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Research Note

Potential Use of DNA Barcodes in Regulatory Science: Identification of the U.S. Food and Drug Administration's ''Dirty 22,'' Contributors to the Spread of Foodborne Pathogens

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ABSTRACT

The U.S. Food, Drug, and Cosmetic Act prohibits the distribution of food that is adulterated, and the regulatory mission of the U.S. Food and Drug Administration (FDA) is to enforce this Act. FDA field laboratories have identified the 22 most common pests that contribute to the spread of foodborne disease (the ''Dirty 22''). The current method of detecting filth and extraneous material (tails, legs, carcasses, etc.) is visual inspection using microscopy. Because microscopy can be time-consuming and may yield inaccurate and/or nonspecific results due to lack of expertise, an alternative method of detecting these adulterants is needed. In this study, we sequenced DNA from the 5′ region of the cytochrome oxidase I gene of these 22 common pests that contribute to the spread of foodborne pathogens. Here, we describe the generation of DNA barcodes for all 22 species. To date, this is the first attempt to develop a sequence-based regulatory database and systematic primer strategy to identify these FDA-targeted species. DNA barcoding can be a powerful tool that can aid the FDA in promoting the protection and safety of the U.S. food supply.

Regulatory action criteria for filth and extraneous materials are used by the U.S. Food and Drug Administration (FDA) to evaluate the adulteration of food products. The criteria are organized into three categories: health hazards, indicators of insanitation, and natural or unavoidable defects *(41)*. Health hazards include criteria for physical, chemical, and microbiological hazards in relation to filth and extraneous materials. Filth includes ''rat, mouse, or other animal hairs and excreta, whole insects, insect parts and excreta, parasitic worms, pollution from the excrement of humans and animals, as well as other extraneous materials which would not knowingly be eaten or used'' *(54)*. The FDA is one of several public health authorities to recognize rodents, flies, and cockroaches as contributing factors to the spread of foodborne pathogens. FDA field laboratories have identified the ''Dirty 22,'' the 22 most common pests that the FDA recognizes in contributing to the spread of foodborne pathogens, which represent the highest risk under the Compliance Policy Guide 7120.18 *(55)*. The common pests identified are German cockroach, brownbanded cockroach, oriental cockroach, American cockroach, pharaoh ant, thief ant, house fly, stable fly, little house fly, latrine fly, cosmopolitan blue bottle fly, holarctic blue bottle fly, oriental latrine fly, secondary screwworm, blue bottle fly, green bottle fly, black blow fly, redtailed flesh fly, house mouse, Polynesian rat, Norway rat, and roof rat (Table 1) *(41)*.

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The Food, Drug, and Cosmetic Act Section 402(a)(4) considers food adulterated ''if it has been prepared, packed, or held under insanitary conditions whereby it may have become contaminated with filth, or whereby it may have been rendered injurious to health'' *(40)*. Most common foodborne biological hazards are bacterial or microbial pathogens. These pathogens have five attributes that likely contribute to the adulteration: synanthropy, endophily, communicative behavior, attraction to filth and human food, and harborage of pathogens in natural (wild) populations *(40)*. Synanthropic and endophilic insects, such as flies, ants, and cockroaches, breed and feed in animal manure and human excrement and have transferred human pathogens on their mouth parts, body, leg hairs, and feet *(12, 41)*. The four cockroach species and two ant species identified are known carriers of the pathogens *Escherichia coli, Salmonella, Shigella,* and *Staphylococcus (41)*. The German and American cockroaches are transmitters of *Entamoeba histolytica,* a protozoa that infects the intestinal track. The 12 filth flies identified are capable of contaminating food with *Shigella, Salmonella, E. coli, Campylobacter jejuni, Vibrio cholera (40),* and the protozoa *Cryptosporidium (12).* The four rodent species are attracted to excrement and human food and are carriers of *Salmonella (41).*

Food contamination can occur during processing and/or storage. Sanitary and insect-free food processing environments are necessary to control or eliminate pests from food supplies. Animal products such as meat, poultry, seafood, dairy products, and eggs are the foods most likely to cause outbreaks in the United States *(8),* and in recent years

TABLE 1. *Twenty-two common pests contributing to the spread of foodborne pathogens (41)*

