FORUM

Barcoding Lepidoptera: Current Situation and Perspectives on the Usefulness of a Contentious Technique

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Barcoding Lepidoptera: Situação Atual e Perspectivas sobre a Utilidade de uma Técnica Controversa

RESUMO - A necessidade crescente de identificação e delimitação de novas espécies, ou de espécies cripticas já estabelecidas, que estão sendo perdidas a uma taxa também crescente, tem levado diversos especialistas a utilizarem uma variedade de ferramentas moleculares e computacionais. Neste momento, taxonomistas devem estar atentos a toda nova tecnologia disponível na chamada “revolução dirigida pela tecnologia” na sistemática, que tem entre as novas ferramentas moleculares a utilização de “DNA barcodes”. O uso de “DNA barcode” tem sido amplamente discutido por aqueles que aplicam essa abordagem com sucesso para identificar e diagnosticar espécies, e por aqueles que acreditam que são tantos os problemas no uso desse marcador molecular que não se justifica seu emprego. Para insetos da ordem Lepidoptera nenhum lado parece estar totalmente certo ou errado e, embora alguns grupos de lepidópteros tenham sido resolvidos taxonomicamente pelo uso exclusivo ou adicional desse marcador, para outros o “barcode” ajudou pouco a resolver problemas taxonômicos. Aqui nós apresentamos brevemente prós e contras do uso de “DNA barcode” como ferramenta em estudos taxonômicos, com atenção especial para estudos com grupos de Lepidoptera desenvolvidos nos últimos anos.

PALAVRAS-CHAVE: Biodiversidade, DNA, espécie criptica, sistemática, taxonomia

ABSTRACT - Faced by a growing need of identification and delimitation of new and established cryptic species that are being lost at an increasing rate, taxonomists can now more than ever take advantage of an enormous variety of new molecular and computational tools. At this moment they should be open to all new available technologies in the so called “technology-driven revolution” in systematics. The use of the “DNA barcode” has been discussed by those applying successfully this approach to identify and diagnose species and by those who believe that the flaws in the use of this molecular marker are as many as to negate the worth of its employment. For insects of the order Lepidoptera neither side seems totally correct or wrong, and although many groups of lepidopterans have been taxonomically resolved by using exclusively or additionally this marker for diagnoses, for others the “barcode” helped little to resolve taxonomic issues. Here we briefly present some pros and cons of using DNA barcode as a tool in taxonomic studies, with special attention to studies with groups of Lepidoptera developed in the last few years.

KEY WORDS: Biodiversity, cryptic species, DNA barcode, systematics, taxonomy

Taxonomic Diversity, Systematics and the Biodiversity Crisis

The term biodiversity as currently conceived appeared in the literature about 20 years ago (Wilson 1988), and since then it has been widely used in all sectors of society. Even if the original definition is ample, meaning the diversity and variability of life forms, the word is used in many different ways in the literature, amongst them as a synonym of species richness (see DeLong Jr 1996). Regardless of what definition is used, an important component of global biodiversity is the total number of species on earth (Wilson 1988). Even
if this question looks simple, the total number of species of live organisms on earth is still unknown, with estimates varying from five million to a hundred million species (Erwin 1982, May 1990, Stork 1993). Few estimates of total species richness exist for any country and the first attempt for Brazil appeared recently in the literature (Lewinsohn & Prado 2002, 2005). In any case, as Erwin (1991) pointed out, no matter the total number of species that are present in earth, biodiversity is being lost at an astonishing rate, and the humans are responsible.

The biodiversity crisis has been exhaustively discussed in the last two decades (Western 1992, Daily & Ehrlich 1995), but despite the efforts and increased knowledge about species and their habitats, human actions have changed most of earth’s biomes in all levels, leading to an overall impoverishment of earth biodiversity (Ehrlich 1988, Davies et al 2001, Hong & Lee 2006). Given this trend, there is an urgent need for a global strategy for conservation of all remaining biodiversity, and conservation biology must now face up the identification of what needs to be done and in which order of priority (Olson et al 2002). To accomplish this task, it is generally agreed that systematics and taxonomy are prerequisites to effectively describe and understand the worlds’ biodiversity (Savage 1995). At this point scientists are confronted with the challenge of recognizing and describing adequately all living taxa, therefore providing reliable data for conservationists, policy makers and resource managers (Savage 1995).

