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<http://www.italian-journal-of-mammalogy.it>

doi:10.4404/hystrix-00237-2019

Research Article

Maltese bats show phylogeographic affiliation with North-Africa: implications for conservation

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Keywords:

Malta
Pipistrellus kuhlii
 16S DNA
Plecotus gaisleri
Pipistrellus pipistrellus
Hypsugo savii

Article history:

Received: 30 August 2019

Accepted: 23 December 2019

Acknowledgements

This study was supported by the Environment Fund, administered by the Ministry for the Environment, Sustainable Development and Climate Change Malta. F.B. is supported by Research Foundation Flanders (FWO). We thank FKNK and the private landowners Ms. Saliba and Mr. Bugeja for allowing access to their property for this study. We thank Vleermuizenwerkgroep Natuurpunt (Belgium) for logistical support. We thank all the volunteers from Green House and BINCO that helped with the field work. Special thanks to volunteers from Nature Trust, especially Ms. Garrovillo and Ms. Azzopardi who joined the surveys and provided us with samples from animals in their care. Furthermore, we would like to thank Simone Cutajar and Merlijn Jocqué for contributing to the overall study design and logistic support, and Jaap van Schaik for comments on an early draft.

We thank four anonymous reviewers for their constructive comments on an earlier version of the manuscript.

Data Accessibility

Nucleotide sequences for the new haplotypes recorded during this study were deposited in GenBank under accession no. MN685103–MN685107. The final alignment is deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S25351>).

Abstract

In the Mediterranean region, cryptic diversity of bats is common. As distinct genetic lineages should be managed independently for conservation, insight into bat phylogeography is important. The Maltese islands are located in the centre of the Mediterranean between North Africa and Sicily and are densely populated. At present, it is thought that at least seven species of bats are native, but phylogeographic affiliations remain largely unexplored. Therefore, we sequenced a ca. 540 bp fragment of the mitochondrial 16S rRNA gene from 23 bats, which were captured during the citizen-science project Akustika. We found two morphologically cryptic lineages common in North Africa, *Plecotus gaisleri* and a mainly North-African lineage of *Hypsugo savii* (named *Hypsugo cf. darwini* in some recent studies). We also recorded two *Pipistrellus* species. The *P. kuhlii* haplotype belonged to a lineage present in North-Africa and across the Mediterranean. Within *P. pipistrellus* we found two novel haplotypes that clustered within a North-African clade, well distinguished from the European haplotypes. Our results highlight the historic connection between the bat fauna of the Maltese Islands and North Africa. Malta is one of the few regions in the European Union where *P. gaisleri* and the North-African clades of *P. pipistrellus* and *H. savii* occur. Hence, Malta has an exceptionally high responsibility for the conservation of these taxa in Europe.

Introduction

Cryptic diversity is common in bats, and the taxonomy of several clades, as well as phylogeographic patterns, remain to be resolved (Mayer et al., 2007; Mayer and von Helversen, 2001; Çoraman et al., 2019; Amorim et al., 2019). During the Pleistocene glacial maxima, European bat species were restricted to refugia in the Iberian and Italian Peninsula, in the Balkans and in the Caucasus (van Schaik et al., 2018; Razgour et al., 2015; Ruedi et al., 2008). However, North Africa was also an important glacial refugium for European bats (Husemann et al., 2014) and today harbours numerous endemic lineages (Puechmaile et al., 2014; Benda et al., 2004b; De Jong, 1998). As a result, bat lineages evolved, which in many cases were morphologically similar but genetically well differentiated, such as in the Natterer's bat (*Myotis nattereri* complex; e.g. Çoraman et al., 2019; Juste et al., 2019).

