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PERSPECTIVES AND PARADIGMS

Common goals: policy implications of DNA barcoding as a protocol for identification of arthropod pests

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Abstract The globalization of commerce carries with it significant biological risks concerning the spread of harmful organisms. International Standards for Phytosanitary Measures (ISPM) No. 27, "Diagnostic Protocols for Regulated Pests", sets out the standards governing protocols for the detection and identification of plant pest species. We argue that DNA barcoding—the use of short, standardized DNA sequences for species identification—is a methodology which should be incorporated into standard diagnostic protocols, as it holds great promise for the rapid identification of species of economic importance, notably arthropods. With a well-defined set of techniques and rigorous standards of data quality and transparency, DNA barcoding already meets or exceeds the minimum standards required for diagnostic protocols under ISPM No. 27. We illustrate the relevance of DNA barcoding to phytosanitary concerns and advocate the development of policy at the national and international levels to expand the scope of barcode coverage for arthropods globally.

Keywords DNA barcodes · ISPM No. 27 · COI · Insect identification · Agricultural pests · Forestry pests

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Introduction

The movement of non-indigenous species (NIS) as an unintended consequence of global commerce poses major problems worldwide, with significant economic, environmental and human costs (Mooney and Hobbs 2000). This movement can only be expected to increase in the future, due to growing international trade, climate change, and increasing human mobility. A rapid and widely accessible identification system is essential to implement management strategies such as quarantine or eradication with minimal delay (Carnegie et al. 2006; Leung et al. 2002; Pemberton 1988). Yet too often, the diagnosis of pest species is a slow and inefficient process, with the result that by the time a NIS has been detected, it has

already spread and proliferated beyond the ability to contain it. Officials such as port inspectors typically do not have the expertise to recognise the vast range of harmful species which might potentially be intercepted. Furthermore, many species have life-cycle stages which cannot be identified by conventional means (e.g. eggs and larvae for many arthropods, seeds for plants); if found as an egg mass or early-instar larva, most pests of regulatory concern cannot be distinguished from other potentially invasive congeners (Pogue and Schaefer 2007).

The concept of DNA barcoding (Arnot et al. 1993; Floyd et al. 2002; Hebert et al. 2003) postulates that it is possible to rapidly and accurately identify a species by amplifying and sequencing short, standardized regions of its genome—for animals, the mitochondrial gene, cytochrome *c* oxidase subunit 1 (COI; Hebert et al. 2003), while for plants, a two-locus system (*rbcL* + *matK*) was recently recommended by the Plant Working Group of the Consortium for the Barcode of Life (CBOL 2009). This signature sequence can be compared against a database of known sequences from identified specimens in order to obtain an identification. Because this method depends on DNA and not morphology, it is applicable to any life stage, from egg to adult. It also aims to employ standardised protocols that may be applied to a wide range of organisms with a minimum of technical expertise and without requiring extensive knowledge of traditional morphological taxonomy. Used as a component of regular monitoring, DNA barcoding has the potential to detect invasions early, providing authorities with the time to develop containment and eradication strategies before populations of a NIS become unmanageable. With this article we wish to make a more direct case for DNA barcoding to be formally recognised and adopted as a diagnostic protocol for regulated pests.

Background

National Plant Protection Organizations (NPPOs) are charged with a difficult task. They must have the capacity to enforce national legislation developed to prevent the introduction of non-native pests or pathogens that could potentially impact plant health (agriculture and forestry), or human and animal health, as well as to meet their obligations under international treaties. In order to prevent the introduction of harmful NIS, interception of pest species can lead to the rejection or treatment (including destruction) of a consignment by a NPPO, thus it is paramount that identifications be accurate, complete and timely.

Addressing such problems requires coordinated efforts of policymakers and scientists alike from many nations. This was among the reasons for the establishment of the International Plant Protection Convention (IPPC), a treaty administered by the Food and Agriculture Organization of the United Nations (FAO) with the goal of securing action to prevent the spread and introduction of pests of plants and plant products, and to promote appropriate measures for their control. The IPPC has the role of working with NPPOs to develop and harmonise standards internationally, and sets out a series of standards, the International Standards for Phytosanitary Measures (ISPMs), which member countries are encouraged to follow. NPPOs of each country signatory to the IPPC are required to base their phytosanitary legislation on the adopted ISPM's to ensure phytosanitary safety of goods moving in international trade.