At the present time, scientists can take advantage of an enormous variety of molecular and computational tools never seen before, and they should be open to all new available technologies in this so called “technology-driven revolution” in systematics (Wilson 2005). This does not mean that morphology and other traditional methods should be discarded, but instead, they should be combined with new approaches as a way of getting the maximum benefits for systematics and taxonomy (Miller et al 1997, Burns & Janzen 2005, Schlick-Steiner et al 2006, 2007). Especially considering that the availability of traditional morphology-based systematists for many taxa is well below the ideal, rapid molecular techniques such as DNA barcoding can be used in a simple way to uncover cryptic species in a world where species are lost daily (Schlick-Steiner et al 2006, Bickford et al 2007).

In the present forum we briefly discuss the application of DNA barcoding as a useful tool for helping in describing and understanding earth’s biodiversity, discussing some of its benefits and pitfalls, with special attention to which is being reached within Lepidoptera taxonomic analysis.

**DNA Barcoding**

**Concept definition.** The idea behind using DNA variation for species identification is not a new concept (Sperling 2003). It emerged progressively in the last few decades with the development of PCR-based methods and has been successfully used in fields of economic interest (whale hunting, Baker & Palumbi 1994; caviar species diagnose, DeSalle & Birstein 1996), and where traditional taxonomy presents some limitations, like studies of bacterial and microbial biodiversity (e.g. Woese 1996, Zhou et al 1997) and identification of cryptic species (Collins & Paskewitz 1996), invasive and forensic species (e.g. Sperling et al 1994) and pathogens and vectors (e.g. Walton et al 1999). Molecular tools for species identification, however, were not implemented in a broader scope until the beginning of this century.

In 2003, Hebert et al (2003a) proposed the creation of a universal system for species inventory based on a standard molecular approach. They proposed the development of a standardized, rapid and inexpensive species identification method, accessible to non-specialists (i.e. non-taxonomists), compiling data in a public library of “DNA barcodes” that would be linked to named specimens (DNA Barcode of Life Project; http://www.barcoding.si.edu). This conception is based on the assumption that every species is expected to have a unique “DNA barcode” and that genetic variation between species exceeds variation within species (Hebert et al 2003a, b). The “DNA barcode” itself, as proposed by Hebert et al (2003a), is a small gene region and consists of 648 bp from the 5’-end of the *cytochrome c oxidase I* (COI) mitochondrial DNA gene.

The main ambitions of DNA barcoding are to assign unknown specimens to species and to enhance the discovery of new species, particularly in organisms with complex or inaccessible morphology (Hebert et al 2003b). The project does not have the goal to build the tree of life nor to perform molecular taxonomy (Ebach & Holdrege 2005, Gregory 2005), but rather to produce a simple diagnostic tool based on taxonomic knowledge that is integrated in the DNA barcode reference library (Schindel & Miller 2005).

The “Barcode of Life Data Systems” (BOLD, Ratnasingham & Hebert 2007) is a database especially dedicated to DNA barcodes (i.e. a DNA barcode reference library) that is central to the DNA barcoding approach. It has been designed to record not only DNA sequences from several individuals per species, but also complete taxonomic information, place and date of collection, and images of all specimens. This reference library is expected to become increasingly useful in the near future, enabling rapid identification of any organism to a low taxonomic level using specific short-DNA sequences (Hajibabaei et al 2006a).

**Usefulness and advantages.** The usefulness of DNA barcoding has been widely discussed in the scientific community (Tautz et al 2003, Moritz & Cicero 2004, Rubinoiff 2006a) and its relevancy is now supported by numerous successes and by the increasing amount of DNA barcoding projects (Consortium for the Barcode of Life – CBOL).

