

Barcoded DNA: Application to Rotifer Phylogeny, Evolution, and Systematics

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This paper has not been submitted elsewhere in identical or similar form, nor will it be during the first three months after its submission to *Hydrobiologia*.

Abstract

DNA barcoding is the use of segments of gene sequences to assign individual organisms to species. Thus it can be used to define species and to identify specimens. Barcoding has been applied as an aid to systematics with little controversy in both monogonont and bdelloid rotifers, and also in environmental sequencing projects designed to determine the diversity of microscopic organisms. In contrast, a great deal of controversy has arisen over the creation of the Consortium for the Barcode of Life, a major initiative to barcode all the species in several major groups of animals, with the long-range goal of barcoding all species of organisms. This is a very brief review of DNA barcoding, especially as applied to rotifers, and a summary of the results of a workshop held at the 11th International Workshop on Rotifera.

History of DNA Barcoding in Rotifers and Other Organisms

Rotifers pose special problems for taxonomists and evolutionary biologists. Their microscopic size makes it necessary to observe their morphology and behavior in the laboratory. It is difficult to know which, if any, phenotypic characters are correlated with reproductive isolation and adaptation to different habitats. As a result, morphology is not always a reliable guide to the identification of species, let alone distinguishing among populations, and there are likely to be many cases of cryptic speciation. This led to a number of rotifer workers to apply molecular methods, initially allozyme analyses and then DNA sequences (reviewed by Gómez, 2005).

A segment of the mitochondrial *cox1* (also known as *COI*) has proved to be especially useful for discriminating species in both monogonont and bdelloid rotifers (including cryptic species difficult or impossible to distinguish by phenotype) and for studying within-species variation and phylogeography (Birky *et al.*, 2005; Gómez, 2005). At the same time, a group of evolutionary biologists and systematists led by Paul Hebert were using sequences of this same DNA segment to study species in other animals. They suggested that these sequences could be used “as the core of a global bioidentification system for animals” (Hebert *et al.* 2003a; *et al.* 2003b) and referred to them as “DNA barcodes”. Colorful examples of DNA barcodes in which the base sequence is represented by bars of four different colors can be found on the CBOL website (<http://barcoding.si.edu/>).

Barcoding Methods for Rotifers

The mitochondrial *cox1* gene that encodes subunit 1 of cytochrome oxidase was selected for phylogenetic analyses in rotifers and also for the most barcoding experiments in other animals for several reasons. One is the availability of universal primers. The Folmer primers (Folmer *et al.*, 1994) work very well on a wide variety of taxa, including all of the monogonont and bdelloid rotifers that we have tried (Birky *et al.*, 2005 and unpublished). It is a good idea to stick with this

gene because of the very large database of rotifer sequences already accumulated and the extensive experience with this gene in other organisms. The rate of evolution of *cox1* seems to be ideal for differentiating species in rotifers and many other groups. We have also had excellent success with a segment of the mitochondrial *cob* gene that encodes the cytochrome *b* apoprotein; the combined *cox1* and *cob* sequences provide better resolution of deeper branches and can distinguish populations within species that might be in the first stages of speciation. Using a protein-coding gene has the advantage over other kinds of sequences that one can distinguish synonymous from nonsynonymous sequence changes in order to separate effects of natural selection from mutation and random drift in separating species. Another advantage is that sequences of protein coding genes can be used to answer evolutionary questions such as whether detrimental mutations tend to accumulate in asexual lineages (Mark-Welch & Meselson, 2001; Birky *et al.*, 2005). Also amino acid sequences of protein-coding genes can often resolve some of the deeper branches in a tree.

An important advantage of mitochondrial genes is that they are effectively haploid. Consequently one can sequence amplification products without first having to clone them to distinguish between the two alleles of a gene. Phylogenetic analyses are not likely to be confounded by paralogous genes because gene duplication and deletion events are rare in animals (Boore, 1999). Exceptions in some groups, especially birds, are genes transferred to the nucleus (Numts) which could seriously compromise the reconstruction of evolutionary history if undetected (Ballard & Whitlock, 2004). Fortunately these are usually easily recognized because sequences of PCR amplification products will have sites with strong signals for two different bases; if amplification products are then cloned and sequenced, the nuclear sequence will usually be recognized as a pseudogene. In contrast, nuclear genes in monogonont rotifers show the Meselson effect in which alleles of a gene diverge greatly in sequence (Mark-Welch &

Meselson, 2000). Phylogenetic information can be lost when the Meselson effect is accompanied by infrequent mitotic recombination or nondisjunction (Birky, 1996). In sexual rotifers, mitochondrial genes have a smaller effective population size than nuclear genes due to their haploidy and maternal inheritance and also due to more frequent hitchhiking events. The smaller effective population size may result in deeper and more easily detected gaps between species.

