Published by Associazione Teriologica Italiana
Hystrix, the Italian Journal of Mammalogy
Volume 26 (1): 13–24, 2015
OPEN ACCESS
Available online at:
http://www.italian-journal-of-mammalogy.it/article/view/11347/pdf
doi:10.4404/hystrix-26.1-11347

Commentary

DNA barcoding in mammals: what's new and where next?

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Keywords:
Biological databases
integrative taxonomy
molecular identification
theriology
wildlife forensics

Article history:
Received: 4 June 2015
Accepted: 25 June 2015

Acknowledgements
The authors are indebted with Monica Pozzi for the linguistic revision of the manuscript. The authors are also grateful to the anonymous referees for their useful comments that substantially improved the final version of this work.

Abstract

DNA barcoding is a universal molecular identification system of living beings for which efficacy and universality have been largely demonstrated in the last decade in many contexts. It is common to link DNA barcoding to phylogenetic reconstruction, and there is indeed an overlap, but identification and phylogenetic positioning/classification are two different processes. In mammals, a better phylogenetic reconstruction, able to dig in fine details the relationships among biological entities, is really welcomed, but do we need DNA barcoding too? In our opinion, the answer is positive, but not only for the identification power, nor for the supposed ability of DNA barcoding to discover new species. We do need DNA barcoding because it is a modern tool, able to create an integrated system, in which it is possible to link the many aspects of the biology of living beings starting from their identification. With 7000 species estimated and a growing interest in knowledge, exploitation and conservation, mammals are one of the best animal groups to achieve this goal.

We organised our review to show how an integrative approach to taxonomy, leaded by DNA barcoding, can be effective in the twenty-first century identification and/or description of species.

Introduction

Mammals represent a relatively small animal group, with 5564 species listed in the Catalogue of Life (ITIS database, http://www.catalogueoflife.org). Being our own class, it is thought that these species are among the most known animals, especially regarding taxonomic aspects (Wilson and Reeder, 2006).

Generally speaking this is correct, but there are relevant exceptions, even on (presumably) well-established species. The case of African bush and forest elephants is emblematic. In 2001 the populations of bush and forest elephants were split in two distinct species, *Loxodonta africana* (Blumenbach, 1797) and *L. cyclotis* (Matschie, 1900), using molecular data to support this separation (Roca et al., 2001). It is clear that there is a hidden biodiversity within the mammal record, the extent of which is still under discussion, but surely in some groups like chiroptera, it has a deep impact on the taxonomy (see for example Galimberti et al., 2012b and Bogdanowicz et al., 2015). On the whole, the estimation of the unknown biodiversity in mammals is not so trivial, but there is an agreement on the number of about 7000 species (Reeder et al., 2007). The question is now simple: how to discover them?

Since 2003, DNA barcoding has been claimed to be an innovative and revolutionary approach to identify living beings, and a way to speed up the writing of "the encyclopedia of life" (Savolainen et al., 2005). In other words, the technique would be a system to increase the efficiency in species discovery. DNA barcoding has many advantages, but criticisms raised against the ability to discover new species (see for a review Casiraghi et al., 2010). The signature of the success of DNA barcoding is evident from the many group-specific or environment-specific campaigns launched in the past years (see an updated list of them at the international Barcode of Life initiative, www.ibol.org). Figure 1 shows a simplistic analysis of the publications on DNA barcoding in vertebrates since the seminal paper by Paul Hebert was issued in 2003 (Hebert et al., 2003). The figure has to be carefully taken into consideration because it does not represent a full bibliometric analysis as many articles do not include barcoding keywords in their title or abstracts (see Fig. 1 caption for more details), making this schematization certainly incomplete. However, Fig. 1 clearly shows that DNA barcoding in vertebrates is still largely diffused among fishes (probably for their importance in the global food market and for the frequent occurrence of frodes, mislabelling, species substitution to which they are subjected, see for instance Barbuto et al., 2010), whereas this tendency is not found in other vertebrates.

The DNA barcoding of mammals is ongoing under the auspices of the iBOL. According to the BOLD System (http://www.boldsystems.org) at the end of May 2015 about 2850 mammal species have been barcoded, and at least 300 unnamed clusters (i.e. not assigned taxonomic rank) are recognised on MammaliaBoL. In Fig. 2, the DNA barcoding coverage in mammal known species is plotted. As a consequence, given the 7000 presumed mammal species, there are DNA barcodes for about 45% of them. This also means that even if we believe in the species discovery power of DNA barcoding, it is difficult to think that this would be the main support for the mammal initiative. It could be a relevant drive in other animals, but not in mammals. In the modern taxonomy, identification and classification are two different processes (Casiraghi et al., 2010) and in mammals the main problem is related to the phylogenetic reconstruction, that is not, in a strict sense, DNA barcoding (Rodrigues et al., 2011; Huang et al., 2012).

DNA barcoding is more than a simple identification system and its major strength is beyond the discrimination power. In this context, DNA barcoding in mammals moved forward from the identification, becoming a "service system" useful for several aspects originating from taxonomy, but being relevant in other areas of the biology of mammals, ranging from distribution to behaviour and conservation.

So, the time is ripe to ask a fundamental question: do we still need DNA barcoding in mammals? We wrote our essay to solve this ques-

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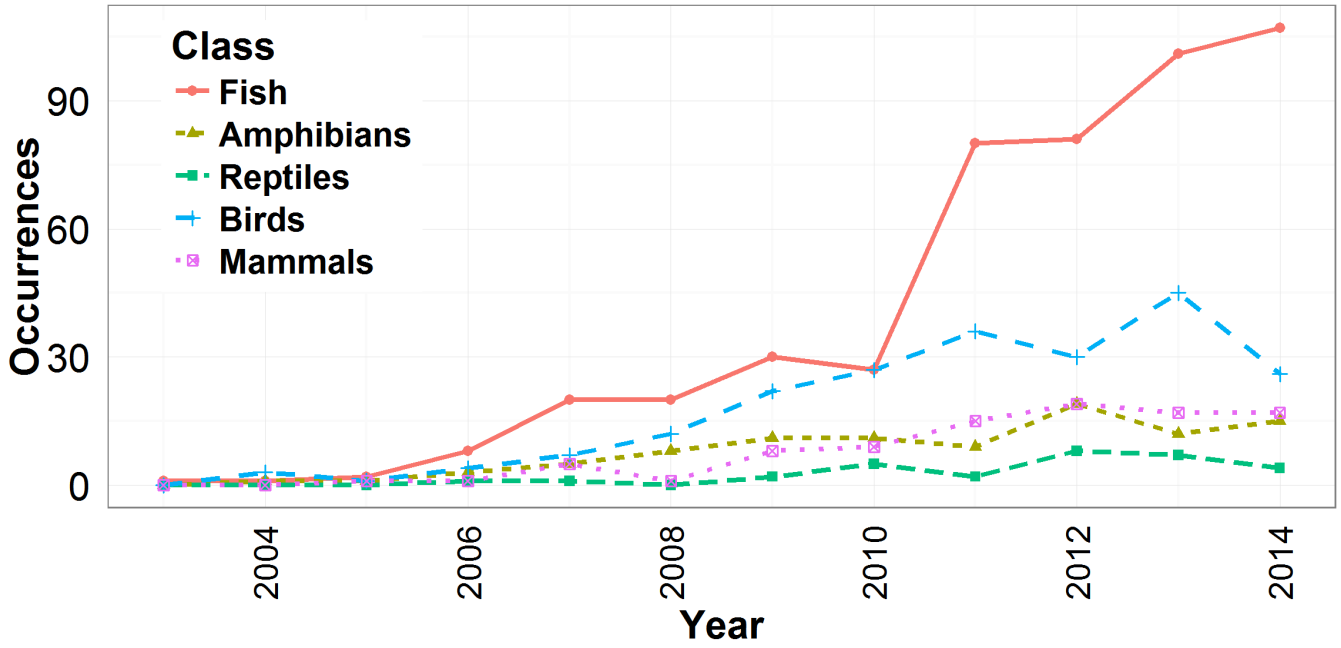