The COI gene has proved to be suitable for species identification in a large range of animal taxa, including butterflies and moths (Hebert et al 2004a, Janzen et al 2005, Hajibabaei et al 2006b, Burns et al 2008), birds (Hebert et al 2004b, Kerr et al 2007), mayflies (Ball et al 2005), spiders (Greenstone et al 2005), fishes (Ward et al 2005), ants (Schlick-Steiner et al 2006), Crustacea (Costa et al 2007), gastropods (Remigio & Hebert 2003), mosquitoes (Kumar et al 2007), and wasps (Smith et al 2008). The efficacy of COI-based barcoding is also documented for few other kingdoms like fungi (e.g. Seifert et al 2007), macroalgae (McDevit &
One unquestionable advantage of DNA barcoding is the rapid acquisition of molecular data. While morphological data are usually time consuming, and in some cases almost impossible to work with (e.g. earthworms, Huang et al. 2007; diatoms, Evans et al. 2007), the time and cost-effectiveness of DNA barcoding enables rapid and automated species identification (Hebert et al. 2003a) (presuming that at some point the barcodes are ultimately anchored to species that have been identified on the basis of traditional features). In this way, it could, for example, improve large surveys aiming at unknown species detection and identification of pathogenic species with medical, ecological and agronomical significance (Armstrong & Ball 2005, Ball & Armstrong 2006).

Another obvious advantage of DNA barcoding comes in situations where species identification must be molecular-based. One of these situations is when it is virtually impossible to match adults with immatures (e.g. amphibians, Randrianaiana et al. 2007; coleopterans, Ahrens et al. 2007; lepidopterans, Hebert et al. 2004a; fish larvae, Pegg et al. 2006). Other situation occurs when it is necessary to determine specimens identity based on a fragment or in damaged organisms (e.g. Pons 2006). Identifications also need to be molecular-based in cases where morphological traits do not clearly discriminate species (e.g. red algae, Saunders 2005; mosquitoes, Kumar et al. 2007), when species are difficult to visualize (e.g. Blaxter et al. 2005, Webb et al. 2006) or if species have polymorphic life cycles or present high phenotypic plasticity (e.g. Lane et al. 2007).

The efficiency of DNA barcoding is well documented in the detection and description of new cryptic species (Gomez et al. 2007, Pfenninger et al. 2007, Tavare & Baker 2008) and of sibling species (Hogg & Hebert 2004, Amaral et al. 2007). In addition, the compiling of DNA barcode sequences improves studies at large geographic scales and across numerous genera (Hajibabaei et al. 2007), and information on the global distribution of species, their genetic diversity and structure may enhance the speed and effectiveness of local population studies.

DNA barcoding may also be used in many conservation studies and can be informative at many different levels of analysis (e.g. Rubinoff 2006a). Some authors suggest that it can provide important insight into the role of historical habitat fragmentation, in species diversification and may potentially contribute to the identification of priority areas for conservation (e.g. Linares et al. 2009, but see Gompert et al. 2006). In most cases, however, barcoding can be used as a simple way to optimize diversity assessments and uncover hidden biological diversity (Swartz et al. 2008).

Beyond the construction of a standard approach based for species inventory, the Barcode of Life project has enhanced communication between different scientific communities. Moreover, DNA barcoding initiative re-opens the debate on species concepts and this by itself is an important issue (Rubinoff 2006b, Miller 2007).

**Limitations and pitfalls.** Despite the great promise of DNA barcoding, this proposal is controversial (Tautz et al. 2003, Will et al. 2005) and some crucial pitfalls must be mentioned. Limitations to DNA barcoding should be clearly identified in the aim to become a universally relevant database.

One clear limitation arises from the inherent characteristics of the mitochondrial DNA. Because mtDNA genes are maternally inherited (but see Ladoukakis & Zouros 2001, Tsousis et al. 2005) some phenomena can lead to misleading results. These phenomena are, for example, the occurrence of interspecific hybridization or endosymbiotic infections that generate transfer of mitochondrial genes outside the species (Hurst & Jiggins 2005, Dasmahapatra & Mallet 2006); the occurrence of indirect selection on mitochondrial DNA arising from male-killing microorganisms and cytoplasmic incompatibility-inducing symbionts (e.g., Wolbachia) (Johnstone & Hurst 1996, Funk et al. 2000, Whitworth et al. 2007), which are particularly common among arthropods (Jeyaprakash & Hoy 2000, Werren & Windsor 2000); or any other reticulate evolutionary phenomena that can occur in a lineage (e.g. intra-specific geographical structure or host specificity, Hulcr et al. 2007).

