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INVITED TECHNICAL REVIEW

An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding

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Abstract

DNA barcoding has become a well-funded, global enterprise since its proposition as a technique for species identification, delimitation and discovery in 2003. However, the rapid development of next generation sequencing (NGS) has the potential to render DNA barcoding irrelevant because of the speed with which it generates large volumes of genomic data. To avoid obsolescence, the DNA barcoding movement must adapt to use this new technology. This review examines the DNA barcoding enterprise, its continued resistance to improvement and the implications of this on the future of the discipline. We present the consistent failure of DNA barcoding to recognize its limitations and evolve its methodologies, reducing the usefulness of the data produced by the movement and throwing into doubt its ability to embrace NGS.

Keywords: barcoding, genetic diagnostics, genetic distance, next generation sequencing

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The rise of DNA barcoding

In 2003, Paul Hebert *et al.* from the University of Guelph, Ontario, Canada, published a paper in the Proceedings of the Royal Society stating that the mitochondrial gene COI could serve as a genetic barcode for all animal life (Hebert *et al.* 2003a). This was not the first time that DNA barcoding had been proposed as a concept. Using short DNA sequences to discriminate amongst microbial species was proposed as early as 1982 (Nanney 1982) and had subsequently been tested on a variety of taxa from nematodes to elephants and even that most famous of extinct species, the dodo (Eggert *et al.* 2002; Floyd *et al.* 2002; Shapiro *et al.* 2002).

Hebert *et al.* (2003a,b) went further than previous studies, however, proposing a single gene barcode locus as a silver bullet to identify species across the whole animal kingdom. More than that, the DNA barcoding system promised a better taxonomic resolution than that which could be achieved through morphological studies and a partial solution to the decline in traditional taxonomic knowledge. Thus, DNA barcoding was proposed as a tool not only to identify species but also to define species boundaries and aid in species delimitation (Hebert *et al.* 2003a).

This promise to revolutionize taxonomy and species discovery received a mixed reception (Blaxter 2003; Janzen 2004; Prendini 2005). Nevertheless, today DNA barcoding is a global enterprise (Box 1), attracting large amounts of funding and operating three international websites encompassing a raft of partner organizations from around the world. The implementation of the idea has seen a rapid rise in that time (Fig. 1), and publications on the topic have been numerous with 411 papers published that mention DNA barcoding in their titles between 2003 and 2010 (Box 2). In its role as a species identifier, DNA barcoding has been used to tackle a wide variety of problems, from resolving adult and larval stages within species (Gossner & Hausmann 2009) to policing fish for sale in supermarkets (Rasmussen *et al.* 2009).

The practicalities of a universal barcode for all life have proved problematic, however, (Vences *et al.* 2005; Rubinoff *et al.* 2006; Eberhardt 2010) and the distance-based methodologies employed in many barcoding studies have been repeatedly criticized (DeSalle *et al.* 2005; Kelly *et al.* 2007). It also remains unclear whether the usefulness of DNA barcoding is restricted to species identification or if it is a general tool that can be used for species discovery and delimitation (Rubinoff 2006a).

More pertinently, although the concept and application of DNA barcoding are simple, there is, as yet, no solid system in place to deal effectively with the enormous volumes of data this field is generating in its attempts to create a genetic reference library (Lucking 2008; Packer *et al.* 2009). The advent of high-throughput next generation sequencing (NGS) technology is already

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Box 1: The main DNA barcoding bodies and resources

Consortium for the Barcode of Life (CBOL)
http://www.barcodeoflife.org
Established in 2004, CBOL promotes DNA barcoding through over 200 Member Organizations from 50 countries. Operates out of the Smithsonian Institution's National Museum of Natural History in Washington and is chaired by Dr. Scott E. Miller of the SI.

International Barcode of Life (iBOL)
http://www.ibol.org
Launched in October 2010, iBOL represents a not-for-profit effort to involve both developing and developed countries in the global barcoding effort, establishing commitments and working groups in 25 countries. The Biodiversity Institute of Ontario is the project's scientific hub and its director, Dr Paul Hebert, is also the scientific director of iBOL.

The Barcode of Life Datasystems (BOLD)
http://www.boldsystems.org
The Barcode of Life Datasystems is an online workbench for DNA barcoders. Combines a barcode repository, analytical tools, interface for submission of sequences to GenBank, a species identification tool and connectivity for external web developers and bioinformaticians. Established in 2005 by the Biodiversity Institute of Ontario.

ushering in a new era for molecular ecology (Ellegren 2008) by making rapid access to fully sequenced genomes a distinct possibility. With such technology becoming increasingly accessible (Kircher & Kelso 2010), this availability of data from the entire genome has obvious implications for surrogate measures such as DNA barcoding and it will be important for its proponents to find a way to evolve their methods to encompass these new developments.

The aim of this review is to examine the progress made thus far by the still relatively new field of DNA barcoding. In doing so, this review also considers the capacity and the motivation of the DNA barcoding movement to evolve in response to the challenges of its critics and explores whether it has the potential to meet the decidedly heavyweight challenge of obsolescence posed by NGS technology.

A taxonomic bias in barcoding?

