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A Genomic Perspective on the Shortcomings of Mitochondrial DNA for "Barcoding" Identification

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Abstract

Approximately 600-bp sequences of mitochondrial DNA (mtDNA) have been designated as "DNA barcodes" and have become one of the most contentious and animated issues in the application of genetic information to global biodiversity assessment and species identification. Advocates of DNA barcodes have received extensive attention and promotion in many popular and refereed scientific publications. However, we suggest that the utility of barcodes is suspect and vulnerable to technical challenges that are particularly pertinent to mtDNA. We review the natural history of mtDNA and discuss problems for barcoding which are particularly associated with mtDNA and inheritance, including reduced effective population size, maternal inheritance, recombination, inconsistent mutation rate, heteroplasmy, and compounding evolutionary processes. The aforementioned could significantly limit the application and utility of mtDNA barcoding efforts. Furthermore, global use of barcodes will require application and acceptance of a barcode-based species concept that has not been evaluated in the context of the extensive literature concerning species designation. Implementation of mtDNA barcodes in spite of technical and practical shortcomings we discuss may degrade the longstanding synthesis of genetic and organism-based research and will not advance studies ranging from genomic evolution to biodiversity assessment.

There is a growing misconception that scientists opposed to DNA barcoding are opposed to the use of molecular tools in systematics and taxonomy. This notion misconstrues the criticisms of DNA barcodes and requires an explanation of promolecular opposition to the barcoding paradigm. DNA barcoding is an ambitious proposition. Originally, Hebert et al. (2003) advocated the use of limited (approximately 600 bp) mitochondrial DNA (mtDNA) sequence data as an inexpensive, easy way to "scan" and identify all of life. This "DNA barcoding" would supersede mundane morphological work and more extensive mitochondrial and nuclear DNA sequencing for taxon identification. Such superiority of barcodes is based on the claims that they would be less expensive, much faster—only a small segment of mtDNA is needed—and would require no taxonomic experts (who are expensive to train and too few in number given the task at hand). However, recent papers intended to exemplify the success of barcoding (Hebert, Penton, et al. 2004; Hebert, Stoeckle, et al. 2004; Hajibabaei et al. 2006) demonstrated the reliance on extensive morphological and ecological data collected by expert ecologists and morphologists. These papers closely follow the integrative approach we would recommend (Rubinoff et al. 2006) but are clearly a violation of the basic premise of the practical barcode methods currently being promoted. The expense and expertise required to complete the aforementioned "barcoding" analysis contradicts the stated benefits of barcoding and raises the unaddressed questions: from a molecular perspective, how well would barcoding work and what are the theoretical issues with using such a limited set of data?

The purpose of this paper is to elucidate the molecular basis for problems with DNA barcoding and to demonstrate how these problems manifest and prevent barcoding from contributing to a meaningful understanding of global biodiversity. Herein we examine 3 issues. First, we explore the contentious definitional issues surrounding DNA barcoding (e.g., identification vs. description, phylogenetics vs. clustering) that have, in the past, clouded discussions of the theoretical basis of DNA barcoding when opponents and proponents have argued at cross-purposes. If DNA barcoding is to achieve

the aims of its supporters, then in addition to delivering accurate identifications it will also need to deal effectively with both discriminating novel species and placing them within a wider biodiversity. Secondly, we examine the assumptions that underlie the utility of using single mitochondrial genes in DNA barcoding and the specific conditions which could lead to large differences in molecular clock rates between closely related species and thereby obfuscate the standardized species boundaries required by the barcoding system. Finally, we discuss whether recent empirical barcoding papers support the notion that DNA barcoding really can achieve its aims based on DNA data alone or if it is critically dependent on the efforts of classical taxonomists and classical data sets.

Conflated Issues: DNA Identifications, DNA Descriptions, Phylogenetics, and Barcoding

One of the difficulties surrounding discussion of DNA barcoding is the considerable confusion about what it is and is not. For the purposes of this paper, we define DNA barcoding using the operational criteria applied by the vast majority of papers supporting this approach as follows:

1. A single gene is sequenced for use as the barcode.
2. The same gene and region are to be used universally allowing standardization of protocols.
3. Gene sequences are analyzed by distance methods to identify specimens as belonging to a given taxon.

Some DNA barcoding efforts may diverge from this paradigm; however, if this debate is to move forward, it is necessary to discuss specifics and for that reason DNA barcoding needs to be defined explicitly. The official Web sites for the Consortium for the Barcode of Life (http://barcoding.si.edu/DNABarCoding.htm) and the Guelph Centre for DNA Barcoding (http://www.dnabarcoding.ca/research.php) do not give an explicit definition of what barcoding is, and until they do, it can only be defined by the papers using a barcoding paradigm. Although some proponents of barcoding have departed from the definition above by advocating the use of different standardized genes (e.g., Vences, Thomas, Bonet, et al. 2005), the use of multiple genes (e.g., Kress et al. 2005), or different analytical approaches (e.g., Matz and Nielsen 2005), the vast majority of DNA empirical barcoding papers follow this structure in that CO1, and only CO1, is used and analyzed by Neighbor Joining (N-J) (Saitou and Nei 1987) and only N-J tree construction. A recently announced GenBank special submission tool for DNA barcoding, which will label sequences with the definition BARCODE, is specifically designed to take CO1 sequences, only further signaling the barcoding effort's commitment to this single gene.

The second major difficulty in discussing DNA barcoding is the conflation of various interpretations of the results of barcode studies that proponents have reached using the general procedure outlined above. At least 3 applications of DNA sequencing to taxonomic issues have been considered under the barcoding paradigm, and unfortunately they are rarely clearly separated; purported success in one area is used as evidence of support for all types of barcoding studies, and any criticism directed at one use of mtDNA may be dismissed because they are not relevant to all other uses of it. This conflation of ideas and misunderstanding of criticism date back to the very first papers advocating DNA barcoding (Hebert et al. 2003), and we argue it is in large part the unrealistic and ambitious agenda of the proponents that leads to this confusion. The 3 major taxonomic goals to which DNA barcoding has been applied to are as follows:

1. The identification of species previously defined by other criteria. This includes both speeding identifications which might have been made on morphological grounds alone, as well as linking specimens unidentifiable by other means to established species identification for purposes such as forensics or life cycle elucidation.
2. The description of new species by interpretation of DNA diversity as indicative of species diversity.
3. The definition of operational units for ecological studies.

