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Barcode BULLETIN

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DNA Barcoding Uncovers the True Toll of Wind Turbines on Bats

Policy

Access and
Benefit-sharing
Considerations
for Barcoders

Research

Colour
Patterns
in Arctic
Bumblebees



News Briefs

Welcome to our March 2016 issue of the Barcode Bulletin.

This issue once again features a wide assortment of articles on DNA barcoding research and applications, with topics ranging from high-level school projects to applications in conservation, freshwater assessment, and saffron identification. Add exciting research and some important advice on access and benefit-sharing and you have yet another diverse issue of our quarterly newsletter.

Enjoy reading.

Dirk Steinke
Editor-in-chief

By creating an international network of researchers, water managers, politicians, and other stakeholders, a recently funded EU COST Action will support the development of novel genomic tools for biodiversity assessments in European water bodies. This EU COST Action will involve a number of iBOL researchers and will allow for a concerted effort in the protection of aquatic ecosystems. For more information about this project, [click here](#).

The sixth offering of the DNA Metabarcoding Spring School will be held in Kunming, China from August 22 to 26, 2016. It is co-organized by the metabarcoding.org team and the Kunming Institute of Zoology, with support from the Chinese Academy of Sciences, Yunnan. The course is divided into two parts, starting with two days of lectures with no limit on the number of participants, and followed by three days of practical training with participation limited to 24. For more information and to apply, [click here](#).

From April 4 to 5, 2016, the Museum of Natural History in Geneva, Switzerland will host a workshop on the application of genomic tools for the biomonitoring of marine environments. The workshop will highlight ecological, legal, and socio-economic perspectives, and participants will learn about the advantages and challenges of the eDNA method. The workshop is free and open to everyone. For more information, [click here](#).

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Barcoding's Role in Bat Fatality Monitoring at Wind Farms

Written by: Dean Williams, Jennifer Korstian, Victoria Bennett, and Amanda Hale (Texas Christian University, United States)

Bats are killed in large numbers by wind turbines each year, and yet, we do not have a full understanding of which factors are contributing to bats coming into contact with wind turbines. Data collected from fatality searches can be used to determine the vulnerability of different species, sexes, and ages. Nevertheless, this depends upon reliable identification of carcasses based on morphology, and this method may not be possible for partial remains or badly decomposed carcasses. Incorrect identification decreases the amount of useable data and can have potentially serious implications when threatened and endangered species are involved (e.g. the Indiana bat, *Myotis sodalists*, the northern long-eared bat, *Myotis septentrionalis*, and the Hawaiian hoary bat, *Lasiurus cinereus semotus*).

The incorrect identification of bats killed by wind turbines "...can have potentially serious implications when threatened and endangered species are involved..."

In this study, we wanted to determine the reliability of DNA barcoding to identify all 47 known bat species in the United States and Canada, and to assess whether it can improve the identification of bat carcasses salvaged at wind farms. Using barcode sequences from GenBank and sequences we generated ourselves, we found that 80% (N = 38) of bat species had distinct barcodes. The remaining 20% (N = 9) of these species belonged to two *Myotis* species complexes. The species within these complexes have traditionally been difficult to separate using only mtDNA sequence data, which suggests that these species have either recently diverged or represent a single phenotypically variable species. The use of an additional nuclear DNA locus or, in some cases, geography could help to identify these nine species.

We then applied DNA barcoding to samples taken from 892 bats salvaged from wind farms across four states. We identified six species, with the three migratory tree bat species comprising the majority of samples (722 specimens of *L. borealis*, *L. cinereus*, and *Nycticeius humeralis*). We were able to identify 14 carcasses to species that could not be identified in the field due to extensive decomposition and scavenging, and determined that another 18 carcasses had been misidentified in the field.

Bat identification based on DNA barcodes indicated that uncommon species (e.g. *Lasionycteris noctivagans*) can easily be misidentified in the field, as well as species with

“We found that species identification based on morphology in the field is most reliable for fresh carcasses...” but “daily fatality monitoring is economically unfeasible.”

similar body size and coloration (e.g. *N. humeralis* and *Perimyotis subflavus*, small-bodied bats with similar pelage).

We found that species identification based on morphology in the field is most reliable for fresh carcasses (i.e. bats ≤ 1 day old), with a higher likelihood of

degradation in carcasses left for longer periods. This is particularly relevant as daily fatality monitoring is economically unfeasible, and so searches often occur at intervals of four or more days.

Our study therefore indicates that DNA barcoding has practical applications for bat conservation, such as identifying remains from wind turbine collisions, remains from hibernacula, and fecal samples.

For more information on the results discussed in this article, see DOIs: [10.1007/s12686-015-0509-4](https://doi.org/10.1007/s12686-015-0509-4), [10.1007/s12686-015-0488-5](https://doi.org/10.1007/s12686-015-0488-5), [10.1111/1755-0998.12118](https://doi.org/10.1111/1755-0998.12118)

Images by Victoria Bennett and Amanda Hale.



Above: Wing membrane samples.

Below: Fresh and partially decomposed bat carcasses.



Highlighting Earthworm Biodiversity Hotspots in French Guiana

Written by: Tribaud Decaëns, David Porco (CEFE, University of Montpellier, France), Samuel W. James (University of Iowa, United States), Elodie Da Silva (EMBRAPA Florestas, Brazil), Lise Dupont, Virginie Roy (IEES, Université Paris Est Créteil, France), Emmanuel Lapiéd (NIBIO, Norwegian University of Life Sciences, Norway), and Rodolphe Rougerie (ISYEB, Muséum national d'Histoire naturelle, France)

Earthworms usually represent a significant, if not the dominant, part of the soil biomass. They are involved in a number of soil processes of vital importance for terrestrial ecosystems and play a major role in agroecosystems, where they improve soil fertility and plant growth. In different parts of the world, several earthworm species have become invasive, with significant impact on their new environment. Nevertheless, earthworm taxonomy remains largely unresolved, and the nearly 6000 species currently recognized worldwide probably represent, at best, half of the actual biodiversity of the group. This taxonomic deficit is particularly critical in the tropics, resulting in questionable species identifications and a lack of ecological studies of earthworm communities.

In 2010, we conducted a first survey of the earthworm communities at two sites of the Nouragues' natural reserve in French Guiana (supported by a CNRS grant). A rapid screening of earthworm communities in a selection of habitats was achieved by using a standardized protocol based on the systematic harvesting of specimens in all types of microhabitats available in a 1 ha area. DNA barcodes obtained for a selection of specimens sorted by morphospecies were clustered to delimit molecular operational taxonomic units (MOTUs),

the number and composition of which was further used to describe species richness and community patterns at different spatial scales. During the following six years, the same approach was used at six additional study sites (DIADEMA project of the Labex CEBA), resulting in the production of 2826 additional DNA barcodes representing 166 MOTUs within 5 families. This represents an unprecedented sampling effort, and a great improvement of our knowledge of regional diversity, when compared to the 22 species that were reported for French Guiana in a recent checklist.

Beta-diversity among sites was high, with up to 70% of the MOTUs only found in a single study site. While part of this variability could be attributed to the high proportion of singletons in our dataset, another part certainly results from the high rate of micro-endemism expected in low-dispersing organisms such as earthworms. As a consequence, the number of species accumulates steadily with the number of study sites sampled, and a rough estimate suggests that at least 400 species can be found in French Guiana. This region of the Amazonian forests could therefore represent one of the richest hotspots of earthworm diversity, and additional research is critically needed to progress toward documenting the actual number of species in this region.

“Moreover, our results offer a unique opportunity to scrutinize the structure of tropical earthworm communities at a local scale.”

