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**RESPONSE TO COMMENTARY BY MORITZ AND CICERO  
ON**

Hebert PDN, Stoeckle MY, Zemplak TS, Francis CM 2004  
*Identification of birds through DNA barcodes*. PLoS 2(10): e312

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We are pleased that the editors of PLoS Biology have highlighted our study by inviting a 2000-word commentary (Moritz and Cicero 2004) on our 3500-word paper (Hebert et al 2004). Importantly, we concur with Moritz and Cicero's statement in their final paragraph, namely:

*“There is little doubt that large-scale and standardized sequencing, when integrated with existing taxonomic practice, can contribute significantly to the challenges of identifying individuals and increasing the rate of discovering biological diversity.”*

In fact, we agree with much of their commentary, particularly as many of their apparent objections are re-statements of observations made in our paper. These include the “need to examine groups with frequent hybridization [and] recent radiations”, and tropical taxa; that a standard sequence threshold will overlook recently diverged species; the importance of “taxonomically extensive analyses that extend beyond the focal geographic region and include potential sister taxa”; and the necessity of multiple lines of taxonomic evidence in addition to genetic data in determining species boundaries. Each of these important issues is discussed in our original paper.

Leaving aside many areas of agreement, where do we differ?

1. *“There is nothing fundamentally new in the DNA barcoding concept, except increased scale and proposed standardization.”*

If there was nothing new in the concept, DNA barcoding would not have attracted the intense, albeit misplaced, criticism from some biologists that Moritz and Cicero describe (and the editors would not have invited their commentary). DNA barcoding is based on the remarkable observation that a short DNA sequence from a uniform locality on the genome enables the identification of diverse animal species, ranging from birds to bees and from crustaceans to cetaceans. By establishing a public library of sequences linked to named specimens, a large-scale DNA barcoding effort will create an entirely new scientific resource. The DNA barcode library will facilitate a wide variety of studies that are not possible with current taxonomic practice, including identifying species in all life stages, recognizing species from bits and pieces, and species identification by non-specialists.

2. “[T]he very term DNA barcoding is unfortunate, as it implies that each species has a fixed and invariant characteristic—like a BarCode on a supermarket product”

Any short description involves simplification. **DNA fingerprinting** has gained wide usage to describe the sequence profiles that distinguish individuals, despite that fact that it involves a protocol radically divergent from ink-stamping digits. The metaphorical **Tree of Life**, cited by Moritz and Cicero, is conceptually engaging, but evolutionary trees bear little physical resemblance to the arboreal namesakes. In a similar fashion, **DNA barcodes** may differ from those in the retail sector, but this term easily conveys the essential nature of the approach: a rapid, standardized, potentially automatable method for identification. Most people recognize that there is variation within species (different humans rarely look alike; not all sheep are clones of Dolly); there is little danger that DNA barcoding will erase this understanding.

3. “Potential limitations of using mtDNA to infer species boundaries include retention of ancestral polymorphism, male-biased gene flow, selection on any mtDNA nucleotide (as the whole genome is one linkage group), introgression following hybridization, and paralogy resulting from transfer of mtDNA gene copies to the nucleus.”

Our data indicate that these theoretical limitations are unlikely to seriously restrict application of a COI barcode to identify birds. First, most paralogous mitochondrial genes (Numts) are shorter than the barcode amplicon and the few exceptions regularly possess diagnostic nucleotide attributes. Second, introgression following hybridization, although it may confound the species tree, will not interfere with identification unless it has been so recent that species share congruent sequences. As all 260 species that we examined had different COI barcode(s), it appears that this is uncommon in North American birds. Third, although the retention of ancestral polymorphisms can result in paraphyly in the species tree (some individuals more closely related to another species than to other members of their own species), this was not observed among the 130 species in which we sampled multiple individuals. We conclude that this is unlikely to be a major limitation. Additionally, if a DNA barcode library includes the relevant paraphyletic sequences, paraphyly will not necessarily limit species identification. Male-biased gene flow and selection on mtDNA nucleotides can confound deriving phylogeny from mtDNA, but they do not affect the recognition of species. Finally, as emphasized by our results and earlier DNA barcoding studies, there is a dearth of deeply ancestral polymorphisms that might complicate species discrimination.

4. “Although Hebert et al. (2004) sampled a large number of species, a true test of the precision of mtDNA barcodes to assign individuals to species would include comparisons with sister species—the most closely related extant relatives.”

