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DNA barcoding in floral and faunal research

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22.1 Introduction

As I write this chapter in mid 2012, the context in which floral and faunal research is done is changing rapidly. Demand for biodiversity information, especially to understand global change, is increasing. The technologies that are available for carrying out biodiversity research and for disseminating the results are changing dramatically. The funding processes and organisational cultures of the institutions that have been the traditional homes of such research are evolving. This creates new challenges and opportunities for floral and faunal research. This chapter will focus on the intersection of DNA barcoding with floral and faunal research, the opportunities that DNA barcoding offers for increasing the quality and quantity of such work, and its connectivity with related activities. I will discuss the opportunities for DNA barcoding and then provide an example from my own research. The essay focuses on plants and animals, but will include some reference to other organisms. Because of the rapid evolution of the underlying technologies, and the related social changes, this essay represents a 'slice of time', and I expect some of the conclusions will be out of date before it is published. While challenges remain, I believe this is an exciting time of renaissance for taxonomy (Miller, 2007).

A DNA barcode is a short gene sequence taken from standardised portions of the genome, used to identify species. Being DNA based, it can be used for specimens

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without morphological characters necessary for traditional identifications, such as roots or immature insects. Being a short sequence, it can be extracted from material that has not been specially preserved, and can often be extracted from standard museum or herbarium specimens. Being from a standardised region allows the use of universal primers for unknown taxa and allows rapid compilation of a global reference library. The choice of gene regions has focused on species level identification. While barcoding is not intended as a tool for higher classification research or for population genetics research, sometimes the barcoding gene regions have useful information at those levels (Craft et al., 2010).

22.2 The present status of DNA barcoding

While molecular diagnostics have been used for many years, DNA barcoding as a global initiative started by Hebert et al. (2003), who, among other things, pointed out that a standardised choice of gene region for species-level diagnosis allows individual projects of whatever scale to contribute to a global reference library that becomes increasingly more powerful over time. Mitochondrial Cytochrome c oxidase I (COI) had been widely used in animals since Folmer et al. (1994) and was quickly adopted as a community standard following Hebert et al. (2003). Standardised gene regions for plants proved more challenging for technical reasons, but are now designated as rbcL and matK, with additional research on trnH-psbA (Hollingsworth et al., 2009). The nuclear ribosomal internal transcribed spacer (ITS) region has been selected as a universal DNA barcode marker for Fungi (Schoch et al., 2012). Explorations are under way in other taxa for appropriate standardised gene regions. The Consortium for the Barcode of Life (CBOL) was established in 2004 as an international membership organisation to help develop barcoding methodology and set standards, such as the 'barcode' reserved keyword in GenBank (Benson et al., 2012). GenBank[1] and its international partner databases, the European Molecular Biology Laboratory (EMBL) and the DNA Data Bank of Japan (DDBJ), provide the archival database for barcode data, and the Barcode of Life Database[2] (BOLD) at the University of Guelph, Ontario, provides an informatics workbench aiding the acquisition, storage, analysis and publication of DNA barcode records, as well as providing public identification tools and other services (Ratnasingham and Hebert, 2007, 2013). At this time, BOLD includes over 1 700 000 barcode sequences from 160 000 named species and many as yet unidentified species, and growing rapidly. Recent reviews of the state of development of barcoding include Waugh (2007), Valentini et al. (2008), Casiraghi et al. (2010), Teletchea (2010), and Kato et al. (2012).