Common name	Scientific name
German cockroach	*Blattella germanica* (L.) (Dicityoptera: Blattellidae)
Brownbanded cockroach	*Supella longipalpa* (Fabriculus) (Dicityoptera: Blattellidae)
Oriental cockroach	*Blatta orientalis* L. (Dictyoptera: Blattidae)
American cockroach	*Periplaneta americana* (L.) (Dictyoptera: Blattidae)
Pharaoh ant	*Monomorium pharaonis* (L.) (Hymenoptera: Formicidae)
Thief ant	*Solenopsis molesta* (Say) (Hymenoptera: Formicidae)
House fly	*Musca domestica* L. (Diptera: Muscidae)
Stable fly	*Stomoxys calcitrans* (L.) (Diptera: Muscidae)
Little house fly	*Fannia canicularis* (L.) (Diptera: Muscidae)
Latrine fly	*Fannia scalaris* (Fabricius) (Diptera: Muscidae)
Cosmopolitan blue bottle fly	*Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae)
Holarctic blue bottle fly	*Calliphora vomitoria* (L.) (Diptera: Calliphoridae)
Oriental latrine fly	*Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae)
Secondary screwworm	*Cochliomyia macellaria* (Fabricius) (Diptera: Calliphoridae)
Blue bottle fly[a]	*Cynomya cadaverina* Robineau-Desvoidy (Diptera: Calliphoridae)
Green bottle fly[a]	*Lucilia coeruleiviridis* (Meigan) (Diptera: Calliphoridae)
Black blow fly	*Phormia regina* (Meigen) (Diptera: Calliphoridae)
Redtailed flesh fly	*Sarcophaga haemorrhoidalis* (Fallen) (Diptera: Sarcophagidae)
House mouse	*Mus musculus* (Mammalia: Muridae)
Polynesian rat	*Rattus exulans* (Mammalia: Muridae)
Norway rat	*Rattus norvegicus* (Mammalia: Muridae)
Roof rat	*Rattus rattus* (Mammalia: Muridae)

[a] Scientific name has changed *(57)* since published by Olsen et al. *(41)* in 2001.

spinach, lettuce, and fruits have caused foodborne illnesses *(10, 39)*. Synanthropic insects contribute to the spread of foodborne diseases in both developing and developed countries, such as the United States *(12)*. Because filth flies exhibit clustering and swarming behaviors at sites of attraction (i.e., food sites), the resulting high density of flies proportionally increases pathogens on surfaces frequented by the flies *(12)*.

The FDA utilizes visual inspection via microscopy to identify insect carcasses and parts for the detection of these Dirty 22 species, following the AOAC official method 970.66 *(3)*. This meticulous method involves sieving, using a Wildman trap flask with a magnetic stirrer, filtration, and examination under a widefield stereoscopic microscope *(3, 4)*. Because microscopy can be time-consuming and may yield inaccurate and/or nonspecific identification through lack of appropriate expertise, this study was conducted to explore a molecular method for identification of these 22 species. Recently, the FDA has developed and validated a PCR–restriction fragment length polymorphism (RFLP) method based on the small subunit rRNA gene that can identify members of group I (cockroaches) of the Dirty 22 *(50)*. A new regulatory approach to species identification of fish is DNA barcoding, a technique that exploits DNA sequence diversity of the selected 5′ region of the cytochrome *c* oxidase subunit I mitochondrial region (COI) *(19, 60)*. Although the idea of using DNA sequence analyses to identify species is not new, the standardization of analysis of sequence diversity in a single gene region for the recognition of species in large groups is new *(20)*. The success of a COI-based identification system is based on the satisfaction of two criteria: (i) the COI sequence for each specimen must be more similar to other COI sequences of that species than to sequences in other species and (ii) there must be a general correspondence between species assignments based on COI divergences and those based on conventional taxonomy. To test these criteria, COI barcodes were generated for all 22 authenticated species of the Dirty 22 currently recognized by the FDA.