Figure 1 – A schematic overview of the tendencies in published papers on DNA barcoding in vertebrates from the beginning of the initiative (2003) to the end of 2014. Please note that the graphic is not exhaustive and it has been generated interlinking different keywords searches on ISI WEB of Science. Mammalia: barcode mammals; barcoding mammals; barcode mammal; barcoding mammal; barcoding mammalia; barcode mammalia. Aves: barcode bird; barcoding bird; barcode birds; barcoding birds; barcoding aves; barcode aves. Amphibia: barcode amphibian; barcoding amphibian; barcode amphibians; barcoding amphibians; barcoding amphibia; barcode Amphibia. Reptilia: barcode reptiles; barcoding reptile; barcode reptiles; barcoding reptiles; barcoding reptilia; barcode reptilia. Fish: barcoding fishes; barcode fishes; barcoding cartilaginous fish; barcode cartilaginous fish; barcoding fish; barcode fish; barcoding Agnatha; barcode Agnatha; barcoding Osteichthyes; barcode Osteichthyes; barcoding bony fishes; barcode bony fishes.

tion, and the different sections listed below are the different answers we can give.

The importance of reference databases

In DNA barcoding, the identification procedure involves the assignment of taxonomic names to unknown specimens using a DNA reference library of vouchers, previously identified trough different criteria. Such reference accessions and the international platforms in which they are organized, constitute the scaffold of the DNA barcoding initiative. Reference DNA barcodes often derive from natural history museums or private collections (Puillandre et al., 2012) as the role of these institutions has always been that of storing, univocally labelling and sharing the reference biological material for taxonomists. In the not-molecularized biology, most of the work of taxonomists was entirely based on the comparison between newly collected or already archived material and the one of other collections. In the case of mammals, one of the main challenges for a taxonomist relies on the fact that the largest reference collections are scattered among museums. This generated some paradoxes with researchers working in tropical biodiversity hotspots that have to move to North America and Europe to examine the largest collections of mammals inhabiting their own species-rich areas (Francis et al., 2010).

The advent of DNA barcoding moved forward allowing contemporary taxonomists to make comparisons with other taxonomic material, even at a distance with consequent benefits in terms of time and resources saved. In addition, ongoing improvements in molecular technology permit to cheaply obtain high quality sequences from very small and long-time preserved tissue samples like those stored in museums (Mitchell, 2015). These advances boosted the researches in mammalogy for several reasons. First, the possibility of confirming the identification of specimens through DNA barcodes allows museums to establish reference collections that can serve as a basis for future research including the description of new biological entities (Puillandre et al., 2012). Second, the standardized molecular reexamination of museum-deposited voucher specimens and the comparison with other reference data permits to rapidly "flag" the identification mistakes typically occurring during field surveys. As pointed out by Francis and co-workers 2010, field determinations for many mammal species are difficult, because they require the analysis of internal morphology (e.g., skull or dentition) and are often biased by age/sex variations, undescribed/extralimital species and lack of comparative material. Finally, the digital nature of genetic information (the so-called "computerization" *sensu* Casiraghi et al., 2010) makes DNA barcoding data readily comparable through publicly accessible online databases thus providing a wide panel of potential applications ranging from progresses in taxonomy to the fields of forensics and food traceability (see dedicated paragraphs of this review).

Concerning this last point, in the framework of the International Barcode of Life (iBOL) initiative, the building of a comprehensive public library of DNA barcodes, the Barcode of Life Data System (BOLD), was launched to provide a global identification system freely accessible (Ratnasingham and Hebert, 2007, 2013). This platform consists of several components, among which the Identification Engine tool (BOLD-IDS) is one of the most useful. BOLD-IDS provides a species identification tool that accepts DNA barcode sequences and returns a taxonomic assignment at the species level whenever possible.

Unlike other international sequence databases (such as EMBL and GenBank), BOLD has a quality control system built in, and specific information is required to store and publish a specimen or barcode. To be included in BOLD, specimens have to be properly vouchered following the protocol specified by the Global Registry of Biodiversity Repositories (http://grbio.org), and the data standards for BARCODE Records (Hanner, 2009). Moreover, required details on the sample include the collection date and location with GPS coordinates, and the PCR primers used to generate the sequences. Finally, submission of the original trace files is also needed. Noteworthy, barcode sequences in BOLD are associated with specimen records linked to institutional (e.g., museum) material making them the most valuable among putative reference accessions.

The accuracy of DNA barcoding species assignment relies upon the level of taxonomic representation for each group of metazoans and the amount of intraspecific genetic diversity represented in the databases (Gaubert et al., 2014).

In the case of mammals, assembling a reference database of DNA barcode sequences is fundamental for the goals of the iBOL initiative, also considering that the rate of species discovery within this class has recently accelerated due to the growing use of molecular techniques (Reeder et al., 2007).

Differently from larger DNA barcoding campaigns focusing on fishes (i.e., FISH-BOL, Becker et al., 2011), birds (i.e., ABBI, Hebert et al., 2004), insects (Jinbo et al., 2011) and others, there have only been a few references on mammals, generally focusing on a limited number of taxa or geographic areas. As of 2015, more than 69000 barcode mammalian sequences from over 2800 species have been archived in BOLD with more than 50% assembled at the Biodiversity Institute of Ontario in collaboration with the Royal Ontario Museum (ROM) and other institutions. The most part of these data belong to bats, rodents and primates from the Neotropical Region and other tropical biodiversity hotspots (Lim, 2012 and Fig. 2).

