

Limited performance of DNA barcoding in a diverse community of tropical butterflies

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DNA ‘barcoding’ relies on a short fragment of mitochondrial DNA to infer identification of specimens. The method depends on genetic diversity being markedly lower within than between species. Closely related species are most likely to share genetic variation in communities where speciation rates are rapid and effective population sizes are large, such that coalescence times are long. We assessed the applicability of DNA barcoding (here the 5′ half of the *cytochrome c oxidase I*) to a diverse community of butterflies from the upper Amazon, using a group with a well-established morphological taxonomy to serve as a reference. Only 77% of species could be accurately identified using the barcode data, a figure that dropped to 68% in species represented in the analyses by more than one geographical race and at least one congener. The use of additional mitochondrial sequence data hardly improved species identification, while a fragment of a nuclear gene resolved issues in some of the problematic species. We acknowledge the utility of barcodes when morphological characters are ambiguous or unknown, but we also recommend the addition of nuclear sequence data, and caution that species-level identification rates might be lower in the most diverse habitats of our planet.

Keywords: DNA barcoding; Amazon; biodiversity; Lepidoptera; mimicry

1. INTRODUCTION

There has been considerable recent interest in the use of short sequence tags known as ‘barcodes’ for the documentation and identification of a species (Blaxter 2003; Hebert *et al.* 2003a,b; Hebert & Gregory 2005; Savolainen *et al.* 2005; Dasmahapatra & Mallet 2006; Hajibabaei *et al.* 2007). In arthropods and vertebrates the DNA sequence used as a barcode is the 5′ half of the mitochondrial gene *cytochrome c oxidase I* (*CoI*). The utility of barcodes to accurately pigeonhole species has been successfully demonstrated in a number of studies (e.g. birds, Hebert *et al.* 2004b; Kerr *et al.* 2007), and has been offered as a tool for the discovery of cryptic butterfly and dipteran species (Hebert *et al.* 2004a; Smith *et al.* 2006, 2007; van Velzen *et al.* 2007). However, barcoding has been criticized from both theoretical and practical perspectives (Will & Rubinoff 2004; DeSalle *et al.* 2005; Ebach & Holdrege 2005; Meyer & Paulay 2005; Will *et al.* 2005; Brower 2006; Meier *et al.* 2006). Notably, successful barcode identification depends upon genetic diversity being markedly lower within than between

species (Hebert *et al.* 2004b). This assumption is likely to be broken sometimes, especially when rates of speciation are greater than coalescence times of the gene in question (Pamilo & Nei 1988; Brower *et al.* 1996; Monaghan *et al.* 2006; Nielsen & Matz 2006; Wiemers & Fiedler 2007). While the success rate of barcoding undoubtedly varies among groups, some taxa and ecosystems are particularly likely to be subject to difficulties—in particular, groups in which recent speciation rates are high and effective population sizes large and reasonably stable, as is probable in many tropical insects. Here we carry out one of the first community-level barcoding studies in the most diverse terrestrial ecosystem in the world—the upper Amazon basin.

Our study group are the Ithomiinae, an entirely Neotropical subfamily of butterflies containing approximately 360 species confined to moist forest habitats. Adults are distasteful to predators and have warning wing colour patterns, and virtually all species are involved in Müllerian mimicry (Müller 1879; Brown 1984). Mimicry is likely to cause speciation (Jiggins *et al.* 2001, 2006) and thus to be involved in the diversification of ithomiine genera. The alpha taxonomy of ithomiines has a relatively long history of study and seems to be fairly well resolved, being largely based on abundant morphological characters including wing pattern, venation, androconia and genitalia

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structures (Lamas 2004; Willmott & Freitas 2006). The Ithomiinae are thus a good test case to assess the effectiveness of barcoding.

Our primary aim is to assess the utility of DNA barcoding in the identification of specimens from an exceptionally diverse ithomiine community in eastern lowland Ecuador. Previous studies based on a larger fragment of mitochondrial DNA (mtDNA) revealed that some ithomiine species in Amazonian Peru were not monophyletic (Whinnett *et al.* 2005), thus questioning the utility of barcoding in this group. Here we measure the frequency of misidentification at the species and genus levels, based on *CoI* barcode sequences only. To take into account geographical diversity, we include also conspecifics from more distant sampling sites, and congeners and other ithomiine genera not represented in the community. We also investigate whether the accuracy of identification is improved by increasing the length of mtDNA sequence and by including a nuclear gene.

2. MATERIAL AND METHODS

(a) Study sites and sampling

Our main sampling effort is focused on two study sites located in eastern Ecuador, across the approximately 1 km wide Río Napo—Garza Cocha/La Selva Jungle Lodge near the Quechua communities of Pilche and Sani Isla on the north bank (0° 29.87' S, 76° 22.45' W, 6 km² surveyed) and around the Añangu Quechua community/Napo Wildlife Center on the south bank (0° 31.41' S, 76° 23.73' W, 15 km² surveyed). Ithomiine populations were studied between 2000 and 2007 at Garza Cocha and in 2005 and 2007 at Añangu. We collected all the 58 species present locally (table 1, electronic supplementary material 1). Specimens were identified by the authors using morphology of wing pattern, venation and genitalia, and were collected during each field season for genetic analyses. Bodies or legs were preserved in salt-saturated DMSO (20% DMSO, 0.25 M EDTA, saturated with NaCl), and wings were kept in envelopes. Rarely, individuals were dried as complete specimens and a single leg was used for genetic analysis. Two to nine local individuals per species were used in the analyses (except for the rare species *Brevioleria seba* and *Dircenna dero*, table 1).

(b) DNA extraction and sequencing, and GenBank sequences

DNA was extracted using the QIAGEN DNeasy Kit, according to the manufacturer's protocol. Two hundred and seventy three local specimens and 80 non-local specimens belonging to the same species or genera (electronic supplementary material 1) were sequenced at the arthropod 'barcode' region, namely the 5' half of the mitochondrial gene *CoI* (653 bp fragment). Primers, PCR and sequencing reaction conditions are given in the electronic supplementary material 2. In addition, 70 sequences of conspecifics, congeners or other ithomiine genera were downloaded from GenBank and aligned with our sequences (electronic supplementary material 1). *Tellervo zoilus* (Tellervinae) was used as an out-group in the analyses detailed below.

