Does the traditional conceptual basis hold its promise? Lessons learnt from Metazoa

James Mallet, with Kanchon Dasmahapatra

Below the species level, there are abundant ecotypes and ecological races that are nearly what we might want to call species, but which we prefer to regard as "races" for one reason or another. Above the species level, hybridization and genetic introgression is a regular occurrence. Hybridization is, of course, very rare on a per-individual basis, but >=10% of all taxonomic species seem to hybridize with at least one other species, and not just with sister species. Furthermore, "legitimate" introgression, as well as "illegitimate" horizontal transfer may both have important consequences for evolution in general, especially low down in the tree of life. "Reproductive isolation" among species declines continuously (albeit noisily) with genetic distance. Theoretical analyses, together with recent historical treatments of 20th Century misunderstandings of Darwin's own idea of species, back up the empirical evidence for this continuum, and provide a coherent understanding of species and speciation for the age of genomics. Empirically speaking, speciation is easy! This means in practice that we constantly see evidence for all the intermediate stages, with the result that we are always going to have difficulties agreeing on a consistent species concept. We therefore argue that a merely practical, rather than a conceptually perfect solution is required. For populations that overlap, we argue that this is best provided by a population genetically validated method of cluster analysis, using multilocus genomic data from individuals, analysed by statistical procedures related to "assignment tests."

Species delimitation in the "higher" eukaryotes for comparison: the impetus for the "biological species concept" of the 1930s and 1940s

By contrast with protists, macro-organisms may seem, at least from the outside, to have well-behaved and easily delimited species. In practice, there are still many problems, and a careful examination of current macro-organism research may help with the elucidation of the perhaps more extreme problems for protist systematics. Although there are clear

gaps between some species of macro-organisms, the fact that species evolve means that there are also many unclear boundaries as well.

Mayr's "biological species concept" (BSC) is among the best known ideas of species in macro-organisms (Mayr 1942). However, it is really suitable only for sexual species, and depends on the idea that species are reproductively isolated from one another, while variants, ecotypes, and geographic races within species are not. One of Mayr's major aims with his promotion of the biological species concept was not, as might be imagined from more recent texts, merely to ensure that reproductive isolation was the definition, but to do away with previous "typological" views of species that depended on only a few morphological characters as practised by taxonomists often referred to as "splitters" (because they split life up into many small groups). The taxonomic result of Mayr's suggestions meant that widely distributed and diverse morphological forms could be united as geographical races or subspecies within a single "polytypic" species if they were all part of the same lineage, and played a similar role in the economy of nature. Mayr was in essence a "lumper" who wanted to promote ideas that reduced unnecessary naming of trivial varieties (Mayr 1942).

Furthermore, cryptic or "sibling" species that are sympatric and morphologically identical, but which do not mate together and are clearly distinct biologically could also be separated via the biological species concept. Dobzhansky had earlier argued with his colleague Sturtevant about whether "race A" and "race B" of *Drosophila pseudoobscura* were distinct species (Dobzhansky 1937); today, we recognize race B as *D. persimilis*, a distinct sibling species from *D. pseudoobscura*. Mayr and Dobzhansky both felt that a species concept based on reproductive isolation was more biologically meaningful than the chaos of naming based purely on morphology that had gone on before.

The impact of the phylogenetic species concept on taxonomy

I think it is true to say that most biologists and practising taxonomists from the 1950s onwards, at least in zoology, agreed both with the idea and with the practice of the biological species concept. In birds and butterflies, this biological justification for polytypic species, in particular, led to a great reduction in the numbers and simplification of species names, and considerable nomenclatural stabilization from about the 1960s to

the 1980s: previously named species often became absorbed as geographic races into more inclusive polytypic species.

