Bats out of Africa: disentangling the systematic position of bats in Cabo Verde

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Todas as correcções determinadas pelo júri, e só essas, foram efectuadas.

O Presidente do Júri,

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Abstract

Cabo Verde Archipelago presents one of the largest knowledge gaps in the distribution and taxonomy of bats in the world. Some data published in the 1960's and 1980's indicate that there are five species in the archipelago. The majority are classified as European taxa, and considered with recent colonization and reduced distribution. The species were identified as the naked-rumped tomb bat *Taphozous nudiventris* Cretzschmar 1830; the Savi's pipistrelle *Hypsugo savii* Bonaparte 1837; the Kuhl's pipistrelle *Pipistrellus kuhlii* (Kuhl 1817); the gray big-eared bat *Plecotus austriacus* (Fischer 1829) and the Schreiber's bent-winged bat *Miniopterus schreibersii* (Kuhl 1817). There is also an observation of the African straw-coloured fruit-bat *Eidolon helvum* (Kerr 1792), which is probably a vagrant species, and an indeterminate molossid species.

An integrative taxonomy was conducted in order to revise the systematic position and distribution of the bats from Cabo Verde based on molecular markers, morphological measurements, and ecological data. The *Hypsugo* bat appear to be a complex of sympatric and cryptic species widespread throughout Europe and North Africa. The results showed that *Hypsugo* from Cabo Verde is close related to those from Canary Islands, presenting unique haplotype structure both at mitochondrial and nuclear level. The identification of distinct lineages based on morphological and acoustic data is a challenge because there is a lack of a modern morphological description and the similarities between the ecolocation calls. However, our results demonstrated values expected for European taxa. Genetic studies and scarce morphological data suggested the *Hypsugo* from Canary Islands, North Africa, Sicily, Sardinia, and Montecristo Islands might be considered *Hypsugo cf. darwinii* (Tomes 1859), which taxonomic status is under debate. The results also expanded the distribution of *T. nudiventris* for Fogo Island through pellets and acoustic identification. These species showed unique haplotypes and might be considered a unique Evolutionarily Significant Unit for conservation planning, even though its native status is still to be proven. Regarding *M. schreibersii*, it shares the same haplotype with specimens from Europe, North Africa and Western Asia. More studies and fieldwork need to follow to access the systematic of these and other species in the Archipelago.

Keywords

Conservation, *Hypsugo savii*, *Miniopterus schereibersii*, oceanic islands, Phylogenetics, remote areas, *Taphozous nudiventris*, Taxonomy.

Resumo

O Arquipélago de Cabo Verde apresenta uma das maiores lacunas de conhecimento na distribuição e taxonomia dos morcegos no mundo. Alguns estudos publicados na década de 1960 e 1980 indicam que existem cinco espécies no arquipélago. A maioria está classificada como taxa europeus, e são consideradas de recente colonização e distribuição geográfica reduzida. As espécies foram identificadas como *Taphozous nudiventris* Cretzschmar 1830; *Hypsugo savii* Bonaparte 1837; *Pipistrellus kuhlii* (Kuhl 1817); *Plecotus austriacus* (Fischer 1829) e *Miniopterus schreibersii* (Kuhl 1817). Há também uma observação do morcego africano *Eidolon helvum* (Kerr 1792) e um molossídeo indeterminado que são provavelmente espécies vagantes.

Um estudo de taxonomia integrada foi conduzido para rever a posição sistemática e distribuição geográfica dos morcegos de Cabo Verde com base em marcadores moleculares, medidas morfológicas e dados ecológicos. O género *Hypsugo* parece ser um complexo de espécies simpátricas distribuídas por toda Europa e norte da África. Os resultados mostram que o *Hypsugo* de Cabo Verde é proximamente relacionado com indivíduos das Ilhas Canárias, apresentando uma estrutura haplotípica única, tanto ao nível de DNA mitocondrial quanto nuclear. A identificação de linhagens distintas com base em dados morfológicos e acústicos é um desafio, pois não existe uma decrição morfológica moderna e os sons de ecolocalização são semelhantes. No entanto, nossos resultados monstram valores esperados para espécies européias. Estudos genéticos e escassos dados morfológicos sugerem que *Hypsugo* das Ilhas Canárias, norte da África, e das ilhas de Sicília, Sardenha e Montecristo pode ser considerado *Hypsugo cf. darwinii* (Tomes 1859), cuja taxonomia ainda está em debate. Os resultados também ampliam a distribuição geográfica de *T. nudiventris* no arquipélago, sendo registrado para ilha do Fogo através de identificação acústica e DNA de amostras fecais. Essas espécies apresentam haplotipos únicos e podem ser consideradas uma unidade evolucionária significativa para futuros estudos de conservação, mesmo que a condição de nativo ainda deva ser comprovada. A respeito de *M. schreibersii*, esta espécie compartilha o mesmo haplotipo com espécimens da Europe, norte da África e oeste da Ásia. Mais estudos e trabalhos de campo precisam ser feitos para acessar a sistemática dessas e de outras espécies no arquipélago.

Palavras-chave

áreas remotas, Conservação, Filogenética, *Hypsugo savii*, ilhas oceânicas, *Miniopterus schereibersii*, *Taphozous nudiventris*, Taxonomia.

List of tables

List of figures

List of Abbreviations

BEAST **–** Bayesian Evolutionary Analysis by Sampling Trees

BLAST **–** Basic Local Alignment Search Tool

bp **–** Base pairs

CF **–** Constant frequency

cyt *b* **–** Cytochrome b

D1 **–** Thumb

D3 **–** Third finger

D5 **–** Fifth finger

DNA **–** Deoxyribonucleic Acid

DUR **–** duration

ESU **–** Evolutionarily Significant Unit

FA **–** Forearm

FM **–** Frequency Modulation

FME **–** Frequency of maximum energy

HF **–** Hind foot

IPI **–** inter-pulse intervals

IUCN **–** International Union for Conservation of Nature

kHz **–** Kilohertz

LF **–** low frequency

Ma **–** Million years ago

MCSNG **–** Museo Civico di Storia Naturale, di Genova, Italy

min **–** Minutes

mm **–** Milimeters

MNHN **–** Muséum National d'Histoire Naturelle, Paris

ms **–** Mili seconds

mtDNA **–** Mitochondrial DNA

My **–** Million years

MZS **–** Museo Zoologico de La Specola, Florence, Italy

NCBI **–** National Center of Biotechnology Information

ND1 **–** NADH-ubiquinone oxidoreductase chain 1

nDNA **–** Nuclear DNA

P3.2 **–** Second phalange of the third finger

P3.3 **–** Third phalange of the third finger

P4.1 **–** First phalange of the fourth finger

P4.2 **–** Second phalange of the fourth finger

PCR **–** Polymerase Chain Reaction

qCF **–** Quasi Constant Frequency

RAG2 **–** Recombination Activating Gene 2

rpm **–** Rotation per minute

rRNA **–** Ribosomal Ribonucleic Acid

s **–** Seconds

SECEMU **–** Spanish Association for the Conservation and Research of Bats

Tib **–** Lower leg

µl **–** microliters

Table of Contents

Introduction

Biodiversity loss is one of the major environmental problems, threatening valuable ecosystem services (Koh *et al*., 2004). Currently, records of extinction have been documented and the world is losing species faster than would be expected from the fossil record (Pimm & Raven, 2000; Thomas *et al*., 2004), suggesting that a sixth mass extinction may be happening (Barnosky *et al*., 2011; Costello *et al*., 2013). In order to have an accurate picture of the biodiversity loss, accurate species inventories and their distribution are needed (Bini *et al*., 2006; Meyer *et al*., 2015). The assessment of global biodiversity is a challenge because most species were still not formally described and the geographical distribution of most species are poorly understood (Bini *et al*., 2006). In fact, the lack of knowledge regarding species richness, abundance and distribution has always been considered an obvious problem for conservation planning, usually referred as the Linnean and Wallacean shortfalls (Bini *et al*., 2006; Brito, 2010; Diniz-Filho *et al*., 2013).

Given the limited resources and urgency for biodiversity rescue, a rapid assessment method is required and the species inventories need to be designed around the use of effective sampling and estimation procedures (Colwell & Coddington, 1994). Phylogenetic analysis has a huge impact on the understanding of relationships among lineages, adding power and robustness to biodiversity assessment (Diniz-Filho *et al*., 2013). It helps to predict where biodiversity hotspots may be located, by informing how many distinct lineages an area may hold, and identifying unique evolutionarily significant units (ESUs) that preserve critical genetic diversity (Purvis *et al*., 2005). The number of bats described has been increasing dramatically since the beginning of the 20th century due to advances in DNA sequencing. Spectacular levels of cryptic diversity have been frequently reported in Europe (Castella *et al*., 2000; Mayer & von Helversen, 2001; Ibáñez *et al*., 2006; Hulva *et al*., 2007; Mayer *et al*., 2007; García-Mudarra *et al*., 2009; Bogdanowicz *et al*., 2015). Although bats have a high potential for dispersion, they can exhibit unexpected levels of genetic differentiation and strong population structure on islands (Ruedi & McCracken, in press; Ibáñez *et al*., 2006). The traditional morphological analysis for the description of species have been widely used by taxonomists. However, it has a lower resolution than molecular analysis in cases of cryptic species, when the morphological variation unable to distinguish them due to the lack of synapomorphic characters (Padial *et al*., 2010).

In all habitats, acoustic sampling is a very effective methodology to survey and monitor bats (MacSwiney *et al*., 2008). Bats emit signals of high frequency and analyze the returning echoes to detect, characterize and localize the reflected objects (Fenton & Bell, 1981). They use a wide variety of species-specific signal types differing in frequency structure, duration, and sound pressure level that can be used for species identification (Fenton & Bell, 1981; MacSwiney *et al*., 2008). Although traditional procedures will remain useful in many cases, taxonomy needs to be integrative with new approaches for species delimitation (Padial *et al*., 2010). The combination of morphological measures, population genetics, ecological and phylogeographic data provide to taxonomists a larger arsenal to face better the huge challenge of inventorying biodiversity (Dayrat, 2005; Padial *et al*., 2010).

Biodiversity is not homogeneously distributed; it varies widely throughout different regions. In general, the diversity of plants and animals increases dramatically from the polar regions to the tropics (Gaston, 2000; Myers *et al*., 2000). Biodiversity is particularly higher on oceanic islands, which never had connection to continental landmasses. They are well-known centers of endemism and range-restricted species, hosting some unique lineages and some of the most threatened species (Myers *et al*., 2000; Mittermeier *et al*., 2011). In these localities, the Linnean and Wallacean shortfalls are even more profound because more scientific investments and effort are needed to elucidate cryptic diversity and species ranges (Myers *et al*., 2000; Mittermeier *et al*., 2011).

Oceanic islands receive their biota only through dispersal, product of oversea colonization and local diversification (Mayr, 1965; MacArthur & Wilson, 1967; Whittaker, 1998). Those islands usually lack several taxa such as amphibians and non-volant mammals, even though a suitable environment may be present, because these species do not have means of dispersion over large water masses (Whittaker & Fernández-Palacios, 2007). However, bats have powerful flight allowing their widespread distribution and they are generally assumed to have high migration and dispersal rates among isolated habitats (Lomolino, 1984; Lomolino, 1986; Lawlor, 1986; Carvajal & Adler, 2005). Hence, bats are a major component of mammalian biodiversity and are often the only native mammals in oceanic islands (Courchamp *et al*., 2003; Jones *et al*., 2009; Fleming & Racey, 2010). Nevertheless, bats are often underrepresented in conservation and management plans because of the knowledge gaps on population structure, distribution, and habitat requirements (Weller & Lee, 2007). Furthermore, bats are not iconic mammals and usually neglected because of their appearance (Voigt & Kingston, 2016).

The Cabo Verde Archipelago is composed by ten oceanic islands (Fig. 1), included in the Mediterranean biodiversity hotspot (Conservation International 2005). In the context of the geographic perspective, the isolation of the archipelago led to the occurrence of several endemics (e.g. reptile: Vasconcelos *et al*., 2013; birds: Hazevoet, 2014b; fish: Brito *et al*., 2007; gastropods: Duda & Rolán, 2005; vascular flora: Duarte & Romeiras, 2009).

Very few studies and field surveys have focused on bats from Cabo Verde. The archipelago possesses one of the largest knowledge gaps in the distribution and taxonomy of those species (Vasconcelos, 2018). Data published in the 1960's and 1980's indicate that there are five species in the archipelago, all considered with recent colonization and reduced distribution. Moreover, the majority are classified as European taxa. The species were identified as the naked-rumped tomb bat *Taphozous nudiventris* Cretzschmar 1830; the Savi's pipistrelle *Hypsugo savii* Bonaparte 1837; the Kuhl's pipistrelle *Pipistrellus kuhlii* (Kuhl 1817); the gray big-eared bat *Plecotus austriacus* (Fischer 1829) and the Schreiber's bent-winged bat *Miniopterus schreibersii* (Kuhl 1817) (Dorst & Naurois, 1966; Pucetti & Zava, 1988; Ibañez & Fernández, 1989; Masseti, 2010; Hazevoet, 2015; Vasconcelos, 2018). There is also an observation of the African straw-coloured fruit-bat *Eidolon helvum* (Kerr 1792), which is probably a vagrant specie and an indeterminate molossid species (Jiménez & Hazevoet, 2010; Hazevoet, 2014a; Hazevoet, 2015; Vasconcelos, 2018). There is also a lack of information about how they reached the archipelago, if they have colonized the islands by passive transport, as boats, or if they have reached the islands by their own means.

The systematic status of the Cabo Verdean bats is still controversial, especially on those complexes of sympatric and cryptic species widespread throughout Europe and North Africa. One of those species is *H. savii,* which belongs to the family Vespertilionidae and has a wide geographic distribution in the Palaearctic and marginally in Indomalayan region. It extends from Southern Europe and North Africa through the Middle East and the Caucasus to Central Asia and Northern India (Horáček & Benda, 2004; Wilson & Reeder, 2005; Juste & Paunović, 2016). During the last decades its geographic range has been expanding northward in Central Europe (Paunović *et al*., 2015, Uhrin *et al*., 2015). More recently, *H. savii* was reported in Slovakia and Czech Republic, and as a vagrant in the Great Britain (Reiter *et al*., 2010, Jahelkovà *et al*., 2014). In the Macaronesia archipelagos, *H. savii* has been reported on La Palma, Tenerife, La Gomera, Gran Canaria and El Hierro in the Canary Islands, and on Fogo, Brava, São Nicolau, São Vicente, and possibly Santo Antão in the Cabo Verde Islands (Pestano *et al*., 2003; Vasconcelos, 2018).

