FORUM

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

Karina L Silva-Brandão[1], Mariana L Lyra[2], André V L Freitas[3]

[1]*Depto. de Entomologia e Acarologia, Escola Superior de Agricultura Luiz de Queiroz, Univ. de São Paulo – ESALQ-USP, Av. Pádua Dias 11, C. postal 9, 13418-900, Piracicaba, SP, klsilva@gmail.com;*
[2]*Centro de Biologia Molecular e Engenharia Genética e Instituto de Biologia, marillyra@gmail.com, Univ. Estadual de Campinas - UNICAMP, 13093-870, Campinas, SP;*
[3]*Depto. de Biologia Animal, Instituto de Biologia, baku@unicamp.br, Univ. Estadual de Campinas - UNICAMP, 13083-870, Campinas, SP*

Edited by Fernando L Cônsoli – ESALQ/USP

Neotropical Entomology 38(4):441-451 (2009)

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 crípticas 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 críptica, 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 earths' 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 1* (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, Rubinoff 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 &

Saunders 2009) and ciliates (Chantangsi *et al* 2007).

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, Randrianiaina *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, Tavares & 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, Tsaousis *et al* 2005) some phenomena can lead to misleading results. These phenomena are, for example, the occurrence of interspecific hybridization or endosymbiont 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, Willams & 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 lack strong biological support and could not become a universal criterion suited to animal species delimitation (Meyer & Paulay 2005, Hickerson *et al* 2006, Wiemer & 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 fully 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 later 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 (Õunap & Viidalepp 2009), and the results showed that a phylogeny-based approach allowed the delimitation of *C. ocultaria* and the new species *C. tasmanica* Õunap & 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. pseudotsugella* 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 *Astraptes '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. neoptolemus* (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'* Hübner 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 patters 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 altitudinal 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 Cytb (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. panthonus jaguarae* (Foetterle), 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 pseudaethalia* 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 lymantriid 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 use of 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.

Acknowledgements

We are in debt with Andrew V Z Brower and Niklas Wahlberg for comments and suggestions on the manuscript. AVLF acknowledges FAPESP (grants #00/01484-1 and #04/05269-9), the Brazilian CNPq (fellowship # 300282/2008-7), and the National Science Foundation (DEB grant #0527441). This research is part of BIOTA-FAPESP program (grant #98/05101-8). KLSB acknowledges the Brazilian CNPq (grant # 480619/2008-5) and FAPESP (grant #06/60127-0 and fellowship #07/53919-0).

References

Abdo Z, Golding G B (2007) A step toward barcoding life: a model-based, decision theoretic method to assign genes to preexisting species groups. Syst Biol 56: 44-56.

Agapow P M, Bininda-Emonds O R P, Crandall K A, Gittleman J L, Mace G M, Marshall J C, Purvis A (2004) The impact of species concept on biodiversity studies. Q Rev Biol 79: 161-179.

Aguiar A P, Santos B F, Couri M S, Rafael J A, Costa C, Ide S, Duarte M, Grazia J, Schwertner C F, Freitas A V L, Azevedo C O (in press) Entomologia. In Rocha R M, Boeger W A P, Zoologia: estado da arte e perspectivas para os próximos 10 anos. Curitiba, Editora da Universidade Federal do Paraná.

Ahrens D, Monaghan M T, Vogler A P (2007) DNA-based taxonomy for associating adults and larvae in multi-species assemblages of chafers (Coleoptera: Scarabaeidae). Mol Phylogenet Evol 44: 436-449.

Amaral A R, Sequeira M, Coelho M M (2007) A first approach to the usefulness of cytochrome c oxidase I barcodes in the identification of closely related delphinid cetacean species. Mar Freshwater Res 58: 505-510.

Armstrong K F, Ball S L (2005) DNA barcodes for biosecurity: invasive species identification. Philos Trans R Soc Lond B Biol Sci, 360: 1813-1823.

Baker C S, Palumbi S R (1994) Which whales are hunted? A molecular genetic approach to monitoring whaling. Science 265: 1538-1539.

Ball S L, Armstrong K F (2006) DNA barcodes for insect pest identification: a test case with tussock moths (Lepidoptera: Lymantriidae). Can J For Res 36: 337-350.