In addition to these glacial refugia, the Mediterranean islands are also hotspots for the biodiversity of bats, which makes them particularly

interesting regions to study cryptic diversity (Puechmaile et al., 2012a; Bruyndonckx et al., 2010; Benda et al., 2008). For example, in Sardinia there are three species of *Plecotus* (Mucella et al., 2002), one of which is endemic to the island (Sardinian long-eared bat, *P. sardus*), while another has a unique genetic lineage in Sardinia (brown long-eared bat, *P. auritus*). On the same island, Veith et al. (2011) demonstrated an unexpected diversity in the genera *Pipistrellus* and *Hypsugo*.

Insight into phylogeographic affiliations of bats is crucial to adequately establish conservation plans, as Evolutionarily Significant Units (ESUs) should be managed independently (Crandall et al., 2000). Endemic and cryptic species are often habitat specialists and locally adapted, making them especially susceptible to land-use change and other anthropogenic stressors (Grill et al., 2007; Munguira, 1995). These effects act increasingly on islands, where habitat availability is limited and populations are generally small (Grill et al., 2007). Bat populations on islands can have a different habitat use than conspecifics on the mainland, which can be highly relevant for conservation (Davy et al., 2007; Ancillotto et al., 2014). Conenna et al. (2017) concluded that bat species endemic to islands tend to be more threatened than continental ones. However, sufficient knowledge is still lacking.

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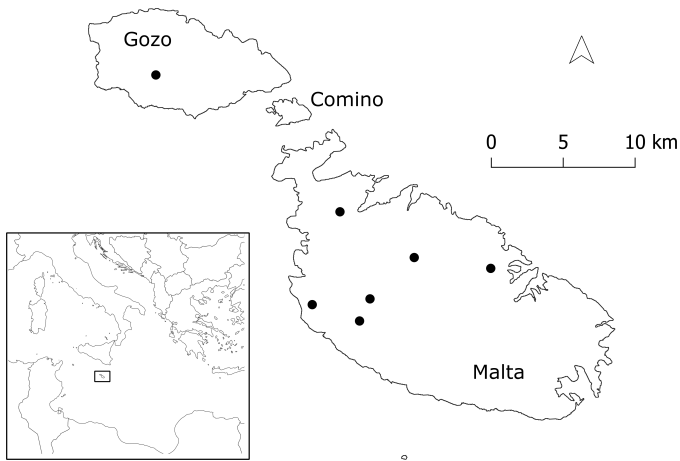


Figure 1 – Map of Malta. Mist netting locations are indicated with dots. Details of the sampled bats, including geographic coordinates, can be found in the supplementary material (Tab. S2).

Malta is an archipelago of islands (316 km²; Fig. 1) between Sicily and North Africa. It ranks among the world's top ten most densely populated countries, with a density of more than 1500 people per square kilometre in 2018 (World Bank, 2018). The intensification of agriculture as well as urbanisation are the largest anthropogenic stressors to Malta's natural environment; both are expected to even increase in the future (Cassar et al., 2008). Malta's fauna and flora consists of a unique mixture of South-European, North African, as well as endemic species. For example, the painted frog (*Discoglossus pictus*) occurs only in the Maghreb, Sicily and the Maltese islands, while four subspecies of the Maltese wall lizard (*Podarcis filfolensis*) are only found in specific parts of the Maltese Archipelago (a fifth subspecies is confined to the Italian islands of Linosa and Lampione; Speybroeck et al., 2016). Within the Maltese butterflies, several species such as the wall brown (*Lasionmata megera*) and the swallowtail (*Papilio machaon*) belong to European lineages, while others, such as the southern blue (*Polyommatus celina*) belong to North African clades (Vodá et al., 2016, 2015).

Currently, seven resident bat species are known to occur on the Maltese islands. Recent scientific publications and reports list the common pipistrelle (*Pipistrellus pipistrellus*), Kuhl's pipistrelle (*P. kuhlii*), Savi's pipistrelle (*Hypsugo savii*), the lesser horseshoe bat (*Rhinolophus hipposideros*), the Maghreb mouse-eared bat (*Myotis punicus*), Gaisler's long-eared bat (*Plecotus gaisleri*) and the European free-tailed bat (*Tadarida teniotis*) as confirmed species (J.J. Borg, unpublished data; and see Mifsud and Vella, 2019; MEPA, 2010; Borg et al., 1997). The noctule (*Nyctalus noctula*) and the soprano pipistrelle (*Pipistrellus pygmaeus*) have also been listed in a governmental report (ERA, 2018), which in addition refers to the grey long-eared bat (*Plecotus austriacus*) rather than to *P. gaisleri*. Such discrepancies among publications confirm the need for research on bat taxonomy and diversity in Malta, an important requisite for adequate conservation efforts.