Other limitation of COI-based DNA barcoding is the possible presence of nuclear copies of COI in the nuclear genome (Nuclear Mitochondrial DNAs – NUMTs, Williams & Knowlton 2001). However, in the sequence acquisition stage, these NUMTs can be probably detected by the sequence checking process proposed in BOLD (Ratnasingham & Hebert 2007), although recently integrated NUMTs are difficult to detect (Thalmann et al. 2004).

Different rates of genome evolution of COI gene are also a limitation, since they are not equal for all living species (e.g. poriferans x cnidarians, Erpenbeck et al. 2006). Indeed, the COI based identification sometimes fails to distinguish closely related animal species, underlining the requirement of other mitochondrial or nuclear regions (e.g. Sevilla et al. 2007). In addition, in higher plants, the COI gene showed to be inappropriate for species distinction (Kress et al. 2005, Chase et al. 2005, Pennisi 2007).

An important issue to be addressed is the frequent lack of monophyly of several groups studied. Due to a range of reasons raised to explain polyphyly in particular clades, Funk & Omland (2003) found that 23% of animal species are polyphyletic if their mtDNA data are accurate, indicating that using a mtDNA barcode to assign a species name to an animal will be ambiguous or erroneous in 23% of the time (see also Meyer & Paulay 2005, Meier et al. 2006).

Besides the biological limitations, DNA barcoding raises analytical and statistical issues (Brower 2006, DeSalle 2006, Nielsen & Matz 2006, Elias et al. 2007). At present, few studies have compared different algorithms for species assignment (Elias et al. 2007, Ross et al. 2008), and comparisons between the approaches are needed.

Delimiting species (or identifying new species) is a controversial objective of DNA barcode analyses and it is also a limitation. Hebert et al. (2004b) first proposed the use of a divergence threshold to delimit species. They proposed a standard sequence threshold to define new species, the so-called “barcoding gap”, which was defined as 10 times the mean intraspecific variation for the group under study. Despite the reported efficiency of this approach for some species (birds, Hebert et al. 2004b; lepidopterans, Hajibabaei et al. 2006b; fishes, Ward et al. 2005), the results are not
universal. Indeed, some authors argue that this approach lacks strong biological support and could not become a universal criterion suited to animal species delimitation (Meyer & Paulay 2005, Hickerson et al 2006, Wiener & Fiedler 2007, Townzen et al 2008). Some alternative approaches to delimit species have been developed (Pons et al 2006, Nielsen & Matz 2006, Abdo & Golding 2007), but they are not yet tested and no single option was found.

The sampling shortage across taxa can be also a limitation and can lead to ‘barcoding gaps’ (Meyer & Paulay 2005). The individuals chosen to represent each taxon in the reference database should cover the major part of the existing diversity. Indeed, identification constraints in BOLD commonly arise when the unknown specimens come from a currently under-described part of biodiversity (Rubinoff 2006a,b). Meyer & Paulay (2005) showed that the DNA barcode exclusively promises robust specimen assignment in clades for which the taxonomy is well understood and the representative specimens are thoroughly sampled.

### Lepidoptera and the DNA Barcoding

Lepidoptera (moths and butterflies) includes ca. of 160,000 species distributed worldwide in 124 families (Kristensen et al 2007). Lepidoptera comprises nearly 17% of all insects, and some estimates propose that the actual number of Lepidoptera will reach 500,000 species (Heppner 1998, Kristensen et al 2007). The number of species is not evenly distributed between butterflies and moths, with the latter including the bulk of species richness (only ca. 17,000 are butterflies), but the former comprising the subject of the vast majority of published studies (Brown & Freitas 1999).

Species of Lepidoptera are part of many important processes and food webs in all earth biomes, and several species are models for studies in genetics, evolution, chemical ecology, insect-plant interactions and mutualisms (Brown & Freitas 1999). Nevertheless, even if Lepidoptera appear to be a very well known group, there are dozens of new species being described each year (Aguiar et al in press), and many complexes of cryptic species have been revealed only recently by using molecular techniques (see below).