Despite the restricted distribution of most species, a few exotic ones were observed in French Guiana. This includes the pantropical species *Pontoscolex corethrurus* (Müller, 1857), which originated at the Guiana Shield but is known to have

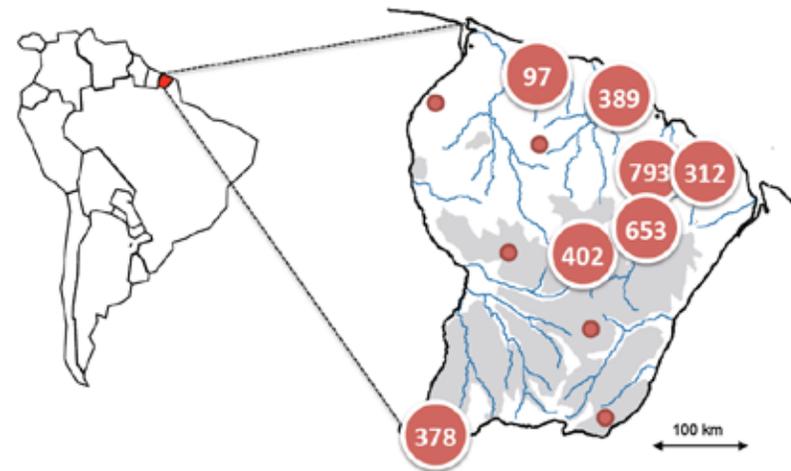


Image credit: T. Decaëns

Above: A striking – yet undescribed – 70-centimetre-long yellow earthworm from the genus *Martiodrilus*. It was found associated with old termite mounds and only in two southern localities of French Guiana to date.

invaded the whole intertropical region. More surprisingly, we also found a few African representatives from the genus *Dichogaster*, even in remote forests deprived of any current anthropogenic activities. This enigmatic distribution could be due to ancient movements of human populations between the littoral and the south of French Guiana.

Moreover, our results offer a unique opportunity to scrutinize the structure of tropical earthworm communities at a local scale. For instance, assemblages are dominated by specialists, with only a small fraction of generalists able to colonize a broad range of habitats or microhabitats. In Nouragues, we found 17 MOTUs on epiphytic plants, which exemplify the extraordinary ability of some species to colonize vacant microhabitats even by climbing into trees. The number of species co-existing in a given habitat never exceeded 15 MOTUs, suggesting that interspecific competition may drive niche saturation during the process of community assembly.

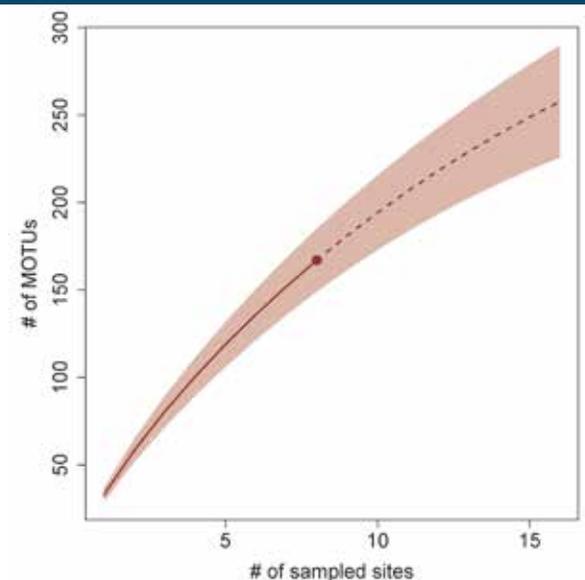


Above: Distribution of study sites in French Guiana, including the number of specimens with DNA barcodes for each site. Small dots represent sites that will be sampled over the course of 2016.

Below: Rarefaction curve showing how MOTUs accumulate with an increasing number of study sites (solid line); extrapolation (dotted line) is shown for a hypothetical duplication of the sampling effort.

The ongoing development of a functional trait database will allow the combination of functional, phylogenetic, and taxonomic diversity data in order to disentangle the relative contributions of habitat filters, biotic interactions, and neutral processes in the structuring of earthworm communities in the rainforest of French Guiana.

For more information about the results discussed in this article, see DOIs: [10.1016/j.ejsobi.2012.09.001](https://doi.org/10.1016/j.ejsobi.2012.09.001), [10.1016/j.soilbio.2015.10.009](https://doi.org/10.1016/j.soilbio.2015.10.009)





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ABS for DNA Barcoders, Part 1: Alphabet Soup

Written by: Kate Davis (Independent ABS Consultant, Canada) and Dirk Neumann (Zoologische Staatssammlung München, Germany)

DNA barcoding can rather resemble cooking, with shopping lists and cookbooks for the lab or recipes for DNA clean-up. Barcoding also combines many actions – field collecting, sampling, sequencing, transfer between labs, publishing research results – but the Convention on Biological Diversity (CBD) and its new ancillary Nagoya Protocol (NP) require us to update some of our traditional recipes. Their provisions on ‘access to genetic resources and benefit-sharing’ (ABS) call for new spices, some hot, some bitter, some sweet – with the intention of achieving a balanced finish.

The CBD recognises that countries hold sovereign rights over their biological resources; they can choose to regulate or to grant free access to the genetic resources (GRs) within their borders. The CBD commits nations to three objectives: biodiversity conservation; sustainable use of biological resources; and, in an effort to balance the costs of conservation and support socio-

economic development of the biodiverse Global South, the fair and equitable sharing of benefits that arise from utilisation of GRs. The CBD also introduces the concepts of *Prior Informed Consent* (PIC) for access and *Mutually Agreed Terms* (MAT) between providers and users for how material may be accessed and used, and what benefits may be shared.

DNA barcoders need to heed the Convention on Biological Diversity, the Nagoya Protocol, and resulting national laws.

Finding the right balance has been problematic. CBD Parties (ratifying countries) now number 196, excluding only the USA and the Vatican among UN-recognised states, and they have taken differing approaches. Following the coming into force of the

CBD in 1993, numerous biodiverse countries introduced laws to regulate access, but the expected partnerships and benefits largely failed to materialise; frustrations and suspicions grew on all sides with the lack of clarity. Soon after the helpful but voluntary ‘Bonn Guidelines’ were adopted, years of negotiations began towards a legally-binding solution.

The NP was established to provide both transparency and force, obliging countries to set out clear measures on access, benefit-sharing and – a new feature – user compliance with providers' national laws. The NP entered into force on 12 October 2014 with the ratification of 50 states and the European Union. EU and Swiss ABS legislation came into force on the same date, marking for the first time a move en masse by industrialised countries to ensure that users within their borders utilise GRs in compliance with provider countries' laws.

DNA barcoders take note: the CBD's scope covers all biological material except human GRs and GRs collected beyond national jurisdiction (e.g. in the high seas), and includes those associated with other samples (e.g. plague strains that might sit on anthropological objects). The NP broadly defines '*utilisation*' as 'to conduct research and development on the genetic and/or biochemical composition of genetic resources, including through the application of biotechnology...' In the

barcoding context, genes, PCR products, and other DNA isolates or derivatives are all likely to be considered GRs by most countries, and DNA barcoding to be considered '*utilisation*.'

On the bright side, after sustained engagement by research institutions in the ABS negotiations, the NP requires countries to create conditions to encourage conservation-supporting research via 'simplified measures' on access for non-commercial research, taking into account possible changes of intent. The CBD's Global Biodiversity Outlook recognises the important trend towards data and information sharing, including networks to promote and facilitate access to natural history collections and the development of DNA barcoding as a means of identifying species. Thus, there is some hope that non-commercial DNA barcoding and biodiversity research will be treated more kindly in some countries.

Stay tuned for the second part of this article in the June 2016 issue. It will introduce a few more ingredients to the mix.

Butter bei die Fische. Adding content to the soup: taxonomic training with fishery experts in Sudan.