We here explore Malta's bat diversity using mitochondrial DNA sequences, focusing on the genera *Plecotus*, *Hypsugo* and *Pipistrellus*. For this purpose, we collected faecal samples for non-invasive DNA barcoding (Puechmaile et al., 2007).

Material and Methods

Fieldwork

Bats were captured during a mist netting survey within the citizen science project Akustika, which aims to map and monitor Maltese bat populations through the recruitment and training of volunteers. Mist nets (Ecotone, Poland) were put up at seven sites, of which six were in Malta and one in Gozo (Fig. 1). Each site was sampled for one night between the 3rd and 11th of September 2018. Basic biometrics such as forearm length (measured with a calliper to the nearest 0.1 mm), body mass (measured with a field balance to the nearest 0.01 g), as well as sex and age were collected on site. Age was determined as either adult or young-of-year (YOY) based on the ossification of epiphyseal joints (Brunet-Rossini and Wilkinson, 2009). If possible, faecal samples were collected and stored in pure ethanol. All bats were released unharmed at the capture site within 10 minutes of capture. Mist netting was performed with the permission of the Environment and Resources Authority (ERA) in Malta (permit number NP 0221/18) and samples were obtained with a Nagoya protocol permit from the ABS Competent Authority in Malta (reference number ABS-PIC-2018-0002). Additional samples were obtained from volunteers from Nature Trust Malta, who rehabilitate wounded bats under license.

DNA extraction, sequencing

DNA was extracted following Veith et al. (2011). We sequenced ca. 540 bp of the mitochondrial 16S rRNA gene using the primers 16SPle1+ and 16SPle4- of Spitzenberger et al. (2006) and following the PCR protocol of Dondini et al. (2016).

We combined one representative of each haplotype found by us with sequences from European and North African species for the genera *Pipistrellus* and *Hypsugo* (from Veith et al., 2011) and *Plecotus* (from Benda et al., 2004b) into a single alignment, with special emphasis on species known to occur in the Mediterranean region. For species outside this region, we added only one or two representative sequences. Homologous sequences of Bechstein's bat (*Myotis bechsteinii*) and Schreiber's bent-wing bat (*Miniopterus schreibersii*) were included as outgroups (for GenBank accession numbers see Supplemental Material S1). Sequences were aligned with the ClustalW algorithm in Mega X (version 10.0.5; Kumar et al., 2018). The beginning and end of the alignment were trimmed in order to compare sequences of equal length. We afterwards improved the alignment by eye for each genus separately in regions where the automatic alignment failed to align unambiguously. This allowed us to keep all base positions rather than to delete such regions as bad alignment. We afterwards deposited the alignment in TreeBASE (<http://purl.org/phylo/treebase/phyloWS/study/TB2:S25351>). We determined the best fitting out of 24 substitution models in Mega X and by applying the AICc criterion for model selection (the selected model is specified in Fig. 2–4). We calculated a maximum likeli-

Table 1 – Species identification of the 17 samples that gave good sequence quality (n=number of individuals); sex and age (adult or YOY) as well as mean and range of right forearm length (FA) and body mass are given.