Although barcoding cannot be considered as a replacement for comprehensive taxonomic analysis, this marker has been successfully used in well-studied groups of Lepidoptera, and earlier studies had demonstrated that the DNA barcode region exhibits a considerable sequence divergence in this group, although some overlap can be seen when comparing intraspecific and interspecific divergences, as found in Parnassinae (Nazari & Sperling 2007). The ability of COI sequences to correctly identify species of lepidopterans was primarily established for five families of moths (Hebert et al 2003a). According to this study, 100% of 150 newly sampled individuals were successfully identified after a “profile” of 200 closely related species. In a simultaneous study, from the 882 individuals of Lepidoptera analyzed, about 96% of closely allied species presented more than 2% sequence divergence (Hebert et al 2003b), suggesting the use of this marker to consistently diagnose close species in this group of insects.

The first massive application of DNA barcode to identify and diagnose species within Lepidoptera has been carried out on the ongoing inventory of caterpillars and their parasitoids and host plants in northwestern Costa Rica, on the “Area de Conservación Guanacaste” (ACG) (Janzen et al 2005, 2009). This initiative can be considered a “guinea pig” to evaluate the employment of DNA barcoding as a consistent marker to diagnose species, since a huge and expressively well documented survey of several groups of Lepidoptera is available. Since 2003, about 100,000 specimens of about 3,500 morphologically defined species of adult butterflies, moths, and their parasitoids flies and wasps were precisely identified using DNA barcoding (Hebert et al 2004a, Hajibabaei et al 2006b, Janzen et al 2009). These results yielded 99.57% success in identifying morphology-based species, and DNA barcoding greatly increased the efficiency to recognize the species inventoried (Janzen et al 2009).

From these first studies on, some groups of butterflies provided very clear patterns of barcode differentiation, while others are still under investigation, since preliminary results using this marker were inconclusive, as in the case of the Pieridae Phoebis argante (Fabricius) (see Janzen et al 2009 for more examples).

The geometrid Crypsiphona ocultaria (Donovan), for example, shows external morphological differences but was previously considered a single species. The COI barcode was used to test the hypothesis of two separated species (Ӧnup & Viidalepp 2009), and the results showed that a phylogeny-based approach allowed the delimitation of C. ocultaria and the new species C. tasmanica Önup & Viidalepp, though distance-based delimitation is problematic due to substantial overlap in intra- and interspecific genetic distances.

Phylogenetic analysis of mtDNA sequences of Grammia tiger moths (Noctuidae), indeed, failed to group haplotypes according to species taxonomy (Schmidt & Sperling 2008). Several phenomena can lead to species-level polyphyly (Funk & Omland 2003, Schmidt & Sperling 2008), one being the inadequate phylogenetic signal when the short mtDNA barcode sequences do not contain enough variability to resolve the relationships among closely allied species. In the specific case of Grammia, mtDNA data pointed out that most discordance between this marker and traditional taxonomy can be explained by extensive ongoing hybridization events resulting in mtDNA introgression (Schmidt & Sperling 2008).

Roe & Sperling (2007) also found that mitochondrial DNA haplotypes do not form reciprocally monophyletic clades, although forewing variation and host plant associations support delimitation of the cryptic Pyralidae species Dioryctria reniculelloides Mutuura & Munroe and D. pseustisgella Munroe, leading the authors to propose an integrative approach by using several characters instead of using only the COI region.

Linares et al (2009) found support for the taxonomic recognition of several geographically widespread morphologically based species of the Satyrinae Heteropsis, although not all clades have strong statistical support. DNA barcoding reinforced some but not all morphology-based species, and can be problematic for recently diverged taxa.

The applicability of DNA barcode was also tested in a
diverse community of butterflies from the upper Amazon, where only 77% of well-established morphological species could be precisely identified using this marker. When more than one geographical race was analyzed, this proportion dropped to 68% (Elias et al. 2007).

Among the many barcoding studies, maybe the most emblematic was the one focusing on the Astrapias 'fulgerator' (Walch) (Hesperiidae) complex. After being barcoded in ACG, this apparently polyphagous and variable species was first divided in 10 (Hebert et al. 2004a), and more recently in 11 different species with different immature forms and using different species as larval host plants (Janzen et al. 2009). The approach applied to this study was criticized however, and it was claimed that further evidence should be presented to support the existence of 10 or 11 species within this complex (Brower 2006).