The systematic and taxonomy of the genus is still far from being sufficiently resolved. The number of known lineages has been increased through molecular analyses (e.g., Mayer & von Helversen, 2001; Hulva *et al*., 2004; Ibáñez *et al*., 2006; Bogdanowicz *et al*., 2015). *Hypsugo savii* was first considered as a monophyletic clade after taxonomic revisions with molecular markers and phylogenetic analyses of the family Vespertiolinidae (Van der Bussche & Hoofer, 2004) and the species appeared to be the only representative of the genus in Europe. Only two subspecies were distinguished within the Western Palearctic realm: *Hypsugo savii savii* (Bonaparte 1837) in southeastern Europe, including Italy and the Alps, and *Hypsugo savii ochromixtus* (Cabrera 1904), probably restricted to the Iberian Peninsula (Horáček *et al*., 2000). However, within mitochondrial (mtDNA) markers cytochrome *b* (cyt *b*) and NADH-ubiquinone oxidoreductase chain 1 **(**ND1), three main lineages in Europe diverged by over 7% (Ibáñez *et al*., 2006; García-Mudarra *et al*., 2008). One lineage was found on southern Iberia populations that may be recognized at subspecific level, which is also widespread in Morocco, whereas another Iberian lineage that occurs as far north as Switzerland (Ibáñez *et al*., 2006). Both lineages appear in sympatry in the southern Iberia Peninsula (García-Mudarra *et al*., 2008). The third lineage corresponds to *H. savii* from eastern Mediterranean (Ibáñez *et al*., 2006). Results based on nuclear markers (nDNA) such as RAG2 also suggested the existence of a distinguished southern Iberian lineage (Ibáñez *et al*., 2006). Bogdanowicz *et al*. (2015) analyzing the mtDNA of populations from Italy and the rest of western Palaearctic found three haplogroup of *H. savii*: one Italian that clusters along with haplotypes from Maghreb, another Southern Iberian, and another east European.

According to morphological data proposed by Ellerman & Morrison-Scott (1966) the taxonomic status of the population of the Canary Islands is considered as *Hypsugo cf. darwinii* (Tomes 1859) based on significant differences between *H. savii* from mainland Spain. Mayer *et al*. (2007) suggested that the African lineage corresponds to *Hypsugo cf. darwinii*, which is highly divergent from the Iberian *Hypsugo s. ochromixtus*. The relationship between Macaronesia bats and this African mainland lineage needs to be assessed before naming the species. In the Sardinia Island, two *Hypsugo* haplolineages occurs in sympatry. One lineage refers to *H. savii* (*sensu strictu*), which grouped together with the European haplotypes and the other lineage refers to *H. cf. darwinii*, which connected to the North-African/Canarian group of haplotypes, also occurring in Turkey (Veith *et al*., 2011). The *H. cf darwinii* also occurs in Tuscany, more specifically in Montecristo Island (Gianna *et al*., 2016), suggesting the hypothesis that this taxon might also be present in other Mediterranean and Macaronesian islands.

Pestano *et al*. (2003) found that the divergence of Canary Islands population compared with mainland Iberia was 6.3–7.2% for cyt *b* and 3.8–4.4% for 16S ribosomal RNA (rRNA), the latter also confirmed by Veith *et al*. (2011). The taxonomic status of the population of the Canary Islands is considered as *H. cf. darwinii* by some authors based on significant morphological differences from mainland Spain (Ellerman & Morrison-Scott, 1966; Dietz & Kiefer, 2014). Considerable differentiation was found in *H. savii* from El Hierro Island that has undergone a relatively long period of isolation since colonization and could be considered an ESU for conservation purposes or even considered a new species (Pestano *et al*., 2003). However, the taxonomic status remains unclear and further studies are needed to establish the pattern of bat biodiversity within Macaronesian archipelagos to underpin local conservation strategies for these taxa. There is no genetic information confirming the taxonomic status of *Hypsugo* from Cabo Verde, so it could be related to Canary Islands and be considered as *H. cf. darwinii* or to *H. savii* from mainland Spain, or even be another distinct lineage (Benda *et al*., 2004; Juste *et al*., 2004; Vasconcelos, 2018).

Regarding the taxonomic status of *Pipistrellus kuhlii*, there is also some debate. The species belongs to the family Vespertilionidae and it is a typical representative of the Mediterranean bat fauna. It is widespread in North Africa, Europe and Central Asia (Sachanowicz *et al.*, 2006; Barti, 2010; Wawrocka *et al.*, 2012). In Macaronesia, *P. kuhii* is recorded on Gran Canaria, Fuerteventura and Lanzarote in Canary Islands (Pestano et al., 2003) and on São Vicente, São Nicolau, Fogo, and Santiago in Cabo Verde (Vasconcelos, 2018). Regarding *P. kuhlii* from Europe there are two major lineages with genetic distances around 6% for cyt *b* and ND1 mtDNA makers (Ibáñez *et al.*, 2006). The first lineage includes most Iberian samples and extends its distribution to Switzerland, whereas the second lineage is apparently found throughout Europe from southern Iberia to Greece (Ibáñez *et al.*, 2006). Both mt DNA lineages are sympatric in southern Iberia, with Switzerland being a contact zone, and the RAG2 nDNA supports the existence of gene flow between them (Ibáñez et al., 2006). Those results are somehow different from those present by Bogdanowicz *et al.* (2015), which recovered based on ND1 three clades, one in Southern Spain, other occurring in Ibero-Maghrebian region across the Balkans and Anatolia, and another in Anatolia. According to more recent genetic studies, European population of *P. kuhlii* consists of two main lineages (western and eastern) diverged by 6.1% for COI (Andriollo *et al*., 2015) and 1.9% for 16S rRNA markers (Sachanowicz *et al.*, 2017). The western/southern lineage is widely distributed across the Mediterranean basin, Canary Islands and in the Balkans and it is referred as *Pipistrellus kuhlii kuhlii* (Kuhl 1817)

while the eastern lineage is referred as *Pipistrellus kuhlii lepidus* Blyth 1845 and it occurs in Poland, Ukraine, Russia, Caucasus, Middle East, and Central Asia (Sachanowicz *et al.*, 2017). In addition to the genetic distances between the lineages, some morphological differences in the dorsal pelage, face, ear, wing margin, and penis colouration, as well as in body mass, and wing margin and forearm width were found (Sachanowicz *et al.* 2017). The presence of two lineages could underlie the existence of distinct species, but more taxonomic considerations, morphological and ecological studies are needed to be ascertained.

Pestano *et al*., (2003) suggest that *P. kuhlii* and *Pipistrellus madeirensis* (Dobson 1878) from Canary Islands form a monophyletic clade in respect to *P. kuhlii* from mainland Spain. Thus, *P. kuhlii* appears to be paraphyletic species due to the nesting of *P. maderensis* within it, which is more closely related to the Kuhl's bats from Canary Islands than those are related to Kuhl's bats from outside the archipelago (Pestano *et al*., 2003). The first records that indicating *P. kuhlii* occurs in the eastern Canary Islands, appears to be a mistake due to morphological similarities between these species. Observations have been made in La Palma, indicating that *P. kuhlii* may be present in most Canary Islands, while *P. madeirensis* has only been detected on the western islands (La Palma, El Hierro, La Gomera, and Tenerife) and Madeira Archipelago (Pestano *et al*., 2003). Results found by Pestano *et al*. (2003) indicate that these two species diverged 3.7–4.7% in cyt *b* mtDNA and 2.0–2.8% in 16S rRNA. However, some intermediate phenotypes have been detected in the islands where both species co-occur (sometimes in the same sites) opening the hypothesis that both lineages may interbreed (Pestano *et al*., 2003). The same authors suggest that each population of *P. madeirensis* from western islands of La Palma, El Hierro together with La Gomera, and Tenerife could be different ESUs for conservation strategies if different lines of evidence (apart from mtDNA) support those differences. However, morphological differences among *P. madeirensis* individuals from distinct islands might be very difficult to detect given the morphological conservatism in bats at low taxonomic levels (Pestano *et al.*, 2003).

Plecotus austriacus belongs to the family Vespertilionidae and it is essentially restricted to the Palearctic, even though it extends to the Ethiopian and the Indomalayan regions (Spitzenberger *et al*., 2006). The genus *Plecotus* has proved to be good at colonizing distant islands, as it occurs on several Mediteranean and Atlantic Islands (e.g., Balearic Islands, Sardinia, Corsica and Sicily). In Cabo Verde, it was recorded on Maio and Santiago (Vasconcelos, 2018).

Historically, a number of taxa have been described in the genus *Plecotus* based on morphological measurements (Ellerman & Morrison-Scott, 1966), but there is a lack of clear diagnostic characters. Classic systematic studies recognized only three species *Plecotus auritus* Linnaeus 1758 and *Plecotus austriacus* in Palearctic and one endemic from Canary Islands *Plecotus teneriffae* Barret-Hamilton 1907 (Juste *et al*., 2004). However, recent molecular studies have revealed three new species: *Plecotus macrobullaris* Kuzjakin 1965 (a senior synonym for *Plecotus alpinus* Kiefer & Veith 2002 and *Plecotus microdontus* Spitzenberger 2002) from Caucasus, Asia Minor, the Alps and the Balkans to the Pyrenees (Spitzenberger *et al.*, 2003; Kiefer *et al*., 2002); *Plecotus kolombatovici* Dulic 1980 (originally described as subspecies of *P. austriacus*) from the Balkans and North Africa (Kiefer *et al*., 2002; Mayer & von Helversen, 2001; Spitzenberger *et al*., 2002) and *Plecotus sardus* Mucedda, Kiefer, Pidinchedda & Veith 2002 endemic taxon from Sardinia Island (Mucedda *et al.*, 2002).

Juste *et al*. (2004) analyzing mtDNA identified two major lineages, the '*austriacus'* and '*auritus*' group, each one is subdivided into two further subgroups. Within the '*auritus*' group, one subclade corresponds to *P. auritus* (*sensu strictu*) from western Europe eastwards at least to Russia and *P. sardus* found on the Sardinia Island (Juste *et al*., 2004). The other subclade corresponds to *P. macrobullaris* present at the Pyrenees, the Swiss Alps and Crete intermixed with sequences from Middle Eastern long eared bats (Juste *et al*., 2004). Within the '*austriacus*' group, there is a clade attributed to *P. austriacus* (*sensu strictu*) from Central Europe, and the molecular evidences suggests that this species extend its distribution throughout Southern Europe, on Madeira and the Balearic Islands (Juste *et al*., 2004). The other subclade includes three very well differentiated lineages, the Canarian long-eared bat *P. teneriffae* from the Canary Islands, an undescribed species occurring in North Africa, and *P. kolombatovici* occurring in the Balkans and Anatolia (Juste *et al*., 2004). The strong geographic component and close phylogenetic relationship between Canarian and North African long-eared bats with those representing *P. kolombatovici* from the Balkans and Anatolia suggest that these bats have an ancient origin south of the Mediterranean Basin (Juste *et al*., 2004). However, the results found by Juste *et al.* (2004) are not robust enough to elaborate a more detailed scenario on the possible colonization routes. It is unkown if the *Plecotus* that occurs in Cabo Verde is actually *P. austriacus* (*sensus strictu*) or *P. teneriffael P. kolombatovici*, or even a distinct lineage resulting from long period of isolation. There are no clear morphological discriminatory characters described for Cabo Verde population, and no previous molecular studies in the archipelago.

The *Miniopterus schreibersii* belongs to the family Miniopteridae and has a geographic distribution across south-western Europe, North and West Africa, Anatolia and the Middle East to Caucasus. The genus *Miniopterus* Bonaparte 1837 has a widespread distribution throughout most of Africa, Europe, Asia, New Guinea, Australia, and the Pacific (Corbet & Hill, 1980; Nowak, 1991; Tan, 1992). The species of this genus are often difficult to identify morphologically given the lack of a discriminate characters (Tate, 1941; Maeda, 1982; Wilson & Reeder, 1993). Several classifications have been constantly reviewed and changed over the years. More recently, Appleton *et al*. (2004) recognized two major clades inside *M. schreibersii* complex, which distributions correspond to the Ethiopian-Palearctic and the Oriental-Australasian zoogeographical regions. Each lineage splitting further into more geographically structured branches.

The *M. schreibersii* complex in Turkey is represented by the nominotypic form *Miniopterus schreibersii schreibersii* (Kuhl 1817), and its subspecies *Miniopterus schreibersii pallidus* Thomas 1907 (Bilgin *et al*., 2012). Some author suggested that *M. s. schreibersii* is a typical representative to Europe, North Africa and Asia Minor and *M. s. pallidus* is present in the Asian part of Turkey (Corbet, 1978; Albayrak & Coskun, 2000). Others placed the division line somewhere in the Marmara and the western Black Sea regions (Steiner & Gaisler, 1994; Karataş & Sözen, 2004). Bilgin *et al*. (2006) studied the genetic differentiation in the transition zone between south-eastern Europe and Anatolia and the results suggest that the western Anatolian and south-eastern European samples are marginally differentiated, while there is a greater differentiation between these and the samples from eastern Anatolia. The Gibraltar Strait appears to have no effect on dispersal rate for this species, which show no genetic discontinuities between one continent and the other (García-Mudarra *et al*., 2008). Unfortunately, no samples from Cabo Verde were included in these studies, so it is unknown which geographic affinities those individuals, only known from Santo Antão Island, could present.

Furman *et al*. (2010) found a shallow genetic differentiation in *M. schreibersii*, suggesting a recent and rapid postglacial re-colonization of Europe, probably from a single glacial refuge in the north-western Anatolia. Unlike the pattern of *H. savii*, *P. kuhlii* and *P. auritus*, which re-colonized Europe from two glacial refugia, the extant population of *M. schreibersii* seem to originate from a single location. Bilgin *et al.* (2016) found out tree possible explanations for the shallow genetic differentiation in *M. schreibersii.* Either all European populations went extinct during the last glacial maximum or contracted to localized refuges and did not contributed to the re-

colonization; the Eastern and Southern coast of the Levant acted as the oldest refuges; or Anatolia emerged as an intermediate region in the process of stepping stone of the Caucasus and mainland Europe from this Levantine refuge.