Species	n	♀-♂	Adult-YoY-NA	mean FA (mm) [range]	mean mass (g) [range]
<i>Plecotus gaisleri</i>	5	5–0	5–0–0	40.1 [39.8–40.5]	8.27 [8.01–8.61]
<i>Hypsugo savii</i>	3	2–1	1–1–1	32.0 [31.1–32.9]	6.24 [5.75–6.73]
<i>Pipistrellus pipistrellus</i>	8	5–3	6–2–0	30.23 [27.4–31.3]	4.14 [3.45–4.49]
<i>Pipistrellus kuhlii</i>	1	0–1	1–0–0	33.28	4.65

hood (ML) phylogenetic tree in Mega X using the inferred substitution model, with 2000 bootstrap replicates, an automatically generated initial tree and the nearest neighbour interchange (NNI) branch swapping algorithm being applied. In addition, we calculated a neighbour-joining (NJ) tree in Mega X using the Tamura and Nei (1993) evolutionary distances estimated by the maximum composite likelihood approach (Tamura et al., 2004) and using the estimated gamma model; again we calculated 2000 bootstrap replicates.

Results

We collected 17 faecal samples from 25 bats caught during mist netting, three faecal samples from rehabilitated bats, and two tissue samples from previously deceased bats. After DNA barcoding, 17 gave sequences of good quality. They were identified as belonging to four species (Tab. 1 and S2) (GenBank accession numbers MN685103–MN685107).

Our *Plecotus* samples belong to *P. gaisleri* (Fig. 2); they correspond to a single haplotype, PtenaisHT2 (AY531624), previously recorded only in Tripolitania, Libya (Benda et al., 2004b).

The three *Hypsugo* samples belong to *H. savii* (Fig. 3) and are identical to haplotype Hsav-II-7 (HQ848778) of Veith et al. (2011), which has previously been found on Sicily (Veith et al., 2011) and the island of Montecristo (Dondini et al., 2016). This haplotype clusters within a clade of North African, Canarian and Turkish haplotypes (Hsav-II-group; Fig. 3) and is well differentiated from the European haplotypes of *H. savii sensu stricto* (Veith et al., 2011).

Our eight *Pipistrellus* samples belong to two species, namely *P. kuhlii* and *P. pipistrellus*. The single *P. kuhlii* sample is identical to the haplotype P cf. des-1 and clustered together with samples from North Africa and from across the Mediterranean (Fig. 4). This haplotype was previously found in Morocco, Italy and Albania (HQ848768; KU058655; MF078006). The samples of *P. pipistrellus* belong to two new haplotypes that are most similar to Ppip-II-6 (HQ848759) and Ppip-II-7 (HQ848760) (Fig. 4), both belonging to a Sicilian, Sardinian and North African clade (Ppip-II; Veith et al., 2011).

Discussion

Our genetic analysis of samples of Maltese bats of *Plecotus gaisleri*, *Hypsugo savii*, *Pipistrellus kuhlii* (cf. *deserti* according to Veith et al., 2011) and *Pipistrellus pipistrellus* showed, across all species, relations to respective intra-generic clades with strong biogeographic affiliations to North Africa. This highlights the historic connection between the bat fauna of the Maltese Islands and North Africa. Our results thus complement the confirmed resident bat *Myotis punicus*, a North African species within the *M. myotis* group (Dietz and Kiefer, 2016; Baron and Borg, 2011). Future studies, using multiple markers and a broader sampling across North Africa and Mediterranean islands could clarify the timing and patterns of these colonisations. Additionally to genetic markers, acoustics patterns, especially using social calls can help to resolve the history and evolution of insular bat taxa (e.g., Nardone et al., 2016; Georgiakakis and Russo, 2012; Bohn and Gillam, 2018).

Apart from the mist-netting, we also obtained samples from rehabilitated bats. This study provides an example of the importance of wildlife rescue and rehabilitation in providing valuable data for scientific research (Ancillotto and Russo, 2015; Khayat et al., 2019; Pyke and Szabo, 2018).