The definition of sister species, as noted by Moritz and Cicero (2004), is a species and its closest living relative. However, there is no general method for identifying sister species. For example, the Osprey (*Pandion haliaetus*) is the only member of its genus; its sister species must be among the hawks, but which one? In practice, the term is commonly used to refer only to pairs of very closely related species. The important point is that closely related sister species are the exception among birds; Moritz and Cicero’s analysis is restricted to this exceptional subset and is thus misleading about birds in general. For example, although they determined mtDNA sequences of

all 15 *Empidonax* flycatchers, they designated only the 4 most closely related pairs (out of 105 possible comparisons) as sister species (Johnson and Cicero 2002, 2004; Moritz and Cicero 2004). Furthermore, as they note, *Empidonax* is an exceptional avian genus comprised of a recent radiation of species showing close morphological congruence. Even within this genus, when all 15 species are considered, the average minimum interspecific p-distance is 4.5% (range 0.7 to 10.3%) and 11 of the 15 (73%) have minimum interspecific distances greater than 2.7% (Johnson and Cicero 2004). As well, all species possess different mtDNA sequences that form monophyletic lineages without paraphyly. In their review of sister species, Johnson and Cicero (2004) used mtDNA distances to determine relatedness within avian genera, and then restricted their analysis to pairs with low divergence. Hence their finding of low divergences among the 39 sister species pairs is not only unsurprising, but essentially circular. The critical issue lies in ascertaining the incidence of such closely-related sister species pairs, not in demonstrating their existence. Our findings are likely to be more representative of birds in general, as we sampled a large number of species in a variety of orders. We expect that additional data will support our finding of large genetic distances between most avian species.

5. *“However, [Hebert et al.’s (2004)] analysis makes the assumption that there is a common underlying pattern of phylogeographic structure, which is unlikely for North American birds (Zink 1996, Zink et al. 2001)”*

Of course, there is not a common phylogenetic structure in North American birds. It is therefore significant that we sampled 130 different species, using specimens collected from distant sites (average 1080 km apart). From this sample we made two observations. First, that intraspecific genetic distances were mostly extremely low (excluding polytypic species, average K2P distance 0.27%). This finding alone indicates that large intra-specific divergences in North American birds are uncommon. Second, we observed no relationship between intra-specific genetic distances and geographic distance, further supporting the absence of high levels of intra-specific variation in North American birds. It is surprising that Moritz and Cicero (2004) cite Zink et al. (1996, 2001) in support of their argument, as other papers by these authors propose that the bird ‘species’ where they found large intra-specific mtDNA divergences should actually be split into two or more species (Zink et al 2000; Zink and Weckstein 2003).

In conclusion, we believe that our paper is one step toward “large-scale and standardized sequencing...integrated with existing taxonomic practice”, i.e. a DNA barcode system for all animal and plant life. An international effort to achieve this goal is already underway. The Consortium for the Barcode of Life (CBOL) recently (May 24-25, 2004) held its inaugural meeting at the Smithsonian Institution in Washington DC ( <http://www.barcoding.si.edu/> ). CBOL is an international collaboration of natural history museums and herbaria together with academic and commercial experts in genomics, electronics, taxonomy, and computer science. CBOL’s mission is to both rapidly compile DNA barcodes of known and newly discovered species and to promote the development of portable devices for DNA barcoding. We hope that our work on birds will encourage diverse persons to join this effort and, in the process, answer the scientific questions raised by our paper and by Moritz and Cicero’s commentary.

## REFERENCES

- Hebert PDN, Stoeckle MY, Zemplak TS, Francis CM (2004) Identification of birds through DNA barcodes. *PLoS Biology* 2(10) e312.
- Johnson NK, Cicero C (2002) The role of ecologic diversification in sibling speciation of *Empidonax* flycatchers (Tyrannidae): multigene evidence from mtDNA. *Molecular Ecology* 11: 2065-2081.
- Johnson NK, Cicero C (2004) New mitochondrial DNA data affirm the importance of Pleistocene speciation in North American birds. *Evolution* 58: 1122-1130.
- Moritz C, Cicero C (2004) DNA barcoding: promise and pitfalls. *PLoS Biology* 2(10): in press.
- Zink RM 1996. Comparative phylogeography in North American birds. *Evolution* 50:308-317.
- Zink RM, Blackwell-Rago, Rachelle C (2000) Species limits and recent population history in the Curve-Billed Thrasher. *The Condor* 102: 881-886.
- Zink RM, Kessen AE, Line TV, and Blackwell-Rago RC (2001) Comparative phylogeography of some aridland bird species. *Condor* 103:1-11.
- Zink RM, Weckstein JD (2003) Recent evolutionary history of the Fox Sparrows (Genus: *Passerella*). *The Auk* 120: 522-527