Little is known about the systematic of the *Taphozous nudiventris*, which belongs to the family Emballonuridae. It has been recorded throughout the North Africa from Morocco to Egypt and southern desert and sub-desert Saharan region and through the Middle East to southern Turkey and more arid areas of Indian subcontinent (Aşan & Albayrak, 2007; Monadjem *et al*., 2017). The southernmost distribution report is from northern Tanzania and there are two isolated records from Myanmar. In South Asia this species is presently known from Afghanistan, Bangladesh, India and Pakistan. This species is found in arid and semi-arid zones, tropical forests and wet evergreen forests (Bates & Harrison, 1997). In Cabo Verde, at the western limit of its distribution, it was recorded on Santiago and Maio islands (Vasconcelos, 2018).

Objectives

The objective of this master thesis is to revise the systematic position and distribution of the Cabo Verde bats based on molecular markers, morphological and acoustic data. More specifically, this project aims to: 1) collect samples of Cabo Verde bats from the field and available museum collections; 2) identify the systematic position of Cabo Verde bats in relation to the Macaronesian region, Europe and Africa; 3) update bat distribution in the Cabo Verde Archipelago. This study is a breakthrough for starting bat research in that country, while also giving support for conservation actions for bat unique evolutionarily units.

Methods

1. Study Area

The Cabo Verde Archipelago is located in the North Atlantic Ocean, close to the West African coast and the West Mediterranean region (Fig. 1). The archipelago is formed by ten volcanic islands and several islets situated between 14°45′-17°10' N and 22°40′-25°20' W (Fig. 1). It lies circa 570 km from African mainland (coast of Senegal), 1.500 km from Canary Islands, and 2.500 km from Azores, covering a combined area slightly over 4.000 km^2 and spreading over 58.000 km^2 (Duarte & Romeiras, 2009).

The islands are usually classified into three groups: Northern Islands: Santo Antão, São Vicente, Santa Luzia and São Nicolau; Eastern Islands: Sal and Boavista and Southern Islands: Maio, Santiago, Fogo and Brava (Fig. 1). Moreover, they are also classified as the Windward Islands (Barlavento), which comprised the North-western and Eastern Islands and the Leeward Islands (Sotavento), comprising the Southern Islands. Cabo Verde is included in the African Sahelian arid and semi-arid climatic region, experiencing climatic ranges from tropical dry to semi-desertic (Duarte & Romeiras, 2009). Mean annual temperatures range from 23-27 °C at sea level to 18-20 °C at high altitudes, but higher temperatures (35-40 °C) can occur in inner regions of the arid Eastern Islands (Duarte & Romeiras, 2009). Annual precipitations are usually low ranging from 80-300 mm in the arid coastal zones and 1200-1600 mm in the highlands of the mountain islands (Duarte & Romeiras, 2009).

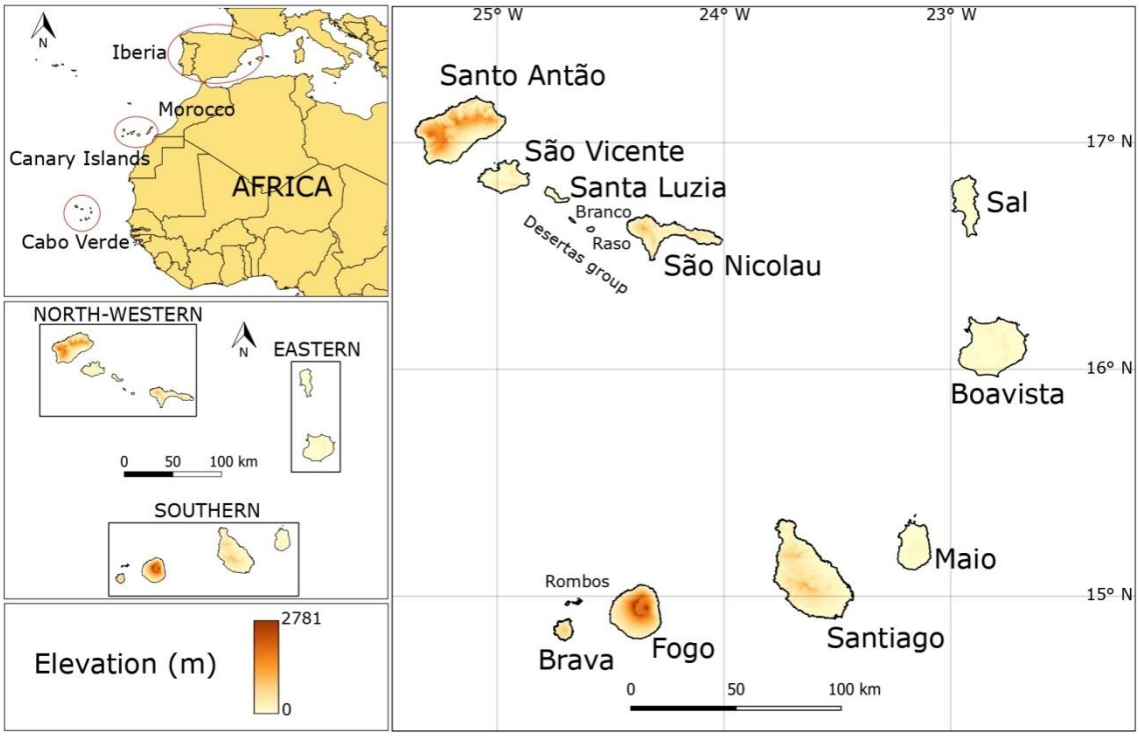

Fig. 1 – Map of the Cabo Verde Islands, showing the geographic location and the elevation of the archipelago and its islands.

Sal, Boavista and Maio islands have a flat landscape (Fig. 1) and a more arid climate because of the infrequent occurrence of rainfall due to their exposure to dry and hot winds coming from the Sahara (Duarte & Romeiras, 2009). The vegetation consists of savannah or steppe vegetation and some areas supports semi-desert plants (Diniz & Matos, 1988a; Diniz & Matos, 1988b; Diniz & Matos, 1993). In contrast, the more western and geologically younger islands have a rugged landscape (Fig. 1) of high peaks, ridges, plateaus and deep valleys. Coastlines of these islands are often cliff-lined bound by steep slopes that descend to rocky shores of very difficult access, which usually host many bat individuals. The climate of the wetter islands, such as Fogo, is suitable for the development of a dry forest in the higher elevations. However, much of the terrain is devoid of vegetation that could be used as shelters by bats due to the intense past and present volcanic activity and deforestation.

All Cabo Verde Islands are volcanic in origin and considered result of magmatism generated by ascending mantle plumes under a gradually moving African plate (Ali *et al*., 2003). The volcanic activity probably started sometime in the Late Oligocene or Early Miocene (Ali *et al*., 2003). The age of the islands is not well known, but most works suggest that islands closer to the mainland are the oldest (12-26 Million years,

My), while the most apart are the youngest (2-8 My) (Mitchell *et al*., 1983; Torres *et al*., 2002; Duprat *et al*., 2007). Brava is thus probably the youngest island, which can be dated to 1.95 ± 0.38 My (Madeira *et al*., 2010), while Maio is thought to be the oldest with 21.8 ± 5 My (Holm *et al*., 2008).

2. Sample and data collection

Fieldwork was performed on São Nicolau Island, at Monte Gordo (16°37'14" N, 24°20'36" W) in November 2015; Fogo Island, at São Filipe (14°53'42" N, 24°29'50" W) and Fogo Natural Park (14°59'44" N, 24°20'39" W) in June 2018; and Santiago Island, at Rocha Preta (15°02'20" N, 23°36'18" W), Monte Caleirão (15°04'42" N, 23°36'26" W) and Calabaceira (14°55'52" N, 23°35'45" W) also in June 2018. All the permits were emitted by Direcção Nacional do Ambiente (permit nr. 117/2018). Mist-nets were set up (1x9 m, 1x10 m, 1x6 m) close to the entrance of roost and hibernation sites and pellets were collected for DNA analyses when present. Once a bat was captured, a small piece of the skin was removed to posterior DNA analysis, and all the individuals were released after morphological measurements.

The acoustic sampling was performed every night from 5:30 to 12 pm with a Pattersson D1000 bat detector with a sampling rate of 512 kHz (Pettersson Elektronik AB, Uppsala, Sweden). We set the bat detector during the first period at dusk, which is usually the period of greater activity and until midnight to hear and record echolocation calls and feeding buzz. The sound was recorded using a microphone Zoom H1 Handy Recorder (www.zoom.co.jp) in time-expansion (10 times). In order to identify species, calls were analyzed with the BatSound software (Pettersson Elektronik AB, Uppsala, Sweden). The variables analyzed were frequency of maximum energy (FME), low frequency (LF), high frequency (HF), duration (DUR) and inter-pulse intervals (IPI). The results were compared with the available literature for the five species that occurs in the archipelago. The sounds were deposited at Morphobank (P3514). All coordinates from new observations were recorded and mapped using DivaGiS (Hijmans *et al*., 2001).

We also visited the *Museo Civico di Storia Naturale*, *di Genova* (MCSNG), Genoa, Italy, the *Museo Zoologico de La Specola* (MZS), Florence, Italy and the *Muséum National d'Histoire Naturelle* (MNHN), Paris, France for collecting DNA samples and basic morphological measurements. The vouches analyzed are represented in Table 1. Tissue samples were collected using the wing punch method, which consist on removing a small circle of the skin (normally smaller than 3 mm) from the wing

membrane using a biopsy punch. The morphological measurements (see table 2) were taken using a caliper according to the identification key (Dietz & von Helversen, 2004). The variables used were the lengths of forearm (FA), fifth finger (D5), third finger (D3), thumb (D1), lower leg (Tib), hind foot (HF), length of the 1st and 2nd phalange of the 4th finger (P4.1 and P4.2), and the 2nd and 3rd phalanges of the 3rd finger (P3.2 and P3.3) (Table 2). Both DNA and morphological data collection were performed wherever animals were captured in the fieldwork or the curator allowed it. All measurements were taken following the recommendations proposed by Dietz & von Helversen (2004).

3. Molecular analyses

3.1 DNA extractions

The faeces were preserved in ethanol 96% and frozen. The DNA from the samples collected with wing punch method was extracted followed by a standard protocol of DNA extraction with silica. The samples from museums were processed followed by the ancient DNA extraction protocol and the DNA extraction of wing punches samples was proceed followed the protocol of saline extraction (Appendix I – Supplementary Material).

3.2 Markers choice

The mitochondrial marker chosen for this study was the cytochrome *b* (cyt *b*). This gene is widely used in systematic studies to resolve divergences at many taxonomic levels. It has been considered one of the most useful genes for phylogenetic studies, and is probably the best-know mitochondrial gene with many sequences on National Center of Biotechnology Information (NCBI) data base (Esposti *et al*., 1993). We design the cyt *b* primers for each species (see table S1). The nuclear gene chosen was the Recombination Activating 2 (RAG2), widely used as phylogenetic marker with highly heterogeneous base composition.

3.3 PCR conditions

PCR conditions I

The PCR for samples from museums and pellet were carried-out in volumes of 12 µl, comprising 5 µl of PCR Master Mix, 0.4 µl of forward primer, 0.4 µl of reverse primer, 2.2 µl of ultra-pure water, and 4 µl of DNA extraction. Cycling conditions used an initial denaturing at 95°C during 15 min, followed by 55 cycles of denaturing at 95°C

for 30 s, annealing at a gradient temperature ranging from 46–54°C during 50 s, and extension at 72°C for 90 s and final extension at 72°C for 10 min.

PCR conditions II

To amplify a fragment of the RAG2, we used the primers RAG2-F1 and RAG2-R2. After the first PCR, a nested PCR was done with the primers RAG2-R1 and RAG2-F1int (Baker *et al*., 2000). The PCR reactions were carried-out in volumes of 10 µl, comprising 5 µl of PCR Master Mix, 0.4 µl of forward primer, 0.4 µl of reverse primer, 2.2 µl of ultra-pure water, and 2 µl of DNA extraction. Cycling conditions used an initial denaturing at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at a gradient temperature ranging from 50–53°C for 50 s and extension at 72°C for 90 s, and the final extension at 72°C for 10 min.

PCR conditions III

For the cyt *b* fragment of *Hypsugo savii*, we used the primers CB1 and cytb2 for the first fragment and cb2F and CB3H for the second fragment. The PCR conditions for samples of *H. savii* for this gene were carried-out in volumes of 10 µl, comprising 5 µl of Master Mix, 0.4 µl of each primer, 2.2 µl of ultra-pure water and 2 µl of DNA extraction. Cycling conditions used an initial denaturing at 95°C during 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 50°C during 50 s and extension at 72°C for 90 s, and final extension at 72°C for 10 min.

3.4 Sequencing

In order to proceed the Sanger sequencing, 8 µl of the amplified product of the PCRs were transferred to a new PCR plate and was added 1 µl of EXO+SAP (24 µl of EXO and 96 µl of SAP). The samples were cleaned with the following thermocycling program: 37°C for 15 min, 85°C for 15 min and 10°C for infinite holding. The sequencing reactions were carried-out in volumes of 10 µl, comprising 7 µl of ultrapure water, 1 µl of TRR buffer, 0.5 µl of TRR, 0.5 of primer and 1 µl of the EXOSAP reaction product. The initial temperature was 94°C during 3 min, followed by 25 cycles of 96°C for 10 s, 52°C for 0.05 s and 60°C for 4 min. The samples were cleaned with 400 µl of Sephadex per sample, and sequenced amplified from both strands on anautomated sequencer.

4. Phylogenetic analysis

After sequencing, every fragment was checked using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) to find regions with higher similarity. The sequences were align using ClustalW multiple alignment application (Thompson *et al*., 1994) and edited in the Geneious Prime v2019.2. Each fragment was inspected visually for ambiguities. Sequences were trimmed for each marker to a fragment in which peaks could unequivocally be assigned for all individuals. Incongruent sequences, stop-codon/indels and double peaks were corrected manually to minimize alignment gaps. The uncertainty positions were filled up with the IUPAC nucleotide ambiguity codes. The amplification success varied greatly among samples due to differences in the quality of the DNA regarding to the conservation conditions and origins. The dataset used for the phylogenetic analyses of the gene cyt *b* consisted of an alignment of 702 base pairs (bp) for 38 *Hypsugo* specimens and four *Pipistrellus* used as outgroup based on published evidence (Roehrs *et al*., 2010). The phylogenetic analyses of the gene cyt *b* for *Taphozous nudiventris* consisted of an alignment of 278 bp for 12 *Taphozous* specimens, and *T. perfomatus* and *T. mauritianus* were used as outgroup (see table S2 for more details).

In order to select the best-fit partitions schemes and models of evolution to each gene the JModelTest v2.1.10 was used (Darriba *et al*., 2012). The best-fit models of evolution were selected according to Akaike Information Criterion (AIC). Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian inference (BI) analyses. Maximum Likelihood analyses were performed with MEGAx v10.0.5 (Kumar *et al*., 2018). A GTR+I+G model was used with parameters estimated independently for partitions and reliability was assessed by 1000 bootstrap replications.