More recently, Cracraft's "phylogenetic species concept" (PSC, Cracraft 1989) whereby species are declared whenever populations have fixed diagnostic characters, has led to yet another revolution in the practice of systematics. A reassessment of many of the previously lumped species has occurred as a result. Since the 1990s, mitochondrial DNA sequencing has often contributed potential fixed characters for the phylogenetic species concept. In the best-studied vertebrate groups, this has led to renewed period of taxonomic inflation. For instance, while numbers of recognized primate species remained fairly constant from 1965-1985, the number recognized has approximately doubled since then, from around 180 to nearly 400 species. Few of the extra species are newly-described taxa; most are formerly known subspecies that have been elevated to species rank (Isaac et al. 2004).What seems to have happened is that the assessment of what differences are "biologically important" in species concepts has changed. This is a rather confusing and possibly unfortunate situation for conservationists and their interaction with the public: on the one hand, primates contain among the most endangered species of any animal group; on the other, scientists apparently can no longer say for certain what it is they mean by species, or whether a single endangered species will be recognized as two species next year.

Mitochondrial DNA "barcoding" and species delimitation

Meanwhile, the newer molecular technologies, particularly mitochondrial DNA studies, are undoubtedly leading to discoveries of a number of cryptic taxa that cannot be classified as geographic races, since they occur together in the same areas. Regardless of one's view of taxonomic inflation due to the recent wave of splitting up of geographic populations, populations that are in sympatry and yet maintain genetic distinctness across their genome are undoubtedly "biologically real" in some sense. In some cases, hybridization among the forms is common, and these forms are generally viewed as ecotypes, or ecological races, below the species level. In other cases, gene flow is absent or minimal, and scientists tend to regard the forms as cryptic species.

A recent movement in macroorganismal systematics has recommended the use of a fragment of a single mitochondrial gene, ~700 base pairs of the 5' end of the cytochrome oxidase I gene (*CoI*), as a "DNA barcode" to delimit and identify species (Hebert et al. 2003). The utility of this procedure is that it can be standardized among many different organisms (although not plants, for instance, where different genes must be used); that it evolves rapidly leading to great discriminatory ability among closely related species, and that it has well-known conserved priming sites for PCR. Barcoding seems to promise discoveries of many cryptic species in groups for which morphological characters are lacking, and it is touted as a method to speed up systematic work, and to be suitable for automated identification. Barcoding is also useful for identification of morphologically uninformative stages such as larvae, for environmental sequencing, or to identify traces in forensic or historical material.

The name "DNA barcoding", however, rests on an analogy that is not entirely correct. A single supermarket barcode corresponds to a single product. In contrast, speciation certainly does not depend in any way on a change in *CoI* sequence (hereafter "barcode"). Thus, a *CoI* barcode may correspond to one or more species and, there is also often considerable variation in the barcode within species. If one has studied mtDNA variation in the groups and species under study, using a barcode is often a good way of making an identification. When one finds new barcode in a group of individuals, it may be an indication of separate species status, and those organisms may repay further study. But the barcodes can often mislead as well; here I give examples from work in my own laboratory.

In Lepidoptera, barcodes are especially useful (Hebert et al. 2003; Hebert et al. 2004). In part this is likely to be a result of general obedience to Haldane's Rule in the Lepidoptera (Presgraves 2002). In Haldane's Rule, the heterogametic sex (i.e. the sex with heterozygous sex chromosomes) tends to be the sex that most quickly becomes sterile in hybrids between a pair of diverging species. In Lepidoptera and birds, the heterogametic sex is the female (ZW), which also is the sex that transmits the mitochondrion to the next generation. If female hybrids between species are sterile, the barcode will be difficult to transmit between species. For mammals and fruitflies, the heterogametic sex is the male (XY); this is not so useful for barcoding, since it is the

male that suffers Haldane's rule sterility, while hybrid introgression of mtDNA may still occur via the females. Thus the abundant female hybrid sterility in Lepidoptera means a reduced possibility for mitochondrial introgression among species, and therefore a smaller likelihood for mitochondrial DNA barcodes to be shared among species (Sperling 1990).