Investigating Ant Biodiversity in the Big City

Written by: Akansha Thakur, Indu Puthenkalam, and Ilona Petrychyn (Forest Hills High School, New York, United States)

In the summer of 2015, we participated in a two-week program with the Urban Barcoding Project in New York City. Along with our biology research teacher, we went to the Environmental Study Center, a special lab facility funded by the New York City Department of Education. There, we worked with Dr. Christine Marizzi from Cold Spring Harbor Laboratory to learn the fundamental steps of DNA barcoding. We went into this program with very limited knowledge, hoping to learn more about the barcoding process and its applications for real-world problems.

At the Environmental Study Center, Dr. Marizzi introduced us to the idea of using barcoding as a conservation genetics tool. We learned about a study by Pećarević *et al.* (2010, DOI: [10.1371/journal.pone.0013222](https://doi.org/10.1371/journal.pone.0013222)) that used barcoding to study the biodiversity of ants within small medians in Manhattan. After analyzing this article, we decided to further investigate ants because of their important role in ecosystems and because they are difficult to identify morphologically.

Dr. Marizzi took us to Marine Park, a nearby salt marsh of which only a small section had undergone ecological restoration. We then designed a project to determine how the level of restoration in a salt marsh affects ant biodiversity. We hypothesized that there would be higher ant biodiversity in the restored part of the salt marsh. Such information then could be used to stress the importance of efficient salt marsh ecosystems.

We collected ants by baiting them with Pecan Sandies cookies. Specimens were obtained from 20 sites in total from the restored and non-restored sections of the park. We then took the ants back to the Environmental Study Center and extracted their DNA by following the barcoding protocol. Successfully extracted and amplified DNA was sent for sequencing and paired with known sequences in GenBank using the *DNA Subway* website (<http://dnasubway.iplantcollaborative.org/>).

“We then designed a project to determine how the level of restoration in a salt marsh affects ant biodiversity.”

We concluded that the level of restoration played a small role in ant biodiversity. We found the same number of species on both sides of the marsh, contradicting our previous assumptions. We also noticed that there was no overlap in the species found on either side of the salt marsh, which was surprising since the two sides were in such close proximity. Finally, our results showed that the percentage of invasive species on both sides was approximately the same. This finding was also unexpected as we had hypothesized that the restored area would have fewer invasive species. While intriguing, our conclusions were limited by the small sample size.

Although “we found the same number of species on both sides of the marsh”, all of the species were unique to either the restored or the non-restored side.

Over the course of this project, we learned that science does not always provide us with definitive answers. It was often hard to tell whether or not we had successfully extracted DNA from the ants. We also had a hard time matching our sequences with those already in the GenBank database. We noticed that there were many mismatches between our DNA samples and previously published sequences, which made it difficult to obtain an exact identification for each ant. In fact, four of the sequences we obtained were eventually

published in GenBank, which will allow researchers to access these sequences when trying to identify their own DNA samples.

We would like to thank the Environmental Study Center and Dr. Christine Marizzi for guiding us through this project. We would also like to thank our research teacher, Ms. Camila Lock, as well as the administration at Forest Hills High School, for allowing us to have such an opportunity.

Images by Camila Lock.





Image credit: Romana Salis

Barcoding Mayflies to Improve Stream Quality Assessments

Written by: Jan N. Macher and Florian Leese (Ruhr University Bochum and University of Duisburg-Essen, Germany)

Streams and rivers are highly important ecosystems, providing people with drinking water and food, serving as transport routes in all parts of the world, and harbouring highly diverse plant and animal communities. Due to the increasingly intense use of freshwaters and their surrounding areas for agriculture and transport, riverine ecosystems are often subject to multiple anthropogenic stressors, e.g. chemical inputs, eutrophication, and high fine sediment loads as a result of increased erosion. All of these stressors have been shown to strongly impact biodiversity in streams.

To monitor these impacts, many countries around the globe have set up surveillance programs for their freshwater ecosystems. These programs often aim to identify changes of so-called biological quality elements, such as the macroinvertebrate

community living in the stream, and to evaluate the ecological status of a stream by assigning ecological values to species.

Assessment results based on higher-level taxonomic data can be biased because “even closely related species can differ greatly in their ecological demands.”

However, exact information on the number, identification, and ecology of species is scarce, even in relatively well-studied regions. Thus, higher-level taxonomic groups (e.g. genus or family) are often used for practical reasons when identifying animals.

This approach can greatly bias the results of assessments because even closely related species can differ greatly in their ecological demands. Species-level data are thus urgently needed to improve bioassessments of streams and rivers. DNA barcoding can reliably generate these species-level data, subsequently allowing the study of species responses to stressors in their environment, and thus improving our understanding of their ecologies.

In our study (DOI: [10.1016/j.ecolind.2015.08.024](https://doi.org/10.1016/j.ecolind.2015.08.024)), we investigated the impacts of two agricultural stressors, fine sediment and nutrient levels, on members of the common mayfly genus *Deleatidium* in the heavily farmed Southland region of New Zealand. After sampling 43 streams that span a wide gradient from near-pristine to

“We reassessed stressor responses of *Deleatidium* in light of these new data and found highly opposing patterns...”

heavily impacted, we first used morphological methods to determine the number of *Deleatidium* species, and we were able to identify only one morphotype. We subsequently sequenced the standard COI barcoding marker for 520 *Deleatidium* specimens to determine if overlooked species were present at the sampling sites. In doing so, we greatly increased the accuracy of identification by identifying 12 molecular clades, which most likely represent distinct species.

We reassessed stressor responses of *Deleatidium* in light of these new data and found highly opposing patterns: while, at the genus level, the abundance of *Deleatidium* was independent of nutrient and fine sediment loads in the water, some of the molecularly identified species showed strong differences in the magnitude and direction of their responses to stressors. The most abundant species, which we informally named Clade 1, showed no response to either nutrients or fine sediment. However, Clade 2 abundance strongly decreased with increasing nutrient load, and Clade 3 abundance decreased strongly with higher fine sediment levels in the stream.

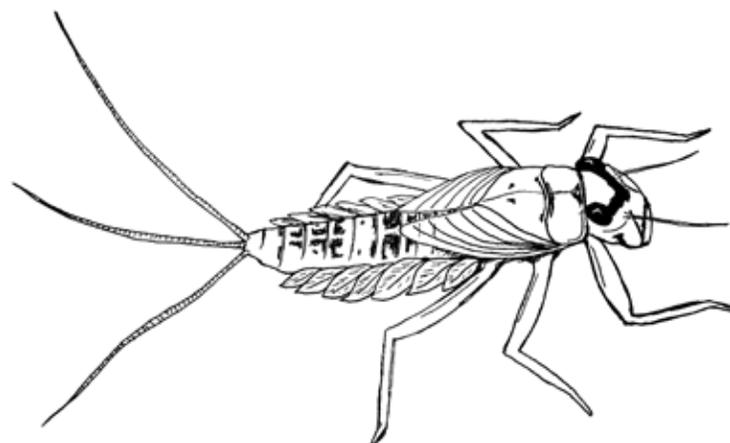
Our findings show that even closely related freshwater invertebrate species can differ substantially in both stressor tolerance and ecology. Thus, working at the genus level can lead to wrong and potentially costly bioassessment results, e.g. if restoration programs are started based on erroneous results. Our study thus highlights the importance of including DNA barcoding in routine freshwater ecosystem assessments and biomonitoring programs.



Image credit: Florian Leese



Image credit: Jan Macher

Above: Close-up of the head of *Deleatidium*.Below: Drawing of a mayfly larva of the genus *Deleatidium* by Romana Salis (University of Otago).

Unidentified *Ptychoptera* species.

Can DNA Barcoding Solve Nomenclatural Problems?

Written by: Gunnar Mikalsen Kvirfte (Universities of Bergen, Norway and Kassel, Germany)

No, but it can solve taxonomic problems, which is more important and can sometimes illuminate nomenclatural problems.