Ball S L, Hebert P D N, Burian S K, Webb J M (2005) Biological identifications of mayflies (Ephemeroptera) using DNA barcodes. J North Am Benthol Soc 24: 508-524.

Bickford D, Lohman D J, Sohdi N S, Ng P K L, Meier R, Winker K, Ingran K K, Das I (2007) Cryptic species as a window on diversity and conservation. Trends Ecol Evol 22: 148-155.

Blaxter M, Mann J, Chapman T, Thomas F, Whitton C, Floyd R, Abebe E (2005) Defining operational taxonomic units using DNA barcode data. Philos Trans R Soc Lond B Biol Sci 360: 1935-1943.

Brower A V Z (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc Natl Acad Sci USA 91: 6491-6495.

Brower A V Z (1996) A new mimetic species of *Heliconius* (Lepidoptera: Nymphalidae), from southeastern Colombia, as revealed by cladistic analysis of mitochondrial DNA sequences. Zool J Linn Soc 116: 317-332.

Brower A V Z (2006) Problems with DNA barcodes for species delimitation: 'ten species' of *Astraptes fulgerator* reassessed (Lepidoptera: Hesperiidae). Syst Biodiver 4: 127-132.

Brower L P (1984) Chemical defence in butterflies, p.111-134. In Vane-Wright R I, Ackery P R (eds) The biology of butterflies. New York, AcademicPress, xxv + 429p.

Brown Jr K S, Freitas A V L (1999) Lepidoptera, p.225-243. In Brandão C R F, Cancello E M (eds) Invertebrados terrestres, v. 5, xvii + 279p. In Joly C A, Bicudo C E de M (orgs) Biodiversidade do estado de São Paulo, Brasil: síntese do conhecimento ao final do século XX. São Paulo, Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP.

Burns J M, Janzen D H (2005). Pan-Neotropical genus *Venada* (Hesperiidae: Pyrginae) is not monotypic: four new species occur on one volcano in the Area de Conservación Guanacaste, Costa Rica. J Lepid Soc 59: 19-34.

Burns J M, Janzen D H, Hajibabaei M, Hallwachs W, Hebert P D N (2008) DNA barcodes and cryptic species of skipper butterflies in the genus *Perichares* in Area de Conservacion Guanacaste, Costa Rica. Proc Natl Acad Sci USA 105: 6350-6355.

Caterino M S, Cho S, Sperling F A H (2000) The current state of insect molecular systematics: a thriving Tower of Babel. Ann Rev Entomol 45: 1-54.

Chantangsi C, Lynn D H, Brandl M T, Cole J C, Hetrick N, Ikonomi P (2007) Barcoding ciliates: a comprehensive study of 75 isolates of the genus *Tetrahymena*. Int J Syst Evol Microbiol 57: 2412–2425.

Chase M W, Salamin N, Wilkinson M, Dunwell J M, Kesanakurthi R P, Haidar N, Savolainen V (2005) Land plants and DNA barcodes: short-term and longterm goals. Philos Trans R Soc Lond B Biol Sci 360: 1889-1895.

Collins F H, Paskewitz S M (1996) A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. Insect Mol Biol 5: 1-9

Costa F O, deWaard J R, Boutillier J, Ratnasingham S, Dooh R T, Hajibabaei M, Hebert P D N (2007) Biological identifications through DNA barcodes: the case of the Crustacea. Can J Fish Aquat Sci 64: 272-295.

Daily G C, Ehrlich P R (1995) Population extinction and the biodiversity crisis, p.45-55. In Perrings C A, Mäler K-G, Folke C, Holling C S, Jansson B-O (eds) Biodiversity conservation: problems and policy. Amsterdam, Kluwer Academic Publishers, xvi + 404p.

Dasmahapatra K K, Mallet J (2006) DNA barcodes: recent successes and future prospects. Heredity 97: 254-255.

Davies K F, Gascon C, Margules C R (2001) Habitat fragmentation, p.81-97. In Soulé M E, Orinas G H (eds) Conservation biology. Research priorities for the next decade. Washington, D.C., Island Press, xvii + 307p.

DeLong Jr D C (1996) Defining biodiversity. Wildl Soc Bull 24: 738-749.