[1] http://www.ncbi.nlm.nih.gov/genbank/ [2] http://www.barcodeoflife.org/

22.3 How does barcoding change the approach to an inventory?

DNA barcoding provides an important tool to improve the quality or speed of floral and faunal studies, while, at the same time, such studies contribute to development of the global sequence library that is becoming an important community resource. The large-scale inventory of caterpillars, their hosts and their parasites in Costa Rica has provided an excellent example of how barcoding has changed the basic approach to an inventory project, starting with sampling, processing, identification, analysis, and even changing the approach to publication of results (Janzen et al., 2009; Janzen and Hallwachs 2011a, 2011b; see also Strutzenberger et al., 2010). In addition to the changes in work flow there have been significant impacts, from finding cryptic species to matching dimorphic males and females, which have substantially improved the quality and depth of the inventory, but also greatly multiplied the number of situations requiring further taxonomic work for resolution. Although the workflow issues differ between different habitats and taxa, other studies have demonstrated the use of barcoding in inventories of diverse taxa, including poorly known freshwater invertebrates (Zhou et al., 2009; Laforest et al., 2013), tropical sand flies (Azpurua et al., 2010; Krüger et al., 2011), bats in Southeast Asia (Francis et al., 2010), difficult to distinguish agricultural pest moths (Roe et al., 2006), pollinating insects in Africa (Nzeduru et al., 2012), diverse radiations of tropical weevils (Pinzón-Navarro et al., 2010a, 2010b; Tänzler et al., 2012), freshwater fishes in Africa (Swartz et al., 2008; Lowenstein et al., 2011), butterflies at country scales (Dinca et al., 2011; Hausmann et al., 2011), amphibians in Panama (Crawford et al., 2010) and trees in forest plots (Kress et al., 2009, 2010; Costion et al., 2011). Perhaps even greater opportunities for improving the speed and quality of inventories exist in the marine realm, where poorly known larval stages exist in vast quantities, and species concepts must be compared across vast oceanic distances (Goetze, 2010; Heimeier et al., 2010; Hubert et al., 2010; Stern et al., 2010; Plaisance et al., 2011; Ranasinghe et al., 2012). But for most efficient use of DNA barcoding, the appropriate sampling and data management needs to be incorporated from the beginning of the fieldwork (Leponce et al., 2010; Dick and Webb, 2012; Puillandre et al., 2012).

22.4 How does barcoding change the use of morphospecies?

Even in floral and faunal studies, in which most identifications are well-resolved to named species, there are usually a few species designated as informal morphospecies, because the samples available lack the diagnostic characters for morphological

identification or the state of taxonomic knowledge does not allow identification. Traditionally, these informal designations are useful only within the local study, cannot be verified except for examination of voucher specimens and cannot be compared in a regional or temporal context. Regional and continental-scale comparisons are starting to become routine with DNA barcodes (see examples below), and Fernandez-Triana et al. (2011) recently used barcoding to compare changes in a local wasp community over 70 years using museum specimens.

DNA barcoding provides new tools for dealing with morphospecies, beyond the obvious facilitation of matches of unidentified specimens with named specimens (e.g. matching immature specimens with adults, or matching males and females). DNA barcoding provides a character-based diagnosis of the morphospecies (the DNA sequence) that can be made public (through GenBank) and can be compared with other species, whether identified or not. Pinzón-Navarro et al. (2010a, 2010b) were able to integrate historic knowledge of a diverse weevil group with larvae and adults reared from fruit in Panama, even though most of the species lacked taxonomic names. DNA barcoding can also provide an interim taxonomic designation, by way of the sequence, the GenBank accession number or the BOLD cluster reference number (BIN) (Schindel and Miller, 2010; Ratnasingham and Hebert, 2013).

22.5 Contribution of barcoding to quality control

The DNA barcode standard (and the restricted keyword barcode in GenBank, EMBL and DDBJ) bring a new standard of documentation and transparency to taxonomy, placing not only the sequence, but the metadata about the sequence and the voucher specimen, in the public record. This will become increasingly important in meeting standards of national and international regulation in fields such as agriculture, forestry and fisheries (Jones, F. C., 2008; de Waard et al., 2010; Floyd et al., 2010; Boykin et al., 2012), as well as emerging rules for access and benefit sharing under the Convention on Biological Diversity (Schindel, 2010). Newmaster and Ragupathy (2010) have applied DNA barcoding to quality control on traditional knowledge in ethnobotany, to test identifications of plants and to explore the presence of cryptic species.