In our own work, we have on several occasions discovered new cryptic species based on barcodes. An example is a group of cryptic species or geographic forms allied to *Heliconius cydno* and *H. timareta* found on the Eastern slopes of the Andes from Colombia to Peru (Brower 1996; Giraldo et al. 2008; Mallet 2009). Butterflies of the genus *Heliconius* (as well as those in the unrelated tribe Ithomiini discussed below) are famous for their unpalatability to predators, bright warning colour patterns and cooperative or Müllerian mimicry. The extremely accurate colour-pattern mimicry makes even distantly related species hard to identify. Cryptic, closely related species like those in the *cydno*/*timareta* group are especially challenging. For all of these, the key or initial clues came via mtDNA work (although not using the "barcode" sequence fragment itself, as it happened), and later confirmed via correlated differences at nuclear loci, biology, subtle morphological details, and ecology.

Recently, we have been studying a variety of other species in the nymphalid butterfly tribe Ithomiini. Here we find every combination of success and problem with mtDNA barcoding. There are cases where multiple "good" species share the same or overlapping barcode sequences, in at least five recognized species of the genus *Melinaea* (Dasmahapatra et al. 2010b; Elias et al. 2007; Whinnett et al. 2005). Further investigation with a multilocus fingerprinting technique, Amplified Fragment Length Polymorphism (AFLP), revealed that the *Melinaea* species were "real," in the sense that the AFLP loci correlated strongly with morphology involved with the Müllerian mimicry colour patterns used to identify the species (Dasmahapatra et al. in prep). This contrasts with similar studies on *Heliconius numata*, which has up to 10 locally sympatric colour pattern morphs, each of which is a Müllerian mimic of a different species of *Melinaea*. Apart from strong differences at the colour pattern "supergene" genomic region itself, no genetic differences (including barcodes) were found among sympatric mimetic morphs of

Heliconius numata, and mating among the morphs appears approximately random (Dasmahapatra et al. 2010b; Joron et al. 2001).

In other cases, some strongly divergent mtDNA fragments (~2% divergent) were found to be widespread within single species of the genus *Mechanitis* (Dasmahapatra et al. 2010a; Elias et al. 2007); note, in these studies we checked >2 kbp of *CoI*, *tRNA-leu*, and *CoII*, rather than just the barcode region). In this genus, there are eight distinct clusters of mtDNA haplotypes (A-H, each differing by 1.6%-4.1%) among five previously recognized species. The mismatch is due to the existence of three mitochondrial haplotype groups in each of two widespread species *Mechanitis polymnia* (B,C,D) and *Mechanitis mazaeus* (A,F,H) (Dasmahapatra et al. 2010a).

Further study of the larval biology, morphology of the adult and young stages, and of nuclear gene loci helped us reach a decision about which species were real and which were not. The larval foodplants, subtle morphological differences, and multilocus AFLP differences correlated with and revealed strong differences with haplotype H (*Mechanitis mazaeus* sensu stricto) versus haplotypes A + F (*M. messenoides*) (Hill et al. in prep.). However, we could find no correlated genetic, morphological, or ecological differences between individuals bearing haplotypes A and F within *M. messenoides*, even though these barcodes are strongly divergent (3.6%), while many species in the group differ by as little as 2%. AFLP studies also revealed no correlated multilocus differences between haplotypes B, C and D, and we therefore conclude that there is no evidence for more than one species within *M. polymnia*. Although there is considerable variation and overlap, most individuals of *M. mazaeus* sensu stricto and *M. messenoides* can be identified via subtle colour pattern differences of the adult wings, while individuals carrying haplogroups A vs. F in *M. messenoides*, and B vs. C vs. D in *M. polymnia* are not distinguishable on the basis of colour pattern (Hill et al. in prep.).

So barcoding is sometimes useful, but sometimes the barcode/DNA analogy fails, and the barcode may "lie" to us, even in Lepidoptera where Haldane's Rule acts in its favour. Why and when does this happen? While we do not fully know the answer, we may speculate. *Melinaea* and *Mechanitis* are widespread genera each consisting of several, very abundant species in the Amazon basin, where most of the polymorphism is found. In