DeSalle R (2006) Species discovery versus species identification in DNA barcoding efforts: response to Rubinoff. Cons Biol 20: 1545-1547.

DeSalle R, Birstein V J (1996) PCR identification of blach caviar. Nature 381: 197-198.

DeSalle R, Egan M G, Siddall M (2005) The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philos Trans R Soc Lond B Biol Sci 360: 1905-1916.

Dayrat B (2005) Towards integrative taxonomy. Biol J Linn Soc 85: 407-415.

Ebach M C, Holdrege C (2005) DNA barcoding is no substitute for taxonomy. Nature 434: 697.

Ehrlich P R (1988) The loss of diversity: causes and consequences, p.21-27. In Wilson E O (ed) Biodiversity. Washington, D.C., National Academy Press, xiii + 521p.

Elias M, Hill R I, Willmott K R, Dasmahapatra K K, Brower A V Z, Mallet J, Jiggins C D (2007) Limited performance of DNA barcoding in a diverse community of tropical butterflies. Proc R Soc Biol Sci Ser B 274: 2881-2889.

Erpenbeck D, Hooper J N A, Worheide G (2006) CO1 phylogenies in diploblasts and the 'Barcoding of Life'– are we sequencing a suboptimal partition? Mol Ecol Notes 6: 550-553.

Erwin T L (1982) Tropical forests: their richness in Coleoptera and other arthropod species. Coleopt Bull 36: 74-75.

Erwin, T L (1991) How many species are there?: Revisited. Cons Biol 5: 330-333.

Evans K M, Wortley A H, Mann D G (2007) An assessment of potential diatom ''barcode'' genes (cox1, rbcL, 18S and ITS rDNA) and their effectiveness in determining relationships in Sellaphora (Bacillariophyta). Protist 158: 349-364.

Freitas A V L (2007) A new species of *Moneuptychia* Forster (Lepidoptera: Satyrinae: Euptychiina) from the highlands of Southeastern Brazil. Neotrop Entomol 36: 919-925.

Funk D J, Helbling L, Wernegreen J J, Moran N A (2000) Intraspecific phylogenetic congruence among multiple symbiont genomes. Proc R Soc Biol Sci Ser B 267: 2517-2521.

Funk D J, Omland K E (2003) Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. Ann Rev Ecol Evol Syst 34: 397-423.

Gomez A, Wright P J, Lunt D H, Cancino J M, Carvalho G R, Hughes R N (2007) Mating trials validate the use of DNA barcoding to reveal cryptic speciation of a marine bryozoan taxon. Proc R Soc Biol Sci Ser B 274: 199-207.

Gompert Z, Nice C C, Fordyce J A, Forister M L, Shapiro A M (2006). Identifying units for conservation using molecular taxonomy: the cautionary tale of the Karner Blue butterfly. Mol Ecol 15: 1759-1768.

Greenstone M H, Rowley D L, Heimbach U, Lundgren J G, Pfannenstiel R S, Rehner S A (2005) Barcoding generalist predators by polymerase chain reaction: carabids and spiders. Mol Ecol 14: 3247-3266.

Gregory T R (2005) DNA barcoding does not compete with taxonomy. Nature 434: 1067.

Hajibabaei M, Janzen D H, Burns J M, Hallwachs W, Hebert P D N (2006b) DNA barcodes distinguish species of tropical Lepidoptera. Proc Nat Acad Sci USA 103: 968-971.

Hajibabaei M, Singer G A C, Hebert P D N, Hickey D A (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet 23: 167-172.

Hajibabaei M, Smith M A, Janzen D H, Rodriguez J J, Whitfield J B, Hebert P D N (2006a) A minimalist barcode can identify a specimen whose DNA is degraded. Mol Ecol Notes 6: 959-964.

Hebert P D N, Cywinska A, Ball S L, DeWaard J R (2003a) Biological identifications through DNA barcodes. Proc R Soc Biol Sci Ser B 270: 313-321.

Hebert P D N, Ratnasingham S, deWaard J R (2003b) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc Biol Sci Ser B 270, S96-S99.

Hebert P D N, Penton E H, Burns J M, Janzen D H, Hallwachs W (2004a) Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Natl Acad Sci USA 101: 14812-14817.