22.6 Value added from the ability to compare regional biotas

DNA barcode data allows comparison of species concepts across geographical boundaries. For example, the taxonomy of the insect faunas of Australia and New Guinea were traditionally studied independently, but extensive DNA barcode

surveys of Lepidoptera in both places are now allowing comparisons of the biogeographical relationships in new depth (Holloway et al., 2009). It has always been challenging to assess small morphological variations across geographical barriers, such as defining the boundaries of species across islands. This was the case in the moth *Homona salaconis* (Meyrick), described from the Philippines but found on nearby islands including New Guinea (Hulcr et al., 2007), where addition of DNA data made the difference between the species as found in the Philippines and New Guinea clear, resulting in the recognition of the New Guinea species as *Homona auriga* (Durrant) (Miller et al., 2010). The Center for Tropical Forest Science's (CTFS's) global network of forest dynamic plots is now implementing barcoding across all the tree species in the plots (early results have been reported by Kress et al., 2009, 2010, 2012) and the National Ecological Observatory Network (NEON) is implementing the barcoding of selected insect groups across North America (Gibson et al., 2012).

Benefits go both ways between floral and faunal work and DNA barcoding. The 18-volume faunal work *The moths of Borneo* completed in 2011 (Holloway, 2011), while based on morphology, began to use DNA data to resolve some taxonomic problems and also pointed out taxonomic and biogeographical problems to which others have started to apply DNA barcode data. The existence of *The moths of Borneo* series itself encouraged Erik Van Nieukerken to undertake a faunal study on moths in Borneo testing the application of DNA barcoding (E. Van Nieukerken, pers. comm.).

While many barcoding studies to date have focused on patterns of local diversity, including recognition of cryptic species, barcoding shows great promise for the analysis of supposedly widespread species, and the identification of invasive species (Smith and Fisher, 2009; Hulcr et al., 2007; Floyd et al., 2010). One of the great values of the creation of global DNA barcode libraries is the opportunity for unexpected matches across the world.

22.7 Opportunities for meta-analysis: phylogeography and community phylogenies

Floral and faunal studies have always provided the raw data for biogeographical studies, and increasingly provide the raw data for the coalescence of the fields of ecology and evolution in phylogeography. While phylogeography might often best be done with longer sequences and more genes, DNA barcode data offer distinct advantages in many cases, being relatively inexpensive to produce with high throughput methods and having the benefit of large comparative libraries for many taxa (Craft et al., 2010). For example, Craft et al. (2010) were able to sample COI haplotypes from 28 Lepidoptera species and 1359 individuals across 4 host

plant genera and 8 sites in New Guinea to estimate population divergence in relation to host specificity and geography, a much larger dataset than most previously published phylogeographical analyses, which had tended to focus on single species. The possibilities for community phylogenetic analyses using large quantities of DNA barcode data are exciting, especially in organisms such as hosts and their parasites, and herbivorous insects and their host plants (Kress et al., 2009, 2010; Emerson et al., 2011).

22.8 Comparing trophic interactions across multiple levels

Traditionally, understanding ecological relationships across multiple trophic levels required time-consuming and expensive rearing or field observations. DNA barcoding is now being used to characterise food items from insect guts (Greenstone et al., 2005; Jurado-Rivera et al., 2009), fish stomachs (Valdez-Moreno et al., 2012) or mammal faeces (Bohmann et al., 2011; Clare et al., 2011; Pompanon et al., 2012), dead hosts of parasites from their larval remains (Hrcek et al., 2011), insect parasitoids dissected from caterpillars (Hrcek et al., 2011), hosts from DNA remaining in parasites (Rougerie et al., 2011) and bloodmeals in ticks (Gariepy et al., 2012). Kaartinen et al. (2010), Hrcek et al. (2011) and Smith et al. (2012) have applied DNA barcoding to the analysis of complex insect food webs. As techniques for handling degraded DNA improve and DNA libraries expand, we can expect these techniques to be widely used. In the near future, I expect that a single caterpillar can be sampled and, in addition to identification of the caterpillar itself, DNA barcoding will allow identification of its plant diet, its insect parasites, and its microbial symbionts.