If DNA barcoding limited itself to the first of these goals it would be relatively uncontroversial. DNA-based identification tools were one of the first applications of molecular biology to taxonomy to be developed (e.g., DeSalle and Birstein 1996; Jousson et al. 1998, 1999) but were unreferenced in the "novel" proposal of DNA barcoding (Hebert et al. 2003). DNA identification has clear and obvious value in forensics, quarantine (e.g., Armstrong and Ball 2005), and life cycle studies (e.g., Miller et al. 2005). However, DNA barcoding proponents have consistently crossed the line between trying to identify known species and identification of new species. Finding new species was going to be a major benefit according to Hebert et al. (2003):

> CO1-based identification systems can also aid in the initial delineation of species.

and later in the same paper:

> Newly encountered species will ordinarily signal their presence by their genetic divergence from known members of the assemblage.

and still further:

> The prospect of using a standard CO1 threshold to guide species diagnosis in situations where prior taxonomic work has been limited is appealing.

The ambitions for this new technique are clear, DNA barcoding will identify those species which we know and reveal those that are undescribed. Given that some 90% of biodiversity consists of undescribed, unknown species, simply limiting DNA barcoding to identifying the known 10% will not respond to the biodiversity crisis. For DNA barcoding to be a useful and novel concept, it must somehow apply to the 90% of unknown biodiversity. Barcoding papers have devoted considerable attention to any instance in which "cryptic diversity" has been uncovered, for example, within the skipper butterfly *Astrapes fulgerator* (Hebert, Penton, et al. 2004), or the parasitic flies that seem to specialize on those *Astraptes* butterflies (Smith et al. 2006); within the bird species *Tringa solitaria*, *Sturnella magna*, *Cisthorus palustris*, and *Vireo gilvus*

(Hebert, Stoeckle, et al. 2004); within the dogfish *Squallus* (Ward et al. 2005); and within neotropical Lepidoptera (Hajibabaei et al. 2006) for example. But such cryptic species identifications are contingent upon a preexisting understanding of species in those groups based on other sources of data and are therefore not representative of the unknown biodiversity we are challenged to identify.

Is this conflation of identification and description of species necessary? The answer depends on the goals barcoders wish to set for themselves. Some barcoding proponents have an extremely ambitious agenda, no less than

> . . . enabling the rapid and inexpensive identification of the estimated 10 million species on Earth. (Savolainen et al. 2005)

and that barcoding is the

> . . . sole prospect for a sustainable identification capability . . . (Hebert et al., 2003)

If barcoding is to substantially contribute to the goals quoted above, species definitions will be applied to new barcodes as part of the process. For example, even if barcoding were limited to purely diagnostic applications such as agricultural quarantine (Armstrong and Ball 2005), the issue remains of how to deal with novel haplotypes. Do such barcodes represent unknown haplotypes of a pest species or a different species which is not troublesome? To be of use barcoding procedures need to be able to distinguish between new haplotypes of a known species and a species unknown to the barcode database based on a robust definition of what constitutes a species. The line between species identification and definition is therefore unavoidably blurred, and some form of barcode species concept is an absolute necessity.

Once new barcoding or "gene species" (Nicholls 2003) or molecular operational taxonomic units (Blaxter 2004) are recognized, what do we actually know about them? Attempts to add value to barcodes leads to the third great inconsistency of barcoding—identification versus phylogenetics. Typical barcoding uses one of the most basic phylogenetic method available—simple pairwise distances interpreted through phenetic clustering to produce tree-like representations of species clusters (N-J phenograms). This has lead to sustained criticism that barcoding uses bad phylogenetic practice and therefore its conclusions are suspect (e.g., Will and Rubinoff 2004). These criticisms are apparently misunderstood as seen with statements such as the following:

> While barcoding does not aim to build phylogenetic trees, it is obvious that morphology-based congenerics are often the nearest neighbours in the NJ phenogram. When they are not, it is a signal that the morphological placement may be profitably re-examined. (Janzen et al. 2005)

This statement suggests that because the apparent goal of barcoding is not about producing phylogenies, one need not worry about phylogenetic theory or phylogenetically based criticisms of barcoding methodologies, even though phylogenetic methods and even evolutionary models (e.g., substitution rate corrections) are part of the normal barcoding protocol. Conveniently, it seems, when the tree produced shows nonmonophyly (suggesting a new species or relationship), as Janzen et al. (2005) state in the quotation above, the barcode N-J phenograms are now phylogenetically informative. Arbitrarily picking and choosing when a method gives significant results is not a generally accepted part of science.

There are additional questions beyond why barcoding shuns phylogenetic theory but almost universally requires phylogenetic methods. Namely, is phylogenetics intrinsic to barcoding or are N-J clusters simply convenient visualizations? If one is simply interested in DNA identifications, then plenty of alternatives to phylogenetic methods are available including Blast scores, diagnostic sequence determination (Davis and Nixon 1992; Sarkar et al. 2002; DeSalle et al. 2005), or *Tax*I (Steinke et al. 2005). None of these approaches however are considered appropriate to evaluate species limits, and so they are of limited value for the identification of new species. Statistical approaches to assigning confidence levels to cluster membership have recently been proposed (Matz and Nielsen 2005; Nielsen and Matz 2006) and allow a more quantitative approach to determining species limits. The numbers of barcodes required for statistical confidence using these methods however are several times larger than those proposed to be sufficient for current barcoding programs (Hajibabaei et al. 2006).