Barcoding a rotifer begins with the isolation of DNA from an individual female or egg collected from nature or from a clone grown in the lab from single animal. Large-scale studies are best done with fast, simple methods such as the use of commercial ion exchange spin columns (e.g. QIAmp or Dneasy from QiaGen; (Birky *et al.*, 2005) or Chelex (Gómez *et al.*, 2000). In our experience the columns do not work well with fewer than 10-20 animals, while Chelex has been used on single animals or resting eggs. A gene fragment is then amplified using PCR and an aliquot of the amplification product is subjected to agarose gel electrophoresis to verify that a fragment of the correct size has been amplified. If this test is positive, the amplification product is purified by a fast method such as spin columns (e.g. QIAquick from QiaGen) or treatment with Exonuclease I and Shrimp Alkaline Phosphatase (combined in ExoSAP-IT from USB). Other protocols can be downloaded from the CBOL website (<http://barcoding.si.edu/>). After purification, the gene fragment is sequenced in both directions using the same primers used for amplification and the resulting sequences are proofread. Alignment of *cox1* and *cob* gene sequences within the monogononts or bdelloids can be done manually because there are few or no gaps due to deletions or insertions. However, some gaps may be encountered if the database includes members of two or more different major groups of rotifers (bdelloids, monogononts, acanthocephalans, and seisonideans).

This procedure was applied to *Brachionus plicatilis* to study population structure (Gómez *et al.*, 2000) and discriminate cryptic species (Gómez *et al.*, 2002). The sequences of both the ITS1

nuclear region and the mitochondrial *cox1* segment fell into tight clusters, well separated from each other; these clusters represent cryptic species. The absence of hybridization and long-term persistence of the new species in sympatry showed that they are reproductively isolated from each other (Gómez *et al.*, 2002; Gómez, 2005). In this study, DNA barcoding was used to identify new specimens by assigning them to species already distinguished by phenotype, and also to discover new species by splitting old ones.

At the Rotifera X meeting in 2003 I showed that phylogenetic analysis of *cox1* sequences can be used to discover species, grouping samples of bdelloid rotifers, mostly identified only to genus, into species (Birky *et al.*, 2005). These species are defined as inclusive populations that have been evolving independently because they are adapted to different niches and/or are physically isolated. They are clusters of genotypically similar individuals separated by differences too large to have been produced by random genetic drift (Barraclough *et al.*, 2003). After such species were first defined, additional specimens were either assigned to one of the already-defined species or formed new species. In this project, species are identified as reciprocally monophyletic clades separated from each other by sequence differences at least four times greater than the mean sequence difference within a clade. This procedure ensures that the populations from which the individuals were sampled are themselves reciprocally monophyletic with 95% probability, and have been separated spatially or ecologically. At the Rotifera XI meeting I reported finding 59 species in a sample of 346 bdelloids collected by myself, by Tim Barraclough at Imperial College London, and by our collaborators. Thus the *cox1* barcode sequence has been used successfully with both monogonont and bdelloid rotifers.

Barcoding as a Global Program for Taxonomy and Systematics: the Consortium for the Barcode of Life

