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Integrative approach unravels the evolutionary history of Western Mediterranean small cicadas (Hemiptera: Cicadettini)

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"In the end we will conserve only what we love,

we will love only what we understand,

and we will understand only what we are taught."

Baba Dioum

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Ir-me-ei lembrar destes tempos bem passados!

Gonçalo Costa

Resumo

As cigarras (Hemiptera: Cicadidae) são um grupo bem conhecido pelo seu canto estival. Apenas o macho produz som e fá-lo para atrair a fêmea para o acasalamento. A produção de som é feita com o auxílio dos tímbalos. O canto produzido pelos machos tem valor taxonómico na distinção das espécies e que tem atraído o interesse de inúmeros cientistas. Outra característica igualmente interessante associada ás cigarras é a duração do seu ciclo de vida que pode chegar a 17 anos na fase ninfal.

Tanto a produção de som pelos machos como os longos ciclos de vida, são características que tornam as cigarras um modelo de estudo tão interessante como desafiante. Assim sendo, dado que o canto emitido pelo macho é único de cada espécie, é possível um diagnóstico rápido apenas com recurso a esta característica. Esta informação é crítica na identificação, tanto mais que na sua ausência apenas um taxonomista treinado consegue distinguir espécies de cigarras. No entanto, as últimas investigações têm apontado a necessidade de camadas adicionais de informação. Assim, em meados do último século, a análise de dados de acústica começou a ser grandemente utilizada e inúmeras espécies novas são descritas. Até então, a descrição unicamente morfológica ignorava esta camada informativa, levando à aglutinação de várias espécies morfologicamente semelhantes numa só.

A biodiversidade de cigarras em Portugal e Espanha foi amplamente subestimada, até finais do século XX. Nas últimas décadas várias espécies foram descritas/redescritas, com o auxílio da análise de dados de acústica. É o caso de uma série de espécies do género *Tettigetta* que em 2010 foram atribuídas a um novo género, *Tettigettalna*. Estas cigarras, ainda que de pequeno tamanho e de cores apagadas, possuem uma grande diversidade de cantos, reconhecendo-se actualmente oito espécies de *Tettigettalna* e duas subespécies na Península Ibérica e que estão essencialmente limitadas à porção sul da região. No entanto, *Tettigettalna estrellae* ocorre apenas no centro e norte de Portugal e *Tettigettalna argentata* possui uma distribuição generalizada na Península Ibérica (não tendo sido ainda encontrada na cordilheira Bética) estendendo-se até França, Itália, Suiça e este da Eslóvenia.

Esta riqueza de espécies de *Tettigettalna* no sul da Península Iberica, despertou grande interesse, nomeadamente por levantar questões relativas à sua origem e em particular a possível ocorrência na área a sul do Mar Mediterrâneo, nomeadamente em Marrocos. Ainda que existam várias descrições de espécies de cigarras dadas para este país, estas descrições não possuem qualquer informação relativamente ao som produzido pelos machos, sendo baseadas em exemplares de museu muito antigos. Como tal, em 2014 efectuou-se uma expedição, financiada pela Linnean Society e pela FCT, que permitiu explorar o Rife e o Médio Atlas. Nesta expedição foram recolhidos vários espécimes de cigarras, assim como várias gravações de som e vídeo de espécies não conhecidos/identificados

Estes novos dados permitiram efectuar o trabalho desenvolvido nesta tese. A primeira parte do trabalho foca-se na descrição com uma abordagem integrativa (dados de morfologia, genética e acústica) de morfótipos seleccionados de cigarras de Marrocos.

Assim, a identificação inicial dos exemplares foi efectuada com recurso à morfologia externa e da genitália. Nesta abordagem inicial confrontou-se a descrição morfológica de 68 espécies e subespécies dadas para o Oeste Mediterrânico. Seguidamente, a análise acústica efectuada às gravações de som de ambas espécies revelou que os exemplares de *Tettigettalna* sp. (atribuídos a este género pela análise morfológica) possuíam uma estrutura da sinal acústico diferente das espécies congenéricas e como tal, poder-se-ia tratar duma espécie nova. Os morfótipos do outro grupo (sem correspondência morfológica a um género já estabelecido) revelaram uma estrutura da som muito distinto e peculiar com modulação em frequência. Ou seja, o sinal acústico possui dois máximos de amplitude distintos nas frequências de ≈14 kHz e ≈8 kHz, curiosamente fazendo lembrar o som dum flato.

Finalmente, a análise genética com o fragmento do gene mitocondrial Citocromo C Oxidase I, (COI-Lep). apoiam a monofilia de cada espécie, bem como a inclusão da nova espécie de *Tettigettalna* na base do género.

A abordagem integrativa destes dados permitiu assim a descrição da agora designada *Tettigettalna afroamissa* Costa *et al*, 2017, cujo epíteto significa "a deixada em África". Adicionalmente, o outro grupo de espécimes foram atribuídos a um novo género *Berberigetta*, significando "a cigarra dos Berbéres", pertencentes à espécie *Berberigetta dimelodica* Costa *et al*, 2017, cujo epíteto, "duas melodias", se refere ao curioso som emitido. Os resultados obtidos foram publicados na revista internacional indexada *Zootaxa*, líder em publicações de taxonomia e sistemática.

Com a descrição formal da primeira espécie de *Tettigettalna* a ocorrer naturalmente fora da Europa foram levantadas novas questões sobre a história, origem e diversificação deste género e que foram analisadas e discutidas na segunda parte da presente tese. Em particular, a questão de como *T. afroamissa* ocorre em África e se este padrão actual de distribuição das *Tettigettalna* se deve a dispersão a partir da Península Ibérica ou de Marrocos ou se algum evento vicariante está na base desta separação. Por forma a responder a esta pergunta, optou-se por uma abordagem multilocus, sequenciando-se em adição ao COI outros quatro fragmentos, dois mitocondriais (COI-CTL e *ATPase*) e dois nucleares (*Elongation factor 1α* e *Calmodulin*), com uma maior amostragem.

As árvores filogenéticas obtidas por máxima verossimilhança e inferência Bayesiana são largamente congruentes apoiando e reforçando resultados previamente obtidos com apenas o fragmento COI-Lep. Estas análises multilocus apoiam: (1) a monofilia do género *Tettigettalna* com a inclusão de *T. afroamissa*; (2) a posição basal de *T. josei*; (3) uma politomia formada pelas três espécies mais recentes: *T. argentata*, *T.mariae* e *T. aneabi* e; (4) *T. armandi* e *T. defauti* como táxones irmãos e subsequente estruturação entre as populações amostradas de ambas espécies.

Para estimar os tempos de divergência entre espécies, recorreu-se à estimação de relógios moleculares através do programa **BEAST*. Assim, recorrendo-se à calibração de relógios moleculares para este grupo as árvores obtidas apoiam a posição basal de *T. josei* e a inclusão de *T. afroamissa* num "clade" em conjunto com a linhagem de espécies de *Tettigettalna* europeias. A posição basal de *T. josei* também é suportada por estudos prévios que, visando a morfologia e acústica desta espécie, colocaram *T. josei* como o táxon mais divergente do grupo

As estimativas obtidas relativamente à separação de *T. josei e T. afroamissa* da linhagem de *Tettigettalna* europeias são coincidentes com o início do Messiniano e com a crise salínica do Messiniano (5.97-5.33 Ma). Durante este período uma extensa ponte terrestre entre a Europa e o Norte de África, que cortou a ligação do Atlântico ao mar Mediterrâneo resultando na dessecação quase total deste.

A reconstrução dos eventos que levaram à diversificação de *Tettigettalna* inicia-se no Tortoniano (11 -7 Ma). Nesta altura, uma população ancestral estaria dispersa pelo sul do maciço Ibérico. No início do período do Messiniano (7 Ma), esta população ter-se-á expandindo para Sul, pela zona que agora corresponde à cordilheira Bética. O isolamento e especiação de *T. josei* no sul de Portugal, estará associado a este fenómeno. A restante população terá colonizado o Norte de África durante a crise salínica (5.9 Ma – 5.3 Ma). Com a abertura do Estreito de Gibraltar , as populações em ambas as margens ficaram isoladas, originando *T. afroamissa* no Norte de África e as restantes espécies de *Tettigettalna* na Península Ibérica. Assim, o cenário de vicariância é o mais provável para explicar a distribuição de *T. afroamissa.*

As distribuições actuais das *Tettigettalna* que compõem a linhagem europeia (*excl. T. josei*) são concomitantes com os múltiplos refúgios glaciais encontrados na Península Ibérica para flora e fauna. Durante as glaciações, as populações poderão ter ficado isoladas em vales e evoluído separadamente das restantes, dando origem ao actual padrão de distribuição reticulado destas cigarras.

Resumindo, com o exercício da tese descreveu-se duas novas espécies de cigarras marroquinas, *Berberigetta dimelodica* e *Tettigettalna afroamissa* com o uso integrativo de três camadas informativas: morfologia, acústica e genética. Também conseguiu-se explicar a distribuição trans-Mediterrânica da espécie africana, *T. afroamissa*, relacionando o seu isolamento e especiacão com a crise salínica do Messiniano.

Concluindo, esta tese permitiu melhorar o conhecimento sobre a biodiversidade críptica que é característica do género *Tettigettalna*, e em particular revelar e descobrir a origem deste grupo.

Palavras-chave: Cigarras, Oeste Mediterrânico, descrição de espécies integrativa, filogenia, Messiniano.

Abstract

Cicadas are no strangers to people in summer. Despite being difficult to spot among the vegetation they are well-known for the loud songs males produce to attract a potential mate and which are useful to tell species apart. They are also well-known for their long nymph stages that may last up to 17 years. In Portugal and Spain the diversity of cicadas has been long underestimated until last decades. It is the case of the formal description of several species of *Tettigettalna* in 2010. These cicadas are morphologically very similar, therefore only with the recent inclusion of acoustics and genetics data several taxa were unraveled from a single taxon. *Tettigettalna* is a genus of small and dull-colored cicadas, composed of nine taxa (eight species and two subspecies) and are present mainly in the Iberian Peninsula. Several of these species are endemic to the southern portion of the Iberian Peninsula, which led us to ask if this genus could also occur in Morocco. Morocco has a rather large number of recorded cicada species, but these species are the result of only a handful of expeditions, that only delivered morphological data. Therefore, in the summer of 2014, the CoBIG2 group, organized an expedition to the Rif and Middle Atlas of Morocco, in order to collect and record these poorly-known group of insects.

This trip yelded several unidentified morphotypes, two of which were of particular interest. These two morphotypes were studied with an integrative taxonomic approach, with the inclusion of morphology, genetics and acoustic data. One of these morphotypes was identified as belonging to the genus *Tettigettalna*. Acoustic and genetic analyses confirmed this taxa as an acoustically-distinct and monophyletic biological entity. This *Tettigettalna, T. afroamissa*, was named as "*afroamissa*" which can be translated as "the one left in Africa", because this is the first cicada of this genus to be found outside Europe. The second taxa could not be directly ascribed to a known genus on the grounds of morphologic analyses alone, so a new genus had to be erected. *Berberigetta*, the Berberian cicada, its type species has a curious and rather unique calling song. The calling song has two distinct call pitches, one of which can be roughly compared to the sound of "blowing a raspberry", thus the name *B. dimelodica*, meaning "two melodies". This frequency modulation of the calling song is an unlikely pattern to be found amongst Mediterranean cicadas, which may be of interest to further investigate.

The description of *T. afroamissa* as the first *Tettigettalna* outside Europe raised important questions on the origin and diversification of this group of cicadas. To ascertain the evolutionary history of these species, sampling was geographically expanded and five genetic loci were sequenced. We followed a multilocus approach alongside a Bayesian framework to generate a robust species tree alongside estimates for species divergences. The resulting species tree supports the inclusion of *T. afroamissa* while placing *T. josei* as the basal taxon of the group. Divergence estimates of the separations of *T. afroamissa* and *T. josei* from the remainder of the European *Tettigettalna* lineage are mostly concurrent to the early Messinian and the Salinity Crisis (5.97-5.33 Ma) when extensive land bridges formed between North Africa and Europe closing the connection between the Mediterranean sea and the Atlantic ocean. We suggest that during this period, *T. josei* was separated from other lineages by the Guadalquivir basin and *T. afroamissa* is the remnant of a large population that occurred between both continents that divided with the opening of the Gibraltar Strait, separating the *T.afroamissa* lineage from the European *Tettigettalna* lineages. By the end of the Messinian there were (at least) three separately evolving lineages.

In conclusion, this theses allowed a better knowledge on the cryptic diversity of Iberian cicadas and contributed to unravel its origins and rediscover the Moroccan cicadas, that only now we have begun to listen.

Keywords: Cicadas, Western Mediterranean, integrative species description, phylogeny, Messinian.

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branch nodes. Scale bar represents the number of estimated changes per branch length. *C. barbara* (Cba203) and *C. orni* (Cor298) were set as outgroup for A) and B). *H. varipes* (Hva608) was set as outgroup for C) and D). *M. cassiope* was set as outgroup for E). (Cont. Fig. 5.S4) Additional taxa details are included on supplementary information Table S1. Root was truncated with double dashes. Some trees were collapsed due to sampling volume and the remaining samples shown under .i) bullets. PAGE 90

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Chapter I

1. General Introduction

1.1. The classical taxonomy and shifting species concepts

Classical taxonomy followed a path of everyday utilitarian purposes to distinguish poisonous from non-poisonous, edible versus inedible, predator from non-predator. The philosophical concept of *scala naturae*, introduced by the Greek philosophers Plato and Aristotle, ranking the elements of nature from minerals to plants to animals to humans can be seen as a first attempt of hierarchical division, and forming a system for classification still in use in everyday life *c.f* plants divided into trees, bushes and herbs. Aristotle also deepens this categorization of life dividing animals based on the physiological similarities.

In the pre-Linnean era there really was no arrangement on species nomenclature. Latin was the language utilized by scholars and names were often short descriptions of an organism. This rather unpractical fashion of nomenclature was improved by Gaspard Bauhin, botanist and author of Phytopinax (1596), who began to introduce a binomial nomenclature to some plant species, which would be later improved by Carl von Linné.

Named as the father of modern taxonomy, Carl von Linné, latinized to Carolus Linnaeus, a Swedish botanical taxonomist, was the first person to formulate and adhere to a constant, binomial, system for nomenclature of plants and animals. In 1758, Linnaeus publishes the 10th edition of Systema Naturae formally introducing the binomial name to animal nomenclature. Linnaeus also established some of the systematic ranks namely kingdom, class order, genus and species effectively nesting on the previous although clarifying that only the last two are considered "natural" and the former are considered as constructs (Larson 1968).

Linnaeus although writing several notes regarding a species concept, officially never published a definition of the term. Early in his career, Linnaeus speculates that a species is a fixed, immutable entity – a rather creationist view– that could see some degree of phenotypical variation. Later on life, when confronted with indubitable fertile hybrid specimens of plants (Gustaffson 1979), he began to change his own views on the immutability of species, calling these hybrids "daughters of time" (Ramsbottom 1938). Linnaeus, therefore, by stressing the species as a stable basic unit of nature and, later on, on the possibility of the emergence of new species, helped set the stage for the discussion of the idea of species in time (Mayr 1963).

It isn't until the publication of Darwin's essays "On the Origin of Species" in 1859 that the species concept problem takes a new interesting view.

"I look at the term species as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms"(Darwin 1872).

"[…] It all comes, I believe, from trying to define the undefinable." (MS DAR 114:187, letter from Darwin to Hooker)

With Darwin seeing species as transmutable entities through time, on which natural selection acts upon intraspecific variation, it causes species to be seen as a continuum through time and therefore impractical to clearly define where species and populations begin or end.

"Nor shall I here discuss the various definitions which have been given of the term species. No one definition has as yet satisfied all naturalists; yet every naturalist knows vaguely what he means when he

speaks of a species." (Darwin 1872).

In the 20th century, the Modern Synthesis brought a major breakthrough for taxonomy with the biological species concept (BSC), proposed by Ernst Mayr (see Table 1. for the definition of the BSC). Through the years, it has been increasingly evident that a typological species concept was impractical. Morphologically indistinguishable cryptic species were being described and even in sympatry, these taxa would not interbreed and maintain relatively cohese and distinct gene pools. Amongst others, these discoveries facilitated the increasingly wide adoption of a species concept that instead would incorporate and be compatible with contemporary evolutionary theories – an evolutionary species concept.

The Biological Species Concept emphasizes reproductive isolation as the mechanism for speciation. For illustrative purposes, two populations on the edges of a species range may become disconnected, thus reducing gene flow between them, and over time, separate into two dissimilar gene pools – allopatric speciation. This separation can be due to reproductive barriers which were described by Dobzhansky separating these into pre- and post-zygotic mechanisms (Dobzhansky 1954). Although, widely adopted, a main criticism remaining was the inability to test the interbreeding criterion on allopatric populations. Also, parapatric populations of closely-related species, or of species complexes, may have some degree of interbreeding on contact zones, thus, with a strict enforcement of the BSC, these species would be lumped under a single taxon.

The proposition of the BSC by Mayr, one of the most popular species concept, caused a chief response by the scientific community, acting as catalyst for disagreement and new species concepts to be published. Over than 20 alternative species concepts have been published, ever since Mayr named, summarized and categorized previous concepts (Hey 2006). These species concept, still in force to this day, usually fall under six categories (see Table 1.1).

Table 1.1. Categories of species concepts and the properties considered to be necessary to species recognition. Categories summarized from Mayden (1997), Winston (1999), De Queiroz (2007, 2016) and Hausdorf (2011).

Species concept (main advocates)	Definition
Biological (Mayr and Dobzhansky)	*"[...] groups of interbreeding natural populations that are reproductively isolated from other such groups."* (Mayr 2000)
Ecological (Van Valen)	Species are lineages (or sets of closely related lineages) occupying same niche or adaptive zone.
Phenetic (Cronquist)	Species are the smallest groups that are consistently and persistently distinct and distinguishable by morphology.
Cohesion (Templeton)	*"[...] the most inclusive population of individuals having the potential for phenotypic cohesion through intrinsic cohesion mechanisms."* (Templeton 1998).
Phylogenetic – monophyly (Donoghue)	Species are a single lineage consisting of an ancestor and all of its descendants;

	commonly inferred from possession of sinapomorphies. (Donoghue 1985).
Evolutionary (Simpson)	A species is a lineage of ancestral descendant populations which maintains its identity from other sister lineages and which has its own evolutionary tendencies and historical fate (Simpson 1951).

"No term is more difficult to define than "species," and on no point are zoologists more divided than as to what should be understood by this word." (Nicholson 1876).

Taxonomy, defined as the science of species delimitation, can be perceived being torn between ignited arguments of taxonomical experts on different study groups, to whom a "species" can mean different things, depending on the characteristics of the organisms involved. It portrays different significances in solitary sexually reproducing animals, than it is in sexually reproducing plants, clonal plants, and clonal or colonial invertebrates, and even harder to define in bacteria and viruses (Donoghue 1985; Amann & Rosselló-Mora 2001; Zimmermann & Radespiel 2014; Has *et al.* 2015). The existence of species is even questioned to exist in asexual organisms (Fontaneto & Barraclough 2015). The "best" species concept is accesory to utilitarian purposes and can be, thus, subject to the inherent problems of the study group, and may need adjustments if required.

An agreement on species concepts is fundamental in order to achieve nomenclatural stability. This will minimize confusion in scientific communications between fundamental investigators and the applied research areas. Several other disciplines need to have a consensus on what a species is, and how to delimit it, otherwise several problems may arise if misidentifications occur. Moreover, misidentifications from closely related species may lead to problems to other areas, such as public health with misidentification of pathogens or vectors (Singh *et al.* 2010), food supply (Beerkircher *et al.* 2009), ecology (Shea *et al.* 2011; Austen *et al.* 2016), niche modeling (Costa *et al.* 2015), and so on.

Difficulties deepen when studying cryptic species. These are groups of taxa that are morphologically very similar to the point that the boundaries separating these entities become unclear and only with additional layers of biological data, are these species readily separated. Cryptic species may suffer also from usage of alternate species concepts as different concepts may recognize different entities (Baum & Donoghue 1995; Peterson & Navarro-Siguenza 1999; Kwon-Chung & Varma 2006; Liti *et al.* 2006; Liu *et al.* 2012).

A general species concept must be followed in order to falter these concerns.

Advancements towards a general species concept have been made in recent years (De Queiroz 2007, 2016; Hausdorf 2011), and these progresses are geared towards efficiently separate and disentangle misperceptions between species delimitation from species definition. The source of the problem for the different species concepts is due to the intrinsic different biological properties that each concept emphasizes as the most important towards recognizing a species: the BSC highlights reproductive isolation, the ecological species concept emphasizes niche occupation, the phylogenetic species concepts favors diagnosability or monophyly. De Queiroz (2007) perceives the previous species concepts not as concepts *per se* but, nevertheless as necessary properties of species.

De Queiroz proposes a unified species concept – the general lineage concept of species – which states: "*Species are (segments of) separately evolving metapopulation lineages*". Under this proposal, De

Queiroz noticeably separates species conceptualization (the conceptual problem of defining a species category – the *what* to define) from species delimitation (the methods used to infer the existence of a species – the *how* to delimit).

These species criteria for delimitation are the fundamental aspects of the previously defined species concepts, and reformulated to species delimitation rather than species conceptualization.

As illustrated in Fig.1.1, we have an ancestral lineage that splits into two derived lineages over time. As seen in this figure, the species criteria (SC) 1-9 are attained during the divergence of these sister lineages. During this time the lineages are evolving, they gain a set of SC, on which previous alternative species concepts would draw a different cutoff in time on whether we would be dealing with one or two species. This cutoff would be drawn earlier on this continuum if we would consider a phylogenetic rather than a phenetic species concept (the case of cryptic species, for example Hebert *et al.* (2004). On both extremes of this continuum most concepts will agree on the existence of one or two species, as the necessary properties for each species concept have not, or have been, completely fulfilled. It is on this grey area, where conflict amongst previous species concept arises and the concept of subspecies may take hold. With the removal of the source of argument between previous alternative species concepts by making these as necessary properties of the species concept, we are therefore able to describe a species on the basis of these species criteria. If a lineage has been under a sufficiently intense divergence, additional criteria are expected to be met and thus the description is further improved.

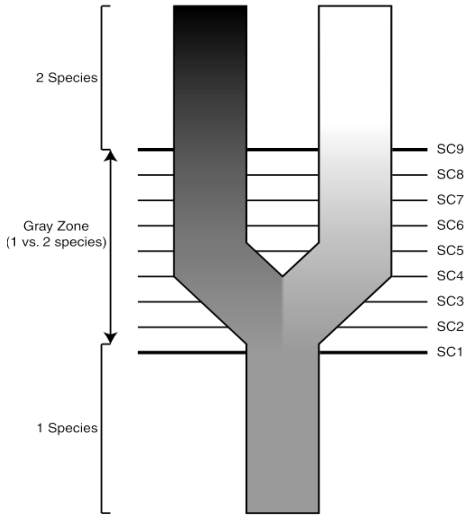

Figure 1.1. Species divergence through time and species concepts. SC1-9 (Species Criterion) are the different biological properties species retain when separately evolving throughout time. This set of SC forms a grey area where alternative species concepts conflict on the number of species. Figure adapted from De Queiroz (2007).

The implementation of this species concept was not without criticism (Hausdorf 2011), but with some taxonomists fully embracing his species concept onto their species descriptions and delimitations (Valcarcel & Vargas 2010; Hertach *et al.* 2015, 2016).

1.1.1 The nonetheless problematic subspecies concept

As a note of remark, the term subspecies has not been the subject of as much work as species has gained throughout the last two centuries, perhaps because it starts to blend between taxonomy and population genetics, making it especially difficult to separate these entities (Mayr 1982).

Formal definitions are given by Mayr (1963), as an extension of the BSC, and Edwards (1954) also provides a definition for subspecies and several other smaller definitions for reproductive barriers that possibly initiate the process of lineage splitting. Both concepts emphasize the geographical race as the source for morphological differences and provide emphasis for introgression on the contact zones of the subspecies populations, yet the criteria Edwards gives to delimit subspecies are purely arbitrarily based on morphological differences – a 75% difference between subspecies (Edwards 1954), which may be useless to apply, as a statistical difference may not translate to an observable biological difference (Mayr 1982; Keita 2014).

A purely phylogenetic approach may also fail to precisely separate specific to infraspecific categories (Braby *et al.* 2012), leading the investigator to over or underspeciate a species group and hamper estimates on global biodiversity, the so-called taxonomy's "lumpers" and "splitters".

The general lineage concept of species (De Queiroz 2007) does help to understand where a subspecies may be in the evolutionary time. Taking again as reference Fig.1.1, in the grey zone, comprehended between SC1-9, it illustrates where a subspecies may be best accommodated, where a lineage has yet to obtain specific criteria to be considered a fully differentiated species – an incipient species, but still has to possess some diagnosable character state to be able to be distinguish from the ancestral lineage and other derived lineages. A subspecies must also exist in a separate time or space scale (allochrony or allopatry scenarios, respectively) so as to occur an incomplete reproductive isolation and allow to evolve, at least, partially separate from these other lineages (Cooley *et al.* 2001; Santos *et al.* 2007). The concept that best fits these hypotheses is proposed by Braby *et al.* (2012) which states:

"Subspecies comprise evolving populations that represent partially isolated lineages of a species that are allopatric, phenotypically distinct, have at least one fixed diagnosable character state, and that these character differences are, or assumed to be, correlated with evolutionary independence according to population genetic structure."

The phenotypical differences stated here must be hereditable and mustn't be subject to environmental variation, of which some species of butterflies may present seasonal polyphenisms (e.g *Araschnia levana* L., *Bicyclus* spp.), that do not account for a genetic pattern (Roskam & Brakefield 1999; Friberg & Karlsson 2010). The authors discuss that the definition can also be applied to parapatric lineages (populations with contact zones) and to sympatric lineages diverging in the ecological space, for example, via differences in the emergence period of short-lived adult forms of insects, such butterflies, cicadas, mayflies (Yoshimura 1997; Cooley *et al.* 2001; Santos *et al.* 2007); spawning events (Rosser 2015) or larval food (Bush 1969; Hebert *et al.* 2004), which may lead to restriction in gene flow and towards an "ecological race" to which, in truth, would likely be described as species undergoing allochronic speciation.

1.2 Females are choosy lovers

"The sight of a feather in a peacock's tail, whenever I gaze at it, makes me sick!" (Darwin, DCP-LETT-2743).

Sexual selection was proposed by Darwin to explain ornate, and often complicated, male body features (eye stalks, leg tufts, colorful feathers or scales, etc...) and elaborate courtship traits (calling songs, dancing rituals, fighting displays, nest decoration…) that seems, at first sight, to reduce rather than enhance survivorship, apparently contradicting natural selection.

For example, cicadas serve as a model on which sexual selection plays a crucial role on acoustic behavior (Karban 1983; Cooley & Marshall 2004). The male cicada will produce a calling song to attract the female. The female after listening to the male will choose whether to approach it or opt by another male. If convinced, the female will approach the male and allow him to court. The male will begin a courtship call and if successful will be allowed to copulate with the female (Boulard & Mondon 1996). Therefore, rather than natural selection, it seems that sexual selection plays a more crucial role in cicada reproductive isolation.

How does the choice of the female impact sexually attractive characters? Can this choice be affected by ambient conditions? If so, do changes to the environment translate into perceivable differences by the females?

A leading theory that addresses these questions is the Sensory Drive hypothesis, placing male and female communication channels into an ecological context, which combines several models for sensory recognition (Endler 1998).

Two concepts should, henceforth, be defined: *signal design*, which refers to the structure and efficacy of the signal, in the way that it is produced by the signaler, transmitted through the environment, detected and received by potential mates or competitors and discriminated from the overall background noise, but also its purpose to elicit a response on the receiver that increases or maintains the fitness of the signaler (Endler 1992); and *signal content*, referring to the information contained within the signal: distance between neighbors, location of food or predators, mate quality, status, intentions of the signaler, etc... (Endler 1998). The Sensory Drive model bridges the gap between natural and sexual selection, in the sense that the signal design is impacted by natural selection, whereas the signal assessment and decision mechanisms that function with content are related to sexual selection mechanisms (Boughman 2002).

It is therefore, a necessary property of Sensory Drive that the signaler and the receiver, often male and female, coevolve together in a sense that changes to the sensory mechanisms of the male signal production will translate the signal transduction abilities of the female (Endler 1998). In other words, the Sensory Drive hypothesis predicts that both the male mating traits and the perceptual systems underlying female preference undergo local adaptation.

Three interrelated processes impact the coevolution of male and female signaling system and are necessary for this hypothesis: *habitat perception*, *perceptual tuning* and *signal matching*.

Habitat perception is key to influence changes to the male-female signaling system. Each habitat has a rather unique set of climatic, biophysical and biochemical variables, and these variables will affect the transmission of male signals by degrading and blurring these against the background noise (Kime 2000). Consequently, signals that preserve their content, are less affected by degrading variables, remain conspicuous and easier to perceive by females are likely to be favored, because these will signal more potential mates from further away from the source, and also be a potential indicator on the male's fitness.

Because habitats are heterogeneous, a signal that transmits well in a habitat may not have the same performance on another environment, and thus, signal design may vary amongst populations of the same species that live in different habitats.

As an example of habitat perception, males of *Habronattus* jumping spiders court females by producing a series of visual displays and seismic signals (substrate-borne vibrations) (Elias *et al.* 2003). Researchers placed the males under three natural-occurring substrates (rock, sand and leaf litter) and measured the signal quality and design reaching the female. Results showed that substrates such as rock and leaf litter allowed for the signals to transmit with little-to-moderate attenuation, with sand rapidly dispersing the vibrations. Because leaf litter transmits signals most extensively, effectively and reliably with few changes to signal design, females would readily mate in this substrate (Elias *et al.* 2004). This experiment shows how habitat can affect signal design and how it can influence female choice.

Concomitant with habitat perception, variations must also be accompanied to female perception – perceptual tuning. Since environmental variables impact signal transmission, these will also have an effect in the ability for the female to perceive these signals. Thus, it is expected that the female perception will also evolve under such selective pressures. Perceptual biases are also likely to impact the direction the perception of female (and male) can evolve (Ryan & Cummings 2013).

For an example regarding perceptual tuning, two populations of cricket frogs (*Acris crepitans*) from a grassland habitat (low ambient noise) and from a pine forest habitat (high ambient noise) were investigated (Witte *et al.* 2005). The males produce a calling song to attract the females into mating. In the forest habitat, the male calls suffer greater attenuation and degradation than in the grassland habitat. Previous works by the authors showed that male calls from the forest population transmit with much less degradation in either habitat than those from open grassland populations (Ryan & Wilczynski 1991). These results suggested that habitat sound variables provided a selective force for the improvement of the male signal design for better transmission reliability. In this study, Witte *et al.* (2005), turned their attention to the female perception. The authors used neurophysiological models as aliases for female perception from both habitats and measured the cross-effect of ambient noise from both habitats (noise x population) on noise filtering abilities. Results indicated that the average forest female model is better than the average grassland female model in filtering ambient noise from both habitats. Thus, it is likely that the forest females have evolved auditory filters that are better at filtering out ambient noise typical of the habitats in which this population lives. Taking also into account the results from Ryan & Wilczynski (1991), we are able to comprehend how a more acoustic challenging habitat provided a sufficient selective force for the evolution of the communication dyad (male call design and female perception) on the forest cricket frogs.