The problem with phantom crane flies

The influence of Johann Wilhelm Meigen in fly taxonomy can hardly be overstated. Most subsequent research in flies builds on his work, and consequently, his interpretations are often more influential than the original, specimen-based research of his predecessors. Unfortunately for nomenclature, this also goes for his misinterpretations.

One misinterpretation concerns a species Carolus Linnaeus described as *Tipula contaminata*. In Meigen's well-established interpretation, this is a slender black and orange fly found near freshwater around Europe, nomenclaturally important as the basis for both the genus name *Ptychoptera* and the family name Ptychopteridae (phantom crane flies). The family name, in particular, is used in countless textbooks and identification keys, besides the taxonomic primary literature.

Meigen unfortunately based his interpretations on Linnaeus' written descriptions rather than on

actual specimens. Thus, when US entomologist Andy Fasbender examined Linnaeus' single remaining *Tipula contaminata* specimen more than 200 years later, he quickly realized that it had nothing to do with *Ptychoptera contaminata* as traditionally understood – it even belonged to the wrong family!

Destroy or rebuild?

The taxonomic lore tells many horror stories of type specimens that clash with current opinion and "accidentally" get destroyed. One distinguished professor tended to drop specimens on the floor and roll over them with his office chair, and so, his interpretations stood unopposed. However, this practice is unpopular among museum curators, who typically prefer that their collections not be destroyed.

Luckily the rules of zoological nomenclature permit taxonomists to deal with the matter in a more civilized fashion. Under the International Code of Zoological Nomenclature (ICZN or *the Code*), you may petition the International Commission for Zoological Nomenclature (also ICZN, confusingly) to set aside the Code on a case-by-case basis, for instance by transferring type status from one specimen to another.

A taxonomist who wants a neotype to be designated first needs to convincingly document that the old type specimen does not fit prevailing usage of the name. The case is then published in the Bulletin of Zoological Nomenclature and left open for at least six months so other scientists may comment. Finally, the Commission votes on the case, based both on the original proposal and the subsequent comments; the opinion they publish in the end is considered the official ruling.

*“The nomenclatural trouble with *Ptychoptera contaminata* is notable because it is the first ICZN case where DNA barcoding-based arguments have been presented.”*

Our very preliminary analysis compared 13 Meigen-type *P. contaminata* with 21 sequences of other *Ptychoptera* species; we found that sequence variation was low within species and high between them. Thus, DNA evidence supports the existing taxonomy built on Meigen’s initial error.

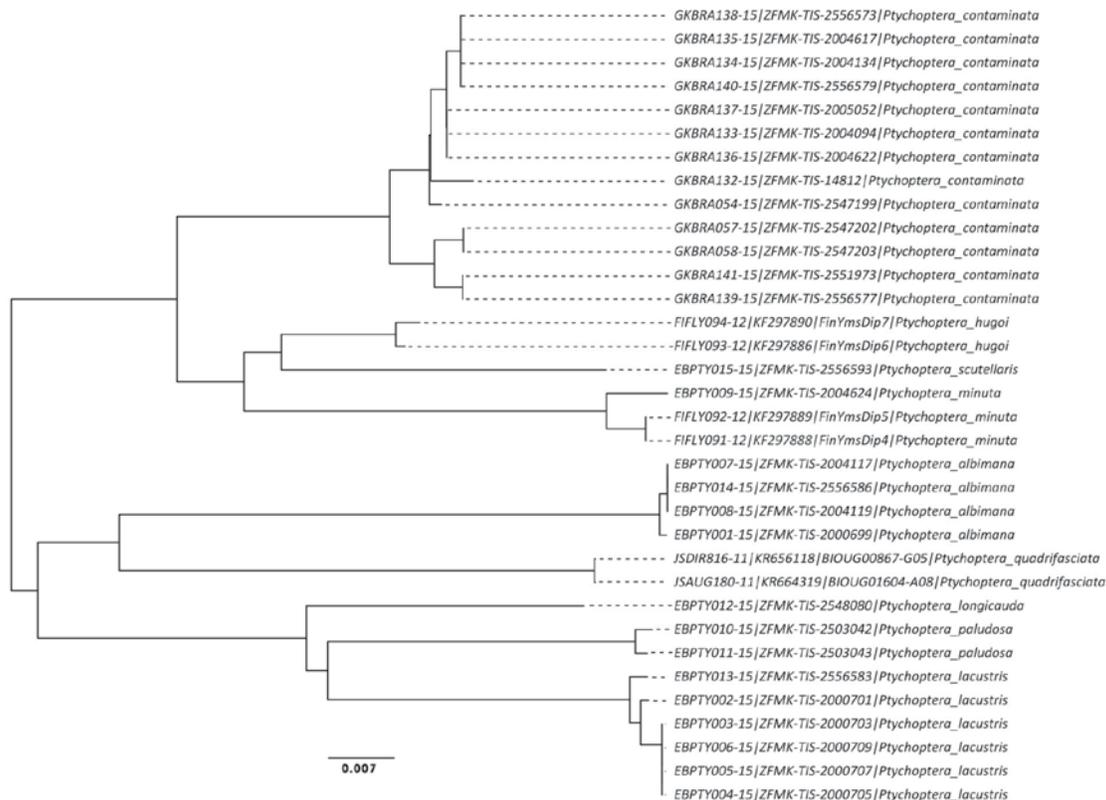
Andy and his coauthor Greg Courtney have already documented that their usage of the name is prevalent in the literature; together with their morphological evidence, our DNA barcode data suggest that this name is consistent with a robust and stable taxonomy.

What does all of this have to do with DNA barcoding?

The nomenclatural trouble with *Ptychoptera contaminata* is notable because it is the first ICZN case where DNA barcoding-based arguments have been presented. One year after Andy’s proposal appeared in print, in December 2014, Linn Hagenlund, Björn Rulik, and I published a comment in support of Andy’s case.

The Commission is known for taking its time and never jumping to rash conclusions, and I still do not know when their final vote will be made. In any case, when the opinion is eventually published, it will be the first time that their case is at least partially informed by evidence from DNA barcoding. With DNA-based methods becoming more and more ubiquitous in the taxonomic community, however, I am sure it won’t be the last.

Unrooted neighbour-joining phenogram of *Ptychoptera* COI sequences in GBOL and NorBOL, published in the Bulletin of Zoological Nomenclature (72: 309–310) in regards to Case 3664.



A view of the Central Park ecosystem, from the southern side, coexisting with the large, bustling city.



Image credit: flickr.com/photos/21962835@N07/

Barcoding Lichens to Assess Air Pollution in Central Park

Written by: Kaitlyn Espiritu, Bryant Lee, and Kang Min Shin (Tenafly High School, New Jersey, United States)

Sulfur dioxide (SO_2), a major pollutant generated from the combustion of sulfur compounds in fossil fuels, is lethal and carcinogenic for humans and other organisms even in minute quantities. In addition, it can form sulfuric acid precipitation, which is perilous to terrestrial, aquatic, and marine ecosystems. Acid rain is also debilitating to the economy since it causes substantial damage to agricultural produce and man-made materials, such as buildings and valuable artifacts.

Lichens are composite organisms formed from the tight-knit mutualism between a photobiont, the algal or cyanobacterial partner, and a mycobiont, the fungal component. The photobionts synthesize essential organic compounds for the mycobionts, and the mycobionts insulate the photobionts from damage and desiccation.

The enzyme nitrogenase, which allows photobionts to process atmospheric nitrogen (N_2) to assemble necessary amino acids, is inhibited

by SO_2 . The degree of inhibition of photobiont nitrogenase by SO_2 and the mycobiont surface hydrophobicity together determine a lichen's specific range of tolerance to SO_2 , which is a highly accurate indication of the SO_2 levels in the region it is inhabiting, as demonstrated by several previous studies. Lichens could be grouped into three categories based on their ranges of SO_2 tolerance: oligotroph (<15 ppb tolerance), mesotroph (~15-30 ppb tolerance), and eutroph (>30 ppb tolerance).