MATERIALS AND METHODS

Specimen samples. Insects were obtained from various universities and academic and research institutions. The National Museum of Natural History (Smithsonian Institution, Washington, DC) loaned vouchered specimens *Mus musculus* (USNM 570605), *Rattus exulans* (USNM 583803), *Rattus norvegicus* (USNM 570865), and *Rattus rattus* (USNM 580439) (Table 1). A vouchered specimen is part of or a complete animal carcass in an accessible collection of species and is used as a reference in a study. Vouchered specimens of the insects are stored at the FDA. One insect leg or an approximately 2-mm-diameter piece of muscle (or the entire ant carcass) was used for analysis.

DNA extraction from tissue and insect legs. At the FDA, the Extract-N-Amp tissue PCR kit (Sigma-Aldrich, St. Louis, MO) was used to extract DNA from all samples. Tissue (2 to 10 mg or two or three insect legs) was mixed with 50 μl of extraction solution and 12.5 μl of tissue preparation solution and incubated for 20 min at room temperature and then at 95°C for 5 min. After incubation, 50 μl of neutralization solution B was added to each sample, and the sample was vortexed and centrifuged at ~13,000 rpm for 30 s (Eppendorf Centrifuge 5415D). Ten to 15 μl of the DNA supernatant was removed and placed into a sterile PCR plate and mailed to the University of Guelph for COI PCR amplification and sequencing.

DNA extraction from tissue/insect legs. At the University of Guelph, total genomic DNA was extracted following the

TABLE 2. *Primer workflow for generating DNA barcodes for 21 species of the Dirty 22*

Primer cocktail	Species	Reference(s)
C_LepFolF + C_LepFolR	Brownbanded cockroach, oriental cockroach, American cockroach, pharaoh ant, house fly, stable fly, little house fly, latrine fly, cosmopolitan blue bottle fly, holarctic blue bottle fly, oriental latrine fly, secondary screwworm, blue bottle fly, green bottle fly, black blow fly, house mouse, Polynesian rat	*11, 21*
C_VF1LFt1 + C_VR1LRt1	Norway rat, roof rat	*25*
LepF1 + LepR1; MLepF1 + MLepR1	German cockroach, thief ant, redtailed flesh fly	*14, 21*

semiautomated glass fiber DNA extraction protocol *(22)* with minor modifications. A differential volume of lysis buffer was used to normalize DNA concentration. Tissue was incubated overnight with 50 μl of insect lysis buffer with proteinase K for insect samples and with 100 μl of vertebrate lysis buffer with proteinase K for vertebrate samples. A 50-μl volume of lysate was then transferred to a clean plate and used for semiautomated DNA extraction on the Biomek FX liquid handling station (Beckman Coulter, Palo Alto, CA) using Acroprep 96-well plates with a 3-μm-pore-size glass fiber membrane over a 0.2-μm-pore-size Bioinert membrane. Insect samples were eluted in 40 μl of 10 mM Tris-HCl (pH 8.0), and vertebrate samples were eluted in 50 μl of 10 mM Tris-HCl (pH 8.0).

Additional DNA from ant samples DIRTT089-11 to DIRTT094-11 was extracted using a quick alkaline lysis procedure *(35)*. This procedure works better for small specimens such as a single ant leg. To eliminate possible inhibition effects from the first series of amplifications, alkaline lysates were purified in spin columns (Epoch Biolabs, Sugar Land, TX) following the glass fiber DNA extraction protocol *(22)*.

Primers. Because of the large number of species being processed, several primer pairs were used for PCR amplification of the barcode region of COI (Table 2). Initially, amplification was attempted for all samples with the C_LepFolF + C_LepFolR cocktail (LepF1, LCO1490 forward primers and LepR1, HCO2198 reverse primers mixed in equal ratios *(11, 21)*). The samples that could not be amplified or sequenced with this system were subjected to a second PCR with C_VF1LFt1 + C_VR1LRt1 M13-tailed cocktail *(25)*. Subsequent failures were reprocessed with LepF1 + LepR1 and finally C_LepFolF + MLepR2 (S. Prosser, unpublished private primer in BOLD *(46)*) in combination with MLepF1 *(14)* + C_LepFolR to amplify two overlapping fragments to recover degraded DNA. Thief ant samples were initially amplified using C_LepFolF + C_LepFolR cocktail followed by LepF1 + LepR1; RonMWASPdeg_t1 + LepR1 in combination with LepF1 + C_ANTMR1D *(48)*. Additional primer pairs used on ant samples after purification were C_LepFolF + C_LepFolR followed by LepF1 + LepR1; RonMWASPdeg_t1 + LepR1 in combination with LepF1 + C_ANTMR1D and, finally, LepF1 + enhANTr1 (M. A. Smith, unpublished public primer in BOLD *(46)*).