Hajibabaei et al. (2006b) also showed that among hesperids, sphingids and saturniids, 97.9% of the 521 species studied were unequivocally distinguished from all other species since their COI sequences formed distinct clusters in a neighbor-joining (NJ) analysis. Vaglia et al. (2008) also found a remarkable congruence in the discrimination of cryptic lineages by both morphological studies and phylogenetic analysis of the COI sequences in the Xylophanes loelia (Druce) and X. neoptolemas (Cramer) (Sphingidae) complexes, which appears to be in fact a complex of five distinct species. Although initially “cryptic”, these species were clearly distinguished both morphologically and genetically.

DNA sequences, including the barcode region, have also been included as part of the description of new species of butterflies both for information purposes only (as in Freitas 2007) or as part of the argument in the description of new species (Brower 1996, Willmott et al. 2009).

There are of course some groups of Lepidoptera where barcodes do not work at all, and this is critical when morphology is also of little help. This appears to be the case in the genus Actinote (Nymphalidae), where DNA barcode is showing to be ineffective to discriminate the strikingly morphologically similar, but geographically disjunct, members of the A. ‘pellenea’ Hübnner species complex, as suggested by the results of Silva-Brandão et al. (2008b). Also in the genus Phyciodes (Nymphalidae), the four morphologically very similar species belonging to the P. tharos-group are not distinguishable based on the DNA barcode (Wahlberg et al. 2003).

Why has Lepidoptera so many examples of cryptic species discerned only or primarily by DNA barcoding?

Among the lepidopterans, butterflies exhibit many examples of mimicry, where a warningly colored and distasteful model is morphologically resembled by a palatable (Batesian mimicry) or equally unpalatable (Müllerian mimicry) mimic (Brower 1984). Probably because of disseminated mimetic rings some color patterns are commoner than others, leading to groups of genetically distinct species to be very similar morphologically, since different patterns can be adaptive in different microhabitats in the forest, as is the case for Ithomiinae butterflies (Nymphalidae) in the Amazonian forest (Joron & Mallet 1998).

Different lineages can also have been apart for a short period of time, showing ecological and behavioral variance without present morphological differentiation in adult characters. The Neotropical Acraeini butterfly Actinote alalia Jordan has a disjunct distribution throughout the south and the north bounds of southern mountains of South America, showing regional differences in morphological and natural history traits. Contrasting to the southern population, found at elevations up to 800 m high, the northern populations are usually found over 1400 m high. In addition, adults from these two regions have minor but perceptive divergences in color pattern, and the larvae are remarkably different, being bluish in southern populations and brownish in the northern. Besides morphological and alitudinal variation, molecular analysis of mitochondrial DNA of individuals sampled from four different populations in southern and northern populations showed a small genetic diversification between them (Silva-Brandão et al. unpublished). This, combined with the morphological differences found among A. alalia populations, seems to suggest that an incipient process of speciation is occurring.

Additionally, insects with less dramatic wing patterns that are not under such intense selection may harbor cryptic DNA diversity. Species within the genus Hermeuptychia, small brownish Satyrinae butterflies, can in fact be composed by two or more species, as suggested by using the mitochondrial DNA Cyb (Marin et al. in press), and this subject has been investigated in more details in populations found in Brazil using DNA barcode (AVLF pers. com.).

Apart from the reason accounting for the discovery of so many new cryptic species, the use of DNA barcoding has improved the original number of morphology-based species of Lepidoptera. Janzen et al. (2009) found that 17 out of 19 families of Lepidoptera surveyed in ACG had an increase in the number of species that can be reliably identified by barcode. Many of these newly discovered ‘hidden’ species have been subsequently studied by traditional morphology-based approaches (e.g. Burns et al. 2008), showing that barcoding species can be a good way to find out where taxonomists should concentrate efforts.

Joining allopatric ‘species’ by DNA barcoding. Taxonomic discussion regarding the separation of closely related allopatric forms can take advantage of DNA barcoding as an additional source of evidence to keep two species apart or together as the same entity. This approach was applied to investigate the taxonomic position of Parides burchellanus (Westwood), an endangered species of Troidini swallowtail butterflies in the family Papilionidae (Silva-Brandão et al. 2008a). DNA barcoding obtained by this study, together with morphological and ecological data, represented strong support of the hypothesis of Mielke et al. (2004) that P. burchellanus are in fact conspecific with P. panthomus jaguarae (Foerterle), although they are both distributed allopatrically. The results were successful in showing that two Brazilian endangered species are in fact a single entity.