To date, there is a taxonomic bias in barcoding (Box 3 and Table 1). Naturally, certain taxa benefit more from barcoding than others. The largest proportion of DNA barcoding work has been conducted with arthropods (Table 1). This is perhaps unsurprising as our taxonomic knowledge of the species within this exceptionally diverse group is considered particularly inadequate (May & Harvey 2009). Additionally, a major role of DNA barcoding is seen as accurately identifying larval stages in species for which this has proved difficult historically such as mayflies (Ball *et al.* 2005), butterflies (Gossner & Hausmann 2009) and stomatopods (Barber & Boyce 2006), identifying tiny organisms that require microscopic examination for morphological characteristics such as zooplankton (Bucklin *et al.* 2007; Elias-Gutierrez *et al.* 2008) or that have a variety of life-stages such as aphids (Foottit *et al.* 2009). Clearly, arthropod taxonomy is a field that stands to benefit from the added clarity promised by DNA barcoding.

Commercially important species are also well represented in barcoding studies. The fact that fish were the third most studied class for DNA barcoding in this review (Table 1) can partly be explained by this being a relatively diverse group with significant morphological variation during development, making DNA barcoding an attractive approach (Hubert *et al.* 2008). More importantly, DNA barcoding has been employed in marketplace seafood authentication (Wong & Hanner 2008; Rasmussen *et al.* 2009; Barbuto *et al.* 2010) and in uncovering species-specific contaminants in fish sold as food that may pose a health risk to humans (Lowenstein *et al.* 2010).

Conversely, mammals have not been a major focus for DNA barcoding to date (Table 1), possibly due to the general feeling that mammal taxonomy is better studied and understood than that of other taxa (Wilson & Reeder 2005). Barcoding work on mammals has tended to centre on less readily identifiable groups such as bats (Clare *et al.* 2007) and opossums and rodents (Borisenko *et al.* 2008). The relatively small number of studies on birds recorded in this review (Table 1) is perhaps misleading as several of the studies conducted represent aggregations of very large numbers of bird species barcodes (Hebert *et al.* 2004b; Yoo *et al.* 2006; Kerr *et al.* 2007, 2009).

Taxonomic biases of one form or another are known to narrow the scope of both ecological and conservation research to particular taxa (Bonnet *et al.* 2002; Clark & May 2002) and occur in taxonomic research as well (Tautz *et al.* 2003). Most DNA barcoding to date has been focussed on animals (Table 1 and Box 3). Fungi are

Box 2: Review method and results summary

A simple literature search was conducted on Web of Science for all articles containing the phrase 'DNA barcod*' in the title of the paper. The asterisk was used to enable the return of results containing the words barcode, barcodes, barcoder, barcoders and barcoding. As no papers prior to 2003 featured the phrase 'DNA barcod*' in the title, the literature search was solely focussed on the period between 2003 and November 2010.

Meeting abstracts and posters were discarded from the search results, but book chapters were retained where the contents of the chapter could be obtained either via a hard copy or electronically.

Individual articles were searched for several categories of information:

1 Year published.
2 Taxa covered (if relevant).
3 DNA sequence region used as barcode (if relevant).
4 Data analysis methods employed (if relevant).

For articles in journals that were not open access or for which Manchester Metropolitan University did not hold a subscription, an email was sent to the correspondence author to request a copy. Where no response was received, any relevant information that could be extracted from the abstract was included in the analysis and the paper excluded from all analyses for which no information was available.

The initial search produced 525 hits that, after de-duplication and removal of meeting abstracts etc.… were reduced to 411 published articles. Of these 411:

1 321 focussed on a particular or several taxa rather than being more general reviews of the topic 'DNA barcod*'.
2 296 were reports of the practical application of one or more DNA barcodes.
3 222 involved analysis of genetic barcode sequences (rather than simply a recording of the barcode).

As different numbers of papers were involved in different analyses for the review, each figure is labelled with the sample size for that statistic.

It is appreciated that many papers on the topic of DNA barcoding do not feature the specific phrase 'DNA barcod*' in their title. A Web of Science search of papers containing 'DNA barcod*' in the topic field returned 1192 results dating back to 1993. However, this wider search included a high number of irrelevant papers and the intention of this review is to provide a snapshot of the DNA barcoding landscape post Hebert *et al.* (2003a). It was felt that the most efficient way to proceed would be to limit the data search to those with 'DNA barcod*' in the title, but take care to refer to relevant papers that fell outside of this search within the body of the review (see References section).

The major results of the literature review are summarized graphically below.

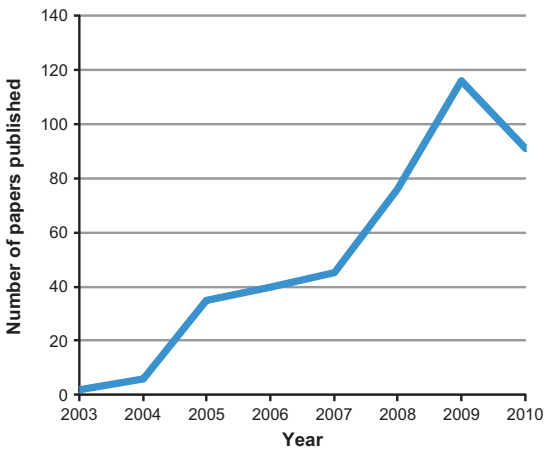


Fig 1. Number of papers published with 'DNA barcod*' in the title (N = 411).