both genera, hybridization among species is known (Lamas 2004; Vasconcellos-Neto and Brown 1982) (Mallet et al. 2007), and this may lead to some transfer of barcodes among species. Although the Amazon went through some climatic disturbances throughout the Pleistocene, it seems likely that many fewer bottleneck events occurred compared with the North temperate zone where most systematics-oriented sequencing studies have been done. Thus it is likely that effective population sizes of these species have remained large for millions of years, which should lead to abundant retention of polymorphism at neutral loci. In the case of both genera, sequencing has detected hardly any additional divergence among closely related species at nuclear loci when compared to the high level of polymorphism within species (Dasmahapatra et al. 2010a, b), thus both genera contain many recent species. In both genera, it is only when we investigate the frequencies of haplotypes at multiple loci (here via AFLP fingerprinting, and the use of assignment tests), that enough signal emerges to enable us to distinguish separate species via nuclear genes.

In the case of the problematic *Melinaea* that share barcodes, the probable reason is that all of the species are very young. This is highly intriguing, because each species has many different geographic races, each of which has strongly divergent mimicry colour patterns. Our sequencing data suggests that the species are less than about 100,000 years old, meaning that the geographic mimicry races are only say ~10,000 years old. Given that *Melinaea* colour patterns are driving the largest Müllerian mimicry rings in the Amazon basin, the "tiger" mimicry rings (Mallet 2001), this implies extremely dynamic mimicry evolution (Dasmahapatra et al. 2010b).

While considerable mtDNA polymorphism exists within each species, we have not detected nearly such strong barcode clustering in *Melinaea* as in *Mechanitis polymnia* and *M. messenoides*. Again, sequence information from nuclear loci was not very informative of *Mechanitis* species status, but AFLP fingerprints depending on multilocus frequency differences at many loci were. So again, we probably have very young species, some with surprisingly large divergences among mtDNA haplotypes perhaps because of large effective population sizes, and perhaps for other unknown reasons, such as mitochondrial selective sweeps, or the effects of cytoplasmic symbiont genomes that could give rise to an equivalent sweeping effect. There is precedence for this in

butterflies: in the genus *Hypolimnas*, cytoplasmically-transmitted bacteria in the genus *Wolbachia* are known to have led to large mtDNA sequence divergences among members of the same species, as well as to have swept mtDNA haplotypes among species (Galtier et al. 2009). While we tend to use mtDNA sequence information as a "neutral marker", there must often be occasions where selection somewhere in the mitochondrial genome can lead to non-neutral effects in our marker sequences.

These are not the only potential problems with barcoding. In Lepidoptera, hybridization and introgression are common, with at least 16% of butterfly species in Europe known to hybridize with at least one other species, and often other species as well. In some groups, hybridization is especially rampant, for example in heliconiine butterflies (36% of species). In flowering plants, approximately ~25% of all species hybridize naturally (in Britain), and in animals at least ~10% on average, although again in certain groups hybridization is much more widespread. Whenever hybridization occurs, barcodes may be transferred, and the barcode is more likely to lie.

In our studies of *Mechanitis*, we titled our paper with the conclusion "Mitochondrial DNA barcoding detects some species that are real, and some that are not" (Dasmahapatra et al. 2010a). In *Melinaea*, we also showed that mitochondrial DNA barcoding does not detect some good species at all. Of course, the barcoding idea need not be restricted to mitochondrial DNA, and indeed in prokaryote studies the sequence of choice seems to have been 16S rDNA, while in protists ITS regions have been suggested. Both of these have been justified on the grounds that transfer is unlikely because they would generate strong incompatibilities, but it should be recognized that stranger things have happened, and also that there may be cryptic variation below the level of these markers which could be as or more important in generating ecologically distinct species than variation above the level of sequence divergence for these markers. In this sense, the microorganismic markers are prone to exactly the same sorts of problems as barcoding is in macro-organisms. In both cases, the markers are extremely useful, but have a tendency to mislead if the barcode analogy is taken too literally.