ISPM No. 27, entitled "Diagnostic Protocols for Regulated Pests" (FAO 2006), describes procedures and methods for the diagnosis of pest species—for example, they must contain basic biological information on the pest (life cycle, morphology, host range etc.), taxonomic information (including synonyms), techniques for detection and identification, and importantly, standards of record-keeping to ensure traceability and data quality (storage of voucher specimens, photographs, DNA extracts, etc.). DNA barcoding is already mentioned briefly in ISPM No. 27 (as an example among several biochemical/molecular-based methods). We will describe the background and methods of barcoding, and specifically discuss how it meets or exceeds the requirements outlined in ISPM No. 27 (FAO 2006).

DNA barcoding: the method

> Each protocol contains the methods and guidance necessary for the regulated pest(s) to be detected and positively identified by an expert (i.e. an entomologist, mycologist, virologist, bacteriologist, nematologist, weed-scientist, molecular biologist)

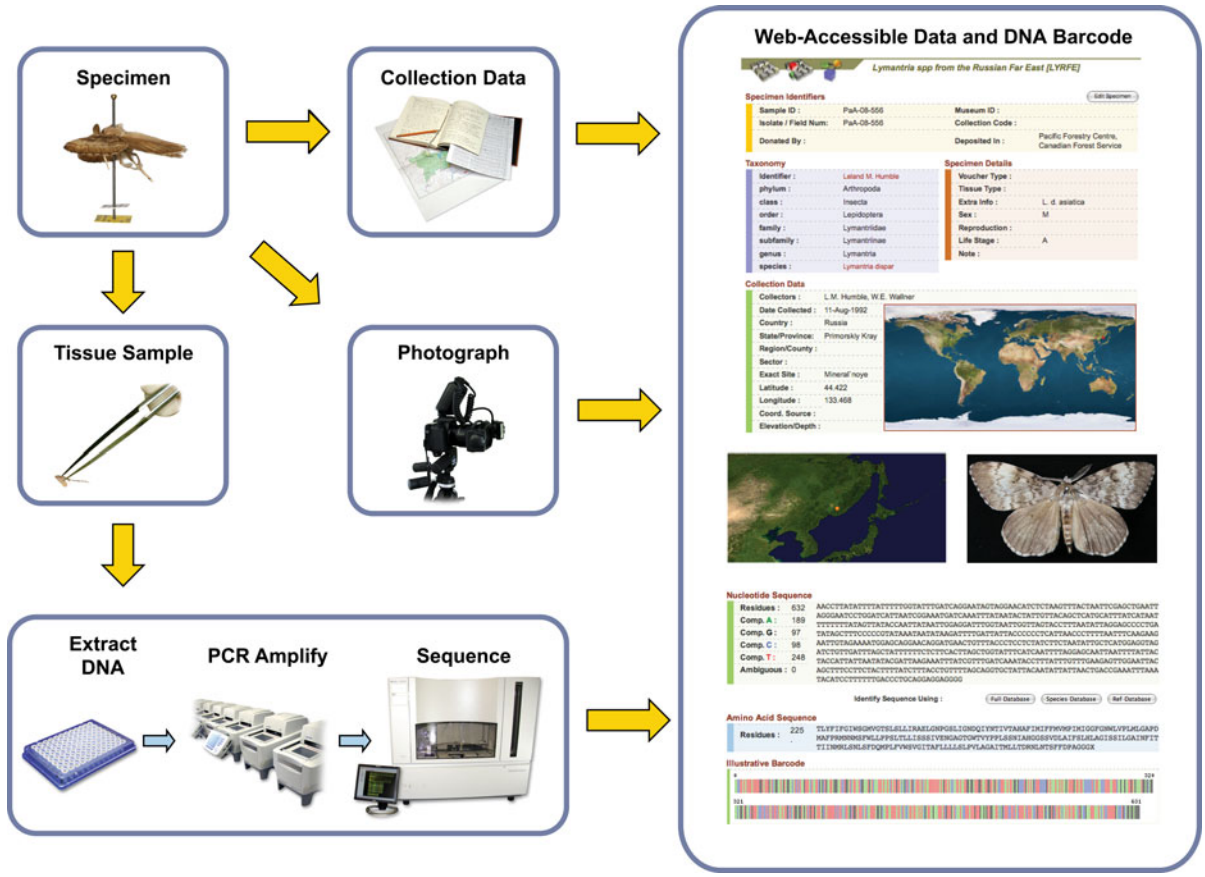

Fig. 1 The workflow of DNA barcoding. A tissue sample (e.g. insect leg) is taken from a specimen, and used to generate a DNA sequence. The original specimen is photographed and kept as a voucher. The image and all collateral information (identification, collection data, etc.) are stored along with the DNA barcode sequence in the BOLD database

> or competent staff that are specifically trained… the availability of equipment, the expertise required for these methods and their practicability (for example ease of use, speed and cost) are taken into account when selecting methods for inclusion in the diagnostic protocol. Usually these methods and their associated information should also be published. ISPM No. 27, General requirements for diagnostic protocols (FAO 2006).