Thirdly, signal matching is expected to happen due to natural selection occurring separately on the male signaling and the female perception. Females are most sensitive to a certain color wavelength or sound frequency, which closely matches the male's signal, be it acoustic or physical (Cummings 2007). Thus, a male that produces a signal that closely links to the female sensitiveness, will be likely be heard better from further away, also by allowing the female to easily detect the male and reduce energy costs relating to the activity of male detection (Endler 1998). Furthermore, signal matching can be biased by the presence of heterospecific mates using a similar communication channel. Reproductive character displacement is expected to occur when males converge independently to a similar communication channel and, in sympatry, changes may occur in one or both species' channels (Poeser *et al.* 2005). This displacement can also be accompanied by changes to the mechanisms underlying female perception. Thus, in this scenario, signal matching occurs not of a female's mating preference among conspecifics, and is unrelated to any strategic aspects of signal design, but arises an incidental consequence of same-species recognition (Ryan & Cummings 2013).

As an example of signal matching, male cicadas of *Tettigettalna josei* produce a calling song with a peak frequency around 17 kHz (Simões *et al.* 2014), and the females have their sensory organs interneurons tuned both for the low-end (1-6 kHz) and high-end (12-25 kHz) of the spectrum allowing the female to fine tune and discriminate the presence or absence of male callings (Hennig *et al.* 2000).

Taken together, the three processes of Sensory Drive (habitat perception, perceptual tuning, and signal matching) can cause female perception and male signaling to coevolve. This might occur because they are shaped by similar environments, or because close matching increases the success of communication (Boughman 2002).

As examples, all three processes of Sensory Drive have been implicated to have shaped the evolution of surfperchs (Cummings 2007), cichlid fishes (Seehausen *et al.* 2008; Maan *et al.* 2017), bamboo-forest birds (Tobias *et al.* 2010), guppies (Endler 1992) and sticklebacks (Scott 2001).

Now, can we relate tendency to speciate and variability in sexually attractive male characters *i.e* in signal design?

Following the Sensory Drive hypothesis, it would be expected that variations of the male signal design should follow environmental changes through local adaption. If there is not a relation between the two, then genetic drift or historical events determine signal design diversity. Nonetheless, should we consider that the variation of signal design follows a normal distribution, then sexual selection through female preference should curtail most of this variability if we would be dealing with a species with a stricter female choice? Consequently, if such design variation occurs through the generations, then, a species with a high degree of variability in signal design should also have more flexible patterns of female choice, in other words, less "choosy" females should allow for a larger intraspecific variation on the design of the sexually attractive signal.

Likewise, incipient species (*i.e* newly formed) are frequently tied to the evolution of behavioral changes, such as novel changes to sexual communication channels. But, as seen above, this variability is constrained by the signal matching of both sexes. The co-evolution of male signaling traits and female choice, as a by-product of ecological shifts, should be critically dependent of genetic linkage on loci that underlie male signal design and female choice phenotypes (Kronforst *et al.* 2006; Wiley *et al.* 2012). Now the obvious question arises: what mechanisms can cause the genetic linkage between loci that underlie signal design and preference?

The reason this question needs to be answered is a corollary of signal matching. It is because females preferring males with a certain signal design will preferentially mate with those males – assortative mating – and their sons must exhibit the same signal design and their daughters must also bear similar mate preferences.

This genetic linkage suggests a mechanism for how signals and preferences might stay coordinated as species diverge, because selective pressure keeping sexual communication behaviorally coupled would be aided by common genetic factors. When a new mutation affects the phenotype of one sex, it simultaneously affects the complementary phenotype of sex the other, facilitating signal matching through pleiotropic mutations (Shaw & Lesnick 2009). This mutation on the other hand, should be controlled by environmental variables, by natural selection. Consequently, individuals with a novel mutation that affects signal design, and likely species recognition, are no longer recognizable as same-species by the remainder of the population, diverging by sexual selection. This consequence will likely keep maintain the mutation on this lineage, reduce hybridization, and generate a distinct gene pool overtime, giving rise to a new species.

A simple and likely prevalent mechanism to genetic linkage of signal design and preference is the

presence of genetic structures that prevent recombination between incipient species (Kronforst *et al.* 2006). Examples from the literature come from *Drosophila pseudoobscura* and *D. persimilis*, two species that occasionally hybridize in North America (Noor *et al.* 2001; Machado *et al.* 2002) and also from the sunflowers, *Helianthus annuus* and *H. petiolaris* which form hybrid zones (Rieseberg *et al.* 1999). These studies show correspondence between the genomic regions associated with reproductive isolation and chromosomal inversions that show little or no evidence of gene flow and can result in hybrid sterility. In more extreme scenario, *D. melanogaster* with a point mutation in the gene *desat1* influences both production and discrimination of cuticular hydrocarbons that act as sex pheromones, thus forming a novel reproductive barrier (Labeur *et al.* 2002; Marcillac *et al.* 2005).

Other cases that offer compelling evidences for physical linkage in loci that lie beneath signal design and preference are found in *Heliconius* butterflies and *Laupala* crickets. Male *Heliconius* butterflies will approach and choose a female via visual cues. Assortative mating between *H. pachinus* (yellow wings) and *H. cydno* (white wings) is mainly driven by wing coloration which is controlled by *wingless*, of Mendelian inheritance (Kronforst *et al.* 2006). Male preference for female coloration is explained by the same locus, possibly caused by pleiotropic effects (Kronforst *et al.* 2006).

Instead of the male driving sexual selection, in the *Laupala* crickets it is the female that chooses amongst the males by their calling song. Wiley *et al.* (2012) working on two recently diverged species *L. kohalensis* and *L. paranigra* co-located a Quantitative Trait Loci (QTL) that determines male signal design and another QTL determining female preference to the same physical chromosomal domain. Rather than a Mendelian inheritance (as is the case for the *Heliconius* spp. abovementioned), signal design and preference, in *Laupala*, are quantitative, multi-genic traits (Shaw & Lesnick 2009).

In conclusion, Sensory Drive is a hypothesis of sexual selection that likely applies to organisms that use sexual communication channels that: (1) are affected by environmental variables and; (2) rely on close sensory matching for mate assessment and species recognition. Sensory Drive pieces on three interrelated levels: habitat perception, perceptual tuning and signal matching. Each of these levels are affected on some varying degree by natural and sexual selection. When an organism moves to, or suffers, new environmental variables, in the presence of standing variation of signal design, natural selection will likely favor signals that disperse further, more reliably and effectively. As environmental variation changes signal design, changes must also occur to the receiver's preference. These individual, but mutual changes to the signal design and receiver's perception must also be closely matched. Matching of signal and preference is facilitated when these phenotypes are somehow linked in the genome, be it by chromosomal inversions, which greatly reduce recombination or even pleiotropy by mutational events on a single gene. When a mutation occurs to these loci and is favored by natural selection (i.e habitat perception) both male and female offspring can express complementary phenotypes that close-match each other, but not to the remainder of the population. Ultimately, this will reunite the necessary conditions to form distinct gene pools and lead to the formation of new species.

1.3 Taxonomy and species concepts in cicadas

Cicadas (Hemiptera: Cicadoidea) are a successful group of insects with nearly 2,500 species described on every continent except Antarctica. These insects are well-known for the male ability to produce loud sounds to attract females into mating. Cicadas are also known for their impressively long nymph stages, remarkably by the periodical cicadas, *Magicicada* spp. that have up to 17 years long, synchronized nymph cycles (Yoshimura 1997; Cooley *et al.* 2001). After the nymph stages feeding on root xylem vessels, the last instars climb out of the ground and moult giving rise to a winged adult, short-lived, living usually a few weeks. In Europe, most cicadas have a life cycle of 2 to 6 years and may be of gregarious (e.g. *Cicada orni*) or solitary nature (e.g. *Lyristes plebejus*) (Boulard & Mondon 1996).

The sound produced by the males can be of several types (Boulard & Mondon 1996):

- Calling song, the most common call of male cicadas that is used to call the females;
- Courtship call, that is emitted when a singing male is approached by an interested female which also involves the use of the anterior wings to produce some degree of stridulation;
- Alarm call, when a cicada senses something unusual in its environment, like a bird or a passing collector;
- Protest calls that are subdivided, between others, in:
    - Opposition calls, when multiple males of same species are present in the same area or tree;
    - Distress calls, when a male cicada is caught.

This variety of calls is produced by a sound-specialized organ, the tymbal, (Fig. 1.2) an abdominal membrane attached to the tymbalic muscles (Fig. 1.2C) which, with each round of contraction and relaxation, is able to vibrate on frequencies between 35 and 100 Hz producing sounds with frequencies up to 25 kHz, past the human audible frequency (Wessel *et al.* 2014). These callings can become very loud, with the loudest insect, the cicada *Brevisiana brevis* (Walker, 1850) having a calling song peaking at some ear-numbing 106.7 dB (Petti 1997). The tymbal structure is very interesting and diverse amongst

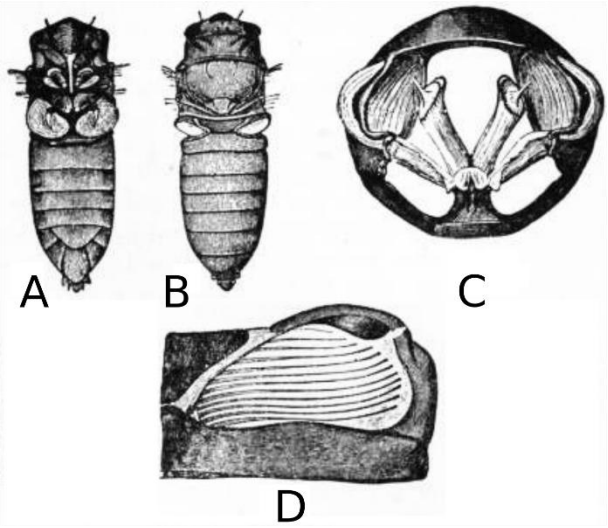

Figure 1.2. Morphology of a cicada tymbal. (A) Ventral view of a male cicada showing the fan-shaped opercula covering the tympana, which the cicada uses to hear. (B) Dorsal view of a male cicada showing the tymbals between the thorax and abdomen. (C) Abdominal cross-section of the male cicada showing the tymbalic muscles connected to the tymbals and anchored to the sternal cuticle. (D) Tymbal membrane, a convex layer of the cuticle, and often possessing several thin and resilin-coated portions intercalated by thickened ribs, as shown. Adapted from Carpenter (1911).

cicada genera, and is composed of several parallel ribs that may divide into sub-ribs (Fig.1.2D) and membranous parts of different shapes, making it a taxonomically valid structure to distinguish cicada genera (Ahmed *et al.* 2015).

The idea that the calling song of male cicadas is species-specific was first introduced by Myers (1929):

"Every cicada with which we are familiar may be recognized with certainty by its song".

Myers published descriptions of several species with a detailed calling song analysis (Myers 1926) and is most likely one of the first authors to add the male calling song to the description of cicada species. This procedure is in vigor to this day, with the majority of novel cicada descriptions including a detailed calling song analysis in conjunction with the typical morphologic and/or genetic analyses (e.g. Gogala & Trilar 2004; Sueur & Puissant 2007; Puissant & Sueur 2010). Myers, therefore, introduced early on a prototype idea, that cicadas may recognize conspecifics via the calling song.

This idea would be further generalized to other taxa with the introduction of the Specific Mate Recognition System (SMRS) species concept of Paterson in 1985. The SMRS (Paterson 1985) states that species are populations of individuals which share a common mate recognition (or fertilization) system. Being a system that must be recognizable by both sexes, it is predicted to be under a strong stabilizing selection. According to Paterson, it is only when a fragment of the original population becomes isolated in a new habitat (with different environmental conditions), that natural selection may act upon the SMRS in small steps, with each step re-establishing the co-adaptation of male and female recognition patterns (Paterson 1980), which can be seen as a form of sexual selection. Under Paterson's model of evolution, speciation occurs only when the members of the derived lineage have been so extensively modified that they are no longer able to recognize and interbreed with the ancestral lineage, in a similar manner, leading towards the definition of the BSC of Mayr. Paterson (1980) also states that these modifications, over time, to the SMRS of a derived lineage may be caused by pleotropic effects, thus speciation may be an incidental effect of adaptation to a new habitat by the derived lineage.

The idea that specific mate recognition systems should be invariant within species, a typological view, attracted dense criticism from Mayr at the time (Mayr 2000), whom vigorously defended an evolutionary view on species concept. It is this very idea that Paterson conveys that some authors consider this species concept as a misconception (Mayden 1997; Mayr 2000; Mendelson & Shaw 2012). The idea that speciation arises from modifications to a lineage's SMRS addresses the *how* – the pattern –, but not the *why*, – the process – of the question of how species are formed. Also, it does not address speciation in uniparental organisms, or even how speciation can occur in sympatry, as according to Paterson, it must happen as an adaptation to a new environment.

Mayr (2000) also discusses that males are ready to mate even with heterospecific females, and females are more selective tending to prefer only intraspecific matings, in order to reduce the effort of producing possibly wasteful hybrids (Peacock *et al.* 2004; Wilson 2006). Thus, under Paterson's species concept the males with a different, broader SMRS would be considered of a different species that of the female's stricter SMRS.

Paterson's concept may not be as broad and encompassing as the BSC, however using it, nested under De Queiroz species concept, makes it, currently, the top species delimitation criterion for cicadas.

Although the great number of described species of cicadas and their naturally conspicuous noisy nature, most cicadas are yet poorly known. With the new advances on genetic analysis and acoustic data collection, new species were recently described and separated from previously existing taxa (Hertach *et al.* 2015, 2016). Particularly interesting is the recent data on new species complexes formed by very morphologically similar species. In Europe, the *Cicadetta montana* (Scopoli, 1772) *sensu lato* once

thought to be a single widespread species of cicada is now divided in several complexes: *C. montana sensu stricto* group (including *C. brevipennis*), *C. cerdaniensis s. l.* and *C. macedonica s. l.* (Gogala *et al.* 2008). Acoustics also played a major role in discriminating another complex within the *C. montana s. s.* complex (Hertach *et al.* 2016), although typical fast-evolving molecular markers (COI and COII) were not able to clearly delimit these taxa, with an integrative approach with acoustic, morphological, genetic, ecological and spatial data the authors were able to recognize five distinct lineages assigned to the species and subspecies level within the *C. brevipennis s. l.* complex .

Bioacoustics, the field of study for the dispersion, production and reception of sound, is a good approach for the study of song production in cicadas. An acoustic analysis can have two levels: spectrum and time variables domains. The use of both levels of analyses of the calling song provide a recommended approach towards several studies in cicadas, such as song character displacement (Cooley *et al.* 2001), acoustic interference (Seabra *et al.* 2006) and species discrimination (Gogala 1995). Fig. 1.3 depicts an example of a typical acoustic analysis of the calling song of *C. orni*. The oscillogram (Fig. 1.3A) displays the structure of the song, which consists of a simple repetition of echemes, a group of pulses, in a rapid succession; the spectrogram (Fig. 1.3B) gives details on spectrum variables, which comprise the limits and peak frequencies, bandwidth and frequency quartiles; and the mean amplitude spectrum (Fig. 1.3C), depicting graphically the distribution of the mean amplitudes as a measure of sound intensity on the frequency range.

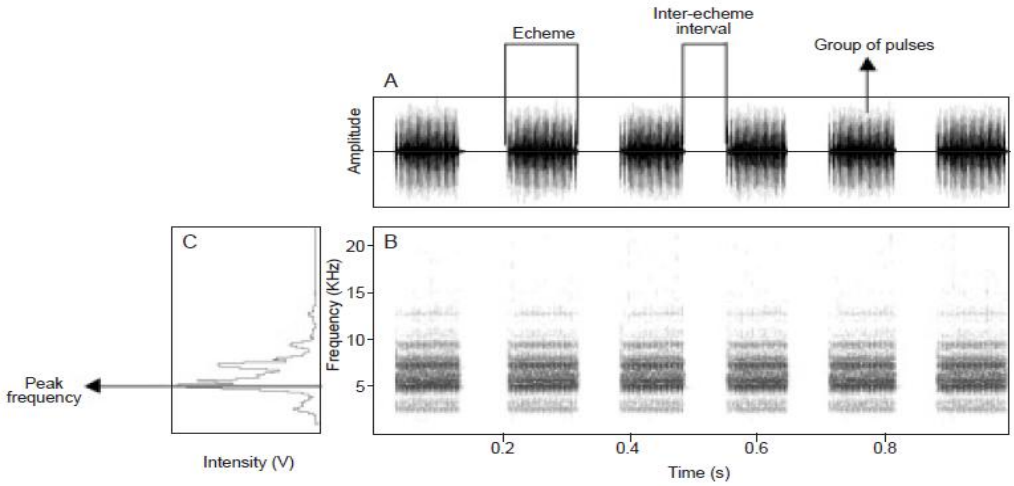

Figure 1.3. Example of a calling song analysis of *C. orni*. (A) Oscillogram (amplitude vs. time); (B) Sonogram or spectrogram (frequency vs. time); and (C) Mean amplitude spectrum (frequency vs. amplitude). Adapted from Pinto-Juma *et al.* (2005).

Cicadas make up for a taxonomically challenging group. In the previous century, many cicada expeditions where prepared with few regards to acoustic or *post hoc* genetic analysis (Villiers 1943; Boulard 1980, 1981, 1987), as the methods for collection or preserving these data were not fully developed or were unavailable at the time, leaving only room for morphologic analysis aimed at species description. Consequently, the earliest descriptions of many cicada species predating the general application of genetic and acoustic analysis do not contain this type of precious information and most are severely deficient on other informative layers. Therefore, the comparison of the type specimens to previously recorded species is, many times, a daunting challenge and add increased difficulty to the description of new species. Furthermore, the descriptive papers of some species sometimes do not follow the ICNZ code for nomenclature, holotypes may be lost or invalid and thus, as a consequence, neotypes must be reassigned as type specimens (Puissant & Sueur 2010).

1.4 Cicadas: a fine model for studying evolution

Cicada populations can occur in regimens of allopatry, parapatry or sympatry and considering their peculiar specific mating recognition systems associated with their low dispersal capabilities (Karban 1981; Simões & Quartau 2007), varying host-specificity, these cicadas can potentially be useful models to test a variety of ecological and evolutionary hypotheses.

The best well-documented example of evolution in cicadas is undoubtedly the broods of periodical cicadas of the eastern USA. The *Magicicada* genera, composed of seven spp., is divided into three monophyletic species groups: the *Decula, Cassini* and *Decim* (Cooley *et al.* 2001). With rare exceptions, at any given population, these species groups can have synchronized life cycles, emerging at a similar schedule, forming one-year localized clusters, termed "broods". There are currently 15 extant broods and these can be composed of any species of the three species groups (Sota *et al.* 2013). These broods can emerge at a 13- or 17-year rate, if found on the southern or northern part of the *Magicicada* spp. range, respectively.

This parallel evolution of the obtainment of lengthy and prime-numbered life cycles have been attributed to Pleistocene climatic cooling as a way to avoid hybridization and increase mating success in populations with reduced mobility and low number of adults (Yoshimura 1997). Another view to explain these life cycles is attributed to the avoidance of predators or parasitoids through high density emergences and longer nymph stages to avoid predator synchrony (Williams & Simon 1995). Within each group, the species are extremely similar in appearance, behavior, male song, and genetics, only distinguishable by emergence rates (Sota *et al.* 2013). Also, intriguingly, emergence rate counterparts (13 vs. 17) are the most closely related taxa, suggesting that speciation in *Magicicada* is, in part, caused by permanent shifts in life cycles allowing lineages to diverge by allochronic speciation (Cooley *et al.* 2003).

Species description can also be based on the study of evolutionary patterns. Marshall & Cooley (2000) found populations of unidentified *Magicicada* singing in chorus along *Magicicada tredecim (*Walsh and Riley, 1868) with two distinct call pitches, an indicative of possible reproductive character displacement caused by the presence of two distinct biological entities. Both entities also present small differences in morphology only when in sympatry, when it is not the case they are indistinct without the assessment of genetic information, thus remaining so long unrecognized as a single taxon, *M. tredecim*. By analyzing acoustic recordings, the authors found that the shift of call pitch was being produced solely by the new species (about two octaves above *M. tredecim*). This asymmetric shift was being perceived by receptive females of the new species and remained unresponsive to calling song of *M. tredecim* males. Prior analysis of phylogeographic data revealed the presence of two distinct mitochondrial lineages without introgression in the sympatric zone (Martin & Simon 1988). Due to these discoveries, this new species was formally described as *M. neotredecim* Marshall & Cooley, 2000. This study is one of the first and few to document the occurrence of reproductive character displacement in cicadas.

A great deal of studies relating to rapid evolutionary adaptive radiations in cicadas can also be found, namely in the colonization of New Zealand's South and North Islands. These island where exposed to two independent colonization events of the islands by two overwater lineages, *Maoricicada – Kikihia – Rhodopsalta* (MRK) clade (with 50 estimated spp. and ssp.), closely related to the New Caledonia cicadas, and by the *Amphipsalta – Notopsalta* (AN) clade (only 4 spp.) related to Australian taxa (Marshall *et al.* 2016). Both lineages ancestors colonized the islands at about the same time during the Miocene (ca 23 – 5 Mya) and experienced similar landscape and climate changes since then (Marshall *et al.* 2012).The great diversity of species in the MRK clade may be due to early rapid radiation to the response of the availability of suitable habitats.

Maoricicada spp. exhibit an alpine to subalpine distribution with few species occupying lowland habitats. Ancestral state reconstruction analysis hints towards the lineage's ancestor being of dark coloration and dense pilosity adapted to alpine, colder climates. By combining altitudinal biogeographic patterns and molecular clocks, it is shown that the alpine species' main radiation (4.5 – 4.8 Mya) corresponded to the uplift and acceleration (ca. 5 Mya) of the Southern NZ Alps (Buckley & Simon 2007; Marshall *et al.* 2008), with some species migrating back to more suitable habitats at lower altitudes.

The *Kikihia* cicadas show even greater specific differentiation triggered by the adaptation to several habitats. *Kikihia* spp. do not habit alpine habitats, preferring subalpine (3 spp.) to lowland forest and open meadows (25 hypothesized spp.) (Arensburger *et al.* 2004). This diversification occurred roughly at the same time as the *Maoricicada* spp. (3 – 5 Mya) and is concurrent with the uplift of the Southern Alps. Because many *Kikihia* spp. are habitat-specific it is likely that species are formed by adapting to new environments. Hence, is it expected that the opening of several suitable habitats in a short time period, caused by tectonic forces rather than glacial events, was accompanied by the rapid explosion in diversification of several *Kikihia* spp. (Marshall *et al.* 2008).

Abnormally, the MRK clade shows greater and earlier divergence than the AN clade. This clade presents only four ancestral lineage splitting events, giving rise to the four extant species. With the support of molecular clocks, the authors hypothesize that these differences in the current pattern result from a stasis with little to no speciation or extinction episodes that thinned derived lineages of the AN clade. Only more recently did these species start to diverge (less than 1 Mya) with the last glacial events shaping speciation events more evidently than of the MRK clade (Marshall *et al.* 2012).

In South Africa, the *Platypleura plumosa s. l.* comprises several species from semi-arid habitats (*P. plumosa s.s, P. hirtipennis* and five other proposed species). These species are confined to river basins, and most occur in a regimen of allopatry. This pattern of close relationship with river basins was never linked to species with non-aquatic stages and it is very likely that these cicadas disperse very little outside these basins, where plant hosts are restricted. The seven mitochondrial lineages found, each corresponding to a species, were dated and splitting likely occurred during the Plio-Pleistocene boundary (≈1.9 Ma). The authors pose the hypothesis that this radiation was caused by the Pleistocene climatic cycles with arid glacial periods reducing a population's distribution to more restricted river basins, decreasing gene flow between populations, and resulting in the observed vicariant speciation events, each linked to a river basin (Price *et al.* 2010). The same climatic events also led to sea level changes and are a likely candidate to the current distribution patterns of the coastal *Platypleura stridula* species complex, which has several mitochondrial lineages distributed across the South African coast and southern mountain ranges (Price *et al.* 2007).

The effects of the Pleistocene climatic cycles can also be observed in European cicadas. The species complexes *Cicadetta brevipennis s.l* and *C. cerdaniensis s.l.* are readily separated by the male calling song (Hertach *et al.* 2015). Because both groups share a similar ecology, it is possible that these taxa could occur in parapatry or sympatry during the Ice Ages. During the colder periods, these taxa were allocated to the main southern peninsular refugia: the northern and southern Apenninians, Iberia and the Balkans. It is observed extensive mtDNA introgression between both song-delimited groups, occurring in all four refugia, likely caused by the occasional contacts that occurred during these periods. Both song-delimited groups have been the subject of similar, but complex, evolutionary patterns, stemming from distribution range reductions during the glacial periods that concentrated gene flow, but not entirely, between these song groups (Hertach *et al.* 2016).

1.5 The case study: *Tettigettalna* Puissant, 2010

In the Iberian Peninsula, the diversity of cicadas was largely underestimated until the recent description and revision of ten small sized cicada taxa belonging to the genus *Tettigettalna* (Puissant & Sueur, 2010). This genus arose as a response to the recent redescription of the genus *Tettigetta* Kolenati, 1857 by Lee (2008).

Eight of these species are endemic to Iberian Peninsula, with seven of them occurring only in the southern portion of the peninsula and with *T. estrellae* restricted to the north of Portugal(Fig. 1.4). *T. argentata* has the broadest known distribution ranging from the south of Portugal to the south of Switzerland and Slovenia, reaching Italy and France (Puissant & Sueur 2010; Simões *et al.* 2013).

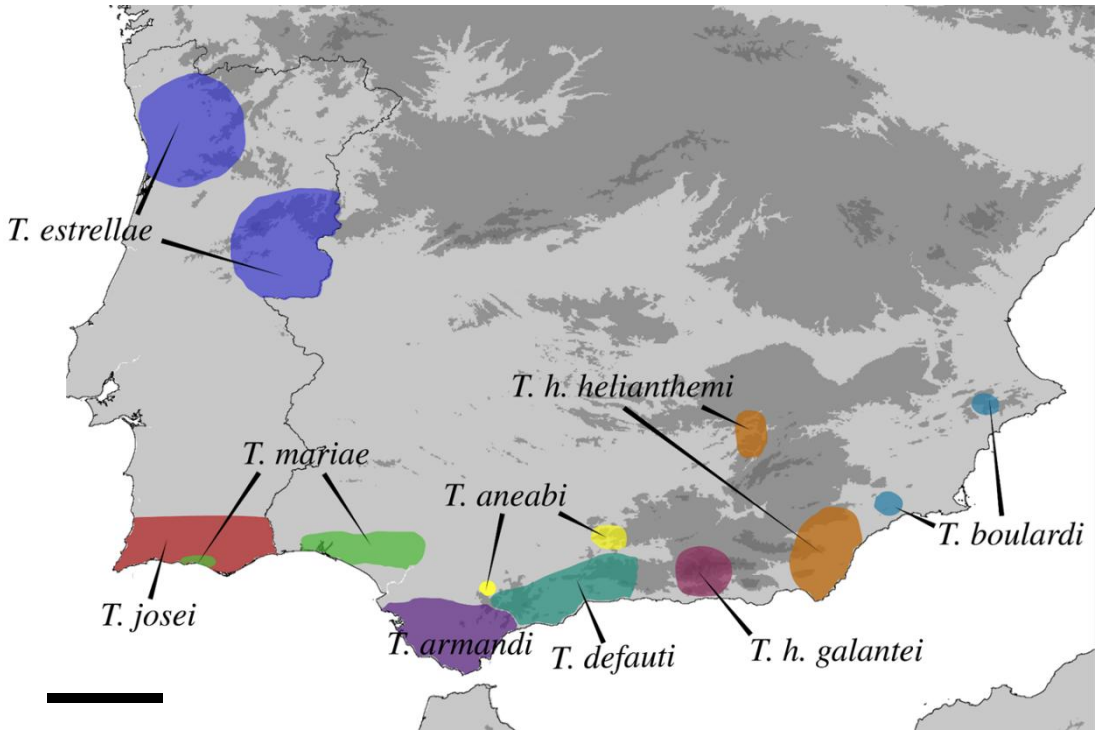

Figure 1.4. Distribution map of the genus *Tettigettalna* with approximate distribution areas extracted from Puissant & Sueur (2010); Simões *et al.* (2013); Nunes *et al.* (2014). The distribution *of T. argentata* is not shown as it is widespread across the Iberian Peninsula, only exempt from the Betic ranges. Scale bar equal 100 km.

Amongst others, a notable case of sympatry found in this genus is between *T. mariae* and *T. argentata* populations. In fact, *T. mariae* is only found in the south coastal region of Portugal and Spain and is often found in sympatry or parapatry with *T. argentata* (Simões *et al.* 2013). These two species are morphologically indistinct but analysis of mitochondrial COI sequences separates *T. argentata* in northern and southern clades. Surprisingly the latter cannot be genetically distinguished from *T. mariae*, further sharing the most common haplotype. However these two species have a distinguishable acoustic behavior, possibly contributing to their reproductive isolation, making it unclear whether the shared haplotypes between Southern *T. argentata* and *T. mariae* populations are due to introgression (existence of gene flow between populations) or incomplete lineage sorting (imperfect segregation of alleles into well-defined lineages) (Mendes *et al.* 2014). Additional studies are underway to determine the cause of the shared haplotypes based on pattern analysis of the mitochondrial sequences.

Such a great diversity of cicadas of the *Tettigettalna* genus especially on the southernmost part of the Iberian Peninsula has raised questions on the origin of the genus. The Western Maghreb region of North

Africa has often been reported as the centre of origin of several Iberian species (Schmitt 2007) and therefore, is an area where the closest relatives are likely to be found.

Being a mostly south Iberian genus, it is also very likely to find *Tettigettalna*'s closest relatives in North Africa. The occurrence of these small sized cicadas in North Africa has not yet been properly accessed. Some species were described for Morocco and surrounding countries, yet these descriptions are only based on morphological analysis without additional layers of relevant data, such as acoustics, genetics, ecology or distribution. Also, no recent attempts have been made on the area to survey species of cicadas, leaving an important gap in the knowledge on the biodiversity and evolution of this group of cicadas. In order to improve our data on the North African cicadas, a fieldtrip to Morocco was performed in 2014 in order to collect and record acoustic data on these poorly known cicadas, and to search for the *Tettigettalna'*s closest taxa. This fieldtrip yielded several unidentified morphotypes and, as predicted, a preliminary analysis pointed to an undescribed species closely related to *T. argentata*.

1.6 Objectives of the thesis

There are two main objectives of the present thesis:

The first objective is to apply a three-pronged approach methodology to the description of undescribed cicada species from Morocco with morphology, acoustics and genetics.

The second objective is to construct a species-tree of the *Tettigettalna* genus and use divergence times estimates to study the impact of major geological and bioclimatic events on the speciation patterns of this group.

The thesis will be separated into four main chapters. This first chapter has abridged the main aspects of the current panorama of species concepts and how it is of crucial importance to have in mind when describing new species, specifically cryptic species, such as this marvelously complex group of cicadas I have partaken to study and share the findings. Cicadas, particularly their calling song, can give clues to how genetically inherited behavior traits can evolve alongside with habitat adaptation.

The second chapter will partake in a three-pronged approach to cicada taxonomy with morphology, acoustics and genetics providing a recommended methodology for a general and clear description of cicada species. In this chapter, I resorted to an initial morphological overview, with the qualitative and quantitative measurement of 23 morphological variables of all 27 individuals of two unidentified taxa collected from the expedition to Morocco, to first try to identify or ascribed these taxa to a genus. A critical comparison of the obtained morphologic data of these taxa to the descriptions of the 68 recorded Cicadidae species and subspecies of the Western Mediterranean pointed towards the hypothesis that we would be dealing to hitherto unknown and undescribed genus and a species belonging to a known Iberian group, the *Tettigettalna*. Acoustic analyses performed on the *Tettigettalna* sp. morphotype revealed a dissimilar calling song pattern of its congeners further strengthening the hypothesis of a new species. A preliminary acoustic analysis on the calling of the other undescribed species (and genus) revealed two distinct song patterns to which a finer acoustic scrutiny was applied, showing a calling song pattern much unlikely heard. Finally, a phylogenetic analysis of mtDNA barcodes supported the monophyly of the *Tettigettalna* sp. nov. and also the new genus/ species, all the while separating the latter, from closely related taxa.