Brief interlude on species concepts, species taxa, and species delimitation

Publications by philosophers and historians of science as well as some biologists are often to be found labouring the logical distinctions between *species concepts*, *species categories*, and *species taxa*. In current usage, the term "species concept" seems to imply some real or functional characteristics whereby nature itself uses to define the species category. Species concepts are usually descriptions of the species category which include a mechanistic explanation of "speciesness." One form of the biological species concept (and the recognition concept of species is similar), for example, proposes that species are cohesive wholes maintained internally by gene flow, and protected externally by isolating mechanisms. What we mean by "concept" in this sense is an attempt to define this truth or reality about species. Without going into much detail, I believe that a lot of the problems and disagreements in the species concept literature stem from an other-worldliness of arguing about something as nebulous as the "true species reality:" different scientists believe different processes are important. Interestingly, there is a link with essentialism here. A species concept can be disobeyed in actual instances, without disproof of the concept or essence itself. For example, reproductive isolation might be held to be the *vera causa*, or true essence of species, even though there are obvious exceptions: gene flow and hybridization does occur between many sexual species, and asexual organisms cannot have reproductive isolation at all.

In contrast, a method of "species delimitation" attempts to use a set of practical criteria to divide individual organisms into large groups of populations that we then call "species taxa", the actual instances of species. The criteria may depend on one or more species concepts; but the important emphasis here is on the practical method of alpha-taxonomy.

The "species category" consists of the total of all the species taxa defined by a given set of criteria. It is the class of all populations we have delimited as species. It seems fairly obvious that the current debate in protistology is largely a practical and scientific one about whether there is a category of taxa, "species" in actual organisms called protists equivalent to the "species" categorized in multicellular organisms. This is really a question about delimitation of species, rather than about concepts, and indeed, I would argue that most answerable questions about species are rooted in the practical matter of species limits, rather than being purely arguments about concepts. I therefore tend to see

understanding speciation as a search for generalities of how individual taxa speciate, or pass the threshold where they become delimited as separate species. In the past, focusing on actual instances of species taxa has been made to seem, quite unfairly, I think, a lower sort of activity than concentration on conceptual matters of species reality. Alpha-taxonomy is in my view quite wronged by this viewpoint.

My argument is that a simple-minded character-based delimitation comes close to how we use words for objects such as “table” and “chair”. Although we know that there are different sorts of essential functions for both tables and chairs, we are really using a quick multivariate analysis of their external characteristics when we say “that is a chair”, or “this is a table”. Therefore, what I am advocating with respect to species delimitation is close to Wittgenstein’s view of the meaning of words: their meaning is in their use; there is no good essential meaning for any word.

Species delimitation and assignment tests to detect multilocus genotypic clusters

There are many ways of delimiting sexual, eukaryotic species practically, and the different methods can produce mutually inconsistent results (Sites and Marshall, 2003). Some of these methods may require tree-building; others are purely phenetic or depend on crossing studies. Today, the major focus is on molecular genetic data, which is potentially much richer than morphological data, and being digital rather than analogue, can give more reliable and repeatable results between different observers laboratories.

As I have argued above, I do not think it is entirely necessary to sort out what is the best concept of species if we merely want to delimit individual groups as species for the sake of scientific discussions. Most definitions of sexual species require some genetic differences (probably multilocus) to exist between two species that occur together in one area; and this forms a minimal set of characteristics, which, it seems, we use instinctively as a basic delimitation method (see above re: tables and chairs). I focus on the case of sympatry or togetherness in this minimalist set of conditions, because most of the disagreements about concepts and delimitation break down when dealing with distant or completely separated populations. An example was given above: the PSC relies on fixed differences, and tends to split geographically separated populations into separate species; in contrast, the BSC relies on reproductive isolation, and would tend to lump similar but

geographically differentiated taxa together as subspecies or geographic races within an overarching polytypic species. But when two genetically differentiated populations in sympatry, with no overlap, and a gap is present between them in the distribution of multilocus genotypes, the two taxa are clearly separate species under both definitions.