COI PCR amplification and sequencing. PCR was run using the DNA extracted from each species at the FDA and the three or four DNA samples for each species extracted at the University of Guelph. All PCR had a total volume of 12.5 μl and included 6.25 μl of 10% trehalose, 2.00 μl of ultrapure water, 1.25 μl of 10× PCR Platinum *Taq* buffer (500 mM KCl, 200 mM Tris-HCl [pH 8.4]), 0.625 μl of 50 mM $MgCl_2$ (Invitrogen, Carlsbad, CA), 0.125 μl of each primer (0.01 mM), 0.0625 μl of each deoxynucleoside triphosphate (10 mM), 0.06 μl of Platinum DNA polymerase (Invitrogen), and 2.0 μl of DNA template as described in the Canadian Centre for DNA Barcoding (CCDB) protocols for COI amplification *(23)*. The thermocycle profile in the gradient thermocycler (Mastercycler Eppendorf, Brinkmann Instruments, Westbury, NY) for all PCR (with the exception of that for the thief ant) consisted of 94°C for 1 min; 5 cycles of 94°C for 40 s, 45°C for 40 s, and 72°C for 1 min; 35 cycles of 94°C for 40 s, 51°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. PCR products were visualized on a 2% agarose gel in an E-Gel96 pre-cast agarose electrophoresis system (Invitrogen) and bidirectionally sequenced using corresponding primers with the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3730xl genetic analyzer (Applied Biosystems) as described in the CCDB protocols for sequencing *(24)*. Bidirectional sequences were assembled and aligned with CodonCode (CodonCode Corporation, Dedham, MA) and manually edited. Primer combinations used to amplify each sample, sequence data, electropherograms, and specimen details are all available within the project files (www.boldsystems.org) under ''FDA RFLP Dirty Twenty Two [DIRTT]'' on the BOLD site *(46)*.

Thief ant barcode recovery. The thief ant was dissected under a stereomicroscope, and six separate DNA extractions were performed on various body parts to negate the effects of PCR inhibition due to the high levels of formic acid. The DNA extractions (standard invert lysis as per the protocol via individual spin columns) were done on (i) rinsate from a thief ant rinsed in 10.0 μl of sterile water, (ii) one antenna, (iii) the head with other antenna, (iv) all trunk and petiole with all but two legs, (v) two legs, and (vi) gastor. Each DNA extract was first amplified with four different primer sets of varying lengths: C_LepFolF + C_LepFolR, LepF1 + C_ANTMR1D, RonWASPdeg_t1 + LepR1, and LepF1 + C113R; this approach helps accommodate for DNA degradation. DNA extracts from each body part with each primer set were amplified at 2.0 μl, 2.0-μl (1:10) dilution, and 2.0-μl (1:100) dilution to further negate PCR inhibitory effects. The same DNA extracts were amplified a second time using LepF1 + LepR1 and a more stringent PCR profile of 94°C for 1 min; 40 cycles of 94°C for 40 s, 51°C for 40 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. All PCR products were visualized on E-gels and sequenced as described.

RESULTS AND DISCUSSION

Morphology (the appearance and structure of an organism) can be utilized for species identification, but this method has disadvantages *(44)*. Sibling species (morphologically similar but reproductively isolated) identification can be complicated, and important morphological characters

may be missing when there is only a small amount of biological material available.