The third chapter will tackle the phylogeny of the *Tettigettalna* genus. With the prior formal description of the first *Tettigettalna* outside of Europe, it led us to question the genus's history and origin. We ask whether Morocco is the point of origin of the genus and then crossing to the Iberian Peninsula where it diverged into most of the species, or if part of the ancestors' populations of *T. afroamissa* crossed the Gibraltar Strait to Morocco and there it evolved separately from its congeners.

We will also search for evidences of large vicariant geographical events at the root of the separation of *T. afroamissa* from the rest of the genus' species or if it was due to overseas dispersal. To answer this question we will build upon – and confront – the published phylogenies of the genus with the additional sequencing of mitochondrial and nuclear markers to construct a species tree and apply divergence time estimates with **BEAST*. Our results point towards the hypothesis that the *Tettigettalna* main lineages have been shaped by a major geological event: the Messinian Salinity Crisis.

In the fourth chapter I will be discussing the results from the two previous chapters in the context of the original proposed question and with those, propose new lines of work and discuss the adequacy of the adopted approaches and new questions that this work opened.

Chapter II

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Morphology, songs and genetics identify two new cicada species from Morocco: *Tettigettalna afroamissa* sp. nov. and *Berberigetta dimelodica* gen. nov. & sp. nov. (Hemiptera: Cicadettini)

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Abstract

Morocco has been the subject of very few expeditions on the last century with the objective of studying small cicadas. In the summer of 2014 an expedition was carried out to Morocco to update our knowledge with acoustic recordings and genetic data of these poorly known species. We describe here two new small-sized cicadas that could not be directly assigned to any species of North African cicadas: *Tettigettalna afroamissa* sp. nov. and *Berberigetta dimelodica* gen. nov. & sp. nov. In respect to *T. afroamissa* it is the first species of the genus to be found outside Europe and we frame this taxon within the evolutionary history of the genus. Acoustic analysis of this species allows us to confidently separate *T. afroamissa* from its congeners. With *B. dimelodica*, a small species showing a remarkable calling song characterized by an abrupt frequency modulation, a new genus had to be erected. Bayesian inference and maximum likelihood phylogenetic analyses with DNA-barcode sequences of Cytochrome C Oxidase 1 support the monophyly of both species, their distinctness and revealed genetic structure within *B. dimelodica.* Alongside the descriptions we also provide GPS coordinates of collection points, distributions and habitat preferences.

Key words: Cicada, new genus, new species, Morocco

Introduction

Cicadas (Hemiptera: Cicadoidea) are a successful insect group with a unique sound production system and thousands of species worldwide (Sanborn 2014). Males produce species-specific acoustic signals, mainly to attract females for pairing and reproduction. These signals have influence in reproductive isolation and thus can be used as important taxonomic characters (Claridge 1985; Boulard 2006; Quartau & Simões 2006; Simões & Quartau 2006), enabling taxonomists to confidently diagnose a specimen even when belonging to cryptic species (Simões *et al.* 2000; Sueur & Puissant 2007; Mendes *et al.* 2014; Hertach *et al.* 2015).

As for a wide range of biological groups, the Mediterranean basin was recently confirmed as a hotspot for cicada diversity. There, the Iberian Peninsula is particularly relevant, and recent studies on the group have unveiled new species and provided novel contributions in distribution and ecology (Puissant & Sueur 2010; Simões *et al.* 2013; Nunes *et al.* 2014a). However, the underlying idea is that our knowledge is far from complete, particularly in North Africa, where despite an initial boost in species' description and collection of samples in the past century, little has been investigated—or published—during the last decade. In fact, specimens from the Maghreb countries of Morocco, Algeria and Tunisia are available in several museum collections and represent a rather large number of cicada species (Villiers 1943; Boulard 1980, 1981, 1987). Regrettably, associated with this invaluable data is neither ecology nor the recordings of specific acoustic signals produced by the males, as these descriptions were based almost exclusively on external morphology. Cryptic species complexes, such as *Cicadetta brevipennis* s. l. or *Tettigettalna* are extremely difficult to distinguish this way (Mendes *et al.* 2014; Hertach *et al.* 2016). Therefore, in

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order to truly understand this biodiversity hotspot, and other relevant biological data, such as genetics, multivariate morphometric analyses, habitat preferences, distribution range, emergence periods or phenology should be assessed.

In particular, the genetic data coupled with behavioral sound analysis may provide a recommended approach for a more accurate and thorough species description and delimitation in cicadas. This is still missing for many cicadas, namely from North Africa, compromising comparative studies with those from other regions. On what concerns molecular genetics, sequence data is highly desirable in modern taxonomy, as these enable clarification of the taxonomic status of closely-related taxa, such as in the recognition of sibling species, and in addition offering useful phylogenetic information (Hebert *et al.* 2004). More recently, DNA barcoding (Hebert *et al.* 2003) and massive sequencing of large amounts of specimens have fostered a renewal of taxonomic procedures and applications. This is particularly relevant for groups with several, very similar species, as trained specialists are currently in high demand but in short supply.

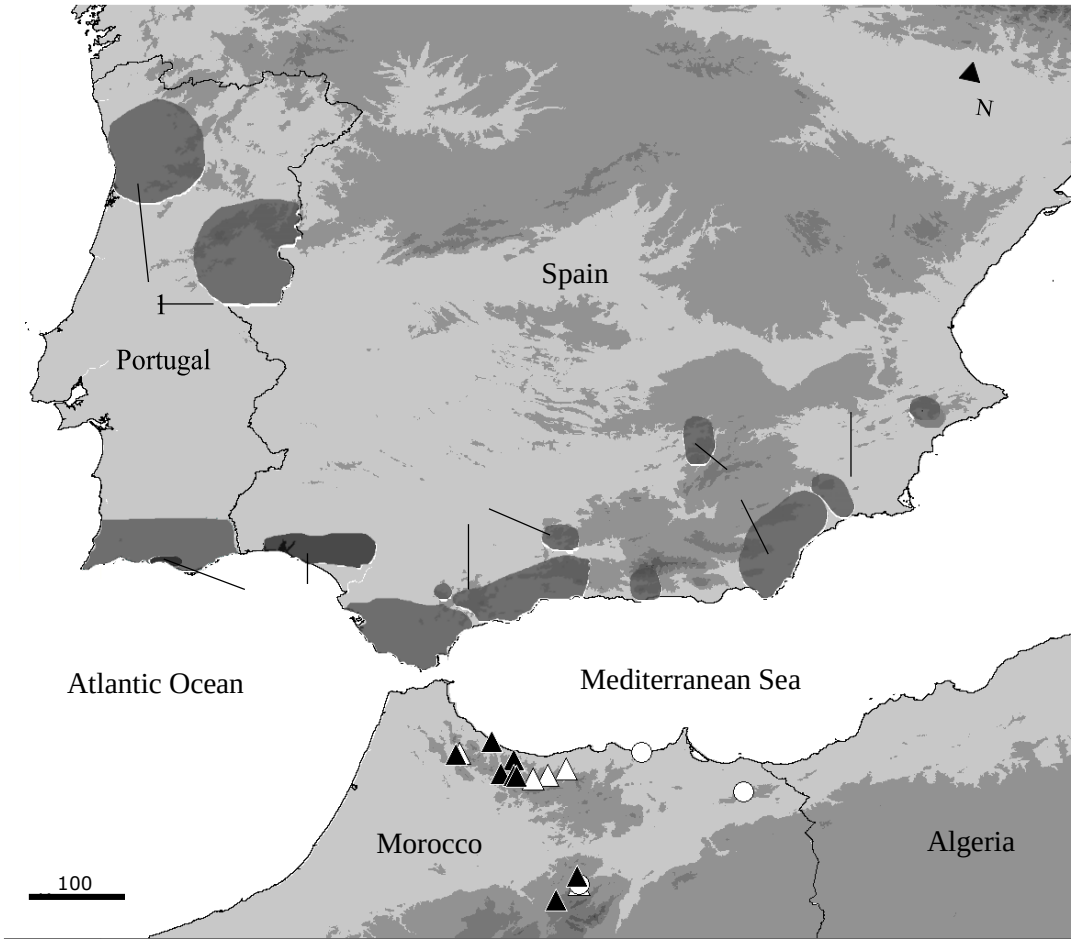

FIGURE 1. Distribution map of the genus *Tettigettalna* with approximate distribution areas extracted from bibliography. The distribution of *T. argentata* is not shown as it is widespread across the Iberian Peninsula. Collection points in Morocco of *T. afroamissa* (white triangle) and *B. dimelodica* (white circle). Black triangles indicate sites where *T. afroamissa* was heard but not collected. Distributions' code: 1—*T. estrellae*; 2—*T. josei*; 3—*T. mariae*; 4—*T. armandi*; 5—*T. defauti*; 6—*T. aneabi*; 7—*T. helianthemi galantei* 8—*T. h. helianthemi*; 9—*T. boulardi*. Scale bar indicates 100 km.

A paradigmatic case within cicadas is the European genus *Tettigettalna* Puissant, 2010. Using the current concept of the genus, it is known to comprise several, usually parapatric, species. This genus shows a pattern of increased diversity in the southern area of the Iberian Peninsula (Figure 1), with many narrow endemics bordering the coastline with the Mediterranean sea (Puissant & Sueur 2010; Simões *et al.* 2013, 2014; Nunes *et al.* 2014b) but a widespread member reaching Slovenia to the east (*Tettigettalna argentata* (Olivier, 1790)). The current knowledge on the distribution boundaries of *Tettigettalna* spp. is far from being properly known, and extensive

field surveys for these cicadas are still needed. Given this southern increased species diversity in the genus, its presence in North Africa had long been expected but not yet investigated.

Fieldwork towards a first screening of cicada biodiversity in the northern part of Morocco (Rif and Middle Atlas mountains) was carried out during the summer of 2014. Among the several Cicadettini collected and recorded, there was a medium-sized species phenotypically similar to the European *T. argentata* (Olivier, 1790), singing on holm-oaks and tall shrubs. In the understory there was sometimes a smaller species, mostly singing among middle-sized shrubs. Further analysis of both entities revealed they belong to two undescribed species, namely the first African member of the genus *Tettigettalna*, *Tettigettalna afroamissa* sp. nov., and a second one, belonging to the new genus *Berberigetta* gen. nov., *i.e.*, *dimelodica* sp. nov. Descriptions of both species are here provided and are based on distinctive morphological, bioacoustic and genetic information.

Materials and methods

Collection of specimens was performed by hand or sweeping net and GPS data was assigned to each capture site. Acoustic data was recorded whenever possible with a CANON EOS 70D camera with an upper frequency limit of over 20 KHz. Distance of the insect to the camera varied between close recordings to up to 0.5–1 m of total distance.

Specimens were photographed or filmed and respective habitats were characterized *in loco*. In the lab, each specimen was assigned a tracking number, pinned and assigned to a morphotype. For most specimens, a front leg was removed and preserved in alcohol for posterior genetic analysis. Acoustic recordings and specimens are stored at the Department of Animal Biology of the Faculty of Sciences, University of Lisbon, Portugal.

Morphology Morphological terminology follows Moulds (2005) and higher systematics follows Sanborn (2014). Both species here described belong to the family Cicadidae Latreille, 1802: subfamily Cicadettinae Buckton, 1889 and tribe Cicadettini Buckton, 1889.

Body, pygophore and aedeagus measurement images were taken on a Zeiss SteREO Lumar V.12 coupled to a TIS DFK 5MPixel camera with IC Capture v.2.1 and calibrated with a 0.01 mm Olympus micrometer. Wing measurements were obtained using photographs taken on a CANON EOS 450D. Each measurement was performed on a single image. Images were calibrated and measured on FIJI (Schindelin *et al.* 2012). Measurement codes and procedure explanation are described on Table 1 and S4. Male genitalia were extracted and placed on a heated 0.1M KOH solution for removal of soft tissues and clarification. Pygophore and aedeagus were conserved on Kaiser gelatin.

Sound Acoustic analysis was performed on AviSoft SAS (Specht 2004). Calling songs were initially trimmed to remove bad quality sections of the recordings and a time domain filter (FIR) was applied with a high pass of 4 kHz for the calling song of *T. afroamissa* and of 2.5 kHz for *B. dimelodica* to remove background noise. A frequency domain transformation was also applied at frequencies ranging 15.59–15.80 kHz to remove electromagnetic interference.

For *T. afroamissa* sp. nov., spectrograms were generated with a FFT length of 512, Hamming type window and 50% temporal overlap. Echemes were labeled with a single automatic threshold and temporal and frequency based variables were generated as described in Pinto-Juma *et al.* 2005. For *B. dimelodica* gen. & sp. nov, due to song peculiarities, an additional Hamming type window with FFT length= 128 was generated.

Discrete values are shown as median ± SD and continuous values as average ± SD followed by (minimum–maximum, total number of observations).

Genetics For the genetic analysis, whole-genome DNA was isolated from a front leg of each specimen with the DNeasy Blood & Tissue Kit (Qiagen). Primers LepF and LepR (Hajibabaei *et al.* 2006) were used to obtain 648 bp of the 5' region of the cytochrome C oxidase I (COI) mitochondrial gene (the 'barcode' region), using the same PCR conditions as Nunes *et al.* (2014a). PCR products were purified with SureClean (Bioline) and sequencing was carried out by Macrogen Europe. Sequences were first corrected in Sequencher 4.0.5 (Gene Codes Co.), then aligned with MAFFT 7.273 (Katoh & Standley 2013) and visually inspected in BioEdit 7.0.9.0 (Hall 1999) and trimmed to the final, same length of 581 bp. The alignment has no gaps or stop codons. Sequences were deposited in GenBank (accession numbers KX582146 to KX582168, see Table 2).

TABLE 1. List and description of the 23 morphological variables analyzed in *T. afroamissa* and *B. dimelodica,* described with codes and abbreviations (Abbr.).

Body region	Code	Abbr.	Description
Head and thorax	1	TL	Total length measured from tip of the head to end of the wings in resting position
	2	HL	Head length measured from the front to the end of the head measured by the dorsal median line
	3	HW	Maximum head width measured between exterior eye margins
	4	EO	Eye-ocellum distance between the margin of a compound eye and the margin of the nearest ocellum
	5	OO	Greatest distance between the two dorsal ocelli
	6	LrL	Labrum length measured between the margin of the anteclypeus to the end of the labrum
	7	LiL	Labium length distance between end of labrum and tip of labium
	8	VW	Vertex width measured with the smallest interocular distance
	9	FR	Front length measured along the dorsal median line
	10	PC	Postclypeus length measured along the median line
	11	PL	Pronotum length
	12	PW	Pronotal width measured at the maximum width of pronotal collar
	13	ML	Mesonotum length measured along dorsal midline until end of scutellum
Abdomen	14	OP	Greatest width of operculum as exemplified on Image 1.
	15	LS	Sternite VII length measured along ventral midline
	16	TyL	Tymbal length as exemplified on Image 1.
	17	TyW	Tymbal width as exemplified on Image 1.
Legs	18	PF	Profemur length measured along median line
Wings	19	FwL	Forewing length measured from intersection of costal vein and CuP+1A vein until apex of wing.
	20	FwW	Forewing width measured from intersection of R+Sc vein and node until intersection of CuP+1A vein and CuA_2 vein.
	21	BCL	Basal cell length measured from intersection of costal vein and CuP+1A vein until beginning of M+CuA vein
	22	MCuA	Length of M+CuA vein
	23	RCL	Radial cell length measured from beginning of M+CuA until intersection of R+Sc vein and node.

Genetic distances (Kimura-2-parameter and p-distances) were obtained with Mega 6 (Tamura *et al.* 2013). Sequences generated for this study were aligned with sequences available in GenBank from Mediterranean species published by Nunes *et al.* (2014a) and Simões *et al.* (2014) from genera *Tettigettalna* Puissant 2010, *Tettigettacula* Puissant, 2010*; Tympanistalna* Boulard,1982 and *Cicada* Linnaeus, 1758, (see Table S1 for accession numbers). For comparative purposes, specimens from two additional Mediterranean genera were also sequenced: *Hilaphura varipes* (Waltl, 1837) and *Euryphara contentei* Boulard, 1982.

The complete matrix with 58 taxa was converted from fasta to nexus with Concatenator 1.1.0 (Pina-Martins & Paulo 2008). A Bayesian phylogenetic tree was generated by MrBayes 3.2.1 (Ronquist *et al.* 2012). The best model of sequence evolution (HKY+ G) was selected under the corrected Akaike information criterion (AICc), as implemented in MrModeltest 2.3 (Nylander *et al.* 2004). The Metropolis-coupled Markov chain Monte Carlo analysis was carried out with four chains. The posterior probabilities for each node were generated from 10^8 generations, sampling at every 100th iteration. The burn-in was set to the first 25% trees, and the remaining trees were used to generate a consensus tree by the 50% majority rule. For maximum likelihood analysis, we used RaxML (Stamatakis 2014) with a GTRCAT model and ran with 10 000 generations. *Cicada barbara* (Stål, 1866) and *Cicada orni* L. 1758, two species belonging to tribe Cicadini and occurring both in the Iberian Peninsula and Morocco, were set as outgroup taxa for Bayesian and ML analyses.

TABLE 2. Description of the collection sites and NCBI accession numbers for COI DNA barcoding of the paratypical series of *T. afroamissa* and *B. dimelodica*. Bold sample IDs indicate the type specimens. Collectors name code: EM—E. Marabuto; VN—VL Nunes; TL—T. Laurentino.

Species	Sample ID	Sex	Population	Locality	Coll.	GPS coordinates	GenBank Accession n.
T. afroamissa	SP18_3779	♂	Rif Mountains	Chefchaouane	EM	35º 11' 2.53" N 5º 13' 25.93' W	(1)
	SP18_3780	♀	Rif Mountains	Chefchaouane	EM	35º 11' 2.53" N 5º 13' 25.93' W	(1)
	SP18_3781	♂	Rif Mountains	Chefchaouane	EM	35º 11' 2.53" N 5º 13' 25.93' W	KX582158
	SP18_3782	♂	Rif Mountains	Chefchaouane	EM	35º 11' 2.53" N 5º 13' 25.93' W	KX582159
	SP18_3783	♂	Rif Mountains	Chefchaouane	EM	35º 11' 2.53" N 5º 13' 25.93' W	KX582160
	SP18_3786	♀	Middle Atlas	Afouzar	EM	33º 52' 16.73" N 4º 1' 42.75" W	KX582161
	SP18_3805	♀	East Rif	Bni Hadifa	EM	35º 01' 48" N 4º 9' 51.85" W	(1)
	SP18_3806	♂	East Rif	Bni Hadifa	EM	35º 01' 48" N 4º 9' 51.85" W	KX582162
	SP18_3807	♂	East Rif	Bni Hadifa	VN	35º 01' 48" N 4º 9' 51.85" W	KX582163
	SP18_3808	♂	East Rif	Bni Hadifa	VN	35º 01' 48" N 4º 9' 51.85" W	KX582164
	SP18_3813	♂	East Rif	Targuist	EM	34º 57' 54.58" N 4º 20' 38.73" W	KX582165
	SP18_3814	♂	East Rif	Tizi Tchen	EM	34º 55' 44.18" N 4º 29´31.87" W	KX582166
	SP18_3815	♂	East Rif	Tizi Tchen	EM	34º 55' 44.18" N 4º 29´31.87" W	KX582167
B. dimelodica	SP19_3787	♀	Middle Atlas	Afouzar	VN	33º 52' 16.73" N 4º 1' 42.75" W	(1)
	SP19_3788	♂	Middle Atlas	Afouzar	VN	33º 52' 16.73" N 4º 1' 42.75" W	(1)
	SP19_3789	♂	Middle Atlas	Afouzar	VN	33º 52' 16.73" N 4º 1' 42.75" W	(1)
	SP19_3790	♂	Middle Atlas	Afouzar	EM	33º 52' 16.73" N 4º 1' 42.75" W	KX582146
	SP19_3791	♂	Middle Atlas	Afouzar	EM	33º 52' 16.73" N 4º 1' 42.75" W	KX582147
	SP19_3792	♂	Middle Atlas	Afouzar	EM	33º 52' 16.73" N 4º 1' 42.75" W	KX582148
	SP19_3793	♂	Middle Atlas	Afouzar	TL	33º 52' 16.73" N 4º 1' 42.75" W	KX582149
	SP19_3794	♂	Berkane	Berbers	VN	34º 47' 59.1" N 2º 23' 59.5" W	(1)

.....continued on the next page

TABLE 2. (Continued)

Species	Sample ID	Sex	Population	Locality	Coll.	GPS coordinates	GenBank Accession n.
SP19_3795		♂	Berkane	Berbers	VN	34º 47' 59.1" N 2º 23' 59.5" W	(1)
SP19_3796		♂	Berkane	Berbers	VN	34º 47' 59.1" N 2º 23' 59.5" W	KX582150
SP19_3797		♂	Berkane	Berbers	VN	34º 47' 59.1" N 2º 23' 59.5" W	KX582151
SP19_3798		♂	Berkane	Berbers	TL	34º 47' 59.1" N 2º 23' 59.5" W	KX582152
SP19_3799		♂	Berkane	Berbers	EM	34º 47' 59.1" N 2º 23' 59.5" W	KX582153
SP19_3803		♂	El Hoceima	Assihel	VN	35º 11' 15.86"N 3º 24' 38.93" W	KX582154

(1)These specimens were not sequenced in order to preserve their morphology for collection purposes.

Results

Tettigettalna Puissant 2010

Originally described and diagnosed by Puissant & Sueur (2010), encompasses nine European species: *T. argentata* (Olivier, 1790), *T. aneabi* (Boulard, 2000), *T. armandi* Puissant, 2010, *T. boulardi* Puissant, 2010, *T. defauti* Puissant, 2010, *T. estrellae* (Boulard, 1982), *T. helianthemi* (Rambur, 1840), *T. josei* (Boulard, 1982) and *T. mariae* (Quartau & Boulard, 1995). Only *T. argentata* is widespread, reaching, France, Italy, Switzerland and Slovenia to the east. The remaining are (rather) narrow Iberian endemics (see Figure 1).

Tettigettalna afroamissa sp. nov. Costa, Nunes, Marabuto, Mendes & Simões

Material examined Paratypical series consist of 13 specimens (ten males and three females). Designated holotype is SP18_3779 (♂) and female paratype is SP18_3780 (♀). See Table 2 for additional information on the paratypical series, specimen IDs, collection sites and GPS data. See Figure 2 for images of male holotype, female paratype and for details of the male genitalia.

Male morphology

Head Head slightly less broad than pronotum; Supra-antennal plates nearly meeting the eye and produced into a pointed lobe; Postclypeus rounded to subquadrate in frontal view, rounded between top and sides in lateral view, transversely grooved towards distal ends; Rostrum brown, reaching the center of mid-trochanters (in rest). Antennae dark-brown, 7-segmented. Dorsal surface of head brown with front bearing a yellowish stripe extending to outer borders; Yellowish stripe at beginning of epicranial suture extending to pronotum. Eyes brown, three red ocelli. Postclypeus dark brown, with apical yellowish-brown spot extending to frons, grooves light-brown or yellowish. Supra-antennal plates dark-brown and yellowish-brown towards distal ends. Gena and lorum brown to dark-brown covered in long white pilosity. Anteclypeus brown to dark-brown with a lighter brownish fascia surrounding a central dark-brown spot.

Thorax Pronotal collar slightly larger than head width, widened, sloping laterally and evenly rounded dorsally. Pronotal tooth present mid-laterally. Scutellum wider than long. Epimeral lobe not reaching operculum. Submedian sigillae well defined. Metanotum partly visible at dorsal midline not expanded over tymbals. Pronotum with an olive-green arrow shaped stripe at dorsal midline bordered with dark-brown in fresh specimens (in preserved specimens this fades away to light brown). Remainder of pronotum brown, with dark-brown markings bearing yellowish borders. Mesonotum on overall brown, with a lighter "crown-like" marking, lateral margins of mesonotum yellowish. Scutellum brown, with a longitudinal dark-brown fascia at midline expanding towards the ends, reaching metanotum. Sides of scutellum with a dense pilosity on lateral-anterior ends with a fading gradient

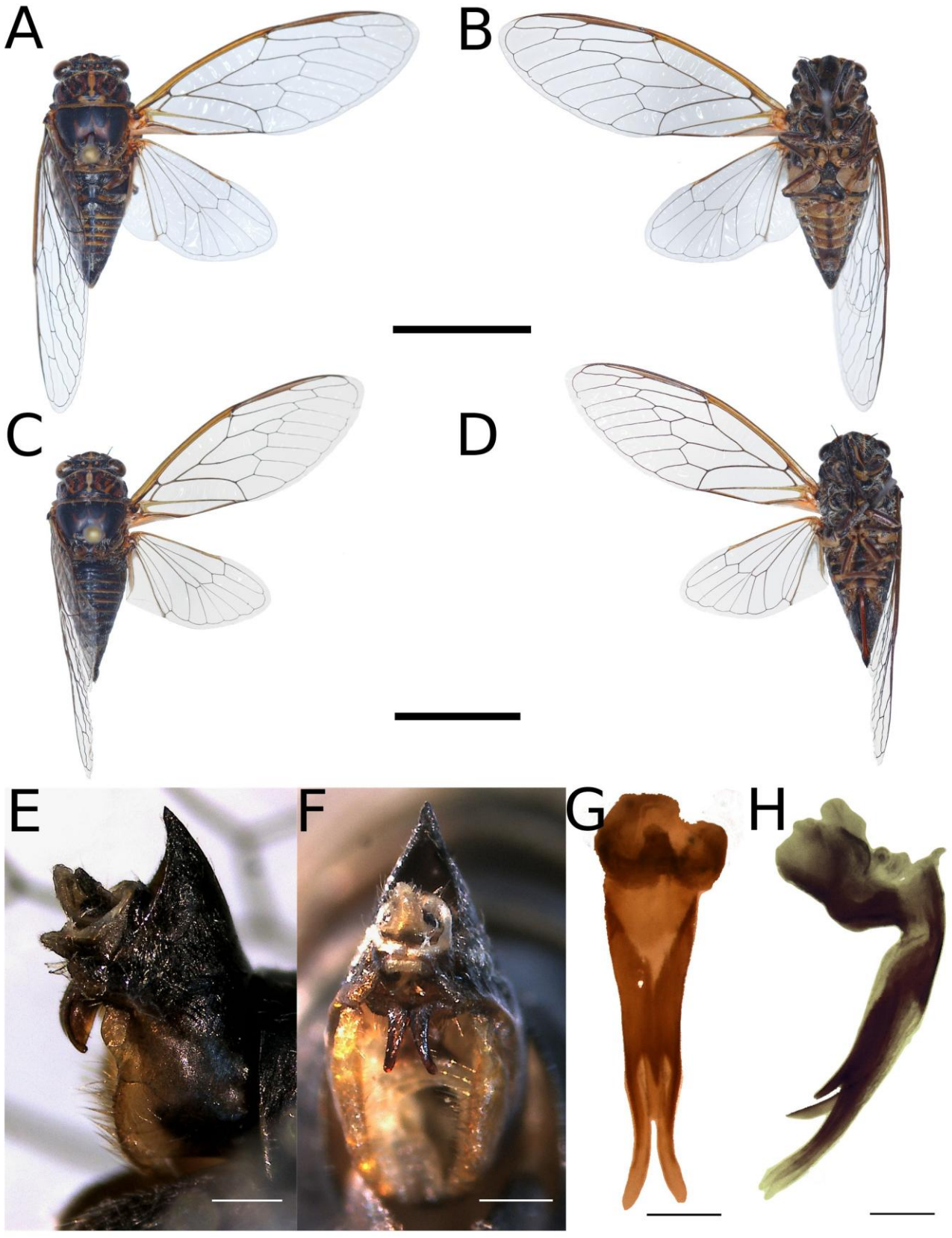

FIGURE 2. Body and male genitalia morphology of *Tettigettalna afroamissa.* A,B—Designated male holotype of *T. afroamissa* in dorsal and ventral views, respectively. Scale bar equals 10 mm; C, D—Designated female paratype of *T. afroamissa* in dorsal and ventral views, respectively. Scale bar equals 10 mm; E, F—Male paratype's pygophore in in lateral and posterior views, respectively. Scale bar equals 500 µm. Photos taken on dry specimens; G, H—Aedeagus in upper and lateral views, respectively. Scale bars equal 200 µm. Photos taken of material preserved in Kaiser gelatin.

of dark-brown to yellowish towards the posterior end with defined, longitudinal, slightly transverse grooves. Metanotum brown, with a dark-brown patch at dorsal midline. Ventral side of thorax brown.

Legs Profemur with three to four dark-brown erect spines. Primary spine clearly separated. Metatibiae with three to four long fine spurs on inner side, and two smaller spurs on outer side with finely dispersed white pilosity. Apex of metatibia surrounded by smaller numerous brown spurs. Tarsal formula: 3-3-3. Legs generally brown in colour. Coxae and trochanters yellowish with a central dark-brown stripe, better defined on the hind legs. Femurs and tibiae brown with two dark-brown longitudinal fasciae. Profemurs with a swollen dark brown fascia surrounded by two yellowish/ light brown stripes, varying somewhat among individuals. Dark-brown border along the spines. Tarsi dark-brown on dorsal side, brown on ventral side. Protarsi darker in colour.

Wings Forewing and hindwing with eight and six apical cells, respectively. Ulnar cell 3 angled towards radial cell; Forewing costa parallel-sided to radial cell; Pterostigma present. CuA vein weakly bowed; M+CuA meeting at basal cell with stems fused. Vein RA_1 aligned closely with subcostal for its length. CuA_1 divided by a crossvein with shorter proximal part. CuP and 1A unfused at their bases. Veins C and R+Sc close together. Outer forewing margin developed for its total length. Hindwing first cubital cell width at distal end much greater than second cubital cell. Hindwing anal lobe broad with 3A vein long and strongly curved at distal end. Hindwing RP and M veins fused at their base. Larger forewing proximal veins yellowish with smaller apical veins brown, same for hindwing. Forewing basal membrane yellow. Hindwing plaga yellow.

Opercula More or less confluent with distal margin of tympanal cavity, well developed towards abdominal midline with sharply rounded apices facing midline. Opercula extending but not reaching posterior border of StII. Opercula distally yellow, dark-brown at base. Meracanthus triangular, following same colour pattern as opercula.

Tymbals Tymbals lacking a tymbal cover. Five ribs, four of which arising from top of a large basal dome, covering about half the tymbal width, and expanding in width towards the posterior side. Fifth rib as an extension of basal dome more or less defined, varying between specimens. First and second anterior ribs, slender, with a transverse break at about halfway of basal dome. Tymbal plate light-grey, ribs and basal dome brownish-grey.

Abdomen Abdomen with somewhat scattered white pilosity. T1 uniformly dark-brown; T2 uniformly dark-brown with a transversal stripe, slightly pointed towards posterior end of abdomen on each side; T3 to T7 dark-brown anteriorly becoming lighter on posterior side; T8 dark-brown. StI mainly dark-brown, yellow posterior margin; StII mainly dark-brown, with yellow lateral borders. StIII to StVI light brown, with a brown spot at midline, forming a well-defined stripe. StVII large, brown, as long as or slightly longer than StVIII; StVIII brown, densely covered in pilosity. Epipleurites brown with yellow posterior border.

Genitalia (Figures 2E to 2H) Pygophore dark-brown on dorsal surface and brown on lateral sides. Pygophore distal shoulder not developed. Pygophore inner tooth absent. Upper lobe flat and moderately developed, distant from dorsal beak with a sharply rounded tip; Basal lobe present, moderately developed and rounded in lateral view. Dorsal beak present and part of chitinized pygophore. Claspers dark-brown, medium-sized, closely aligned ending on a rounded, sharp tip. Uncus brown, duck-bill shaped, small and flat, not dominant. Uncus lateral lobes absent. Aedeagus basal plate, in lateral view, with an undulated ventral surface skewed towards the proximal end; In ventral view, apically broad with a small constriction mid-ventrally expanding afterwards with a midgroove between two longitudinally expanded lobes; Basal portion of basal plate directed forwards and away from thecal shaft; Basal plate ventral rib not apparent; Basal plate attached with a functional membranous "hinge". Theca, in lateral view, curved into a gentle arc; Thecal pseudoparamers present, dorsal of theca, originating closer to theca than its base; Endothecal ventral support present; Thecal aperture upper diagonal in lateral view.