22.9 Identification of immature and dimorphic stages

As noted above, faunal and floral studies are plagued by specimens in immature stages that lack the characters necessary for identification, or sexes that cannot be matched, especially if they are dimorphic such as some insects in which the sexes vary dramatically in size or colour (Pinzón-Navarro et al., 2010a, 2010b). Richard et al. (2010) have used barcoding to identify earthworm juveniles in soil ecology studies. Plants sampled only in ‘vegetative’ stages are a widespread problem in floral studies. Even long-term studies of forest tree plots often have individual trees that remain unidentified for years because of the duration between flowering in many tropical trees (e.g. Lafrankie et al., 2005). The ability to identify plant roots and shoots using DNA will dramatically change the depth of ecological analysis that is possible (Jones, F. A., et al., 2011).

22.10 Environmental samples

One of the exciting horizons for expansion of DNA barcoding is the application of DNA barcode libraries to the identification and quantification of species in environmental samples that have been sequenced using next-generation sequencing techniques (Pfrender et al., 2010; Hajibabaei et al., 2011; Baird and Hajibabaei, 2012). Although the technique has not yet been applied widely, interesting demonstrations are being published, such as the identification of frog DNA in water samples (Ficetola et al., 2008), phylogeography of soil invertebrates (Emerson et al., 2011) and the interesting case of insect DNA from alcohol preservative (the 'worm' in mescal liquor) (Shokralla et al., 2010). Related research is finding that, in many cases, sequences that are shorter than the standard 648 base-pair animal barcodes have almost as much information content as the full-length barcodes, still allowing species identifications (Meusnier et al., 2008; Lees et al., 2010) and opening greater opportunities for the use of next-generation sequencing.

22.11 An example of the impact of DNA barcoding in insect faunistic studies

For many years I have been involved in local inventories of Lepidoptera (butterflies and moths), especially in Papua New Guinea (PNG) and Kenya. The work in PNG involves the large-scale rearing of caterpillars to adults, as part of the analysis of ecological and biogeographical patterns among host plants, caterpillars and their parasites (e.g. Miller et al., 2003, 2013; Craft et al., 2010; Novotny et al., 2012). The PNG research started in 1994 with parataxonomists taking the first pass at morphospecies identifications, with the aid of a database with images as a working identification tool (Basset et al., 2000, 2004), then a review in my laboratory based on external morphology and then extensive dissection of genitalia, to purify species concepts and to associate the species concepts with species names based on published revisions or type specimens. This process worked well, but involved the preparation of large quantities of genitalic dissections and, thus, was relatively expensive and time-consuming. I started collaborating with Paul Hebert in DNA barcoding in 2003, but did not start processing large quantities of DNA barcode samples until 2005. In the early stages, we used DNA barcoding to solve problems that morphology alone could not resolve, such as matching males and females, and clarifying species limits in polymorphic species. Adamski et al. (2010) is an example of a hybrid product during this transitional period – we did a traditional morphology-based analysis of African Blastobasinae moths in the early 2000s, then tested it with DNA barcodes in the mid 2000s and then eventually published the joint results.

In recent years we have shifted the work process to put the emphasis on DNA barcodes, because of their accuracy and cost-effectiveness. We still use genitalic dissections, both for delimiting species concepts and for matching with names, but use the barcode analysis to guide which specimens should be dissected. This has reversed the relative importance of barcodes and genitalic morphology in our work process, allowing faster and higher quality results. Because we are able to do DNA barcodes for more specimens than were dissected under the old system, we have more data for species concept delimitation, and we catch more errors in association of individuals with species concepts (for example, poor quality specimens). Similar projects in Costa Rica and Canada have gone through a similar transition in work process (de Waard et al., 2009; Janzen et al., 2009; Janzen and Hallwachs 2011a, 2011b).