To test whether increasing information improves the accuracy of identification, a larger mitochondrial fragment (the entire *CoI*, 1464 bp, *tRNA-Leucine*, 62 bp and *CoII*, 716 bp) and a fragment of a nuclear gene, *Elongation factor 1α* (*Ef1α*, 1215 bp), were sequenced or downloaded from

GenBank for a subset of the previous specimens (electronic supplementary material 1). Sequences were aligned using CODONCODE ALIGNER v. 1.6.3 (CodonCode Corporation) and checked for reading frame errors using MACCLADE v. 4.07 (Maddison & Maddison 1997). All sequences have been deposited in GenBank under accession numbers EU068763–EU069266.

(c) Data analyses

Relationships among barcode sequences were inferred with three clustering methods. A neighbour-joining (NJ) tree based on Kimura 2-parameter (K2P) distances was computed using the software MEGA v. 3.1 (Kumar *et al.* 2004). Branch support was assessed with 1000 bootstrap replicates. A maximum likelihood (ML) tree was generated with the program PHYLML (Guindon & Gascuel 2003) using a GTR+Γ substitution model. Branch support was assessed with 100 bootstrap replicates. We also performed a Bayesian analysis using the program MrBAYES v. 3.1.2 (Huelsenbeck & Ronquist 2001). We performed two runs of four simultaneous Markov chains each for 1 000 000 generations starting from random initial trees and under a GTR+I+Γ model, and sampled a tree every 100 generations. Data from the first 900 000 generations (9000 trees) were discarded, after confirming that likelihood values had stabilized well before that. The consensus tree and posterior probability of nodes were calculated from the remaining 1000 trees.

Accuracy of identification was also tested by using the BLAST algorithm (Altschul *et al.* 1997) as follows. A FASTA file of the barcode data was first formatted as a BLAST database and a local BLASTN search (v. 2.2.15) was carried out on the entire dataset against this database.

Specimens that did not cluster with conspecifics or congeners in our barcode analysis were re-examined carefully to confirm identification (real specimen or photo) and were sometimes sequenced again to confirm initial results.

Two phylogenetic trees based on *CoI–CoII* and *EF1α* were obtained with MrBAYES v. 3.1.2 (Huelsenbeck & Ronquist 2001) as described previously. Mitochondrial sequence data were divided into eight partitions: *CoI* codon positions 1, 2 and 3, *CoII* codon positions 1, 2 and 3, *tRNA-leucine*, and non-coding DNA upstream of *CoI*; *EF1α* sequence data were partitioned by codon position. The best model of substitution for each region was selected using MrMODELTEST v. 2.2 (Nylander 2004; electronic supplementary material 3). ML and parsimony analyses were also performed, and they showed identical clustering patterns at the genus and species levels (results not shown).

3. RESULTS

(a) Analyses of barcode sequences

The three clustering methods (NJ, ML and Bayesian) gave comparable results for all but three species: *Brevioleria arzialia*, *Napeogenes sylphis* and *Pseudoscada timna* (table 1). Up to 42 out of 56 species present locally and represented by more than one individual (75%) formed well-supported monophyletic groups (bootstrap or Bayesian probability greater than 50%, table 1, figure 1, electronic supplementary material 4a–c). Species monophyly increased to 77% (44 out of 57) when *Napeogenes larina aethra* and *Mechanitis mazaesus deceptus* were considered species separate from *Napeogenes larina otaxes* and *Mechanitis mazaesus mazaesus*, respectively, as these and

Table 1. Checklist and number of local and geographically distant conspecifics, support values (bootstrap or Bayesian posterior probabilities, in percentage) of the three clustering methods of the barcode sequences for genera and species represented by more than one species or individual.