The proposal by Hebert (Hebert *et al.*, 2003) that barcoding could be used as a general method for specimen identification (assigning individuals to known species) and species discovery (assigning individuals to new species) led to the formation in 2004 of the Consortium for the Barcode of Life (CBOL). As described in its website (<http://barcoding.si.edu/>), CBOL is an international initiative devoted to developing DNA barcoding as an international standard for taxonomy, including specimen identification and the discovery of new species. Barcoding is advocated as a tool to make taxonomy more effective. The initiative is funded by grants from the Alfred P. Sloan Foundation to the Smithsonian Institution. The members of the consortium are more than 130 museums, colleges and departments, conservation organizations, and other institutions from more than 40 countries around the world. Projects include the Fish Barcode of Life and All Birds Barcoding Initiative that have the goal of barcoding all species of birds and fish. CBOL has also held a number of workshops and symposia, and established laboratory protocols, data standards, and databases for barcodes. Most barcoding is being done with animals, but efforts are being made to extend this to fungi and plants; in principle it could also be used for protists where traditional taxonomy based on phenotypes is especially difficult. However, each major group of organisms will probably require a different barcode sequence which has the appropriate level of variability to distinguish species (but see Saunders 2005 who successfully used a *cox1* fragment with red algae).|

According to CBOL, barcoding begins with obtaining barcode sequences from one or more individuals already identified to species and adding them to the database. It is intended that all barcodes be linked to voucher specimens deposited in museums or other institutions. CBOL also

emphasizes that barcodes and related data should be public property. Once a large barcode database has been established for a taxonomic group, the barcode of a newly collected specimen is compared to the database; the specimen is either assigned to species with a sufficiently similar barcode or to a new species. The CBOL web site does not specify what is “sufficiently similar”, although various cutoff values have been proposed. Empirical studies of some animal groups suggest that a specimen can be assigned to a species if its barcode sequence differs from that of other barcodes in that species by less than 10X the mean intraspecific variation in the group being studied (Hebert *et al.*, 2004). Note that unlike the 4X rule used for bdelloids, the 10X rule has no theoretical basis. More sophisticated methods of identification and of species discovery are being investigated. For example, (Pons *et al.*, 2006) made a phylogenetic tree of barcode sequences from Australian tiger beetles, assuming a molecular clock. They then used a maximum likelihood analysis of the number of branches at varying distances from the coalescent to detect the transition points where a branch connecting a species with its ancestral species joins the branches representing lineages of genes within that species. The species identified in this way also showed various properties of reproductively isolated biological species including morphological differences. This method has also been applied to the bdelloid rotifer barcode sequences and usually finds the same species that are found with the 4~~X~~^V rule (T. Barraclough, personal communication).

Criticisms of Barcoding

The field of systematics is notorious for controversies over both theory and practice. Some controversies center on the use of sequence data to find and characterize species, although an increasing number of studies combine sequence analysis with traditional methods. Not surprisingly, the proposal that specimens could be identified by their barcodes without detailed

study of phenotypes, or that species could be defined by barcodes alone, and that this method of systematics should be the object of a major grant-supported initiative, unleashed a storm of reaction. Some of the main arguments were summarized in a debate held under the auspices of the Partnerships for Enhancing Expertise in Taxonomy (PEET) initiative; the debate was summarized by (Smith 2005) and arguments pro and con were presented by (Hebert & Gregory, 2005) and (Will *et al.*, 2005) respectively. Some of the arguments on both sides were summarized by (Moritz & Cicero, 2004).

Arguments against barcoding include the following:

1. Barcoding does not use cladistic methods to define species. This is true; the dispute over cladistic vs. other systematic philosophies, including the use of phylogenetic trees based on distance methods, is not unique to barcoding.
2. Barcoding is proposed to replace studies of the biology of organisms (integrative taxonomy). This is true in a limited sense. Presently the barcoding initiative is focused on the use of barcodes to assign specimens to species already defined by integrative taxonomy. However, it is true that specimens that cannot be assigned to such species could be assigned new species on the basis of their barcode sequence alone. On the other hand, this does not rule out subsequent or simultaneous studies of the biology of the organisms. CBOL emphasizes the importance of depositing well- annotated voucher specimens that can be used for this purpose. As described below, this was also emphasized in the Rotifer XI barcoding workshop.
3. Barcodes invoke a typological species concept. This is not true; barcoders explicitly recognize the diversity of DNA sequences within species.
4. Barcoding is a phenetic approach to systematics. This is true, to the extent that barcoding relies on sequence similarity criteria such as the 4X rule that are based on empirical

observations. Whether the absence of a theoretical justification in the form of a species concept is bad depends on one's philosophy of systematics. This issue is discussed further below.