A collection of Lepidoptera reared from native fruits in PNG in 2008–2009 provides an example of the new process of using barcoding. While there are some lineages of Lepidoptera that specialise in using fruit as larval hosts, rearing Lepidoptera from fruit is a time-consuming, messy and relatively low-yield activity, so it is rarely done on a mass scale, but the results are interesting. In addition to the ecology of fruit feeding, Lepidoptera in PNG being poorly known, many of the Microlepidoptera that are fruit specialists are very poorly known globally and the PNG fauna is almost unknown. While a few of the butterflies are well-known, even family-level identifications prove challenging for some of the tiny moths. Thus, I was both delighted and daunted to receive the first instalment of Lepidoptera reared from about 4000 lots of native fruit in PNG, in a programme organised by Richard Ctvrtecka.

The collection had been sorted into 70 morphospecies by the parataxonomists in PNG, and included between 1 and 20 specimens for each of the morphospecies, with many morphospecies represented by only a few specimens. After an initial assessment by external morphology (including a few splits in morphospecies), we sent up to five individuals per morphospecies to the University of Guelph for sequencing using their standard protocols (Craft et al., 2010; Wilson, 2012). This yielded 227 sequences, covering all morphospecies, deposited in GenBank as accession numbers GU695412–5, GU695431–2, GU695434–66, GU695468–9, GU695504–46, GU695548–58, GU695561, GU695575–80, GU695623–36, GU695639–701, GU695716–7, GU695720–1, GU695745, HM376367–75, HM376381–4, HM422448–56, HM902704–15, HQ947496–7, HQ956600–1 and HQ956613–4. The result of barcoding, confirmed by genitalic morphology, is 79 species. Two morphospecies were represented by unique individuals in poor condition that were shown by barcoding to belong to existing morphospecies. Eleven morphospecies were split, in seven cases because of unique individuals in poor condition that were incorrectly identified based on external morphology. This confirms the high quality of the sorting of our PNG parataxonomists, given that they are using only external morphology.

The barcode library in BOLD is becoming rich enough in many parts of the Lepidoptera to be useful in identification of unknowns, especially North America (Hebert et al., 2010), but also other regions such as Costa Rica and Australia. For example, one of the species was identified as the cosmopolitan tineid pest *Phereoeca uterella*, and several Phycitinae, a very difficult group taxonomically, were immediately identified to genus because of samples from Australia that Marianne Horak had provided to BOLD. By contrast, BOLD has been less useful for associating weevils reared from the same fruit, because the taxonomic sampling in BOLD remains small (some 13 000 sequences of Curculionidae in BOLD in July 2012 compared to 114 000 sequences of Geometridae, for example). But this shows how increasingly useful BOLD will become as more projects contribute reference sequences.

Following purification of species concepts, we have targeted genitalic dissections to confirm the barcoding results and to allow comparison with type specimens. About two-thirds of the species have been positively identified (either as known taxa or as new species) through consultation with literature, the collections in Washington, D.C. and London and assistance from colleagues. The identification process will continue for some time, because many of the Microlepidoptera types have never been dissected and properly placed in modern generic concepts.

22.12 Conclusions

DNA barcoding builds on the best of the traditional strengths of floral and faunal research based on morphological identifications of voucher specimens, especially in enhancing the quality of identifications for named species and in enhancing the information associated with informal morphospecies designations. While DNA barcoding can be applied 'after the fact,' it is most cost-effective to plan the appropriate sampling and data management from the beginning of fieldwork. Floristic and faunistic works have traditionally been descriptions of areas at a given point in time, limited by the species concepts available at the time. DNA barcodes, along with modern information management systems for other characters, allow the local species (or morphospecies) concepts to be seen as part of a dynamic global taxonomy that can be repeatedly queried in the context of changing questions.

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