Hebert P D N, Stoeckle M Y, Zemlak T S, Francis C M (2004b) Identification of birds through DNA barcodes. Plos Biol 2: 1657-1663.

Heppner J B (1998) Classification of Lepidoptera: Part I. Introduction. Trop Lepid 5 (suppl. 1): 1-148.

Hickerson M J, Meyer C P, Moritz C (2006) DNA barcoding will often fail to discover new animal species over broad parameter space. Syst Biol 55: 729-739.

Hogg I D, Hebert P D N (2004) Biological identification of springtails (Hexapoda: Collembola) from the Canadian Arctic, using mitochondrial DNA barcodes. Can J Zool 82: 749-754.

Hong S K, Lee J A (2006) Global environmental changes in terrestrial ecosystems. International issues and strategic solutions: introduction. Ecol Res 21: 783-787.

Huang J, Qin Xu Q, Sun Z J, Tang G L, Su Z Y (2007) Identifying earthworms through DNA barcodes. Pedobiologia 51: 301-309.

Hulcr J, Miller S E, Setliff G P, Darrow K, Mueller N D, Hebert P D N, Weiblen G D (2007) DNA barcoding confirms polyphagy in a generalist moth, *Homona mermerodes* (Lepidoptera: Tortricidae). Mol Ecol Notes 7: 549-557.

Hurst G D, Jiggins F M (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc Biol Sci 272: 1525-1534.

Janzen D H, Hajibabaei M, Burns J M, Hallwachs W, Remigio E, Hebert P (2005) Wedding biodiversity inventory of a large complex Lepidoptera fauna with DNA barcoding. Philos Trans R Soc Lond B Biol Sci 360: 1835-1845.

Janzen D H, Hallwachs W, Blandin P, Burns J M, Cadiou J M, Chacon I, Dapkey T, Deans A R, Epstein M E, Espinoza B, Franclemont J G, Haber W A, Hajibabaei M, Hall J P W, Hebert P D N, Gauld I D, Harvey D J, Hausmann A, Kitching I J, Lafontaine D, Landry J F, Lemaire C, Miller J Y, Miller J S, Miller L, Miller S E, Montero J, Munroe E, Green S R, Ratnasingham S, Rawlins J E, Robbins R K, Rodriguez J J, Rougerie R, Sharkey M J, Smith M A, Solis M A, Sullivan J B, Thiaucourt P, Wahl D B, Weller S J, Whitfield J B, Willmott K R, Wood D M, Woodley N E, Wilson J J (2009) Integration of DNA barcoding into an ongoing inventory of complex tropical biodiversity. Mol Ecol Resour 9: 1-26.

Jeyaprakash A, Hoy M A (2000) Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Mol Biol 9: 393-405.

Johnstone R A, Hurst G D D (1996) Maternally inherited male-killing microorganisms may confound interpretation of mitochondrial DNA variability. Biol J Linn Soc 58: 453-470.

Joron M, Mallet J L B (1998) Diversity in mimicry: paradox or paradigm? Trends Ecol Evol 13: 461-466.

Kerr K C R, Stoeckle M Y, Dove C J, Weigt L A, Francis C M, Hebert P D N (2007) Comprehensive DNA barcode coverage of North American birds. Mol Ecol Notes 7: 535-543.

Kress W J, Wurdack K J, Zimmer E A, Weigt L A, Janzen D H (2005) Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci USA 102: 8369- 8374.

Kristensen N P, Scoble M J, Karsholt O (2007) Lepidoptera phylogeny and systematics: the state of inventorying moth and butterfly diversity. Zootaxa 1668: 699-747.

Kumar N P, Rajavel A R, Natarajan R, Jambulingam P (2007) DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae). J Med Entomol 44: 1-7.

Ladoukakis E D, Zouros E (2001) Direct evidence for homologous recombination in mussel (*Mytilus galloprovincialis*) mitochondrial DNA. Mol Biol Evol 18: 1168-1175.

Lane C E, Lindstrom S C, Saunders G W (2007) A molecular assessment of northeast Pacific *Alaria* species (Laminariales, Phaeophyceae) with reference to the utility of DNA barcoding. Mol Phylogenet Evol 44: 634-648.