The real indispensability of phylogenetics to barcoding, however, becomes apparent when one considers higher taxonomic levels other than just species membership tests. If a newly recovered barcode is determined, by whatever means, to represent a new species, then, without some method of "placing" this barcode within all of life, simply recognizing it as a new species is next to worthless. To really contribute to biodiversity studies, species discovered by barcoding need to be assigned to genera, families, etc. For example, the discovery of cryptic species is exciting because they are immediately relevant to other, previously known, species and genera. For applied barcoding uses, such as quarantine identifications, knowing whether a new barcode belongs to a genus which includes noxious pest species or one which is harmless will have tremendous practical and financial value and determine how intercepted material can be treated. Phylogenetic methodology is the only way to address these questions. Despite disagreements over analytical methods used to arrive at phylogenies, evolutionary-based criteria for recognizing groups and in particular the absolute necessity of monophyly at taxonomic levels above the species level are today almost universally accepted. Empirical barcoding papers demonstrate this point. Hogg and Hebert (2004) attempted to barcode a group of collembolans (springtails), small soil-dwelling arthropods, for which genetic-based methods of recognizing cryptic diversity would likely be very useful (Figure 1). Unfortunately, this analysis reveals apparent rampant paraphyly among the genera, with the genus *Folsomia* being rendered paraphyletic on 4 separate occasions! In this situation the chances are remote that an undescribed species of *Folsomia* will be correctly identified based on a barcode because the number of ambiguous placements near *Folsomia* rivals those that would lead to proper identification. A prerequisite to placing species newly discovered by barcoding is accurate phylogenetics. If *Folsomia* is not monophyletic, this

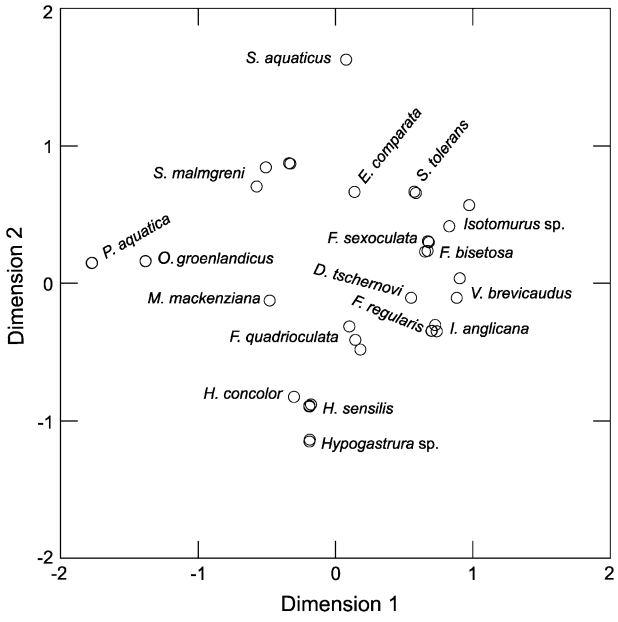

Figure 1. Euclidean 2-dimension distance matrix of Collembola from Hogg and Hebert (2004). Species and genus plots demonstrate inconsistencies of distance-based methods and mitochondrial DNA barcodes to effectively delineate species or genus boundaries. For example, *Folsomia regularis* and *Isotoma anglicana* share the same dimensions, making the species and genera indistinguishable and any new sample that falls within the region unidentifiable.

needs to be determined by rigorous criteria, including appropriate analytical methods and determining which data sources are able to robustly resolve relationships within the group. Although N-J methods may be sufficient for some applications of barcoding to identify species that have already been well described by other means, for more sophisticated and more useful applications—such as biodiversity discovery programs—good phylogenetics are absolutely essential. Proponents of DNA barcoding need to carefully consider methodological criticisms of their phylogenetic methods, or the lack of robustness will impoverish their results.

The conflation of issues surrounding DNA barcoding is thus germane to any realistic discussion of the merits and drawbacks of a full-blown DNA barcoding program to address the diversity of life. Although initially touted as a method of species identification (Hebert et al. 2003), to properly achieve this goal, barcoding needs to go further. Without a robust methodology for defining and identifying novel species and making predictive statements about these gene species (Nicholls 2003) regarding what they are, that is, to which higher taxonomic ranks they belong, DNA barcoding is limited to making clusters of similar DNA sequences that may or may not be biologically important.

There are 3 aspects of barcoding: taxon identification, species delimitation, and phylogenetic placement; the first is promoted by an argument about efficiency—what is the fastest and most accurate method of getting IDs—and the third, producing phylogenies, is a massive area of ongoing research onto itself. It is the second, species delimitation, which is currently the most contentious and the area for which DNA barcoding has been touted as having the most promise. How does one infer species diversity from the observed DNA diversity? The favored method among barcoding proponents is the application of threshold values (Countway et al. 2005; De Lay et al. 2005; Hebert et al. 2003; Hebert, Stoeckle, et al. 2004; Hogg and Hebert 2004; Smith et al. 2006; Vences, Thomas, Bonett, et al. 2005, Vences, Thomas, van der Meijden, et al. 2005; Ward et al. 2005). Barcodes from predetermined species identifications are used to determine the mean within- and between-species genetic distances, and a threshold value for species boundaries is developed: for example, if the mean within-species divergence for identified taxa is 0.5% and between species is 4%, anything greater than 3% may be arrived at as diagnostic of a new species. Most empirical barcoding papers have used this methodology for calculating variation within taxonomically well-studied groups to arrive at threshold values that are applied to understudied groups. For example, by examining Canadian day-flying moths, Hebert et al. (2003) arrived at 3% as an appropriate threshold value for species across a wide range of paraphyletic taxa. At other times, Hebert, Stoeckle, et al. (2004) have advocated flexible species limit thresholds, a threshold value of 10 times the mean for within-species variation for a group was proposed. However, as pointed out by Meyer and Paulay (2005) for mollusks and Vences, Thomas, van der Meijden, et al. (2005) for amphibians, this approach often results in considerable overlap between taxonomically well-defined species and a failure of the discriminating power of barcoding. There have also been instances when barcoding advocates have abandoned thresholds all together. Neither, Hebert, Penton, et al. (2004) nor Hajibabaei et al. (2006) employed thresholds in examining cases of cryptic species within neotropical lepidoptera assemblages. Instead when "substantially" divergent CO1 lineages could be correlated with morphological or life-history variation, they were regarded as cryptic assemblages. Truly cryptic assemblages were ignored:

> Some showed sequence diversity rivaling that found between very similar species. However, because we found no evidence of morphological or life history covariation with the barcode variants within these taxa, we here regard each of them as a single species. (Hajibabaei et al. 2006)

This approach is close to what has been termed "integrative taxonomy" (Dayrat 2005; Will et al. 2005); DNA sequences in combination with traditional character sets are used in a complementary fashion to define and describe species. The point is that the "traditional" (nonmolecular) characters lead the interpretation of the DNA sequences. In the cases noted above, the nonmolecular characters are provided by the nearly 30 years of studying the lepidopteran fauna of the Area de Conservación Guanacaste (ACG) in Costa Rica (Janzen 2004), which has produced one of the largest morphological and life-history data sets for a group of tropical invertebrates ever assembled. Situations like the ACG are, unfortunately, vanishingly rare in biology, and for most

groups, extensive background information is lacking. Can barcoding function without morphology or act as a first-pass assessment to group biodiversity and speed taxonomic studies? In short, can it substantially contribute to the biodiversity crisis, as its proponents claim?