Molecular techniques have been widely used for the identification of various organisms to the species level *(2, 30, 37, 38, 45, 56, 61)*; however, methods for the identification of regulated pest species must meet certain criteria. Immunological *(52, 53)* or protein-based *(34, 49)* methods are highly taxon specific, too complicated for use in a regulatory setting, vulnerable to environmental factors, and dependent on good quality or fresh tissue. Regulatory assays must be quick, easy to perform, and inexpensive to operate. In most cases, regulatory work will be done by a trained technician who is not necessarily an expert. Most molecular diagnostic methods include PCR-based DNA analyses, which are not affected by these limitations *(5)*. Molecular methods for species identification have several advantages: they are fast, accurate, cost-efficient, and reliable. Molecular analyses have been a popular alternative to conventional morphology-based species identification. For more than 20 years, allozyme and DNA sequence data have been used to elucidate taxonomic relationships in groups in which morphology-based approaches are difficult *(1, 18, 36, 42)*. Even for groups with well-established taxonomy, molecular approaches have frequently been applied in situations in which traditional methods provide inconclusive data, e.g., linking various life stages *(7, 43),* recognizing prey items in predator gut contents *(47, 51),* and identifying tissue remains *(27, 29, 31)*. Similar methods also have been recognized for their potential in forensic applications within the marketplace, e.g., forensically informative nucleotide sequencing *(6)*. The molecular assay approach taken in the present study was designed to complement morphological taxonomy used for species identification.

This study was conducted to investigate the principal DNA barcoding function of assigning an unknown sample to a known species. Criticisms have been made regarding the inability of DNA barcoding to assign an unknown sample to a known species and the ability to detect previously unsampled species as distinct *(9, 13, 32, 58)*. The criticism is justified where taxonomic scrutiny has not been thorough and species recognition is limited to a few traditional character sets untested by additional studies and tools *(33)*. We recognize that the Dirty 22 database does not contain a complete collection of all sister taxa, but that lack does not necessarily preclude the effectiveness of a DNA barcoding system for accurate species identification. The data support our claim that the regulatory application of DNA barcoding could be valuable because the species involved are well-known, comprehensively sampled groups of insects *(26, 59)* and mammals *(15, 16, 28)* that have been extensively characterized by both genetic and morphological methods.

Despite the criticisms, DNA barcoding has shown its utility for species identification based on the COI gene. Because no two species are assumed to share an identical COI sequence, with rare exceptions of introgression and hybridization, the efficiency of a DNA barcoding system functions on this basis. The assumption is that the COI sequences of individuals in the same species will more closely resemble one another than they will resemble the sequences of closely related sister taxa. Results of previous studies *(19, 60)* support the use of the barcode data for regulatory applications. In these studies, each species sequenced possessed a unique and diagnostic COI sequence, which acted as a reference library for species identification. The patterns of sequence divergence reported in the present study support the effectiveness of COI as a suitable marker for distinguishing the FDA's Dirty 22.

DNA was extracted from all specimens, and PCRs were performed four or five times to ensure experiment repeatability. The DNA extraction at the FDA was a control method used as a basis to ensure that specimen samples sent to the University of Guelph were not mislabeled or contaminated. Regardless of the DNA extraction method, the same DNA sequences were obtained using the same primer sets.

DNA barcodes of the Dirty 22 were generated successfully; however, unique protocols were used to obtain DNA barcodes from the thief ant and the German cockroach. The thief ant required a special protocol to extract DNA from several different body parts. Sequencing of the PCR products generated from the first amplification attempt resulted in a mixed signal or two types of poor quality sequences slightly different from one another (e.g., dilutions of the head extract yielded two sequences that both matched *Solenopsis molesta* at either 100 or 98.85%), suggesting the presence of nuclear pseudogenes (genomic DNA sequences similar to normal genes but nonfunctional). The second amplification attempt with more stringent primers and thermocycling conditions yielded four types of clean sequences: 658-bp sequence of *S. molesta* from the head (LepF1 + LepR1 primers), collembolan sequence (*Lepidocyrtus cyaneus,* possibly prey) from the gastor (LepF1 + LepR1 primers), 657-bp pseudogene sequence with stop codons from antenna, legs, and all trunk and petiole (LepF1 + LepR1 and MLepF1 + MLepR1 primers), and short 168-bp nonspecific sequence from head and antenna (MLepF1 + MLepR1 primers). Pseudogene sequencing still enabled correct species identification with a 98.7% match to *S. molesta*. The German cockroach yielded only a 238-bp product because of coamplification of a shorter nonspecific PCR product, possibly a nuclear pseudogene. However, even short DNA barcode sequences have been sufficient for species discrimination *(17)*.