Female morphology Females overall slightly darker than males. Pronotal posterior border light-brown. Mesonotal "crown-like" mark much more faded and smaller than males. Scutellum light-brown. Meso- and metatarsi lighter in colour, light-brown turning brown towards claws. Opercula almost reaching posterior border of StII but much smaller. T1 and T2 totally dark-brown. Abdominal ventral midline fascia dark-brown very well defined. StVII yellowish and split, with a light-brown groove on each side. Stigma dorsal beak dark-brown. Ovipositor brown with dark-brown tip.

Body measurements for *T. afroamissa* males (n=10) Total length: 27.17 ± 1.25 mm; Pronotal length: 2.79 ± 0.13 mm; Mesonotal length: 4.35 ± 0.26 mm; Forewing length: 21.26 ± 0.97 mm; M+CuA length: 1.26 ± 0.19 mm. Female and additional body measurements can be found on Table 3.

Bioacoustics The male acoustic signals here described are based on the analysis of the calling song of six males recorded at T= 38–40 ºC (see Figure 3). The typical calling song is composed by the repetition of a phrase

subdivided into two parts: A—a first single, short echeme and B—a longer group composed of 9 ± 7.461 echemes (6–50, n=124) and the interval between parts A and B has a duration of 155±53 ms (112–539 ms; n=99). In 23.6% of the phrases part A was absent. We also report a single calling song with a continuous phrase without any apparent pauses.

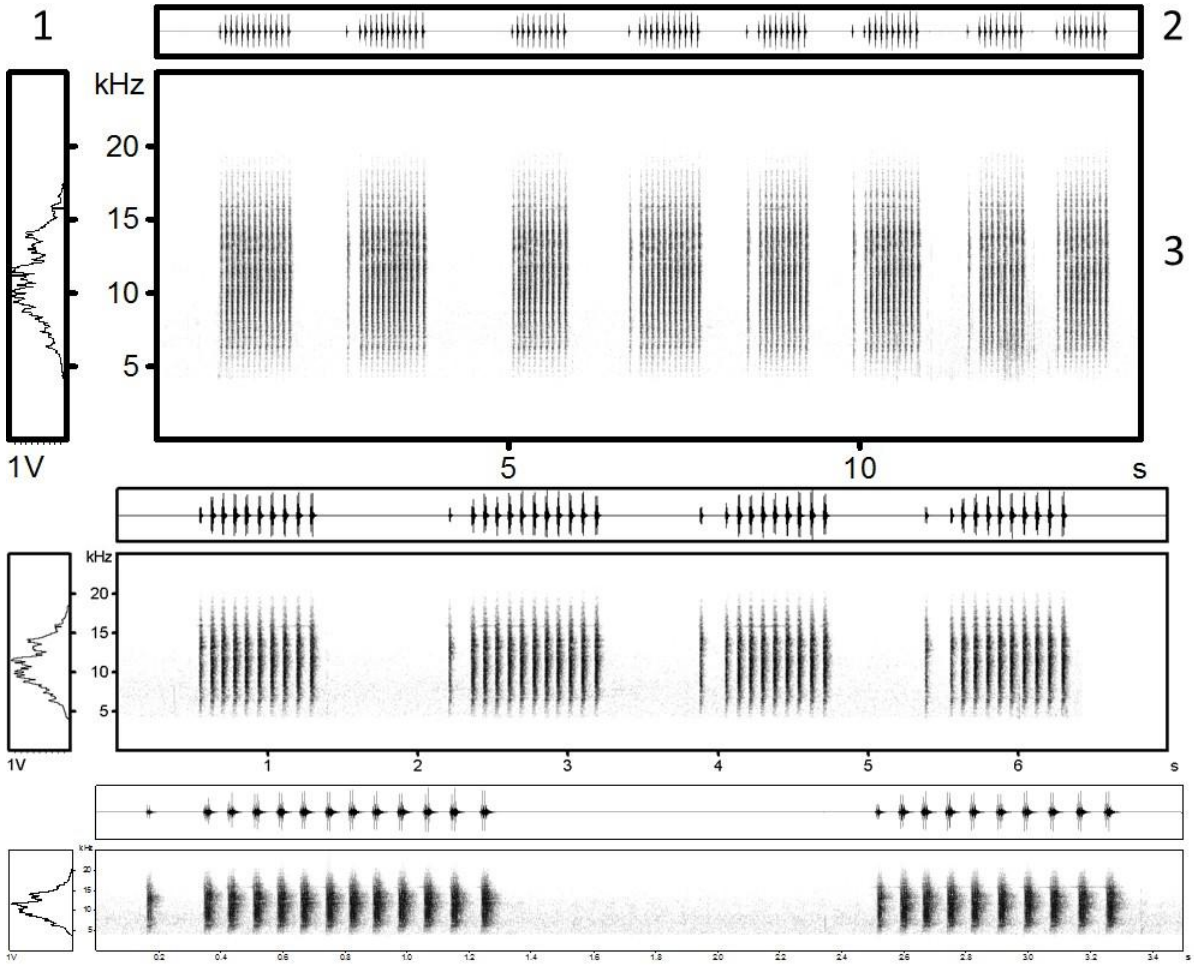

FIGURE 3. *Tettigettalna afroamissa* nov. sp. calling song profile with successive ampliation of recorded phrases. Mean frequency spectrum (1), oscillogram (2) and spectrogram (3). Calling song recorded on Afouzar, Middle Atlas, Morocco at 39-40ºC.

Peak frequency of all calling songs is at 11.72 ± 0.79 kHz, maximum and minimum frequencies are 18.45 ± 1.74 kHz and 4.14 ± 0.44 kHz, respectively.

Additional temporal and frequency-based variables are indicated in Table 4. Because of the similarities in frequencies of parts A and B, these were grouped in the same analysis.

Diagnosis *T. afroamissa* is morphologically similar to all other *Tettigettalna* spp. but presents some peculiarities, allowing for its ready separation from its closest relatives. With an average total body length of 27 mm, it seems to be the genus' largest species (Mendes *et al.* 2014, Simões *et al*. 2014, Puissant & Sueur 2010). *T. afroamissa* shows unique colour traits: all examined specimens have a black stripe running across the entire length of the ventral surface of the abdomen and an olive-green arrow-shaped stripe in the pronotum midline, which, upon death, fades over time to a paler shade of green in dry specimens (see image S5 for a live male bearing the typical olive-green stripe on the pronotum).

TABLE 3. Descriptive statistics of morphological variables performed on samples of *T. afroamissa* and *B. dimelodica*. Body measurements are presented as average ± SD in mm. Please refer to Table 1 for name code variables and explanation.

		T. afroamissa				*B. dimelodica*		
		Male (n=10)		Female (n=3)		Male (n=l3)		Female (n=l)
Body region	Code	Mean ± SD	Min–Max	Mean ± SD	Min–Max	Mean ± SD	Min–Max	
Head and thorax	TL	27.31 ± 1.11	25.93 – 29.21	25.84 ± 1.16	24.72 – 27.03	16.99 ± 0.78	15.59 – 18.30	17.30
	HL	2.05 ± 0.12	1.86 – 2.20	1.98 ± 0.06	1.93 – 2.02	1.47 ± 0.11	1.24 – 1.61	-
	HW	5.97 ± 0.21	5.72 – 6.29	5.64 ± 0.32	5.41 – 5.86	3.86 ± 0.15	3.56 – 4.13	-
	EO	0.77 ± 0.04	0.71 – 0.84	0.76 ± 0.03	0.73 – 0.78	0.54 ± 0.05	0.46 – 0.61	-
	OO	1.37 ± 0.05	1.3 – 1.46	1.34 ± 0.18	1.22 – 1.47	0.89 ± 0.04	0.81 – 0.96	-
	LrL	1.15 ± 0.11	1.01 – 1.37	1.05 ± 0.16	0.94 – 1.16	0.85 ± 0.08	0.76 – 1.04	-
	LiL	2.92 ± 0.14	2.66 – 3.10	2.62 ± 0.07	2.57 – 2.67	1.97 ± 0.11	1.70 – 2.15	-
	VW	2.91 ± 0.12	2.71 – 3.10	2.82 ± 0.25	2.64 – 2.99	1.91 ± 0.12	1.68 – 2.06	-
	FR	0.62 ± 0.05	0.55 – 0.69	0.6 ± 0.03	0.59 – 0.62	0.40 ± 0.06	0.31 – 0.52	-
	PC	2.36 ± 0.11	2.23 – 2.50	2.28 ± 0.01	2.28 – 2.29	1.54 ± 0.09	1.36 – 1.66	-
	PL	2.82 ± 0.09	2.67 – 2.95	2.66 ± 0.25	2.48 – 2.83	1.74 ± 0.15	1.46 – 1.98	-
	PW	6.71 ± 0.37	6.20 – 7.34	6.31 ± 0.43	6.01 – 6.62	4.31 ± 0.26	3.65 – 4.58	-
	ML	4.40 ± 0.19	4.15 – 4.68	4.12 ± 0.52	3.76 – 4.49	2.67 ± 0.11	2.43 – 2.85	-
Abdomen	OP	3.92 ± 0.17	3.64 – 4.17	1.76 ± 0.22	1.60 – 1.91	2.55 ± 0.16	2.09 – 2.73	-
	LS	1.62 ± 0.09	1.52 – 1.77	-	-	1.32 ± 0.13	1.03 – 1.49	-
	TyL	1.53 ± 0.06	1.43 – 1.64	-	-	0.99 ± 0.07	0.90 – 1.14	-
	TyW	2.84 ± 0.06	2.76 – 2.95	-	-	1.93 ± 0.10	1.67 – 2.06	-
Legs	PF	3.20 ± 0.11	3.05 – 3.36	3.08 ± 0.06	3.04 – 3.13	2.10 ± 0.15	1.78 – 2.26	-
Wings	FwL	21.37 ± 0.85	20.16 – 22.84	20.28 ± 0.91	19.42 – 21.23	13.39 ± 0.54	12.42 – 14.27	13.59
	FwW	7.50 ± 0.27	7.13 – 7.88	7.11 ± 0.48	6.67 – 7.62	5.37 ± 0.84	4.87 – 8.10	5.10
	BCL	1.85 ± 0.14	1.68 – 2.06	1.72 ± 0.10	1.62 – 1.81	1.23 ± 0.11	1.09 – 1.42	1.24
	McuA	1.26 ± 0.18	0.92 – 1.48	1.34 ± 0.21	1.10 – 1.50	1.21 ± 0.18	0.94 – 1.41	1.22
	RCL	8.53 ± 0.36	7.93 – 9.16	8.32 ± 0.39	7.95 – 8.72	6.12 ± 0.36	5.20 – 6.59	6.24

TABLE 4. Time and frequency based parameters of the analyzed phrases of *T. afroamissa*. Frequency variables values are presented in kHz.

T. afroamissa	Phrase			Part A			Part B		
Time variables	Mean±SD	Min–Max	n	Mean±SD	Min–Max	N	Mean±SD	Min–Max	n
Duration (ms)	726 ± 582	314–3749	124	10 ± 4.5	5–27	97	720 ± 580	309–3733	124
Echeme duration (ms)	-	-	-	Same as above			20.97 ± 8.26	5–43	1364
Echeme rate (echeme.s^{-1})	-	-	-	-	-	-	16.21 ± 1.73	10.88–19.42	1364
Interval (ms)	326 ± 116	186–906	94	-	-	-	51.20 ± 7.07	26–63	1340
Frequency variables	Peak frequency	Min frequency	Max frequency	Bandwidth	Quartile 25	Quartile 50	Quartile 75	Quartile (75%-25%)	
Mean ± SD	11.72 ± 0.79	4.14 ± 0.44	18.45 ± 1.74	14.30 ± 1.87	9.93 ± 0.56	11.50 ± 0.48	12.82 ± 0.45	2.89 ± 0.55	
Min–Max	7.21 – 14.25	3.93 – 8.81	15.46 – 23.81	7.68 – 19.87	7.59 – 10.96	10.03 – 12.75	11.25 – 14.34	1.41 – 4.69	

TABLE 5. Mean pairwise genetic distances (%) between the taxa considered for phylogenetic analysis: P-distances in the upper diagonal and Kimura 2-parameter distances in the lower diagonal. Highlighted values in bold belong to genus *Tettigettalna*.

		1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.
1.	*Cicada barbara*		12.0	18.6	19.6	18.6	18.9	20.0	19.5	19.6	19.4	19.5	19.3	18.4	19.4	19.1	19.4	19.9	20.2
2.	*Cicada orni*	13.3		17.2	19.3	19.3	19.1	20.0	19.7	19.5	19.4	20.6	18.4	19.3	19.2	18.9	18.7	19.0	18.7
3.	*Hilaphura varipes*	21.5	19.6		11.4	12.4	11.2	11.7	12.4	12.3	12.0	12.0	11.2	10.9	11.3	11.4	10.9	12.9	9.8
4.	*Euryphara contentei*	22.9	22.4	12.5		9.6	8.1	10.8	10.7	10.6	10.3	9.7	10.0	9.2	9.0	9.6	9.4	11.4	9.8
5.	*Tympanistalna gastrica*	21.5	22.4	13.8	10.4		9.8	12.4	13.5	13.2	13.3	12.7	11.4	11.4	11.6	12.4	11.3	12.4	11.5
6.	*Tettigettacula baenai*	21.9	22.2	12.3	8.7	10.7		10.4	10.6	10.6	10.3	9.6	11.0	9.0	8.6	9.0	8.9	11.5	9.7
7.	*Tettigettalna estrelae*	23.4	23.3	12.9	11.7	13.7	11.3		**7.7**	**7.4**	**7.8**	**5.3**	**9.2**	**5.8**	**5.1**	**5.4**	**4.8**	**9.5**	11.8
8.	*Tettigettalna argentata*	22.7	23.0	13.7	11.7	15.0	11.6	**8.3**		**1.7**	**1.9**	**7.4**	**9.6**	**6.3**	**6.6**	**6.4**	**7.3**	**10.4**	11.1
9.	*Tettigettalna mariae*	22.8	22.7	13.7	11.6	14.7	11.6	**8.0**	**1.8**		**1.2**	**7.1**	**9.4**	**6.1**	**6.2**	**6.1**	**6.9**	**10.8**	10.7
10.	*Tettigettalna aneabi*	22.7	22.6	13.3	11.2	14.8	11.2	**8.4**	**1.9**	**1.2**		**7.3**	**8.8**	**5.9**	**6.7**	**6.0**	**7.2**	**10.4**	10.0
11.	*Tettigettalna boulardi*	22.8	24.2	13.2	10.5	14.0	10.3	**5.6**	**7.9**	**7.6**	**7.8**		**9.2**	**5.6**	**4.3**	**5.4**	**4.9**	**9.8**	11.6
12.	*Tettigettalna josei*	22.5	21.1	12.2	10.7	12.5	11.9	**10.0**	**10.4**	**10.2**	**9.5**	**10.0**		**8.0**	**8.7**	**7.9**	**8.5**	**9.6**	10.0
13.	*T. helianthemi helianthemi*	21.3	22.4	11.9	9.9	12.5	9.7	**6.1**	**6.7**	**6.4**	**6.3**	**5.9**	**8.5**		**3.4**	**5.2**	**5.2**	**9.2**	10.5
14.	*T. helianthemi galantei*	22.6	22.3	12.4	9.6	12.7	9.2	**5.3**	**7.0**	**6.6**	**7.1**	**4.5**	**9.4**	**3.6**		**5.0**	**4.3**	**9.0**	11.0
15.	*Tettigettalna armandi*	22.2	21.9	12.5	10.4	13.7	9.6	**5.7**	**6.8**	**6.5**	**6.4**	**5.7**	**8.5**	**5.5**	**5.2**		**3.7**	**9.0**	10.4
16.	*Tettigettalna defauti*	22.7	21.6	11.9	10.1	12.3	9.5	**5.0**	**7.8**	**7.3**	**7.7**	**5.2**	**9.1**	**5.5**	**4.5**	**3.9**		**8.3**	10.9
17.	*Tettigettalna afroamissa*	23.4	22.0	14.3	12.5	13.6	12.6	**10.3**	**11.5**	**11.9**	**11.5**	**10.7**	**10.4**	**10.0**	**9.8**	**9.8**	**8.9**		11.9
18.	*Berberigetta dimelodica*	23.8	21.6	10.6	10.6	12.7	10.5	13.0	12.1	11.6	10.9	12.8	10.8	11.4	12.0	11.3	11.8	13.1	

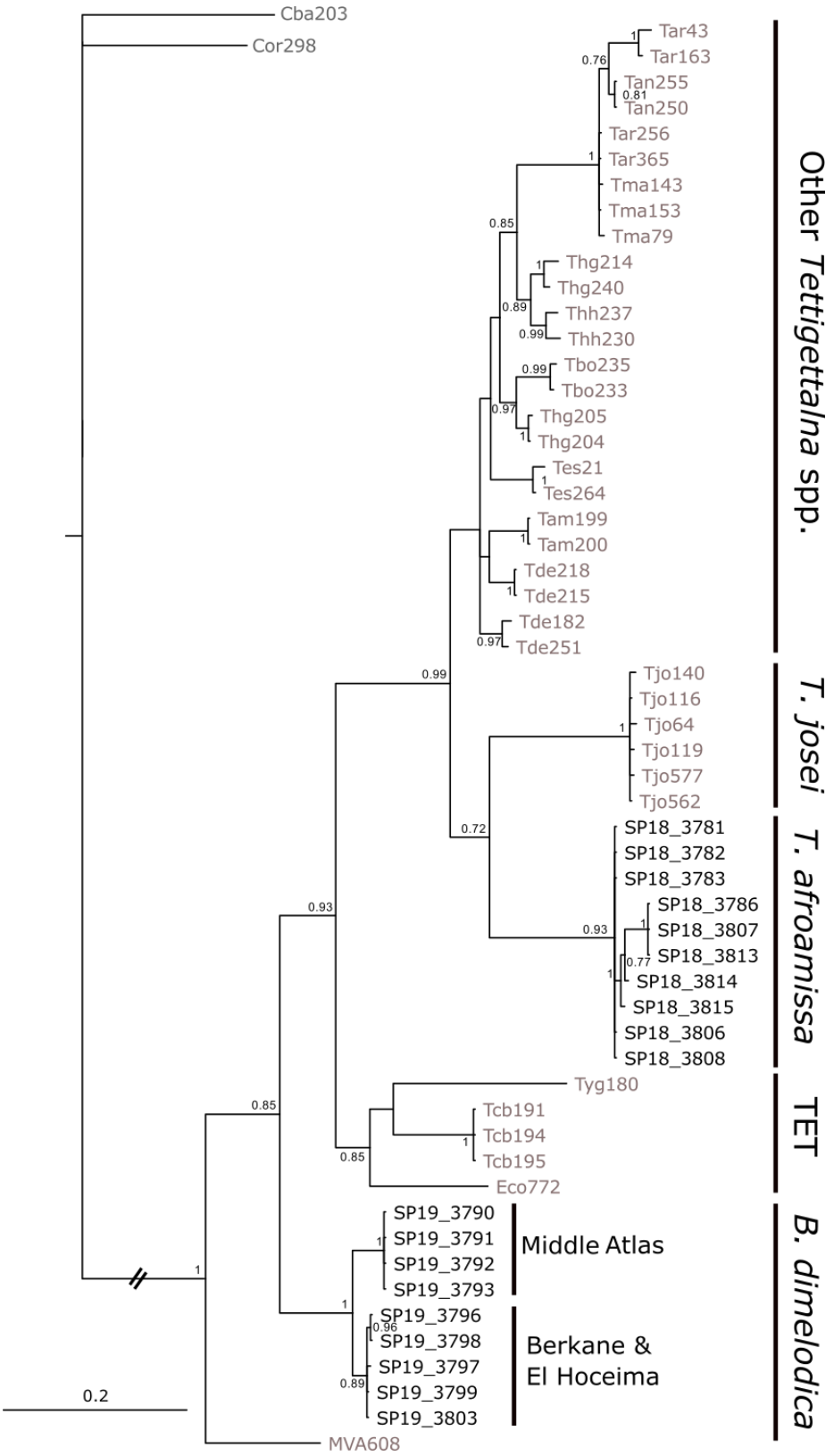

FIGURE 4. Bayesian inference phylogenetic tree of Cytochrome C oxidase subunit I mitochondrial DNA of *T. afroamissa* and *B. dimelodica* with other previous published taxa. Posterior probabilities are shown next to branch nodes. TET stands for *Tettigettacula—Euryphara—Tympanistalna* clade. Scale bar represents the number of estimated changes per branch length. *C. barbara* (Cba203) and *C. orni* (Cor298) were set as an outgroup. *T. afroamissa* and *B. dimelodica* taxa IDs are detailed on Table 2. Additional taxa details are included on supplementary information Table S1. Root was truncated with double dash totalling 0.6 changes per branch length.

Acoustic analysis enables easy and accurate identification of all *Tetigettalna* species. *T. afroamissa* is no exception. Its calling song is structurally different from all other *Tettigettalna* spp., although reminiscent of *T. argentata* (Olivier, 1790) and *T. boulardi* Puissant, 2010.

The song of *T. afroamissa* can be distinguished from *T. argentata* for it has higher echeme rate (t= 16.21 ± 1.73 echemes.s^{-1} vs t= 12.82 ± 1.49 echemes.s^{-1}) and a shorter inter-echeme interval (t= 51.20 ± 7.07 ms vs t= 71.00 ± 13.00 ms) (Mendes *et al.* 2014).

T. boulardi has a typical calling song with a short echeme (t= 200 ± 110 ms) followed by a long echeme (t= 2.17 ± 0.30 s), whereas in *T. afroamissa* this initial echeme is even shorter (t= 10 ± 4.5 ms), followed by a succession of very short echemes (t=720 ± 580 ms), instead of a single one. Inter-phrase interval is also much shorter for *T. afroamissa* (t= 326 ± 116 ms) than for *T. boulardi* (t= 3270 ± 680 ms). For additional time and frequency measurements regarding *T. boulardi* see Puissant & Sueur (2010).

DNA barcoding Males from all sampled locations were sequenced for COI. Four haplotypes were recovered in a total of 10 sequences. The dataset includes one non-synonymous mutation and a total of 14 polymorphic sites, corresponding to a nucleotide diversity of π=0.1075. All *T. afroamissa* sequences grouped in a fully supported monophyletic clade (Figure 4) and intraspecific pairwise distances (K2P) varied from 0.5 to 2.1 %. This clade clusters with remaining *Tettigettalna* spp. in an unresolved polytomy. Mean genetic distances among *T. afroamissa* and all other species of the genus are shown in Table 5, and vary from 8.9% (with *T. defauti*) to 11.9 % (with *T. mariae*). Thus, the genetic distance associated with the fragment of COI used here, the "barcode gap", is high enough to be used for DNA barcoding of *T. afroamissa*.

FIGURE 5. Habitats of *T. afroamissa* (A-D) and *B. dimelodica* (D-F) in Morocco: Rif mountains near Chefchaouane (A), Bni Hadifa (B) and Taferka (C); Middle Atlas near Taza (D); Berkane (E) and El Hoceima (F). Specimens were captured in all locations but C (see supplementary Table, S2). Photos by VL Nunes.

Habitat (Figure 5) An arboreal species, inhabiting open Mediterranean-type woodland and tall scrubland. This species has been scored singing mainly on holm-oak trees (*Quercus rotundifolia*) and bushes such as *Pistacia lentiscus* and *Cistus* spp. but locally, in the Rif, it was found on pine trees (*Pinus* spp.) (Figure 5B), *Abies pinsapo* var. *marocana* and *Cedrus atlantica* (Figure 5C) and almond trees *(Prunus dulcis)*.

Distribution Northern Morocco, along the Rif Mountains and nearby Mediterranean coastline between Tetuan and Al Hoceima. Also found in the northern parts of the Middle Atlas, near Taza (Figure 1). Not found near Ceuta or Tangier.

Etymology Specific epithet formed by combining the suffix *afro* (pertaining to Africa) and the prefix *amissa*, feminine of the latin āmissus, meaning "having been lost" or "let go". Literal translation would be "cicada (of the genus Tettigettalna) left / lost in Africa" as this new species is the only *Tettigettalna* spp. known so far to occur in Africa, the remaining being European.

Berberigetta nov. gen. Costa, Nunes, Marabuto, Mendes & Simões

Diagnosis This genus can be readily distinguished from other morphologically similar genera by the analysis of the male genitalia. The type species has a very large tube-like aedeagus with two pseudoparamers fused until three quarters of total thecal length, ending in a sharp-tip and about of the same length as the endotheca (see Figure 6F). Therefore, it can be distinguished from the similar genus *Tettigettacula* (type species: *T. baenai* (Boulard, 2000)) for the latter has two unfused thick pseudoparamers arising dorsally from base of the theca, and separate from the endotheca (Puissant & Sueur 2010). *Berberigetta* differs from *Cicadetta* Kolenati, 1857 (type species: *Cicadetta montana* (Scopoli, 1772)) in aedeagus morphology: *C. montana* shows a similarly long aedeagus, yet the pseudoparamers are exceedingly long and partly unfused, surpassing the distal end of theca by about half its length (Moulds 2012).

Type species *Berberigetta dimelodica* designed by monotypy.

Etymology Name formed by combining the suffix *Berber* (pertaining to the Maghrebian Roman region, Barbaria, and the prevailing ethnic group in northern Maghreb) and the prefix –getta, an arbitrary combination of letters associated with small cicada species, as in *Tettigetta*.

Berberigetta dimelodica sp. nov. Costa, Nunes, Marabuto, Mendes & Simões

Material examined Paratypical series consists of a total of 14 specimens (13 males and one female). Designated holotype is SP19_3795 (♂), and female paratype is SP19_3787 (♀). See Table 2 for additional information on paratypical series, specimen IDs, collection sites and GPS data. See Figure 6 for images on male holotype, female paratype (see supplementary image, S6 for live specimens) and details of the male genitalia.

Male morphology

Head Supra-antennal plate produced into a pointed lobe; Supra-antennal plate nearly meeting the eye. Postclypeus subquadrate to round in front view; Postclypeus transversely grooved towards distal ends. Rostrum brown, reaching the center of mid-trochanters when in resting position. Antennae brown, 7-segmented. Postclypeus dark brown, with apical yellowish-brown spot, grooves light-brown or yellowish; Anteclypeus yellowish with a brown central spot. Gena and lorum brown to light-brown covered with white long pilosity. Supra-antennal plates light brown distally near the eye, becoming dark-brown towards midline. Three red ocelli. Eyes light-brown. Dorsal surface of head dark-brown, supraocular border brown, with yellowish stripe on epicranial suture.

Thorax Pronotal collar broad, slightly greater than eye width; Pronotal lateral development ampliate, sloping in lateral view, evenly rounded in dorsal view. Pronotal mid-lateral tooth absent. Scutellum wider than long. Epimeral lobe not reaching operculum. Metanotum partly visible at dorsal midline, not expanded over tymbals. Pronotum brown with a dark-brown stripe along dorsal midline, ending posteriorly in dark-brown spot. Mesonotum with two yellowish fasciae bordering between parapsidal suture and submedian sigillae prolonging to anterior arms of scutellum; Mesonotal lateral dorsal margins yellowish. Central area of scutellum brown with yellowish arms. Metanotum yellowish, brown at dorsal midline.

Legs Profemur with a large primary erect spine plus two smaller secondary spines dark-brown/ brownish in colour, some individuals with a much smaller fourth spine. Meracanthus triangular. Tarsal formula 3-3-3. General brown to yellowish in colour. Metatibiae with four long fine reddish spurs on inner side and two smaller reddish spurs on outer side. Coxae yellowish, with a central dark-brown stripe, becoming gradually browner and less yellowish towards metacoxae. Trochanters brown. Meso and metafemurs yellowish with dark-brown to brownish stripes. Tarsi and tibia light-brown.

Wings Forewing with eight apical and four subapical cells. Ulnar cell 3 angled to radial cell. Costal vein parallel-sided to node. Pterostigma present becoming darker towards distal end. CuA weakly bowed. M and

CuA meeting at basal cell with stems completely fused. RA_1 slightly diverging from subcostal at subapical region before crossvein. C and R+Sc close together. CuP and 1A non-fused at their bases. Forewing outer margin developed for its total length. Membrane hyaline. Hindwing vein 2A with an infuscation running alongside total length of vein. First cubital cell width at distal end much greater than second cubital cell. Anal lobe broad, with vein 3A bowed at distal end. Larger forewing proximal veins yellowish with smaller apical veins brown, same vein colour pattern for hindwing. Costal vein yellowish. Basal membrane and plaga yellowish.

Opercula More or less confluent with distal margin of tympanal cavity, well developed towards abdominal midline with sharply rounded apices facing midline. General opercula colour yellowish becoming brown at the base. Meracanthus following the same colour pattern as opercula.

Tymbals Tymbal covers absent. Four to five ribs, broadening apically, three of which arising from anterior proximal part of a large basal dome covering over half total length of tymbal. First anterior rib is slender, with a break at about a third of its length. Fourth rib arising from anterior distal side of basal dome more or less evident amongst individuals. Some specimens present a fifth less defined rib arising from posterior distal end of the basal dome, transversal to fourth rib and converging in a sharp end. Tymbal ribs and basal dome brownish-grey; tymbal plate light-grey.

Abdomen Tergites T2 and T3 much enlarged accounting for about a third of total abdominal length. StVIII greater in length than StVII. T1 and T2 dark-brown; T4–7 dark-brown on dorsal midline, sides red and covered in fine silvery pubescence; T8 dark-brown on dorsal midline, sides yellowish. Sternite I brown; StII yellowish with a brown patch on elevated central area; StIII–VIII yellowish. Epipleurites yellowish.

Genitalia (Figure 6C to 6F) Pygophore distal shoulder not developed; Pygophore inner tooth absent; Upper lobe present, small and rounded, distant from dorsal beak; Basal lobe small to moderately developed ending in a sharp, rounded tip, in lateral view. Dorsal beak well developed, sharp and part of chitinized pygophore. Ventrobasal pocket absent. Claspers small-medium sized, hooked slightly outwards on distal end, rounded tip. Uncus duck-bill shaped, small and flat, not dominant and retractable within pygophore; Uncus lateral lobes absent. Aedeagal basal plate, undulated in lateral view, weakly depressed on dorsal midline; Basal plate apically broad, flat and rounded in ventral view, with a medial small sharp-tipped lobe on both sides, followed by a tube- like constriction leading to theca, gradually narrowing, slight medial lateral depression; Basal plate bearing a ripple-like pattern in dorsal view. Basal portion directed forwards away from thecal shaft; Ventral rib not apparent; Basal plate completely fused to theca without mobility. Theca very long and J-shaped in lateral view. Thecal pseudoparamers lateral of theca, dorsally fused until two thirds of theca length, very flat, as long as endotheca, ending on an upward pointed, sharp tip; Ventral support absent. Pygophore dorsal surface light- brown to yellow. Claspers dark-brown. Uncus brown.

Female morphology Only one female known so far (see supplementary image, S6 for the live specimen). Generally lighter in colour than male. Postclypeus yellowish with brown grooves, genae and lora light brown; Legs generally light brown; Dorsal surface of head light-brown with brown patterns; thorax and scutellum light- brown. Abdomen light brown laterally, with a lighter brown on dorsal midline.

