

Molecular Identification of Italian Mouse-eared Bats (genus *Myotis*)

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Abstract — Despite the fact that the genus *Myotis* (Mouse-Eared bats) is one of the most investigated microchiropteran groups, recent molecular studies highlighted the presence of several cryptic species with substantial implications for ecological and conservation issues. Our dataset includes 55 *coxI* sequences from 11 morphologically-identified Italian Mouse-Eared bats species. We applied an integrated approach comparing data from a traditional morphological identification and molecular variability in a fragment of the mitochondrial *coxI* gene (DNA barcoding). Our results clearly show a strong coherence between the two identification approaches for almost all of the examined species, and revealed interesting patterns of intraspecific variability within the species *M. nattereri*. Finally, we successfully tested the efficacy of our identification method on undetermined individuals sampled in the field.

Index Terms — cryptic species, DNA barcoding, integrated taxonomy, Vespertilionidae.

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1 INTRODUCTION

Mammals are usually considered as one of the best-known animal groups. However, several studies provided clear evidences that bats (order Chiroptera) are characterized by a high incidence of overlooked taxa due to their cryptic morphology and habits [1]. A clear example of this situation is given by the recent taxonomic changes within the family Vespertilionidae (one of the best-studied taxonomic group of bats) in the Western Palearctic. Thanks to the development of recent molecular techniques, the number of species within

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this family has risen from 37 to 54, with at least 8 new cryptic species identified in Europe [2], [3], [4].

Within the Mediterranean basin, the Italian peninsula is one of the most important biodiversity hotspots for bats and other taxa [5]. It has been hypothesized that this peninsula would have provided stable habitats during ice ages, where species survived leading to the generation of new cryptic lineages [5]. 33 microchiropteran species out of the almost 40 currently known to live in Europe are reported for Italy [5], the family Vespertilionidae being the most diverse and abundant (8 genus and 27 species). As showed in recent studies, the family Vespertilionidae is characterized by high levels of cryptic diversity [3], [4] and in particular in Italy, at least 11 different species are included in the group of Mouse-eared bats (genus *Myotis*). This taxon is the most problematic concerning species identification, due to the presence of cryptic species like in the 'Whiskered-bats' complex (i.e.: *M. mystacinus*, *M. Brandtii*, *M. alcathoe*), peculiar biogeographical histories (i.e.: *M. Myotis* and *M. Blythii*; [6]) and genetically-uncharacterized lineages (i.e.: *M. Nattereri*; [2], [4]).

Despite the fact that the genus *Myotis* includes several threatened species, the compilation of realistic action plans for their conservation is biased by some practical difficulties: bats are hard to observe because of their elusive habits. Moreover, several species are cryptic and sometimes it is impossible to reach a correct identification in the field, especially for juveniles or females [5], [7]. Despite the fact that morphological identification keys are available for European bats (e.g. [7]), integration with molecular approaches has proven to be efficient in detecting morphologically cryptic species [2], [3], [4], [8], [9]. An efficient and widely used molecular tool in species identification is DNA barcoding [10]. This technique is based on the analysis of the variability in the nucleotide sequence of a short, standardized region of the genome (among metazoans the 5'- end of the mitochondrial subunit 1 of cytochrome c oxidase), to evaluate differences among species [10]. A few studies have shown the efficacy of *cox1* in identifying bats species, but any (e.g.: [1]) work was conducted with a standardized DNA barcoding approach for European and Italian *Myotis* and other Vespertilionids.

The main objectives of our study are: i) to compile a reference dataset of *cox1* sequences from all the Italian *Myotis* species; ii) to test the coherence between a molecular approach and the morphologically-based taxonomy and iii) to investigate the intraspecific molecular variability of the barcode region to reveal the presence of undescribed cryptic lineages.

2 MATERIALS AND METHODS

2.1 SAMPLING, DNA EXTRACTION, SEQUENCING AND ALIGNMENT

The samples analysed in this study derive from 55 bats belonging to 11 *Myotis* species collected during 2006-2007 from 16 Italian localities distributed along the Italian peninsula. Bats were identified by researchers of the GIRC (Italian Chiroptera Research Group). Tissue samples (i.e.: 'punches' of patagium, 3mm large) were stored in ethanol 96%. According to the protocol specified by the

Biorepositories initiative (<http://www.biorepositories.org>) each sample was vouchered with the id Institution name 'MIB:ZPL:' followed by a progressive numeric code. Sixteen unidentified individuals belonging to the genus *Myotis* were also collected.