The simplicity of the primer workflow (Table 2) used to generate DNA barcodes and the development of a regulatory database using vouchered specimens makes this approach potentially useful for either supplementing or replacing microscopy. Future goals are to expand the COI database to generate a comprehensive library of DNA barcodes of other common pests that contribute to the spread of foodborne disease. DNA barcoding has the potential to be a useful regulatory tool for the identification of the Dirty 22 with the goal of protecting the U.S. food supply by preventing the spread of foodborne pathogens and disease.

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REFERENCES

1. Allander, T., S. Emerson, R. Engle, R. Purcell, and J. Bukh. 2001. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. *Proc. Natl. Acad. Sci. USA* 98:11609–11614.
2. Anderson, D., and M. Morgan. 2007. Genetic and morphological variation of bee-parasitic *Tropilaelaps* mites (Acari: Laelapidae): new and re-defined species. *Exp. Appl. Acarol.* 43:1–24.
3. AOAC International. 2006. Official method 970.66: light and heavy filth, p. 2. *In* Official methods of analysis. AOAC International, Gaithersburg, MD.
4. AOAC International. 2010. Official method 945.75: extraneous materials (foreign matter) in products, p. 4. *In* Official methods of analysis. AOAC International, Gaithersburg, MD.
5. Armstrong, K., and S. Ball. 2005. DNA barcodes for biosecurity: invasive species identification. *Philos. Trans. R. Soc. B Biol. Sci.* 360: 1813–1823.
6. Bartlett, S., and W. Davidson. 1992. FINS (forensically informative nucleotide sequencing)—a procedure for identifying the animal origin of biological specimens. *BioTechniques* 12:408–411.
7. Besansky, N., D. Severson, and M. Ferdig. 2003. DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends Parasitol.* 19:545–546.
8. Buzby, J., P. Frenzen, and B. Rasco. 2001. Product liability and microbial foodborne illness. U.S. Department of Agriculture, Washington, DC.
9. Ebach, M., and C. Holdrege. 2005. DNA barcoding is no substitute for taxonomy. *Nature* 434:697.
10. Falkenstein, D. 2011. Lettuce, spinach and *E. coli*: the devastating fall of 2006. *Food Poison J*. Available at: http://www.foodpoisonjournal.com/foodborne-illness-outbreaks/lettuce-spinach-and-e-coli-the-devastating-fall-of-2006/. Accessed April 2012.
11. Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3:294–297.
12. Graczyk, T., R. Knight, and L. Tamang. 2005. Mechanical transmission of human protozoan parasites by insects. *Clin. Microbiol. Rev.* 18:128–132.
13. Gregory, T. 2005. DNA barcoding does not compete with taxonomy. *Nature* 434:1067.
14. Hajibabaei, M., D. Janzen, J. Burns, W. Hallwachs, and P. Hebert. 2006. DNA barcodes distinguish species of tropical Lepidoptera. *Proc. Natl. Acad. Sci. USA* 103:968–971.
15. Hajibabaei, M., G. Singer, E. Clare, and P. Hebert. 2007. Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. *BMC Biol.* 5:24.
16. Hajibabaei, M., G. Singer, and D. Hickey. 2006. Benchmarking DNA barcodes: an assessment using available primate sequences. *Genome* 49:851–854.
17. Hajibabaei, M., M. Smith, D. Janzen, J. Rodriguez, J. Whitfield, and P. Hebert. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. *Mol. Ecol. Notes* 6:959–964.