Table 1 Taxa covered by papers with DNA Barcod* in the title

Taxa (with kingdom animalia broken down into classes)	*N*	(%)
Invertebrates	186	(53.14)
Plants	55	(15.71)
Fish	37	(10.57)
Birds	18	(5.14)
Protists	18	(5.14)
Fungi	14	(4.00)
Mammals	9	(2.57)
Amphibians	8	(2.29)
Reptiles	5	(1.43)

N = 321 (NB: some papers covered more than one taxon).

Box 2: Continued

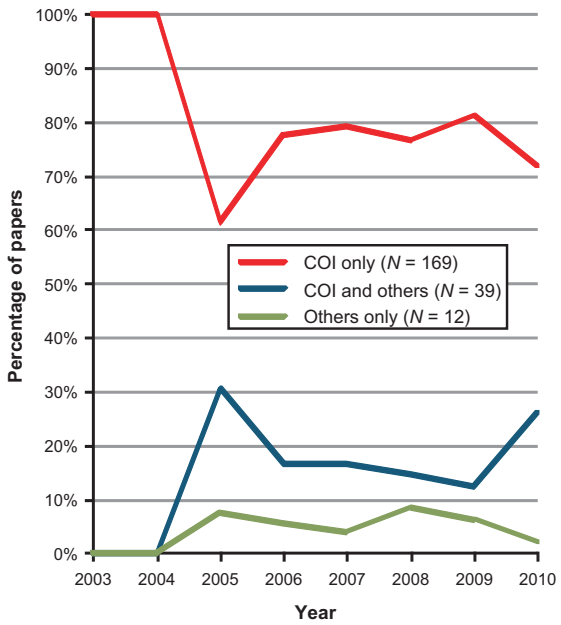


Fig 2. Choice of barcode in 'DNA barcod*' papers—animal studies only (N = 220).

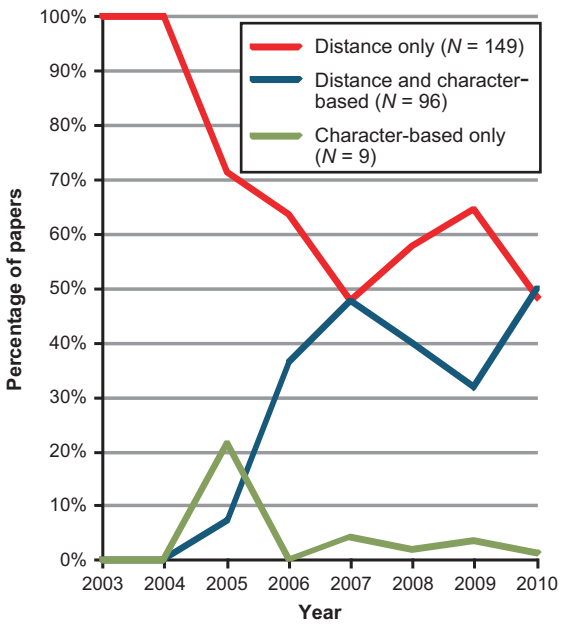


Fig 3. Change in use of distance and character-based methods in 'DNA barcod*' papers over time (N = 254).

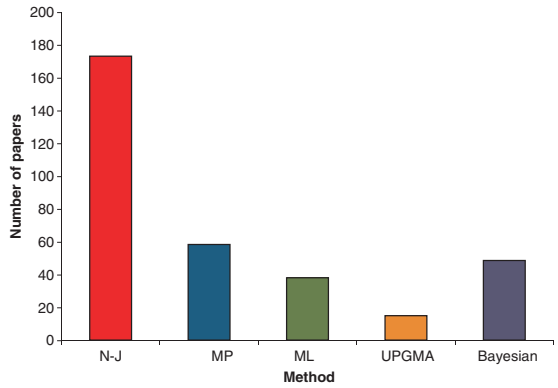


Fig 4. Tree building methods employed in 'DNA barcod*' papers (N = 220) (NB: Some papers employed more than one tree building method).

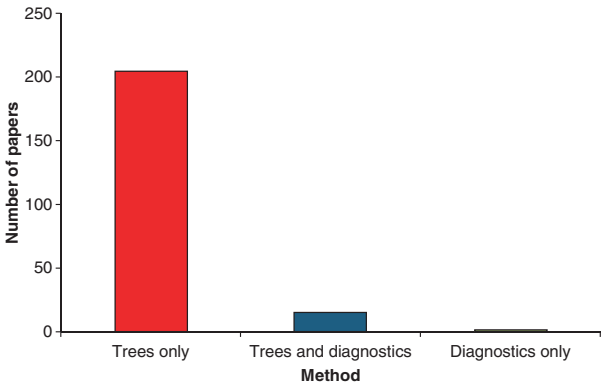


Fig 5. Prevalence of tree building in 'DNA barcod*' papers (N = 222).

thought to be ranked second only to insects in terms of diversity (Webster 1997), yet this group accounts for only 4% of the DNA barcoding work surveyed here (Table 1) and <1% of the species recorded on BOLD (Box 3). Plants and protists too are severely under-represented relative to their taxonomic abundance (Box 3). Yet taxonomic bias alone is not to blame for this imbalance; finding a universal barcode for all life has proved more difficult than previously supposed.