To date, the largest published studies on mammals DNA barcoding are those by Francis et al. (2010) and Clare et al. (2011), where the authors examined 1896 specimens belonging to 157 species from the South East Asia and 9076 specimens belonging to 163 species from the Neotropics respectively. Table 1 provides an updated list of the major studies that contributed to populate the current reference DNA barcoding database for mammals. Although most of these are limited to a reduced number of species or geographical extent, they are important in

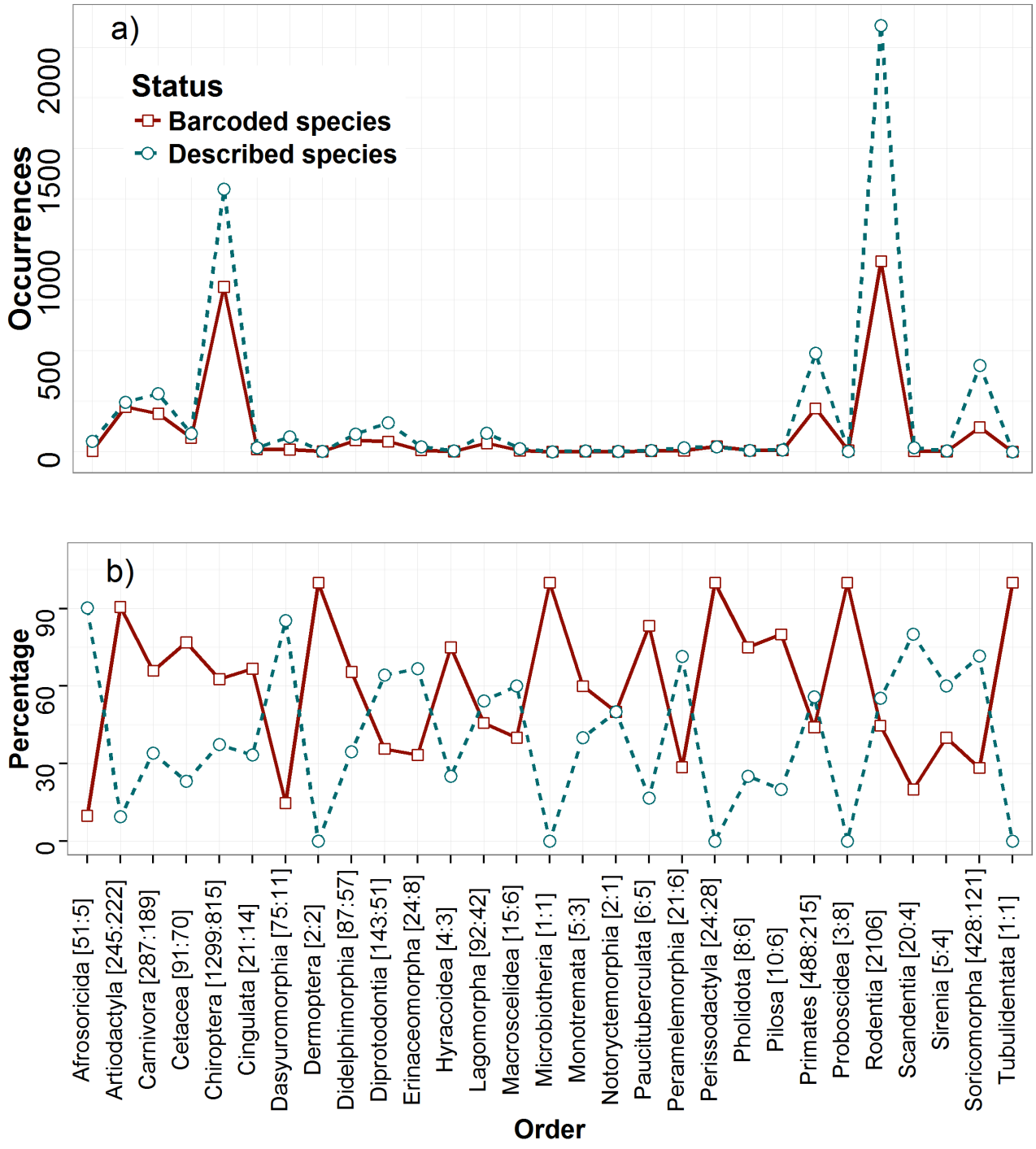

Figure 2 – Overview of the Mammalian DNA barcoding initiative showing the distribution of barcoded species in the different orders. Data on described species is derived from Integrated Taxonomic Information System (ITIS, http://www.itis.gov). Data on barcoded species is derived from the Barcode of Life Data Systems (BOLD System, http://www.boldsystems.org). In a) the number of species described and barcoded is plotted in the various mammal orders. In b) the percentage of species described and barcoded is plotted in the various mammal orders. Dotted line: described species (number or percentage). Continuous line: species with a DNA barcode.

filling the gaps of knowledge for many taxonomic groups, discovering new species or lineages and enabling potential effective conservation planning. The availability of a public database of reference specimens and related genetic data of mammal species is also at the base of wildlife forensics as for example recommended by the International Society for Forensic Genetics Commission (Linacre et al., 2011; Johnson et al., 2014).

Increasing knowledge on biology, distribution and conservation

As a matter of fact, the primary role of DNA barcoding in mammals has been so far, and will long remain, the identification of known species and one of the most rapid approaches to detect new ones, the so-called "DNA barcoding *sensu stricto*". Table 1 provides a list of case studies where DNA barcoding was successfully used in many application contexts to identify mammal species.

However, the "*sensu lato*" face of the approach (see Casiraghi et al., 2010), is even more interesting as it provides new information on the biology, distribution and conservation of mammals.

First of all, DNA-based techniques and consequently DNA barcoding are valid data generators to increase the existing knowledge on rare or poorly investigated taxa. In most cases, the analysis of barcode sequences allowed to confirm the occurrence of certain species in areas out of their known distributional range such as bats (e.g., De Pasquale and Galimberti, 2014) and Artiodactyla (e.g., Wilsonet al., 2014). The implications in a context of conservation are numerous and many studies supported the use of DNA barcoding in recognizing rare or elusive mammal species traditionally monitored with expensive field techniques (i.e., direct observations, captures and camera traps). DNA barcoding proved to be more effective in discriminating morphologically similar species, such as small ungulates and carnivores, which were difficult to recognize using camera traps (Inoue and Akomo-Okoue, 2015). In these cases, great advantage was provided by the possibility of identifying species from a part of the animal (i.e., hair/fur, claws, or skin) or its droppings as well described in recent case studies conducted in Amazonian and other unexplored areas of the planet (Michalski et al., 2011; De Matteo et al., 2014; Stanton et al., 2014; Inoue and Akomo-Okoue, 2015).

In other situations, the DNA barcoding approach could flag the occurrence of newly undescribed lineages that are confined to a certain geographic area or could represent a new taxa. Apart from the light and shadows of the method in a pure taxonomic context, an aspect of primary importance is the possibility of rapidly detecting putative units deserving further investigations to characterize their ecology, distribution and conservation status. Such kind of approach is fundamental to plan early and effective conservation strategies. Several studies proved the role of DNA barcoding in this framework such as in the case of Italian echolocating bats (Galimberti et al., 2012b) where the authors found, starting from DNA barcoding, a new well diverged lineage of *Myotis nattereri* in Southern Italy and several less divergent lineages within *M. bechsteinii* and *Plecotus auritus* from different areas of the Peninsula. A greater diversity was also found within neotropical bats in which Clare and colleagues 2011 found supported evidence of the existence of previously undescribed lineages for at least 44 species out of the 163 examined by DNA barcoding.