Lewinsohn T M, Prado P I (2002) Biodiversidade brasileira: síntese do estado atual do conhecimento. São Paulo, Editora Contexto, 176p.

Lewinsohn T M, Prado P I (2005) How many species are there in Brazil? Cons Biol 19: 619-624.

Linares M C, Soto-Calderon I D, Lees D C, Anthony N M (2009) High mitochondrial diversity in geographically widespread butterflies of Madagascar: a test of the DNA barcoding approach. Mol Phylogenet Evol 50: 485-495.

Mallet J, Willmott K (2003) Taxonomy: renaissance or Tower of Babel? Trends Ecol Evol 18: 57-59.

Marin M A, López A, Freitas A V L, Uribe S I (in press) Caracterización molecular de *Euptychiina* (Lepidoptera: Satyrinae) del norte de la cordillera central de los andes. Rev Colomb Entomol.

Martel C, Réjasse A, Rousset F, Bethenod M-T, Bourguet D (2003) Host-plant-associated genetic differentiation in Northern French populations of the European corn borer. Heredity 90: 141-149.

May R M (1990) How many species? Phil Trans R Soc Lond B 330: 293-304.

McDevit D C, Saunders G W (2009) On the utility of DNA barcoding for species differentiation among brown macroalgae (Phaeophyceae) including a novel extraction protocol. Phycol Res 57: 131-141.

Meier R, Shiyang K, Vaidya G, Ng P K L (2006) DNA Barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification sucess. Syst Biol 55: 715-728.

Meyer C P, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. Plos Biol 3: 2229-2238.

Mielke O H H, Mielke C G C, Casagrande M M (2004) *Parides panthonus jaguarae* (Foetterle) (Lepidoptera, Papilionidae) redescoberto em Minas Gerais, Brasil: sua identidade. Rev Bras Zool 21: 9-12.

Miller J S, Brower A V Z, DeSalle R (1997) Phylogeny of the neotropical moth tribe Josiini (Notodontidae: Dioptinae): comparing and combining evidence from DNA sequences and morphology. Biol J Linn Soc 60: 297-316.

Miller S E (2007) DNA barcoding and the renaissance of taxonomy. Proc Natl Acad Sci USA 104: 4775-4776.

Moritz C, Cicero C (2004) DNA barcoding: promise and pitfalls. PLoS Biol 2:e354.

Nazari V, Sperling F A H (2007) Mitochondrial DNA divergence and phylogeography in western Palaearctic Parnassiinae (Lepidoptera: Papilionidae): How many species are there? Insect Syst Evol 38: 121-138.

Nielsen R, Matz M (2006) Statistical approaches for DNA barcoding. Syst Biol 55: 162-169.

Olson D M, Dinerstein E, Powell G V N, Wikramanayake E D (2002) Conservation biology for the biodiversity crisis. Conserv Biol 16: 1-3.

Õunap E, Viidalepp J (2009) Description of *Crypsiphona tasmanica* sp. nov. (Lepidoptera: Geometridae:Geometrinae), with notes on limitations in using DNA barcodes for delimiting species. Aust J Entomol 48: 113-124.

Pegg G G, Sinclair B, Briskey L, Aspden W J (2006) MtDNA barcode identification of fish larvae in the southern Great Barrier Reef, Australia. Sci Mar 70: 7-12.

Pennisi E (2007) Wanted: a barcode for plants. Science 318: 190-191.

Pfenninger M, Nowak C, Kley C, Steinke D, Streit B (2007) Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. Mol Ecol 16: 1957-1968.

Pons J (2006) DNA-based identification of preys from non-destructive, total DNA extractions of predators using arthropod universal primers. Mol Ecol Notes 6: 623-626.

Pons T, Gonzalez B, Ceciliani F, Galizzi A (2006) FlgM anti-sigma factors: identification of novel members of the family, evolutionary analysis, homology modeling, and analysis of sequence-structure-function relationships. J Mol Model 12: 973-983.

Randrianiaina R -D, Glaw F, Thomas M, Glos J, Raminosoa N, Vences M (2007) Descriptions of the tadpoles of two species of *Gephyromantis*, with a discussion of the phylogenetic origin of direct development in mantellid frogs. Zootaxa 1401: 53-61.