COI—one for all or one for some?

With their 2003 paper, Hebert *et al.* argued strongly for COI as the best candidate for a universal barcode for ani-

mal life. The authors cited the availability of robust universal primers for this sequence, its relatively high rate of molecular evolution and lack of insertions or deletion mutations relative to ribosomal sequences such as 12S and 16S as excellent qualifications for the role of 'universal barcode' (Hebert *et al.* 2003a). Their study demonstrated the promise of this sequence in animals and, following concerns regarding sequence differences between closely related species (Mallet & Willmott 2003), Hebert *et al.* (2003b) went on to demonstrate sufficient divergence of COI for discrimination of closely related species across all animal phyla except the cnidarians.

Subsequently, there have been consistent problems with using COI as a barcode in amphibians, where high variability in the group's mitochondrial DNA and its COI priming sites has made DNA barcoding using this sequence particularly challenging (Vences *et al.* 2005). Although progress has been made in this area, there is a definite need for more research to ensure future success (Smith *et al.* 2008).

The existence of sufficient divergence between related species in the COI sequence (the so called 'barcoding gap') remains an issue for COI as a universal barcode locus (Wiemers & Fiedler 2007) and even the latest methods of barcode gap discovery cannot overcome situations where no gap is present (Puillandre *et al.* 2011). This has led to a slight rise over time in the number of studies employing or testing other barcode loci alongside COI or choosing different sequences entirely to identify animals (Fig. 2), despite the original idea of barcoding being to rely on a single sequence to identify all species (Hebert *et al.* 2003a). Some studies have defined DNA barcoding exclusively as the use of the COI region to identify species (Neigel *et al.* 2007; Ward *et al.* 2008; Holmes *et al.* 2009), and COI has even been termed 'the barcoding gene' (Dove *et al.* 2008). Given the persistent problems with this locus in some animal groups, it would seem that more caution should be used when employing these kinds of generalizations, at least for the time being.

While COI as a universal DNA barcode in animals still faces challenges, it is relatively robust when compared with its utility in protists, fungi and, in particular, plants. There has been some success in barcoding with COI in protists, especially red algae (Robba *et al.* 2006; Saunders 2008) and brown algae (Lane *et al.* 2007; Kucera & Saunders 2008; McDevit & Saunders 2009). However, problems have been encountered with the primers for COI in algae, and the universal plastid amplicon (UPA) has been suggested as an alternative, although it has the drawback of low interspecific divergence (Clarkston & Saunders 2010). Anaerobic protists such as *Blastocystis* have no mitochondria and therefore no COI gene; instead, small subunit ribosomal genes have been trialled here with some success (Scicluna *et al.* 2006). Other protist studies appear to elect to use an alternative barcode sequence purely because of the bad press received by COI (Gile *et al.* 2010).

Short-sequence identification work in fungi pre-dates the current research in DNA barcoding by at least a decade (Bruns *et al.* 1991), yet issues persist in the search for a universal barcode for this group (Eberhardt 2010). COI has been found to produce disappointing results in many fungal taxa, leading to the proposition of the internal transcribed spacer (ITS) nuclear ribosomal sequence as more appropriate barcode for fungi (Seifert 2009). However, problems with primers for ITS sequences have been reported (Bellemain *et al.* 2010) and ITS performs poorly in assigning species-specific barcodes in *Penicillium* moulds (Seifert *et al.* 2007). Thus, no single universally informative locus has emerged for fungal groups and research into the topic is on-going (Eberhardt 2010). Although there are currently around 70 000 ITS sequences on GenBank, these have yet to be verified and incorporated into BOLD (Seifert 2009), contributing further to this kingdom's underrepresentation in barcoding (Box 3 and Table 1).

Barcoding in plants is even more complex than that in the taxa discussed earlier, with COI having been found to be ineffectual because of its low rate of evolution in this kingdom and consequent low divergence levels (Kress & Erickson 2007). No less than 41 different loci have been trialled in the search for a universal plant barcode in the studies recorded in this review alone. Thus, the majority of research in plants has been devoted to identifying a suitable barcode rather than collecting barcoding data for submission to BOLD, explaining the under representation of this group in its database (Box 3). This continuing search has been likened to that for the Holy Grail (Rubinoff *et al.* 2006), an appropriate metaphor given that the latest recommendation from the Consortium for the Barcode of Life (CBOL) is that two sequences (the chloroplast genes rbcL and mat K) be used in tandem as a plant barcode (Hollingsworth *et al.* 2009). Even that approach is still in review (Hollingsworth *et al.* 2011). For plants, at least, the search for a single barcode locus looks set to remain active for the foreseeable future.