Technical Problems with Defining Species Using mtDNA: Major Issues

mtDNA is not adequate as a sole source of species-defining data due to the following factors: reduced effective population size and introgression, maternal inheritance, recombination, inconsistent mutation rate, heteroplasmy, and compounding evolutionary processes.

Effective Population Size and Introgression

Reduced effective population sizes and lineage sorting will not have a predictable impact on barcoding and may, in one case, lump what are largely independent lineages and, in another case, split a single species. mtDNA is maternally inherited and usually single copy, therefore, it has one-fourth the effective population size (Ne) of nuclear genes and a different inheritance pattern. This makes mtDNA more sensitive than nuclear genes to population bottlenecks and isolation at all timescales and may be a major source of its discordance with nuclear gene phylogenies. It is obvious that bottlenecks or periods of isolation and introgression revealed solely by mtDNA are important evolutionary phenomena. However, relationships derived from the nuclear genome, which are often incongruent with those from the mtDNA (Funk and Omland 2003), should not be ignored when identifying species, clades, or their relationships anymore than mtDNA should be discarded (Rubinoff and Holland 2005). mtDNA's more rapid lineage sorting can provide information about population-level relationships, but additional sources of information are needed to understand species-level patterns (Patton and Smith 1994).

mtDNA introgression confounds the boundaries between otherwise distinct lineages, but there are no indications that this confusion exists when mtDNA is used in a vacuum the way that a DNA barcode for species identification would be used. Such introgression between species can lead to inaccurate identifications in a wide variety of animals including fish (Gerber et al. 2001; Schelly et al. 2006), elephants (Roca and O'Brien 2005), deer (Cathey et al. 1998), ducks (Kulikova et al. 2004; McCracken and Sorensen 2005), sea turtles (Karl and Bowen 1999), butterflies (Sperling and Harrison 1994), and beetles (Sota et al. 2001). In a broad survey of phylogenetic studies, Funk and Omland (2003) found that more than 20% of lineages they surveyed showed evidence for such "misleading" mtDNA introgression between otherwise distinct species.

Maternal Inheritance

The full effect of maternal inheritance on rates of molecular divergence in mtDNA is not predictable, and therefore, the failure rate for mtDNA barcoding will also be unpredictable. Most mitochondrial inheritance in animals is maternal, although significant exceptions exist (for a review see Korpelainen 2004). The most extreme exception is bivalve mollusks, which display doubly uniparental mitochondrial inheritance (Hoeh et al. 2002). This means that by tying species definitions to a mitochondrial gene, we are ignoring all evolutionary processes that do not affect females and skewing our interpretation of those processes that affect the sexes differently. Species with sedentary females and highly mobile males will have different diversification patterns depending upon whether maternally or paternally inherited markers are considered (Lavrenchenko et al., 2004; Rubinoff and Sperling 2004). This factor could be theoretically accounted for in such groups by allowing much larger levels of genetic differentiation to be included within a species than for groups where males and females have similar motilities. Even then infrequent paternal inheritance of mitochondria could upset the whole structure. Instances of paternal inheritance of mitochondria are increasingly being noted across a wide range of taxa (Zhao et al. 2004). The real-world variation therefore, in some instances, can lead to larger (in sedentary species) or smaller (due to paternal inheritance) evolutionary distances than expected and questionable accuracy of mtDNA barcode identification and species delimitation.

Recombination

The general absence of recombination in mtDNA will lead to the persistence of population structure long after the barriers which created that structure are removed and gene flow restored. Many models of speciation can result in highly structured populations despite ongoing gene flow between demes of a single species (Moritz et al. 2000), and many empirical examples have been documented (e.g., Schneider et al. 1998; Hugall et al. 2002). The unfortunate result is that one cannot predict or know with certainty when barcoding with mtDNA will over-, under-, or correctly estimate species boundaries that would be best determined from a broader data set.

Conversely, and although still relatively rarely reported, mitochondrial recombination does occur and would produce sequence variation through a process that violates assumptions important to the diagnostic protocol of barcoding. The perception that mitochondrial recombination is impossible (Avise 2000) is mostly due to the early failures to observe recombinant haplotypes in cell cultures or natural populations (e.g., Zuckerman et al. 1986). Recent surveys have found significant evidence of mitochondrial recombination (Piganeau et al. 2004; Tsaousis et al. 2005), and empirical evidence is available for 5 animal species: *Meloidogyne javanica* (Nematoda; Lunt and Hyman 1997), *Mytilus galloprovincialis* (Bivalvia; Ladoukakis and Zouros 2001), *Mytilis trossulus* (Bivalvia; Burzynski et al. 2003), *Platichthus flesus* (Teleost; Hoarau et al. 2002), and humans (Kratysberg et al. 2004). Indirect evidence of recombination has been found across most animal phyla, through a range of temporal scales, in various lineages from interpopulation to interspecific levels among closely related species and in up to 53% of the cases examined in recent studies (Piganeau et al. 2004; Tsaousis et al. 2005). Although not an impediment to DNA-based species definitions per se, recombination has major effects on the methodology by which one would define

those species. Recombination is a major violation of the bifurcating tree model of lineage descent upon which N-J (the topology-building model chosen by barcoders) is based. It also necessitates much more complex models of nucleotide evolution to be used in the calculation of genetic distance than have currently been used in barcoding efforts. In extreme cases, recombination may even result in the complete replacement of genes by introgression, a result which has been noted on many occasions when mitochondrial phylogenies have been compared against morphological or nuclear genetic results (Croucher et al. 2004). Thus, the technical inconsistency of recombination affects our ability to correlate any logical species concept with patterns resultant from recombination, the implication being serious theoretical problems with mtDNA barcode descriptions. Quite simply, DNA barcodes might be uninformative as a result of recombination. Although this phenomenon is still poorly understood and may not be common, the global scale of the barcoding effort magnifies the potential for any inconsistency to have a significant negative impact.

Technical Problems with Defining Species Using mtDNA: Emerging Problems

The following are additional phenomena currently under study that will affect DNA barcodes to varying degrees, the severity of which is currently not well understood.