18. Hamels, J., L. Gala, S. Dufour, P. Vannuffel, N. Zammatteo, and J. Remacle. 2001. Consensus PCR and microarray for diagnosis of the genus *Staphylococcus* species, and methicillin resistance. *BioTechniques* 31:1364–1372.
19. Handy, S., J. Deeds, N. Ivanova, P. Hebert, R. Hanner, A. Ormos, L. Weigt, M. Moore, and H. Yancy. 2011. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *J. AOAC Int.* 94:201–210.
20. Hebert, P., A. Cywinska, S. Ball, and J. DeWaard. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B Biol. Sci.* 270:313–321.
21. Hebert, P., E. Penton, J. Burns, D. Janzen, and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc. Natl. Acad. Sci. USA* 101:14812–14817.
22. Ivanova, N., J. Dewaard, and P. Hebert. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Mol. Ecol. Notes* 6:998–1002.
23. Ivanova, N., and C. Grainger. 2007. CCDB protocols: COI amplification. Available at: http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Amplification.pdf. Accessed 15 July 2010.
24. Ivanova, N., and C. Grainger. 2007. CCDB protocols: sequencing. Available at: http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Sequencing.pdf. Accessed 27 July 2010.
25. Ivanova, N., T. Zemlak, R. Hanner, and P. Hebert. 2007. Universal primer cocktails for fish DNA barcoding. *Mol. Ecol. Notes* 7:544–548.
26. Jung, S., R. Duwal, and S. Lee. 2011. COI barcoding of true bugs (Insecta, Heteroptera). *Mol. Ecol. Resour.* 11:266–270.
27. Karaiskou, N., A. Triantafyllidis, and C. Triantaphyllidis. 2003. Discrimination of three *Trachurus* species using both mitochondrial- and nuclear-based DNA approaches. *J. Agric. Food Chem.* 51:4935–4940.
28. Lorenz, J., W. Jackson, J. Beck, and R. Hanner. 2005. The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. *Philos. Trans. R. Soc. B Biol. Sci.* 360:1869–1877.
29. Mackie, I., S. Pryde, C. Gonzales-Sotelo, I. Medina, R. Perez-Martin, J. Quinteiro, M. Rey-Mendez, and H. Rehbein. 1999. Challenges in the identification of species of canned fish. *Trends Food Sci. Technol.* 10:9–14.
30. Mahani, M., N. Inomata, A. Saboori, B. Tabatabaei, H. Ishiyama, A. Ariana, and A. Szmidt. 2009. Genetic variation in populations of *Allothrombium pulvinum* (Acari: Trombidiidae) from northern Iran revealed by mitochondrial *coxI* and nuclear rDNA ITS2 sequences. *Exp. Appl. Acarol.* 48:273–289.
31. Marko, P., S. Lee, A. Rice, J. Gramling, T. Fitzhenry, J. McAlister, G. Harper, and A. Moran. 2004. Mislabelling of a depleted reef fish. *Nature* 430:309–310.
32. Marshall, E. 2005. Taxonomy—will DNA bar codes breathe life into classification? *Science* 307:1037.
33. Meyer, C., and G. Paulay. 2005. DNA barcoding: error rates based on comprehensive sampling. *PLoS Biol.* 3:2229–2238.
34. Miles, S. 1979. Biochemical key to adult members of the *Anopheles gambiae* group of species (Diptera, Culicidae). *J. Med. Entomol.* 15: 297–299.
35. Montero-Pau, J., A. Gomez, and J. Munoz. 2008. Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. *Limnol. Oceanogr. Methods* 6:218–222.
36. Nanney, D. 1982. Genes and phenes in *Tetrahymena*. *BioScience* 32: 783–788.
37. Navajas, M., J. Lagnel, G. Fauvel, and G. De Moraes. 1999. Sequence variation of ribosomal internal transcribed spacers (ITS) in commercially important Phytoseiidae mites. *Exp. Appl. Acarol.* 23: 851–859.
38. Navajas, M., A. Tsagkarakov, J. Lagnel, and M. Perrot-Minnot. 2000. Genetic differentiation in *Tetranychus urticae* (Acari: Tetranychidae):