genus (ML, Bayesian and NJ support values)	species	local subspecies	no. local individuals	no. distant conspecifics	support values		
					ML	Bayes	NJ
<i>Aeria</i> (only one species)	<i>eurimedia</i>	<i>negricola</i>	6	3	100	100	100
<i>Brevioleria</i> (no, no, no)	<i>arzalia</i>	ssp.	5	2	no	78	98
	<i>seba</i>	<i>oculata</i>	1	no			
<i>Callithomia</i> (100, 100, 100)	<i>alexirrhoe</i>	<i>butes</i>	2	2	no	no	no
	<i>lenea</i>	<i>zelie</i>	5	3	no	no	no
<i>Ceratinia</i> (no, no, no)	<i>tutia</i>	<i>poecila</i>	6	4	99	100	99
<i>Dircenna</i> (only one species)	<i>dero</i>		1	1	100	100	100
<i>Episcada</i> (no, no, 54)	<i>sulphurea</i>	ssp. 1	4	1	99	99	98
<i>Forbestra</i> (98, 100, 84)	<i>equicola</i>	<i>equicoloides</i>	3	1	97	100	100
	<i>olivencia</i>	<i>juntana</i>	6	2	no	no	no
	<i>proceris</i>		2	1	no	no	no
<i>Godyris</i> (95, 100, 99)	<i>zavaleta</i>	<i>matronalis</i>	5	3	95	97	96
<i>Heterosais</i> (only one species)	<i>nephele</i>	<i>nephele</i>	5	1	95	100	100
<i>Hyalyris</i> (no, no, no)	sp.		4	no	90	100	100
<i>Hypoleria</i> (only one species)	<i>lavinia</i>	<i>chrysodonia</i>	5	1	100	100	100
<i>Hyposcada</i> (no, no, 21)	<i>anchiala</i>	ssp.	5	2	84	100	98
	<i>illinissa</i>	<i>ida</i>	5	5	99	100	99
	<i>kena</i>	<i>kena</i>	2	1	100	100	100
<i>Hypothyris</i> (no, no, no)	<i>anastasia</i>	<i>honesta</i>	5	2	100	100	100
	<i>euclea</i>	<i>intermedia</i>	5	1	100	100	99
	<i>fluonia</i>	<i>berna</i>	5	2	100	100	100
	<i>mamercus</i>	<i>mamercus</i>	7	2	100	100	100
	<i>moebiusi</i>	<i>moebiusi</i>	5	no	100	100	100
	<i>semifulva</i>	<i>satura</i>	5	1	95	100	100
<i>Ithomia</i> (27, 50, 29)	<i>agnosia</i>	<i>agnosia</i>	6	1	100	100	100
	<i>amarilla</i>		6	no	100	100	100
	<i>salapia</i>	<i>salapia</i>	5	3	100	100	100
<i>Mechanitis</i> (100, 100, 99)	<i>lysimmia</i>	<i>roqueensis</i>	6	1	80	84	96
	<i>mazaeus</i>	<i>mazaeus</i> ^a	5	2	100	100	100
	‘mazaeus’	<i>deceptus</i> ^a	5	2	no	no	no
	<i>polymnia</i>	ssp.	7	4	no	no	no
<i>Melinaea</i> (100, 100, 100)	<i>marsaeus</i>	<i>macaria/mothone</i>	9	3	no	no	no
	<i>menophilus</i>	<i>menophilus</i>	5	3	no	no	no
	<i>satevis</i>	<i>maeonis</i>	6	3	no	no	no
<i>Methona</i> (100, 100, 98)	<i>confusa</i>	<i>confusa</i>	3	5	98	100	100
	<i>curvifascia</i>		6	1	100	100	100
	<i>grandior</i>	ssp.	5	no	100	100	100
<i>Napeogenes</i> (66, 100, 72)	<i>achaea</i>	<i>achaea</i>	3	no	100	100	100
	<i>duessa</i>	ssp.	6	1	95	100	92
	<i>inachia</i>	<i>pozziana</i>	5	4	69	69	99
	‘larina’	<i>aethra</i> ^b	5	no	100	100	100
	<i>pharo</i>	<i>pharo</i>	5	1	91	100	100
	<i>quadrilis</i>		4	no	100	100	100
	<i>sylphis</i>	<i>corena</i>	5	2	94	no	99
<i>Oleria</i> (18, 100, no)	<i>agarista</i>	<i>agarista</i>	2	1	no	no	no
	<i>assimilis</i>	<i>assimilis</i>	7	no	100	100	100
	<i>gunilla</i>	<i>lota</i>	5	1	100	100	100
	<i>ilerdina</i>	<i>lerida</i>	4	no	100	100	100
	<i>onega</i>	ssp.	5	2	90	100	96
	<i>sexmaculata</i>	<i>sexmaculata</i>	3	no	100	100	100
<i>Pseudoscada</i> (no, no, no)	<i>florula</i>	<i>aureola</i>	4	1	100	100	100
	<i>timna</i>	<i>utilla</i>	5	4	no	59	27
<i>Pteronymia</i> (65, 100, 56)	<i>primula</i>	<i>primula</i>	5	1	no	no	no
	<i>sao</i>	ssp.	4	1	100	100	100
	<i>vestilla</i>	<i>sparsa</i>	5	1	no	no	no
<i>Scada</i> (100, 100, 99)	<i>zibia</i>	<i>batesi</i>	5	1	100	100	100
<i>Thyridia</i> (only one species)	<i>psidii</i>	<i>ino</i>	2	2	100	100	100

(Continued.)

Table 1. (Continued.)

genus (ML, Bayesian and NJ support values)	species	local subspecies	no. local individuals	no. distant conspecifics	support values		
					ML	Bayes	NJ
<i>Tithorea</i> (only one species)	<i>harmonia</i>	<i>hermias</i>	6	2	100	100	100
species that form clusters (all species) ^a					43	44	44
species that form clusters (species with foreign conspecifics and congeners) ^{a,b}					27	28	28

^a *Mechanitis mazaesus* is here regarded as two species, represented by *M. mazaesus mazaesus* and *M. mazaesus deceptus*.

^b *Napeogenes larina aethra* is here considered a species different from *N. larina otaxes*.

other data suggest (Elias *et al.* 2007; R. I. Hill *et al.* 2006–2007, unpublished data). Consequently, *Napeogenes* ‘*larina*’ *aethra* and *M. ‘mazaesus’ deceptus* will be considered ‘good’ species in the analyses below. Considering only those species from the community dataset represented by more than one congener and including remote conspecifics in the analysis (i.e. situations where barcoding would be most useful), only 28 out of 41 species (68%) were monophyletic. Similar results were achieved using the BLAST algorithm. In 13 of the species (23% of all the species, 32% of the species with congeners and remote conspecifics), at least one non-conspecific individual showed a higher BLAST bit score as compared with other conspecifics in the dataset.

In the genus *Melinaea*, the three local species were entirely undifferentiated, confirming previous observations from Peru (Whinnett *et al.* 2005). Similarly, sister species pairs such as *Forbestra olivencia* and *Forbestra proceris*, *Pteronymia vestilla* and *Pteronymia primula*, *Callithomia lenea* and *Callithomia alexirrhoe* were partly or completely undifferentiated with respect to one another. In contrast, *M. ‘mazaesus’ deceptus* and *Oleria agarista* were split into several lineages, thus becoming para- or polyphyletic with respect to closely related taxa. Failure to form monophyletic groups occurred between local conspecifics in 10 species, while only divergence between geographical populations was involved in the remaining three cases (figure 1). There was no differentiation between the two banks of the Río Napo, the only exception being *O. agarista* (electronic supplementary material 4a–c).

Genetic distances within species present locally (0.0085 ± 0.0137) were on average much lower than distances between congeneric species (0.0602 ± 0.0292 , figure 2a). However, the distributions overlapped, with intraspecific distances over 0.08 in *Oleria* and *Hyposcada* contrasting with no interspecific divergence in *Melinaea* and *Forbestra* (figure 2a,b).

At the generic level, 9 (ML) to 11 (Bayesian analysis) of the 18 genera present locally and represented by more than one species (50–61%) were monophyletic (figure 1, table 1, electronic supplementary material 4a–c). Paraphyletic genera were *Brevioleria*, *Ceratinia*, *Episcada*, *Hyaliris*, *Hyposcada*, *Hypothyris*, *Ithomia*, *Oleria* and *Pseudoscada*.

(b) Improved accuracy by using more genes

Inclusion of the entire *CoI–CoII* region resulted in the same relationships within species as with the barcode alone (electronic supplementary material 5a). The only exception to this was *O. agarista*, which was monophyletic in the full mtDNA dataset. The nuclear gene *EF1α* did contribute more to identification accuracy (electronic

supplementary material 5b). *Pteronymia primula*, *F. proceris*, *F. olivencia* and *O. agarista* were monophyletic, sequences of the last species being virtually identical. All *M. mazaesus* clustered together, but there was no distinction between *M. mazaesus mazaesus* and *M. ‘mazaesus’ deceptus*.