Bayesian analyses were conducted using BEAST v2.6.1 (Drummond & Rambaut, 2007), using the same dataset. These analyses were used to infer the phylogenetic relationships and simultaneously estimate the timing of the cladogenese events. All parameters were established using the extension BEAUti v2.6.1 (Drummond *et al*., 2012). The lack of internal calibration points in *Hypsugo* could not provide a realistic calibration for shallow divergences between sibling species. Therefore, the mean substitution rate of the cyt *b* mitochondrial gene calculated for the genus *Myotis*, family Vespertilionidae was used for this purpose (Stadelmann *et al*., 2007). The molecular clock estimation of nodes ages was chosen due to its similarities with *Hypsugo* and pipistrelles in amount of genetic divergence and thus expected divergence time and taxonomic proximity.

The Bayesian analyses was conducted with Yule process (Yule, 1924) as tree prior and random starting trees without constraints, simulations of Markov Carlo Mont Chain (MCMC) were run for 50 million generations, trees were sampled every 1000 generations. Stationarity was assessed by examining the standard deviation of split frequencies and by plotting the -In L per generation using TRACER v1.5 (Rambaut *et al*., 2018) and trees generated before stationarity were discarded as "burn-in". The models were tested in addition to all options for the clock models (strict, uncorrelated relaxed lognormal, gamma and exponential, random local and fixed local) with likelihoods compared in TRACER v1.5. TreeAnnotator v.2.6.0 application (Helfrich *et al*., 2018) was used to summarize the information from a sample of trees produced by BEAST onto a single tree with information about posterior probabilities of the nodes and estimates of highest posterior density intervals. Then, the file generated was open in the FigTree v1.4.4 (Rambaut, 2012) to have a graphical viewer of phylogenetic tree. The genealogical relationships among taxa were assessed with haplotype networks constructed using statistical parsimony (Templeton *et al*., 1992), implemented in the program TCS v.1.21 (Clement *et al*., 2000) with connection limit 95% and deletions treated as a fifth state. The program tcsBU (Santos *et al*., 2015) was used to improve the final network layout. The genetic population analyses were performed in MEGAx v10.0.5 and DNASP v.6 (Rozas *et al*., 2017) to check the value of number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity and within mean group distance of each population.

Results

1. Collected data and samples

We were able to collect the following samples from museums: MCSNG 47910a, MCSNG 47910b, MZS 1399, MZS 12222 (*H. savii*), MZS 12221, MZS 12514 (*M. schreibersii*), MZS 10597 (*P. kuhlii*), MNHN 1986-375, MNHN 1986-376, MNHN 1986-377, MNHN 1986-378, MNHN 1986-379, MNHN 1983-2229 (*T. nudiventris*) and MNHN 1983-1467 (*P. austriacus*) (Table 1 and Fig. 2). We could amplify a small fragment of every sample except the MZS 1399, MNHN 1986-377 and MNHN 1983-1467.

Table 1 – Summary of all Cabo Verde samples used in this study. The species, with the specimen and Morphobank codes, island (I) and locality, sample type and DNA amplification success (DNA) is given. F (Fogo), M (Maio), SA (Santo Antão), SN (São Nicolau), ST (Santiago), SV (São Vicente).

Species	Code	I	Locality	Sample	DNA	Morphobank
H. savii	MCSNG 47910a	F	São Filipe	Museum	Yes	-
H. savii	MCSNG 47910b	F	São Filipe	Museum	Yes	-
H. savii	MZS 1399	SV	Unknown	Museum	No	M675987
H. savii	MZS 12222	SV	Monte Verde	Museum	Yes	M675986
H. savii	SNQ001	SN	Monte Gordo	Wing punch	Yes	M676428-M676509
H. savii	SNQ002	SN	Monte Gordo	Wing punch	Yes	M676428-M676509
H. savii	SNQ003	SN	Monte Gordo	Wing punch	Yes	M676428-M676509
H. savii	SNQ004	SN	Monte Gordo	Wing punch	Yes	M676428-M676509
H. savii	SNQ005	SN	Monte Gordo	Wing punch	Yes	M676428-M676509
H. savii	SNQ006	SN	Cachaço	Wing punch	Yes	M676546
H. savii	InBIO 1806_12	F	São Filipe	Acoustic	-	P3514-Doc 1
P. kuhlii	MZS 10597	SV	Unknown	Museum	Yes	M675984-M675985
P. kuhlii	InBIO 1806_09	ST	Monte Caleirão	Acoustic	-	P3514-Doc 3
P. kuhlii	InBIO 1806_21	F	São Filipe	Acoustic	-	P3514-Doc 2
M. schreibersii	MZS 12221	SA	Ribeira Grande	Museum	Yes	M675988-M675989
M. schreibersii	MZS 12514	SA	Paúl	Museum	Yes	M675990-M675991
P. austriacus	MNHN 1983-1467	M	Vila do Maio	Museum	No	-
T. nudiventris	MNHN 1986-375	M	Unknown	Museum	Yes	M676419
T. nudiventris	MNHN 1986-376	M	Unknown	Museum	Yes	M676420
T. nudiventris	MNHN 1986-377	ST	Pedra Badejo	Museum	No	M676249/M676418
T. nudiventris	MNHN 1986-378	ST	Pedra Badejo	Museum	Yes	M676421
T. nudiventris	MNHN 1986-379	ST	Pedra Badejo	Museum	No	M676422
T. nudiventris	MNHN 1983-2229	ST	Trindade	Museum	Yes	M676250/M676417
T. nudiventris	InBIO 1806_11	F	São Filipe	Acoustic	-	P3514-Doc 4
T. nudiventris	STM1	ST	Calabaceira	Pellet	-	-
T. nudiventris	FM10	F	São Filipe	Pellet	-	-

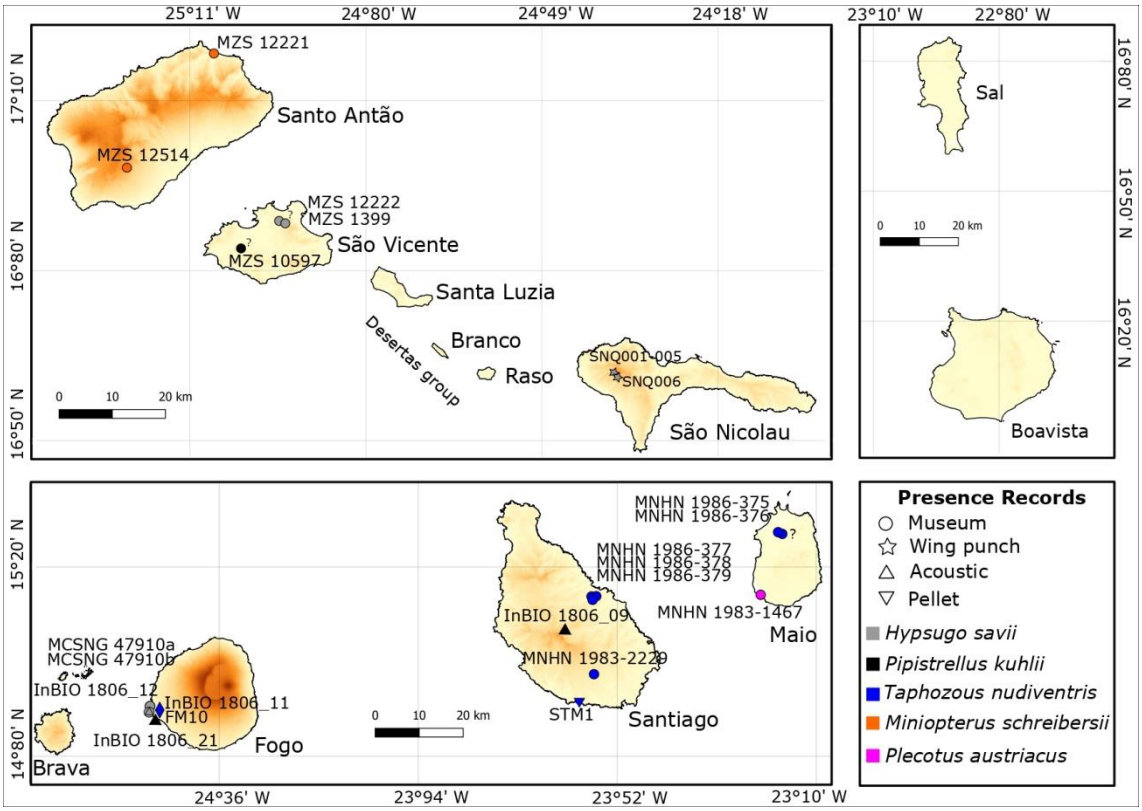

Fig. 2 – Bat samples collected in Cabo Verde Islands. Each species is depicted with a different color and each sample type with a different shape. See Table 1 for more details.

We were unable to amply DNA fragments of *P. austriacus* held in the Natural History Museum of Paris, which is the only known specimen from Cabo Verde. The only specimen of *P. kuhlii* held on the Museo Zoologico de La Specola, Firenze (MZS) is actually *H. savii.* The samples collected using the wing punch method had a great DNA quality and we were able to amplify a larger fragment of the cyt *b* and RAG2. The sample SNQ006 was the only one in which we could not amplify for the RAG2 marker. Regarding the acoustic samples, for Santiago Island we could identify the presence of *P. kuhlii*, *H. savii* and *T. nudiventris* on Fogo Island (see below). The latter represents the first record of *T. nudiventris* species for Fogo. These recording are deposited at Morphobank (project number P3514). One pellet sample collected on São Filipe was amplified and checked at BLAST, it was correspondent to *T. nudiventris*, corroborated the presence of the species on Fogo Island. The morphological data collected from vouchers and São Nicolau specimens are represented in Table 2.

Table 2 – Morphological measurements of specimens. Information about sex (S), length of forearm (FA), fifth finger (D5), third finger (D3), thumb (D1), lower leg (Tib), hind foot (HF), 1st and 2nd phalange of the 4th finger (P4.1 and P4.2), and the 2nd and 3rd phalanges of the 3rd finger (P3.2 and P3.3) are also given.

Species	Code	S	FA	D5	D3	D1	Tib	HF	P4.1	P4.2	P3.2	P3.3
H. savii	MZS 1399	F	37.6	32.2	35.5	8.8	16.6	6.6	10.0	8.0	-	11.1
H. savii	MZS 12222	F	35.4	33.2	35.5	8.8	15.4	6.6	11.1	8.8	-	10.0
H. savii	MZS 10597	F	35.4	32.2	33.3	8.8	14.4	6.6	11.1	7.7	-	10.0
H. savii	MCSNG 47910a	M	35.8	31.5	34.2	7.0	15.7	5.0	10.6	10.4	-	11.9
H. savii	MCSNG 47910b	F	34.5	29.4	30.7	7.5	14.9	5.6	10.7	8.0	-	10.0
H. savii	SNQ001	M	34.9	43.6	58.0	3.1	15.2	4.6	10.5	10.5	11.0	6.0
H. savii	SNQ002	F	37.1	52.8	61.3	4.2	16.0	4.8	10.5	10.5	11.0	7.0
H. savii	SNQ003	M	35.4	42.2	58.3	3.1	15.7	4.7	10.3	10.3	11.2	6.6
H. savii	SNQ004	M	35.3	43.1	58.4	4.6	15.5	4.5	10.6	9.7	10.7	5.2
H. savii	SNQ005	M	35.3	43.4	60.2	3.7	15.2	4.0	11.0	10.3	11.0	6.8
H. savii	SNQ006	M	35.5	32.2	34.4	4.2	15.4	4.3	10.5	8.9	10.1	4.8
M. schreibersii	MZS 12221	F	43.3	36.6	41.1	9.9	19.9	9.9	9.9	14.3	-	28.8
M. schreibersii	MZS 12514	M	45.4	38.7	43.2	9.9	20.0	9.9	9.8	15.5	-	29.9
P. austriacus	MNHN 1983-1467	unk	40.3	35.3	37.0	10.0	18.4	9.0	10.1	10.7	11.8	8.0
T. nudiventris	MNHN 1986-375	unk	68.2	42.7	60.0	17.1	28.2	17.7	14.3	8.3	23.4	-
T. nudiventris	MNHN 1986-376	F	67.4	43.9	61.9	18.7	27.9	15.9	13.5	8.0	25.0	-
T. nudiventris	MNHN 1986-377	F	69.9	45.3	64.4	17.8	30.9	16.3	15.1	8.6	32.5	-
T. nudiventris	MNHN 1986-378	M	69.4	43.1	61.5	17.8	27.3	16.7	13.8	8.4	25.5	-
T. nudiventris	MNHN 1986-379	F	71.3	47.0	68.3	17.3	30.1	16.7	14.1	7.6	27.2	-
T. nudiventris	MNHN 1983-2229	M	74.9	48.9	69.5	17.5	31.0	14.2	16.7	9.6	29.0	-

2. Phylogenies

The phylogenetic tree constructed for estimating the relationships of *Hypsugo* from Cabo Verde with related groups is presented in Fig. 3. The JModelTest program selected the HKY+G model based on the Akaike Information Criterion. The phylogeny with highest likelihood was obtained from Yule species model and strict molecular clock (likelihood= -2255). The results of ML and BI analyses yielded the same topology, with high bootstrap values and posterior probabilities in most clades (Fig. 3). The Cabo Verde samples presented some geographical structure. The sample MZS 12222 from São Vicente was the only sample from Cabo Verde that nested within the Canary Islands clade. The Cabo Verde and Canarian clades split around 0.049 Ma. The Cabo Verde samples grouped together with *Hypsugo* from Canary Islands in a monophyletic well supported group (Bayesian posterior probabilities > 95%) (Fig. 3). The sample AJ426626 from El Hierro Island represented a slightly different lineage (Pestano *et al.*, 2003). Nevertheless, it also nested in the Canarian clade with a Bayesian posterior probabilities superior to 90%.

The samples of *Hypsugo sp.* from Morocco and two samples of *H. savii* from Southern Iberia formed a well supported sister clade of *Hypsugo* from Cabo Verde and Canary Islands (Fig. 3). However, this clade is polyphyletic. The group of Cabo Verde, Canary Islands and the samples from Southern Iberia and Morocco split about 0.65 million year ago (Ma). The other group is represented by samples from mainland Europe (Iberian Peninsula and Switzerland), with two clear subgroups with geographic structure: one of samples from southern Iberia, and another with samples from northern Iberia and Switzerland that split circa 0.22 Ma (Fig. 3). Finally, the Eastern Europe clade composed by sequences from Greece, Montenegro, Iran, Turkey, Syria and Cyprus also present a clear geographic grouping of samples and split from the other clades around 0.7 Ma (Fig. 3).