Mutation Rate

For DNA barcodes to be used without additional information from other data sources, mutation rates would have to be consistent for threshold values, such as the proposed 2–3% divergence (Hebert et al. 2003), to correlate with species limits on a consistent basis. Unfortunately, there is no such obvious consistency, and neither is speciation uniquely driven by changes in mtDNA nor does the speciation event necessarily alter the mtDNA haplotype. In mitochondria the mutation rate of a gene is a function of its physical location in the genome (Saccone 1999; Gibson et al. 2005) such that the further it is from the origins of replication the faster mutations accumulate. This is due to the asymmetrical replication of the mitochondrial genome, which makes the lagging strand single stranded until approximately two-thirds of the leading strand has been replicated (Shadel and Clayton 1997). Thus, variations in genome arrangement will affect the mutation rate of *CO1*, which, again, can be accounted for only if genome arrangement is consistent across a group. Full mitochondrial genomes are available for a handful of taxa outside the phyla Chordata and Arthropoda, and so levels of genome variability remains impossible to realistically predict. Even some better sampled groups, like insects, vary widely in their levels of rearrangements (Dowton and Austin 1999; Dowton et al. 2003). Lineages with high levels of rearrangements will have multiple mutation regimes operating in different portions of the group such that 2% divergence between species might be appropriate for one group of taxa but 6% is more accurate for another. There is no way to determine this degree of variation prior to investing a significant amount of taxonomic and molecular expertise. After that investment, a *CO1* barcode would be just one possible tool that may or may not reflect the taxonomy of the group. We expect that biological processes that alter mutation rate, such as various types of genetic systems like parthenogenesis or haplodiploidy, sociality, and even life-history strategies such as generalists versus specialists, will have similarly unpredictable effects. Imposition of any single threshold measure of genetic distance for species designation is fraught with difficulty and inconsistency.

Heteroplasmy

The classical view is that the mitochondria are functionally haploid, with multiple, but identical, copies. There is growing evidence that single nucleotide differences between different mitochondrial haplotypes within individuals are common and in some species abundant. Significant levels of heteroplasmy have been reported in bats (Petri et al. 1996), fish (Hoarau et al. 2002; Hilsdorf and Krieger 2004), humans (Grybowski et al. 2003), insects (Nardi et al. 2001; Farge et al. 2002; Frey JE and Frey B 2004), and nematodes (Tsang and Lemire 2002). Most of these studies have been limited to detailing heteroplasmy at restriction sites. This focus may underestimate the potential number of heteroplasmic sites as these studies examine variation in only 4- to 12-bp stretches within the amplified gene. In studies of mitochondrial sequence variation within goniodid lice, we have found that, within individual louse, over 10% of sites were heteroplasmic (Cameron S, unpublished data), a higher level of variability than has previously been reported between species within this group (Johnson et al. 2001). This variability includes both sequence variability and length heteroplasmies due to insertion/deletions (indels) within coding genes. The phenomenon of indels has not previously been addressed by proponents of barcoding who use unspecified models of DNA alignment. Even a single indel would wreak havoc on simplistic models of DNA evolution such as N-J if not accounted for in the alignment phase. Extensive fine-tuning of alignments and the subjective decisions that may entail is yet another violation of the "fast and simple" benefit of a barcoding approach. Additionally, heteroplasmy means that the mitochondria of an individual may thus represent a sampling of the alleles within a population just like any nuclear gene. Therefore, for barcoding to be accurate, the set of those alleles must be nonoverlapping with the alleles of any other species. For many species, this nonoverlapping allelic set is probably small and the assumption of haploidy approaches reality, and for other species, particularly those with very fast molecular evolutionary rates, this assumption is violated (Korpelainen 2004). Large-scale barcoding is vulnerable even to relatively rare problems because they are magnified at the global level.

A second source of apparent mitochondrial heteroplasmy is nuclear pseudogenes (Lopez et al. 1994), which, if of recent origin, will be difficult to differentiate from the mitochondrial copies of the same gene. Nuclear pseudogenes of mitochondrial origin (NUMTs) may be very common (Zhang and Hewitt 1997) and can amplify to the exclusion of the "true"

mitochondrial copy of the gene. There is also the secondary issue of how one collects the barcode; conventional automated sequencing gives a consensus of the population of DNAs which were amplified, so it is perfectly conceivable that a species may have two or more significantly different haplotypes in each individual, but if the dominant haplotype is not consistent then conspecific individuals will appear to be different species. Clearly there is the possibility for widespread retention of ancestral polymorphisms or rampant independent adoptions of the same haplotype; either scenario will cause large problems for barcode species identification. It will be impossible to work in such a system unless a very high level of knowledge about the group is available prior to barcoding, rather than the single sequence from small number of individuals typical of barcode efforts to date. Thus, to ensure that heteroplasmic range is handled properly, routine cloning of every individual and sequencing of a representative collection of clones from each individual would be necessary, but doing so would go against the fast and inexpensive barcoding ethos.

An example of heteroplasmy in a barcoding study has already been found (Hebert, Penton, et al. 2004, p.14815), which the authors attempted to provide for in their skipper butterfly data set:

> Adults with two different CO1 sequences came from caterpillars collected on [host plants]. Because of the strong associations between caterpillar food plants and CO1 sequences in other individuals, it was possible to ascertain the likely genotypic characteristics of these "heterozygotes." By assuming that one of the CO1 sequences in each individual matched the typical sequence for other *A. fulgerator* found feeding on its food plant, the second sequence could be determined by subtraction.

The authors then go on to detail how they determined that one of the cases was a nuclear pseudogene (NUMT) and the other was a "heteroplasmy" (MYST), although it is unclear why this uncontroversial heteroplasmy requires quotation marks in their paper. Neither case could have been identified without extensive rearing and host plant association data collected by coauthor Janzen. Once again an "integrated approach" is necessary and demonstrates the shortcomings of dependence on a barcoding system.