At the generic level, *Ceratinia* remained paraphyletic with respect to *Episcada* with both gene regions. Species from the genera *Greta* and *Pseudoscada* were intermixed in the mtDNA analysis, but with *EF1α* *Pseudoscada* species formed a monophyletic group, close to a paraphyletic *Greta*. *Oleria* became monophyletic with the longer mitochondrial region but not with *EF1α*. *Hyaliris*, *Hyposcada* and *Ithomia* formed monophyletic groups using each of the additional regions.

4. DISCUSSION

Barcoding has triggered a passionate debate between proponents who aim to ‘tag’ the diversity of life (Hebert *et al.* 2003a,b; Hebert & Gregory 2005; Savolainen *et al.* 2005; Hajibabaei *et al.* 2007), and detractors who highlight the pitfalls of the single-gene approach and are concerned about competition for funds with traditional taxonomy (Will & Rubinoff 2004; Ebach & Holdrege 2005; Wheeler 2005; Will *et al.* 2005; Brower 2006; Cameron *et al.* 2006; Rubinoff 2006). It has been claimed that mtDNA barcodes achieve an accuracy close to 100% in delimiting species in some groups (Hebert *et al.* 2004b; Hajibabaei *et al.* 2006a; Clare *et al.* 2007; Ekrem *et al.* 2007; Kerr *et al.* 2007), although in some cases it has proved less successful (Meyer & Paulay 2005; Meier *et al.* 2006; Wiemers & Fiedler 2007; Whitworth *et al.* 2007). Our own study shows a rather poor success of DNA barcoding, with only 77% of species unambiguously identified.

Previous barcoding work in the tropics has mainly focused on the Guanacaste National Park in Costa Rica (Hebert *et al.* 2004a; Janzen *et al.* 2005; Hajibabaei *et al.* 2006a; Smith *et al.* 2006, 2007), a mosaic of mainly dry forest and cloud forest habitats which is less diverse than the habitat studied here. For example, in the whole of Costa Rica (51 100 km²) there are 61 species of ithomiine butterflies (DeVries 1997), similar to the diversity found in the 21 km² sampled in the Napo community. The relatively aseasonal and continuous habitat of the upper Amazon probably maintains large and stable population sizes of these butterflies, with correspondingly longer coalescence times when compared with more seasonal habitats such as dry forest, or temperate regions where populations have been reduced to small refugia during glacial periods. Moreover, butterfly genera of the upper Amazon tend to

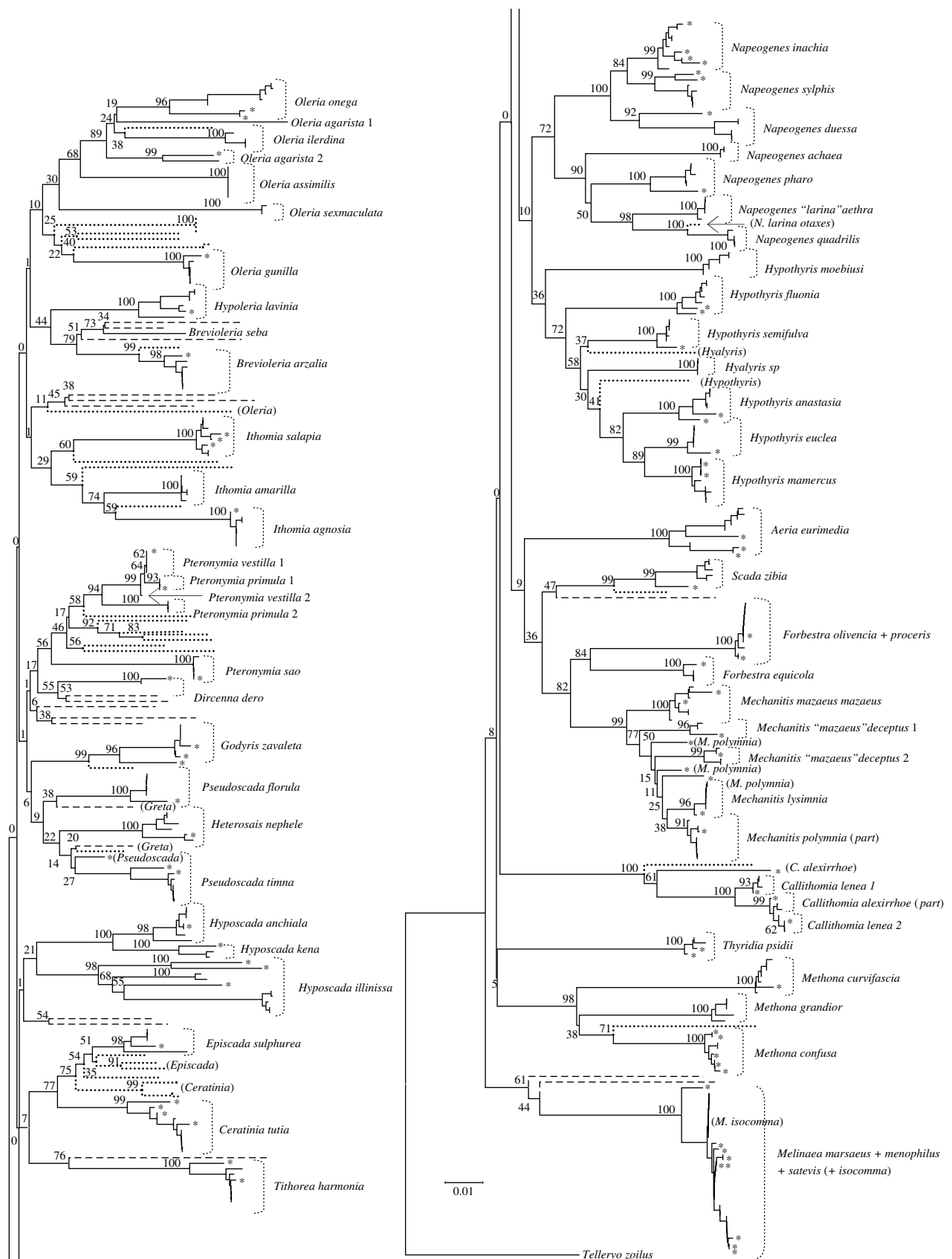


Figure 1. Neighbour-joining clustering of barcode sequences, with bootstrap values shown on the nodes. Geographically distant conspecifics are represented by an asterisk. Dotted and dashed lines represent congeners and genera that are not represented in the Rio Napo community, respectively. Names of non-local specimens are indicated in brackets in case of ambiguity or particular interest. Complete trees obtained with the three clustering methods are available in the electronic supplementary material 4a–c.