This gap between genetic clusters is, I believe, the same as the underlying pre-Mendelian idea put forward by (Darwin 1871):

> *Independently of fusion from intercrossing, the complete absence, in a well-investigated region, of varieties linking together any two closely-allied forms, is probably the most important of all the criterions of their specific distinctness*

In my own work, I had been studying hybrid zones between races and species, and also sympatric ecological races, as well as hybridization among sympatric taxa most people want to call species. I argued that two species are, at minimum, clusters of multilocus genotypes that are distinguishable genetically in zones of overlap: they form bimodal genotypic clusters, where the gap between the clusters may be complete or may contain a few "hybrids" (Jiggins and Mallet 2000; Mallet 1995). Some saw these ideas as an attempt to define "a new species concept": however, my intent was practical and anti-conceptual (in the sense I use "concept" here); it was a method of delimitation, which is inherently likely to be true under most "species concepts." I demonstrated how one could separate sibling species of *Anopheles* based on gene frequencies at only a few allozyme loci using these methods (Mallet 1995).

One weakness with my proposed delimitation method is that, although I explicitly formulated the ideas in manner suitable for likelihood-based testing, I failed to provide an implementation. However, independently of these basic ideas about species concepts, extremely useful statistical packages known as "assignment tests" soon started to appear. Assignment tests identify genetically distinguishable populations using multilocus genotypic data, and assign individuals to those populations (Corander et al. 2004; Corander et al. 2003; Falush et al. 2007; Falush et al. 2003; Huelsenbeck and Andolfatto

2007; Pritchard et al. 2000). All of these implementations use inhomogeneities in the data, particularly linkage disequilibrium, to detect groups; one does not expect to see heterozygote deficits or linkage disequilibrium within populations (see Pritchard et al. (2000). The results are usually displayed as a bar graph, with the bars for each individual coloured to represent Bayesian posterior probabilities, the assignment probabilities, of belonging to each group. If the samples are all taken from a single area, species delimitation via genotypic clusters in sympatry consists of detecting inhomogeneities of population structure in multilocus genetic data; the resulting assignment probabilities represent probabilities of belonging to a particular species. I regard assignment tests as the method of choice when implementing the genotypic cluster idea of species as a delimitation procedure.

Possible applications in protist systematics and evolution

Three problems:

1) too little recombination and frequent asexuality

2) geographic population structure not related to species status

3) large effective population size of protists, and consequently higher levels of polymorphism

1) The former problem seems the major difficulty with using assignment tests to detect genomic cluster species in protists. Assignment tests will typically detect a great deal of population structure in organisms that breed asexually and have little recombination. An example is given in Jukka Corander's analysis of *Neisseria* bacteria. His work detected 35 forms in multilocus data from 3,175 strains. Notice that the nominal species (mainly *N. meningitidis* and *N. lactamicus*) each have many genetically detectable subgroups (Fig. 1). Assignment tests cluster groups by genotypes, and detect inhomogeneities in the population structure. Thus if there are low rates of recombination, epidemic outbreaks of particular haplogroups can lead to structuring and high levels of disequilibrium (Maynard Smith et al. 1993). It seems to me that in this situation there is no particular way

to decide which haplogroups are “species,” and which can be assigned to variants within a species. It seems most sensible to call them separate species if they display major disease-causing differences or have other ecological or biological differences that seem worth recognizing as species. Although this might seem arbitrary, this arbitrariness is also inherent in how species delimitation is actually performed in supposedly well-behaved organisms like primates and birds as well (see above). In vertebrates, a difference in interest in different biological parameters over the years has led to different treatments of geographically separated and genetically identifiable populations.