Compounding Genetic Factors

Finally, there is the problem of coinherited factors that can bias simple mitochondrial inheritance. The 2 most obvious factors are mitochondrial selection, either on the barcoding gene itself or on other, linked, genes and cytoplasmically inherited bacteria such as *Wolbachia* and some *Rickettsia* species, which can alter inheritance patterns. Selection is an underappreciated factor in mitochondrial evolution but plays a significant role in shaping the variability of mitochondrial gene sequences within a species (Ballard and Whitlock 2004; Korpelainen 2004; Ruiz-Pesini et al. 2004). Additionally, as mitochondrial genomes are generally inherited as a unit (the full effects of low-level recombination on this have yet to be evaluated), each gene is considered to be in genetic linkage with others. Therefore, strong purifying selection on one gene will cause equivalent purifying selection on all mitochondrial genes. In general, purifying selection will act to reduce variation between individuals and upset assumptions of a linear molecular clock that would allow for threshold calculations. Of course, as previously stated, such local selective regimes cannot be predicted without extensive background knowledge.

Cytoplasmically inherited bacteria are another factor that can upset linearity of mitochondrial molecular clocks. *Wolbachia* sweeps due to cytoplasmic incompatibility (Wade 2001; crossings between infected and uninfected individuals result in nonviable offspring) have been demonstrated to cause the rapid fixation of the mitochondrial haplotype in which the sweep arose. This fixation resembles purifying selection by reducing molecular variability, but the timing of such sweeps is entirely random and thus the final mitochondrial population structure within a group of closely related species cannot be predicted. Given that *Wolbachia* infects upward of 76% of arthropods (Jeyaprakash and Hoy 2000), which are the most diverse phylum on earth, such effects cannot be ignored for groups in which barcoding is supposed to have the most important applications.

The aforementioned problems challenge the notion that any single mitochondrial gene is a global panacea that can be applied to the problem of species identification and/or definition using standardized threshold values. Furthermore, although many of these factors could be accounted for if they were consistent or predictable across taxonomic groups (a possibility within well-circumscribed small groups), the problems rarely show such consistency and thus cannot be accounted for a priori. The inability to formulate meaningful "rules" for defining species impairs an automated approach to species definitions and limits the utility of automation for species identification if it operates blind, that is, molecular data without recourse to extensive morphological, ecological, or behavioral data. In recent reviews of species delimitation methods, Sites and Marshall (2003, 2004) list the classes of data that can be used with each method. They show explicitly that several cannot be used with mitochondrial data and that all benefit from the use of multiple unlinked data sources. The reasons for the exclusion or relegation of mitochondrial data are simple: there are problems with mitochondrial data which limit their usefulness in species determination. These problems appear to have been underestimated by proponents of barcoding who may be relying on oversimplifications of how mitochondrial data behave.

Barcode Species Limit Thresholds Are Inoperable in Practice

Hebert et al. (2003) designate 2–3% mtDNA sequence divergence as a "threshold" to constitute species for insects and mammals (p. 319). Does that mean that taxa which are 1.8% diverged should not be considered separate species from a conservation or management perspective (Rubinoff 2006)? This is not a trivial point because rates of change

between taxa are a dynamic process; we can expect that most divergences will not be roughly equal to 2%. A wide ranging or ring species complex (Moritz et al. 1992; Irwin et al. 2001) would have taxa on the edges which are over 2% diverged, but when all the populations are sampled (which would happen in no systematic way with randomly harvested barcodes), there is much lower divergence between adjacent populations. Thus, taxa for which mtDNA varies geographically (a ubiquitous phenomenon) may not be recognized as conspecific. Individuals from disparate populations will be significantly diverged, though such ring species when viewed in context are genetically contiguous. What kind of rubric could mechanical barcoding use to address this problem? Depending on which populations were considered at any time, the species boundaries will change. What if the species boundaries dictated by a blindly sampled barcode miss major ecological differences (Crandall et al. 2000)? There is no room for considering ecology (unless this work has already been done—which is the situation for some of the barcode test case papers, (e.g., Hebert, Penton, et al. 2004, but not for most organisms), nor radical divergence in other parts of a genome if these patterns are not reflected in the 600 bp of mtDNA sequence. For example, polar bears render brown bears paraphyletic (Talbot and Shields 1996), and therefore, polar bears would be unlikely to receive any special protection were it not so obvious that they differ from brown bears in many other important characteristics (marine ecology, morphology, etc.). It is likely that a similar pattern of cryptic diversity not represented in mtDNA occurs in other, poorly known taxa. This would be especially true for arthropods (the bulk of biodiversity and also the majority of gaps in our knowledge). Because it is extremely unusual to have a wealth of additional data for such mitochondrially indistinct species, they would go unrecognized, though additional data, typical of an integrative approach, would clearly show their divergence. Barcoding simply fails to be a diverse and predictable source of data on which its users can confidently depend.

Is a barcode enough to defend major conservation efforts for supposed new cryptic taxa, which are genetically diverged, but do not show differences in morphology and ecology (Rubinoff and Sperling 2004)? Defining and protecting species solely based on the mtDNA barcode may not be received with enthusiasm by the public or policy makers who cannot see a difference (Rubinoff 2006). Practically speaking, anyone with a large economic stake in developing an area that might be considered for protection based on "gene species" could readily select a "suitable" threshold to justify their actions. Some proponents of the barcoding model may not have considered these issues adequately or at least have not detailed potential downsides.

Given the wide range of reasons why threshold values for species limits might theoretically fail, how do they perform in reality? The figures used to define species vary widely between studies on even closely related groups such as the heavily barcoded crown-group Lepidoptera (Kristensen and Skalski 1999). In the skipper butterfly paper (Hebert, Penton, et al. 2004), the threshold is as low as 0.32%, but it was as high as 3% in the original paper of Hebert et al. (2003) examining Canadian day-flying moths. In a third instance, Whinnett et al. (2005) examined a series of butterfly sister species from the same subfamily, Ithominae, whose speciation was driven by a single biogeographic boundary (disjunct Pleistocene Amazon rainforest refugia). They found divergences from as low as 0.23% to as high as 6.40%, demonstrating that species thresholds can be extremely fluid, even within a single analysis. Boundaries between intra- and interspecific variation become impossible to distinguish, especially when informed by only a single locus. This inconsistency has even been demonstrated by papers purported to be examples of barcoding success. For example, Smith et al. (2006) determine that their samples of parasitic flies had "intraspecific" divergence which overlapped in divergence with their choice of "interspecific" units. If the divergences of such biologically similar species pairs, created at the same time by the same speciation mechanism, can vary so much, what hope is there for establishing threshold values that can be used across even modest taxonomic distances? This demonstrates the "flexibilities"(or arbitrary nature) needed for barcodes to differentiate the groups that are a priori defined as significant units, for example, host plant associates (Hebert, Penton, et al. 2004) or previously described and accepted species of arctiids, notodontids, and sphingids (Hebert et al. 2003). What approach will be taken when the sample for identification is a canopy fogging event (e.g., Erwin 1995) or stream drift samples or pitfall traps where there are not just multiple insect orders but the full range of invertebrate phyla present with untold variation in the amount of mitochondrial differentiation within what would classically be termed a species within each group? This scenario illustrates the simplicity, and therefore inherent difficulty, of barcoding.