The Nymphalidae butterflies Adelpha pseudaeathalia Hall and A. melanthe (Bates) differ in their barcodes by only 0-1 base pairs (Janzen et al. 2009), and their larvae and pupae are very similar. The adults however present different color patterns (Willmott 2003). Heliconius erato (L.) also has
different geographical races with almost identical mtDNA (Brower 1994). In these two examples, different selection pressures can have been imposed on each lineage to involve on different mimicry rings. Mutations responsible for the adaptation of these forms to different mimicry pattern can have a weak link with mutations in the DNA barcode region. In this way, this marker can show so few differences that these forms can be considered the same entity, although they can be very dissimilar morphologically.

**DNA barcoding pest species of Lepidoptera.** The identification, diagnose and naming of species of Lepidoptera are important issues not just when conservation aspects of natural populations are discussed, because many species of the group are considered important agricultural crop pests. Mitochondrial DNA has been used to determine polymorphism (Roehrdanz et al. 1994) and to identify closely related species of pest species of Lepidoptera (Roehrdanz 1997). DNA barcoding was 100% efficient in identifying species of the defoliators of trees, tussock moths (Lymantriidae), in 93 lymantrid specimens tested (Ball & Armstrong 2006). Indeed, the ability of this marker to correctly distinguish among different closely allied pests species still needs further evidences, but the use of mtDNA can be very useful in the determination of genetic isolation due to selective different host plants, as in populations of European corn borer (Martel et al. 2003).

**General Impressions of DNA Barcoding in Lepidoptera**

The use of the DNA barcode marker as additional data for diagnosing and identifying new or taxonomic problematic species in Lepidoptera has offered enough good results to support its employment. In this way, it is clear that this identification tool can improve classifications and can be used to re-examine the accuracy of morphological traits commonly used in taxonomy. Its application as a unique marker to propose new species is a more controversial issue however.

Clear limitations arise from the incomplete coverage of the existing diversity, the inherent characteristics of the mitochondrial DNA (evolutionary rate, inheritance, introns and neutrality) and the single-locus initial strategy. Therefore, the idea of a multi-locus DNA barcoding approach is progressively emerging and is now commonly accepted (particularly in cases where COI is not species specific or for taxa with low mitochondrial evolutionary rates). Thus, DeSalle et al. (2005), among others, proposed the combination of morphological and molecular characters, which has the advantage of bridging the gap between the classical taxonomy and ‘molecular-taxonomy’ and the DNA barcoding approach. This integrative approach, based on the use of several different markers to carry out combined analyses (Mallet & Willmott 2003) has been suggested to deal with taxonomic problems, such as species boundaries (Dayrat 2005, Will et al. 2005, Roe & Sperling 2007).

It seems that whatever approach is used, every taxonomic decision using DNA barcode data should be corroborated by other independent lines of evidence (Schlick-Steiner et al. 2006, Burns et al. 2008). Non-cryptic species can generally be resolved by either traditional or molecular taxonomy without ambiguity. However, more difficult cases will only yield in a combination of approaches. In fact, for groups of species that diverged in the recent past none marker alone will be enough to clearly determine their taxonomic position or resolve specific limits, no matter the species concept used (Agapow et al. 2004). This can be considered the main constraint to a threshold to delimit species in any group.

The current trend suggests that DNA barcoding needs to be used alongside traditional taxonomic tools and alternative forms of molecular systematics so that problematic cases can be identified and errors detected. But, if the universality of a single locus remains utopian, the use of a few common loci is still a great advance for future diversity assessments within higher taxa (e.g., Caterino et al. 2000).

As a final point, due to the several recent publications, the DNA barcoding is becoming a very useful tool at least for Lepidoptera, and has been used for different purposes even by those researchers that are sensitive to the flaws of the method and its implications. For that reason, probably in a very near future the majority of the butterflies and many moths will have their barcodes sequenced and revealed, and this has already started with the All Leps Barcode of Life Project (http://www.lepbarcoding.org/). This technique will be more often used by ecologists, taxonomists and even amateur butterfly lovers as a relevant tool for studying biodiversity and evolution, or simply for observing and understanding this charismatic group of living organisms.

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