5. Barcoding works poorly with closely-related species (Meyer & Paulay, 2005; Meier *et al.*, 2006). This is true, but of course it is true to varying degrees of all other methods of systematics. It should be noted that studies that test the performance of barcoding generally make the dubious assumption that the traditional classification of organisms used in the test is valid. It would be more appropriate to specify a suitable species concept and criteria in advance then compare the performance of traditional systematics and DNA barcoding under that model.
6. Barcode sequences of genes in mitochondria (or, by extension, genes in chloroplasts) may not accurately reflect the behavior of nuclear genes; a single nuclear gene may not accurately reflect the phylogeny of the rest of the nuclear genes, including those involved in reproductive isolation (Moritz & Cicero, 2004). This is true, and is a significant problem for barcoding organisms that reproduce sexually (but not for asexuals). Presumably, systematists who subscribe to the biological species concept would be willing to use a barcode sequence from a gene involved in reproductive isolation, while those who prefer an ecological species concept would prefer a gene responsible for adaptation to different habitats; other systematists might prefer different genes, or insist that no single gene suffices. Here as elsewhere, systematics is hampered by lack of agreement on a species concept or on operational criteria.
7. Grant support of large-scale barcoding will reduce support for traditional or integrative systematics. This is correct if obtaining grants is a zero-sum game; barcoding supporters suggest that it is not, because barcoding will attract additional funding. The level and

distribution of funding is partly determined by politics, which makes it difficult to know what effect large-scale barcoding would have.

8. An increased emphasis on barcoding will destroy traditional systematics. The analogy to genomics, which has not destroyed other areas of genetics, suggests that this is not necessarily true.

What is Barcoding Good For?

Barcoding shares many of the virtues of other molecular methods for species identification:

1. Barcoding works for all life stages. Of course this is also true of the most thorough systematic studies based on morphology; there are taxonomic keys for butterfly larvae and pupae as well as adults.
2. Barcoding can be applied to fragments of organisms, even pieces of tissue.
3. Barcoding combined with phenotypic studies enables a comparison of phenotypic and genotypic variation. This can reveal homoplasy and convergence in phenotypes, facilitating the evaluation of phenotypic characters for classification and identification.
4. Barcoding is unaffected by sexual dimorphism and phenotypic variation induced by the environment; the latter in particular has created problems for the systematics of monogonont rotifers.
5. Barcoding can reveal cryptic species. But integrative systematics can also reveal cryptic species in sexual organisms by testing for reproductive isolation or, more often, phenotypic or biogeographic features that serve as surrogates for reproductive isolation.
6. Barcoding is the only feasible basis of large-scale surveys of diversity by environmental sequencing..
7. Barcoding is the only game in town for microorganisms that cannot be isolated and grown in the laboratory for phenotypic tests (Blaxter *et al.*, 2005).

8. Because barcoding requires less training, is faster, and is (or soon will be) cheaper than traditional systematics, it is the only way to even approach the goal of determining the number of species of organisms on earth, or even the number of species in any large taxon.

Barcoding With and Without Species Concepts and Criteria

Much of the work in CBOL involved assigning individuals to species that have already been defined by a variety of criteria, principally morphology and other phenotypic traits. Many of these criteria are associated with the Biological Species Concept (BSC). The identification of individuals by DNA barcoding was widely criticized by taxonomists for using a phenetic method (the similarity cutoff), and because the classification did not always agree with the results of cladistic classification (e.g. DeSalle *et al.*, 2005; Will *et al.*, 2005). This illustrates a major problem with the current practice of DNA barcoding and with the field of taxonomy in general: species criteria and concepts are often not specified, and when they are, there are fundamental disagreements as to which ones are appropriate. In fact not all systematists agree on the importance of species as a taxonomic rank (Will *et al.*, 2005), even while they work at defining and discussing species.

In contrast, the use of barcoding by the rotifer community has been relatively well grounded in theory. Cryptic monogonont species identified as distinct lineages on phylogenetic trees tend to be reproductively isolated from each other and thus fit the BSC and also the evolutionary species concept. For bdelloids, we (Birky *et al.*, 2005) use a species concept and criterion based on coalescent theory developed by (Barraclough *et al.*, 2003). This is probably one reason why most or all of the barcode workshop participants seemed to agree that DNA sequences have been effectively and appropriately used to find species in rotifers, including identifying cryptic species.