Further problems remain for using threshold values in species-level identification. Hebert, Penton, et al. (2004) state:

> We emphasize that barcodes differ from the standard traits used for species discrimination in the following important way: they can be obtained in a mechanized manner. Hence they can be used without much background knowledge both for routine identifications and for the detection of hidden species.

However, if their barcodes are used blindly (which would be a necessity for the bulk of species that lack other sources of data), it is doubtful that one would arrive at the same number of species they recognized. See for example their Figure 2 without the codes on the right-hand side (our Figure 2). Indeed, of the 10 "species" identified in this paper, only TRIGGO and CELT are differentiated from the remaining specimens by more than 3%, the value advocated in Hebert et al. (2003). If a 3% threshold had been applied, *A. fulgerator* would have been split into 4 species, TRIGO, CELT, NUMT, and the rest (Figure 2a). Thus, the considerable diversity of caterpillar morphology and host plant use identified by careful ecological and morphological study would never have been revealed by barcodes alone and, conversely, would have accorded a pseudogene species status. In contrast, if we accept the minimum genetic distance deemed to represent a species (0.32% INGCUP to HIHAMP) and apply it across

the tree, we arrive at 14 species (Figure 2b), CELT divides into 2 species, SENNOV into 4, and YESSEN into 2, assuming we can determine what NUMT and MYST represent ahead of time, otherwise they would be species too. Both exercises are meaningless. By a priori determination we can, within vague boundaries, get as many or as few species as are wanted. Meaning is only ascribed to the grouping in a tree a posteriori when the relevant ecological and morphological data are included. A recently published update of this work (Hajibabaei et al. 2006) does not attempt to diagnose clusters on the basis of barcodes alone. In an analysis of 521 species in 3 families (Hesperiidae, Sphingidae, and Saturniidae) 13, or 2.5% of the fauna, were found to consist of "distinct clusters" which correlate with morphological or ecological differences that had been recognized prior to the study. Similar objections (Moritz and Cicero 2004) have been raised about the cryptic bird species "discovered" by DNA barcoding (Hebert, Stoeckle, et al. 2004) wherein each cryptic species had been previously recognized as part of a taxonomically ambiguous species. Although barcodes may seem like a beneficial first screening, the simplicity and rapidity of barcodes result in an inherent and nontrivial risk of barcodes becoming the sole source of information for public policy in a practical application—like conservation (Rubinoff 2006). Further, in Hajibabaei et al. (2006), no definition is given for what constitutes a distinct cluster but some species (the number of species is also not specified) displayed higher levels of variation which could not be correlated with other biological factors and so remain as a single species. This relegates DNA barcoding to secondary importance to morphological or ecological considerations. Indeed, in their closing paragraph, Hajibabaei et al. (2006) suggest as much:

> Barcoding is no substitute for full taxonomic analysis, because the coupling of detailed morphological and ecological investigations with barcode results is critical for a final documentation of species richness.

It appears that DNA barcoders now espouse "integrative taxonomy" as the best way to conduct biodiversity studies. Yet, there continue to be statements that barcoding with fixed thresholds is of value in poorly studied groups, where the aforementioned inconsistencies would not be so obvious:

> Barcoding may also be applied to lesser known groups where a count of barcode lineages showing deep divergences (e.g.>2%) will provide a preliminary signal of species richness. (Hajibabaei et al. 2006)

This quotation shows how one may accept the idea that "barcode lineages" (lineages being a phylogenetic concept) are equal to species and can be used as a basis for important calculations such as "species richness." Again, a barcode species concept has never been explicitly articulated or tested.

Perhaps if the world's biodiversity had already been as well studied as the lepidopteran fauna of the ACG of Costa Rica, then DNA barcoding would be a worthwhile way of catching the crumbs (and 2.5% of a well-studied fauna is surely a crumb) that fall through the cracks of comprehensive taxonomic assessments of the world's biodiversity. But the world is a poorly known place and, until that situation changes, we suggest the development of more AGCs and fewer Centers to Barcode Life. In the meantime, why not target the use of DNA as a part of a more complete taxonomy, rather than the sole source of data? Profitable efforts can target species that appear to be suspect on morphological or ecological grounds already. Janzen and colleagues clearly had suspicions about the butterfly *A. fulgerator* (Hebert, Penton, et al. 2004) which may be why they sequenced 460 specimens of what was originally one species. In contrast, the average number of specimens examined per species in the broader Hesperiidae, Sphingidae, and Saturniidae study was 8 (Hajibabaei et al. 2006). The results of the studies of Hebert, Penton, et al. (2004) and Hajibabaei et al. (2006) continue to exemplify the utility of mtDNA as part of an integrated data set and confirm the inadequacy of using such barcodes in a vacuum.

Most studies to date clearly indicate that there is a need for more basic, fundamental systematic research before a rapid identification method of any kind would be truly useful. It is likely that most poorly known groups will require intensive work and background knowledge without which barcodes will fail to produce meaningful identifications. When funding and attention is shunted to barcodes over integrated research, we only get farther from an assessment of global biodiversity, or worse, we may be misled to believe that the crisis has been met by barcoding data alone.

Conclusion

Ultimately, the way barcoding is currently packaged, it is at best an exapted term for incorporating DNA sequence data into ongoing studies. By collaborating with morphologists (Hogg and Hebert 2004) and ecologists (Hebert, Penton, et al. 2004; Smith et al. 2006), DNA sequence takes part in resolving already established research questions. When barcodes are tested against a truly unstudied group, they produce unsatisfactory results with respect to species identifications (Meyer and Pauley 2005). Barcoding also fails to distinguish between members of closely related species groups (Armstrong and Ball 2005) and morphologically highly similar species (Hajibabaei et al. 2006), 2 instances for which DNA identifications should have had the most value. Thus, barcoding as it actually is functioning is very different from barcoding as it has been promoted (Will et al. 2005).