Genus *Plecotus*

Within the genus *Plecotus*, the recently described *P. gaisleri* (Benda et al., 2004b) has currently been listed for Malta by Mifsud and Vella (2019). This, together with all previously published Maltese *Plecotus* records, is based on external morphology only. However, external morphology largely overlaps among the different *Plecotus* species (Benda et al., 2004b), so an unambiguous discrimination between *P. gaisleri*

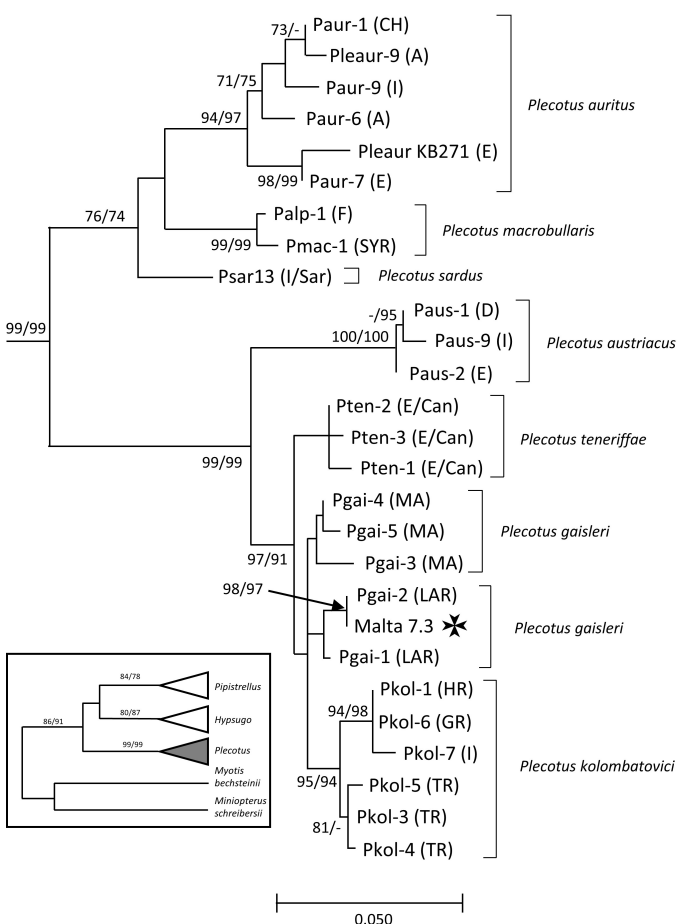


Figure 2 – Maximum likelihood (ML) tree of *Plecotus* haplotypes, based on a 540 bp fragment of the 16S rRNA gene and the GTR+I+G substitution model (gamma shape parameter $\alpha=0.65$, I=41.3%). Maltese samples are marked with the Malta cross. Node support values $\geq 70\%$ for the ML/the Neighbor Joining tree are given next to the branches (2000 bootstrap replicates each; - = the respective node is not sufficiently supported in the ML/NJ tree). This analysis involved 90 nucleotide sequences; the inset shows the position of *Plecotus* within the entire tree. Countries and regions of occurrence are indicated behind haplotype labels (A = Austria, Can = Canary Islands, CH = Switzerland, D = Germany, E = Spain, F = France, GR = Greece, HR = Croatia, I = Italy, LAR = Libya, MA = Morocco, Sar = Sardinia, SYR = Syria, TR = Turkey).