Body measurements for 13 males of *B. dimelodica* Total length: 16.99 ± 0.78 mm; Pronotal length: 1.74 ±0.15 mm; Mesonotal length: 2.67 ± 0.11 mm; Forewing length: 13.39 ± 0.54 mm; M+CuA length: 1.21 ± 0.18 mm. Female and additional body measurements can be found on Table 3.

Bioacoustics The calling song here described is based on the analysis of recordings of three males singing at T=39–40 ºC. A typical phrase is structured into four sequential parts (Figure 7): A, a single echeme; B, a series of 16 ± 2.60 echemes (10–21, n=52) in rapid succession; C, a group of 8 ± 3.68 echemes (5–18, n= 53) ending on D, a single, long echeme. In 21.15% and 9.61% of the phrases part A and part D are missing, respectively.

Calling song frequency-based analysis revealed an interesting frequency modulation in part B. Peak frequency for parts A, C and D is 13.88 ± 0.79 kHz, with maximum frequency of 20.65 ± 0.54 kHz. During part B there is an abrupt reduction of the frequency with a peak frequency of 7.91 ± 1.62 kHz, yet, maintaining the maximum frequency at 21.62 ± 1.11 kHz.

For additional time and frequency variables consult Table 6. Note that, due to frequency modulation in part B, it was separated from parts A, C and D in our analysis.

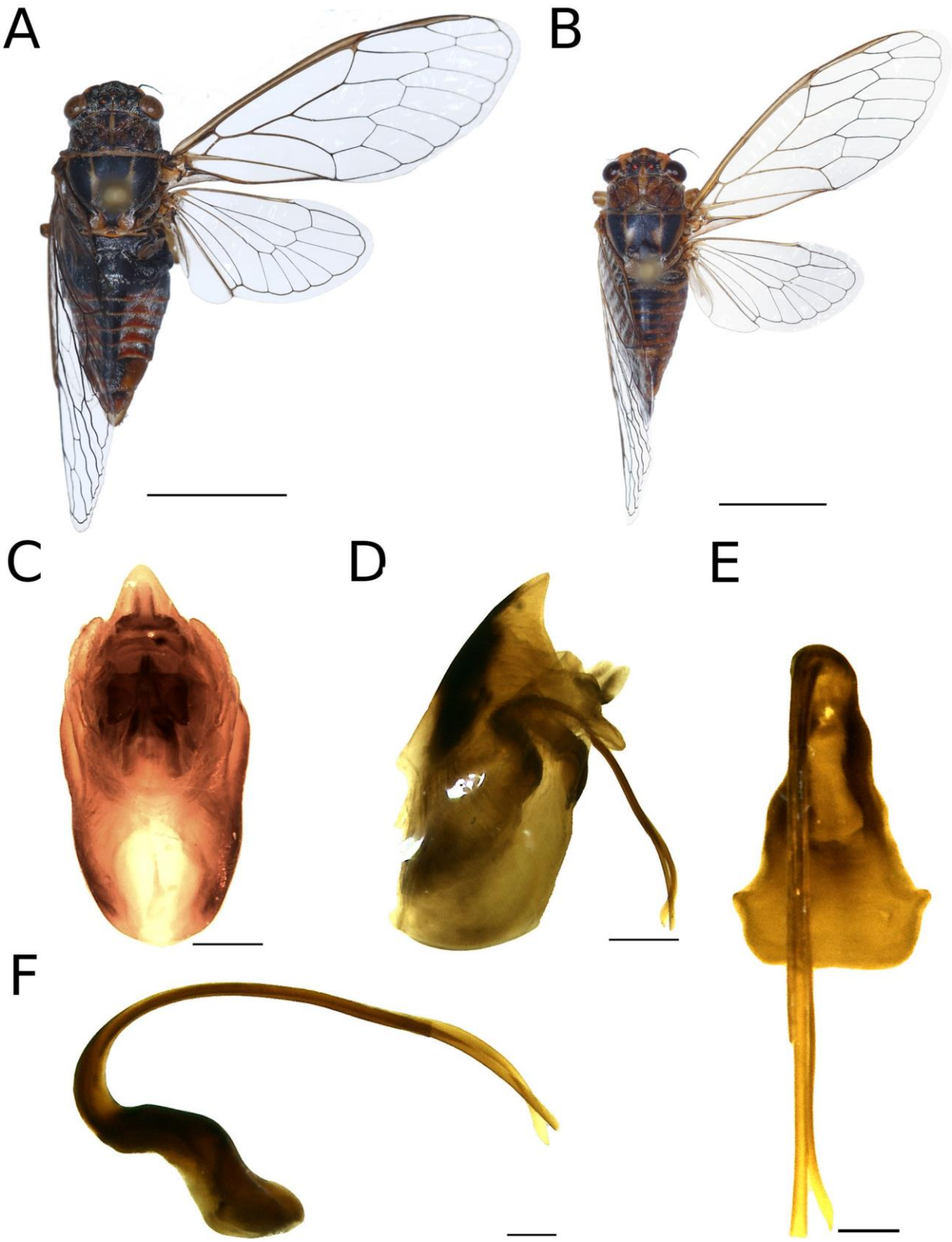

FIGURE 6. Body and male genitalia morphology of *Berberigetta dimelodica*. A—Designated male holotype of *B. dimelodica.* Scale bar equals 10 mm; B—Designated female paratype of *B. dimelodica.* Scale bar equals 10 mm; C, D—Male paratypes' pygophore overview in posterior and lateral views, respectively. Scale bars equal 500 µm. E, F—Aedeagus in upper and lateral views, respectively. Scale bars equal 200 µm. Pygophore and aedeagus photos were taken of material preserved in Kaiser gelatin. Note that the tip of the left pseudoparamer is broken.

TABLE 6. Time and frequency based parameters of the analyzed phrases of *B. dimelodica*. In the frequency analysis, part B of the calling song was separated from parts A, C and D due to significant frequency downshift in part B. Frequency variables values are presented in kHz.

B. dimelodica	Phrase			Part A			Part B		
Time variables	Mean±SD	Min–Max	n	Mean±SD	Min–Max	n	Mean±SD	Min–Max	n
Duration (ms)	2218 ± 559	1357–3448	52	30 ± 10	15–56	47	335 ± 52	212–411	52
Echeme duration (ms)	-	-	-	Same as above			2.14 ± 1.06	0.8–7	849
Echeme rate (echeme.s^{-1})	-	-	-	-	-	-	49.16 ± 6.08	36.08–72.67	52
Interval (ms)	259 ± 82	195–614	49	-	-	-	19.55 ± 5.31	2.8–55	797

	Part C			Part D		
Time variables	Mean±SD	Min–Max	n	Mean±SD	Min–Max	n
Duration (ms)	1364 ± 679	632–2992	53	252. 29 ± 79.23	97–430	41
Echeme duration (ms)	49.2 ±20.6	5–253	487	Same as above		
Echeme rate (echeme.s^{-1})	7.10 ± 1.04	3.34–10.32	53	-	-	-
Interval (ms)	108.83 ± 22.24	34–260	435	-	-	-

continued.

Frequency variables		Peak frequency	Min frequency	Max frequency	Bandwidth
Part ACD	Mean ± SD	13.88 ± 0.79	4.65 ± 0.96	20.65 ± 0.54	15.94 ± 1.31
	Min–Max	11.50–15.50	1.96–6.00	18.70–22.40	12.80–19.78
Part B	Mean ± SD	7.91 ± 1.62	4.39 ± 1.01	21.62 ± 1.11	17.14 ± 1.59
	Min–Max	5.60–16.50	0.30–5.80	13.92–23.40	9.04–22.80
Frequency variables		Quartile 25	Quartile 50	Quartile 75	Quartile (75%–25%)
Part ACD	Mean ± SD	11.91 ± 0.22	13.48 ± 0.27	14.89 ± 0.32	2.98 ± 0.26
	Min–Max	10.70–12.50	12.28–14.40	13.40–15.80	1.87–4.10
Part B	Mean ± SD	7.54 ± 0.61	9.57 ± 0.79	11.61 ± 1.26	4.07 ± 0.93
	Min–Max	6.30–12.00	7.50–13.50	9.70–17.50	2.70–8.20

DNA barcoding Four haplotypes were recovered among the COI sequences of nine males of *B. dimelodica* sp. nov., with a nucleotide diversity of π= 0.0164. Sequences were clustered into two well supported sister clades (Figure 4) diverging by 2.9 % (K2P distance). These clades are, according to our currently knowledge, geographically segregated. Among the 18 segregating sites observed, 16 are fixed for each clade, being two of them non-synonymous mutations. Mean interspecific genetic distances for *B. dimelodica* are presented in Table 5. The new species is clearly distinguishable within the Cicadettini (*Tettigettalna*, *Tettigettacula*, *Tympanistalna*, *Euryphara* and *Hilaphura*), with mean pairwise genetic distances >10%. The COI fragment is therefore apparently proficient for DNA barcoding of *B. dimelodica*, though the genetic structure reported here must be taken into account.

Distribution (Figure 1) Morocco, in the northern parts of Middle Atlas Mountains, near Taza and along the eastern Rif mountains (Al Hoceima), eastward to Berkane (Beni-Snassen Mountains), as the extreme western foot of the Tellian Atlas Mountains. On biogeographical grounds it is possible that this species is also in western Algeria.

Habitat (Figure 5) Open scrubland or light xerothermophilous woodland dominated by holm-oak (*Quercus rotundifolia*) in the northern Middle Atlas or mixed pinewoods of *Pinus halepensis* and *Tetraclinis articulata* with a rich understory of *Pistacia lentiscus*, *Chamaerops humilis*, *Rosmarinus officinalis* and *Stipa* spp. Males sing mainly perched on these shrubs, and sometimes on the lower branches of trees (< 3 m height).

Etymology Specific epithet *dimelodica* arises from the dual sound production during the calling song of this species, meaning "two melodies". It consists of two distinct sound patterns, with the second part severely

downshifted in frequency and resembling a human-produced unvoiced linguolabial trill, often referred as "Blowing a raspberry".

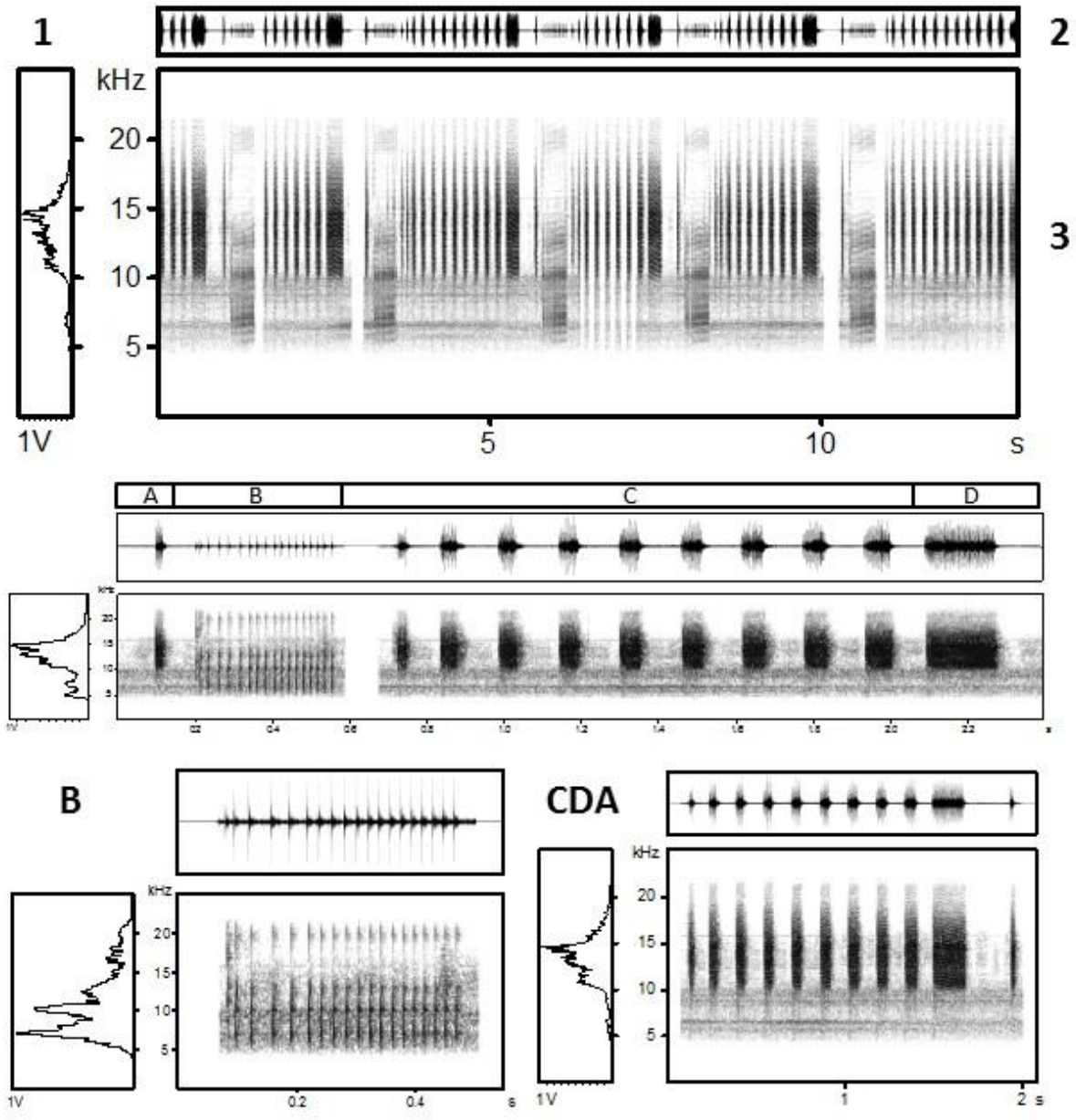

FIGURE 7. *Berberigetta dimelodica* calling song profile. Mean frequency spectrum (1), oscillogram (2) and spectrogram (3). Letters A, B, C and D refer to the structural divisions found in a typical phrase. Individualized analysis of part B and parts C, D and A (sequentially) are displayed in the bottom graphs. Calling song recorded on Middle Atlas, Afouzar at 38–40ºC.

Discussion

The two new species described in this paper based on acoustic, morphological and genetic data, used a more comprehensive species concept according to the contemporaneous perspective on species delimitation (De Queiroz 2007, 2016; Hausdorf 2011). For cicadas in general, the male calling song is thought to act as a pre-zygotic barrier which leads to specific-mate recognition and pairing (Paterson 1985), allowing for a reproductive, sometimes semipermeable, separation broadly considered as one of the early stages of species differentiation (Mayr 1963; Nosil 2008).

The placement of *T. afroamissa* sp. nov. under *Tettigettalna* is supported by aedeagus morphology (Figs. 3D and 3E), size, behaviour and genetic distance. *Tettigettalna* spp. are all morphologically similar but are confidently distinguished through the analysis of their calling songs (Puissant & Sueur 2010). While most *Tettigettalna* species have small distribution ranges in the Iberian Peninsula, *T. argentata* is an outlier, spreading elsewhere in SW Europe (Puissant & Sueur 2010; Nunes *et al.* 2014b). Despite the limited knowledge on the distribution limits of *T. afroamissa*, the species apparently shows a broad distribution range in Northern Morocco and bears some COI genetic variation, but unlike *T. argentata* (Nunes *et al.* 2014a), it constitutes a monophyletic clade, with no evidence of geographically structured genetic differentiation.

Although the use of the 5' end of the COI gene as DNA barcode has been proven relatively inefficient in the unambiguous identification of European *Tettigettalna* spp. (Nunes *et al.* 2014a), this was not the case for *T. afroamissa*. Mean pairwise distance between *T. afroamissa* and all other *Tettigettalna* is > 9%, which is well beyond commonly used thresholds for species differentiation with this marker (Hebert *et al.* 2004; Wiemers & Fiedler 2007; Linares *et al.* 2009).

Both phylogenetic trees obtained by Bayesian Inference and Maximum Likelihood (Figure 4 and S3, respectively) agree on the branch topology of the most recent taxa within *Tettigettalna*, but such cannot be said about the deeper-level relationships. The new species found in Morocco appears basally segregated in the genus, alongside *T. josei.* Asserting which is the basal taxon will need the inclusion of slower-evolution, nuclear genes. A recent work by Marshall *et al.* 2015, includes a dated global phylogeny from the tribe Cicadettini with mitochondrial and nuclear genes, placing *Tettigettalna* very far from all other European genera included in our analyses (*Tettigettacula*, *Euryphara*, *Tympanistalna*, *Hilaphura* and *Cicada*). Conversely, it is interesting to note that *Tettigettalna* forms a well-defined clade with American, continental Asia, Philippines and Micronesian species. The discovery of the first species of *Tettigettalna* out of Europe is an important step towards understanding the place and time of origin of this genus, its evolution and diversification. Further phylogenetic analyses are thus required, with the inclusion of additional genetic data and divergence time estimates.

Berberigetta had to be erected as a new genus to accommodate a new species found so far only in Morocco. The type species, *B. dimelodica*, can be readily separated from other closely related genera (*Cicadetta*, *Tettigettacula*) with a set of characters, which include genital morphology and a deep genetic divergence. However, the acoustic behaviour of this species turns up as the most striking feature. The very particular calling song shows a downshift in frequency (about 43% reduction) in part B of the phrase. Frequency shifts inside a phrase have also been reported for Dundubinii and Platypleurinii cicadas of Southern Asia, amongst others, such as *Meimuna tavoyana* (Distant, 1888), *Purana metallica* Duffels & Schouten, 2007, *Maua albigutta* (Walker, 1857) and *Kalabita operculata* Moulton, 1923 (Gogala 1995; Gogala & Trilar 2004; Gogala *et al.* 2004; Trilar 2006; cf. *P. metallica* as *P. aff. tigrina*).

Some European Cicadettini also reveal some degree of frequency modulation within a phrase, namely *Pagiphora aschei* Kartal, 1978, *P. annulata* (Brullé, 1832), *Euboeana castaneivaga* Gogala *et al*., 2011 and *H. varipes* (calling songs and spectrograms available at www.cicadasong.eu) but neither as pronounced nor with an abrupt downshift as seen in *B. dimelodica*. Video recordings of a calling male (see video in appendix, S7, credits to E. Marabuto) reveal that during the downshifted portion of the phrase, the male will slightly raise and tighten its abdomen probably with the help of longitudinal ventral muscles, in a similar fashion as *M. albigutta* (Gogala *et al.* 2004), a species with portions of a phrase with abrupt downshifts in frequency. Although it is difficult to uncouple the effect of tympanal gap, opercula and abdominal muscles have in the production and frequency regulation in cicadas, further studies are still needed to better understand the general mechanisms of frequency modulation in cicadas.

Finally, phylogenetic analysis of *B. dimelodica* revealed evidence of population structure. Populations from Berkane and Middle Atlas were recovered as genetically divergent (2.9%) and well resolved sister clades, suggesting two isolated distribution areas. Further fieldwork is required to confirm if they can be separated into different taxa, despite their seemingly alike calling songs. As Berkane is located near the international Morocco-Algeria border, the presence of *B. dimelodica* in this latter country cannot be dismissed.

The two new species here presented confirm the need for more data and effort to properly assess and update our knowledge of biodiversity and evolution of the rich cicadofauna of North Africa. Thus, taking into consideration that the Western Mediterranean area encompasses important biogeographical barriers and each part has been differentially affected by climate changes in the recent geological past, understanding the role of the

Maghreb as a reservoir of biodiversity in general (Schmitt 2007, Husemann *et al*. 2014), or referring to cicadas in particular, is of the utmost importance.

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Chapter III

3. The role of the Messinian Salinity Crisis on the diversification of the Mediterranean cicadas of the genus *Tettigettalna* (Hemiptera: Cicadettinii)

3.1. Abstract

The distribution patterns of many species of flora and fauna in the Mediterranean are a direct consequence of a just a few large and impactful past geoclimatic events. The flow of organisms between the continents of Africa and Europe mainly occurs in the Gibraltar Strait and between Sicily and Tunisia. Two major events during the past 10 Ma abridged the distance between – even connecting – both continents: the Pleistocenic "Ice Ages" and the Messinian Salinity Crisis (MSC). *Tettigettalna* is a genus of small cicadas that occur mostly in the Southern Iberia, with the striking exception of *T. afroamissa* Costa *et al*., 2017, occurring in the North Rif and Middle Atlas of Morocco. The discovery of an African *Tettigettalna* raised questions on the phylogeny and origin of the genus. Working with an expanded dataset with the inclusion of nuclear and mitochondrial loci, we provide a more comprehensive phylogeny of the genus alongside divergence estimates for the separation of the basal species, *T. josei* and *T. afroamissa*. Our results provide an insight for the impact of the MSC, and earlier events leading to it, in the isolation of these two taxa from the main European clade, first by the Guadalquivir basin and then by the opening of the Gibraltar Strait.

Keywords: Cicada, *Tettigettalna*, Messinian Salinity Crisis, West Mediterranean, multilocus, time estimates.

3.2. Introduction

The role of the Iberian Peninsula and Maghreb as harbors of biodiversity during the Pleistocene has been thoroughly discussed and an assemblage of glacial refugia have been recently discovered with the help of phylogeographical studies (consult Feliner 2011; Husemann *et al.* 2014 and Petit 2003 for reviews on this subject). The contrasting orography and climate during these Ice Ages allowed several animal and plant taxa to thrive under a range of suitable conditions and gathering under several smaller refugia spread throughout the Iberian Peninsula and Maghreb (see Fig. 3.1D), with several authors supporting the term coined by Gómez & Lunt (2007) as "refugia within refugia".

The relative proximity of the Europe and Africa by the Gibraltar Strait, (14km overseas distance), and to a lesser extent between Sicily and Tunisia (~140km distance), may have allowed the passage of terrestrial and freshwater species during the Pleistocene Ice Ages. During this period, sea levels were up to 150 m lower (Rohling *et al.* 2014), surfacing small islands on the Mediterranean Sea (Thiede 1978) (see Fig. 3.1D). Insects in general have had recent colonization events during the Pleistocene or experience to this day gene flow between these landmasses due to overseas dispersal (Franck *et al.* 2001 for honeybees; Habel *et al.* 2009, 2011 for butterflies; Rodrigues *et al.* 2014 for *Philaenus* spittle bugs; Sýkora *et al.* 2017 for *Meladema* diving beetles). It, therefore, seems that the Strait of Gibraltar acts as

a permeable barrier to dispersal, and extending to other animal and plant taxa, allowing these to aptly disperse between the two continents in both directions (Jaramillo-Correa *et al.* 2010 for *Pinus halepensis* trees; Paulo *et al.* 2008 for *Lacerta* lizards; Stuckas *et al.* 2014 for *Emys orbicularis* pond turtles; Harris *et al.* 2002 for *Podarcis* lizards; Ortiz *et al.* 2007 for *Hypochaeris* dandelions) or, on the other hand, isolating or greatly restricting gene flow between populations on either sides (Batista *et al.* 2004 and Fonseca *et al.* 2009 for *Acanthodactylus* lizards; Fromhage *et al.* 2004 for *Discoglossus* frogs; Hulva *et al.* 2004 for *Pipistrellus* bats; Jaramillo-Correa *et al.* 2010 for *Pinus pinaster* pines).

Two past major geological events connected the European and African landmasses: the first, the closing of the Tethys Sea, dated around 19 Ma, late Oligocene (Okay *et al.* 2010), was evoked to have impact in the distribution of some extant taxa (Hrbek & Meyer 2003; Sanmartín 2003). The majority of the present vicariant scenarios, however, are attributed to the second geological event: the Messinian Salinity Crisis (MSC) (5.97-5.33 Ma), a period in which the Mediterranean Sea almost dried up and allowed the formation of an extensive land bridge between Europe and the Maghreb (Krijgsman *et al.* 1999).

The MSC should be considered as the culmination of a chain of events. First, the Tortonian (11.62–7.25 Ma) marks the beginning of the formation of the Southern Iberia and ends with the closing of the ancient Mediterranean Sea (Fig. 3.1A). The mostly marine area which now corresponds to the Betic cordillera saw an uplift of its basin basement, forming a large island and being delimited northwards by the Guadalhorce and Betic corridors (Fig.3.1A) (Braga *et al.* 2003). Southwards, this island is delimited by another forming island roughly corresponding to the Moroccan northern portion of the Rif cordillera and extending to the Gibraltar Strait, and in turn delimited southwards by the Rifian corridors (Warny *et al.* 2003) (Fig.3.1A). Then, these corridors progressively close, first by the northern Betic and Guadalhorce corridors, (approximately at 7.3 Ma and 6.8 Ma, respectively) (Martin *et al.* 2001) and followed by the Rifian corridors, beginning at 7.4 – 7.2 Ma, totally closing at 6.0 Ma (Warny *et al.* 2003) (Fig.3.1A). When these corridors closed the Atlantic Ocean influx ceased with the Mediterranean. Nonetheless, there still exists a large saltwater body, the Guadalquivir Basin extending inland and maintained even during the Messinian (Fig.3.1B), sustained by the Atlantic ocean, and only receding on the Early Pliocene (Elez *et al.* 2016).

Through these tectonically-driven declines of the hydrological exchanges with the Atlantic ocean, the MSC was finally triggered by glacial conditions in the northern hemisphere and by arid conditions in northern Africa (Fauquette *et al.* 2006). It began with a series of evaporation cycles at ≈5.97 Ma (Manzi *et al.* 2013), climaxing in the nearly total desiccation of the Mediterranean Sea basin (Fig.3.1B). This led to the eastward expansion of the previous land bridge between North Africa and the Iberian Peninsula, extensively uniting these landmasses for over 0.64 Ma, allowing the passage of fauna and flora between the two continents. This land bridge was suddenly divided at the Gibraltar Strait around 5.33 Ma, which reconnected and completely refilled the Mediterranean basin with Atlantic waters, in the span of just a few decades (Blanc 2002). With this very sudden refill and separation of the now discontinuous North African and Iberian landmasses, by the late Pliocene, the Western Mediterranean obtained its current coastal boundaries (Jolivet *et al.* 2006) (Fig.3.1C).

Tettigettalna Puissant, 2010 is a group of nine cicada species that have only been reported to occur in Europe (Puissant & Sueur 2010; Nunes *et al.* 2014). As all European cicadas, adult *Tettigettalna* emerge each summer and males sing to attract females for mating. They often live in parapatry or sympatry (Mendes *et al.* 2014) and though morphologically very similar, these species can be easily recognized by their unique male calling song, which is species-specific (Puissant & Sueur 2010).

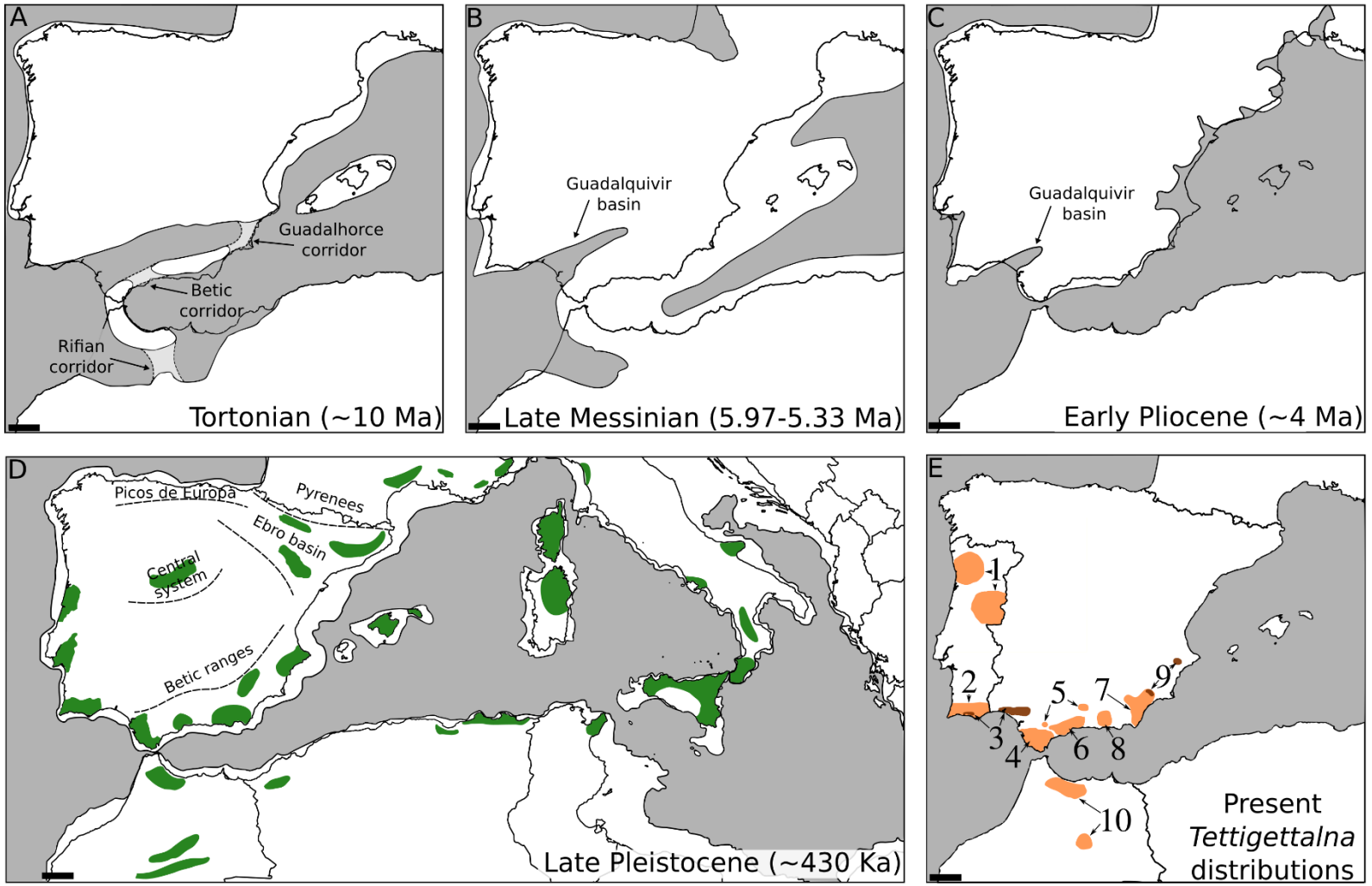

Figure 3.1. Major geological events of the Western Mediterranean, Pleistocenic glacial refugia and *Tettigettalna* spp. distributions. Panels A – D show a schematic of the evolution of the West Mediterranean region from the Tortonian to the Late Pleistocene. A – Mid Tortonian, depicting the three Eurafrican corridors that later closed, between 7.8 to 6.0 Ma. B – Late Messinian, during the Salinity Crisis an extensive land bridge formed between Iberia and North Africa. Arrow points to the Guadalquivir basin, a large saltwater basin. C – Early Pliocene, land bridge is now disrupted and the Guadalquivir basin has almost retreated. D – Late Pleistocene, during the period when sea level was lowest, according to Rohling *et al*. (2014), approx. 150 m lower. No land bridges are present during this period. Putative Pleistocenic glacial refugia of the Western Mediterranean inferred for flora (Médail & Diadema 2009) in green, and terrestrial fauna and flora (Gómez & Lunt 2007) delimited with broken lines. E – Present day *Tettigettalna* spp. distributions in light brown, according to Costa *et al.* (2017). Legend: 1–*T. estrellae*; 2–*T. josei*; 3–*T. mariae*; 4–*T. armandi*; 5–*T. aneabi*; 6–*T. defauti*; 7– *T. helianthemi helianthemi*; 8–*T. h. galantei*; 9–*T. boulardi*; 10–*T. afroamissa*. Species' distributions in orange overlap with those of other species. The distribution of *T. argentata* is not shown as it is widespread across the Iberian Peninsula, but exempt from the Betic ranges. Scale bar equals 100 km.

Most *Tettigettalna* spp. are restricted to the Southern part of the Iberian Peninsula (see Fig. 3.3E), with the exception of two species: *T. estrellae*, occurring in the north of Portugal, and *T. argentata*, which is widespread across the Iberian peninsula, but exempt from the Betic ranges, and extending its distribution to France, Italy and Slovenia to the east (Costa *et al.* 2017).

