

NATURAL HISTORY NOTE

First record of *Myotis crypticus* (Ruedi, Ibanez, Salicini, Juste and Puechmaille, 2019) for Portugal

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ABSTRACT

Myotis crypticus is a recently described bat species belonging to the *Myotis nattereri* species complex. Its distribution range is not entirely known and so far has only been found across Italy, southern France, and central and northern Spain. Here we present the first record of *M. crypticus* for Portugal. In October 2018, one individual of this species was captured at a swarming site within the Natura 2000 Site of Community Importance (SCI) Alvão/Marão in northern Portugal. The morphological identification of the bat in the field was later confirmed molecularly.

The Natterer's bat, *Myotis nattereri*, was supposed to be a morphologically uniform taxonomic entity and widespread across most of the Western Palearctic (Ruedi et al. 2019). However, molecular research carried out over the last few years has revealed that this species is a complex of several lineages considered as unnamed cryptic species (Ibáñez et al. 2006, Salicini et al. 2011, Puechmaille et al. 2012, Çoraman et al. 2019). As a result, the existence of two different species has been recently confirmed for the Iberian Peninsula (Juste et al. 2019): *Myotis escaleraei*, which is mainly endemic to the Iberian Peninsula (Spain and Portugal), the Balearic Islands, and reaching the eastern French Pyrenees, and *Myotis crypticus*, which is found across Italy, southern France, and the north of Spain (Salicini et al. 2011), however the limits of its distribution, particularly in the north, are still unclear (Juste et al. 2019).

Our bat record was collected as part of a long-term bat mist-netting survey in the north of Portugal. During the mating season (September and October), we regularly monitor a swarming site located within the Natura 2000 Site of Community Importance (SCI) Alvão/Marão. This site is classified in the scope of the Natura 2000 network achieving the favourable conservation status of Mediterranean habitats and species of community importance. This site comprises approximately 58,788 ha and includes two distinct zones: the highlands (Marão: 1,416 m a.s.l. and Alvão: 1,330 m a.s.l.) localized between the Lamas de Olo plateau and the lowlands (Ermelo, Fervença and Régua, near the Douro

river) where the lowest point of the SCI is located at 50 m a.s.l.. The Alvão/Marão site is located in an intermediate zone influenced by the Atlantic and the Mediterranean climates, which gives rise to a large diversity of habitats. Oak forests of the species *Quercus pyrenaica* and *Quercus robur* are predominant although agricultural areas (mainly pastures) can also be found. The SCI also encompasses an extended hydrologic network, characteristic of montane areas (Moreira et al. 2008), that offers favourable conditions to a high percentage of Portugal's inland bat community: supporting at least 22 of the 25 species known to occur in the country (Barros et al. 2012, Rainho et al. 2013).

Bats were captured at the swarming site using 2.5 x 3 m and 2.5 x 9 m mist-nets (16x16 mm, 70/2 Nylon: Ecotone, Poland) and a harp trap (Bat Conservation and Management, EUA) placed at the caves' entrances or in the immediate surroundings.

The nets were opened one hour after sunset and kept open for five hours. Forearm length and body mass of each bat captured was measured using digital callipers (± 0.01 mm) and a digital scale (± 0.01 g). Reproductive status was checked according to Haarsma (2008). Bats belonging to a cryptic complex or a species of particular interest (e.g. *M. escaleraei*/*M. crypticus*, *Plecotus auritus*/*Plecotus austriacus*, *Myotis daubetonii*) were subject to a skin biopsy sampling using a sterile puncher of a 3 mm diameter from the wing membrane (mesopatagium) (Ibáñez et al. 2006). Samples

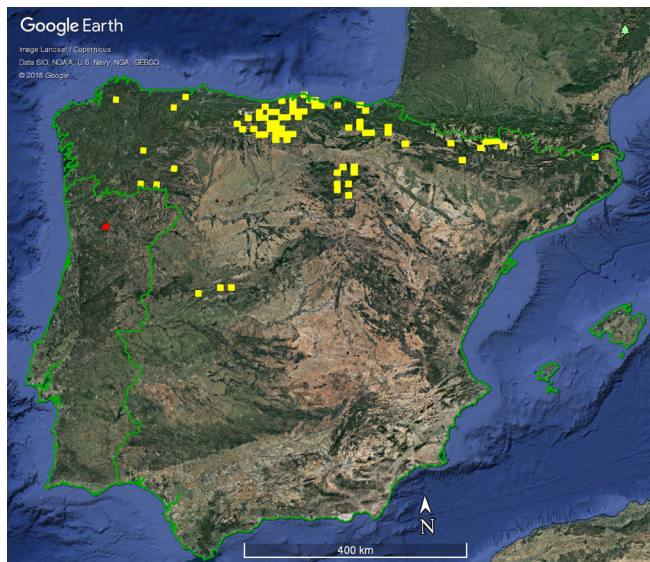


Fig. 1 – First record of *Myotis crypticus* for Portugal (red square), distribution of *Myotis crypticus* in Spain (yellow squares) (Salicini et al. 2013, Lorenzo et al. 2018, Molleda & Fombellida 2018, Razgour et al. 2019).

were stored in sterile tubes containing 70% ethanol for subsequent molecular analysis. Bats were then released at the same capture site. Samples were sent for DNA analyses to the Laboratory of Molecular Ecology (LEM) at the Estación Biológica de Doñana (EBD-CSIC) in Seville, Spain.