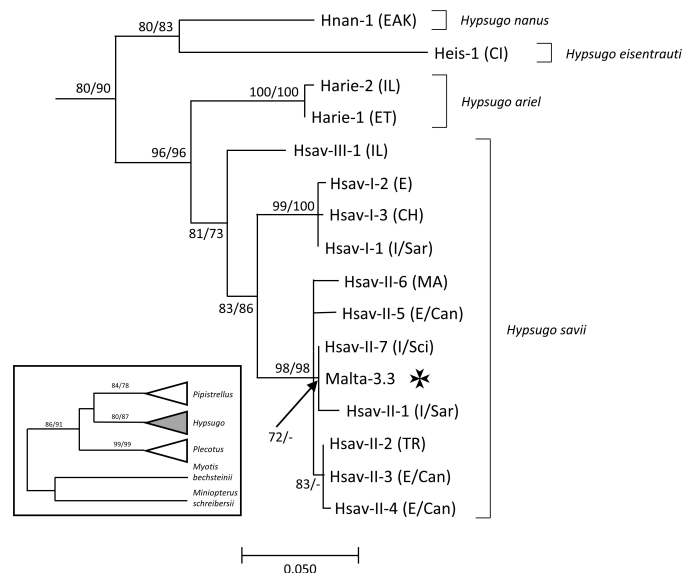


Figure 3 – Maximum likelihood (ML) tree of *Hypsugo* haplotypes, based on a 540 bp fragment of the 16S rRNA gene and the GTR+I+G substitution model (gamma shape parameter $\alpha=0.65$, I=41.3%). Maltese samples are marked with the Malta cross. Node support values $\geq 70\%$ for the ML/the Neighbor Joining tree are given next to the branches (2000 bootstrap replicates each; - = the respective node is not sufficiently supported in the NJ tree). This analysis involved 90 nucleotide sequences; the inset shows the position of *Hypsugo* within the entire tree. Countries and regions of occurrence are indicated behind haplotype labels (Can = Canary Islands, CH = Switzerland, E = Spain, EAK = Kenya, ET = Egypt, I = Italy, IL = Israel, MA = Morocco, Sar = Sardinia, Sci = Sicily, TR = Turkey).