Total genomic DNA was extracted using a guanidinium thiocyanate and diatomaceous earth protocol [11]. *coxI* amplification and sequencing were obtained following the laboratory protocols provided by [1]. Sequences were checked and aligned following the approach described in [12] and, after checked for the presence of pseudogenes and numts (i.e. nuclear mitochondrial pseudogenes), alignment was cut to 560 bp in order to have all the sequences of the same length.

2.2 MOLECULAR DATASET DEFINITION AND DNA BARCODING ANALYSES

To evaluate the efficacy of a DNA barcoding approach as a molecular tool to identify Italian Mouse-Eared bats species, we assembled a 'Reference Dataset' including only *coxI* sequences from Italian morphologically identified bats belonging to the genus *Myotis*. An 'Optimum Threshold' value of molecular divergence (OT) was then calculated (following [12]) directly from the whole range of molecular variability of the molecular dataset. OT value maximizes the coherence between the morphological identification and the molecular divergence minimizing, at the same time, the total amount of possible mismatches (see Minimum Cumulative Error analysis in [12]). To avoid biases during OT calculation, we removed *Myotis nattereri* barcode sequences because of its high intraspecific variability, probably due to the presence of undescribed cryptic lineages (as clearly shown in [2] and [4])

OT was then used to perform a DNA barcoding analysis on the 'comprehensive molecular dataset', containing all reference barcodes and also the *coxI* sequences of the 16 unidentified Italian *Myotis* sp. individuals and the 9 *Myotis nattereri* samples. Intraspecific molecular variability was then analyzed to test the congruence with previously described species. OT has also been used to predict potentially new taxa within the dataset. Finally, a neighbor-joining (NJ) tree (Fig. 1) was generated from the comprehensive molecular dataset using MEGA 4.1 according to the parameters provided in [12].

3 RESULTS AND DISCUSSION

Any *coxI* sequence exhibited indels, stop-codons or numts interferences. The 'Reference Dataset' included 46 barcode sequences 560 bp long belonging to all the Mouse-eared bat species distributed in Italy, except for *M. nattereri* (average number of *coxI* sequences for every single species: 5, standard deviation: 3.05, range: 1-9).

Bioinformatic analyses on the 'Reference dataset' allowed us to infer the following parameters: mean K2P distance within species 0.6% (standard deviation: 0.7%; range: 0% – 2%), mean K2P distance between species 15.8% (standard deviation: 2.8%; range: 2% – 20.1%) and *coxI* overall mean diversity 14% (standard deviation: 1.1%). Concerning the OT calculation, we obtained