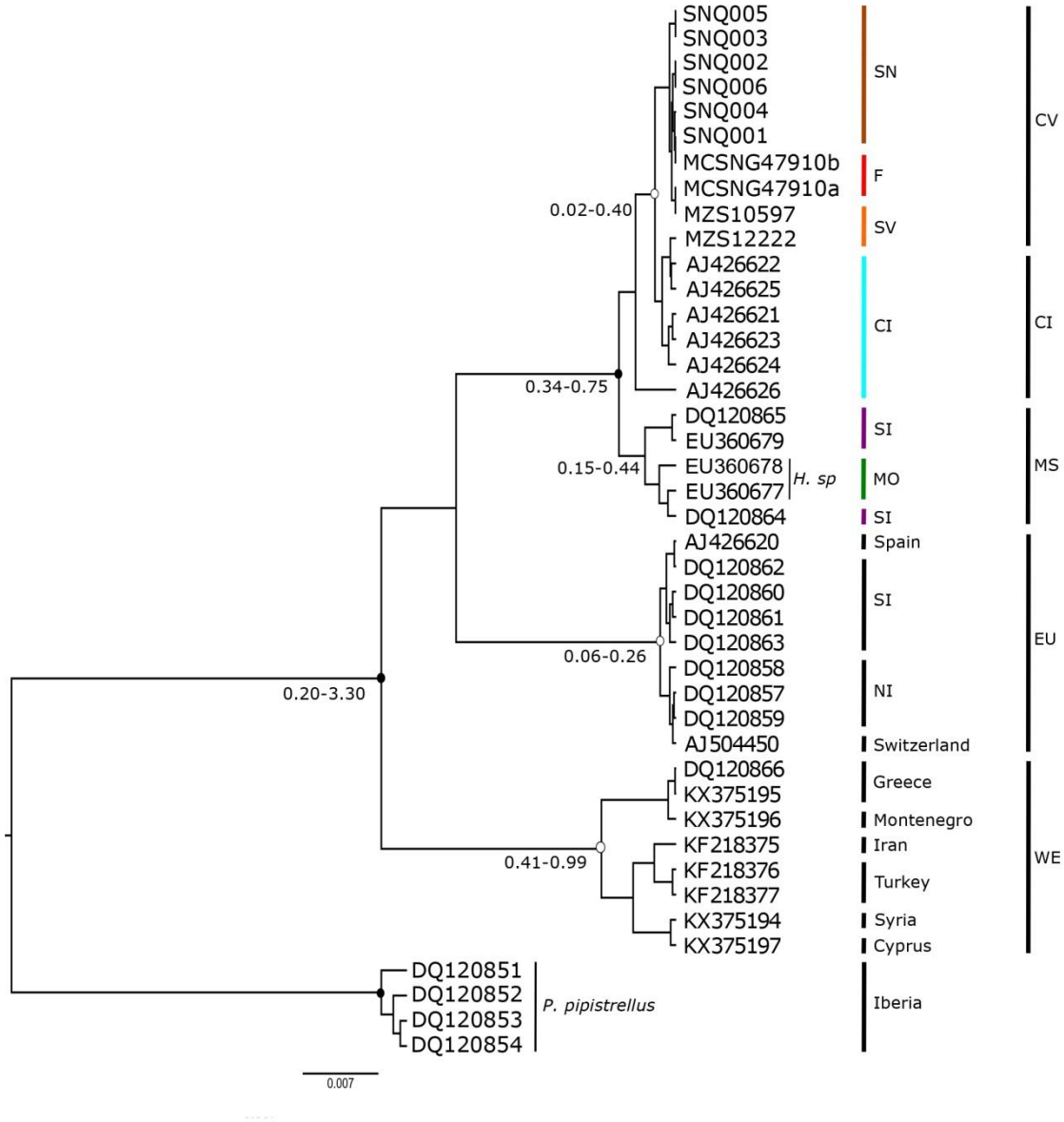

Fig. 3 – Maximum Likelihood (ML) phylogenetic tree of *Hypsugo* individuals rooted with *P. pipistrellus* (outgroup). Black dots on the nodes indicate both ML bootstraps values ≥ 70 and Bayesian Inference (BI) posterior probability ≥ 0.95, while white dots show BI ≥ 0.95. The 95% highest posterior density intervals of the nodes estimated with BEAST dating analysis are indicated below the branches. Clades Cabo Verde (CV), with the following islands: SN (São Nicolau), F (Fogo) and SV (São Vicente); CI (Canary Islands); MS (Morocco, MO and Southern Iberia, SI); EU (Europe) and EE (Eastern Europe) are colored according to their geographic location corresponding to Fig. 4. For further details on the samples, see Fig. 1, and Table 1 and S2.

Regarding the cyt *b* haplotype network of *Hypsugo* (Fig. 4), the results showed one haplogroup corresponding to Cabo Verde samples, which is composed by two haplotypes unique from Cabo Verde. One of them is more common, being present on Fogo, São Vicente and São Nicolau islands, and the other composed by samples only from São Nicolau Island (Fig. 4). However, there is a third haplotype present in Cabo

Verde samples. The latter one is closer related to the Canarian haplogroup, which showed five haplotypes. The sample MZS 12222 from São Vicente exhibit the same haplotype of the most frequent haplotypes from Canary Islands (Fig. 4). Considerable differentiation was found in the sequence from El Hierro Island (AJ426626), which exhibit four mutational steps, being more distant than the haplogroup of Cabo Verde (two mutational steps). The samples from mainland Europe and Africa showed differentiation by five mutational steps from both haplogroups. The haplotype network of RAG2 corroborates with the view that sequences from Cabo Verde correspond to a nique haplotypes, two mutational steps apart from *H. sp.* from Morocco, *H. savii* from Switzerland, and *H. cadornae* from Laos and Cambodia (Fig. 4).

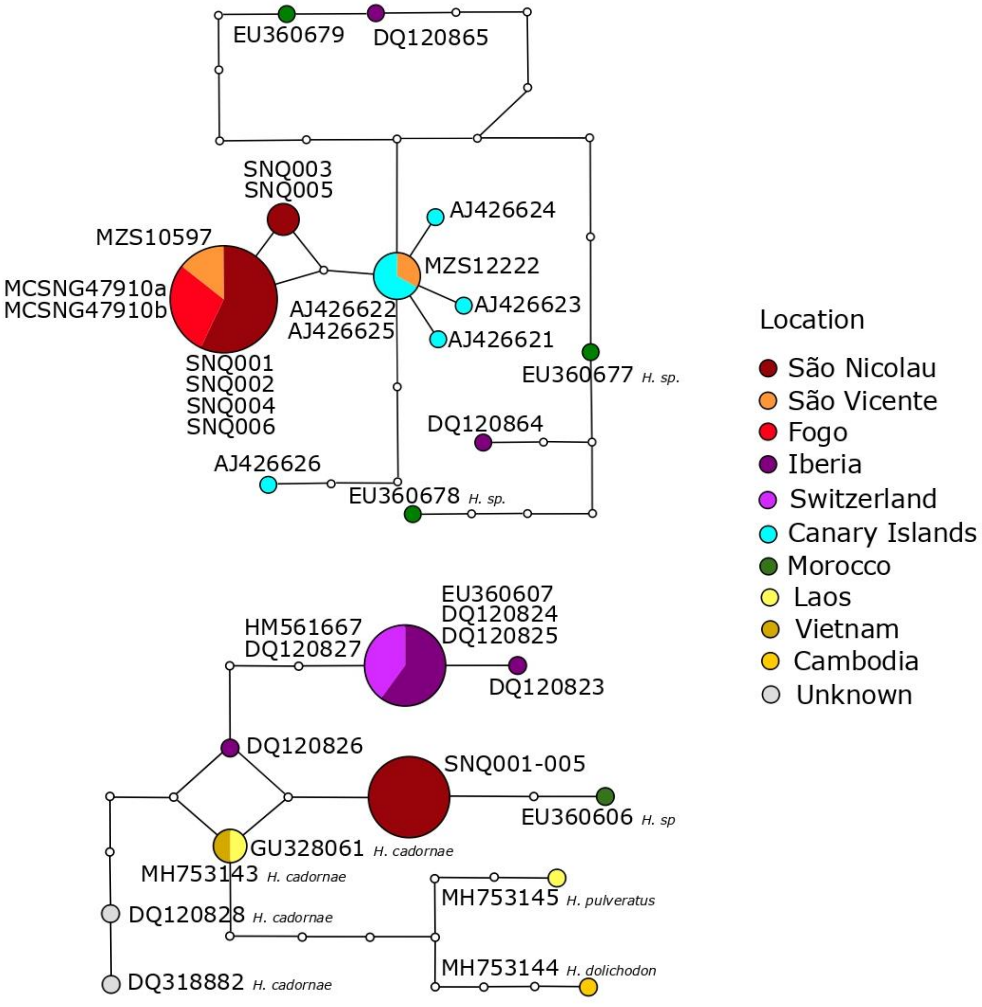

Fig. 4 – Parsimony networks including sequences similar to those of Hypsugo from Cabo Verde Islands. A) Network corresponding to the cyt *b* mtDNA and B) RAG2 nDNA sequences variation. Lines represent mutational steps, circles indicate haplotypes, and dots show missing haplotypes. The size of the circle is proportional to the number of individuals, and different colors correspond to different locations. For further details on the samples, see Fig. 1, Table 1 and S2.

A total of 37 polimorphic sites and 23 haplotypes were found in *Hypsugo* sequences (Table 3). The genetic diversity of each population regarding the haplotype (Hd) and nucleotide diversity (π) was high in the population of Canary Islands (CI) and the group of Morocco and South Iberia (MS) samples. The genetic diversity was relatively high in Europe (EU) and Eastern Europe (EE) populations (Table 3). The haplotype diversity of the Cabo Verde (CV) population was 0.200 (± 0.154) and the nucleotide diversity 0.00063 (± 0.0048).

Table 3 – Genetic Genetic diversity parameters calculated for *H. savii* cyt *b* mtDNA sequences for each clade represented in the phylogenetic tree. The number of sequences (N), polymorphic sites (S), and haplotypes (h), as well as haplotype (Hd) and nucleotide (π) diverssity, and Within Mean Group Distance (WMGD) with respective standard error (SD) are given.

Clade	Description	N	S	h	Hd ± SD	π ± SD	WMGD ± SD
CV	Cabo Verde	10	2	2	0.200 ± 0.154	0.00063 ± 0.00048	0.00 ± 0.00
CI	Canary Islands	6	11	6	1.000 ± 0.096	0.00612 ± 0.00197	0.01 ± 0.00
MS	Morocco and Southern Iberia	5	13	5	1.000 ± 0.126	0.00922 ± 0.00161	0.01 ± 0.00
EU	Europe	9	4	5	0.722 ± 0.159	0.00176 ± 0.00049	0.00 ± 0.00
EE	Eastern Europe	8	7	5	0.857 ± 0.108	0.00491 ± 0.00072	0.01 ± 0.00

The value of evolutionary divergence between groups showed that the most divergent was the EU clade when compared with MS. The closest related population was the CV and CI (Table 4).

Table 4 – Estimates of evolutionary divergence over sequence pairs between clades. Standard error estimates are shown above the diagonal.

Clade	Description	CV	CI	MS	EU	WE
CV	Cabo Verde	-	0.0025	0.0043	0.0098	0.0090
CI	Canary Islands	0.0065	-	0.0033	0.0086	0.0079
MS	Morocco and Southern Iberia	0.0188	0.0125	-	0.0099	0.0096
EU	Europe	0.0664	0.0503	0.0711	-	0.0090
EE	Eastern Europe	0.0616	0.0470	0.0668	0.0631	-

For *T. nudiventris* cyt *b* aligment, JModelTest program selected the GTR+I based on Akaike Information Criterion. The results of ML and the values of bootstrap are represented in Fig. 5. Samples from Cabo Verde formed a monophyletic well-supported clade (bootstrap values > 70).

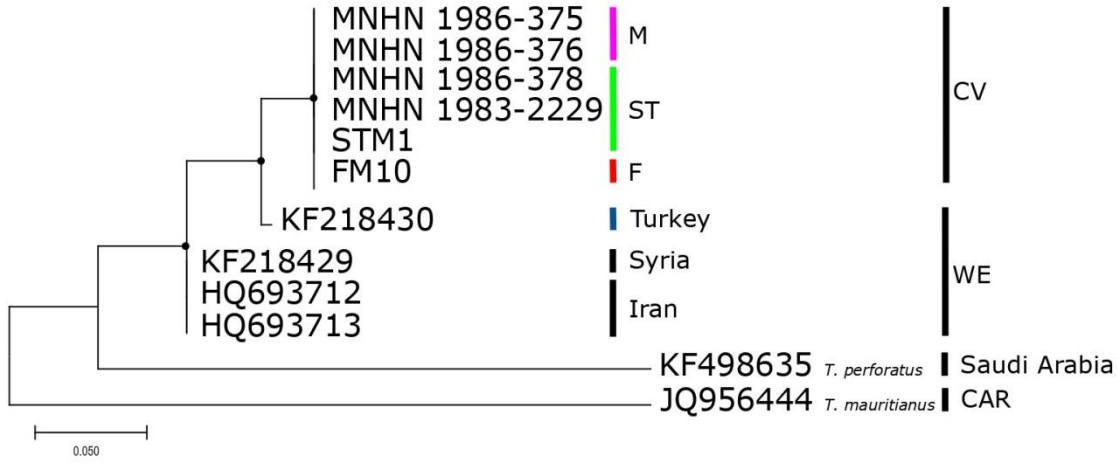

Fig. 5 – The evolutionary history was inferred by using the Maximum Likelihood phylogenetic tree rooted with *T. mauritianus* (outgroup). Black dots on the nodes indicate ML bootstraps values ≥ 70. Clades (M, Maio; ST, Santiago; F, Fogo; CV, Cabo Verde; EE, Eastern Europe) are coloured according to their geographic location corresponding to Fig. 6. CAR stands for Central African Republic.

The cyt *b* network of *T. nudiventris* showed that Cabo Verde samples from three different islands have the same unique haplotype separeted by four mutational steps from the close related sample from Turkey (Fig. 6).