contain more species (Lamas 2004). Both factors point to a greater challenge for gene-based identification in the Amazon. Not surprisingly, the six species without

congeners in our analysis were accurately diagnosed. However, adding congeners and geographical populations decreased the success of barcoding: 32% of such

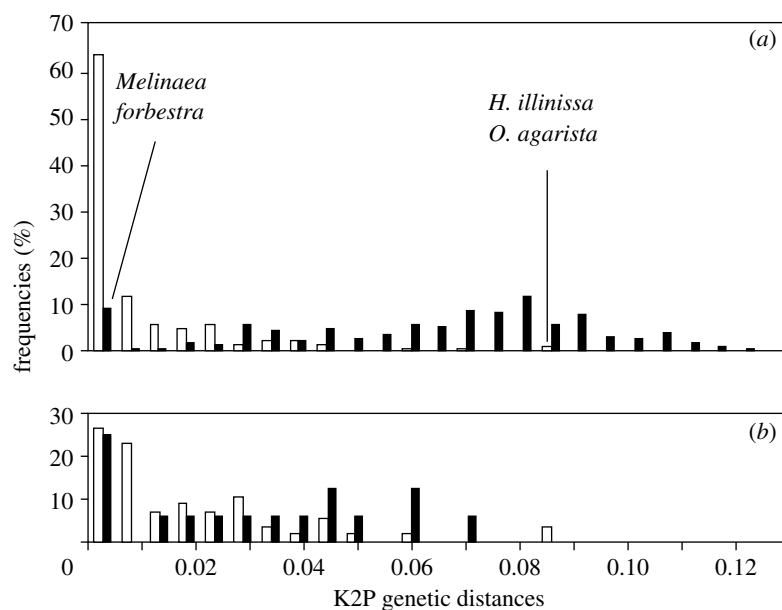


Figure 2. Distribution of within-species (white) and between congeneric species (black) K2P distances of barcode sequences. Only species and genera represented locally are considered. (a) All pairwise distances and (b) only maximum intraspecific and minimum interspecific distances.

species could not be diagnosed. In other words, the barcode method of identification becomes significantly less reliable when groups of closely related (congeneric) species are examined, or geographical populations of the same species are included, exactly the circumstances where barcoding would be most useful as a tool. Thus, biogeographic and evolutionary history also plays a role in the success of the identification of specimens using barcoding.

Hebert *et al.* (2004b) suggested the use of a threshold in sequence divergence in the discovery of new species, claiming that barcoding could be an outstanding tool for this purpose (Gomez *et al.* 2007). Yet the existence of the so-called 'barcode gap' has been challenged both on theoretical (Hickerson *et al.* 2006) and empirical grounds (Cognato 2006; Wiemers & Fiedler 2007). The pattern of barcode evolution in ithomiine butterflies, with highly variable levels of divergence within different species complexes and genera (Whinnett *et al.* 2005; figure 2), adds fuel to these criticisms. We agree that barcoding should not be relied on as the sole basis for species discovery, except as a preliminary effort in the case where ecological, morphological or additional genetic data are absent.

(a) Generic level identification

At best 61% of currently recognized genera represented by more than one species formed stable clusters. Some generic circumscriptions are likely to be revised in the near future on the basis of ongoing morphological and molecular work (Brower *et al.* 2006; Willmott & Freitas 2006; K. R. Willmott 2001–2007, unpublished data), and some taxa could be moved from one genus to another. This might affect the pairs of genera *Pseudoscada* and *Greta*, *Hypothyris* and *Hyalyris*, and *Ceratinia* and *Episcada*. In the most optimistic hypothesis where these pairs of genera in our study are pooled, only 11–13 of the rearranged genera represented locally (69–81%) are monophyletic. Thus, even at the generic level, the barcode performs rather poorly in assigning names to specimens.

(b) Limitations of existing databases

DNA barcodes cannot be a useful identification tool without a comprehensive and reliable reference database (Meyer & Paulay 2005; Brower 2006; Hajibabaei *et al.* 2006b; Scheffer *et al.* 2006). To investigate the extent to which existing databases can be used to identify specimens in our study, we tested the Barcode of Life Data Systems identification engine (BoLD-ID) in August 2007 (Ratnasingham & Hebert 2007; <http://www.barcodinglife.com/views/idrequest.php>), with mixed results. We used a subset of 61 sequences of local specimens representing all species and major lineages within species. While 74% of the specimens were correctly identified at the genus level using the full database (but only 41% using the reference database), a number of specimens (18 and 44%, using the full and reference databases, respectively) were assigned to an incorrect (and unrelated) genus with a probability of placement of 1. The remaining specimens could not be assigned to any genus. In the latter cases, the paucity of ithomiine sequences in the database (15 and 9 ithomiine genera found in the full and reference databases, respectively) explains the failure to hit the correct genera. Moreover, the decision system that generates the probabilities of placement tends to overestimate these values for taxa poorly represented in the database. Thus the BoLD-ID engine is currently not suitable for a reliable identification of ithomiines (and that of other relatively poorly represented groups), although the identification accuracy should improve when the sequences of this study are included in the BoLD database. As a comparison when BLASTing the same sequences against the GenBank nucleotide database, where all the tested genera are represented, the correct genus was identified in 88% of the cases.

(c) Analytical methods for barcode data

As no consensus has been reached yet concerning the analysis of barcode data, there is a growing literature testing and describing analytical methods (e.g. Little & Stevenson 2007). A number of authors have proposed or

tested novel methods based on diagnostic molecular characters relying either on the presence/absence of short fragments of sequence (DasGupta *et al.* 2005; Little & Stevenson 2007) or on parsimony informative sites or combinations of sites referred to as 'characteristic attributes' (Sarkar *et al.* 2002; Kelly *et al.* 2007). We have not used any of these methods as we believe that the use of diagnostic molecular characters to identify species is likely to result in lower accuracy due to incomplete conspecific reference material (see previous paragraph), especially from across the geographical ranges of the species (Little & Stevenson 2007). To establish a sequence database for the Ithomiinae over the entire range of the subfamily would require collecting and sequencing more than 1600 morphologically divergent subspecific taxa, which is a daunting task.