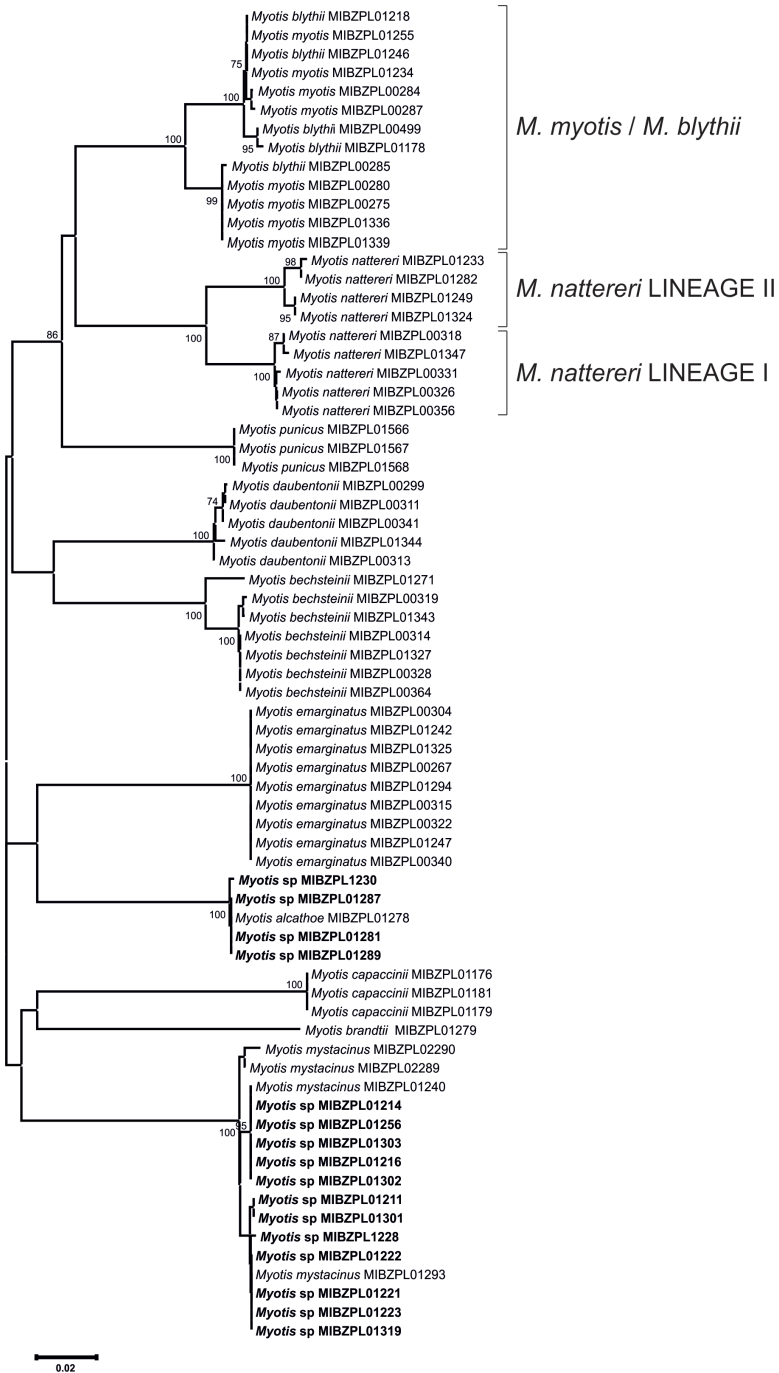


Fig. 1 – NJ Tree. Neighbour joining tree based on *coxI* sequences of Italian Mouse-eared bats generated with MEGA 4.0 (Tamura et al, 2007). Unidentified samples are indicated as “*Myotis* sp.”. Cryptic molecular lineages inferred using OT Threshold are indicated with square brackets.

a minimum cumulative error of 4.36% at a Optimum Threshold value of 4.2%. Only one identification mismatch occurred, due to the low interspecific variability (lower than OT) observed between the species *M. myotis* and *M. blythii* (mean K2P distance between species: 2.0%, standard deviation: 1.7%, range: 0% – 4.1%). A similar result has been previously reported in other molecular studies on these taxa (e.g.: [6], [8]) and relies on the fact that a series of introgression events having occurred repeatedly during the recent colonization of Europe by *M. blythii* from Asia. Hybridization is still ongoing in the areas of sympatry (e.g.: in Italy), therefore suggesting an unclear taxonomic status of these taxa in the Western Palearctic.

The application of the OT value on the 'comprehensive dataset' allowed to assign the unidentified specimens (Fig.1) to 12 *M. mystacinus* and 4 *M. alcathoe*. These are two cryptic sympatric species of Mouse-eared bats one of which (*M. alcathoe*) has been recently described [13] and which status in Italy is almost unknown.

Moreover, OT revealed the presence of two cryptic lineages within the taxon *M. nattereri* (here tentatively named 'Lineage I' and 'Lineage II') exclusive of Northern and Southern localities of the peninsula respectively (Fig.1). The mean K2P distances within each lineage are 0.7% and 0.4% for Lineage I and II respectively, while the mean K2P distance between the two lineages is higher than OT: 5.6%. Garcia-Mudarra and colleagues [4] recently identified at least four European cryptic molecular lineages within this taxa and they concluded that ecological as well as morphological studies would be desirable before any definitive conclusions can be drawn about its taxonomic status. Moreover, preliminary molecular comparisons among our lineages and other mitochondrial sequences available in GenBank (i.e.: ND1 and cytb) revealed that the Southern Italian 'Lineage II' discovered by our DNA barcoding approach is completely undescribed and could represent a new cryptic *Myotis* species for the Western Palearctic (data not shown).

4 CONCLUSIONS

Our study provides clear evidences that DNA barcoding is a reliable and efficient tool for the discrimination of almost all the Italian Mouse-eared bats, showing a high strength of coherence between data based on classical morphology and variability in the mitochondrial *cox1* barcode region. OT value calculated from our dataset allows to infer a clear taxonomic assignment for all the morphologically-unidentified individuals collected in the field. Moreover, the OT value inferred from the molecular dataset is efficient to reveal the presence of undescribed cryptic lineages within known species, like the case of *M. nattereri*. These results suggest that DNA barcoding could be successfully used as a reliable support to ecological studies in order to develop efficient conservation strategies for endangered bats populations.

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