Invaluable data on mammal ecology and their conservation also derive from the characterization of their diets which has been conducted in many cases with a DNA barcoding approach. Understanding trophic interactions is fundamental also to assess the importance of certain species for ecosystems functioning and how they respond to variation (Clare et al., 2014a). The recent exploitation of High Throughput DNA Sequencing techniques (see below) allowed to characterize mixed DNA samples (e.g., stomach contents or faecal samples) and to identify the preys consumed by a given predator (Boyer et al., 2015). Such analyses revealed for example temporal and spatial variation patterns in the use of arthropod resources by different bat species (Clare et al., 2014b; Rasgour et al., 2011; Alberdi et al., 2012; Vesterinen et al., 2013; Hope et al., 2014) or diet differentiation between species and/or during different phenological periods (Bohmann et al., 2011; Burgar et al., 2014; Krüger et al., 2014a,b; Sedlock et al., 2014).

In conclusion, we are now aware that in mammals, even more than in other animals, we need to collect complementary data to better understand their biology. The system generated by DNA barcoding has the possibility to rapidly increase these knowledge.

Forensic applications

Given its peculiarities as a universal identification tool, DNA barcoding naturally acquired a role of primary importance in forensic (Dawnay et al., 2007; Iyengar, 2014), including case studies on animal derived foodstuff (e.g., Barbuto et al., 2010; Galimberti et al., 2013). In particular, wildlife forensic is a wide-ranging discipline covering more forms of crimes compared to human forensic. Concerning mammals, typical investigations include: trafficking in live specimens or parts of them, poaching or hunting out of season, cruelty to animals, habitat destruction and species substitution of food products (e.g., the bushmeat). These phenomena are of major concern also considering their economic impact at the global scale. For instance, recent estimates highlighted that a significant portion of the international trade of wildlife and wildlife products is illegal (i.e., 5–8 billion US $ of the total 6-20 billion US $, Baker, 2008) and includes species that are protected by national laws and international conventions (Eaton et al., 2010). Given the illicit nature of these activities, it is almost impossible to monitor and quantify the exact volumes and species involved as well as the real impact on wildlife populations (Gavin et al., 2010; Conteh et al., 2015). However, in the last century, the tremendous global collapse of some species that are object of illegal trade confirms the emerging problem of wildlife crimes (see for example the cases of *Panthera tigris* and *Diceros bicornis* which populations have decreased of 90% and 96% respectively in few decades; Linacre and Tobe, 2011). The biological material that is traded and analyzed in wildlife forensic is vast, ranging from whole animals (live, hunted or inadvertently killed) to skins, skeletons or animal body parts (e.g., meat, horns and teeth) (Huffman and Wallace, 2012; Johnson et al., 2014). In other cases, the only available material is blood, hairs and trace DNA or mixtures of genetic material (Johnson et al., 2014). Apart from clearly unmistakable species (e.g., an elephant tusk or a skin of a big carnivore), the morphological approach used for identification has usually to be undertaken by an expert mammalogist (Huffman and Wallace, 2012). Also microscopy of hairs or the analysis of bones require high-skilled experience to achieve a reliable identification, and even so, in some cases they failed to go further from a general group of putative species (see examples in Moore, 1988). Indeed, the strong processing of the wildlife raw material that can be finally traded as fillets, powders, potions or oils, often impedes unequivocal identification with morphology. In addition, both general operators and specialists are sometimes required to investigate on species that have not previously been studied in a forensic context and therefore lacks of accurate morphological reference data.

Given these premises, it is clear that universal, fast and accurate methods of species identification are necessary to improve the ability of detecting, monitoring and controlling the trade in mammals and other groups of animals (and their processed products).

In the last decades, the advent of DNA-based technologies revolutionized the field of wildlife forensic as DNA tools offered the possibility of overcoming the limits described above. Concerning species identification, several approaches and loci were selected, but in the last 10 years, DNA barcoding and the use of the mitochondrial cytochrome-c-oxidase subunit (i.e. mt-*coxI*) rapidly affirmed their utility in those cases involving crimes against mammals. Literature and examples are numerous, and three main categories of wildlife forensic investigations where DNA barcoding is successfully adopted can be identified:

Illegal hunting and traceability of wild game

The unregulated hunting of wildlife is an emerging issue as it involves the harvesting of millions of tons of wild game -– mostly mammals -– per year (Eaton et al., 2010; Gaubert et al., 2014). Conservation problems are typically referred to the "bushmeat" hunting which includes

Table 1 – Updated list of case studies dealing with mammals DNA barcoding. For each study, the context of application, the taxonomic order of target mammals, the aim of the work and the number of species involved are reported .