The DNA barcoding approach depends upon rigorous standardisation of both laboratory methods and data handling. The typical workflow of DNA barcoding is shown in Fig. 1. From each specimen to be barcoded, a tissue sample (e.g. insect leg) is taken, and used to generate a DNA sequence, a procedure which may be divided into six steps: DNA extraction from the tissue; PCR amplification of the barcode region (COI) using universal primers; PCR product check using gel electrophoresis (which may be followed by "hit picking", i.e. selective processing of successes vs. failures); cycle-sequencing to generate dye-labelled products; cleanup of the labelled product; and analysis on an automated DNA sequencer to generate the final read. Most of these steps are amenable to automation for high-throughput screening (Hajibabaei et al. 2005; Ivanova et al. 2006), allowing increasingly fast and inexpensive processing of specimens.

The COI gene is a sound choice for use in a global arthropod diagnostic system for several reasons: it lacks introns, rarely possesses indels, readily amplifies by polymerase chain reaction (PCR) due to the availability of robust universal primers across many taxa (Folmer et al. 1994), and is easily aligned after sequencing (Hebert et al. 2003). Because each

eukaryote cell contains multiple copies of COI, only small amounts of a tissue (e.g. a leg or antenna) are required from a specimen for successful amplification and sequencing. In addition, COI sequences can be obtained from highly degraded specimens (Meusnier et al. 2008).

It is important to distinguish between two types of application which may both be referred to by the term "barcoding". The first is sequencing an unknown specimen as a method of making routine identifications by comparison against the database of known sequences. The second is the actual construction of the reference library, which requires the storage of the specimen and capture of data according to clearly defined process standards (Hanner 2005). The original specimen is photographed and kept as a morphological voucher (and must be deposited with a traceable ID in a museum or other recognised scientific repository (Hanner and Gregory 2007)); the frozen DNA extract is also archived for potential future analysis. The image and all collateral information (identification, collection data, etc.) are stored along with the DNA barcode sequence in a custom database: the Barcode of Life Data Systems, or BOLD (Ratnasingham and Hebert 2007), a web-based platform which acts as both a universal repository for DNA barcode data and an online workbench with tools for data analysis. Thus, a reference database of COI barcode sequences matched to known species names and images is built up; any novel COI sequence from an unknown specimen may be searched against the database and, if a match is found, a species identification may be rapidly made.

There are seven basic data requirements for a specimen to meet the formal barcode reference sequence standard (Hanner 2005):

- Species name (although this may be interim and subject to revision);
- Voucher data (catalogue number and institution storing);
- Collection record (collector, collection date and location with GPS coordinates);
- Identifier of the specimen (name and contact details);
- COI sequence of at least 500 base pairs (bp) with fewer than 1% ambiguous base calls derived from 2× (e.g. bidirectional) sequence coverage;
- PCR primers used to generate the amplicon;
- Trace files (electropherograms generated by sequencer).

Records meeting these standards that are submitted to the International Nucleotide Sequence Database Collaboration (e.g. GenBank) are annotated with the reserved keyword "BARCODE", as well as being stored in BOLD. These BARCODE sequences, derived from expert-identified taxonomic reference specimens, are recommended for use as the basis for making routine identifications through generating equivalent DNA sequences from unknown specimens. The existence of morphological and DNA vouchers for all BARCODE sequences is of critical importance in maintaining reliability of identifications over time as they allow future resolution of species identifications as morphological and systematic knowledge advances.

Therefore, considered as a diagnostic protocol, DNA barcoding meets or exceeds the specific guidelines laid out in ISPM No. 27, which are in place for similar reasons of traceability and scientific validation. Indeed, ISPM No. 27 only requires that materials such as specimens and DNA extracts must be archived for a period of 1 year[1] (FAO 2006), whereas the data standards adopted by the DNA barcoding community call for permanent archiving of both vouchers and DNA for all barcoded specimens (Hanner 2005).