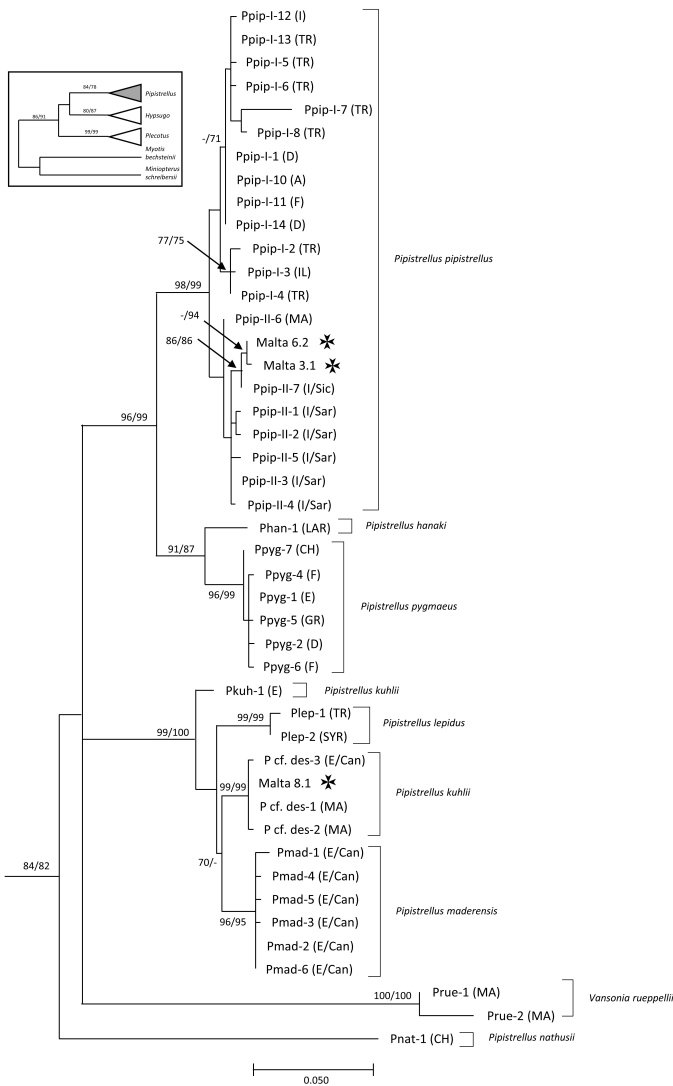


Figure 4 – Maximum likelihood (ML) tree of *Pipistrellus* haplotypes, based on a 540 bp fragment of the 16S rRNA gene and the GTR+H+G substitution model (gamma shape parameter $\alpha=0.65$, $I=41.3\%$). Maltese samples are marked with the Malta cross. Node support values $\geq 70\%$ for the ML/the Neighbor Joining tree are given next to the branches (2000 bootstrap replicates each; - = the respective node is not supported in the ML/NJ tree). This analysis involved 90 nucleotide sequences; the inset shows the position of *Pipistrellus* within the entire tree. Countries and regions of occurrence are indicated behind haplotype labels (A = Austria, Can = Canary Islands, CH = Switzerland, D = Germany, E = Spain, F = France, GR = Greece, I = Italy, IL = Israel, LAR = Libya, MA = Morocco, Sar = Sardinia, SYR = Syria, TR = Turkey).

and its sister taxon *P. kolombatovici* (Mediterranean long-eared bat) is only possible using molecular methods or skull characteristics (Dietz and Kiefer, 2016). *Plecotus gaisleri* occurs in the Maghreb, but details are unknown, while *P. kolombatovici*, *P. auritus*, and *P. austriacus* occur in the Balkans and on the Apennine Peninsula (Ancillotto et al., 2019; Dietz and Kiefer, 2016).

Our study for the first time genetically confirms the presence of *P. gaisleri* in Malta and thus in the European Union (Hutson, 2018). This has important implications for conservation and highlights the special responsibility of Malta for this species in Europe. We caught our five specimens in one of the few forested areas in Malta, Mizieb in Melièħa, where this species has not been recorded before. Insights into the habitat use of *P. gaisleri*, e.g. through radio-tracking studies, is urgently needed in order to understand its habitat requirements and hence could lead to more directed conservation measures.

Genus *Hypsugo*

Within the genus *Hypsugo*, *H. savii* has recently been listed for the Maltese islands by Mifsud and Vella (2019). *Hypsugo savii* has long been considered the only European species within the genus. However, due to a sequence divergence of 9.6% in the mitochondrial ND1 gene,

Mayer et al. (2007) considered *H. savii sensu stricto* and *H. cf. darwini* as separate species. This latter lineage is known from the Canary Islands, the Maghreb, Sicily, Sardinia, and Montecristo (Dondini et al., 2016; Veith et al., 2011). Since the Maltese islands fall within its distribution range, it is not surprising that the *Hypsugo savii* samples collected by us actually belong to this lineage, whose taxonomic status is still unclear (Hutson, 2018). More research, for instance by combining nuclear markers, mitochondrial sequences (Dondini et al., 2016; Veith et al., 2011; Mayer et al., 2007) and sampling throughout the entire distribution range, is needed to finally clarify the taxonomic status and the geographic range of this mainly North-African *H. savii*-lineage. However, despite this unclear taxonomic status, *H. cf. darwini* clearly constitutes an evolutionary significant unit (ESU) (Veith et al., 2011). Mifsud and Vella (2019) reported *H. savii* to be present in Malta and cited an observation based on a mtDNA sequence. However, this record was based on a short sequence only (104 bp fragment of cytb, pers. comm. David Dodds). In our study, a much longer fragment (540 bp) and a more extensive reference database was used, which allows a clear discrimination between the two lineages. Since our study only contained three samples, we cannot exclude the presence of both *H. savii sensu stricto* and the North-African lineage (*H. cf. darwini sensu* Mayer et al., 2007) on the Maltese islands, as is the case in Sardinia (Veith et al., 2011). More extensive sampling in Malta would therefore be highly interesting.

Genus *Pipistrellus*

Of the seven European *Pipistrellus* species described up to now (Dietz and Kiefer, 2016; Mayer et al., 2007), several cryptic clades or lineages exist within the Mediterranean realm (Hulva et al., 2007).