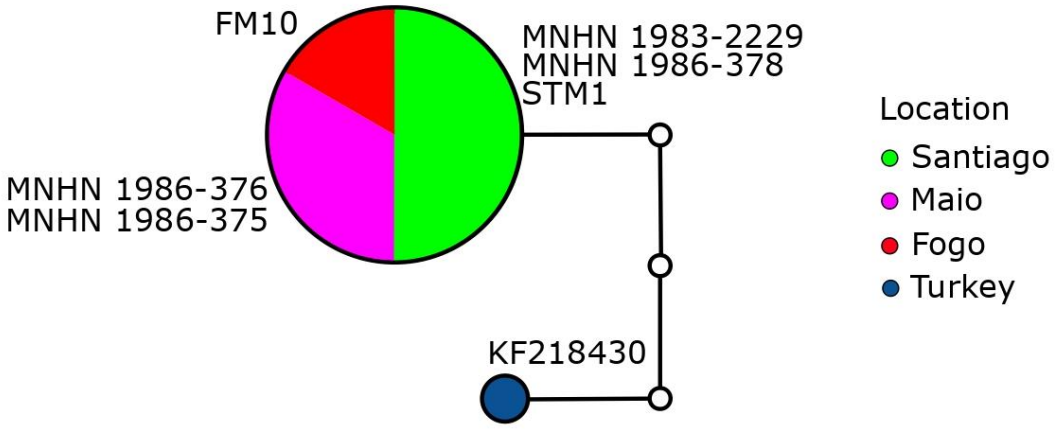

Fig. 6 – Parsimony network corresponding to the cyt *b* mtDNA sequences in *Taphozous nudiventris*. Lines represent mutational steps, circles indicate haplotypes, and dots show missing haplotypes. The size of the circle is proportional to the number of individuals, and different colours correspond to different locations. For further details on the samples, see Fig. 1, Table 1 and S2.

Regarding the cyt *b* network of *Miniopterus*, 21 haplotypes were found. The Santo Antão sample shared the same and most common ancestral haplotype with samples from Russia, Cyprus, Lebanon, France, Iberia, North Africa and Balkans (Fig. 7).

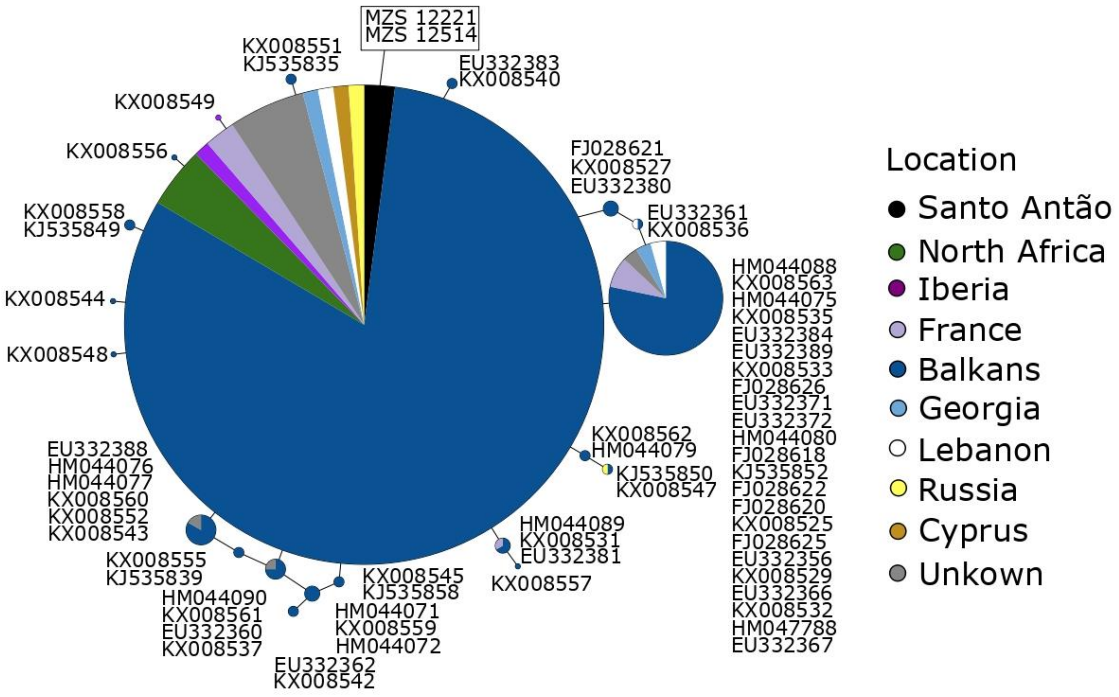

Fig. 7 – Parsimony network corresponding to the cyt *b* mtDNA sequences in *Miniopterus schreibersii*. Lines represent mutational steps, circles indicate haplotypes, and dots show missing haplotypes. The size of the circle is proportional to the number of individuals, and different colours correspond to different locations. The only amples codes corresponding to the main central haplotype that are given the ones from Cabo Verde; for further details on the samples, see Fig. 1, Table 1 and S2.

3. Acoustic analyses

Echolocation calls were recorded during free-flight and we could identify a typical frequency modulation (FM) and quasi constant frequency (qCF) conponents of *Pipistrellus kuhlii* calls for Santiago and Fogo islands. The peak frequency was around 39–42 kHz, the pulse duration was about 6ms, and the interval between pulses was between 100–200 ms (Fig. 8). We were also able to identify the species *Hypsugo savii* for Fogo Island. This pulse was characterized by a CF with an explosive start, peak frequency around 36 kHz, and pulse duration of 12 ms (Fig. 8). The species *Taphozous nudiventris* also appears in the records for Fogo Island, and was characterized by the CF pattern and the peak frequency around 25 kHz and interval between pulses of 300 ms (Fig. 8).

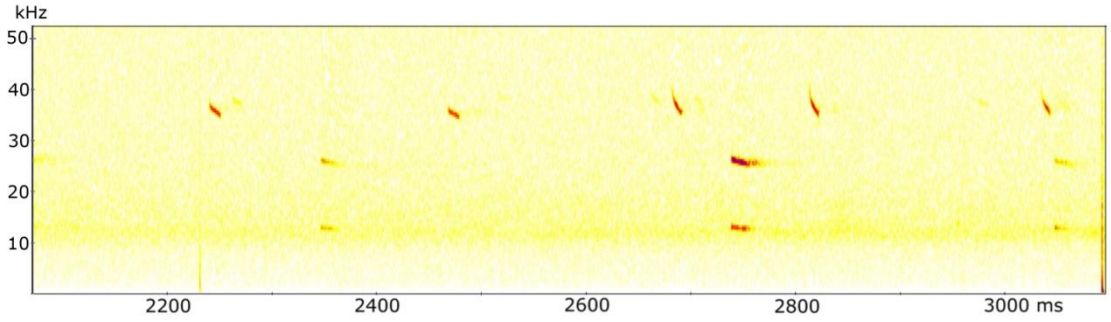

Fig. 8 – Examples of the recorded sonograms for *Pipistrellus kulli* (higher frequency) and *Taphozous nudiventris* (lower frequency). Records are available in Morphobank (P3514), for more details see table 1.

Discussion

1. Major highlights and findings

This study presents the first phylogenetic and network reconstruction of the *Hypsugo*, *Taphozous* and *Miniopterus* for the Cabo Verde Archipelago. It also expands the geographic distribution of *T. nudiventris* to Fogo Island and represent new morphological and acoustic data for this and the other bats species that may be useful for future systematic studies.

By the time that the first field expeditions were conducted in this archipelago, the species identification was based only on morphological characters using the European taxa as reference. The only exception was *Taphozous nudiventris*, which is associated with more arid zones of Africa continent. In Europe, high levels of cryptic diversity are associated to the climatic oscillations during the Pleistocene (Gómez & Lunt, 2006; Feliner, 2011). The Iberian, Italian and the Balkans peninsulas have long been reported as Pleistocene glacial refugia (Hewitt, 1996; Taberlet *et al.*, 1998; Jaarola & Searle, 2004; Pfenninger & Schwenk, 2007). Mediterranean Islands, including Sardinia, Corsica and the Balearics have also been described as centers of endemism (Maldonado, 1985; De Jong, 1998). For instance, the *P. auritus*, *P. kuhlii*, *H. savii* and *M. schreibersii* seem to have re-colonized Europe from Iberia and the Balkans (Ibáñez *et al*., 2006; Bilgin et al. 2016). These species appear to be a complexes of sympatric and cryptic species widespread throughout Europe, North Africa and parts of Asia.

Our results based on mtDNA and nDNA corroborate the presence of a divergent lineage of *H. savii* complex in the western Palaearctic detected by Mayer *et al.* (2007), which are spread across Canary Islands and North Africa to Turkey (Veith *et al*., 2011). Samples of *Hypsugo* from Cabo Verde Islands do not share haplotypes with specimens from Canary Islands, but form a monophyletic clade with those (see Fig. 3 and 4). These results points to an independent colonization event of Cabo Verde by Canarian ancestral about 0.049 Ma. However, one specimen from São Vicente Island (MZS 12222) grouped with individuals from Gran Canaria (AJ426622) and La Gomera (AJ426625). This specimen could have reached Cabo Verde by its own means, suggesting a second recent colonization event or it could have been introduced in the archipelago by passive transport from the Canary Islands, thus explaning no genetic differentiation between them. The genetic divergence between Cabo Verde samples and the Canary Islands, even though low comparing to other major clades, suggested that those lineages should be treated, at least, as different ESUs. Pestano *et al*. (2003)

found a significant differentiation between the Canarian population compared with mainland Spain, in particular on El Hierro Island, which showed more distant haplotypes within the Canarian haplogroup than from the most common Canarian haplotype to samples from Cabo Verde. Therefore, either there are more than one undescribed taxa on Canary Islands, or Canarian and Cabo Verdean populations should be described within the same taxon.

As explained in details in the introduction, some authors recognized *H. savii* as two or even more species (Ellerman & Morrison-Scott, 1966; Mayer *et al*., 2007; Dietz & Kiefer, 2014). There is a current discussion regarding the taxonomic status of *Hypsugo cf. darwinii*, which was first described to the North-Africa/Canarian group. Mayer *et al*. (2007) suggested provisional use of the name *Hypsugo cf. darwinii* for the Canary Islands and Morocco *Hypsugo* individuals based on significant mtDNA differences in respect to European mainland individuals. Dietz & Kiefer (2014) treated *H. darwinii* as a separated species and stated that it 'resembles' most closely the Savi's pipistrelle bat of Northern Africa, the Canary Islands, Sicily and Sardinia (Veith *et al.*, 2011). In the absence of formal designation of *H. darwinii* as a separated taxon, the species is currently not accepted as occuring in Europe. The fact that most Cabo Verde specimens presents unique haplotypes opens the question of considering them a different taxon, as they are as distant to the mainland European *H. s. ochromixtus* than individuals from Canary Islands, or treat them within *H. darwinii*. Our mtDNA and nDNA results support the validity of a Macaronesian-Magrebian taxon as a solution to resolve partially the current *H. savii* species complex.

The migratory behaviour of *H. savii* is unknown (Hutterer *et al*., 2005). Migration for long distances is suspected in Europe, even though the longest recorded movement of 250km has an unreliable origin (Dietz *et al*., 2009). The species may be good at colonizing islands, as it occurs in many Mediterranean islands, and it also have been report as a vagrant in the Great Britain (Reiter *et al*., 2010, Jahelkovà *et al*., 2014). In the Cabo Verde Archipelago *Hypsugo* was reported for northwestern and southern islands (Pestano *et al*., 2003; Vasconcelos, 2018). In this study, we prove that individuals from both island groups share the same haplotype, supporting the hypothesis of gene flow among relatively long-distanced populations. Thus, *Hypsugo* individuals from Southern Iberia Peninsula may have colonized the North of Africa by crossing the geographic barrier of Gibraltar Strait, and then the Canary and Cabo Verde Islands by stepping-stone in the Saharan Islands, existing between both archipelagos during the Pleistocene glaciations (Fernández-Palácios *et al*., 2010). In fact, García-Mudarra *et al*. (2008) found two samples of *H. savii* from Southern Iberia

clustering within the North African lineage. For this species, the Strait of Gibraltar represents a barrier in the distribution of lineages because it showed distinct haplotypes on each side of the Strait. However, some permeability occurs in a very low proportion of the haplotypes from Iberia that clustered within the Moroccan lineages (García-Mudarra *et al*., 2008). For bats, almost 30% of the species present in both continents showed discontinuities in their mtDNA (García-Mudarra *et al*., 2008).

As a biogeographic barrier, the Strait of Gibraltar and the Atlantic Ocean played important roles in shaping the diversity of the bat communities in both sides of the Mediterranean Sea and in the Macaronesia, respectively. Alternatively, *Hypsugo* individuals from Italian peninsula could have reached the north of Africa using Sicily as stepping-stone, as suggested by ND1 mtDNA data (Bogdanowicz *et al*., 2015) and then the Canary and Cabo Verde islands. As, unfortunately, we could not include Italian specimens in our mtDNA analyses, neither Canarian nor Italian specimens in our nDNA analyses, it was impossible to untangle between the two alternative scenarios.

Unfortunately, other lines of evidence do not help clarifying the taxonomic status of *Hypsugo* individuals from Cabo Verde. The echolocation records of *Hypsugo cf. darwinii* are closely related to those of *H. savii* (Russo & Jones, 2002; Papadatou *et al*., 2008; Barataud, 2015; Gianna *et al*., 2016). These two taxa seem to echolocate using almost identical calls, increasing the difficulties to identify *H. cf. darwinii* based only in acoustic surveys. Moreover, the identification through morphological measurements is difficult due to the lack of a modern morphological description (Gianna *et al*., 2016). Hair coloration is usually used as a taxonomic character, and *H. cf. darwinii* apparently has a dorsal lighter brown pelage with a small reddish brown spot between the ears, the corners of mouth and the shoulders (Gianna *et al*., 2016). The specimens caught in São Nicolau have a dark and long dorsal pelage with contrasting light golden tips. However, the reddish brown spot is not so evident as reported by Gianna *et al*., 2016. Another character very used to distinguish Hypsugo is the size and shape of the ear and tragus. Tragus are short and slightly broadening above, the length of the front margin of the tragus almost corresponding to its width, and the tip of the ear is broadly rounded in *H. savii* (Dietz & von Helversen, 2004). Unfurtunatly, we could not compare the measure of the ear in the museum specimens related to the individuals caught in the field. The material preservated was not in a good condition to see the details of the ear shape. The lenght of the forearm, fifth and third finger is within the range expected for *H. savii* from Iberia. And other measurement is not discriminative between *H. savii* and *H. cf. darwinii*.