Here, we compared three alignment-based clustering methods and an alignment-free similarity method, which gave similar results. Since the NJ clustering and the similarity method (BLAST) perform considerably faster than the others, we believe they are a good choice for barcode analyses (Little & Stevenson 2007). NJ clustering has indeed been used in the great majority of published barcoding studies. However, the most promising area for future research is methods that take into account population size and therefore coalescence times in the estimation of barcoding probabilities (Nielsen & Matz 2006).

(d) *The value of nuclear data*

Despite extensive use of the mitochondrial gene *CoI* in vertebrates, arthropods and, more recently, fungi (Seifert *et al.* 2007), there are potential drawbacks to using the mitochondrial genome (Rubinoff *et al.* 2006). Notably, maternal inheritance, the absence of recombination or the spread of cytoplasmically inherited symbionts can lead to misleading identification using the mtDNA barcode (Hurst & Jiggins 2005; Rubinoff *et al.* 2006; Whitworth *et al.* 2007).

Our data have demonstrated the value of adding nuclear sequence data to the mtDNA barcode. Despite the relatively slow rate of evolution at *EF1 α* , it has nonetheless resolved several cases where the mtDNA barcode failed. Notably, species with very divergent mtDNA sequences between geographical populations showed nearly identical *EF1 α* sequences. As already suggested (Dasmahapatra & Mallet 2006; Smith *et al.* 2006, 2007; Gomez *et al.* 2007) we would therefore recommend that the mtDNA barcode be supplemented where possible by one or more nuclear genes. *EF1 α* has been used here as it is widely studied in insect phylogenetics and easily amplified across many insect groups (Wahlberg *et al.* 2005; Danforth *et al.* 2006), although its slow rate of evolution means that other genes, such as rapidly evolving introns (Beltrán *et al.* 2002), might be more informative for closely related species.

5. CONCLUSION

Our work on ithomiine butterflies has demonstrated that the barcode method of identification, as currently applied, may be significantly less successful in certain circumstances than proponents have suggested. In particular, diverse tropical invertebrate faunas with multiple

congeneric species and geographical races, in other words the bulk of biodiversity, pose a particular challenge. Despite the limitations of barcoding for species identification and discovery, the use of mitochondrial sequence data is clearly a valuable tool for taxonomy. Insect molecular systematics employing mtDNA has enjoyed great success for two decades (DeSalle *et al.* 1987; Brower 1994; Caterino *et al.* 2000). Unexpected mtDNA *CoI* patterns have led to the discovery of cryptic species (Brower 1996; Hebert *et al.* 2004a; Mallarino *et al.* 2005; Smith *et al.* 2006, 2007). Similarly, barcode patterns have encouraged us to search for covarying morphological, genetic and ecological characters in the genus *Mechanitis* (R. I. Hill *et al.* 2006–2007, unpublished data) and in the species *Napeogenes larina* (Elias *et al.* 2007), where divergent lineages probably represent unrecognized cryptic species. We are also using barcode sequences to recover the identity of immature stages and thus host plant records, where larvae were collected in the field but died before reaching the adult stage (K. R. Willmott *et al.* 2005–2007, unpublished data), and to identify the sources of blood meals from engorged mosquitoes (Townzen *et al.* 2005, unpublished data). Finally, mitochondrial barcode sequences are sometimes the best sequence data that can be obtained from old, degraded museum specimens. The growing public databases containing a homologous sequence region from a diversity of taxa will undoubtedly continue to be a valuable tool for the study of biodiversity.