In *P. kuhlii*, two distinct mitochondrial lineages occur in Europe: a “Western lineage” in the Iberian Peninsula and France and an “Eastern lineage” in North Africa, the Balkan Peninsula and the central Mediterranean region (Ibáñez et al., 2006; Andriollo et al., 2015; Benda et al., 2015). The arid North African population of the latter was described as *P. deserti* (desert pipistrelle) by Thomas (1902). However, recent molecular studies suggest that the “*P. deserti*” morphology is likely a recent adaptation to arid habitats rather than reflecting a long independent evolutionary history (Andriollo et al., 2015; Benda et al., 2015). The microsatellite data of Andriollo et al. (2015) show that the African *P. kuhlii* population, including the “*P. deserti*” morphotype, seems to share a common nuclear genepool. Our *P. kuhlii* samples showed a haplotype previously recorded in Morocco (Veith et al., 2011), Italy (Locatelli et al., 2016), and Albania (Sachanowicz et al., 2017), and thus belongs to the mitochondrial eastern lineage of Andriollo et al. (2015) and, more specifically, to the sub-cluster of *P. cf. deserti* haplotypes.

Phylogeographic studies of *P. pipistrellus* identified a distinct lineage found in North Africa and on several Mediterranean islands (Boston et al., 2015; Veith et al., 2011; Hulva et al., 2010) including a single sample from Malta (Hulva et al., 2010). The eight Maltese *P. pipistrellus* samples studied by us nested within a North African clade (Ppip-II, Veith et al., 2011). Apart from North Africa, haplotypes of this clade were previously recorded in Sicily, Corsica and Sardinia (Veith et al., 2011; Hulva et al., 2010), which are well-differentiated from the haplotypes encountered in Europe and Asia Minor. Through this study, we found two very similar haplotypes of *P. pipistrellus* that have not been recorded elsewhere. Extensive sampling in North Africa is needed to elucidate if these haplotypes are unique for Malta or if they may also occur in North Africa. Morphologically, the animals captured by us were smaller than European specimens (Tab. 1); however, their forearm length corresponds to measurements from Tunisia, Morocco and Algeria (Puechmaile et al., 2012b; Benda et al., 2004a). Interestingly, at the mitochondrial level the North African lineage is closely related to other *P. pipistrellus* lineages, but microsatellites indicate that on the nuclear genetic level this lineage is more closely related to Hanaki’s dwarf bat (*P. hanaki*, Hulva et al., 2010). Further molecular and morphological studies are needed to clarify the taxonomic status of the *P. pipistrellus* populations in North Africa and the central Mediterranean islands, including Malta (Puechmaile et al., 2012b).

Conclusions

Bat species are threatened by several pressures, including loss, degradation and disturbance of roost sites, habitat loss and fragmentation, as well as prejudices against bats arising from lack of awareness (Barova and Streit, 2018). Bats are therefore under strong legal protection in the European Union: all bat species are listed in Annex IV of the European Habitats Directive (Directive 92/43/EEC). According to this directive, Member States must implement specific conservation plans and protect habitats where these species are known to occur. Insights into the phylogeographic affiliation of bat species is important to develop appropriate conservation plans. This is very relevant for Malta, as urbanisation and agriculture are especially high anthropogenic stressors. Urban areas can negatively impact bats, especially *Plecotus* species, but can also provide roosting and foraging opportunities for other species (Gili et al., 2019). Yet, consequences of urbanisation for the local bat fauna in Malta are not well documented and are understudied. In this study we show that two cryptic lineages from North Africa, *P. gaisleri* and a North-African *H. savii* lineage, as well as lineages of *P. kuhlii* (with a *P. cf. deserti* haplotype) and *P. pipistrellus* (with two unique haplotypes) are present on Malta. As the distribution of the North-African *H. savii* lineage, *P. gaisleri*, and the haplotypes found in *P. pipistrellus* and *P. kuhlii* in Europe is limited, Malta has an important responsibility for the conservation of these species within the European Union. ☞

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Associate Editor: P. Colangelo

Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Identification, localities, haplotypes and GenBank accession number of samples.

Table S2 Detail of analysed samples from Malta (locality, haplotype, biometrics).