polymorphism, host races or sibling species? *Exp. Appl. Acarol.* 24: 365–376.
39. Neale, T. 2009. Medical news: poultry, fruit, veggies lead the way in foodborne illness. *MedPage Today*. Available at: http://www.medpagetoday.com/PublicHealthPolicy/PublicHealth/14655. Accessed April 2012.
40. Olsen, A. 1998. Regulatory action criteria for filth and other extraneous materials. III. Review of flies and foodborne enteric disease. *Regul. Toxicol. Pharmacol.* 28:199–211.
41. Olsen, A., J. Gecan, G. Ziobro, and J. Bryce. 2001. Regulatory action criteria for filth and other extraneous materials. V. Strategy for evaluating hazardous and nonhazardous filth. *Regul. Toxicol. Pharmacol.* 33:363–392.
42. Pace, N. 1997. A molecular view of microbial diversity and the biosphere. *Science* 276:734–740.
43. Paquin, P., and M. Hedin. 2004. The power and perils of 'molecular taxonomy': a case study of eyeless and endangered Cicurina (Araneae: Dictynidae) from Texas caves. *Mol. Ecol.* 13:3239–3255.
44. Pereira, F., J. Carneiro, and A. Amorim. 2008. Identification of species with DNA-based technology: current progress and challenges. *Recent Pat. DNA Gene Seq.* 2:187–199.
45. Potenza, L., M. Cafiero, A. Camarda, G. La Salandra, L. Cucchiarini, and M. Dacha. 2009. Characterization of *Dermanyssus gallinae* (Acarina: Dermanissydae) by sequence analysis of the ribosomal internal transcribed spacer regions. *Vet. Res. Commun.* 33:611–618.
46. Ratnasingham, S., and P. Hebert. 2007. BOLD: the barcode of life data system (www.barcodinglife.org). *Mol. Ecol. Notes* 7:355–364.
47. Rosel, P., and T. Kocher. 2002. DNA-based identification of larval cod in stomach contents of predatory fishes. *J. Exp. Mar. Biol. Ecol.* 267:75–88.
48. Smith, P. J., S. M. McVeagh, and D. Steinke. 2008. DNA barcoding for the identification of smoked fish products. *J. Fish Biol.* 72:464–471.
49. Soares, R., M. Santanna, N. Gontijo, A. Romanha, L. Diotaiuti, and M. Pereira. 2000. Identification of morphologically similar *Rhodnius* species (Hemiptera: Reduviidae: Triatominae) by electrophoresis of salivary heme proteins. *Am. J. Trop. Med. Hyg.* 62:157–161.
50. Sulaiman, I., M. Anderson, M. Khristova, K. Tang, N. Sulaiman, E. Phifer, S. Simpson, and K. Kerdahi. 2011. Development of a PCR–restriction fragment length polymorphism protocol for rapid detection and differentiation of four cockroach vectors (group I ''Dirty 22'' species) responsible for food contamination and spreading of foodborne pathogens: public health importance. *J. Food Prot.* 74: 1883–1890.
51. Symondson, W. 2002. Molecular identification of prey in predator diets. *Mol. Ecol.* 11:627–641.
52. Symondson, W., T. Gasull, and J. Liddell. 1999. Rapid identification of adult whiteflies in plant consignments using monoclonal antibodies. *Ann. Appl. Biol.* 134:271–276.
53. Trowell, S., N. Forrester, K. Garsia, G. Lang, L. Bird, A. Hill, J. Skerritt, and J. Daly. 2000. Rapid antibody-based field test to distinguish between *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Helicoverpa punctigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 93:878–891.
54. U.S. Food and Drug Administration. 1984. Requirements of laws and regulations enforced by the U.S. Food and Drug Administration. U.S. Food and Drug Administration, Washington, DC.
55. U.S. Food and Drug Administration. 2002. CPG Sec. 555.600. Filth from insects, rodents, and other pests in foods. U.S. Food and Drug Administration, Washington, DC.
56. Vargas, M., M. Garcia-Varela, J. Laclette, and T. Perez. 2005. Application of ITS-2 sequences as markers for identification and phylogenetic inference within the genus *Geomylichus* (Acari: Listrophoridae). *Exp. Appl. Acarol.* 35:223–238.
57. Whitworth, T. L. 2010. Keys to the genera and species of blow flies (Diptera: Calliphoridae) of the West Indies and description of a new species of *Lucilia* Robineau-Desvoidy. *Zootaxa* 2663:1–35.
58. Will, K., and D. Rubinoff. 2004. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics* 20:47–55.
59. Wilson, J. 2012. DNA barcodes for insects. *Methods Mol. Biol.* 858: 17–46.
60. Yancy, H., T. Zemlak, J. Mason, J. Washington, B. Tenge, N. Nguyen, J. Barnett, W. Savary, W. Hill, M. Moore, F. Fry, S. Randolph, P. Rogers, and P. Hebert. 2008. Potential use of DNA barcodes in regulatory science: applications of the Regulatory Fish Encyclopedia. *J. Food Prot.* 71:210–217.
61. Zahler, M., and R. Gothe. 1997. Evidence for the reproductive isolation of *Dermacentor marginatus* and *Dermacentor reticulatus* (Acari: Ixodidae) ticks based on cross-breeding, morphology and molecular studies. *Exp. Appl. Acarol.* 21:685–696.