Context	Order	Aim	N°species	References
DNA taxonomy	Chiroptera	Characterization of Guyana bat species	87	Clare et al., 2007
DNA taxonomy	Chiroptera	Identification of a new species of Malaysian bat	1	Francis et al., 2007
DNA taxonomy	various	Characterization of small mammal communities of Suriname	74	Borisenko et al., 2008
DNA taxonomy	Didelphimorphia	Identification of cryptic species of opossum	2	Cervantes et al., 2010
DNA taxonomy	Primates	Characterization of primates species	50	Nijman and Aliabadian, 2010
DNA taxonomy	Chiroptera	Characterization of Malaysian wolly bats	6	Khan et al., 2010
DNA taxonomy	Chiroptera	Characterization of Southeast Asian bats	165	Francis et al., 2010
DNA taxonomy	Soricomorpha	Characterization of white-toothed shrews from Vietnam	6	Bannikova et al., 2011
DNA taxonomy	Artiodactyla	Characterization of Tanzanian antelopes	20	Bitanyi et al., 2011
DNA taxonomy	Artiodactyla	Characterization of Chinese bovidae	18	Cai et al., 2011
DNA taxonomy	Chiroptera	Characterization of Neotropical bats	163	Clare, 2011
DNA taxonomy	Chiroptera	Characterization of Ecuadorian bats	45	McDonough et al., 2011
DNA taxonomy	Soricomorpha	Characterization of shrews from Guinea	10	Jacquet et al., 2012
DNA taxonomy	Didelphimorphia	Characterization of opossum species in Brazilian Atlantic Rainforest	2	Sousa et al., 2012
DNA taxonomy	Cetacea	Characterization of Cetacean species	61	Viricel and Rosel, 2012
DNA taxonomy	Rodentia	Characterization of Chinese small mammals	11	Lu et al., 2012
DNA taxonomy	Rodentia	Characterization of species in the Praomyini tribe	40	Nicolas et al., 2012
DNA taxonomy	Chiroptera	Characterization of Neotropical *Myotis* bats	18	Larsen et al., 2012
DNA taxonomy	Chiroptera	Characterization of Italian echolocating bats	31	Galimberti et al., 2012b
DNA taxonomy	Chiroptera	Charachterization of the Mexican funnel-eared bats	2	López-Wilchis et al., 2012
DNA taxonomy	Chiroptera	Characterization of Yucatan phyllostomid bats	20	Hernández-Dávila et al., 2012
DNA taxonomy	Didelphimorphia	Characterization of atlantic forest didelphid marsupials	11	Agrizzi et al., 2012
DNA taxonomy	Rodentia	Characterization of minibarcode regions for rodents identification	103	Galan et al., 2012
DNA taxonomy	Chiroptera	Characterization of genetic diversity of northeastern Palearctic bats	38	Kruskop et al., 2012
DNA taxonomy	Rodentia	Characterization of Brazilian Sigmodontine Rodents	45	Müller et al., 2013
DNA taxonomy	various	Identification of marine mammals along the French Atlantic coast	15	Alfonsi et al., 2013
DNA taxonomy	Chiroptera	Identification of cryptic species in the New World bat *Pteronotus parnellii*	1	Clare et al., 2013
DNA taxonomy	Chiroptera	Identification of a new bat species in Vietnam	1	Kruskop and Borisenko, 2013
DNA taxonomy	various	Identification of Brazilian forest mammals	7	Cerboncini et al., 2014
DNA taxonomy	Chiroptera	Identification of cryptic bat species in French Guiana and Brazil	2	Thoisy et al., 2014
DNA taxonomy	Primates	Characterization of Peruvian primate species	2	Ruiz-García et al., 2014
DNA taxonomy	Chiroptera	Characterization of Kerivoula bats in Thailand	7	Douangboubpha et al., 2015
DNA taxonomy	Chiroptera	Identification of Malaysian bat species	9	Wilsonet al., 2014
DNA taxonomy	Rodentia	Identification of alien *Callosciurus* squirrels in Argentina	5	Gabrielli et al., 2014
DNA taxonomy	Rodentia	Characterization of Chinese species of Murinae and Arvicolinae	54	Li et al., 2015b
DNA taxonomy	Artiodactyla	Characterization of Chinese Cervidae	21	Cai et al., 2015
DNA taxonomy	Chiroptera	Characterization of two Southeast Asian *Miniopterus* species	2	Li et al., 2015a
DNA taxonomy	Rodentia	Characterization of Eurasian Ground Squirrels	16	Ermakov et al., 2015
Forensic	various	Traceability of bushmeat origin from Central African and South American countries	12	Eaton et al., 2010
Forensic	Artiodactyla	Identification of wildlife crime cases in South Africa	2	Dalton and Kotze, 2011
Forensic	various	Investigation of illegal hunting cases of Brazilian wildlife	2	Sanches et al., 2012

Table 1 – Updated list of case studies dealing with mammals DNA barcoding. For each study, the context of application, the taxonomic order of target mammals, the aim of the work and the number of species involved are reported *(continued)*.

Context	Order	Aim	N°species	References
Forensic	Artiodactyla	Identification of African bushmeat items	15	Bitanyi et al., 2013
Forensic	various	Identification of organs of threatened species	10	Luo et al., 2013
Forensic	Primates	Identification of primate bushmeat in Guinea-Bissau markets	6	Minhós et al., 2013
Forensic	Artiodactyla	Traceability of animal horn products in China	10	Yan et al., 2013
Forensic	various	Authentication of South African wild meat products	10	D'Amato et al., 2013
Forensic	Artiodactyla	Identification of ungulates used in traditional chinese medicine	8	Chen et al., 2015
Forensic	various	Development of a traceability system for African forest bushmeat	59	Gaubert et al., 2014
Non-invasive sampling	Artiodactyla	etection of Kenyan mountain bongo from faecal samples	1	Faria et al., 2011
Non-invasive sampling	Carnivora	Identification of Carnivore species from faecal samples	33	Chaves et al., 2012
Non-invasive sampling	Carnivora	Identification of felid species from scat samples	4	De Matteo et al., 2014
Non-invasive sampling	various	Species identification from faeces	14	Inoue and Akomo-Okoue, 2015
Non-invasive sampling	various	Species identification from blowfly guts content	40	Lee et al., 2015
Parasitology investigation	various	Identification of bloodmeal hosts of ectoparasite species	16	Alcaide et al., 2009
Parasitology investigation	various	Identification of bloodmeal African hosts of tsetse flies	7	Muturi et al., 2011
Parasitology investigation	various	Identification of bloodmeal hosts of biting midges	3	Lassen et al., 2011
Parasitology investigation	various	Development of a rapid diagnostic approach to identify bloodmeal hosts of mosquitoes	5	Thiemann et al., 2012
Parasitology investigation	various	Identification of bloodmeal hosts of ticks	10	Gariepy et al., 2012

most mammals. Although considered illegal, the bushmeat hunting is an increasing economic activity in many countries among which Western and central Africa and other tropical regions (Nasi et al., 2008). In these countries the practice has historically been conducted for subsistence consumption or for local trade and now has reached unsustainable levels (Jenkins et al., 2011; Harrison et al., 2013; Borgerson, 2015).

Several studies, recently examined the utility of DNA barcoding as a standard tool to monitor the traffic of mammals (i.e., whole animals, meat, and other products), with particular emphasis on species commonly traded in bushmeat markets or to determine the species of unknown samples deriving from local cases of poaching or species substitution (see for example Eaton et al., 2010; Dalton and Kotze, 2011; Gaubert et al., 2014). These studies encompassed different groups of mammals such as: bovids (Bitanyi et al., 2011; Cai et al., 2011), suids (Eaton et al., 2010) and primates (Minhós et al., 2013) or covered a wider panel of taxa in an attempt to generate reference datasets for future applications. Concerning this last category, a clear example is given by the DNABUSHMEAT dataset developed by Gaubert and colleagues (2014). Four mitochondrial gene fragments (including the barcode *coxI*), were sequenced in more than 300 African bushmeat samples belonging to nine orders and 59 species. Sequences were then included as references in a query database, called DNABUSHMEAT, which provides an efficient DNA typing decision pipeline to trace the origin of bushmeat items. The DNABUSHMEAT project also contributed in filling the existing gap of African mammals representations in the international archives (i.e., NCBI and BOLD). The availability of a well populated reference dataset is a necessary condition for a successful application of DNA-based identification techniques. The relevance of reference databases has been underlined in recent studies, where a DNA barcoding survey on bushmeat food items traded in Tanzania (Bitanyi et al., 2013) and South Africa (D'Amato et al., 2013) revealed a low correctness of species identification by consumers (i.e., 59% of 124 analysed samples, Bitanyi et al., 2013) and a high rate of species substitution in local markets (i.e., 76.5% of 146 samples, D'Amato et al., 2013). Such problem is not uncommon in the context of the global food market and many published works highlighted the suitability of DNA barcoding in monitoring and hopefully reducing the overexploitation of wildlife species (see for example, Barbuto et al., 2010; Ardura et al., 2013).

Use of animal parts in traditional medicine

The use of animal organs or parts in traditional medicine involves many mammalian species that are currently known for their threatened or endangered conservation status. Among the most frequent cases there is the illegal hunting and trading of rhinoceros horn, saiga antelope horn, bear bile crystals and many others which are commonly used as ingredients in traditional Asian medicine (Luo et al., 2013; Yan et al., 2013). Despite the existing international legislation for the safeguard of these species (i.e., the CITES and the IUCN Red List), the trade of organs still remains an issue of major concern for wildlife conservation and is accelerating the extinction of many species.