The global wind circulation pattern may explain the routes colonization for flying animals. In the northern hemisphere, the wind flows to the right due to the Earth's rotation. It moves southwards towards the equator where the wind is deflected westwards. Because of that, the pattern of the colonization of Cabo Verde Archipelago may have occurred from north to southwestern from the Canary Islands. Thus, the most likely hypothesis is that the northern islands as Santo Antão were the first to be colonized followed by a stepping-stone colonization to other northwestern islands. In fact, there is an unconfirmed record of *Hypsugo* on Santo Antão (Vasconcelos, 2018) already present in all other northwestern Cabo Verde Islands, expect the extremely dry Desertas Islands. If, alternatively the closer ancestral is coming from mainland Africa. The colonization pattern of Cabo Verde Islands by bats hence appear to be different from expected by the theory of island biogeography, which predicts that larger islands and islands closer to the mainland receive more migrants, and present fewer extinctions and lower degree of divergent evolution (Whittaker & Fernández-Palacios, 2007).

Although Sal and Boavista islands are closer to the African mainland, few observations of bat were recorded, and all probably of vagrant individuals and none *Hypsugo* specimen (Vasconcelos, 2018). In fact, the migratory *E. helvum* is a common species broadly distributed across the lowland rainforest and savanna zones of Africa from Senegal in the west, through to South Africa in the south and Ethiopia in the east. However, only a single individual was found in Cabo Verde, Boavista (Hazevoet, 2014). These islands appear to have an unsuitable environment for the referenced bat species, as the climate is drier and the terrain is flatter compared with the northwestern and southern islands (Fig. 1). Little is known about the habitat requirements for *H. savii/ H. darwinii*, but these are found mainly in uplands and mountains, forages over open woodland, pasture and wetlands, what may explain the absence of *Hypsugo* records on those eastern islands.

Regarding the phylogeny of *T. nudiventris*, the lack of samples from Canary Islands or mainland Africa preclude any biogeographical interpretation of our results. The fact that Cabo Verde individuals present all the same unique haplotype could be interpreted as recent colonization of the islands or introduction of individuals from any of those geographical areas. It could be also the result of unsampled intra-archipelago diversity. When analyzing the distribution pattern of *T. nudiventris*, it appears to occur only in the southern islands as there is no record for this species in the northwestern or eastern islands until now. However, the distribution range of this species in the archipelago could be greatly underestimated due to the poor number of bat surveys in the country.

As this species is a typical representative of the African mainland, the most possible scenario is that the first islands to be colonized were the eastern or non western islands. This species is found in arid and semi-arid regions, and populations in northern Africa are found in Sahelian and Sudanian savanna zones (Bates & Harrison, 1997). The desert-like characteristics of Sal and Boavista and other dryer northwestern islands, such a São Vicente and the Desertas might provide a suitable habitat for *T. nudiventris* and settlement for immigrants from the mainland that could present different haplotypes from the ones sampled if speciation occurred.

In our acoustic data we found a typical call of *T. nudiventris* on Fogo Island, characterized by a peak frequency around 24 kHz CF, a pulse duration of 20 ms, and interval between pulse around 400 ms. This record, together with pellet samples, expanded the geographic range for this species westward. The morphological features important to discriminate species of *Taphozous* are the shape, length and width of the *baculum*. In addition to the pelage colour, which is pale light brown, tinged very light gray, while the ventral color is dirty white and tinged somewhat light pale brown (Aşan & Albayrak, 2007). Unfortunatelly, we could not analyze the *baculum* or even pelage colour from species held in the museum because of the poor conditions of the preserved material (see photos on Morphobank).

Hille *et al*. (2003) analyzing the genetic structure of other flying vertebrates (kestrel) found a reduced gene flow between Leeward and Windward populations and the same pattern was found in reptiles (Vasconcelos *et al*., 2013). Due to the lack of samples from Leeward Islands, we are hence unable to distinguish if *Taphozous* population of Cabo Verde represent an exotic native or taxa, what would represent diametrically opposite results for conservation purposes.

In conclusion, the scattered distribution of bats in Cabo Verde could be explained by recent colonization or introduction events. Most species did not had enough time to differentiate and colonize other islands. Individual might have reached the archipelago involuntarily by boats or storms (Chevalier, 1935; Pucetti & Zava, 1988). The bat colonization could be, in fact, only a few centuries old, considering the recent human occupation of the islands in the 15th, 16th, and 17th century. Alternatively, it is possible that adaptation to an arid oceanic island could have led to speciation of native cryptic species due to the large distance from the Africa mainland and between islands (Juste *et al*., 2004). Poor sampling effort also could explain their scattered distribution. In fact, the bat fauna has been continuously neglected in biodiversity surveys because of the difficulty to capture and identify bat species due to their low abundances and reduced

habitat availably. Each new sampling in the archipelago results into new occurrence records for some species or even new observations of Chiroptera taxa for the archipelago (e.g., Vasconcelos, 2018). Future studies are very important to fill the sampling gaps and to understand better the systematic position of chiropteran species.

2. Caveats and limitations

We were unable to amplify the DNA of MZS 1399, MNHN 1986-377, MNHN 1986-379 and MNHN 1983-1467. They were collect and deposited in 1909, 1968, 1969 and 1965, respectively, and the information about the methods used for conservation the biological material is not clear. The *P. austriacus* specimen captured in 1965 was probably kept in a liquid not appropriated for DNA preservation, as Juste *et al.* (2004) also tried to amplify fragments from the same specimen without success. Regarding the specimen SN0006, it was found and collect by local population, and it was already in a degraded stage. The amplification failure of MZS 1399 (*H. savii*) and MNHN 1983-377 (*T. nudiventris*), as well as SN0006, was not so detrimental to the analysis given that there are two other samples from the same island, and so we would probably recover the same haplotypes as the ones already detected. In the case of the latter two species, their presence in the islands was confirmed with acoustic records.

Mitochondrial DNA (mtDNA) has been a marker of choice for reconstructing historical patterns of population demography, admixture, biogeography and speciation (Hurst & Jiggins, 2005). Because the mtDNA has a high evolutionary rate, it allows to recovery the pattern of recent historical events without an extensive sequencing effort. Moreover, it has low recombination, the whole molecule can be assumed to have the same genealogical history. In fact, the use of mtDNA has been suggested for species taxonomic differentiation. However, recent review and theoretical studies showed the role of hybridization and introgression in the diversification of animals (Dowling & Secor, 1997). The gene exchange among related sexual species is documented for bats (Berthier *et al*., 2006). It should be kept in mind that taxonomic conclusions based only on mitochondrial genes may be misleading due to effects such as incomplete lineage sorting, introgression, and selection on mitochondrial loci. As a mitochondrial DNA is maternally transmitted, it reflects only a part of the genetic structure of species. This is especially true in the case of bats, in which males and females often exhibit contrasting dispersal pattern (Ruedi & McCracken, 2009). It would thus be necessary sequencing more nuclear makers and including samples from all identified clades to

better understand the evolutionary history of *Hypsugo*, *Taphozous* and *Miniopterus* in the western Palearctic.

Ancient DNAs hold tremendous potential for studies of phylogeny, biogeography and molecular evolution (Soltis & Soltis, 1993). However, extraction of DNA from preserved vouchers from museums is a challenge because not all preserved material yields DNA and the majority of the studies have reported substantial degradation, with most fragments ranging in size from 50–500 bp (Soltis & Soltis, 1993). The amplification generally proves more difficult, because of the presence of several PCR inhibitors. In fact, we could amplify only 121 bp of *Hypsugo* specimens from Museum, 120 bp of *Taphozous*, and 114 bp of *Miniopterus*. The size of the fragment is direct related to the amount of information for phylogenetic reconstruction.

3. Future studies

Additional sampling in Cabo Verde is essential to study the levels of genetic diversity in the archipelago and between islands. For *Hypsugo*, samples are required from the northern islands, mainly on Santo Antão where there are no records. However, is likely that the species occurs on this islands due to the habitat similarities and proximity to São Vicente and São Nicolau. In southern islands, more capture effort is necessary on Santiago Island. *Hypsugo* has similar environment requirements as *Pipistrellus*, so the latter species might also occur on this island. This would expand the geographic range for this species in the archipelago and may clarify the genetic diversity within the archipelago. Additional sampling is also necessary on Canary Islands, especially regarding nDNA. Our results based on mtDNA showed that Cabo Verde samples have a unique haplogroup, but one sample is included in the Canary Islands haplogroup. However, our results of nDNA lack information of this archipelago. In order to have a better picture of the genetic structure of *Hypsugo* species complex, more samples from Morocco, Iberia (both northern and southern), Italy (including the Mediterranean islands of Sicily and Sardinia), and the Balkans, mainly from Turkey, is required. Our results revealed that Hypsugo from Cabo Verde is close related to Hypsugo from Canary Islands, strongly suggesting that they represent different species. For future studies, the taxonomic status of *Hypsugo cf. darwinii* should be clarified in order to proceed conservation and ecological studies.

Regarding *Taphozous* additional information of the African population, especially from the western mainland, is crucial to identify potential distinct lineages in the Cabo Verde. In the archipelago, there are records only on southern islands. However, the species might also occurs on eastern islands. Thus, more fieldwork, mainly on Sal and

Boavista is fundamental to establish the geographic range and better understand the genetic structure within the archipelago. Regarding *Miniopterus*, unfortunately the DNA fragment amplified was very short to distinguish different haplotypes, due to the limitations of using voucher species. Because of that, we are not able to reconstruct a phylogenetic tree and establish the genetic relationships related to the lineages from western and eastern Europe, Middle East, Africa and Asia. Fresh samples would allow higher phylogenetic resolution.

This study also highlights the importance of the combination of different methodological approaches. Several studies have concluded that a combination of mist net and acoustic-monitoring surveys provide more complete bat inventories than employing one or the other technique alone (Kuenzi & Morrison, 1998; O'Farrell & Gannon, 1999). The use of acoustic method would decrease the number of surveys required to record the presence of some species. However, the species identification using echolocation calls may be difficult because of the environmental plasticity and similarities between some species (O'Farrell & Gannon, 1999). Capture surveys are vital component of bat inventories. Furthermore, techniques improvements of fossil DNA extraction and amplification could provide a better resolution of the systematic for those species by analyzing the genetic samples from museums, given the difficulty of bat sampling in Cabo Verde. In addition to the genetic and acoustic data, more studies in the morphological level would help clarify the differences between insular populations and those from mainland.

Recognizing distinct lineages is fundamental for conservation actions and little is known about the phylogenetic relationships and the genetic diversity of species present in Cape Verde. The archipelago may host a unique evolutionary lineage that is not yet recognized. In this study, we emphasize the importance of phylogenetic studies of bats in the Cabo Verde Islands, because they may be the only native mammals in the archipelago. We also highlight the importance of systematic status establishment as the first step for species conservation.

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Supplementary material

Appendix I– details on the molecular methods

S1 – Summary of all primers used by gene with the sequence, species in which they were used, the reference and type of PCR protocol.

Gene	Primers	Sequence (5'-3')	Species	Reference	PCR
cyt *b*	F_Hs	CCTTGCAATACACTATACATCAGAC	*H. savii*	This study	I
cyt *b*	R_Hs	CCCATAGTAAAGGCCCCGTC	*H. savii*	This study	I
cyt *b*	F_PPk_m	CGGATCCCTACTGGGCATTT	*P. kuhlii*	This study	I
cyt *b*	R_PPk_m	TCCTACGTGCAGGTATAAGCA	*P. kuhlii*	This study	I
cyt *b*	F_PPh	ACGCAATTCTACGATCAATCCCTA	*P. kuhlii*	This study	I
cyt *b*	R_PPh	AACTGGCTGTCCTCCAATTCAT	*P. kuhlii*	This study	I
cyt *b*	F_PLar_teSP	GCCATACACTACACATCAGACAC	*P. austriacus*	This study	I
cyt *b*	R_PLar_teSP	GATCCGTAGTAAAGGCCTCGG	*P. austriacus*	This study	I
cyt *b*	F_PLar_teCI	AGCACTACAAATCTTAACAGGACTT	*P. austriacus*	This study	I
cyt *b*	R_PLar_teCI	TGGAGGCTCCGTTAGCATGA	*P. austriacus*	This study	I
cyt *b*	F_PLar_teTR	AGCACTACAAATCTTAACAGGACTT	*P. austriacus*	This study	I
cyt *b*	R_PLar_teTR	GGAGGCTCCGTTAGCATGAA	*P. austriacus*	This study	I
cyt *b*	F_PLma	ATCATGATGAAACTTTGGATCTCTC	*P. austriacus*	This study	I
cyt *b*	R_PLma	TCTCGGCAAATATGGGTGACA	*P. austriacus*	This study	I
cyt *b*	F_PLas	CCTTTTGAGGGGCAACCGTA	*P. austriacus*	This study	I
cyt *b*	R_PLas	GAGTTAGCGTGGCCTTGTCT	*P. austriacus*	This study	I
cyt *b*	F_Msc_ma1	TCCAAAGACTCAAGGAAAGAGCA	*M. schreibersii*	This study	I
cyt *b*	R_Msc_ma1	ACATGCTACATATTCATGGGGA	*M. schreibersii*	This study	I
cyt *b*	F_Msc_ma2	TCCTGCTCTTCGCTGTAATAG	*M. schreibersii*	This study	I
cyt *b*	R_Msc_ma2	AGAATCGGGTAAGGGTTGCT	*M. schreibersii*	This study	I
cyt *b*	F_Tn	ACGCAAATGGGGCTTCCATA	*T. nudiventris*	This study	I
cyt *b*	R_Tn	CGGTTGCGCCTCAAAAAGAT	*T. nudiventris*	This study	II
RAG2	RAG2-F1	GGCYGGCCCAARAGATCCTG	*H. savii*	Baker *et al*., 2000	II
RAG2	RAG2-R2	GRAAGGATTTCTTGGCAGGAGT	*H. savii*	Baker *et al*., 2000	II
RAG2	RAG2-R1	AACYTGYTTATTGTCTCCTGGTATGC	*H. savii*	Baker *et al*., 2000	II
RAG2	RAG2-F1int	GRACAGTCGAGGGAARAGCATGG	*H. savii*	Baker *et al*., 2000	II
cyt *b*	CB1	CCATCCAACATCTCAGCATGATGAAA	*H. savii*	Unknown	III
cyt *b*	cytb2	CCCTCAGAATGATATTTGTCCTCA	*H. savii*	Unknown	III
cyt *b*	cb2F	TGAGGACAAATATCATTCTGAGGG	*H. savii*	Unknown	III
cyt *b*	CB3H	GGCAAATAGGAARTATCATTC	*H. savii*	Unknown	III
cyt *b*	Molcit-F	AATGACATGAAAAATCACCGTTGT	*M. schreibersii*	Puechmaille *et al*., 2014	I
cyt *b*	MVZ-16	AAATAGGAARTATCAYTCTGGTTTRAT	*M. schreibersii*	Puechmaille *et al*., 2014	I

Saline extraction protocol

A small amount of tissue was taken and cut into fine pieces using a sterile scalpel blade, the contents were transferred into a labeled 1.5 ml eppendorf tube. Then, 600 µl of lysis buffer and 10 µl of proteinase K were added to each tube. The samples were vortexed several times and incubated at 56°C overnight. After that, samples were put in the fridge at 4°C for 10 min, and 300 µl of ammonium acetate was added. The samples were centrifuged for 15 min at 14000 rpm and kept at 4°C. The supernatant was transferred to a new 1.5 ml eppendorf tube, and 600 µl of ice-cold isopropanol was added and mixed by inverting the tubers several times. The samples were put in the freezer overnight and then centrifuged for 30 min at 14000 rpm. The supernatant was discarded and 1000 µl of ice-cold ethanol (70%) was added and mixed by tapping with the finger at the bottom until the DNA pellet was released. The tubes were centrifuged for 15 min at 14000 rpm. The supernatant was rapidly discarded and the tubes were incubated for evaporation at room temperature until complete evaporation. After that, 50 µl of ultra-pure water was added and the samples were leaved to hydrate at ambient temperature overnight.