Recently, a new *Tettigettalna* species was found, *T. afroamissa* Costa *et al.*, 2017, being the first species of the genus found in Africa (in the Northern Rif and Middle Atlas, Morocco), presenting a similar ecology to *T. argentata*.

The discovery of *T. afroamissa* confirmed the hypothesis that the presence of *Tettigettalna* genus on the other side of the Gibraltar strait, in the Maghreb region, would be very likely, given its present pattern of distribution in southern Iberia. The newly discovered species raised critical questions on the processes that led to the diversification of the genus and its current distribution, as phylogenetic analyses of the genus relied on a single mitochondrial marker and did not clarify the basal species (Nunes *et al.* 2014; Costa *et al.* 2017).

Three hypothetic processes can then be drawn for the current distribution pattern of *Tettigettalna* spp.:

- Pre-MSC dispersal: Under this scenario, overseas dispersal before the MSC was responsible for colonization events in both continents, in either direction, resulting in the splitting of the European and Moroccan lineages before 5.9 Ma.
- Post-MSC vicariance: Under this hypothesis, a large population existed across the land bridge during the MSC, then, with the opening of the Gibraltar Strait ending the MSC, *T. afroamissa'*s lineage got separated from the rest of the European *Tettigettalna*. It is therefore expected that these lineages' split occurred between 5.9 and 5.3 Ma.
- Post-MSC dispersal: This scenario is similar to the pre-MSC dispersal, but dispersal occurred after the MSC, and European-African lineage splitting should take place after 5.3 Ma and most likely during the Pleistocene, when sea-level was remarkably lower. Colonization events are expected to have occurred in either direction.

In this study we will determine how the past geological and bioclimatic events shaped the diversification of *Tettigettalna* spp. Sampling from previous molecular works (Nunes *et al.* 2014; Simões *et al.* 2014; Costa *et al.* 2017) was expanded and four additional markers (two nuclear and two mitochondrial fragments) were sequenced for each species. This comprehensive phylogeny was produced with a Bayesian framework for species tree reconstruction and a molecular clock was employed to estimate divergence dates and we will link these divergence estimates to major geological events and test whether scenarios of vicariance or dispersal are the most-parsimonious to explain *Tettigettalna* spp. trans-Mediterranean distribution.

3.3. Materials and Methods

3.3.1. Sampling, DNA extraction and sequencing

Collection of specimens was performed by hand or sweeping net and GPS data was assigned to each capture site (see Fig. 3.2 for collection points and Table S1 for detailed locations, GPS coordinates of the captured specimens and respective accession numbers of analyzed loci). Specimens were photographed and respective habitats were characterized *in loco*. In the field, each specimen was assigned a tracking number and assigned to a species according to the male calling song. In the lab, each specimen was pinned and a front leg was removed and preserved in alcohol for posterior genetic

analysis. Dry specimens are stored at the Department of Animal Biology of the Faculty of Sciences, University of Lisbon, Portugal.

DNA extraction was performed by isolating whole-genome DNA from a front leg of each specimen with the DNeasy Blood & Tissue Kit (Qiagen). A total of five fragments were amplified, hereafter named after the codes: (i) COI-Lep: 5' region of the cytochrome C oxidase I (COI) mitochondrial gene; (ii) COI-CTL: 3' region of the cytochrome C oxidase I (COI) mitochondrial gene; (iii) ATP: mitochondrial locus comprising tRNA-Asp gene, complete sequence; ATPase subunit 8 gene, complete cds and ATPase subunit 6 gene, partial cds; (iv) CAL: calmodulin, nuclear intronic sequence; (v) EF1α: nuclear locus of Elongation Factor 1α (EF-1α) comprising: exon2, partial cds; intron2, complete sequence; exon3, complete cds; intron3, complete sequence and exon4, partial cds. Amplification of each locus by PCR was performed in a total volume of 15 or 20 µl containing 1xPCR buffer (Promega), 0.6 U *Taq* polymerase (Promega), 2.8 mM MgCl2, 0.10 mM dNTPs and 0.4 µM of each primer (see Table S5.2 for primers sequences and sources). The standard cycling conditions used were 94ºC for 3 min, 35 x (30 s at 94º C, 30 s at the specific annealing temperature as in Table S2 and 30 s at 72ºC) followed by a final elongation step at 72ºC for 10 min. PCR products were purified with Sureclean (Bioline) following the manufacturer's instructions. Purified fragments were sequenced using standard protocols with Big Dye Terminator v.3.1 (Applied Biosystems) on an ABI PRISM 310 (Applied Biosystems) at FCUL's Evolutionary Biology laboratory or shipped for external companies for Sanger sequencing service (Macrogen or Beckman Coulter Genomics). Sequences generated by this study were deposited in GenBank (accession numbers xxxxxx-xxxxxx, see Table S1).

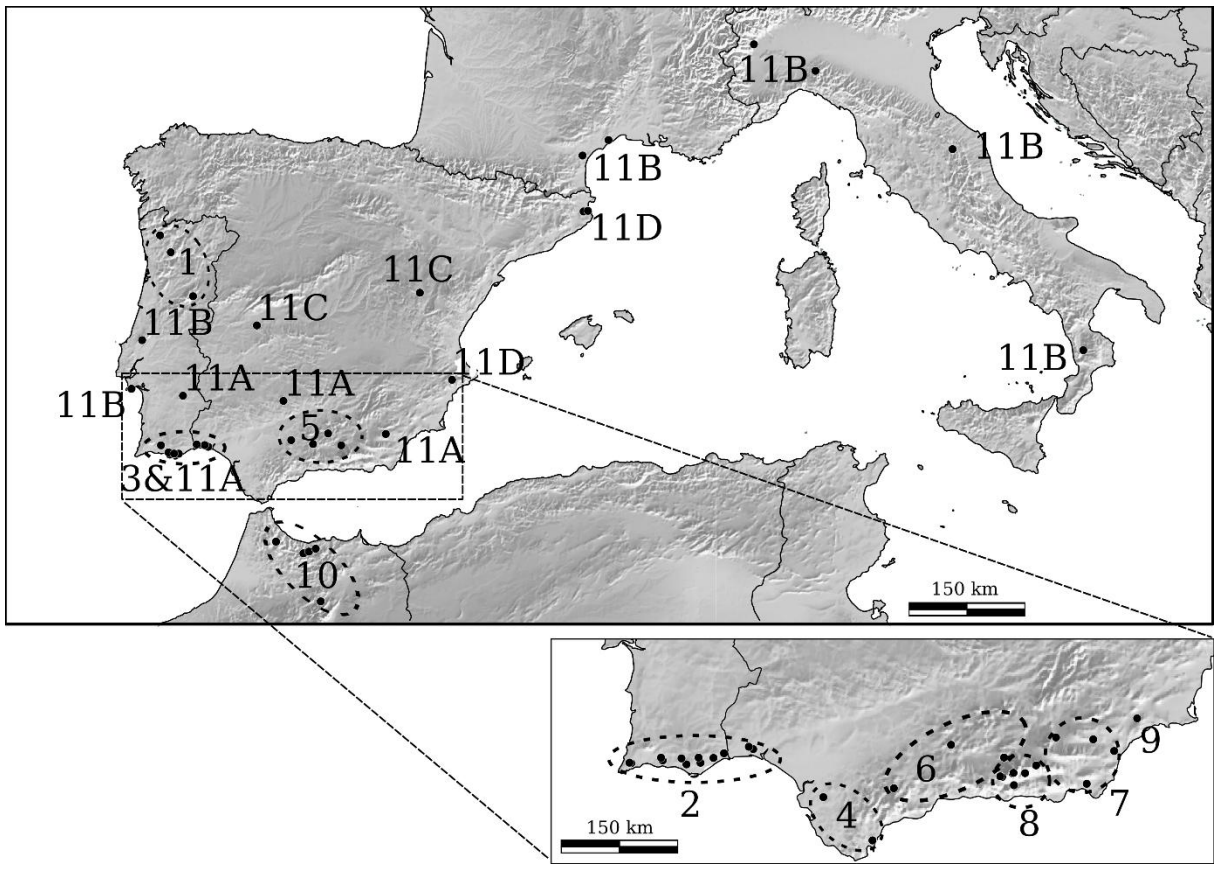

Figure 3.2. Sampling of *Tettigettalna* spp. Circles indicate same-species collection points. Due to the volume of sampling from the Southern Iberian Peninsula, the smaller box below shows additional sampling points for other species annotated for that area. Legend: 1–*T. estrellae*; 2–*T. josei*; 3–*T. mariae*; 4–*T. armandi*; 5–*T. aneabi*; 6–*T. defauti*; 7– *T. helianthemi helianthemi*; 8–*T. h. galantei*; 9–*T. boulardi*; 10–*T. afroamissa*; 11A –*T. argentata* South Clade; 11B – *T. argentata* North Clade; 11C – *T. argentata* Central Clade; 11D – *T. argentata* Catalonia Clade.

3.3.2. Sequence treatment

Sequences were assembled and edited in *Sequencher* v4.0.5 (Gene Codes Co.), to correct noisy and ambiguous base calling. Missing data was coded as N. Mitochondrial sequences were translated with the mitochondrial invertebrate genetic code in *DnaSP* v5.10 (Librado & Rozas 2009) to check for stop codons. Nuclear gene phasing was necessary and determined with *PHASE* v1.0 (Stephens *et al.* 2001; Stephens & Donnelly 2003), when allele phase probability was below 0.70. ambiguities where assigned as N. Sequences generated for this study were aligned with sequences previously made available in GenBank for COI-Lep by Nunes *et al.* 2014, Simões *et al.* 2014 and Costa *et al.* 2017 (see Table S1 for a detailed list and accession numbers). Sequences were aligned with *MAFFT* v7.273 (Katoh & Standley 2013) and visually inspected and trimmed in *BioEdit* v7.0.9.0 (Hall 1999) to reduce missing data from the 5'- and 3'- ends. Our full dataset contained 387 sequences (excluding nuclear haplotypes) and 2976 bp in length. The proportion of samples represented within each individual gene matrix varies meaningfully (100% of the 154 samples were sequenced for COI-LEP; 40% for COI-CTL; 36% for ATP; 41% for EF-1α; and 34% for CAL), revealing the heterogeneous coverage of our dataset.

3.3.3.Single-gene and concatenated phylogenies

Single-gene trees were obtained for each of the five sequenced loci. Site substitution saturation was tested in *DAMBE* (Xia & Xie 2001; Xia *et al.* 2003) for each codon position of coding sequences and found to be non-significant (p-value>0.05) for all tested loci. For the concatenated loci analysis each loci was concatenated in *TriFusion* (available at https://github.com/ODiogoSilva/TriFusion). Maximum likelihood trees were obtained by assigning each separate locus dataset a GTRCAT model, 1000 replicates and a rapid bootstrap analysis on *RAxML-HPC* v.8 (Stamatakis 2014) as implemented on the CIPRES Science Gateway (Miller *et al.* 2010). For the Bayesian inference, each dataset was partitioned into loci subsets and coding sequences were further partitioned into codon positions. These partitions were subsequently tested and assigned an evolution model on *PartitionFinder* v2 (Lanfear *et al.* 2016) under the corrected Akaike information criterion (AICc). Each matrix was converted from FASTA to NEXUS or PHYLIP formats with *TriFusion*. Bayesian inference trees were generated on MrBayes v3.2.6 (Ronquist *et al.* 2012) as implemented on the CIPRES Science Gateway (Miller *et al.* 2010). Each dataset was assigned with two independent runs with four chains, $5x10^7$ generations with burn-in set to the initial 25% trees and the evolution models previously selected in *PartitionFinder2* (Lanfear *et al.* 2016). Parameters confluence was checked in *TRACER* and if confluence was not attained, runs were assigned additional $2x10^7$ generations and rechecked again. The selected outgroup for all single-gene analyses was *Hilaphura varipes*, (see table S1 for accession numbers), with the exception of the CAL which was *Maoricicada caciope*, coded as "Mcass14", from Buckley & Simon (2007) (DQ178585.1). Tree outputs were visualized in *FigTree* (http://tree.bio.ed.ac.uk/software/figtree/) and imaged in *Inkscape*. Bootstrap support and posterior probabilities below 70% and 0.90, respectively, were removed from tree figures.

3.3.4.Estimation of Divergence Times

Estimation of divergences times was performed in **BEAST* (Heled & Drummond 2010), an extension package of *BEAST* v.1.8.4 (Drummond *et al.* 2012). Preliminary runs with the full mitochondrial and nuclear dataset mixed poorly, resulting in very low effective sample sizes (ESS), with the trait set following the group's taxonomy. This is likely due to the reduced number of variable sites of the nuclear loci and the inability to support the monophyletic entities defined in the trait set and required for **BEAST*. Thus, in order to obtain a true species-tree based on the combination of mitochondrial and

nuclear data, one would have to look for the entities the nuclear data could resolve as monophyletic. EF-1α only resolves *T. josei* and *T. afroamissa* as monophyletic clades, thus the remainder of the *Tettigettalna* need to be under a single entity, named *T.* other. With this reduced trait set, (*T. josei*, *T. afroamissa* and *T.* other) model optimization was rapidly obtained.

The final dataset for the ***BEAST*** analysis includes 4 partitions: COI-CTL, COI-LEP, EF-1α intron and EF-1α exon) and the reduced trait set. After preliminary runs, the site models selected were: K3Puf+G for COI-CTL and COI-LEP, HKY+G for EF-1α exon and HKY for EF-1α intron. Tree models were linked for the COI and the EF-1α partitions, with a Yule process prior. Clock models were linked for the COI partitions. Because the parameter "std.dev" of the EF-1α exon partition on preliminary runs abutted 0 the clock model was changed from uncorrelated relaxed to a strict clock with a lognormal distribution. The remaining partitions were assigned an uncorrelated relaxed clock, with a lognormal distribution with "mean in real time" checked. Clock rate estimates follow Marshall *et al.* (2016) for the COI estimates (M=0.01172, S=0.288) and the inferred clock rates as obtained in Subclade I (see supplementary information of Marshall *et al.* (2016) in which the *Tettigettalna* are included): M=0.001965; S=2.0 for EF-1α intron and M=0.0075 for EF-1α exon. MCMC chain length was set for $5x10^8$, logging every 50000^{th} iteration and ran in triplicate to check for repeatability in BEAST v.1.8.4 as implemented on the CIPRES Science Gateway (Miller *et al.* 2010). *Tracer* v1.4 was used to assess convergence and correct mixing of all parameters by visually inspecting the .log trace files and assessing the Effective Sample Size (ESS) of each informative parameter. *Logcombiner* was used to combine the cloned runs and *Treeannotator* were used to extract maximum clade credibility consensus trees. Trees were visualized in *Densitree*.

3.4. Results

3.4.1.Single-tree phylogenies

Bayesian inference and maximum likelihood trees were performed for each sequenced loci. Regarding the aligned datasets, Table 3.1 indicates the gene information used for the multilocus analyses.

Table 3.1 Gene information for multilocus analyses. Locus information includes locus name, sequence length (in bp), number of sequences (N), number of haplotypes, number of variable sites (V) and number of parsimony-informative sites (P).

Locus name	Locus Size	N	Haplotypes	V	P
COI-Lep mtDNA	581	149	83	208	175
COI-CTL mtDNA	683	55	49	243	154
ATP mtDNA	668	51	42	211	162
CAL nuDNA	472	48	28	50	38
EF1-α nuDNA	572	56	61	100	34

Phylogenetic trees produced with mitochondrial loci by maximum likelihood and Bayesian inference are mostly concordant and these loci successfully retrieve most song-delimited species (see Supplementary Figs S3 and S4. for individual loci trees, by the Bayesian inference and maximum likelihood methods, respectively) The same unresolved taxa remain as in previous studies (Nunes *et al.* 2014; Costa *et al.* 2017), namely: *T. argentata*, *T. aneabi* and *T. mariae* clade, with these markers unable to accurately delimit these taxa, but nonetheless forming a well-supported clade (COI-Lep: 92% BS, ≈1pp; COI-CTL: 96% BS, 1 pp; ATP: <70% BS, ≈1pp).

Furthermore, all three mitochondrial loci reinforce the apparent polyphyly of *T. helianthemi* (see Figs S3 and S4, in Supplementary Material); these taxa are split into two separate and well-supported clades, with samples of *T. h. galantei* coming from Lanjarón, Sierra Nevada (per Nunes *et al.* (2014), defined as type II) as sister taxa of *T. boulardi* (COI-Lep: 82% BS, ≈1 pp; COI-CTL: 100% BS, 1 pp; ATP: 92% BS, 0.976 pp) and the remainder of the *T. h. galantei* samples (type I) forming a well-supported clade with the *T. h. helianthemi* subspecies (COI-Lep: 82% BS, ≈1 pp; COI-CTL: <70% BS, not applicable; ATP: 97% BS ≈1 pp).

Whereas mitochondrial loci are efficient to reconstruct more derived relationships, they fail to reconstruct the deep nodes amongst the *Tettigettalna* (see Figs. S3 and S4, in Supplementary Material). Bootstrap supports and posterior probability are relatively low in the most basal nodes, particularly for resolving the position of *T. josei* and *T. afroamissa*. Their relationship remains unclear, as ML trees point towards a well-defined clade but BI tends to create a paraphyly with *T. josei* as basal taxon of the *Tettigettalna*.

CAL has a low resolution overall, being only able to confidently separate *T. afroamissa* and *T.estrellae* from the remainder of taxa (with >70% BS; >0.9 pp), which form a large polytomy. EF-1α provided a greater resolution on tree topology than CAL, fully retrieving *T. afroamissa* as well as *T. josei* as monophyletic entities. The remainder of the specimens form a large, low supported, polytomy or interspecific groupings (see Figs. S3 and S4, in Supplementary Material).

3.4.2.Concatenated nuclear and mitochondrial phylogenies

Combined mitochondrial phylogenetic tree (Fig.3.3B) retrieved most previously defined clades by Nunes *et al.* (2014). The increased sampling proved efficient in resolving *T. defauti* and *T. armandi* as sister taxa (92% BS; 1 pp), previously a polytomy, and also supporting within each separate species, two mitochondrial lineages formed by the Sierra Nevada and Ronda & Sagra populations of *T. defauti* (71% BS; 0.892 pp) and the Jerez and Gibraltar populations of *T. armandi* (94% BS; 1 pp). Basal relationships are also well supported by this analysis. *T. josei* was retrieved as the basal species of the genus by both analyses (99% BS; 1 pp), followed by *T. afroamissa* and the remainder of the *Tettigettalna* (<70% BS; 0.96 pp).

The concatenated nuclear loci retrieved similar topologies for the ML and BI trees with the support of well-defined clades of *T. josei* (96% BS; 0.98 pp), *T. estrellae* (85% BS; 0.95 pp) and *T. afroamissa* (98% BS; 0.99 pp) (see Fig. 3.3 A). Again, the nuclear data shows little variability and cannot resolve the more recent phylogenetic relationships.

3.4.3.Divergence time estimates

The program **BEAST*, besides providing time estimates on species divergences, also enables the user to provide information on the ploidy of the loci – *i.e* differentially weighing the mutations that occur in the slower-evolving nuclear loci and the faster-evolving mitochondrial loci. This approach is more recommendable than unweighted concatenation because the nuclear information will be easily overwhelmed by the volume of data that the mitochondrial loci provides, losing much of the resolution the nuclear loci provide, especially on the deeper phylogenies.

**BEAST* allowed to estimate the posterior distribution of the time to the most recent common ancestor (tMRCA), including 95% credibility intervals of highest posterior density (HDP), mean and clade support was obtained in *Densitree.* In order to make a *bona fide* estimation of the chain of events of the diversification of the *Tettigettalna*, we estimated tMRCA of the clades definable by the nuclear dataset:

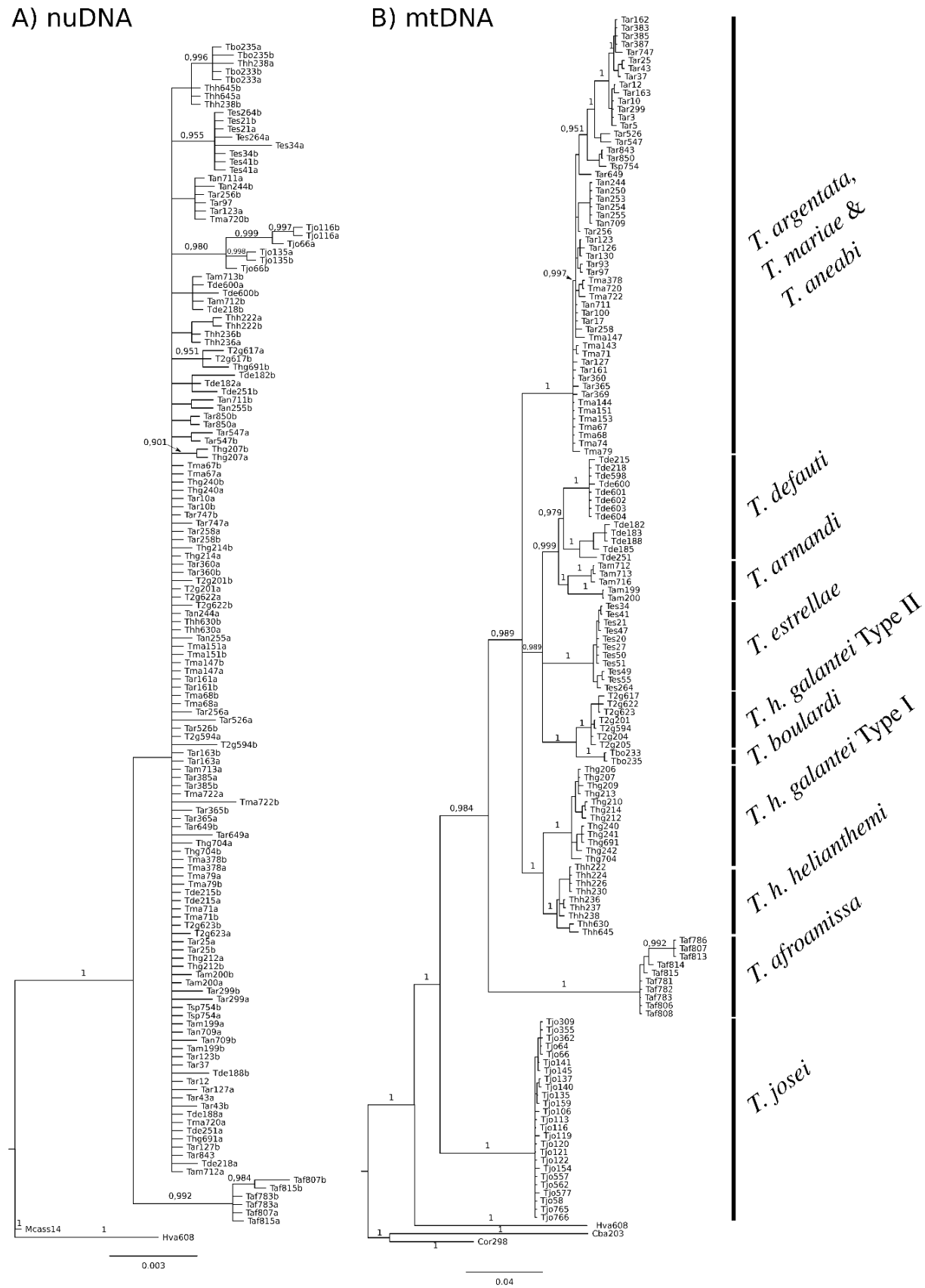

Figure 3.3. Bayesian inference phylogenetic trees for the concatenated nuclear (A) and mitochondrial (B) datasets. Posterior probabilities are shown next to branch nodes. Scale bar represents the number of estimated changes per branch length. *H. varipes* (Hva608) was set as outgroup for A). *C. barbara* (Cba203) and *C. orni* (Cor298) were set as outgroup for B). Monophyletic clades are annotated for B). Additional taxa details are included on supplementary information Table 3.S1. Root was truncated with double dashes.

T. josei, *T. afroamissa* and the remainder of the European *Tettigettalna, T.* other. These results are summarized in Table 3.2.

Because we are working with a reduced number of target clades, we can also ponder all the three possible phylogenetic relationship scenarios between these three clades. The probabilities of such sub-clades are also presented in Table 3.2. The results show that the clade *Tettigettalna* is monophyletic in the present analysis, regarding the outgroups, (95.12% support), and placing *T. josei* as the basal species with a tMRCA of 7,039 ± 0.080 Ma. Of the three likely phylogenetic relationship scenarios, this is the one with the highest bootstrap support (81.59%) with the remaining having a combined reduced probability (<20%). The tMRCA for the *T. afroamissa – T.* other split is dated to 5,308 ± 0.054 Ma. Relating these divergence estimates with geological events, the split of *T. josei* is dated to the early Messinian and the split of *T. afroamissa* from end of the Messinian, coinciding with the ending of the MSC.

Table 3.2. Mean age estimates in million years ago (Ma) and 95% highest probability density intervals of tMRCA, including standard error. Clade support is given in percentage of trees that support that topology, post-burnin.

Clade	Lower 95% HPD	Mean ± Std Error	Upper 95% HPD	Support
Tettigettalna	2.638	7.039 ± 0.080	12.274	95.12%
T. *afroamissa – T.* other	2.047	5.308 ± 0.054	9.565	81.59%
T. josei – T. other	2.548	6.965 ±0.080	12.297	8.4%
T. afroamissa – T. josei	2.429	6.952 ± 0.080	12.410	9%

3.5. Discussion

The placement of *T. josei* as the basal taxon for this group is expected, as previous works suggested that *T. josei* was a likely outgroup for this genus. These studies support *T. josei* as the most divergent taxa at the molecular, morphology and also acoustic level (Mendes *et al.* 2014; Nunes *et al.* 2014). DNA barcoding for *T. afroamissa* placed this taxa as a sister species of *T. josei*, and thus leaving the *Tettigettalna* clade, unresolved, as paraphyletic (Costa *et al.* 2017).

Our concatenated multilocus approach retrieved the *Tettigettalna* as a monophyletic entity with the African species, *T. afroamissa*, and *T. josei* as the basal species, well supported within this clade by the concatenation approach. Corroborating the well-supported phylogeny obtained from the concatenated analyses, the *BEAST species tree supports the same basal topology of the *Tettigettalna*. Nonetheless the other two phylogenetic scenarios (i.e subclades *T. josei – T.* other and *T. afroamissa – T. josei*) have some degree of support (8.4 and 9%, respectively), which can't be discarded (see Table 3.2).

This pattern may be evidence for a vicariant or dispersal scenario for *T. afroamissa*'s lineage. Divergence times estimate the separation time for *T. afroamissa* (5.308 ± 0.054 Ma) to be after *T. josei* (7.039 ± 0.080 Ma). These estimates place the separation of *T. josei* and *T. afroamissa* from the remainder of the European *Tettigettalna* lineage during the Messinian. We therefore advance the hypothesis that the Messinian had a double-effect on the diversification of the *Tettigettalna*. Our reconstruction for the most-parsimonious biogeographic scenario has three steps: Firstly, *T. josei* was separated from the remainder of the *Tettigettalna* lineage, the divergence estimate is concurrent with the closing of the Betic and Guadalhorce corridors (7.3 Ma and 6.8 Ma, respectively) and the formation of the Guadalquivir Basin (Fig. 3.4A). Secondly, the *T. afroamissa* – European *Tettigettalna* lineage composed a widespread population that spread to both continents with the early onset of the MSC (Fig. 3.4 B). And thirdly, the sudden separation of the *T. afroamissa* lineage from the European lineage (5.308

± 0.054 Ma) with the opening of the Gibraltar Strait (5.33 Ma). The opening isolated the *T. afroamissa* and the European *Tettigettalna* lineages very rapidly, leaving these lineages separately evolving from each other (Fig. 3.4 C). Thus, under the present results, the biogeographic hypothesis of post-MSC vicariance is, so far, the most-parsimonious to explain the current distribution pattern of *T. afroamissa*.

The physical separation of the two geological masses, the Iberian Massif and the Betic Cordillera was, during the late Tortonian, a large seawater strait – the Betic strait – that connected the Mediterranean Sea to the Atlantic Ocean. With the closure of the Betic and Guadalhorce corridors during the Messinian, this strait turned to a large seawater basin – the Guadalquivir basin. This separation, during the early Messinian, coincides with the genetic split between *T. josei* and the main ancestral lineage of the *Tettigettalna*, estimated to have occurred at 7.039 ± 0.080 Ma. The formation of the Eurafrican land

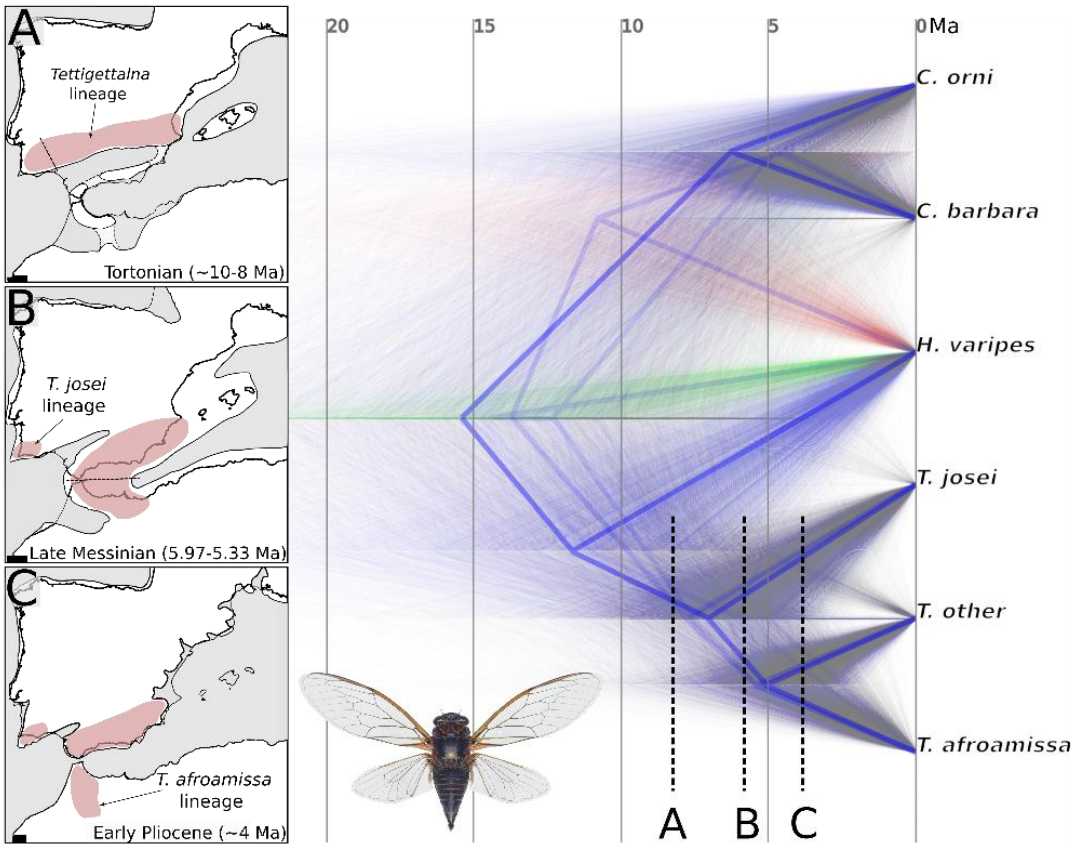

Figure 3.4. DensiTree output of the Bayesian inference species tree of *Tettigettalna* with the partitioned mtCOI and nuEF-1α dataset. The consensus trees are shown by the bold blue line. Uncertainty of node heights and topology is shown by the transparent green, purple and red lines. *T.* other refers to the clade composed of the remainder of the *Tettigettalna* (see methods for explanation). Scale bar indicates Ma. The broken lines refer to key moments in time illustrated in the left panes. A) Mid-Tortonian (10~8 Ma) when the ancestral population of the *Tettigettalna* occurred in the southern Iberian Peninsula; the broken line indicate the latter separation of the *T. josei* lineage in the south of Portugal with the main ancestral population. B) Late Messinian, during the Salinity Crisis (5.97-5.33 Ma), when the main population disperses to North Africa, via the formed landbridge; the broken line indicates the rupture caused by the opening of the Gibraltar Strait by end of the Messinian. C) Early Pliocene (~4 Ma), showing the three lineages: *T. josei* in Southern Portugal; *T. afroamissa* in Morocco and the remainder of the European *Tettigettalna* lineage which would later diverge into the current species. In the lower left corner, a male of the Morrocan species *T. afroamissa* is shown.

bridge was a consequence of the progressive uplift of the Betic basement basin. With this, the main *Tettigettalna* ancestral population was able to migrate southwards and separate from the *T. josei* lineage population. This lineage in turn could not migrate southwards, thus being isolated in the south of Portugal (see Fig. 3.4B). Presently there is little recorded evidence for the role of the Guadalquivir basin as a biogeographical barrier, within the Iberian Peninsula, especially for insect taxa. This barrier has been implicated in the divergence of two subspecies of the Iberian salamander, *Salamandra salamandra*

morenica and *S. s. longirostris*, although the divergence time estimated is much younger, dated to the early Pliocene (García-París *et al.* 1998).