As reported in several studies, animal organs/parts are usually processed to obtain powder, tablets, capsules and oils (Coghlan et al., 2012; Cao et al., 2014). Such processes impede any kind of morphological identification and therefore it is almost impossible to set up a suitable traceability pipeline along the supply chain. A method to characterize the biological origin of processed materials is thus mandatory to overcome the limits of morphological-based approaches. In recent

years, some studies highlighted the efficacy of DNA barcoding in authenticating mammal traded organs/parts or their occurrence in traditional medicine products (Luo et al., 2013; Yan et al., 2013). Most of these studies focused on the identification of horns and horn powder, mainly belonging to Cervidae and Bovidae such as the Saiga antelope (*Saiga tatarica*), a protected migratory ungulate living in central Asia and south-eastern Europe, whose horns are one of the main ingredients of the "Lingyangjiao", a traditional Chinese remedy (Chen et al., 2015).

Also in this case, DNA barcoding shows great potentials and should be considered as a valid tool for enforcing local and international legislation and to prosecute cases of illegal trade of mammal organs/parts.

Pet trade and monitoring of alien species

Another issue of major concern involving wildlife conservation and in particular mammals is the trade of organisms as pets. Nowadays, the pet trade is a common pathway of species introduction at the global scale (Bertolino, 2009; Bomford et al., 2009; Genovesi et al., 2012). Frequently, traded individuals are able to establish wild populations as a consequence of either accidental escapes or deliberate releases thus provoking severe problems to the indigenous communities. As a matter of fact, the introduction of alien species is one of the most important causes of biodiversity loss and represents a long-term threat to ecosystem functioning (Mack et al., 2000; Ehrenfeld, 2010; Strayer, 2012). When monitoring or preventive actions are required to control the spread of invasive species, as well as tracking their potential pathways of introduction, the first step is the correct identification of the invasive taxon (Boykin et al., 2012; Pisanu et al., 2013).

In this context, DNA barcoding showed great potential, for instance in the case of squirrels. Many squirrel species belonging to different continents have been introduced through the international pet trade for aesthetic reasons, or to increase hunting opportunities (Long, 2003), and in most cases they established as successful invaders (Bertolino, 2009; Martinoli et al., 2010). Some studies also suggested a lack of taxonomic knowledge within this well studied groups of mammals (Gabrielli et al., 2014; Ermakov et al., 2015). *coxI* barcode sequences were used to investigate the taxonomic status of a group of invasive tree squirrels belonging to the genus *Callosciurus* introduced in Argentina. Unexpectedly, the captured animals were found to be grouped in a previously uncharacterized molecular lineage closer to *C. finlaysonii* rather than to *C. erythraeus* as initially expected from morphological comparisons (Gabrielli et al., 2014). Ermakov and co-workers (2015) used DNA barcoding to characterize the whole diversity of Eurasian ground squirrels. They found unexpected levels of *coxI* divergence in four species out of the 16 investigated, suggesting the occurrence of undescribed cryptic species.

In conclusion, the system generated from DNA barcoding is really useful in the forensic field, and mammals indeed represent a group of organisms in which this application is really welcomed.

Parasitological analyses

Mammals are the natural hosts for a wide panel of parasites. In a broader vision, the parasites typically harbored by mammals could be grouped in macroparasites (e.g., helminths and arthropods) and microbial pathogens (e.g., viruses and bacteria) (Price, 1980; Pedersen et al., 2007; Hatcher and Dunn, 2011). The attack by one or more group of parasites can negatively affect the fitness of the host and even cause significant population declines or boost the extinction risk in already threatened species (Pedersen et al., 2007). In addition, it has been estimated that since the end of 20th century, at least 75% of the emerging infection diseases for humans were zoonotic (Taylor et al., 2001). For this reason, the monitoring and control of zoonotic diseases is nowadays one of the most important concerns in global economies and human health (Daszak et al., 2000; Chomel et al., 2007; Thompson et al., 2009; Rhyan and Spraker, 2010). Another factor influencing the spread of parasites and therefore affecting the conservation status of mammal species is the interaction of indigenous populations with alien taxa. Alien species can indeed carry along with them non-indigenous parasites and these may be transmitted to native species usually lacking an appropriate defense mechanism (Dunn and Hatcher, 2015; Romeo et al., 2015).

Knowledge of the exact species of parasite and/or of the mammal that is carrying harmful pathogens is fundamental to shed light on the factors influencing the occurrence, proliferation, and transmission mediated by animal vectors of such agents (Besansky et al., 2003; Criscione et al., 2005; Kent, 2009). In this framework, molecular methods and in the last decade the DNA barcoding approach, have been playing a key role to understand the complex relationships occurring among mammal hosts, parasites and their intermediate vectors. Most parasites are indeed difficult to discriminate based on morphology, for different reasons (lack of discriminating features, very different life stages, recovery of damaged or partial specimens, see for instance Ferri et al., 2009). For example in the case of endoparasites, their identification is often based on post-mortem examination of the hosts, because less-invasive approaches (e.g., the collection of eggs, larvae or pieces in host blood, tissue samples or faeces) cannot permit an easier identification owing to the loss of many diagnostic tracts (Ondrejicka et al., 2014). DNA barcoding approach contributed to overcome these limits and successful protocols have been developed to identify the principal classes of parasites affecting mammals such as filarioid nematodes (Ferri et al., 2009), cestodes (Galimberti et al., 2012a), ticks (Zhang and Zhang, 2014) and mosquitoes (Cywinska et al., 2006). In other cases, DNA barcoding has been largely applied to identify the mammal hosts of important parasites / pathogens. These case studies especially involved rodent species complexes characterized by a high number of cryptic taxa inhabiting poorly studied areas of the planet. Specifically, in 2012, Lu and co-workers, studied the relationships between *Rickettsia* bacteria (i.e., the agent responsible for the spotted fever) and ten rodent hosts of China (Lu et al., 2012). DNA barcoding was used to differentiate host species and the values of molecular divergence highlighted the need for further taxonomic investigations on some species groups. Similarly, in 2013, Müller and co-workers used *coxI* barcode sequences to recognize members of Sigmodontinae subfamily in Brazil which are reservoirs of zoonoses including arenaviruses, hantaviruses, Chagas disease and leishmaniasis (Müller et al., 2013).