In the laboratory, DNA was extracted from the wing biopsies following Higuchi et al. (1988). A fragment of subunit 1 of the mitochondrial NADH dehydrogenase (ND1) gene was amplified using primers ND1-F2 and ND1-R (Kawai et al. 2002). The PCR mix (20 µl final reaction volume) included 2 µl of DNA extract, 1 µl of each primer (10 mM), 0.8 µl of MgCl₂ (50 mM), 0.16 µl dNTP (25 mM), 0.5 unit of taq-polymerase with appropriate buffer and H₂O. Thermocycling consisted of 5' initial denaturation at 94°C, followed by 40 cycles at 94°C (30''), 52°C (30'') and 72°C (1'30''), with a final extension at 72°C (5'). The fragment was sequenced directly from purified PCR product using an ABI 3,100 automated sequencer (Applied Biosystems, Warrington, UK), following the manufacturer's protocols. The partial sequences were aligned and edited using the program Geneious R7 (Biomatters Ltd.) and visually inspected. For species identification purposes, the obtained sequences were compared to available sequences in the GenBank database (<http://www.ncbi.nlm.nih.gov/GenBank>) using the BLAST tool.

On October 12th 2018 in a swarming site at the SCI Alvão/Marão (UTM WGS84 NF97; elevation: 760 m a.s.l.) (Fig. 1), one male belonging to the species *M. crypticus* was caught (Fig. 2). The individual was measured (FA = 37.44 mm), weighed (W = 5.50 g) and its reproductive status was checked, showing signs of sexual maturity (epididymis appeared swollen and black). During the same survey, we also caught 42 *M. escalerai* (the other species of the Natterer's cryptic species complex present in Portugal) and further nine species (*Myotis myotis*, *Myotis blythii*, *Myotis bechsteinii*, *Myotis emarginatus*, *M. daubentonii*, *P. auritus*, *P. austriacus*, *Rhinolophus ferrumequinum* and *Rhinolophus hipposideros*).



Fig. 2 – Individual of *Myotis crypticus* (© Paulo Barros).



Fig. 3 - Ventral views of the insertion of the plagiopatagium at the base of the toe in the *Myotis crypticus* captured (© Paulo Barros).

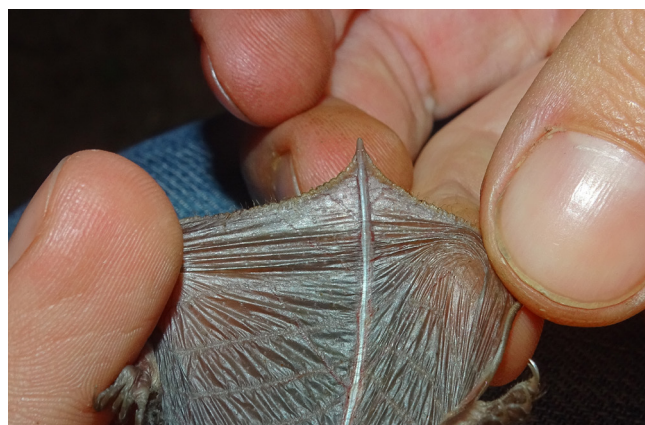


Fig. 4 - Ventral views of fringing stiff hairs directed outwards present along the tail membrane in the *Myotis crypticus* captured (© Paulo Barros).

The species *M. crypticus* was morphologically distinguished from the *M. escalerai* by the observation of the wing membrane insertion at the base of the toe (Fig. 3) and the stiff hairs along the uropatagium margin (Fig. 4). Molecular analysis of the sample collected allowed the unequivocal taxonomic confirmation of the identification carried out in the field. The sequence of around 950 bp of the ND1 fragment showed > 95% identity with available homologous ND1 sequences of *M. crypticus* in GenBank in a BLAST comparison, confirming unambiguously that the small *Myotis* was the recently described *M. crypticus* (GenBank accession number:MT090964).

Around the roost where *M. crypticus* was first found in Spain (Cueva Cerraúco, El Rasillo de Cameros, La Rioja [ca. 42°11'0" N, 2°44'20" W], 1,400 m a.s.l.) the species is commonly found in dense forests of Pyrenean oak (*Q. pyrenaica*) and beech (*Fagus sylvatica*), in forests cleared for pasture but which retain scattered old-growth trees and also in subalpine prairies up to 2,000 m a.s.l. (Juste et al. 2019). The swarming site where we caught the individual of *M. crypticus* is surrounded by goat grazing pastures composed of *Ericaceae* shrubs with sparse pines (*Pinus sylvestris* and *Pinus pinaster*). Adjacent areas are predominantly composed of broadleaf woodland (*Q. pyrenaica*, *Q. robur* and *Castanea sativa*) and agricultural land. Therefore, our record (considering the surrounding habitat and elevation of 760 m a.s.l.), is in keeping with the ecological requirements observed in other studies and localities, including Western Switzerland (Beck 1991, Arlettaz 1996), France (Puechmaille et al. 2012) and Italy (Salicini et al. 2013).

The adult male of *M. crypticus* found at the swarming site showed evidence of sexual maturity and was swarming together with ten other bat species, including the sibling *M. escalerai*. A similar pattern was described in other studies, mainly in Switzerland, France, Italy, and Spain, where *M. crypticus* has been observed gathering in large numbers with other *Myotis* species at swarming sites in autumn (Juste et al. 2019).

The lack of knowledge about the distribution, behaviour and ecology of *M. crypticus* stems from its recent recognition as a separate species. Therefore specific studies concerning its ecology and the potential interactions between this species and *M. escalerai* are yet to be conducted (Juste et al. 2019). The recent recognition of *M. crypticus* as an independent species results in the need to evaluate its population's status and distribution under the criteria of the IUCN (Juste et al. 2019) and for the inclusion of this new species in the Portuguese Red Data Book for Vertebrates to receive appropriate protection.

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Conflict of interests

The authors declare that they have no conflict of interest.

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