Barcoding in Sexual vs. Asexual Organisms

In sexual organisms where the BSC applies, DNA barcodes can be used in at least some cases as an adjunct to species discovery, but because the genes used as barcodes are not the genes that cause reproductive isolation, and may in fact recombine and re-assort with those genes, their use as a primary method of species discovery is open to criticism. In particular, it is likely that mitochondrial genes will have more recent coalescents than nuclear genes, including the nuclear genes that are responsible for reproductive isolation (or for the genes responsible for ecological isolation) (Birky, 1991). Whether this happens often enough to make mitochondrial barcoding impractical is an empirical question and the answer may vary among species and populations. Thus it seems wise to use DNA barcodes and other gene sequences as adjuncts to traditional taxonomic methods for identifying species. As the work on a particular group of organisms progresses, it should become clear whether, or how, barcodes can be used to assign individuals to species. Thus far it seems to work well with monogonont rotifers.

In asexual organisms, on the other hand, the sequence of any one gene can accurately reflect the genealogy of the organisms. This is true provided the gene has not undergone duplication or deletion, which cause gene trees to split at the time of duplication in addition to, or instead of, the time of speciation events. These processes are likely to be rare, and thus might be expected to obscure the relationships of different genera and species but not the clusters that constitute species. Consequently, for asexual organisms one can use any gene sequence as a barcode to define species and then use them to assign future individuals to species.

Barcoding as Big Science: What is CBOL Good For?

Workshop participants pointed out that the barcoding initiative embodied in CBOL is a well-marketed initiative that has been successful in attracting financial support, and it might be helpful for getting funding for research in rotifer phylogenetics and taxonomy. On the downside,

it would be most unfortunate if took away funding from traditional taxonomy. While CBOL has probably increased interest in taxonomy, it appears that barcoding has been over-sold, making promises that are laudable goals but unlikely to be achieved. For example, there is some question whether it is increasing interest in taxonomy in general or just in barcoding. The notion of a handheld barcoder is clever but at this point is science fiction, like the Tricorder of *Star Trek* fame that seems to have inspired it. Certainly it won't replace morphology as the method of first choice for the identification of macroscopic animals and plants in the field.

There seemed to be a consensus at the Rotifer XI barcoding workshop that DNA barcodes must be coupled with more traditional information about the morphology, behavior, ecology, and geographic distribution of organisms. It seems inconceivable that determining DNA sequences from samples could completely replace morphology as a primary method for quickly assigning organisms to genera or species. Equally important, although barcode sequences can accurately track the recent phylogenetic history of organisms, they contain very little information about the biology of those organisms. An extreme case is environmental sequencing in which gene is amplified from DNA isolated from samples of water, soil, or any other habitat, the amplification products are cloned in bacteria, and individual clones are sequenced. The resulting sequences can provide useful information about biological diversity at the genetic level even when individual sequences cannot be assigned to specific organisms. But even in these projects, the payoff is much greater if it can be coupled with information about the biology of the organisms or their relatives.

It is worth noting that CBOL is developing standards for barcode sequences deposited in GenBank and other sequence databases. The standards include links to voucher specimens and associated data. This and other statements from CBOL suggest that the organization

acknowledges the essential role of morphology, biogeography, and other traditional kinds of data in taxonomy.

CBOL may provide some special opportunities for attracting support for rotifer research. In his workshop presentation Hendrik Segers suggested that cryptic species complexes in monogonont rotifers such as *Brachionus plicatilis*, *B. calyciflorus*, *B. angularis-caudatus*, *Keratella cochlearis*, *Epiphanes senta*, *Asplanchna sieboldi*, and *Polyarthra dolichoptera* could be potential showcases for DNA barcoding in sexual organisms. *B. plicatilis* is particularly attractive because of the availability of many *cox1* sequences together with a great deal of information on morphology, reproductive isolation, and biogeography. The *cox1* gene appears to have an appropriate level of resolution for distinguishing species in rotifers. The economic importance of rotifers for aquaculture could help attract funding. What is needed for barcoding rotifers, or any group of rotifers, is an organized project with infrastructure, including the development of centers for identification and reference, and an effort to make nomenclature conform to existing knowledge. The bdelloid rotifers are an exceptionally good example of the simultaneous application of barcoding to species discovery and identification, and might attract funding as a test case that is not complicated by recombination.