One of the most innovative applications of DNA barcoding in the study of host-parasite interactions is the characterization of insect bloodmeals. As a matter of fact, most zoonoses are likely to be vector-borne by blood-feeding arthropods (Jones et al., 2008) which dictate the relationship between host and pathogen (Thiemann et al., 2012). Blood feeding vectors may transmit agents responsible for emerging diseases such as malaria, viral encephalitis, West Nile virus, Chagas disease, Lyme disease or African sleeping sickness (Kent, 2009). By studying arthropods behaviour, it has been possible to understand the evolution of host specificity between vertebrates and their ectoparasites, how the host choice drives pathogen transmission, and the economic and demographic impacts of ectoparasite infestations on wildlife and domestic livestock (Kent, 2009). A deep knowledge of these factors can help improving reliable disease risk models to be used in veterinary and public health contingency plans (Kent, 2009; Gomez-Diaz and Figuerola, 2010; Collini et al., 2015). Several DNA barcoding-based surveys have been conducted in the last years to fill the gaps in the comprehension of such dynamics. Published studies involved a specific group of blood-feeding arthropods such as *Culex* spp. mosquitoes (Muños et al., 2012; Thiemann et al., 2012), ticks (Gariepy et al., 2012; Collini et al., 2015), biting midges (Lassen et al., 2011), tsetse flies (Muturi et al., 2011) as well as the simultaneous analysis of a wide range of vectors (Alcaide et al., 2009).

In all of these case studies, the analysis of *coxI* barcode sequences obtained from the bloodmeal consumed by hematophagous vectors allowed to trace the identity of the "last supper" (i.e., the vertebrate host – often a mammal) on which the vector fed before being collected. Finally, in a recent study conducted in Peninsular Malaysia, a biodiversity hotspot, Lee and colleagues (2015) proposed the DNA barcoding analysis of the stomach content of the saprophagous / coprophagous blow-

flies (Calliphoridae) as a suitable, fast and economic tool to characterize the mammal biodiversity of a study area.

In conclusion, the analysis of parasites is a complex matter and molecular tools, like DNA barcoding, are really welcomed.

Massive DNA sequencing

In the last decade, there has been a great revolution in DNA sequencing technologies. The introduction of the so-called "Next Generation Sequencing", NGS, also better defined as "High Throughput DNA Sequencing", HTS, expanded the universe of DNA sequencing. The rise of DNA barcoding took place in the same years and it was only a matter of time to assist to the encounter of these two worlds. The DNA metabarcoding is the result of this marriage (Taberlet et al., 2012). HTS has revolutionized DNA-based research, especially biodiversity assessment in complex biological matrix (i.e. comprising many species contemporaneously) (Shokralla et al., 2012). In HTS, DNA sequences are accumulated at an unprecedented rate and it is now possible to analyze simultaneously several samples (through multiplexing) identified by custom-designed oligonucleotide tags.

The idea is simple: DNA is everywhere, and this molecule is relatively stable and durable in dry, but even wet conditions (Dejean et al., 2012; Yoccoz et al., 2012). This DNA represents the so-called "environmental DNA" or eDNA (Shokralla et al., 2012; Thomsen and Willerslev, 2015). eDNA is formed by short DNA molecules (i.e., free, cellular debris or particle-bound), which are released by living or dead organisms. eDNA is typically defined by the process used to collect it, and this makes its definition in a some way foggy. Much more clear is the use of eDNA: the living beings present in the environmental sample are not known and HTS allows to identify them. In addition, even if DNA in the environment is relatively stable, it is also usually degraded. In such a condition the classic DNA barcoding approach is often useless, conversely to metabarcoding, due to the possibility of generating a huge amount of data. The first application in mammals was aimed at uncovering the diets composition of elusive animals (Valentini et al., 2009). This approach was successfully adopted in the last 5 years with some group being very well represented, such as Chiroptera (Bohmann et al., 2011; Alberdi et al., 2012; Vesterinen et al., 2013; Krüger et al., 2014a,b; Burgar et al., 2014; Clare et al., 2014a,b; Hope et al., 2014; Sedlock et al., 2014).

Although it is now relatively simple to characterize the diets of herbivorous and insectivorous mammals, the analysis of diets of carnivores is really challenging because predator DNA can be simultaneously amplified with prey DNA (Symondson, 2002; King et al., 2008; Symondson and Harwood, 2014; Boyer et al., 2015). To avoid this problem an interesting approach was the introduction of blocking primers in the analysis of snow leopard (*Panthera uncia*) diet (Shehzad et al., 2012). This molecular approach prevents the amplification of predator DNA allowing the amplification of the other vertebrate groups.

HTS techniques can also be used to identify elusive mammal species from the faeces found on the ground (Michalski et al., 2011; Chaves et al., 2012; Rodgers and Janecka, 2013) or as a general method to identify mammals in complex mixtures (Foote et al., 2012; Galan et al., 2012; Deagle et al., 2013; Tillmar et al., 2013). Noteworthy, the possibility of better defining the areas of distribution of some species with such non-invasive sampling is of particular interest to increase the knowledge of mammals biology and conservation.

In spite of these practical approaches, HTS techniques in mammals have also been used to characterize population structure (Rasgour et al., 2011; Botero-Castro et al., 2013). The rapid developments of these technologies have created new possibilities to build quickly and cost-efficiently reference libraries for whole mitochondrial genomes in a wide range of animal lineages. The accumulation of whole mitogenomes in the public domain covering the Tree of Animal Life will improve our knowledge on evolutionary history of animals and global patterns in genomic features of mitochondria as a sort of future next comprehensive barcode marker.

In conclusion, HTS and the DNA metabarcoding approaches are expanding fields of research that will likely be very fertile for several years to come, particularly considering the rapid increase of reference databases that allows a better characterization of complex cases.

The integrative role of DNA barcoding

As described in the previous sections, DNA barcoding can be successfully involved as a supporting tool for both theoretical and applicative necessities. The presented case studies highlighted the versatility of the approach, and the aptitude of being integrated with other sources of taxonomic information in a highly interconnected environment.

As a matter of fact, species are not unequivocally defined and their designations based on a single category of taxonomic features (morphological, ecological, molecular, or biogeographic) is questionable. In this context, molecular techniques and more recently the DNA barcoding, triggered a small revolution inside taxonomy: the process of identifying biological entities opened the doors to a real integration of knowledge to improve practical purposes (Unit of Conservation *sensu* Dodson et al., 1998) or theoretical approaches (Unit of Evolution or Evolutionarily Significant Unit, ESU, *sensu* Ryder, 1986).

In a framework of integration, divergent molecular lineages do not necessarily reflect distinct species but, in many cases, molecular data remains at the core of current taxonomic approaches. However, the future of taxonomy cannot rely only on molecular markers. Rather, it is more and more oriented towards the definition of the best way to integrate molecular data into multidisciplinary taxonomic approaches.

In an attempt of providing a better understanding of the possible taxonomic outcomes deriving from an integrative DNA barcoding-based approach, Galimberti and colleagues recently proposed a schematization using echolocating bats as a model (Galimberti et al., 2012b). In this schematic view, the taxonomic ranks are grouped based on their information content: from individuals (i.e., the less informative level), to species (i.e., the more informative level), passing through intermediate categories defined by the adoption of a single (i.e., morphotype, Molecular Operational Taxonomic Unit - MOTU and unconfirmed candidate species) or an integrative approach (i.e., Integrative Operational Taxonomic Units - IOTU, deep conspecific lineage and confirmed candidate species).