Ratnasingham S, Hebert P D N (2007) BOLD: The barcode of life data system (http://www.barcodinglife.org). Mol Ecol Notes 7:355-364.

Remigio E A, Hebert P D N (2003) Testing the utility of partial COI sequences for phylogenetic estimates of Gastropod relationships. Mol Phylogenet Evol 29: 641-647.

Roe A D, Sperling F A H (2007) Population structure and species boundary delimitation of cryptic *Dioryctria* moths using an integrative approach. Mol Ecol 16: 3617-3633.

Roehrdanz R L (1997) Identification of tobacco budworm and corn earworm (Lepidoptera: Noctuidae) during early developmental stages by polymerase chain reaction and restriction fragment length polymorphism. Ann Entomol Soc Am 90: 329-332.

Roehrdanz R L, Lopez J D, Loera J, Hendricks D E (1994) Limited mitochondrial-DNA polymorphism in North-American populations of *Heliothis virescens* (Lepidoptera, Noctuidae). Ann Entomol Soc Am 87: 856-866.

Ross H A, Murugan S, Li W L S (2008) Testing the reliability of genetic methods of species identification via simulation. Syst Biol 57: 216-230.

Rubinoff D (2006a) Utility of mitochondrial DNA barcodes in species conservation. Cons Biol 20: 1026-1033.

Rubinoff D (2006b) DNA barcoding evolves into the familiar. Cons Biol 20: 1548-1549.

Saunders G W (2005) Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philos Trans R Soc Lond B Biol Sci 360: 1879-1888.

Savage J M (1995) Systematics and the biodiversity crisis. BioScience 45: 673-679.

Schindel D E, Miller S E (2005) DNA barcoding a useful tool for taxonomists. Nature 435: 17-117.

Schlick-Steiner B C, Steiner F M, Moder K, Seifert B, Sanetra M, Dyreson E, Stauffer C, Christian E (2006) A multidisciplinary approach reveals cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). Mol Phylogenet Evol 40: 259-273.

Schlick-Steiner B C, Seifert B, Stauffer C, Christian E Crozier R H, Steiner F M (2007) Without morphology, cryptic species stay in taxonomic crypsis following discovery. Trends Ecol Evol 22: 391-392.

Schmidt B C, Sperling F A H (2008) Widespread decoupling of mtDNA variation and species integrity in *Grammia* tiger moths (Lepidoptera: Noctuidae). Syst Entomol 33: 613-634.

Seifert K A, Samson R A, Dewaard J R, Houbraken J, Levesque C A, Moncalvo J M, Louis-Seize G, Hebert P D N (2007) Prospects for fungus identification using C01 DNA barcodes, with *Penicillium* as a test case. Proc Natl Acad Sci USA 104: 3901-3906.

Sevilla R G, Diez A, Noren M, Mouchel O, Jerome M, Verrez-Bagnis V, van Pelt H, Favre-Krey L, Krey G, Bautista J M (2007) Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. Mol Ecol Notes 7: 730-734.

Silva-Brandão K L, Azeredo-Espin A M L, Freitas A V L (2008a) New evidence on the systematic and phylogenetic position of *Parides burchellanus* (Lepidoptera : Papilionidae). Mol Ecol Resour 8: 502-511.

Silva-Brandão K L, Wahlberg N, Francini R B, Azeredo-Espin A M L, Brown Jr K S, Paluch M, Lees D C, Freitas A V L (2008b) Phylogenetic relationships of butterflies of the tribe Acraeini (Lepidoptera, Nymphalidae, Heliconiinae) and the evolution of host plant use. Mol Phylogenet Evol 46: 515-531.

Smith M A, Rodriguez J, Whitfield J, Deans A, Janzen D H, Hallwachs W, Hebert P D N (2008) Extraordinary diversity of parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology and collections. Proc Natl Acad Sci USA 105: 12359-12364.

Sperling F (2003) DNA Barcoding: Deus ex Machina. Newsletter Biol Survey Canada (Terrestrial Arthropods) 22: 50-53.

Sperling F A H, Anderson G S, Hickey, D A (1994) A DNA-based approach to identification of insect species used for postmortem interval estimation. J Forensic Sci 39: 418-427.