Clearly, there is still much to be resolved regarding the universal barcodes for the major groups of eukaryotes. Given that a standard marker region is a fulcrum of the DNA barcoding concept, the resolution of this problem would seem vital to the success of barcoding life on earth. However, this is not the only area of DNA barcoding up for debate, with other aspects of the methodology attracting even stronger criticism from some quarters.

Distance vs. diagnostics

Hebert *et al.* (2003a,b) proposed the use of genetic distance as a standard method of analysis of barcode data,

Box 3: A bias in barcoding on bold

The Consortium for the Barcode of Life (CBOL) states on its website that its mission is to 'promote the exploration and development of DNA barcoding as a global standard for species identification' (http://www.barcodeoflife.org). Implicit in this statement is the fact that DNA barcoding should be applicable and applied to all species.

To establish whether this is, indeed, the case, we reviewed the Barcode of Life Database (BOLD) website's specimen record page (as of 26 September 2011) and calculated the number of barcoded specimens recorded for each eukaryotic kingdom. We then compared these numbers with the proportions suggested by recent research on the global species count (Mora *et al.* 2011). The kingdoms 'chromista' and 'protozoa' listed by Mora *et al.* were combined into 'protists' for the purposes of this analysis as the BOLD website does not currently differentiate further than 'protists'.

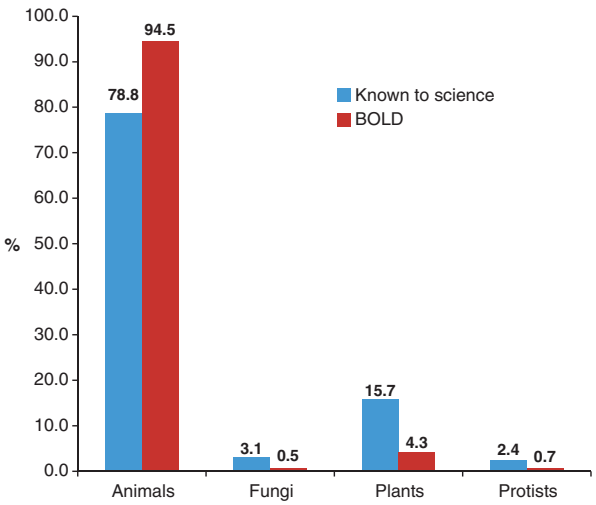


Fig 6. Comparison of the proportions of species in each eukaryotic kingdom currently known to science (from Mora *et al.* 2011) with the specimens in each kingdom listed on the BOLD website.

At first glance, the proportion of animal specimens recorded on BOLD relative to the other three kingdoms appeared substantially higher than that predicted by the number of animal species currently known to science, while the proportions of fungi, plants and protists all appeared lower (Fig. 6).

A chi-squared test of goodness of fit confirmed that there was a significant difference between the frequencies of species in each kingdom predicted by the proportions currently known to science and the number of specimens in each kingdom recorded on the BOLD website ($\chi^2 = 273314.57$, $P < 0.001$). The greatest part of this difference resulted from the discrepancy for plants, with the bias in favor of animals on BOLD being the second biggest contributing factor. Potential reasons for these differences are discussed in the main text.

and the majority of barcoding studies have followed suit (Fig. 3). The use of distances relies on the assumption that intraspecific divergences will be less than interspecific divergences (Meyer & Paulay 2005) and the idea of a 'divergence threshold'—the aforementioned 'barcoding gap' that is been called into question repeatedly in the literature (Meyer & Paulay 2005; Gompert *et al.* 2006; Stribling 2006; Wiemers & Fiedler 2007). It has also been noted that species-level divergence cut-off values can be fairly arbitrary (DeSalle *et al.* 2005; Prendini 2005; Rubinoff 2006b; Vogler 2006), although recent efforts have been made to overcome this problem (Puillandre *et al.* 2011).

Overriding all of these criticisms, however, is the notion that distance should not be used at all in DNA barcoding as it is a phenetic measure that is incompatible with the diagnostic, character-based techniques used by traditional taxonomists (DeSalle *et al.* 2005). DeSalle's continued (yet measured) criticism of the DNA barcoding movement *sensu* Hebert has centred on the argument that barcoding should never be based on genetic distances but, instead, on diagnostic genetic characters (Box 4).

Character-based analysis has proven to be an effective tool for species identification and discrimination (Kelly *et al.* 2007; Rach *et al.* 2008; Bergmann *et al.* 2009; Lowenstein *et al.* 2009), and Characteristic Attributes Organization System (CAOS) software has been developed to facilitate its implementation (Sarkar *et al.* 2008). Character-based analysis schemes seem more analogous to the idea of a barcode than their distance-based counterparts. Indeed, it is far easier to imagine the frequently predicted Star Trek tricorder-style handheld barcoding device (Savolainen *et al.* 2005) based on a diagnostic character string than a distance-based comparison. Character string analysis offers an objective yes or no rather than a relatively subjective continuum of maybes, with no loss of information because of multiple nucleotide differences being reduced to a single distance-based measure. In spite of this, to date, character-based diagnostics have

failed to break into the mainstream of DNA barcoding (Fig. 3). Since DeSalle *et al.*'s 2005 paper, some studies have started to incorporate character-based methodologies into their analysis (Fig. 3), but these are often still tree-based, and very few barcoding studies focus solely on a character-based diagnostic approach, demonstrating an unfortunate resistance to the adoption of new (and arguably improved) methodologies by the barcoding community.