The end of the Messinian by the opening of the Gibraltar Strait and refilling of the Mediterranean Sea also had an important role in separating lineages of terrestrial taxa. It isolated the ancestors of the *Alytes* midwife toads, *Alytes maurus* from *Alytes dickhilleni* and *Alytes muletensis* on opposite shores of the incipient Mediterranean Sea (Martínez-Solano *et al.* 2004).

Although the extent to which cicadas are able to disperse has only been assessed in species that form large choirs (Karban 1981; Simões & Quartau 2007), our results can also point towards the low-dispersal capability of the *Tettigettalna*, as these were only able to reach North Africa when the MSC allowed for an ample land crossing. Even during the Pleistocene when sea-level was remarkably lower, no signs of recent introgression are found between *T. afroamissa* and the more recent species of *Tettigettalna* (although it cannot be totally excluded) and, no other *Tettigettalna* species have been found, so far, in Morocco, with the sole exception of *T. afroamissa*. This could indicate a potentially low potential to disperse over large oceanic bodies for these cicada species. The idea that cicadas are poor dispersers over large distances was also reiterated by de Boer & Duffels (1996) whom correlated the distribution of several Indo-Australian cicadas of the subtribe Cosmopsaltriaria and the tribe Chlorocystinii to the tectonic movements of the area. Contrarily, Arensburger *et al.* (2004) found that certain wind direction patterns favored the long-range dispersal of some New Zealand *Kikihia* cicadas, *Kikihia* spp. near *cutora*, to nearby, outer islands. The cicadas that colonized these islands had "unusually long wings" unlike any other mainland *Kikihia* spp, a phenotype that could help to disperse further. Also, a revision by Holloway & Hall (1998) showed that dispersal can also occur in other cicada taxa, but in a more localized way over the same geological template, even though the *Baeturia bloetei* species group was able to disperse several thousands of miles over the numerous (surrounding) islands of New Guinea, via a stepping-stone manner. Therefore, it seems that long-distance dispersal is a rare event, occurring mostly locally and certain phenotypes (such as a high wing length – body ratio) are needed to take flight over large distances.

It yet remains to explain the concurrent distribution of many *Tettigettalna* species with the putative glacial refugia of plant and animal taxa. *T. afroamissa*, for example, can only be found on two populations which coincide with the refugia of the North Rif and the Middle Atlas. The *Tettigettalna* inhabiting the Betic ranges also coincide with several other refugia. To ascertain the influence of the Pleistocene and obtain divergence estimates, NGS technologies could also help resolve questions on the clade *T. argentata –T. aneabi – T. mariae* alongside ecological niche modeling to provide a full picture of the diversification processes of the *Tettigettalna*.

Several other cicada species share a similar trans-Mediterranean distribution. Genera such as *Cicadetta* Kolenati, 1857, *Euryphara* Hórvath, 1912, *Cicada* L., 1758 and *Pseudotettigetta* Puissant, 2010 possess species on both sides of the Strait of Gibraltar but very little is known about their North-African counterparts. Key elements for a proper and integrative species delimitation are missing for most of these species, such as access to acoustics, preserved specimens for DNA extraction or ecology data.

We hope that in the future, more attention is brought upon the unknown counterparts of the well-known European cicadas and abridge the knowledge gap between the two continents.

4. Final remarks

The description of several species of the *Tettigettalna* genus in 2010 exposed the deep-rooted necessity of exploring and rediscovering our neighboring countries. Being just outside of Europe, it would be expected that Morocco, also minding the present geopolitical context, would be well-explored and with updated faunal records. This is not the case for the Moroccan cicadas, as the last recorded expedition dated to 1983. The descriptions of these species are purely reliant on morphology studies and it leaves to question of how many species are still clumped under a single taxon. The ever-reducing costs of sequencing genetic sequences and availability of professional acoustic recording devices also allows taxonomists to readily and easily bypass traditional morphological barriers. Therefore it greatly expands the possibilities of species description based on an evolutionary view – rather than an antiquated typological view.

In agreement with this context we decided to enlarge our knowledge on the *Tettigettalna* species group, namely in the north of Africa. Therefore, present results lead to the new species described in Chapter II. First, *B. dimelodica*, a unique species in the way that it possesses a calling song with a remarkable frequency modulation (some parts are 43% downshifted). This pattern is unlikely found amongst small cicadas (tribe Cicadettinii) of Europe (of which there are song recordings libraries), but resembles that of some south-Asian cicadas (Dundubinii and Platypleurinii). This pattern leads to question how – the process – that led to it. The double frequency can be an adaptation to: 1) a heterogeneous landscape, or 2) sympatry with other species sharing the same acoustic space. Under both scenarios, the dual peak frequencies may be able to disperse further, more reliably and effectively in noisy environments (see *habitat tuning* under the chapter 1.2). This species, can in turn become a model for studying sensory drive although reuniting the conditions to test the hypothesis can be an ordeal in itself. As with other Moroccan cicadas, next to nothing is known about their biology and nymph stage is probably 2 or 3 years, becoming a longer-than-a-PhD-time commitment only to data collection.

The genetic analysis of *B. dimelodica*, although with a single marker, evidenced structuring between the populations of Berkane & El Hoceima and the Middle Atlas. This structure could be an artifact of our sampling, as these populations are separated by over 200km and it relies on a single mitochondrial marker. If with an expanded sampling and additional acoustic recordings this structuring is still clear, perhaps then we will be able to delimit two evolutionary lineages with subspecies or species statuses.

The Moroccan *T. afroamissa* is an apparent outlier to the *Tettigettalna*. It is the first of its group to be found outside Europe and the performed genetic analysis places this taxon as sister of *T. josei*, but with a low support. It shares a similar calling song with *T. argentata* and is morphologically very similar to the remainder of the *Tettigettalna*, except *T. josei*. This discovery also raised questions on how *T. afroamissa* obtained its current distribution, far from its congeners.

On chapter III, we reconstructed the phylogeny of the *Tettigettalna* with an updated dataset with a greater sampling effort and additional sequenced loci, including nuclear information (3 mtDNA loci + 2 nuDNA loci). We proceeded to reconstruct the phylogeny of each single-loci dataset and the full concatenated dataset (mitochondrial; nuclear and mitochondrial + nuclear) with two popular phylogenetic methods, Maximum Likelihood and Bayesian inference. This approach was successful in: 1) placing *T. josei* as the basal taxa of the genus; 2) resolving *T. defauti* and *T. armandi* as sister taxa and 3) placing *T. afroamissa* at the basis of the European *Tettigettalna*.

However, with this approach we were unable: 1) to resolve the phylogenetic relationship between *T. argentata*, *T. mariae* and *T. aneabi*; 2) to resolve the contradicting *T. helianthemi* taxonomy and polyphyly.

In chapter III, results of time-estimates placed the separation of *T. josei*, *T. afroamissa* and the remainder European *Tettigettalna* to a critical event in the Mediterranean history, the Messinian Salinity Crisis. The species-tree provided by *BEAST is mainly congruent with the concatenation approach. During this period (5.97-5.33 Ma), the Mediterranean was almost desiccated and an extensive land bridge formed between North Africa and the Iberian Peninsula.

Our most-parsimonious reconstruction of the events that led to the separation of these three lineages: 1) places *T. josei* lineage isolated, in the south of Portugal, by the Guadalquivir basin, and is likely to have taken place before the onset of the MSC and well before the *T. afroamissa* – European lineages' split; 2) placing a large population spread across the land bridge and extending to both continents and composed of the *T. afroamissa* and European lineages; 3) the sudden separation of the *T. afroamissa* lineage from the European lineage with the end of the MSC and 4) by the end of the Messinian forming three separately-evolving lineages forming the current pattern of distribution.

Recapping the thesis objectives:

The first objective of applying a three-pronged approach methodology led to the description of two new cicada species from Morocco using morphology, acoustics and genetics.

The second objective, of constructing a species-tree of the *Tettigettalna* genus and studying the divergence time estimates, allowed us to access the impact of major geological and bioclimatic events on the speciation patterns of this group.

In conclusion, this thesis objectives' have been fulfilled having provided the description of two new cicada species from Morocco, with results published in the international taxonomic journal Zootaxa. Moreover, it allowed us to better explain the disparate distribution of the newly described species, *T. afroamissa*. Although it wasn't possible to obtain additional estimates for the divergence of the European *Tettigettalna* lineages using *BEAST, we were able to explain the distribution of this species and link it to a major geological event.

During this time period I shared my findings on two separate occasions. First, during the XVII Iberian Congress of Entomology and the latter in the "Encontros Scientia" at the Faculty of Sciences.

Likewise most scientific works, this thesis opened up more questions than those that have been solved and these beget solving with multiple methods encompassing several disciplines. A proper species delimitation is still needed for the *T. mariae – T. aneabi – T. argentata* clade, and besides improving our capability in discriminating these species, it will also help unveil the history of this particular group. *Berberigetta dimelodica* is another species that requires further inspection, as it may comprise two distinct evolutionary lineages, and therefore, two separate species/subspecies. Many other species of cicadas, and not only from Morocco, need to be properly studied with a variety of methods to extract the most information and to perform an integrative species delimitation. This effort will likely produced new species that, as discussed throughout the thesis, are clumped under a single, typological taxon. The same effort applied in Chapter II could be easily derived, as a starting point, to these other taxa, and be adapted to include more layers of informative aspects of that taxon, as many as necessary to properly define the species boundaries.

I am hopeful that in the future we will be listening to more of these unknown cicadas.

"It is nice to think that there are so many unsolved puzzles for biology, although I wonder whether we will ever find enough graduate students"

Lewis Thomas, 1974

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6. Supplementary Material

CHAPTER II

Table S1. Additional taxa sampling included in our phylogenetic analysis including collection points and GenBank accession numbers.

Taxon	Sample ID	Country	Location	GPS coordinates	GenBank accession n.	Source
Cicada Barbara	Cba203	Spain	Sierra Nevada, Lanjarón	36º54'57.78"N; 3º30'14.4"W	KC807317	Nunes *et al*, 2014
Cicada orni	Cor298	Portugal	Serra d'Aires e Candeeiros	39º27'17.6"N 8º45'07.8"W	KC807318	Nunes *et al*, 2014
Euryphara contentei	Eco772	Portugal	Beringel	38º3'19.5"N; 7º59'50.28"W	-	This paper
Hilaphura varipes	Mva608	Spain	Sierra Nevada, Pinos Genil	37º8'15.5"N; 3º28'34"W	-	This paper
T. armandi	Tam199	Spain	near Gibraltar	36º11'17.7"N; 5º21'33.6"W	KC807277	Nunes *et al*, 2014
T. armandi	Tam200	Spain	near Gibraltar	36º11'17.7"N; 5º21'33.6"W	KC807278	Nunes *et al*, 2014
T. aneabi	Tan250	Spain	Zagra	37º16'59.82"N; 4º14'4.02"W	KC807301	Nunes *et al*, 2014
T. aneabi	Tan255	Spain	Zagra	37º16'59.82"N; 4º14'4.02"W	KC807299	Nunes *et al*, 2014
T. argentata	Tar163	France	Narbonne	43º9'16.92"N; 2º57'49.14"W	KC807234	Nunes *et al*, 2014
T. argentata	Tar256	Spain	Espiel	38º11'3.72"N; 5º1'36.12"W	KC807232	Nunes *et al*, 2014
T. argentata	Tar365	Spain	Ayamonte	37º16'3.3"N; 7º20'32.28"W	KC807246	Nunes *et al*, 2014
T. argentata	Tar43	Portugal	Braga	41º34'54.48"N; 8º19'14.1"W	KC807229	Nunes *et al*, 2014
T. boulardi	Tbo233	Spain	Campico de los López, Murcia	37º34'57"N; 1º34'16.5"W	KC807276	Nunes *et al*, 2014
T. boulardi	Tbo235	Spain	Campico de los López, Murcia	37º34'57"N; 1º34'16.5"W	KC807275	Nunes *et al*, 2014
Tettigettacula baenai	Tcb191	Spain	Grazalema	36º45'24.18"N; 5º24'6.3"W	KC807311	Nunes *et al*, 2014
Tettigettacula baenai	Tcb194	Spain	Grazalema	36º45'24.18"N; 5º24'6.3"W	KC807312	Nunes *et al*, 2014
Tettigettacula baenai	Tcb195	Spain	Grazalema	36º45'39.18"N; 5º22'57.6"W	KC807313	Nunes *et al*, 2014
T. defauti	Tde182	Spain	Puerto del Viento, Ronda	36º47'13.32"N; 5º3'11.88"W	KC807305	Nunes *et al*, 2014
T. defauti	Tde183	Spain	Puerto del Viento, Ronda	36º47'13.32"N; 5º3'11.88"W	KC807307	Nunes *et al*, 2014
T. defauti	Tde185	Spain	Puerto del Viento, Ronda	36º47'13.32"N; 5º3'11.88"W	KC807309	Nunes *et al*, 2014
T. defauti	Tde188	Spain	Puerto del Viento, Ronda	36º47'13.32"N; 5º3'11.88"W	KC807308	Nunes *et al*, 2014
T. estrellae	Tes21	Portugal	Braga	41º34'54.48"N; 8º19'14.1"W	KC807263	Nunes *et al*, 2014
T. estrellae	Tes264	Portugal	Serra da Estrela	40º21'17.76"N; 7º26'24.6"W	KC807265	Nunes *et al*, 2014

Table S1. Continued

T. helianthemi galantei	Thg204	Spain	Lanjarón, Sierra Nevada	36º54’57.78"N; 3º30’14.4"W	KC807281	Nunes *et al*, 2014
T. helianthemi galantei	Thg205	Spain	Lanjarón, Sierra Nevada	36º54’57.78"N; 3º30’14.4"W	KC807280	Nunes *et al*, 2014
T. helianthemi galantei	Thg214	Spain	Capileira, Sierra Nevada	36º57’47.88"N; 3º20’26.52"W	KC807286	Nunes *et al*, 2014
T. helianthemi galantei	Thg240	Spain	Laroles, Sierra Nevada	37º2’57.06"N; 3º1’0.9"W	KC807287	Nunes *et al*, 2014
T. helianthemi helianthemi	Thh230	Spain	Cabo de Gata	36º50’18.3"N; 2º17’35.58"W	KC807297	Nunes *et al*, 2014
T. helianthemi helianthemi	Thh237	Spain	Vera	37º12’48.06"N; 1º53’58.68"W	KC807293	Nunes *et al*, 2014
T. josei	Tjo116	Portugal	Lagoa, Algarve	37º8’9.36"N; 8º23’4.2"W	KC807271	Nunes *et al*, 2014
T. josei	Tjo119	Portugal	Budens	37º4’45.2"N; 8º50’11.6"W	KF977491	Simões *et al*, 2014
T. josei	Tjo140	Portugal	Castro Marim, Algarve	37º11’10.92"N; 7º29’2.1"W	KC807269	Nunes *et al*, 2014
T. josei	Tjo562	Spain	Cartaya	37º15’38.4"N; 7º7’43.5"W	KF977504	Simões *et al*, 2014
T. josei	Tjo577	Spain	Cartaya	37º14’3.7"N; 7º3’56.8"W	KF977505	Simões *et al*, 2014
T. josei	Tjo64	Portugal	Vale Judeu, Algarve	37º7’39.78"N; 8º5’36.06"W	KC807274	Nunes *et al*, 2014
T. mariae	Tma143	Portugal	Vale do Lobo, Algarve	37º3’41.1"N; 8º3’39.12"W	KC807253	Nunes *et al*, 2014
T. mariae	Tma153	Portugal	Vale do Lobo, Algarve	37º3’41.1"N; 8º3’39.12"W	KC807257	Nunes *et al*, 2014
T. mariae	Tma79	Portugal	Vale Judeu, Algarve	37º6’20.88"N; 8º5’42.66"W	KC807256	Nunes *et al*, 2014
Tympanistalna gastrica	Tyg180	Portugal	Sesimbra	38º27’4.5"N; 9º5’27.9"W	KC807314	Nunes *et al*, 2014

Table S2. GPS coordinates and annotated populations where *T. afroamissa* was heard but not collected.

Population	GPS Coordinates	Date	Habitat notes
Chefchaouane	35º 10' 29.34" N 5º 15' 28.93" W	17-07-2014	*Quercus rotundifolia*, *Pinus* sp., *Abies* sp., *Cistus* spp., *Juniperus* sp.
Rif	35º 17' 53.50" N 4º 53' 53.60" W	19-07-2014	Near the seashore, dominated by small shrubs.
	35º 6' 55.68" N 4º 40' 45.13" W	19-07-2014	*Prunus dulcis* orchard, arid habitat.
	34º 59' 6.76" N 4º 48' 35.15" W	19-07-2014	Dominated by *Quercus canariensis.*
	34º 57' 38.06" N 4º 40' 48.76" W	19-07-2014	*Q. rotundifolia*, *Cupressus* sp. and small shrubs.
	34º 57' 32.05" N 4º 39' 2.75" W	19-07-2014	Dominated by *Cupressus* sp.
Taza	33º 57' 23.00"N 4º 3' 5.00" W	17-07-2014	Mainly *Q. rotundifolia* and some *Pinus* sp.
	33º 43' 16.50" N 4º 15' 38.8"W	16-07-2014	*Q. rotundifolia* and various shrubs.

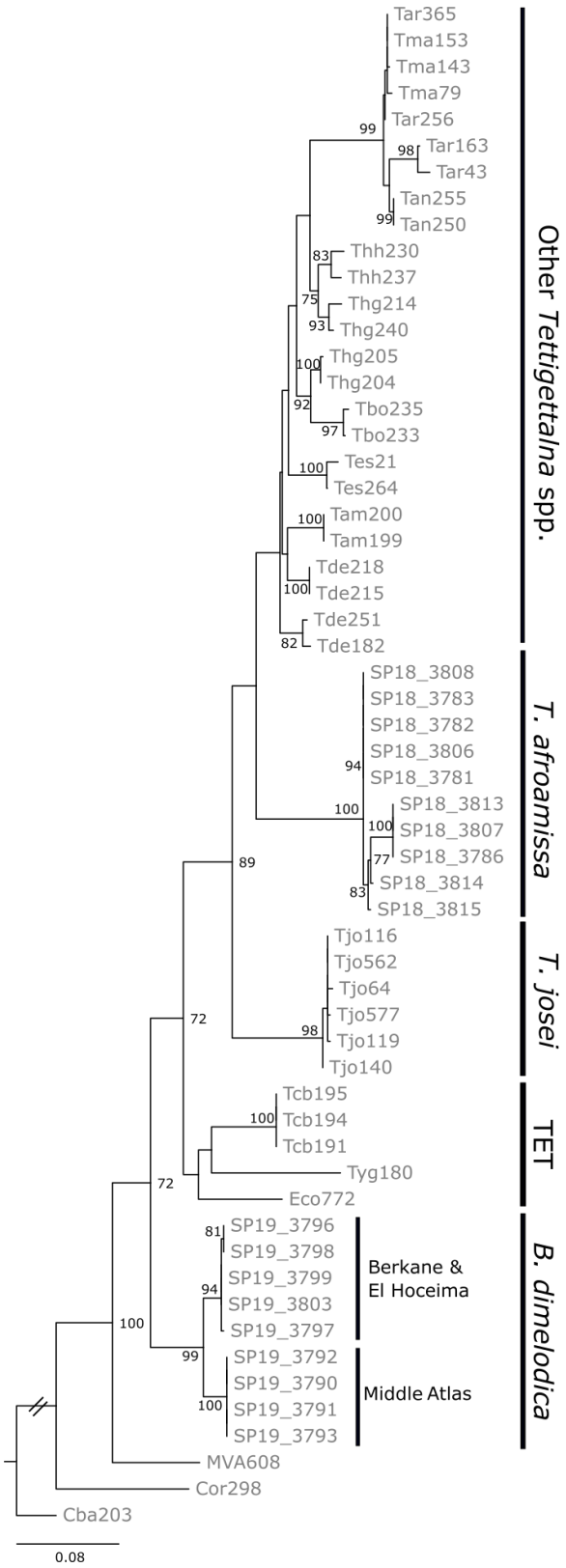

Figure S3. Maximum likelihood phylogenetic tree obtained with Cytochrome C oxidase subunit I mitochondrial DNA of *T. afroamissa* and *B. dimelodica* and with other previous published taxa. Bootstrap values are shown next to branch nodes. TET stands for *Tettigettacula—Euryphara—Tympanistalna* clade. Scale bar represents the number of estimated changes per branch length. *C. barbara* (Cba203) and *C. orni* (Cor298) were set as an outgroup. *T. afroamissa* and *B. dimelodica* taxa IDs are detailed on Table 1.2. Additional taxa details are included on Table S1. Root was truncated with double dash totalling 0.35 changes per branch length.

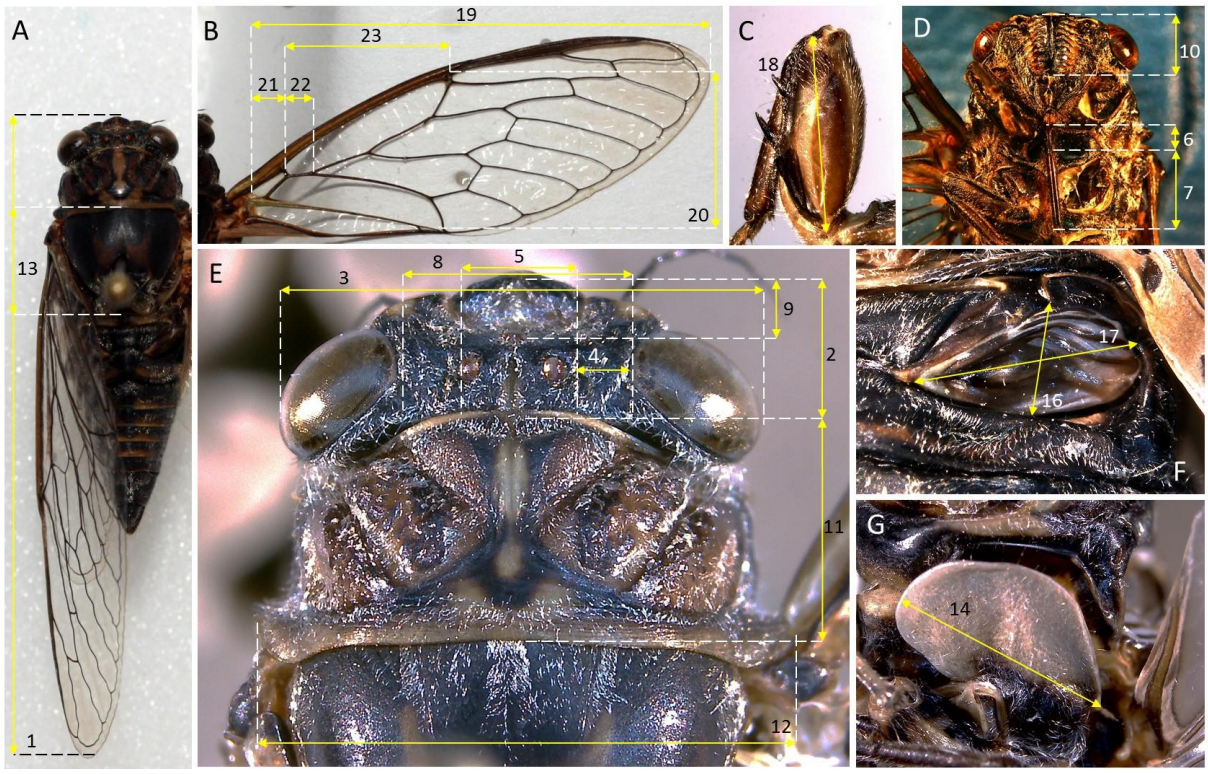

Figure S4. Illustration of the 23 variables of external morphology described on Table 1 (codes used are the same as in Table 1). All images are from paratypical series of *T. afroamissa*. A—Dorsal view; B—Right wing view; C—Right profemur; D—Head and thorax ventral view; E—Head and thorax dorsal view; F—Right tymbal; E—Left operculum.

Figure S5. Image of a *T. afroamissa* sp. nov live male. Notice the olive-green stripe in the pronotum. Image by Eduardo Marabuto.

Figure S6. Image of a live male (left) and a female (right) of *Berberigetta dimelodica* sp. nov. Images by Eduardo Marabuto.

Video S7. Video recording of a male *Berberigetta dimelodica* calling. Note the abdomen tightens during part B of the phrase, resounding as "blowing a raspberry". (available at https://www.youtube.com/watch?v=lYbhnsBYBek).

CHAPTER III

Table S1. Taxa sampling included in our phylogenetic analysis including collection points, codes, GPS coordinates and GenBank accession numbers of previous and of the present study.

					GenBank acession numbers				
Taxa	**Code**	**Country**	**Locality**	**GPS coordinates**	**COI-Lep**	**COI-CTL**	**ATP**	**EF1-α**	**CAL**
T. afroamissa	Taf781	Morocco	Chefchaouane	35,184N; -5,224W	KX582158				
T. afroamissa	Taf782	Morocco	Chefchaouane	35,184N; -5,224W	KX582159				
T. afroamissa	Taf783	Morocco	Chefchaouane	35,184N; -5,224W	KX582160		Taf783	Taf783	Taf783
T. afroamissa	Taf786	Morocco	Afouzar	33,871N; -4,029W	KX582161				
T. afroamissa	Taf806	Morocco	Bni Hadifa	35,03N; -4,164W	KX582162				
T. afroamissa	Taf807	Morocco	Bni Hadifa	35,03N; -4,164W	KX582163		Taf807	Taf807	Ta807
T. afroamissa	Taf808	Morocco	Bni Hadifa	35,03N; -4,164W	KX582164				
T. afroamissa	Taf813	Morocco	Targuist	34,965N; -4,344W	KX582165				
T. afroamissa	Taf814	Morocco	Tizi Tchen	34,929N; -4,492W	KX582166				
T. afroamissa	Taf815	Morocco	Tizi Tchen	34,929N; -4,492W	KX582167		Taf815	Taf815	
T. josei	Tjo106	Portugal	Porches	37,136N; -8,385W	KC807272				
T. josei	Tjo113	Portugal	Porches	37,136N; -8,385W	KF977493				
T. josei	Tjo116	Portugal	Lagoa	37,136N; -8,385W	KC807271	Tjo116	Tjo116	Tjo116	Tjo116
T. josei	Tjo119	Portugal	Budens	37,079N; -8,837W	KF977491				
T. josei	Tjo120	Portugal	Budens	37,073N; -8,812W	KC807267				
T. josei	Tjo121	Portugal	Budens	37,073N; -8,812W	KC807268	Tjo121			
T. josei	Tjo122	Portugal	Budens	37,073N; -8,812W	KF977492				
T. josei	Tjo135	Portugal	Castro Marim	37,186N; -7,484W	KC807270	Tjo135	Tjo135	Tjo135	Tjo135
T. josei	Tjo137	Portugal	Castro Marim	37,186N; -7,484W	KF977502	Tjo137			
T. josei	Tjo140	Portugal	Castro Marim	37,186N; -7,484W	KC807269				
T. josei	Tjo141	Portugal	Moncarapacho	37,078N; -7,821W	KF977499	Tjo141			
T. josei	Tjo145	Portugal	S. Brás de Alportel	37,137N; -7,848W	KF977498				
T. josei	Tjo154	Portugal	Moncarapacho	37,078N; -7,821W	KF977500				
T. josei	Tjo159	Portugal	Tavira	37,134N; -7,635W	KF977501				
T. josei	Tjo309	Portugal	Quinta do Lago	37,06N; -8,021W	KF977495				
T. josei	Tjo355	Portugal	Quinta do Lago	37,06N; -8,021W	KF977496				
T. josei	Tjo362	Portugal	Quinta do Lago	37,06N; -8,021W	KF977497				
T. josei	Tjo557	Spain	Cartaya	37,261N; -7,129W	KF977503				

Table S1. Continued

					GenBank acession numbers				
Taxa	**Code**	**Country**	**Locality**	**GPS coordinates**	**COI-Lep**	**COI-CTL**	**ATP**	**EF1-α**	**CAL**
T. josei	Tjo562	Spain	Cartaya	37,261N; -7,129W	KF977504				
T. josei	Tjo577	Spain	Cartaya	37,234N; -7,066W	KF977505				
T. josei	Tjo58	Portugal	Vale Judeu	37,128N; -8,093W	KC807273				
T. josei	Tjo64	Portugal	Vale Judeu	37,128N; -8,093W	KC807274	Tjo64			
T. josei	Tjo66	Portugal	Vale Judeu	37,128N; -8,093W	KF977494			Tjo66	
T. josei	Tjo765	Portugal	Armação de Pêra	37,105N; -8,361W	Tjo765				
T. josei	Tjo766	Portugal	Armação de Pêra	37,105N; -8,361W	Tjo766				
T. estrelae	Tes21	Portugal	Braga	41,582N; -8,321W	KC807263	Tes21	Tes21	Tes21	Tes21
T. estrelae	Tes20	Portugal	Braga	41,582N; -8,321W	Tes20				
T. estrelae	Tes27	Portugal	Braga	41,582N; -8,321W	KC807261				
T. estrelae	Tes34	Portugal	Braga	41,582N; -8,321W	Tes34	Tes34		Tes34	
T. estrelae	Tes41	Portugal	Braga	41,582N; -8,321W	KC807264	Tes41		Tes41	
T. estrelae	Tes47	Portugal	Amarante	41,243N; -8,034W	KC807262				
T. estrelae	Tes49	Portugal	Amarante	41,243N; -8,034W	Tes49				
T. estrelae	Tes50	Portugal	Amarante	41,244N; -8,034W	KC807260				
T. estrelae	Tes51	Portugal	Amarante	41,243N; -8,034W	KC807259				
T. estrelae	Tes55	Portugal	Amarante	41,243N; -8,034W	KC807266				
T. estrelae	Tes264	Portugal	Serra Estrela	40,355N; -7,44W	KC807265	Tes264	Tes264	Tes264	Tes264
T. galantei type 1	Thg206	Spain	Capileira, Sierra Nevada	36,957N; -3,353W	KC807285				
T. galantei type 1	Thg207	Spain	Capileira, Sierra Nevada	36,957N; -3,353W	KC807282	Thg207	Thg207	Thg207	
T. galantei type 1	Thg209	Spain	Capileira, Sierra Nevada	36,956N; -3,347W	KC807289				
T. galantei type 1	Thg210	Spain	Capileira, Sierra Nevada	36,956N; -3,347W	KC807291				
T. galantei type 1	Thg212	Spain	Capileira, Sierra Nevada	36,956N; -3,347W	KC807284		Thg212		Thg212
T. galantei type 1	Thg213	Spain	Capileira, Sierra Nevada	36,963N; -3,341W	KC807290				
T. galantei type 1	Thg214	Spain	Capileira, Sierra Nevada	36,963N; -3,341W	KC807286	Thg214	Thg214	Thg214	Thg214
T. galantei type 1	Thg240	Spain	Laroles, Sierra Nevada	37,049N; -3,017W	KC807287				Thg240
T. galantei type 1	Thg241	Spain	Laroles, Sierra Nevada	37,049N; -3,017W	KC807283				
T. galantei type 1	Thg242	Spain	Laroles, Sierra Nevada	37,049N; -3,017W	KC807288				
T. galantei type 1	Thg691	Spain	Narila, Sierra Nevada	36,96N; -3,175W	Thg691	Thg691	Thg691	Thg691	
T. galantei type 1	Thg704	Spain	Rubite	36,822N; -3,335W	Thg704			Thg704	Thg704