The occurrence of eutrophic lichens indicates that SO_2 levels must be monitored, as the upper safety limit for SO_2 concentrations is 30 ppb.

Our group decided to investigate the lichen population of NYC Central Park because we were

curious to see if this unique environment, surrounded by buildings and the world's greatest daily influx of traffic, could still maintain low enough SO_2 levels to sustain a healthy ecosystem. Based on our knowledge above, we hypothesized that only eutrophs, the most tolerant lichen types, would be found in Central Park.

"...the identification of the species occurring in a region provides an accurate indication of the level of SO_2 ."

“Eutrophic lichens populated most of southern Central Park, while mesotrophic and oligotrophic lichens inhabited the northern parts...”

In order to conduct our research, thirty lichen samples were collected throughout Central Park. Their DNA was isolated following the Harlem DNA Learning Center protocol, and two separate Polymerase Chain Reactions (PCR)

were performed on the purified DNA using different primers for the photobiont (*rbcL*) and mycobiont (fungal ITS) of the lichen, as both sequences are needed to identify lichen. The samples then underwent gel electrophoresis, and samples of sufficiently high quality, 11 in total, were sent for sequencing at Cold Spring Harbor. In order to determine the correct species of the samples, we uploaded the sequences onto the *DNA Subway* website and used the BLAST algorithm to match them with existing gene databases in Genbank.

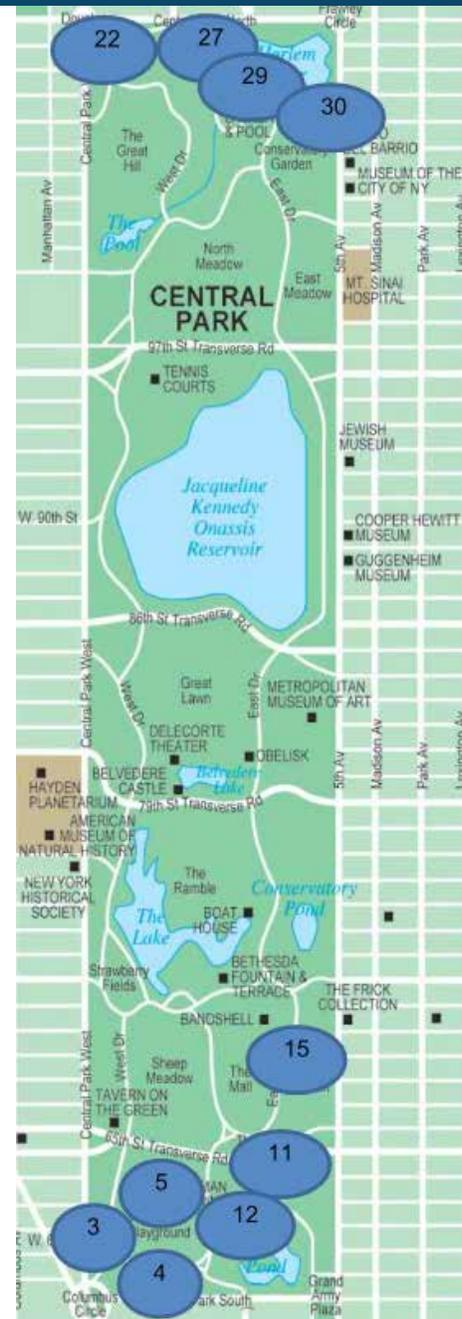
We found that a variety of lichen species existed across Central Park. Eutrophic lichens populated most of southern Central Park, while mesotrophic and oligotrophic lichens inhabited the northern parts, indicating that the northern area had a relatively low SO₂ concentration while the southern area had critical levels of SO₂, which may require monitoring. Afterwards, based on NYCCAS SO₂ maps and NYC traffic data, we discovered that our results corresponded with a greater frequency of cars in the southern area, leading to higher SO₂ levels.

In conclusion, there were different clusters of certain lichen populations in the northern and southern regions of Central Park with different tolerances of SO₂, corresponding with higher traffic levels in the south. Through our research, we not only showed that SO₂ levels in Central Park vary and that the southern area needs to be monitored, but also demonstrated that, through lichen analysis, SO₂ pollution in a region could be gauged highly accurately and potentially more inexpensively than traditional SO₂ detectors.



Above: A picture of the mesotrophic lichen *Phaeophyscia pyrrophora*, one of the lichens found in the northern area of Central Park. It has an intermediate tolerance to SO₂ (~15-30 ppb).

Below: Locations (circled) of each of the successfully sampled specimens. Map from About.com New York City Travel.





DNA Mini-barcodes for Saffron Identification

Written by: Caterina Villa, Joana Costa, Liliana Meira, M. Beatriz P.P. Oliveira, and Isabel Mafra (REQUIMTE-LAQV, University of Porto, Portugal)

Crocus sativus L. is the most important crop plant of the Iridaceae family. Saffron, the most expensive spice in the world, consists of the dried stigmas of *C. sativus*, and they are greatly appreciated in many dishes for their unique color, taste, and aroma. Owing to its high market value, saffron has been associated with an unrivaled degree of adulteration.

Adulterations of both ground and whole stigma samples have made use of diverse materials and strategies including colorants, mineral compounds, spices, and parts of other plants, such as flowers of *Carthamus tinctorius* or stigmas of other *Crocus* spp., with the aim of increasing the volume and weight of commercial lots. Accurate analytical methods for the detection of potential saffron adulterants are therefore crucial for the evaluation of the product's value, ensuring consumer protection against fraudulent practices.

Different methods have been developed to evaluate the quality and authenticity of saffron based on standardized methods for assessing chemical and sensorial parameters. Saffron authentication relies mostly on the microscopic observation of morphological traits, which is

time-consuming, requires experienced personnel, and is susceptible to subjective interpretation.

Among DNA markers, the use of barcodes has recently been proposed as a powerful pharmacognostic tool for the identification of medicinal plants and their adulterants at the species level. However, DNA barcoding entails the amplification and sequencing of relatively large DNA fragments (400-800 bp), which represents a major drawback for the analysis of processed/complex matrices.

“...saffron has been associated with an unrivaled degree of adulteration.”

When analyzing samples with degraded DNA, such as processed plants, poorly preserved plants, and/or those stored for a long period of time, DNA barcodes are probably not the best target sequences. In such cases, species-specific primers based on barcodes but targeting small-size amplicons (e.g. DNA mini-barcodes) have been successfully applied for species identification. The recent approach of high resolution melting (HRM) analysis coupled with specific barcodes represents a potential cost-effective tool to detect small nucleotide differences without requiring further sequencing (Costa *et al.* 2016, DOI: [10.1016/j.foodcont.2015.09.035](https://doi.org/10.1016/j.foodcont.2015.09.035)).

Our study focused on the identification and selection of DNA mini-barcodes to further combine with HRM analysis to identify *C. sativus* and differentiate it from closely related species, thereby assessing the authenticity of saffron. As candidate markers, we chose ITS and the chloroplast *matK* gene. New primers were specifically designed targeting three DNA mini-barcode loci, namely ITS1, ITS2, and *matK*.

Successful amplification of ITS2 was only achieved for *C. sativus* and *Crocus cartwrightianus*, a fact explained by the genetic proximity of these two species. *C. sativus* is believed to have derived from a common ancestor of the current *C. cartwrightianus*.

*“With the application of HRM analysis, it was possible to distinguish *C. sativus* from closely related species...”*

For ITS1 and *matK*, all samples from the *Crocus* genus were successfully amplified. From the tested loci, primers targeting the ITS1 barcode region were selected for real-time PCR coupled with HRM analysis

to determine their suitability for differentiating *C. sativus* from other allied species.

