis of *C. deserticoloides* show that this species is clearly segregated from other cicindelids like *C. littorea*, *M. melancholica* or *G. euphratica*. In the studied locality, *C. deserticoloides* starts to emerge early (from May to July) through the limit between the wet marsh and the arid salt steppe. *C. littorea* is crepuscular and starts to thrive at the end of June, becoming abundant at mid-July, and is relegated to patches next to the water stream.

The sampling of larvae in April and their identification using non-invasive methods reveals a predominance of *C. deserticoloides* at this time of the year, suggesting that the larval activity might also experience a temporal segregation. All this results hint at that *C. deserticoloides* and *C. littorea* have distinct well-defined and delimited ecological roles.

The abundance estimates of *C. deserticoloides* reveal that the population of this species peaks on the first week of July, through a uninterrupted corridor along the habitat. This population would be the largest of those known, with a total of 12000 individuals. This high number of individuals and the reduced available habitat results in a high density, and highlight the necessity of a continued viability of this space for the continuity of this species.

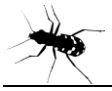
This number of individuals is similar to those of other managed and endangered tiger beetle species, such as *Cicindela puritana*, *Cicindela ohlone* or *Cicindela dorsalis*. These species have a protected status, and are the focus of protection measures and programs of public awareness. The results of this thesis suggest that investing in

characterizing, monitoring, and re-evaluating the conservation status of *C. deserticoloides*, including its inclusion as a candidate species for conservation programs, are fully justified activities.

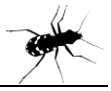
Finally, the design of the sets of primers for discriminating *C. deserticoloides* and *C. littorea*, together with the non-invasive extraction of environmental DNA from the entrance of the larval burrows, promises to be a useful tool for ecological studies, such as the microhabitat segregation analysis of tiger beetle species.

The conclusions of this thesis are:

1. The taxonomy of *Cephalota* must be revised, especially regarding the validity of the subgenus *Taenidia* and the subgeneric identity of *C. hispanica*.
2. The species *C. atrata* and *C. chiloleuca* should be considered as a single species (nominally *C. atrata*), based on their phylogenetic placement and the reduced genetic differences between them.
3. The origin of *Cephalota* is placed back to 13.5 million years ago. Its evolutionary history is related to the ebb and flow of suitable habitats for this halophile group caused by fluctuating levels and profiles of the Mediterranean Sea.
4. The genus *Cephalota* is divided in two lineages that were linked to a vicariance event. The divergences within each linage are related to dispersals in a longitudinal axis, some of them related to events like the Messinian crisis.
5. The Iberian Peninsula was a center of speciation for one of the clades of *Cephalota* that includes several endemic and restricted species. *C. deserticoloides* originated from a lineage that diverged from this clade 12 million years ago.
6. The ancestral population of *C. deserticoloides* lived in the area including Rambla Salada and Alhama. The San Isidro population originated from two independent eastward colonizations.



7. San Isidro has a greater diversity and uniqueness of haplotypes than the other two populations.
8. *C. deserticoloides* has a greater genetic diversity than its related species *C. littorea*. This is due to its specialization and the isolation of its populations, which leads to a higher genetic and morphological differentiation.
9. The geometric morphometrics analysis of the wing shape is a good predictor to assign each specimen of *C. deserticoloides* to its population.
10. DNA extraction from soil collected from the entrance of larval burrows is a valid non-invasive alternative for ascribing larvae to tiger beetle species, by means of differential PCR using species-exclusive primers.
11. *C. deserticoloides* is separated both spatially and temporally from other co-occurrent cicindelid species. *C. deserticoloides* occupies humid but not damp soil distributed along the stream, and shows a peak of abundance in late May and early June.
12. The *C. deserticoloides* population from Rambla Salada is estimated to be composed of 11400 adult individuals, a number similar to other endangered tiger beetle species.



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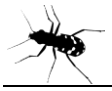

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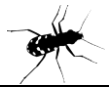

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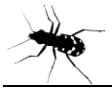

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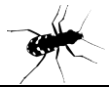

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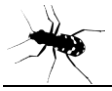

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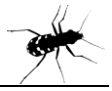

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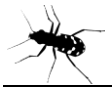

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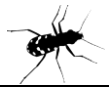

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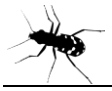

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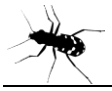

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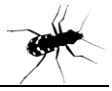

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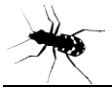

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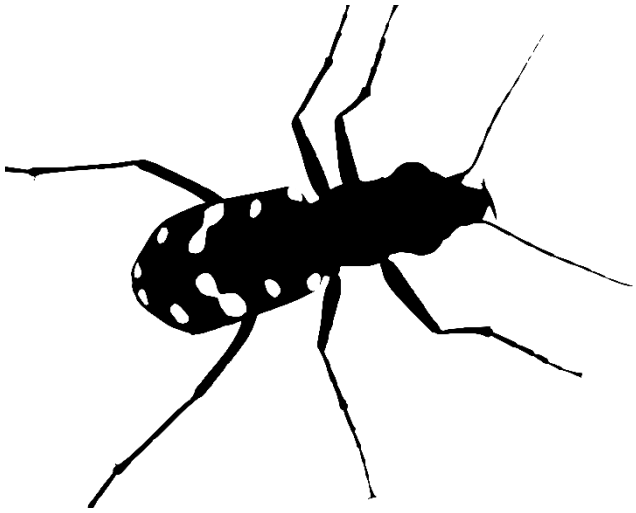
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