This bias is probably due to the methods employed in the original cohort of DNA barcoding papers being replicated by later studies (DeSalle 2007) and the fact that these are the methods supported by the analytical tools on the BOLD website. A character-based tool (BLOG) has been developed for use with BOLD but currently resides on the Barcode of Life Data Portal (BDP) rather than the main BOLD website (Sarkar & Trizna 2011). The current popular methods, then, may be a product of routine rather than informed choice (Casiraghi *et al.* 2010). This suggests an opportunity for systematic appraisal analysis methods both within and between taxa that has, thus far, not been capitalized on by the barcoding movement. Unfortunately, this rejection of constructive criticism and aversion to new methods is a running theme in the DNA barcoding literature.

Branching out: the role of DNA barcoding

Some DNA barcoding studies have attempted to build character-based analysis into their studies by employing maximum parsimony, maximum likelihood or Bayesian trees in tandem with or instead of the more widely used distance-based neighbour joining trees (Fig. 4), sometimes using DeSalle *et al.*'s 2005 paper as justification for this (Locke *et al.* 2010). However, DeSalle *et al.*'s criticism of current DNA barcoding methods did not stop at distances vs. diagnostics. They also proposed avoiding any tree-building analyses, even character-based ones. Species delimitation via phylogenetic reconstruction is a difficult concept for a number of reasons (Goldstein & DeSalle 2011), particularly in the face of incomplete sampling (Will & Rubinoff 2004) and the assumption of monophyly (Zhang *et al.* 2011). Moreover, barcoding via tree-based methods gives the impression of inferring phylogenies and relationships from single gene trees (DeSalle *et al.* 2005), now widely recognized as a problem by phylogeneticists.

This last point encapsulates a continuing debate regarding DNA barcoding: what it can and should be used for. In particular, there has been repeated confusion in the literature of two terms: 'DNA barcoding' and 'DNA taxonomy' (DeSalle 2007). DNA barcoding *sensu stricto* is designed purely to aid the recognition and identification of known species (Valentini *et al.* 2009; Casiraghi *et al.* 2010). Thus, despite its current limitations, the barcoding approach provides a framework for the survey of biodiversity—a crucial task for prioritising conservation efforts given the current extinction crisis.

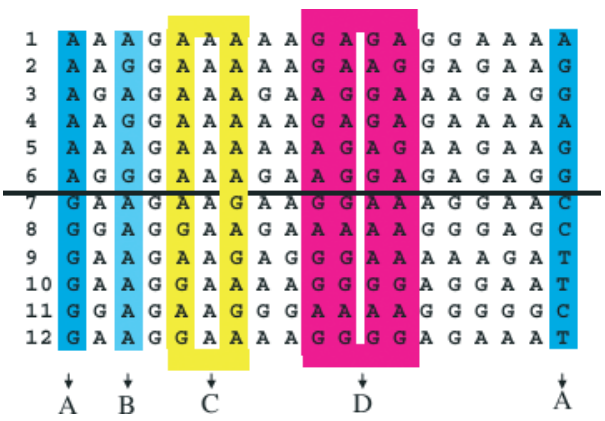
However, DNA barcoding has also been seized upon as a method for both the discovery of new species (Hebert

Box 4: Character-based diagnostics

Suggested as an alternative to more frequently used, distance-based methods, diagnostic barcoding approaches involve identifying species via individual characteristic differences between sequences.

This diagram (from DeSalle *et al.* 2005) demonstrates a hypothetical example of character-based diagnostics.

The solid line divides two populations, with six individuals in each. Several different kinds of diagnostic characters are highlighted:



A—Single pure character attributes—purely diagnostic (*sensu* Davis and Nixon 1992).

B—Single private character attributes—here, the G in the top population is private to that population.

C—Compound pure character attributes—two private diagnostic positions which, when taken together provide the pure diagnostic of AA vs. AG/GA (Sarkar *et al.* 2002).

D—Compound private character attributes—these columns are neither diagnostic nor private when viewed in isolation, but together, they provide a means of diagnosing the top population from the bottom one as the top one always contains combinations of GA, AG and the bottom population GG, AA.

et al. 2004a; Witt *et al.* 2006; Gomez *et al.* 2007; Johnson *et al.* 2008; Moura *et al.* 2008; deWaard *et al.* 2009; Ragupathy *et al.* 2009; Pauls *et al.* 2010) and revising taxonomies (DNA taxonomy) (Blaxter *et al.* 2005; Vogler & Monaghan 2007). It is these uses of DNA barcoding that cause the most concern for some critics. They contend that it would be naïve to describe a new species or infer a phylogeny without any corroborating evidence other than a single locus DNA sequence (DeSalle 2006) and that barcoding should supplement morphological data for species description rather than replace it (Prendini 2005). In the absence of other evidence, DNA barcoding creates hypotheses regarding new species rather than outright discovering them (Goldstein & DeSalle 2011). Indeed, it has been pointed out that the discovery of species using a method designed to barcode all described species is a somewhat tautological concept (Forister *et al.* 2008). It should be noted too that even a character-based methodology such as that suggested by DeSalle *et al.* (2005) would struggle to perform in the face of cryptic species.