With the application of HRM analysis, it was possible to distinguish *C. sativus* from closely related species, namely wild saffron, *Crocus kosaninii*, *Crocus kotschyanus*, *Crocus speciosus*, *Crocus olivieri balansae*, and even *C. cartwrightianus* with high levels of confidence (>99.5%), which was also confirmed by sequencing. This study highlights the successful use of DNA mini-barcodes combined with HRM analysis for saffron authentication.

For more information about this research, see DOI: [10.1016/j.foodcont.2016.01.008](https://doi.org/10.1016/j.foodcont.2016.01.008)

Images by Isabel Mafra.



Family or Fashion? Colour Patterns in Arctic Bumblebees

Written by: Paul H. Williams (Natural History Museum, UK / CAAS Institute for Apicultural Research, China)

Bumblebees are remarkably homogeneous in morphology. More than two centuries of bumblebee taxonomy have led to suspicions that the striking colour patterns of the hair – most often used to describe species – are in fact highly variable within species, making species difficult to recognise. Recently, species have come to be viewed in theory as evolutionarily independent lineages (EILs). Barcodes could be of enormous help because, despite the potential pitfalls and the need for corroboration from nuclear genes, the rapid evolution of barcodes is presenting many new characters that could help in the practice of recognising species as EILs.

Barcodes showed their value for bumblebee taxonomy whilst producing the recent *Bumble Bees of North America, an Identification Guide*, the first of its kind in a century (Williams *et al.* 2014). Barcodes helped reveal patterns of variation that largely corroborated the traditional ideas of species. As a result, barcodes are currently being used extensively in revising the much larger, but far less well-known, bumblebee fauna of China (An *et al.* 2014, DOI: [10.11646/zootaxa.3830.1.1](https://doi.org/10.11646/zootaxa.3830.1.1)). Now,

a research programme is building on these studies to compare evolutionary patterns in the bumblebee groups that specialise in the most extreme alpine and arctic environments worldwide. In the Arctic, barcodes are showing some unexpected results.

In comparison to other bumblebee groups, taxonomic revisions for the primarily arctic bumblebees of the subgenus *Alpinobombus* have been rather unusual in that they have all reached different conclusions on the number of species present. Revisions based on morphology concluded that there are between four and six species, while variation in colour patterns suggested at least seven. In the most recent study (Williams *et al.* 2015, DOI: [10.1371/journal.pone.0144544](https://doi.org/10.1371/journal.pone.0144544)), based on the view of species as EILs and on barcodes of samples from across the Arctic, nine species are supported.

In Alpinobombus, “the resemblances in colour pattern among some parts of many species are due to persistent ancestral polymorphisms.”

In practice, EILs are recognised not simply by a particular genetic distance, but rather from the gene coalecscents that provide direct evidence for the evolutionary independence of their lineages, using techniques based on mixed Yule/coalescent models or Poisson-tree process models. In this case, the same result was also obtained with BOLD's BIN method. Evidence from the nuclear PEPCK gene adds further support for some of the same species and is consistent with the others, although this nuclear gene evolves more slowly, making it less informative. The new species are mostly prevented from interbreeding by oceans, but one new species, which appears to be endemic to mountains in southern Alaska and the Yukon, is overlapping.

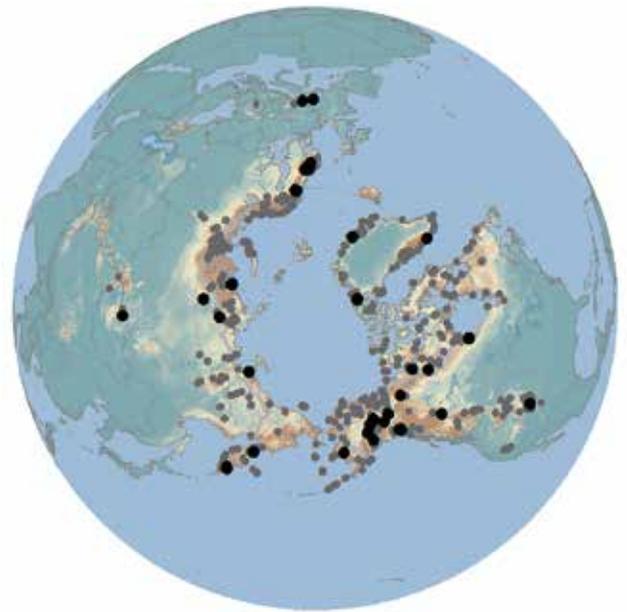
The most unexpected result for *Alpinobombus* is that the resemblances in colour pattern among some parts of many species are due to persistent ancestral polymorphisms. This contrasts with the usual explanation for the common colour-pattern resemblance between parts of different species occurring in the same place – which is usually interpreted as the result of mimetic convergence. For *Alpinobombus*, reconstructing ancestral colour-pattern states shows that speciation is likely to have cut across taxonomically and geographically widespread ancestral polymorphisms. This pattern has not been known previously for bumblebees.

Arctic bumblebees are expected to be under particular threat from climate change. Modelling predicted range shifts depends on understanding their current distributions, so discovering the geographic limits of species is important. For *Alpinobombus* bumblebees, grouping individuals by morphology, by colour pattern, or by barcodes shows little agreement. This helps explain the lack of agreement among previous taxonomic revisions. Barcodes, however, with their rapid evolution, appear to be especially valuable in helping us to get closer to understanding species as EILs (www.nhm.ac.uk/bombus).



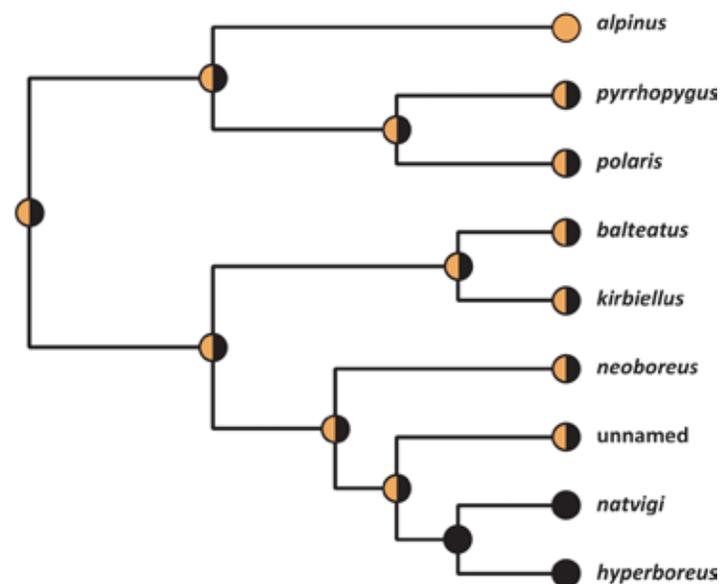
Image credit: Claus Rasmussen

Above: *Alpinobombus* habitat in Greenland.



Above: Map showing climatic suitability (favourable in brown), specimen records (grey), and barcodes (black).

Below: Example of evolution of *Alpinobombus* bumblebee tail colour patterns, with orange spots for pale, black for dark, and mixed for polymorphic.





Assessing Exotic Fauna from Maritime Ports

Written by: Ivana Pejovic, Alba Ardura, Laura Miralles, and Eva Garcia-Vazquez (University of Oviedo, Spain)

Ever since humans started to travel across the oceans, they were not travelling alone. In temperate regions like the European Atlantic coasts, ship traffic is the main pathway for the introduction of exotic species (Molnar *et al.* 2008, DOI: [10.1890/070064](https://doi.org/10.1890/070064)). Consequently, commercial ports with intense marine traffic and disturbed environmental conditions have become “alien hotspots”.

Molluscs are a prevalent group of marine biological invaders and can tremendously impact native resources. Exotic species may remain unnoticed when they are morphologically similar to other co-occurring species, thus making an early visual identification impossible. We investigated the usefulness of DNA barcoding for detecting exotic molluscs that may be otherwise overlooked by routine monitoring in regions where the fauna is already well known.