When dealing with invasive species it is often of particular importance not only to be able to identify species, but to pinpoint the geographic origin of a particular intercepted specimen; this can be important in reconstructing the route that a particular invasion has followed ("pathway analysis"). Barcoding is capable of fulfilling this need to the extent that different populations or subspecies carry distinct COI signatures, which are fully represented in the reference database. For example, differences in COI sequences between the Gypsy moth *Lymantria dispar* (a major forestry pest) from northern Asia and those from eastern North America and western Europe allowed regulatory agencies to conclusively diagnose the presence of established populations of *L. dispar*

[1] "In cases where other contracting parties may be adversely affected by the results of the diagnosis, records and evidence of the results of the diagnosis should be retained for at least 1 year". Section 2.1, Records.

from Russia in Vancouver, British Columbia and Seattle-Tacoma, Washington and Portland, Oregon (Armstrong and Ball 2005; Ball and Armstrong 2006; Bogdanowicz et al. 1993, 2000). Furthermore, Barr (2009) found that a sequence-based approach (using both mitochondrial ND4-ND5 and COI genes) was more effective than the more routinely used PCR–RFLP method for pathway analysis of Mediterranean fruit fly populations, while Simonsen et al. (2008) used COI data to trace the invasion route of cactus moths (*Cactoblastis cactorum*) in the United States.

Because BOLD is actively curated and as the dataset continues to grow in coverage, being populated with ever more sequences carrying full data on locality as well as taxonomy, it will become increasingly effective for regional haplotype matches regardless of taxonomic uncertainty.

The importance of vouchering, and the inadequacy of existing DNA sequence-based resources

A number of authors have recently stressed the importance of retaining voucher specimens in systematic and ecological studies (Por 2007; Ruedas et al. 2000; Wheeler 2003). Incorrect identifications in such studies can lead to "error cascades" (Bortolus 2008) as mistakes are subsequently cited and repeated by others; without voucher specimens that can be re-examined, taxonomic errors are impossible to definitively resolve. Similarly, existing DNA repositories such as GenBank (Benson et al. 2008) house vast amounts of data from biological specimens, and list the taxonomic assignments of those specimens; however, the onus is on the individual making the data submission to ensure that the taxonomy is correct, and few quality control procedures are in place. Several recent studies have uncovered indications that a worrying number of published DNA sequence records contain taxonomic errors (Bridge et al. 2003; Harris 2003; Nilsson et al. 2006). Since voucher specimens or DNA extracts are not routinely archived in most molecular work, it is difficult if not impossible to rectify many of these errors. Many records also lack provenance, geographic information and other significant metadata. Thus, if we were to rely simply on sequence data from public sources such as GenBank as a basis for an identification system, there would remain an unacceptable degree of doubt in the reliability of the results.

In this context, we can see the importance of the process standards adopted as part of the DNA barcoding protocol, as well as the enhanced value of the data generated as a result; all barcode reference sequences will be linked to digital images, as well as being traceable back to a voucher specimen in a museum, which can be re-checked in the event of any disagreement over taxonomy, or revision of taxonomic names. This will ensure that matches made using DNA barcodes are based on reliable data, an essential feature in a diagnostic protocol which could have ramifications affecting international trade.

Involvement of developing countries

International regulations designed to prevent the movement of NIS can significantly impact trade, particularly for developing countries. Frequently, disputes between trade partners involve concerns over the introduction of harmful species; the impact of resulting trade barriers can cost hundreds of millions of dollars (Powell 1997). Two World Trade Organization (WTO) agreements, the Technical Barriers to Trade Agreement[2] (TBT) and the Sanitary and Phytosanitary Measures Agreement[3] (SPS), focus on the protection of food, animal, and plant safety of trade partners while attempting to prohibit trade protectionism and discrimination. Under the SPS, restrictions on trade must be based on objective, scientific criteria (Spreij 2007).

While establishing shared standards for phytosanitary measures is clearly of benefit to international commerce, in practice developing countries often struggle to meet these standards owing to a lack of scientific infrastructure, and consequently suffer restrictions in trade (Campbell 2001). For example, among the requirements under the SPS, exporting countries are obligated to provide the importing country with a list of pests likely to be associated with each commodity, demonstrate competency in gathering local pest data (biology, ecology, and taxonomy), conduct pest risk analyses in areas from

[2] http://www.wto.org/English/tratop_e/tbt_e/tbt_info_e.htm.

[3] http://www.wto.org/English/tratop_e/sps_e/spsagr_e.htm.

which exports originate, and be able to detect NIS and implement control strategies (Gascoine et al. 2000). These activities require the maintenance of properly vouchered specimens that are curated in recognized institutions (government agencies, museums, and universities) so that accessible evidence of a country's plant health status is available. Developing countries are usually limited in technical and taxonomic expertise to comply with TBT and SPS agreements in a timely manner.