Recently, the use of DNA barcoding alone to discover new species has been tempered by some to it being used as a tool to *speed* species discovery (Kress & Erickson 2008) or to 'flag' rather than discover new species (Hajibabaei *et al.* 2011) and species discovery itself receives little mention on the CBOL, International Barcode of Life (iBOL) or BOLD websites. Still, the fact that most DNA barcoding studies to date have remained stubbornly attached to tree-based analytical methods (Fig. 5) (another legacy of the initial cohort of DNA barcoding publications and the methods promoted by BOLD, perhaps) could mean that the confusion and debate over DNA barcoding vs. DNA taxonomy continues for the foreseeable future. Thus, a dogmatic adherence to one particular formula stands in the way of improvements to the initiative.

Valuable reference library or expensive sequence dump?

In essence, DNA barcoding is a method of identifying previously described taxa (Rubinoff 2006a). Reference sequences lie at the very heart of the DNA barcoding initiative (Meyer & Paulay 2005; Begerow *et al.* 2010; Zhang *et al.* 2011). Without verified reference sequences from voucher specimens that have been authenticated by qualified taxonomists, there is no reliable library for newly generated query sequences to be compared with. These voucher specimens also allow the replication of results, making DNA barcoding a proper scientific discipline (Peterson *et al.* 2007). This illustrates the potential for DNA barcoding to be a well-funded imperative for the collection and storage of voucher specimens and/or tissue samples for biodiversity research.

At the time of writing, there were 145 298 formally described species with barcodes on the BOLD database. While this may sound impressive, there were 1 493 132 barcode records uploaded in total, indicating that the number of barcodes being produced is outstripping the number verified by an order of magnitude. As mentioned earlier, for fungi alone, there is a huge amount of 'legacy' sequence data currently stored on GenBank for which there are no voucher specimens, effectively excluding these sequences from use as barcodes (Seifert 2009). Thus, the speed of cataloguing life on earth has arguably not been improved by DNA barcoding (Hajibabaei *et al.* 2011). By admission of its main promoters, it is the front-end curation and verification part of the barcode-processing pipeline that cause bottlenecks rather than the molecular analysis (Borisenko *et al.* 2009).

Rather than replacing traditional taxonomy, DNA barcoding has actually reinforced the need for qualified taxonomists by producing sequence data that needs to be paired with a verified morphological type specimen (Packer *et al.* 2009). This data backlog is just one of the issues faced by DNA barcoding that could be exacerbated by the advent of NGS.

NGS: the destiny or downfall of DNA barcoding?

The future of DNA barcoding will almost certainly be influenced by NGS technologies. These advances are already here in the form of systems such as the Illumina genome analyser, Applied Biosystems' SoLiD platform, Helicos' HeliScope and, most relevant for DNA barcoding because of its longer average sequence read length, Roche's 454 sequencing platform (Kircher & Kelso 2010).

Although the cost of NGS is currently prohibitive for many small labs, some vendors are already offering scaled-down, 'budget' versions of these machines (Kircher & Kelso 2010). A 454 sequencer can process 2 000 000 reads per run compared with the 96 produced by a high-throughput Sanger sequencer (Valentini *et al.* 2009). The technology has already been implemented with some success to investigate single nucleotide polymorphisms in the great tit (Van Bers *et al.* 2010), meiofaunal assemblages (Creer *et al.* 2010) and microbial eukaryotes (Stoeck *et al.* 2010). Heralded as the dawning of a new era for molecular ecology (Ellegren 2008), NGS is being described by some as potentially transformative for the field (Tautz *et al.* 2010) and presents the prospect of readily available, full genomic sequence data in the near future.

The field of 'ecological genomics' has been proposed as an emerging discipline (Tautz *et al.* 2010), calling into question the necessity of indirect measures for estimating genetic distances at all (Avise 2010). Given the frequent

recommendations that single genes should not be relied upon for species delimitation (Puillandre *et al.* 2011; Zhang *et al.* 2011) and the declining cost of NGS, DNA barcoding could soon become obsolete.

One could argue that the existence of a new technology such as NGS does not necessitate its use, especially if costs remain high. This argument holds true if barcode sequences such as COI are proved to be representative of the genome as a whole. Unfortunately, the differences in evolutionary history between nuclear and mitochondrial DNA mean that a mitochondrial barcode such as COI is unlikely to be representative of nuclear divergence and a genetic divergence estimate taken from just one part of the genome does not produce an accurate representation of organismal divergence (i.e. speciation) (Humphries & Winker 2011).

Other issues such as heteroplasmy (the occurrence of multiple mitochondrial haplotypes within a single organism) and numts (nuclear mitochondrial pseudogenes) also reduce the reliability of mitochondrial DNA barcodes as species identifier surrogates (Magnacca & Brown 2010; Moulton *et al.* 2010). Thus, it would only seem sensible to 'make-do' and rely on barcoding as a surrogate for full sequence data if obtaining full sequences is prohibitively expensive. As the cost of NGS plummets, this argument for using barcoding becomes increasingly weak.