Such schematization, tested on Italian bats species, confirmed the risk of erroneous taxonomic interpretations when molecular entities (MOTUs) are used as the only criterion (see the case of *Eptesicus* species in Galimberti et al., 2012b). The authors also proposed a new entity, the IOTU, defined by molecular lineages that have further support from at least one additional line of evidence. This concept links different data sources in taxonomy, allowing morphological, ecological, geographical and other characteristics of living beings to be better combined with molecular data. The application of IOTU concept to the study of echolocating bats showed for example the occurrence a new undescribed species of *Myotis nattereri* inhabiting the southern part of the Italian peninsula.

Known problems of DNA barcoding of mammals

DNA barcoding generated huge controversies, but like any other diagnostic technique it has pros and cons. Since its launch, the practicalities of a universal barcode for all the living beings showed pitfalls, as firstly dependent on the group of organisms under examination (see Casiraghi et al., 2010 and Collins and Cruicsshank, 2013 and references therein). Concerning mammals, three main categories of problems should be taken into account when DNA barcoding is applied to their study. The first concerns the availability of public and well populated reference archives of DNA barcodes and related specimens (see the dedicated paragraph above). Reference sequences constitute the main core of the DNA barcoding initiative and their absence or the lack of control of the correct identification of the source specimens by expert taxonomists, can irremediably affect the assignment of newly generated query sequences. The second problem category is directly related to the processes of molecular evolution, such as the occurrence of NUMTs (i.e., nuclear copies of mitochondrial DNA). NUMTs are usually considered a challenge in those case studies based on mtDNA

due to the fact that they can be inadvertently amplified, thus causing bias in the barcode dataset and in the accuracy of subsequent analyses (e.g., overestimating intra and interspecific variability levels) (Bensasson et al., 2001; Song et al., 2008; Ermakov et al., 2015). Recently, Ermakov and co-workers (2015), described the amplification of NUMTs in a species of Eurasian ground squirrel. This is only one of the multiple documented examples of this problem. NUMTs have been found in over 20 mammalian species belonging to seven different orders (see (Triant and DeWoody, 2007) for more details). To overcome the risk of NUMTs interference, Song et al. (2008) and Buhay (2009) suggested step-by-step procedures in order to identify possible pseudogenes. BOLD itself provides a quality control tool to check sequences for the presence of stop codons and verify that they derive from *coxI* by comparing them against a Hidden Markov Model (Ratnasingham and Hebert, 2007). To avoid NUMTs interference, Triant and DeWoody (2007) suggested three alternative strategies: i) the isolation of the entire mtDNA genome, ii) the use of tissue sources naturally rich in mtDNA (e.g., liver and muscle), and iii) the use of PCR primers that amplify substantial portions of the mtDNA molecule (i.e., > 1 kb). In other cases, the re-extraction of gDNA and the reamplification of the barcode region can help resolving the matter (Ermakov et al., 2015). The last group of issues causing failure of DNA barcoding identification are mainly due to the essence of biological species, rather than in the method, and relies on the criteria adopted to discriminate species. As well as in many other cases, species delimitation in mammals is based almost completely on two strategies: the genetic distance and the reciprocal monophyly (Dávalos and Russell, 2014). However, when dealing with mtDNA, attention is needed when automatically associating divergence values (which are often useful "hypothesis generator") with the extent of gene flow. As discussed by Dávalos and co-workers (2014), such way of thinking can lead to false-positive errors in which distances or monophyly diagnose species despite ongoing gene flow, and false-negative errors when gene flow is taken into account despite its absence. Mitochondrial DNA barcode markers, are indeed prone to problems such as introgression, incomplete lineage sorting and hybridization and this may generate misleading results particularly in mammals (Heckman et al., 2007; Godinho et al., 2011; Melo-Ferreira et al., 2012).

In a DNA barcoding study conducted on the whole panel of species of Eurasian ground squirrels, Ermakov and colleagues (2015), documented the occurrence of mtDNA introgression in four cases due to ancient hybridization events followed by divergence. Similar conditions have been also detected in other groups of mammals such as bears (Hailer et al., 2012), marmots (Brandler et al., 2010) and bats (Berthier et al., 2006; Artyushin et al., 2009).

Moreover, mammals are often characterized by sex-biased gene flow in which males disperse widely and females exhibit natal philopatry (Greenwood, 1980). Such condition also shape the genetic structure of species and populations when maternally-inherited mitochondrial markers are analysed (Clare, 2011; Dávalos and Russell, 2014). To overcome this limit of mtDNA, the selection of complementary loci with independent evolutionary histories can help depicting a more realistic schematization of the divergences at both the intra and interspecific level. For example, in 2011, Clare published a study in which she successfully compared the phylogeographic patterns revealed through the maternally inherited mitochondrial *coxI* and the paternally inherited 7th intron region of the *Dby* gene on the Y-chromosome in eight common Neotropical bat species (Clare, 2011). The combined approach proposed by Clare allowed the author to validate patterns of gene flow and also to find previously unrecognized species.

Similarly, Silva and coauthors (2014) developed a method based on polymorphism of the mitochondrial *cytb* and the nuclear *KCAS* gene to identify nine ungulate species occurring in North Africa.

As a final consideration, it is important to underline that when DNA barcoding investigations reveal the occurrence of new intraspecific lineages, they should be integrated with alternate lines of evidence such as ecological data, morphology and geography to avoid misinterpretation of genetic variability (Galimberti et al., 2012b). DNA barcoding problems are well known, but as underlined above, we do not have to stop at them, and consider the whole system created by this technique.

The future of DNA barcoding of mammals

In spite of an apparent decreasing trend in the rate of publication on the topic "mammals DNA barcoding" (see Fig. 1), this molecular approach is still alive and healthy. Probably, this apparent reduction is due to the fact that the modern taxonomic system is now a matter of fact, and the DNA barcoding approach is often integrated even without naming it. Indeed, DNA barcoding does not rely on the use of a monospecific marker only, as often stated, but is currently referred to as a way of thinking rather than a name of a technique.

In the case of mammals, DNA barcoding is alive and proactive, because these animals represent the principal group in which the scientific community moved from a *sensu stricto* approach to broader applications. Indeed, DNA barcoding *sensu stricto* is designed for not specialized operators in a certain taxonomic field. Generally speaking, the specialist does not have real problems to discriminate among the living beings he/she is studying, because in most cases, he/she himself/herself is the one who created the classification system (hopefully using a robust integrated approach). Consequently, the specialist is the principal actor who has to work to create a solid DNA barcoding system to help other users in achieving a correct identification for purposes ranging from wildlife management, to conservation, eco-ethological studies and so on.

As we underlined in our essay, in many cases DNA barcoding in mammals has already reached this level and we foresee that in the next future this approach will move towards two main branches of application. The first branch (the molecular one) is that of taxonomic studies to fully uncover the hidden biodiversity within this animal group. On the other side, even if strictly connected, there will be the branch of "taxonomic services" in which DNA barcoding is one of the more correct, easier and more sparing (both in terms of money and time) solutions.

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Associate Editor: D.G. Preatoni