Our study focused on molluscs present in maritime ports of the Bay of Biscay in northern Iberia (Asturias region). This temperate region has a coastline with a heterogeneous intertidal environment. Dominated by sharp rocky cliffs and a pronounced shore slope, it also contains several small sandy beaches and a few estuaries. There are

two international trade ports under state control (Aviles and Gijon- El Musel) in the region, as well as 24 small regional ports with an average surface area of 31,900 m². In this area, shellfish fauna has been thoroughly investigated to understand the effects of climate change and anthropogenic pressures. Finding new species here therefore poses a challenge for barcoding projects.

Molluscs were collected from Aviles, Gijon, and Luanco ports. The sampling protocol was modified from the Rapid Survey described by Minchin (2007, DOI: [10.3391/ai.2007.2.1.8](https://doi.org/10.3391/ai.2007.2.1.8)). We targeted molluscs and ignored the remainder of the biota in a sampling surface of approximately 200 m², trying to collect a number of individuals of each phenotype proportional to its abundance.

In total, 506 molluscs were collected and genetic barcodes obtained using universal primers for two mitochondrial genes: cytochrome *c* oxidase subunit I (COI) and 16S ribosomal RNA genes. The sequences were compared with online databases, GenBank and BOLD, and species identifications were based on the closest match (at least 99%) together with a deep analysis of the diagnostic morphological features of the studied mollusc species.

In addition to 14 native molluscs, four potential non-native bivalves were identified. We reported the cryptogenic mussel *Mytilaster minimus* (Figure 1D; the native *Mytilus galloprovincialis* in 1C for comparison) for the first time in this region. The exotics were *Ostrea stentina* (dwarf oyster, 1A)

and the two highly invasive species, *Crassostrea gigas* (Pacific oyster, 1B) and *Xenostrobus securis* (pigmy mussel, 1E). This is the first record of *O. stentina* and the second of *X. securis* in the whole Bay of Biscay. These exotics might have been overlooked due to their phenotypic similarity with co-occurring oyster and mussel species. Alternatively, they could have arrived recently in the ports. In either case, this study illustrates the usefulness of DNA barcoding for cases of morphological plasticity or similarity of native and/or exotic species. The particular case of *Xenostrobus securis* is especially concerning because it is highly invasive and has already been declared a pest for the Atlantic coast of Spain.

The results strongly suggest the need for DNA barcoding in monitoring faunal composition in port and estuary surveys.

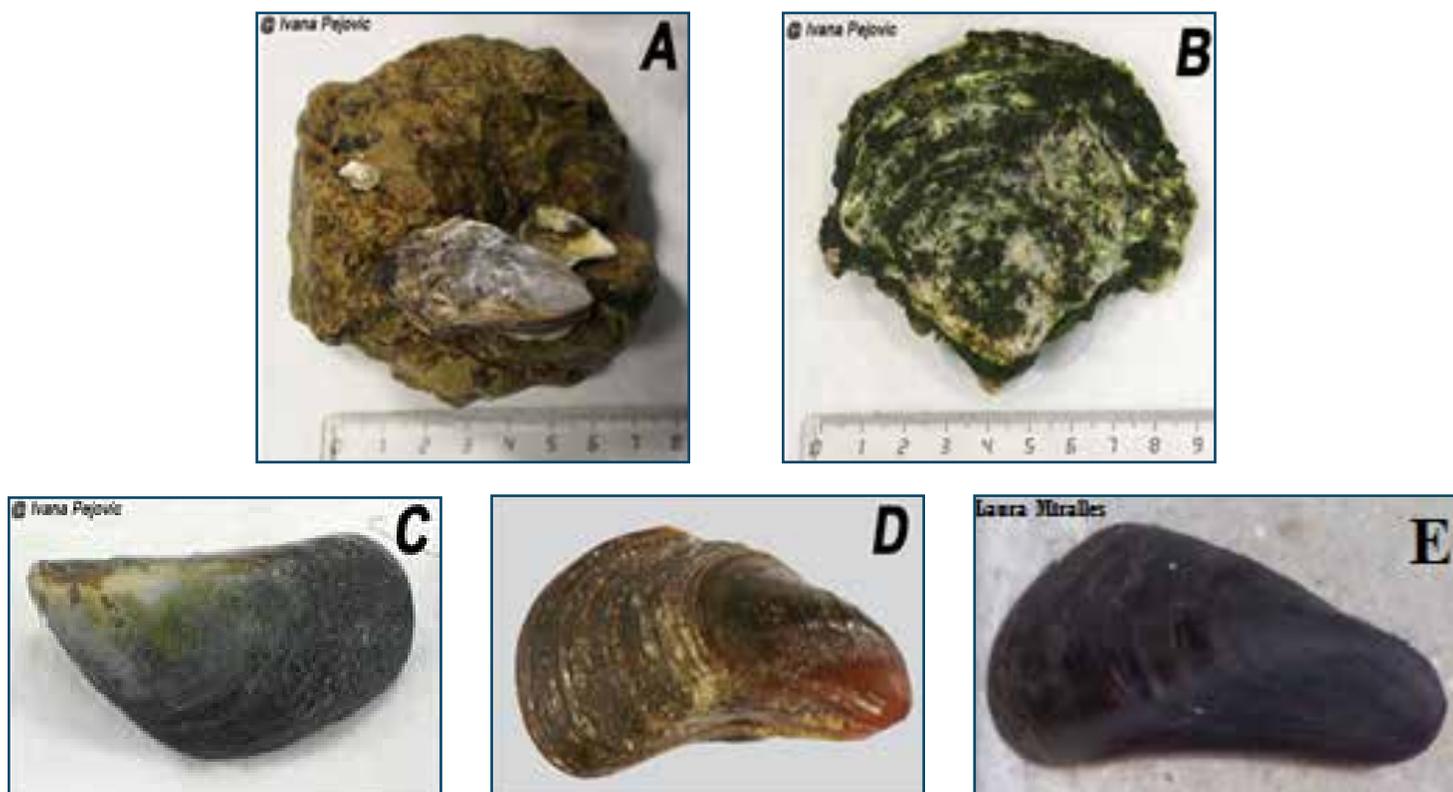
This type of assessment can aid in port and marina biosecurity surveys, which are much needed given the important role of maritime transport in the introduction of exotic species. The molecular protocol is simple and can be applied easily in any laboratory. However, a possible

limitation of the methodology could be the lack of reference sequences in international databases. For instance, two genes were required for the present study because, at the time of the assessment (July 2015), only one reference sequence was available for *Mytilaster minimus* in both the GenBank and BOLD databases, the 16S rDNA sequence DQ836017 (Pejovic *et al.* 2015, DOI: [10.1080/17451000.2015.1112016](https://doi.org/10.1080/17451000.2015.1112016)).

Images by Ivana Pejovic and Laura Miralles.

This study was funded by the Spanish Grant MINECO CGL2013-42415-R and the Principality of Asturias, Grant GRUPIN-2014-093.

Figure 1. Photographs of exotic and cryptogenic molluscs found in the studied region for the first time. A, B, C, D, and E are *Ostrea stentina*, *Crassostrea gigas*, *Mytilus galloprovincialis*, *Mytilaster minimus*, and *Xenostrobus securis*, respectively.





Sample Size Estimation for DNA Barcoding of Fishes

Written by: Jarrett Phillips (University of Guelph, Canada)

The ability of DNA barcodes to detect meaningful genetic variation within and between species is strongly influenced by the scale of specimen sampling. Unfortunately, global barcoding efforts have only been partially successful in this regard due to the majority of studies forgoing deep taxon sampling in favour of maximizing the number of taxa sampled.

A practical sample size of five individuals per species is common in barcoding studies, but such a strategy is by no means sufficient. This has led to sampling schemes in which many more specimens per species are collected.

The following question must therefore be addressed: is there an optimal threshold for specimen sampling above which no new genetic (i.e. haplotype) variation is likely to be observed for a species? That is, can all haplotype diversity for a species be uncovered by simply sampling N individuals?