Current barcoding research proposals seek to involve not only developed countries with established scientific infrastructure and expertise, but also to encourage poorer and developing countries to establish barcoding programmes for their own faunas. The development of species lists and curated biological collections is not only of scientific interest, but in the case of pest species, could provide a real solution to the difficulties faced by developing countries in meeting pest reporting requirements under WTO and IPPC rules. The capacity to ascertain with greater speed and accuracy whether pests or disease are present in countries, whether they are widespread or have a restricted range, and whether product at the border is free of pests and disease may be a key means by which developing countries will be able to gain access to and thereby trade on the international market. Furthermore, the assurance that end-users all over the world are using the same protocols, data standards and identifications system (as opposed to a situation where every country has its own conflicting species lists and keys, with no global database) would offer the kind of unified standards in phytosantitary protection which the IPPC has always sought to achieve, enhancing trade and facilitating the resolution of disputes.

Conclusion

The applicability of DNA barcoding as a diagnostic protocol for regulated pests is appealing because it meets the requirements outlined in ISPM No. 27 (FAO 2006). Specifically, the DNA barcoding protocol is flexible, sensitive, specific, reproducible, and subject to review and amendment. It is also accessible, inexpensive, robust, simple, standardizable, and it can accelerate NIS diagnostics nationally and internationally more than any current molecular method.

Recent developments in the practical adoption of barcoding include the European QBOL project (http://www.qbol.org/UK/), a collaborative effort funded by the EU 7th Framework Program aiming to utilize barcoding in the identification and control of quarantine organisms. Additionally, the USDA (United States Department of Agriculture) and CDFA (California Department of Food and Agriculture) are now using barcoding operationally to track a specific new pest species in California, the light brown apple moth, *Epiphyas postvittana* (an invasive originating from Australia), and have an online sequence-based diagnostic tool available, LBAM-ID.[4]

For NPPOs to adopt barcoding as a diagnostic tool they will need to ensure that the barcode specimen records exist for all taxa of interest (Ekrem et al. 2007), as well as their congeners. This may entail generation of the reference sequences for these taxa in collaboration with systematists, national collections and specialist barcode facilities. Once the reference libraries for the taxa of interest are in place, the routine diagnostics through sequence-based matches will be possible. Generation of these reference libraries could be accomplished as part of routine surveillance and diagnostic activities, if inspectors ensure that their samples are collected in such a way as to preserve both the DNA and the morphological voucher specimen. Regulatory agencies interested in adopting barcoding would require certain modifications to their collection and storage procedures—for example, ensuring that specimens were preserved in a "DNA friendly" format (i.e. freezing or 95% ethanol), and arranging facilities for long-term storage of voucher specimens and DNA extracts (which could be coordinated with museums and/or barcoding facilities). We believe that the advantages brought by DNA barcoding render such efforts worthwhile particularly because barcodes can unite a diverse assemblage of reference specimens and genetic resources under a common registry of genetic sequence accessions (Walters and Hanner 2006). This in turn will help to standardize the application of names across diverse collections and jurisdictions, providing an organizational infrastructure

[4] http://www.keys.lucidcentral.org/keys/v3/LBAM/dna_search.html.

necessary to support a global network of biological resource centers. DNA barcoding does not strive to be the sole method for identifying animal life, but rather serves as a universal baseline; once standardized COI reference sequences are collected, submitted, and approved, supplementary nuclear gene(s) for specific taxa can be agreed upon to further strengthen a global NIS identification system (as of November 2009, BOLD version 2.5 supports additional marker genes including ITS for fungi, and *rbcL* and *matK* for plants; further markers can be registered by users). Thus, DNA barcoding has the potential to complement and unite traditional morphology-based and nuclear gene-based identification methods, and its framework is supportive of integrative taxonomy.

Given the promise that DNA barcoding holds for early detection of invasive animal species, and the fact that it already meets the standards of ISPM-27 and is ready for implementation, we advocate the development of policy at the national and international levels to expand and enhance the global database. The international mobility of goods, vessels, and people necessitates that any DNA barcoding and management program designed for a given country be incorporated into a common global database to facilitate species identification, knowing their region of origin, and risk assessment. DNA barcoding offers the potential not only for enhanced recognition and thereby control of harmful species in the immediate term, it also promises to help us to understand the global movement of species in a level of detail never before attainable.

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