From a molecular perspective, mtDNA barcodes do not answer the same questions as integrated molecular, morphological, and ecological data sets, and therefore, barcoding is not a replacement paradigm for the integrated approach. What barcoding offers is unclear. Barcodes cannot be used solely, or even initially, to understand biodiversity because they are incomplete and often incongruent with other sources of data. For those relatively few groups where barcoding would be useful, the vast majority of the taxonomic work is already done, which is exactly why barcodes can make a contribution —confirmation of earlier work and finding the last few, cryptic species. In these instances, barcoding or other DNA data can make a contribution to species

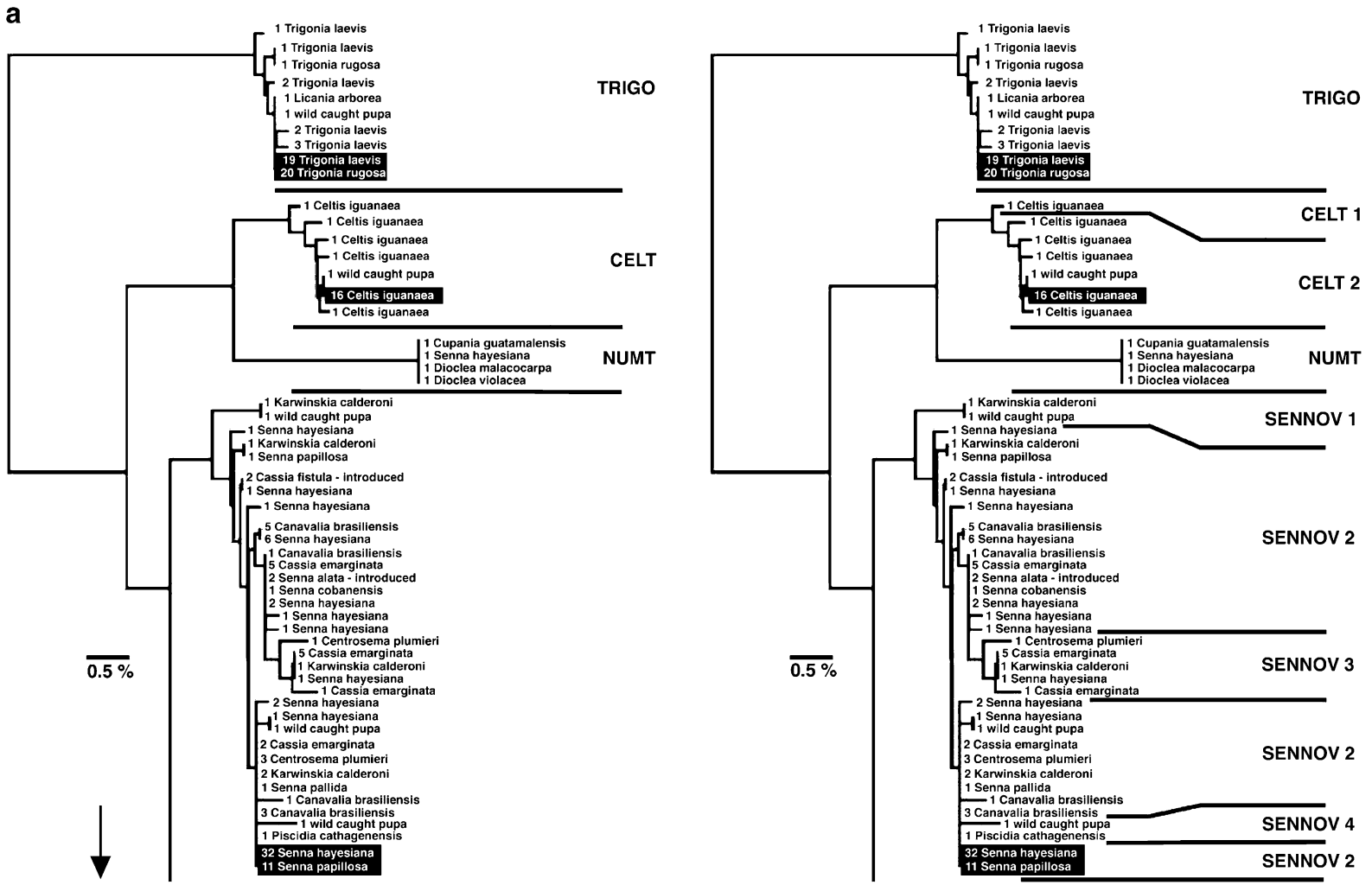

Figure 2. N-J tree of *Astraptes fulgerator* CO1 sequences redrawn after Hebert, Penton, et al. (2004); "species" name codes follow Hebert, Penton, et al. (2004). Topology A indicates those species that would be defined by the 3% divergence limit advocated by Hebert et al. (2003). Topology B indicates those species that would be defined using the minimum divergence identified between ecological feeding types identified a priori by coauthor Jansen, 0.32%, across the entire tree.

Figure 2. Continued.

identification. But for the vast majority of unidentified life, barcoding will not help with the "taxonomic impediment" that can only be solved through an understanding of complex species definitions and effective and accurate biodiversity assessments. For this task integrated taxonomy is essential, and such an integrated research model has already been in effect. So barcoding is either nothing new (as presented in Herbert, Penton, et al. 2004; Smith et al. 2006) or an expensive and incomplete digression that will not be adequate for identifying biodiversity. Although there are problems with the sole use of mtDNA, the genome has long been extremely valuable to a vast array of studies and we are not advocating that genetic data be excluded from systematic use (Rubinoff 2006), but it must be considered in conjunction with other sources of data such as nuclear DNA, morphology, or ecology (Rubinoff and Holland 2005). Barcodes of mtDNA could be useful for confirmation after hypotheses based on other or more complete sources of data have been gathered and analyzed. This was the case for the vertebrate barcoding examples (Hebert, Stoeckle, et al. 2004) and parasitoids (Smith et al. 2006). These are the examples to be followed. Certainly mtDNA could be part of a program of "integrated barcodes," relying on multiple sources of data very much like the current, balanced studies (DeSalle et al. 2005; Brower 2006; Rubinoff 2006). Ultimately, any of these solutions render barcodes as a secondary and perhaps less crucial part of the identification process, and, as such, it should not take resources away from other, more complete research programs.

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