I was very interested in further exploring this paradigm for my Masters studies in Bioinformatics at the University of Guelph using ray-finned fishes (Chordata: Actinopterygii) as a taxonomic focus.

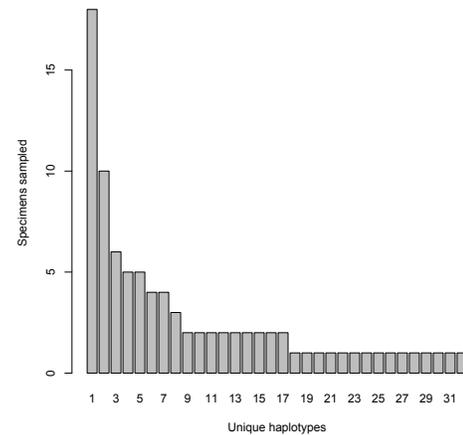
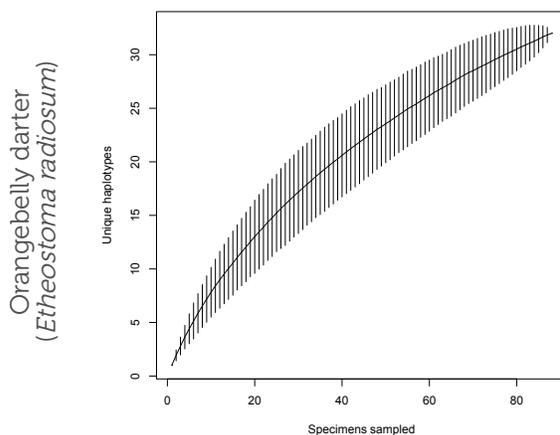
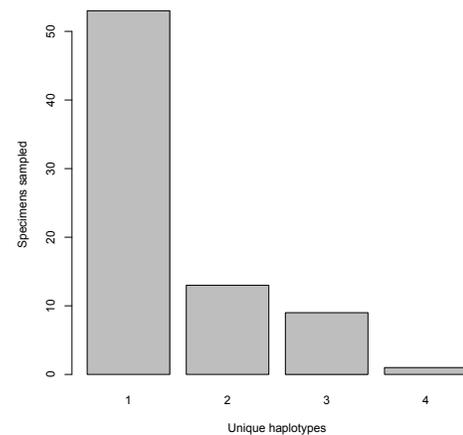
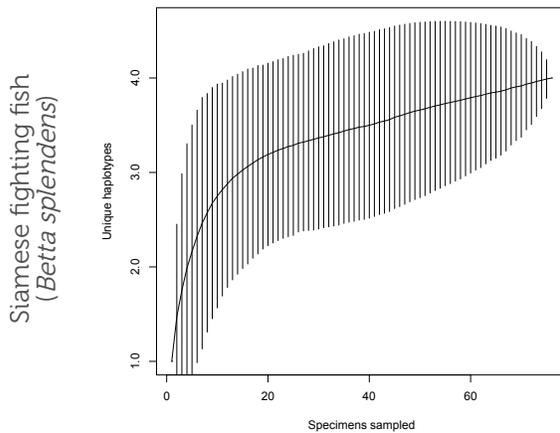
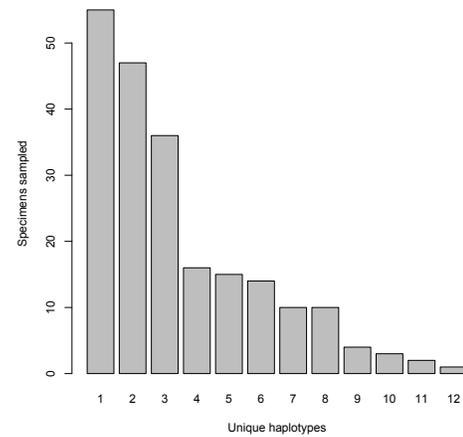
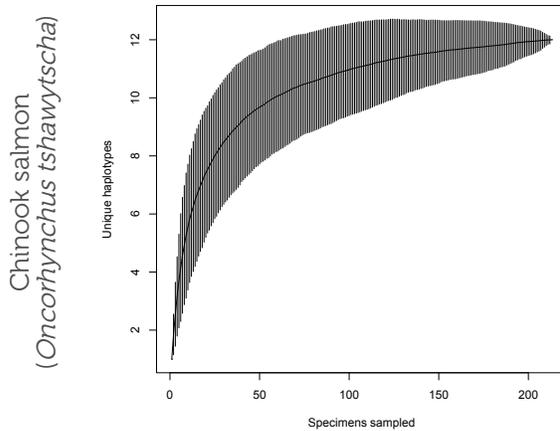
To do this, I used haplotype accumulation curves calibrated by a simple quantitative model that I developed to predict total sample sizes given estimates of observed specimen numbers as well as observed COI haplotype numbers and total haplotype diversity for a species. In formulating the model, one very important assumption was made: haplotypes occur at equal frequency within populations. Such an assumption is not biologically realistic since species abundances are often skewed geographically.

“A practical sample size of five individuals per species is common in barcoding studies, but such a strategy is by no means sufficient.”

Using my model, I found that sample sizes ranging from 150 to 5400 individuals per species are likely needed to uncover all haplotype diversity across the 18 selected species. This is a far cry from sampling intensities currently employed in many barcoding initiatives; however, such numbers may not be practical, and further investigation will be required to fully probe the extent of sampling necessary to gauge existing species genetic diversity in this group and others.

Sample sizes obtained here likely represent underestimates of the true number of individuals of a given species which should be sampled. Furthermore, equal haplotype frequencies are rarely observed in natural populations, and the development of more sophisticated models should explore the use of data simulations to explicitly account for variation in species haplotype frequencies. For more details, see [Phillips *et al.* 2015, DNA Barcodes 3: 66-73.](#)

Haplotype accumulation curves and their corresponding slope values are shown below along with haplotype frequency barplots for several species, demonstrating patterns representative of the 18 species dataset.



Call for Nominations for Training Course in DNA Barcoding

The Secretariat of the Convention on Biological Diversity has announced the second round of the training course on the application of DNA barcoding in the detection and monitoring of priority invasive alien species, pests, and zoonotic disease vectors. Interested Parties to the Convention have been invited to nominate training course participants through their corresponding CBD Focal Points. The course is geared toward professionals involved in national regulatory and monitoring organizations overseeing the quarantine and management of invasive alien species and other organisms of regulatory concern. Selected participants from developing countries will have their transportation, accommodation, and training fees covered through bursaries, thanks to financial support from the Government of Japan. The course is also open to self-funded experts from countries that are not eligible for SCBD financial support.

This activity builds on the success of a similar training event held in 2015 and is reinforced by a Memorandum of Understanding signed by the CBD Secretariat and the University of Guelph. It will facilitate the development of national capacity

to detect and manage invasive alien species, pests, disease vectors, and other organisms of special concern through the adoption of rapid DNA-based diagnostic approaches.

Similar to last year, the course will consist of two training stages: an eight-week distance education course in DNA barcoding for at least 30 participants, administered through Open Learning and Educational Support at the University of Guelph, and a follow-up four-week intense hands-on course in standard DNA barcoding protocols for at least 15 selected trainees, facilitated by the Centre for Biodiversity Genomics, Biodiversity Institute of Ontario. Online training will begin in April 2016, and the hands-on modules are scheduled to start in July.

The full announcement is available [here](#). For inquiries on eligibility, selection criteria, and the application procedure, please contact the CBD Secretariat at secretariat@cbd.int.



Convention on
Biological Diversity

Credits and Contributions

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The Barcode Bulletin owes its success to the valuable contributions of researchers and enthusiasts within the global DNA barcoding community. If you wish to contribute please contact us at barcodebulletin@gmail.com.