Ancient DNA extraction protocol

The samples were cut in small pieces and about 50 mg was added to a 2.0 ml eppendorf tube together with 1 ml of extraction buffer (745 µl H_2O; 9 ml EDTA 0.5 M (pH=8.0); 250 µl Proteinase K 10 mg/ml; 5 µl Tween 20) they were left overnight at 37°C in the incubator. In the next day, the samples were spun for 2 min at maximum speed and the supernatant was transferred to a 50 ml falcon tube containing 10 ml Binding Buffer (23.88 g Guanidine hydrochloride; 30 ml H_2O; 50 ml Isopropanol; 25 µl Tween 20) and 400 µl 3M sodium acetate. A MinElute spin column was separated for each sample and an extension reservoir of a V-spin column was forced into the opening of the MinElute tube. The extension reservoir/MinElute assembly was removed from the collection tube and placed into a 50 ml falcon tube.

The solution with Binding buffer mixture was transferred into the extension reservoir, then it was centrifuged for 4 min at 1500 rpm and then for 2 min at 1500 rpm. The extension reservoir/spin column assembly was placed back into the collection tube and a dry spin was performed for 1 min at 6000 rpm in a centrifuge, then the flow-through was discarded. After that, 750 µl PE buffer was added and the samples were centrifuged at 6000 rpm for 30 s and the flow-through was discarded, this last step was repeated. After that, the spin column was turned and a dry spin was performed for 1 min at 13000 rpm, the spin column was then transferred into a 1.5 ml tube with the cap

ripped off. Next, 25 µl TET buffer (~49.4 ml H_2O; 100 µl EDTA 0.5 M (pH=8.0); 500 µl Tris-HCl 1 M (pH=8.0); 25 µl Tween 20) was added on top of the silica membrane and left standing for 5 min before centrifugation at 13000 rpm during 30 s. After that, more 25 µl of TET was added and the centrifugation was repeated. The 50 µl of the extraction was transferred to a fresh tube labeled with sample code.

DNA extraction with sillica protocol

The feces were preserved in ethanol 96% and frozen. In order to proceed the DNA extraction, the samples were left in the greenhouse overnight to evaporate de ethanol. The entire process was done with filter tips and a negative control was prepared for the set of samples. The first step was adding 20 ml of PBS solution (6.08 g KH_2PO_4; 8.62 g NaCl and 1000 ml H_2O) in a 50 ml falcon with the scat (entire pellet). The material was wash vigorously with a Pasteur pipette to promote the epithelial cells elution. About 15 ml of the supernatant was transferred to a 15 ml falcon and centrifuged at 4000 rpm during 20 min. After that, the supernatant was discarded and 2 ml of buffer L6 (147.8 g Guanidine Thiocyanate; 25 ml Tris-HCl 1 M (pH=6.4); 10 ml EDTA 0.1 M (pH=8.0); 3.25 ml Tríton X-100 and 250 ml H_2O) was added to the pellet and the solution was vortexed. Then, the samples were incubated overnight at room temperature on constant agitation.

In the next day, they were centrifuged at 4000 rpm at 15 min, the supernatant was transferred to a new 15 ml falcon, 250 µl of silica solution was added and vortexed during 2 min to promote the bond of DNA molecules with silica solution. Then, the solution was centrifuged during 2 min at 4000 rpm. After that, the supernatant was discarded and 2.5 ml of buffer L2 (350 g Guanidine Thiocyanate; 59.2 ml Tris-HCl (pH=6.4); 23.7 ml EDTA (pH=8.0) and 592 ml H_2O) was added to each sample and vortexed until the pellet dissolution. The tubes were centrifuged at 4000 rpm during 2 min, this last step was then repeated in order to clean the DNA solution. Then, 4 ml of ethanol 80% was added to dissolve the entire pellet and the samples were centrifuged again at 4000 rpm during 2 min.

The tubes were left in the greenhouse at 60°C to dry the pellet and remove all the ethanol. The DNA elution was made with 500 µl of ultra-pure water, the material was vortexed to dissolve the pellet and incubated at room temperature. After that, the solution was centrifuged at 4000 rpm during 10 min, the supernatant was recovered and transferred to a new eppendorf 1.5 ml. In order to remove all silica residuals, the samples were centrifuged at 8000 rpm during 1 min. The supernatant was then transferred to a column and centrifuged at 13000 rpm during 10 min to purify the DNA.

The fluid was discarded, 500 µl of ultra-pure water was added to the column and centrifuged at 13000 rpm during 15 min. Then, the final step was adding 120 µl of ultra-pure water to a new tube, invert the column and centrifuge at 14000 rpm during 1 min. For the Silica Solution preparation, 6 g of Silica was dissolved in 50 ml of distilled water. The solution was incubated at room temperature wrapped in aluminium foil to avoid light during 24 hours to promote silica precipitation. The supernatant was discarded and 50 ml of distilled water was added, the solution was vortexed until the dissolution of the silica pellet. Then it was incubated at room temperature covered by aluminium foil during 5 hours to promote de silica precipitation. The supernatant was discarded (about 44 ml) and 60 µl of HCL 10 M (37%) was added.

S2 – Summary of GenBank sequences used in this study. The species name, marker, country of origin and corresponding reference of each sequence is also given.

Species	Gene	Code	Country	Reference
H. savii	cyt *b*	KX375197	Cyprus	Benda *et al*., 2016
H. savii	cyt *b*	KX375196	Montenegro	Benda *et al*., 2016
H. savii	cyt *b*	KX375195	Greece	Benda *et al*., 2016
H. savii	cyt *b*	KX375194	Syria	Benda *et al*., 2016
H. savii	cyt *b*	DQ120866	Greece	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120865	Southern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120864	Southern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120863	Southern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120862	Southern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120861	Southern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120860	Southern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120859	Northern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120858	Northern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	DQ120857	Northern Iberia	Ibáñez *et al*., 2006
H. savii	cyt *b*	KF218377	Turkey	Çoraman *et al*., 2013
H. savii	cyt *b*	KF218376	Turkey	Çoraman *et al*., 2013
H. savii	cyt *b*	KF218375	Iran	Çoraman *et al*., 2013
H. savii	cyt *b*	AJ504450	Switzerland	Stadelmann *et al*., 2004
H. savii	cyt *b*	AJ426626	Canary Islands, El Hierro	Pestano *et al*., 2002
H. savii	cyt *b*	AJ426625	Canary Islands, La Gomera	Pestano *et al*., 2002
H. savii	cyt *b*	AJ426624	Canary Islands, La Gomera	Pestano *et al*., 2002
H. savii	cyt *b*	AJ426623	Canary Islands, Tenerife	Pestano *et al*., 2002
H. savii	cyt *b*	AJ426622	Canary Islands, Gran Canaria	Pestano *et al*., 2002
H. savii	cyt *b*	AJ426621	Canary Islands	Pestano *et al*., 2002
H. savii	cyt *b*	AJ426620	Spain	Pestano *et al*., 2002
H. sp. C1	cyt *b*	EU360677	Morocco	García-Mudarra *et al*., 2009
H. sp. C2	cyt *b*	EU360678	Morocco	García-Mudarra *et al*., 2009
H. sp. C4	cyt *b*	EU360679	Morocco	García-Mudarra *et al*., 2009
H. savii	RAG2	EU360607	Northern Iberia	García-Mudarra *et al*., 2009
H. savii	RAG2	DQ120827	Switzerland	Ibáñez *et al*., 2006
H. savii	RAG2	DQ120826	Northern Iberia	Ibáñez *et al*., 2006
H. savii	RAG2	DQ120825	Southern Iberia	Ibáñez *et al*., 2006
H. savii	RAG2	DQ120824	Southern Iberia	Ibáñez *et al*., 2006
H. savii	RAG2	DQ120823	Southern Iberia	Ibáñez *et al*., 2006
H. savii	RAG2	HM561667	Switzerland	Roehrs *et al*., 2010
H. cadornae	RAG2	MH753143	Vietnam	Goerfoel & Csorba, 2018
H. cadornae	RAG2	DQ318882	Unknown	Ibáñez *et al*. unpublished
H. cadornae	RAG2	DQ120828	Unknown	Ibáñez *et al*., 2006
H. cadornae	RAG2	GU328061	Laos	Lack *et al*., 2010
H. pulveratus	RAG2	MH753145	Laos	Goerfoel & Csorba, 2018
H. sp. R1	RAG2	EU360606	Morocco	García-Mudarra *et al*., 2009
H. dolichodon	RAG2	MH753144	Cambodia	Goerfoel & Csorba, 2018
T. nudiventris	cyt *b*	KF218430	Turkey	Çoraman *et al*., 2013
T. nudiventris	cyt *b*	KF218429	Syria	Çoraman *et al*., 2013

T. nudiventris	cyt *b*	HQ693713	Iran	Ruedi *et al*., 2012
T. nudiventris	cyt *b*	HQ693712	Iran	Ruedi *et al*., 2012
T. perforatus	cyt *b*	KF498635	Saudi Arabia	Memish *et al*., 2013
T. mauritianus	cyt *b*	JQ956444	Central African Republic	Maganga *et al*., 2014
M. schreibersii	cyt *b*	FJ028649	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028648	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028647	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028646	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028631	Turkey/Georgia	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028630	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028629	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028628	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028627	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028626	Georgia	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028625	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028624	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028623	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028622	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028621	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028620	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028619	Turkey	Furman *et al*., 2009
M. schreibersii	cyt *b*	FJ028618	Turkey	Furman *et al*., 2009
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Appendix II – Presentations in congresses and conferences

Bats out of Africa: disentangling the systematic position of bats in Cabo Verde

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Introduction

The West African region possesses one of the largest **knowledge gaps** in the distribution and taxonomy of bats, and Cabo Verde Islands are no exception.

Some preliminary data indicate that there are **five species** identified as:

Hypsugo savii (Bonaparte, 1837)	**Vagrants**
Pipistrellus kuhlii (Kuhl, 1817)	Molossidae (indeterminate)
Taphozous nudiventris Cretzschmar (1830)	***Eidolon helvum*** (Kerr, 1792)
Plecotus austriacus (Fischer, 1829)	
Miniopterus schreibersii (Kuhl, 1817)	

The Cabo Verde Archipelago is in the **Macaronesian** region.

Formed by 10 volcanic islands, ~570 km from the African mainland (**Figure 1**).

Objectives

Revise the systematic status of the Cabo Verde bats based on molecular markers, morphological and acoustic data. More specifically, this project aims to:

1) identify the **systematic position** of Cabo Verde bats in relation to their Macaronesian and African counterparts

2) understand the **structure of the populations** within the archipelago

3) determine the **native** vs **exotic** status of the species

Methods

Field work performed on **three islands**: S. Nicolau (November 2015), Fogo and Santiago (June 2018).

Ultrasound surveys made every night; **mist-nets** set close to roost sites, and **faecal samples** collected in roosts.

Museums visited for morphological data and DNA tissue samples: Natural History in Genova, MCSNG, and Firenze, MZS (September 2018).

Calls analyzed with Bat Sound v.4 software (**Figure 2**).

DNA samples in process of analyses.

P. kuhlii captured in São Nicolau Island

P. kuhlii analyzed from MZS Firenze, Italy

Mist-nets set in São Filipe city, Fogo Island

Preliminary Results and Discussion

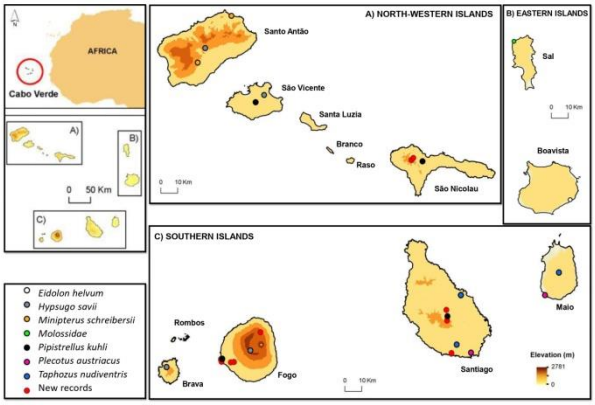

Figure 1: Cabo Verde Islands and summary of bibliographic reviews and new records for all species of bats registered in the arquipelago

- **New records** of *Taphozous nudiventris* for Fogo Island and possibly a species of Molossidae (**Figure 1**).
- The most **widespread** species is *Pipistrellus* c.f. *kuhlii* with scattered distribution.
- Morphology: *H. savii* from São Vicente has forearm longer (average 1.35mm) than maximum values known for the species.
- Preliminary molecular results suggest the **need of taxonomical reviews**.
- This study will be a **milestone** for starting bat research in the country and future work aims promoting **conservation actions** for the group.

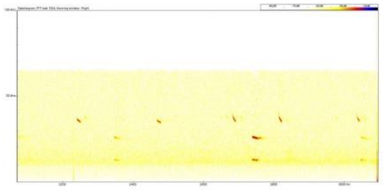

Figure 2: Records of *Taphozus nudiventris* and *Hypsugo savii*

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S3 – Banner presented in the VII SECEMU (Spanish Association for the Conservation and Research of Bats) conference, Gibraltar 2018.