Archiving Barcoded Specimens

The workshop included a great deal of discussion of the need to archive organisms from which barcode sequences are obtained. One purpose of archiving is to tie genetic diversity to morphological diversity and enable species identified by DNA sequence to be assigned to morphospecies in the future. With many animals, different individuals from the same population must be archived and used for DNA extraction, and these will usually not be genetically identical. This is not a problem for rotifers because both monogononts and bdelloids produce parthenogenetic clones. Generally it is easy to rear at least small clones from single females in

the lab, then fix and archive some individuals as microscope slides while extracting DNA from other members of the same clone. Still other individuals can be stored in ethanol at -20° for future extraction of DNA and used to obtain digital still and moving pictures of live animals.

But there are some cases where this is not possible or feasible. Single resting eggs (fertilized eggs produced by the sexual monogonont rotifers) can provide enough mitochondrial DNA to enable amplification and sequencing of the *cox1* or other mitochondrial genes. Consequently resting eggs can be used in surveys of genetic variability in rotifers. Moreover viable resting eggs can be recovered from pond or lake sediments of varying ages, providing a picture of how the rotifer diversity of a body of water changes over time and the role of resting eggs as “seed banks” (Gómez, 2005). There was some discussion at the workshop of how one might archive resting eggs. A major problem is that when barcode sequences are obtained from single resting eggs, those eggs are completely destroyed. The only answer offered was to take digital photographs before the egg is destroyed for sequencing, but it is not clear that resting egg morphology is sufficiently variable to enable an egg to be classified to species.

It is also possible to get barcode sequences from single females. Although this has not been widely done, it may be important when it is necessary to get DNA sequences from very large numbers of individuals collected in nature. In this case one could use digital video microscopy to archive both the morphology and behavior of the female before isolating DNA.

It was pointed out that isolating DNA from single eggs was really like environmental sequencing, in which bulk DNA is isolated from a sample of water or soil or other habitat, a gene is amplified and cloned, and the clones are sequenced. The resulting sequences are anonymous, not tied to any organism known from its phenotype. However, once a sequence is used to assign a resting egg to an already-described species, the biology of the egg can be deduced from that of its conspecifics.

In another workshop at the same meeting (Rotifer Stock-Culture Centres) the participants were discussing the difficulty of archiving live rotifers from important collections so that experimental results obtained with those stocks can be verified and extended by future workers. Long-term cryoscopy is not yet routine with monogonont rotifers. In contrast, I reported that cultures of bdelloid rotifers frozen at -80° without any special preparation are viable for at least two years (this method did not work with monogononts). But the future of frozen cultures is dependent on the willingness of future generations to maintain them. With some organisms, archiving is not a problem because individuals of the same population and species can be collected again and again from nature based on species and location descriptions. This is problematic or impossible in some rotifers where there are cryptic species or clones with important genetic variation. I pointed out that this problem could be avoided or minimized if DNA barcodes and digital photos of important experimental clones were routinely archived. Then clones of the same or similar morphology could be collected from the original site and barcoded to find the closest available genotype.

Conclusions about Barcoding Rotifers

DNA barcoding has been playing an increasingly important role in the systematics of rotifers as well as in studies of their phylogeny and biogeography for some years, and this trend will certainly continue. Rotifer workers will continue to combine sequence analyses with more traditional phenotypic studies to produce the most useful kinds of information about biological diversity. But this work would be greatly facilitated by several kinds of programs:

1. More collaborations are needed to bring together rotifer workers with skills in different relevant disciplines such as molecular phylogenetics, morphometric analysis, behavior studies, and ecological studies.

2. It would be extremely helpful to organize large-scale projects capable of attracting the attention of the public and of policy makers; this would attract more scientists and greater financial and infrastructure support.
3. An infrastructure needs to be developed for archiving rotifer specimens linked to supporting data, including DNA sequences still photos, drawings, and video. Ideally, samples of each clone should be preserved in a state suitable for DNA extraction.
4. A strong effort must be made to achieve a working consensus about what species concepts are most useful and appropriate for understanding rotifer diversity. In particular, if we do not come a consensus on the roles of cladistic and phylogenetic approaches to systematics, we will certainly end up talking past each other and producing taxonomic confusion.

Unfortunately, this may be the most difficult task of all.

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