Some proponents of DNA barcoding have suggested the utility of NGS for 'mini-barcodes'—even smaller fragments of DNA—for use in species identification (Hajibabaei *et al.* 2011), but this seems an illogical shift towards less rather than more information in the face of potentially huge amounts of data, particularly in the light of the unreliability of short sequences for species delimitation discussed here.

NGS could be an opportunity for the DNA barcoding community if it is able to encompass new techniques and evolve to make use of more than just the short sequences currently employed. The Canadian Centre for DNA Barcoding (the hub of the barcoding effort), however, continues to advocate Sanger sequencing, with a one of its most recent studies on techniques and logistics focussed entirely on the adoption of 'high-throughput' 96-well microplates (Borisenko *et al.* 2009).

That is not to say that the barcoding movement is ignoring NGS. Aside from the aforementioned mini-barcoding suggestion, iBOL has a working group focussed entirely on environmental barcoding—a technique for identifying species within bulk environmental samples such as river benthos (Hajibabaei *et al.* 2011). Useful though this may be for biomonitoring, it fails to address the issue of the huge volumes of data that NGS could potentially generate. As discussed earlier, the increasingly rapid generation of data (be it genomic or single sequence) puts taxonomy in danger of being overwhelmed by genetic data that will inform humanity of little, by itself, regarding the diversity of life on earth other than its magnitude. Without the proper tools to verify, organize and analyze this data, it will be difficult for DNA barcoding to take advantage of NGS and remain a relevant discipline.

The barcoding movement can do little to speed the authentication of voucher specimens (aside, of course, from engaging more taxonomists). However, to make proper use of the data provided by NGS, it will be important for the BOLD system to be made NGS capable. If nothing else, this will provide an efficiently organized storage area for longer/full sequence data destined for species identification until it can be verified, encouraging further deposition of tissue specimens. Failure to bring BOLD up to speed with NGS would represent another seemingly counterproductive and stubborn stance in the face of scientific advancement.

DNA barcoding: the wrong tools for an important task?

It is easy to understand why there was so much excitement around the advent of DNA barcoding. With an extinction crisis in progress (Bottrill *et al.* 2008) and a consequent, pressing need to catalogue and protect life on earth, there was bound to be enthusiasm for a method that promised a handheld DNA-based species identification device within the next few decades, if not sooner (Janzen 2004). There can be no denying that DNA barcoding has potential and that its expansion has been explosive, but perhaps this rapid growth is at the heart of its troubles. Without taking time to stop and consider whether or not the methods it has encouraged an increasing group of followers to adopt were up to task, the DNA barcoding movement has rendered its data, analysis and utility questionable, open to repeated criticism and potentially unreliable.

The search for a universal barcode locus continues—most heatedly in plants, but also in fungi, protists and, to a lesser extent, animals. We have provided evidence that barcoding research still relies predominantly on distance-based, tree-building methods, despite the problems of such methods being pointed out repeatedly (DeSalle *et al.* 2005; DeSalle 2006, 2007; Rubinoff 2006b), major criticisms of the analytical tools supported by BOLD (Lowenstein *et al.* 2009) and the suggestion of alternative methods (DeSalle *et al.* 2005), (Figs 3–5).

Such a decisive attitude is perhaps commendable in a field such as conservation science, where quick decision-making is required because of the triage nature of the discipline (Bottrill *et al.* 2008). Conversely, by refusing to consider and integrate other tools and techniques that

may be more appropriate for the task in hand, the DNA barcoding movement could be hampering its own development.

Compared with the bold claims made in Hebert *et al*. (2003a), the more balanced tone adopted by CBOL and iBOL on their respective websites suggests a maturation of the DNA barcoding movement. Those involved in DNA barcoding have recognized (to an extent) the need to work in tandem with traditional taxonomists (Packer *et al*. 2009; Puillandre *et al*. 2011) and have omitted species discovery and delimitation from their aims on these websites at least.

The rise in prevalence of NGS and ecological genomics promises developments in both applied conservation and basic research, offering opportunities for DNA barcoding if it can adapt to capitalize on them. Failure to evolve could result in extinction, and this field's apparent resistance to change in its short history to date is, therefore, of some concern. Responding to some early criticism of DNA barcoding by traditional taxonomists, Hebert & Barrett (2005) stated: 'A DNA-based future can herald several possible fates for the Linnean system, from outright dismissal to revitalization.' Seven years later, the same could be said for DNA barcoding in a genomic future. The ground has shifted, and it is now DNA barcoding, rather than traditional taxonomy, which runs the risk of becoming irrelevant if it refuses to embrace change.

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This article is a result of a literature review conducted by H.R.T. for her MSc thesis while the student of W.E.H. H.R.T. has since moved to Victoria University of Wellington in New Zealand where she is studying for a PhD researching the conservation genetics and ecology of little spotted kiwi (*Apteryx owenii*). W.E.H. remains at Manchester Metropolitan University in the UK where his research interests include the genetic basis of social traits as well as conservation genetics.