Table S1. Continued

					GenBank acession numbers				
Taxa	**Code**	**Country**	**Locality**	**GPS coordinates**	**COI-Lep**	**COI-CTL**	**ATP**	**EF1-α**	**CAL**
T. h. helianthemi	Thh222	Spain	Cabo da Gata	36,838N; -2,293W	KC807292	Thh222	Thh222	Thh222	Thh222
T. h. helianthemi	Thh224	Spain	Cabo da Gata	36,838N; -2,293W	KC807296				
T. h. helianthemi	Thh226	Spain	Cabo da Gata	36,838N; -2,293W	KC807294				
T. h. helianthemi	Thh230	Spain	Cabo da Gata	36,838N; -2,293W	KC807297				Thh230
T. h. helianthemi	Thh236	Spain	Vera	37,213N; -1,9W	KC807295	Thh236	Thh236	Thh236	Thh236
T. h. helianthemi	Thh237	Spain	Vera	37,213N; -1,9W	KC807293				
T. h. helianthemi	Thh238	Spain	Vera	37,213N; -1,9W	KC807298			Thh238	Thh238
T. h. helianthemi	Thh630	Spain	Sierra Filabres, north slope	37,366N; -2,732W	Thh630	Thh630	Thh630	Thh630	Thh630
T. h. helianthemi	Thh645	Spain	Cantoria, Sierra Filabres	37,345N; -2,199W	Thh645	Thh645	Thh645	Thh645	Thh645
T. galantei type 2	T2g201	Spain	Lanjarón, Sierra Nevada	36,923N; -3,531W	KC807279	Thg201	T2g201	T2g201	T2g201
T. galantei type 2	T2g204	Spain	Lanjarón, Sierra Nevada	36,916N; -3,504W	KC807281				
T. galantei type 2	T2g205	Spain	Lanjarón, Sierra Nevada	36,916N; -3,504W	KC807280				
T. galantei type 2	T2g594	Spain	W Lanjarón, Sierra Nevada,	36,923N; -3,531W	T2g594	Thg594	T2g594	T2g594	T2g594
T. galantei type 2	T2g617	Spain	Pinos Genil, Sierra Nevada	37,138N; -3,476W	T2g617		T2g617	T2g617	T2g617
T. galantei type 2	T2g623	Spain	Pinos Genil, Sierra Nevada	37,138N; -3,476W	T2g623	Thg623	T2g623	T2g623	T2g623
T. galantei type 2	T2g622	Spain	Pinos Genil, Sierra Nevada	37,138N; -3,476W	T2g622			T2g622	T2g622
T. boulardi	Tbo233	Spain	Campico de los López, Murcia	37,583N; -1,571W	KC807276	Tbo233	Tbo233	Tbo233	Tbo233
T. boulardi	Tbo235	Spain	Campico de los López, Murcia	37,583N; -1,571W	KC807275	Tbo235	Tbo235	Tbo235	Tbo235
T. armandi	Tam199	Spain	Gibraltar	36,188N; -5,359W	KC807277	Tam199	Tam199	Tam199	
T. armandi	Tam200	Spain	Gibraltar	36,188N; -5,359W	KC807278	Tam200	Tam200	Tam200	Tam200
T. armandi	Tam712	Spain	Estella del Marques	36,685N; -6,063W	Tam712	Tam712	Tam712	Tam712	
T. armandi	Tam713	Spain	Estella del Marques	36,685N; -6,063W	Tam713	Tam713	Tam713	Tam713	Tam713
T. armandi	Tam716	Spain	Estella del Marques	36,685N; -6,063W	Tam716	Tam716	Tam716		
T. defauti	Tde182	Spain	Puerto del Viento, Ronda	36,787N; -5,053W	KC807305	Tde182	Tde182	Tde182	Tde182
T. defauti	Tde183	Spain	Puerto del Viento, Ronda	36,787N; -5,053W	KC807307				
T. defauti	Tde185	Spain	Puerto del Viento, Ronda	36,787N; -5,053W	KC807309				

Table S1. Continued

					GenBank acession numbers				
Taxa	**Code**	**Country**	**Locality**	**GPS coordinates**	**COI-Lep**	**COI-CTL**	**ATP**	**EF1-α**	**CAL**
T. defauti	Tde188	Spain	Puerto del Viento, Ronda	36,787N; -5,053W	KC807308	Tde188	Tde188	Tde188	
T. defauti	Tde215	Spain	Sierra Nevada	37,138N; -3,468W	KC807310	Tde215	Tde215	Tde215	Tde215
T. defauti	Tde218	Spain	Sierra Nevada	37,138N; -3,468W	KC807304			Tde218	
T. defauti	Tde251	Spain	Zagra	37,283N; -4,234W	KC807306	Tde251	Tde251	Tde251	
T. defauti	Tde598	Spain	Sierra Nevada	37,138N; -3,476W	Tde598				
T. defauti	Tde600	Spain	Sierra Nevada	37,138N; -3,476W	Tde600	Tde600	Tde600	Tde600	
T. defauti	Tde601	Spain	Sierra Nevada	37,138N; -3,476W	Tde601				
T. defauti	Tde602	Spain	Sierra Nevada	37,138N; -3,476W	Tde602				
T. defauti	Tde603	Spain	Sierra Nevada	37,138N; -3,476W	Tde603				
T. defauti	Tde604	Spain	Sierra Nevada	37,138N; -3,476W	Tde604				
T. aneabi	Tan244	Spain	Granada	37,256N; -3,482W	KC807300			Tan244	Tan244
T. aneabi	Tan250	Spain	Zagra	37,283N; -4,234W	KC807301				
T. aneabi	Tan253	Spain	Zagra	37,283N; -4,234W	KC807303				
T. aneabi	Tan254	Spain	Zagra	37,283N; -4,234W	KC807302				
T. aneabi	Tan255	Spain	Zagra	37,283N; -4,234W	KC807309	Tan255	Tan255	Tan255	Tan255
T. aneabi	Tan709	Spain	Frailes	37,508N; -3,832W	Tan709	Tan709	Tan709	Tan709	
T. aneabi	Tan711	Spain	Estepa	37,366N; -4,818W	Tan711	Tan711	Tan711	Tan711	Tan711
T. mariae	Tma143	Portugal	Vale do Lobo	37,061N; -8,061W	KC807253				
T. mariae	Tma144	Portugal	Vale do Lobo	37,061N; -8,061W	KC807249	Tma144			
T. mariae	Tma147	Portugal	Vale do Lobo	37,061N; -8,061W	KC807255	Tma147	Tma147	Tma147	Tma147
T. mariae	Tma151	Portugal	Vale do Lobo	37,061N; -8,061W	KC807250		Tma151		Tma151
T. mariae	Tma153	Portugal	Vale do Lobo	37,061N; -8,061W	KC807257				
T. mariae	Tma378	Spain	Cartaya	37,262N; -7,13W	Tma78				Tma378
T. mariae	Tma67	Portugal	Vale Judeu	37,106N; -8,095W	KC807258				Tma67
T. mariae	Tma68	Portugal	Vale Judeu	37,106N; -8,095W	KC807251	Tma68	Tma68	Tma68	Tma68
T. mariae	Tma71	Portugal	Vale Judeu	37,106N; -8,095W	KC807254	Tma71	Tma71	Tma71	Tma71
T. mariae	Tma720	Spain	Huelva	37,226N; -7,035W	Tma720	Tma720	Tma720	Tma720	
T. mariae	Tma722	Spain	Huelva	37,226N; -7,035W	Tma722	Tma722	Tma722	Tma722	Tma722
T. mariae	Tma74	Portugal	Vale Judeu	37,106N; -8,095W	KC807252				
T. mariae	Tma79	Portugal	Vale Judeu	37,106N; -8,095W	KC807256				Tma79
T. argentata North clade	Tar3	Portugal	Sesimbra	38,447N; -9,086W	KC807243				

Table S1. Continued

					GenBank acession numbers				
Taxa	**Code**	**Country**	**Locality**	**GPS coordinates**	**COI-Lep**	**COI-CTL**	**ATP**	**EF1-α**	**CAL**
T. argentata North clade	Tar5	Portugal	Sesimbra	38,443N; -9,089W	KC807245				
T. argentata North clade	Tar10	Portugal	Sesimbra	38,443N; -9,089W	KC807244	Tar10	Tar10		
T. argentata North clade	Tar12	Portugal	Sesimbra	38,445N; -9,091W	Tar12	Tar12		Tar12	
T. argentata North clade	Tar25	Portugal	Braga	41,582N; -8,321W	KC807230	Tar25			
T. argentata North clade	Tar37	Portugal	Braga	41,582N; -8,321W	Tar37	Tar37	Tar37	Tar37	
T. argentata North clade	Tar43	Portugal	Braga	41,582N; -8,321W	KC807229	Tar43		Tar43	
T. argentata North clade	Tar162	France	Bouzigues	43,455N; 3,657W	KC807233				
T. argentata North clade	Tar163	France	Narbonne	43,155N; 2,964W	KC807234				Tar163
T. argentata North clade	Tar299	Portugal	Serra d'Aire & Candeeiros	39,456N; -8,8W	Tar299	Tar299	Tar299	Tar299	Tar299
T. argentata North clade	Tar383	Italy	Benne, Piedmont	45,281N; 7,541W	KC807237				
T. argentata North clade	Tar385	Italy	Serradica, Marche	43,278N; 12,847W	KC807236	Tar385	Tar385	Tar385	Tar385
T. argentata North clade	Tar387	Italy	Cella, Lombardy	44,78N; 9,187W	KC807235				
T. argentata North clade	Tar747	Italy	Pietrafitta, Calabria	39,249N; 16,34W	Tar747	Tar747	Tar747	Tar747	Tar747
T. argentata South clade	Tar17	Portugal	Portel	38,303N; -7,709W	KC807238				
T. argentata South clade	Tar93	Portugal	Portel	38,303N; -7,709W	KC807239				
T. argentata South clade	Tar97	Portugal	Portel	38,303N; -7,709W	Tar97			Tar97	
T. argentata South clade	Tar100	Portugal	Portel	38,303N; -7,709W	KC807248				
T. argentata South clade	Tar123	Portugal	S. Bartolomeu de Messines	37,257N; -8,297W	KC807240	Tar123	Tar123	Tar123	
T. argentata South clade	Tar126	Portugal	S. Bartolomeu de Messines	37,257N; -8,297W	KC807242				
T. argentata South clade	Tar127	Portugal	S. Bartolomeu de Messines	37,257N; -8,297W	Tar127			Tar127	
T. argentata South clade	Tar130	Portugal	S. Bartolomeu de Messines	37,257N; -8,297W	KC807241	Tar130			
T. argentata South clade	Tar161	Portugal	Moncarapacho	37,078N; -7,821W	Tar161	Tar161	Tar161	Tar161	Tar161
T. argentata South clade	Tar256	Spain	Espiel	38,194N; -5,027W	KC807232	Tar256	Tar256	Tar256	Tar256
T. argentata South clade	Tar258	Spain	Espiel	38,194N; -5,027W	KC807231				Tar258
T. argentata South clade	Tar360	Portugal	Mata Lobo	37,08N; -7,949W	Tar360		Tar360		Tar360
T. argentata South clade	Tar365	Spain	Ayamonte	37,276N; -7,342W	KC807246	Tar365	Tar365	Tar365	Tar365
T. argentata South clade	Tar369	Spain	Ayamonte	37,276N; -7,342W	KC807247				
T. argentata South clade	Tar649	Spain	Oria	37,497N; -2,292W	Tar649	Tar649	Tar649	Tar649	Tar649
T. argentata Central clade	Tar526	Spain	Almaraz	39,76N; -5,735W	Tar526	Tar526	Tar526	Tar526	Tar526

Table S1. Continued

					GenBank acession numbers				
Taxa	**Code**	**Country**	**Locality**	**GPS coordinates**	**COI-Lep**	**COI-CTL**	**ATP**	**EF1-α**	**CAL**
T. argentata Central clade	Tar547	Spain	Albarracín	40,425N; -1,381W	Tar547	Tar547	Tar547	Tar547	Tar547
T. argentata Catalonia clade	Tar850	Spain	Catalonia	42,069N; 3,107W	Tar850	Tar850	Tar850	Tar850	Tar850
T. argentata Catalonia clade	Tar754	Spain	Alicante	38,634N; -0,523W	Tar754		Tar754	Tar754	
Cicada barbara	Cba203	Spain	Lanjarón, Sierra Nevada	36,916N; -3,504W	KC807317	Cba203			
Cicada orni	Cor298	Spain	Serra d'Aire & Candeeiros	39,455N; -8,752W	KC807318				
Hilaphura varipes	Hva608	Spain	Pinos Genil, Sierra Nevada	37,138N; -3,476W	KX582168	Hva608	Hva608	Hva608	
Maoricicada cassiope	Mcass14	New Zealand	-	-					DQ178585

Table S2. List of primers used, forward and reverser primer sequences and codes, including source references and annealing temperatures.

Gene	Primers	Primer sequence (from 5' to 3')	References	Product length (bp)	$T_{annealing}$ (ºC)
Mitochondrial loci					
Cytochrome oxidase I (COI-Lep) 5' region	LepF	ATT CAA CCA ATC ATA AAG ATA TTG G	Hajibabaei *et al.* (2006)	650	45
	LepR	TAA ACT TCT GGA TGT CCA AAA AAT CA	Hajibabaei *et al.* (2006)		
Cytochrome oxidase I (COI-CTL) 3' region	C1-J-2195	TTG ATT TTT TGG TCA TCC AGA AGT	Simon *et al.* (1994)	850	53
	TL2-N-3014	TCC AAT GCA CTA ATC TGC CAT ATT A	Simon *et al.* (1994)		
ATP synthetase A6/A8	TK-J-3799_for	GGC TGA AAG TAA GTA ATG GTC TCT	Buckley *et al.* (2001)	800	57
	A6A8_rev	ATG RCC AGC AAT TAT ATT AGC TG	modified from Marshall *et al.* (2008)		
Nuclear loci					
Elongation Factor-1α	EF1a-97_for	ACG CCC CTG GAC ATA GAG AT	Buckley *et al.* (2006)	600	60
	EF1a-189_rev	CAA CCT GAG ATT GGC ACA AA	Buckley *et al.* (2006)		
Calmodulin	Cal-60_F	AAC GAA GTA GAT GCC GAT GG	Buckley *et al.* (2006)	650	55
	Cal-72_R	GTG TCC TTC ATT TTN CKT GCC ATC AT	Buckley *et al.* (2006)		

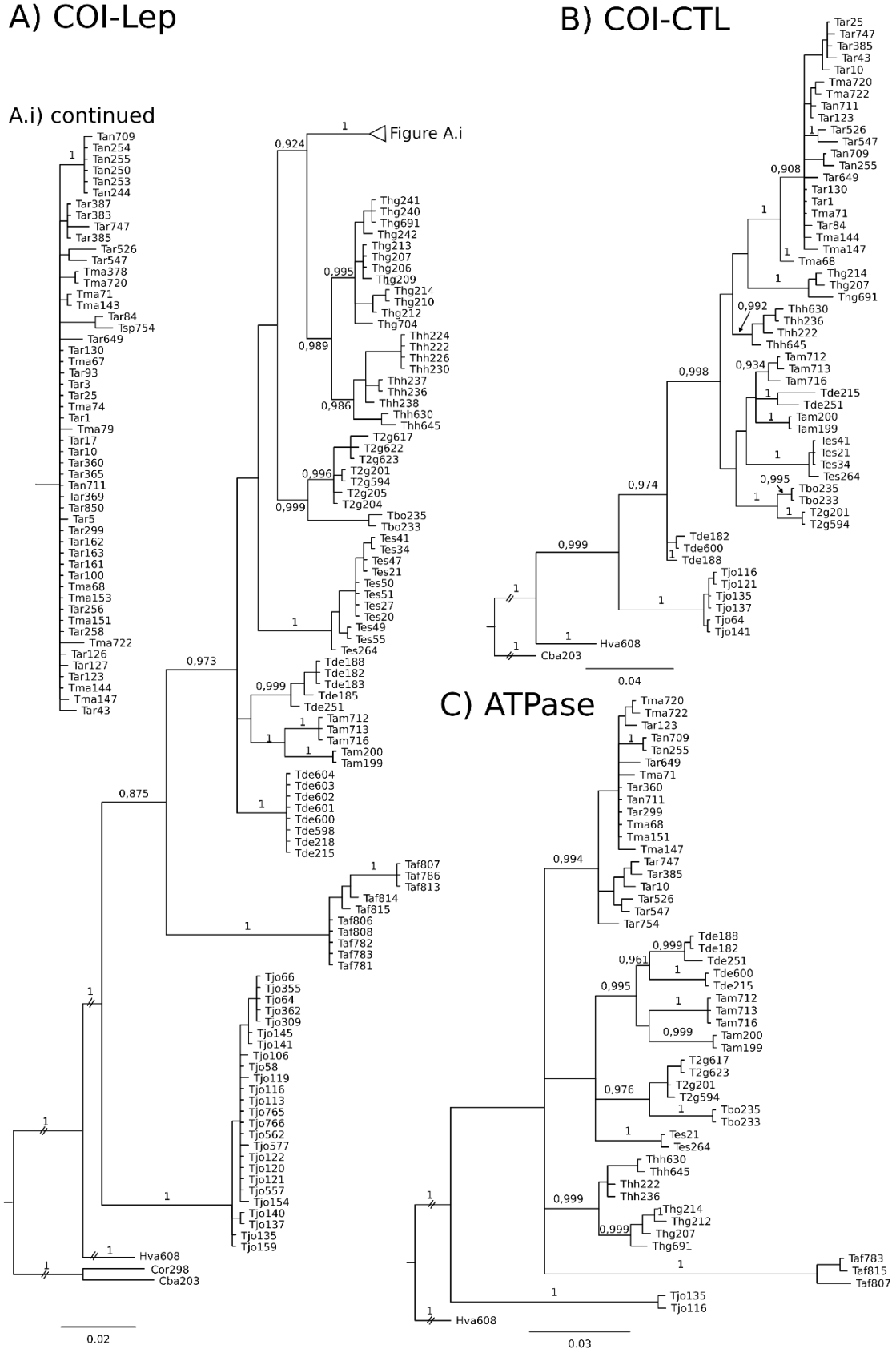

Figure S3. Individual Bayesian inference phylogenetic trees. A) COI-Lep; **B)** COI-CTL; **C)** ATPase; **D)** Elongation Factor 1-α; **E)** Calmodulin. Posterior probabilities are shown next to branch nodes. Scale bar represents the number of estimated changes per branch length. *C. barbara* (Cba203) and *C. orni* (Cor298) were set as outgroup for A) and B). *H. varipes* (Hva608) was set as outgroup for C) and D). *M. cassiope* was set as outgroup for E). Additional taxa details are included on supplementary information Table S1. Root was truncated with double dashes. Some trees were collapsed due to sampling volume and the remaining samples shown under .i) bullets.

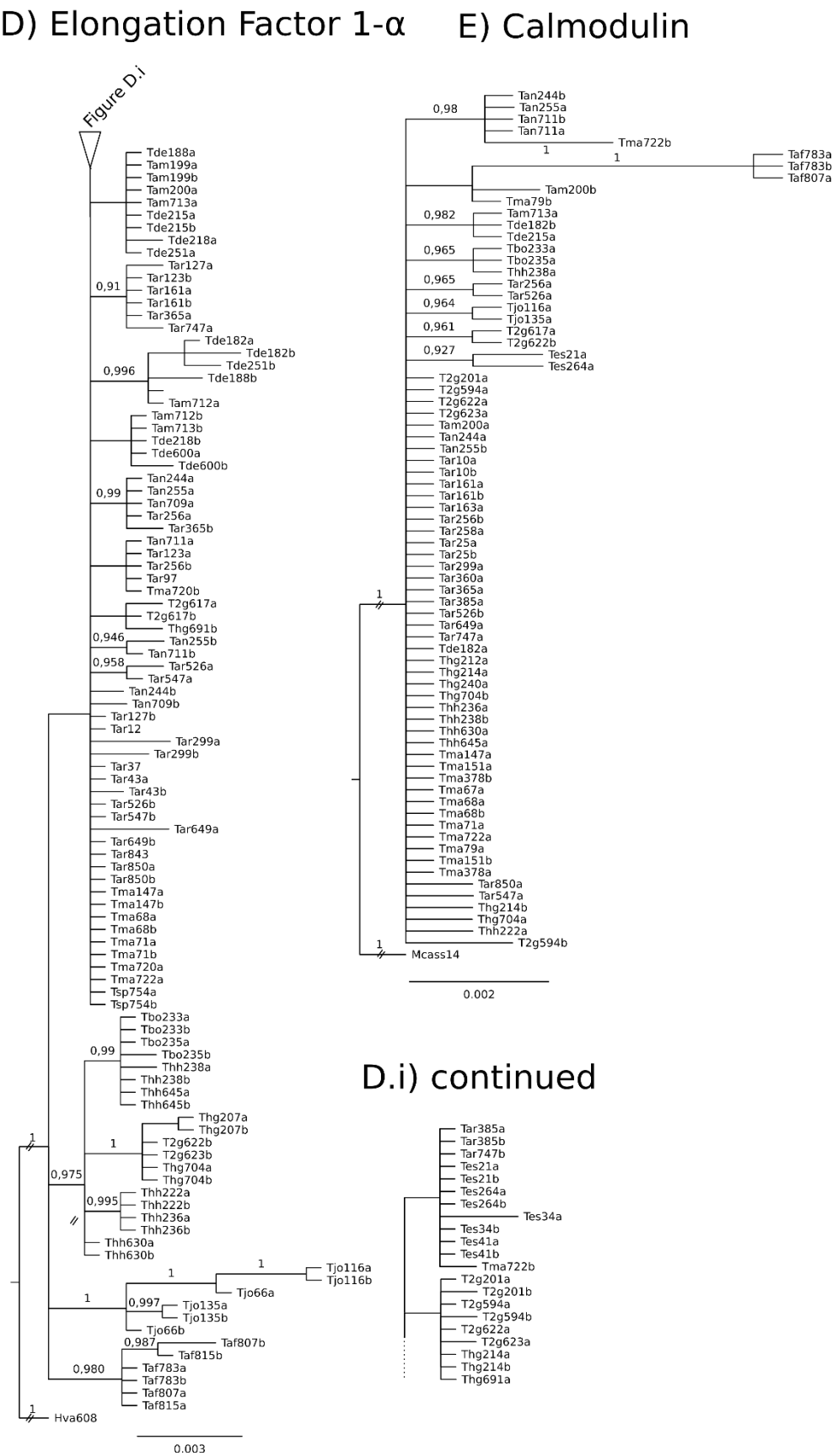

Figure S3. Individual Bayesian inference phylogenetic trees. Continued.

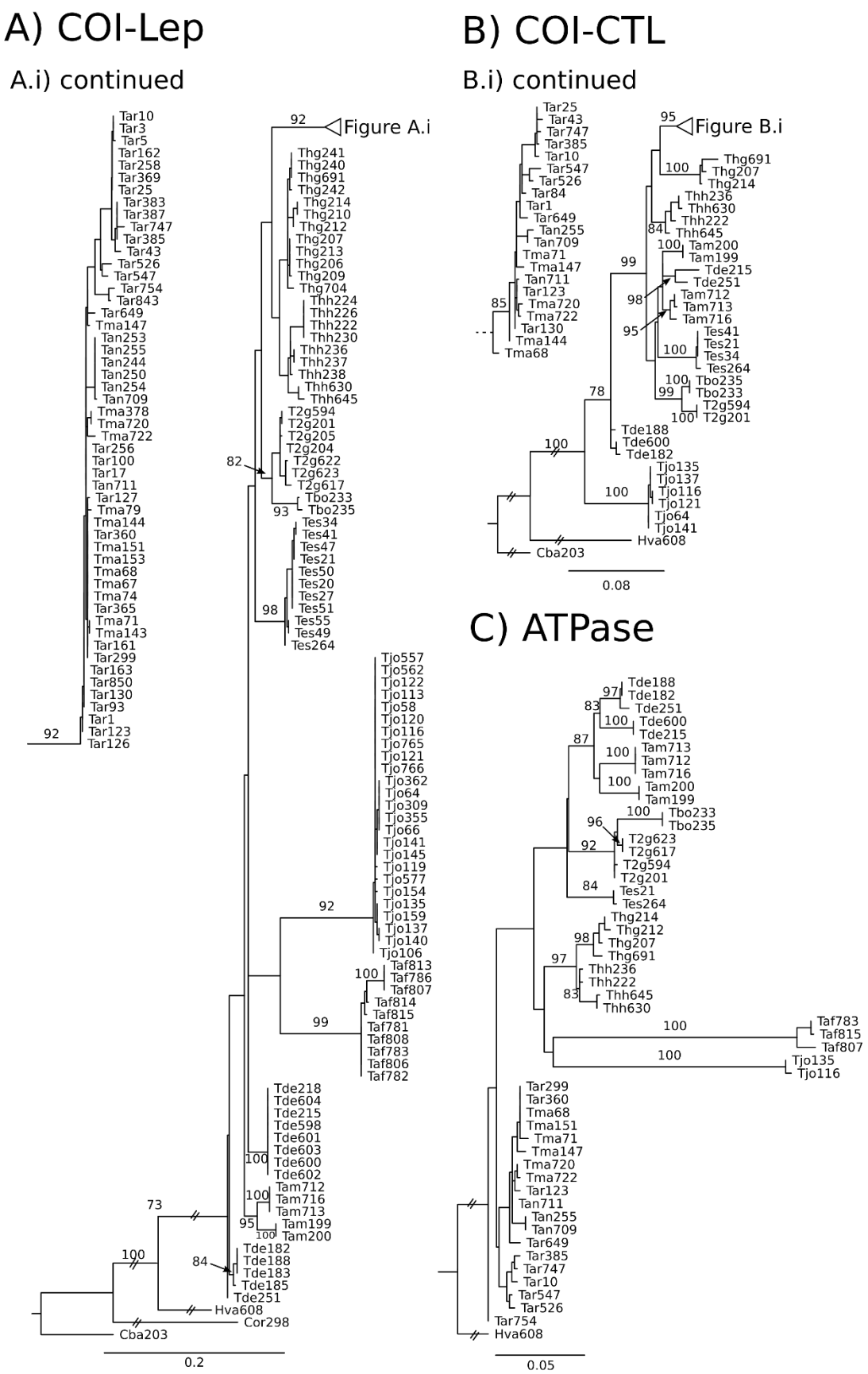

Figure S4. Individual maximum likelihood phylogenetic trees. A) COI-Lep; B) COI-CTL; C) ATPase; D) Elongation Factor 1-α; E) Calmodulin. Bootstrap support is shown next to branch nodes. Scale bar represents the number of estimated changes per branch length. *C. barbara* (Cba203) and *C. orni* (Cor298) were set as outgroup for A) and B). *H. varipes* (Hva608) was set as outgroup for C) and D). *M. cassiope* was set as outgroup for E). Additional taxa details are included on supplementary information Table S1. Root was truncated with double dashes. Some trees were collapsed due to sampling volume and the remaining samples shown under .i) bullets.

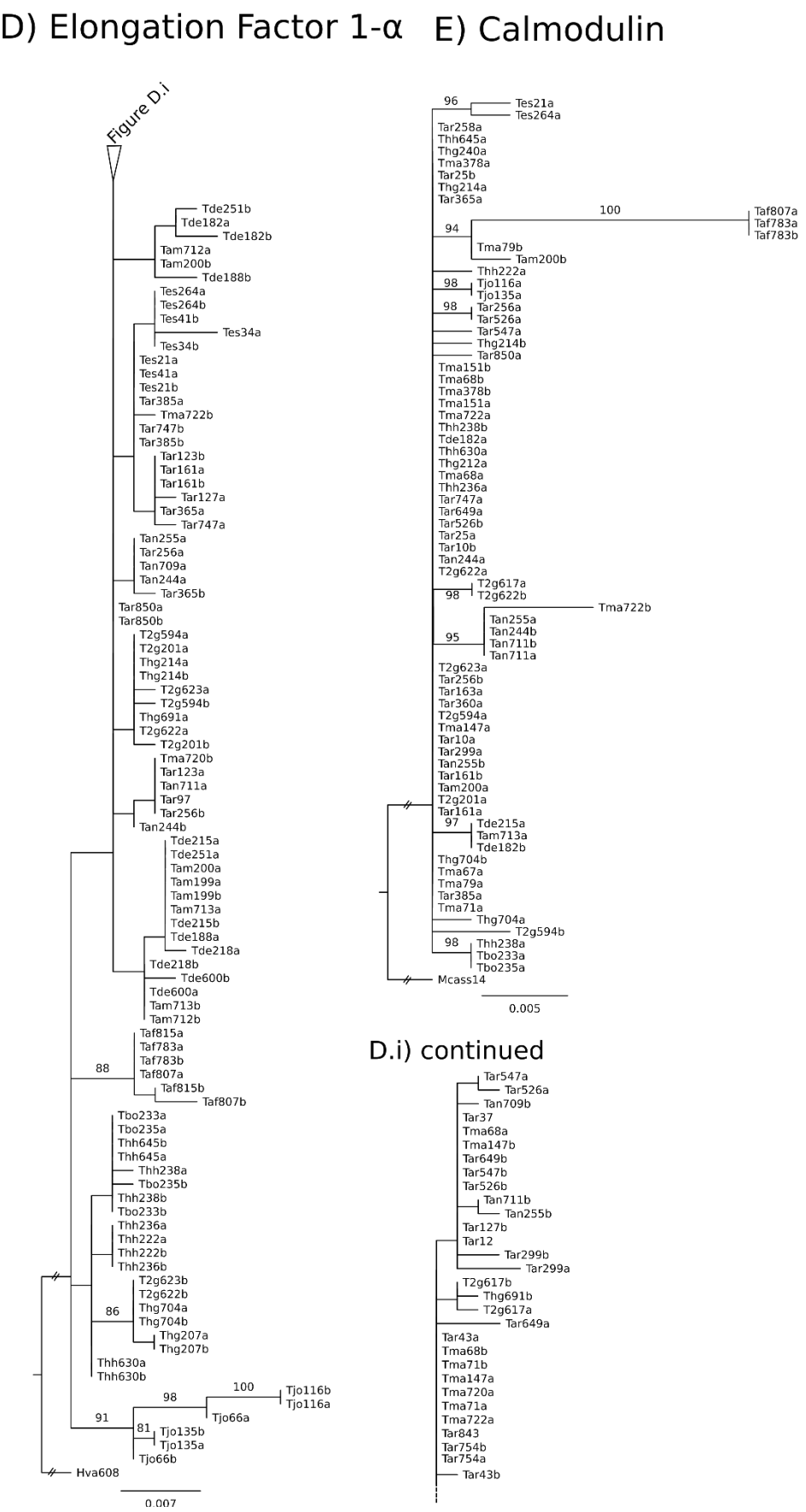

Figure S4. Individual maximum likelihood phylogenetic trees. Continued