Stork, NE (1993) How many species are there? Biodivers Conserv 2: 215-232.

Swartz E R, Mwale M, Hanner R (2008) A role for barcoding in the study of African fish diversity and conservation. S Afr J Sci 104: 293-298.

Tautz D, Arctander P, Minelli A, Thomas R H, Vogler A P (2003) A plea for DNA taxonomy. Trends Ecol Evol 18: 70-74.

Tavares E S, Baker A J (2008) Single mitochondrial gene barcodes reliably identify sister-species in diverse clades of birds. BMC Evol Biol 8: 81-94.

Thalmann O, Hebler J, Poinar H N, Paabo S, Vigilant L (2004) Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. Mol Ecol 13: 321-335.

Townzen J S, Brower A V Z, Judd D D (2008) Identification of mosquito bloodmeals using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences. Med Vet Entomol 22: 386-393.

Tsaousis A D, Martin D P, Ladoukakis E D, Posada D, Zouros E (2005) Widespread recombination in published animal mtDNA sequences. Mol Biol Evol 22: 925-933.

Vaglia T, Haxaire J, Kitching I J, Meusnier I, Rougerie R (2008) Morphology and DNA barcoding reveal three cryptic species within the *Xylophanes neoptolemus* and *loelia* species-groups (Lepidoptera: Sphingidae). Zootaxa 1923: 18-36.

Wahlberg N, Oliveira R, Scott J A (2003) Phylogenetic relationships of *Phyciodes* butterfly species (Lepidoptera: Nymphalidae): complex mtDNA variations and species delimitations. Syst Entomol 28: 257-273

Walton C, Sharpe R G, Pritchard S J, Thelwell N J, Butlin R K (1999) Molecular identification of mosquito species. Biol J Linn Soc 68: 241-256.

Ward R D, Zemlak T S, Innes B H, Last P R, Hebert P D N (2005) DNA barcoding Australia's fish species. Philos Trans R Soc Lond B Biol Sci 360: 1847-1857.

Webb K E, Barnes D K A, Clark M S, Bowden D A (2006) DNA barcoding: a molecular tool to identify Antarctic marine larvae. Deep-Sea Res Part II 53: 1053-1060.

Werren J H, Windsor D M (2000) *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc R Soc London 267B: 1277-1285.

Western D (1992) The biodsiversity crisis: a challenge for biology. Oikos 63: 29-38.

Whitworth T L, Dawson R D, Magalon H, Baudry E (2007) DNA barcoding cannot reliably identify species of the blowfly genus *Protocalliphora* (Diptera: Calliphoridae). Proc R Soc Biol Sci Ser B 274: 1731-1739.

Wiemer M, Fiedler K (2007) Does the DNA barcoding gap exist? – a case study in blue butterflies (Lepidoptera: Lycaenidae). Front Zool 4:8-23

Will K W, Mishler B D, Wheeler Q D (2005) The perils of DNA barcoding and the need for integrative taxonomy. Syst Biol 54: 844-851.

Williams S T, Knowlton N (2001) Mitochondrial pseudogenes are pervasive and often insidious in the snapping shrimp genus *Alpheus*. Mol Biol Evol 18: 1484-1493.

Willmott K R (2003) The genus *Adelpha*: its systematics, biology and biogeography (Lepidoptera: Nymphalidae: Limenitidini). Gainesville, Scientific Publishers, viii + 322p.

Willmott K R, Freitas A V L, Hall J P W, Silva-Brandão K L, Paluch M (2009) A new species of *Actinote* Hübner from the Eastern Andes of Ecuador (Lepidoptera: Nymphalidae: Heliconiinae). Proc Entomol Soc Wash 111: 47-56.

Wilson, E O (ed) (1988) Biodiversity. Washington, D.C., National Academy Press, viii + 521p.

Wilson, E O (2005) Systematics and the future of biology. Proc Natl Acad Sci USA 102: 6520-6521.

Woese C R (1996) Phylogenetic trees: whither microbiology? Curr Biol 1060-1063.

Zhou J, Davey M E, Figueras J B, Rivkina E, Gilichinsky D, Tiedje J M (1997) Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. Microbiology 143: 3913-3919.

Received 20/VII/09. Accepted 22/VII/09.