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REFERENCES

- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W. & Lipman, D. J. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402. (doi:10.1093/nar/25.17.3389)
- Beltrán, M. S., Jiggins, C. D., Bull, V., Linares, M., Mallet, J., McMillan, W. O. & Bermingham, E. 2002 Phylogenetic discordance at the species boundary: comparative gene genealogies among rapidly radiating *Heliconius* butterflies. *Mol. Biol. Evol.* **19**, 2176–2190.
- Blaxter, M. 2003 Molecular systematics—counting angels with DNA. *Nature* **421**, 122–124. (doi:10.1038/421122a)
- Brower, A. V. Z. 1994 Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol.* **3**, 159–174. (doi:10.1006/mpev.1994.1018)
- 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. (doi:10.1006/zjls.1996.0022)
- Brower, A. V. Z. 2006 Problems with DNA barcodes for species delimitation: 'ten species' of *Astraptes fulgerator* reassessed (Lepidoptera: HesperIIDae). *Syst. Biodiv.* **4**, 127–132. (doi:10.1017/S147720000500191X)
- Brower, A. V. Z., DeSalle, R. & Vogler, A. P. 1996 Gene trees, species trees and systematics: a cladistic perspective. *Annu. Rev. Ecol. Syst.* **27**, 423–450. (doi:10.1146/annurev.ecolsys.27.1.423)
- Brower, A. V. Z., Freitas, A. V. L., Lee, M. M., Silva-Brandao, K. L., Whinnett, A. & Willmott, K. R. 2006 Phylogenetic relationships among the *Ithomiini* (Lepidoptera: Nymphalidae) inferred from one mitochondrial and two nuclear gene regions. *Syst. Entomol.* **31**, 288–301. (doi:10.1111/j.1365-3113.2006.00321.x)
- Brown Jr, K. S. 1984 Adult-obtained pyrrolizidine alkaloids defend ithomiine butterflies against a spider predator. *Nature* **309**, 707–709. (doi:10.1038/309707a0)
- Cameron, S., Rubinoff, D. & Will, K. 2006 Who will actually use DNA barcoding and what will it cost? *Syst. Biol.* **55**, 844–847. (doi:10.1080/10635150600960079)
- Caterino, M. S., Cho, S. & Sperling, F. A. H. 2000 The current state of insect molecular systematics: a thriving tower of Babel. *Annu. Rev. Entomol.* **45**, 1–54. (doi:10.1146/annurev.ento.45.1.1)
- Clare, E. L., Lim, B. K., Engstrom, M. D., Eger, J. L. & Hebert, P. D. N. 2007 DNA barcoding of Neotropical bats: species identification and discovery within Guyana. *Mol. Ecol. Notes* **7**, 184–190. (doi:10.1111/j.1471-8286.2006.01657.x)
- Cognato, A. I. 2006 Standard percent DNA sequence difference for insects does not predict species boundaries. *J. Econ. Entomol.* **99**, 1037–1045.
- Danforth, B. N., Sipes, S., Fang, J. & Brady, S. G. 2006 The history of early bee diversification based on five genes plus morphology. *Proc. Natl Acad. Sci. USA* **103**, 15 118–15 123. (doi:10.1073/pnas.0604033103)
- DasGupta, B., Konwar, K. M., Măndoiu, I. I. & Shvartsman, A. A. 2005 DNA-BAR: distinguisher selection for DNA barcoding. *Bioinformatics* **21**, 3424–3426. (doi:10.1093/bioinformatics/bti547)
- Dasmahapatra, K. K. & Mallet, J. 2006 DNA barcodes: recent successes and future prospects. *Heredity* **97**, 254–255. (doi:10.1038/sj.hdy.6800858)
- DeSalle, R., Freedman, T., Prager, E. M. & Wilson, A. C. 1987 Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. *J. Mol. Evol.* **26**, 157–164. (doi:10.1007/BF02111289)
- DeSalle, R., Egan, M. G. & Siddall, M. 2005 The unholy trinity: taxonomy, species delimitation and DNA barcoding. *Phil. Trans. R. Soc. B* **360**, 1905–1916. (doi:10.1098/rstb.2005.1722)
- DeVries, P. J. 1997 *The butterflies of Costa Rica and their natural history*, vol. 1. *Papilionidae, Pieridae, Nymphalidae*. Princeton, NJ: Princeton University Press.
- Ebach, M. C. & Holdrege, C. 2005 DNA barcoding is no substitute for taxonomy. *Nature* **434**, 697–697. (doi:10.1038/434697b)
- Ekrem, T., Willassen, E. & Stur, E. 2007 A comprehensive DNA sequence library is essential for identification with DNA barcodes. *Mol. Phylogenet. Evol.* **43**, 530–542. (doi:10.1016/j.ympev.2006.11.021)
- Elias, M., *et al.* 2007 Phylogenetic hypothesis, pattern of speciation and evolution of wing pattern in neotropical *Napeogenes* butterflies (Lepidoptera: Nymphalidae). In *Seventh Int. Workshop on the Molecular Biology and Genetics of the Lepidoptera 20–26 August 2006, Orthodox Academy of Crete, Kolympari, Crete, Greece*, (eds K. Iatrou & P. Couble), pp. 13–14. *J. Insect Sci.*, **7**, 29.
- 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. B* **274**, 199–207. (doi:10.1098/rspb.2006.3718)
- Guindon, S. & Gascuel, O. 2003 A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**, 696–704. (doi:10.1080/10635150390235520)
- Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W. & Hebert, P. D. N. 2006a DNA barcodes distinguish species of tropical Lepidoptera. *Proc. Natl Acad. Sci. USA* **103**, 968–971. (doi:10.1073/pnas.0510466103)
- Hajibabaei, M., Singer, G. A. C. & Hickey, D. A. 2006b Benchmarking DNA barcodes: an assessment using available primate sequences. *Genome* **49**, 851–854. (doi:10.1139/G06-025)
- 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. (doi:10.1016/j.tig.2007.02.001)
- Hebert, P. D. N. & Gregory, T. R. 2005 The promise of DNA barcoding for taxonomy. *Syst. Biol.* **54**, 852–859. (doi:10.1080/10635150500354886)
- Hebert, P. D. N., Cywinska, A., Ball, S. L. & DeWaard, J. R. 2003a Biological identifications through DNA barcodes. *Proc. R. Soc. B* **270**, 313–321. (doi:10.1098/rspb.2002.2218)
- 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. B* **270**, S96–S99. (doi:10.1098/rsbl.2003.0025)
- 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**, 14 812–14 817. (doi:10.1073/pnas.0406166101)
- 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. (doi:10.1371/journal.pbio.0020312)
- Hickerson, M., Meyer, C. & Moritz, C. 2006 DNA barcoding will often fail to discover new animal species over broad parameter space. *Syst. Biol.* **55**, 729–739. (doi:10.1080/10635150600969898)
- Huelsenbeck, J. P. & Ronquist, F. 2001 MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755. (doi:10.1093/bioinformatics/17.8.754)
- Hurst, G. D. D. & Jiggins, F. M. 2005 Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proc. R. Soc. B* **272**, 1525–1534. (doi:10.1098/rspb.2005.3056)
- Janzen, D. H., Hajibabaei, M., Burns, J. M., Hallwachs, W., Remigio, E. & Hebert, P. D. N. 2005 Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Phil. Trans. R. Soc. B* **1462**, 1835–1846. (doi:10.1098/rstb.2005.1715)
- Jiggins, C. D., Naisbit, R. E., Coe, R. L. & Mallet, J. 2001 Reproductive isolation caused by colour pattern mimicry. *Nature* **411**, 302–305. (doi:10.1038/35077075)
- Jiggins, C. D., Mallarino, R., Willmott, K. W. & Bermingham, E. 2006 The phylogenetic pattern of speciation and wing pattern change in neotropical *Ithomia* butterflies (Lepidoptera; Nymphalidae). *Evolution* **60**, 1454–1466.

- Kelly, R. P., Sarkar, I. N., Eernisse, D. J. & Desalle, R. 2007 DNA barcoding using chitons (genus *Mopalia*). *Mol. Ecol. Notes* 7, 177–183. (doi:10.1111/j.1471-8286.2006.01641.x)
- 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.
- Kumar, S., Tamura, K. & Nei, M. 2004 MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* 5, 150–163. (doi:10.1093/bib/5.2.150)
- Lamas, G. 2004 Ithomiinae. In *Atlas of neotropical Lepidoptera. Checklist: Part 4A. Hesperioidea–Papilionoidea* (ed. J. B. Heppner), pp. 172–191. Gainesville, FL: Association for Tropical Lepidoptera/Scientific Publishers.
- Little, D. P. & Stevenson, D. W. 2007 A comparison of algorithms for the identification of specimens using DNA barcodes: examples from gymnosperms. *Cladistics* 23, 1–21. (doi:10.1111/j.1096-0031.2006.00126.x)
- Maddison, W. P. & Maddison, D. R. 1997 *MACCLADE: analysis of phylogeny and character evolution*. Sunderland, MA: Sinauer Associates.
- Mallarino, R., Bermingham, E., Willmott, K. R., Whinnett, A. & Jiggins, C. D. 2005 Molecular systematics of the butterfly genus *Ithomia* (Lepidoptera: Ithomiinae): a composite phylogenetic hypothesis based on seven genes. *Mol. Phylogenet. Evol.* 34, 625–644. (doi:10.1016/j.ympev.2004.10.021)
- 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 success. *Syst. Biol.* 55, 715–728. (doi:10.1080/10635150600969864)
- Meyer, C. P. & Paulay, G. 2005 DNA barcoding: error rates based on comprehensive sampling. *PLoS Biol.* 3, 2229–2238.
- Monaghan, M. T., Balke, M., Pons, J. & Vogler, A. P. 2006 Beyond barcodes: complex DNA taxonomy of a south pacific island radiation. *Proc. R. Soc. B* 273, 887–893. (doi:10.1098/rspb.2005.3391)
- Müller, F. 1879 Ituna and Thyridia: a remarkable case of mimicry in butterflies. *Trans. Entomol. Soc. London* 1879, xx–xxix.
- Nielsen, R. & Matz, M. 2006 Statistical approaches for DNA barcoding. *Syst. Biol.* 55, 162–169. (doi:10.1080/10635150500431239)
- Nylander, J. A. A. 2004 *MrMODELTEST v2. Program distributed by the author*. Uppsala University, Sweden: Evolutionary Biology Center.
- Pamilo, P. & Nei, M. 1988 Relationships between gene trees and species trees. *Mol. Biol. Evol.* 5, 568–583.
- Ratnasingham, S. & Hebert, P. D. N. 2007 BOLD: the Barcode of Life Data System. *Mol. Ecol. Notes* 7, 355–364. (<http://www.barcodinglife.org>)
- Rubinfoff, D. 2006 Utility of mitochondrial DNA barcodes in species conservation. *Conserv. Biol.* 20, 1026–1033. (doi:10.1111/j.1523-1739.2006.00542.x)
- Rubinfoff, D., Cameron, S. & Will, K. 2006 A genomic perspective on the shortcomings of mitochondrial DNA for “barcoding” identification. *J. Hered.* 97, 581–594. (doi:10.1093/jhered/esl036)
- Sarkar, I. N., Joseph, W. T., Paul, J. P., David, H. F., Bernd, S. & Rob, D. 2002 An automated phylogenetic key for classifying homeoboxes. *Mol. Phylogenet. Evol.* 24, 388–399. (doi:10.1016/S1055-7903(02)00259-2)
- Savolainen, V., Cowan, R. S., Vogler, A. P., Roderick, G. K. & Lane, R. 2005 Towards writing the encyclopaedia of life: an introduction to DNA barcoding. *Phil. Trans. R. Soc. B* 360, 1805–1811. (doi:10.1098/rstb.2005.1730)
- Scheffer, S. J., Lewis, M. L. & Joshi, R. C. 2006 DNA barcoding applied to invasive leafminers (Diptera: Agromyzidae) in The Philippines. *Ann. Entomol. Soc. Am.* 99, 204–210. (doi:10.1603/0013-8746(2006)099[0204:DBATIL]2.0.CO;2)
- Seifert, K. A., Samson, R. A., Dewaard, J. R., Houbbraken, J., Levesque, C. A., Moncalvo, J. M., Louis-Seize, G. & Hebert, P. D. N. 2007 Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. *Proc. Natl Acad. Sci. USA* 104, 3901–3906. (doi:10.1073/pnas.0611691104)
- Smith, M. A., Woodley, N. E., Janzen, D. H., Hallwachs, W. & Hebert, P. D. N. 2006 DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). *Proc. Natl Acad. Sci. USA* 103, 3657–3662. (doi:10.1073/pnas.0511318103)
- Smith, M. A., Wood, D. M., Janzen, D. H., Hallwachs, W. & Hebert, P. D. N. 2007 DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. *Proc. Natl Acad. Sci. USA* 104, 4967–4972. (doi:10.1073/pnas.0700050104)
- van Velzen, R., Bakker, F. T. & van Loon, J. J. A. 2007 DNA barcoding reveals hidden species diversity in Cymothoe (Nymphalidae). *Proc. Neth. Entomol. Soc. Meet.* 18, 95–103.
- Wahlberg, N., Brower, A. V. Z. & Nylin, S. 2005 Phylogenetic relationships and historical biogeography of tribes and genera in the subfamily Nymphalinae (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* 86, 227–251. (doi:10.1111/j.1095-8312.2005.00531.x)
- Wheeler, Q. D. 2005 Losing the plot: DNA “barcodes” and taxonomy. *Cladistics* 21, 405–407. (doi:10.1111/j.1096-0031.2005.00075.x)
- Whinnett, A., Zimmermann, M., Willmott, K. R., Herrera, N., Mallarino, R., Simpson, F., Joron, M., Lamas, G. & Mallet, J. 2005 Strikingly variable divergence times inferred across an Amazonian butterfly ‘suture zone’. *Proc. R. Soc. B* 272, 2525–2533. (doi:10.1098/rspb.2005.3247)
- 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. B* 274, 1731–1739. (doi:10.1098/rspb.2007.0062)
- Wiemers, M. & Fiedler, K. 2007 Does the DNA barcoding gap exist?—a case study in blue butterflies (Lepidoptera: Lycaenidae). *Front. Zool.* 4, 8. (doi:10.1186/1742-9994-4-8)
- Will, K. W. & Rubinoff, D. 2004 Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics* 20, 47–55. (doi:10.1111/j.1096-0031.2003.00008.x)
- 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. (doi:10.1080/10635150500354878)
- Willmott, K. R. & Freitas, A. V. L. 2006 Higher-level phylogeny of the *Ithomiinae* (Lepidoptera: Nymphalidae): classification, patterns of larval hostplant colonization and diversification. *Cladistics* 22, 297–368. (doi:10.1111/j.1096-0031.2006.00108.x)