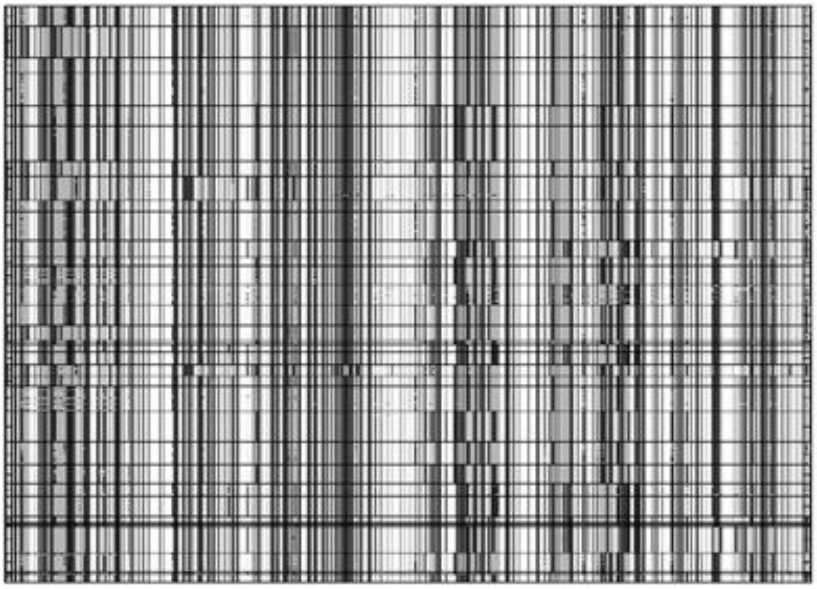

Figure 1. Jukka Corander’s Assignment Test analysis of two ‘species’ of *Neisseria*. 5,175 bacterial multi-locus DNA sequences, from which 35 clusters are inferred. Cluster limits are shown as horizontal lines; columns are sites of aligned DNA sequences; rows are individual strains.

Some asexual clusters have the characteristics of "good" separate species, as has been noted for many years (Hutchinson 1968). In the bdelloid rotifers, meiosis has presumably been lost, and asexuality has been the norm across the whole group for hundreds of millions of years. This asexuality in a large diploid eukaryote group leads to fixed heterozygosity and a situation where different homologues of the same chromosome are more divergent within a species than are the same homologues between species (Mark Welch and Meselson 2000). However, clusters are detectable with long branches in mitochondrial and nuclear 28S ribosomal DNA phylogenies between species, and much shorter branches within species (Fontaneto et al., 2007). It is likely that ecological competition prunes the branches so that only closely related clusters survive, and that bdelloids have speciated very much in a Darwinian way, with ecological factors and disruptive selection driving the species apart. Thus sexuality is not required for the application of biological ideas to delimit species, although severe asexuality and epidemic population structures can cause a problem that too many detectable "species" may result to be convenient for naming.

2) The second problem is population structure. We encountered the problem of geographic population structure in the case of the butterfly *Mechanitis menapis* (Dasmahapatra et al. 2010a), and also in the primates and birds whose species are being split due to application of the phylogenetic species concept to newer genetic data. Geographic populations will differ in allele frequencies (i.e. $F_{ST} > 0$), and so will show up as detectably different genetic groups in assignment tests. It will always be an arbitrary decision to decide whether geographically separated populations are distinct species, because such populations are likely to differ, not only genetically, but also to some extent biologically and ecologically. We do not infer that all inter-individual incompatibilities or "reproductive isolation", or ecological differences, or genetic differences are evidence for species status, there must always be some arbitrariness in our decision. To the extent that population structure in microbes is often limited by extensive gene flow across the planet, this should ease the possibility of detecting clusters in protists; divergence

between clusters is better evidence of species status in taxa with spatially extensive gene flow than it would be if the strong divergence resulted from a lack of effective gene flow over long distances.

3) A third problem is that very high levels of neutral or nearly neutral polymorphism may exist within natural populations of protists, if, as is likely, they have very large effective population sizes compared to multicellular organisms whose population genetics are better known. Suppose a group of sexual protists consist of a number of perfectly good biological species with complete reproductive isolation among the species. Then coalescent theory predicts that coalescence times will be very large, and if speciation is rapid, there could be much sharing of ancestral polymorphism between recent species (Hudson and Coyne 2002). This would mean that the possibility for a "barcoding gap" (the distance between typical levels of divergence within a species and typical levels between the closest pairs of species) would become more remote even than it is in multicellular organisms. This would compromise the use of single sequences to delimit species. Any attempt to use a standard percentage of divergence in a barcode or even perhaps ITS2 sequences would risk creating a lot of apparent species that bear no relation with any patterns at the rest of the genome.
   This problem could again be mitigated by using a multilocus or nuclear genomic approach, such as is adopted in assignment testing (see above). Assignment tests discern signatures in multilocus data that mark breaks between species, essentially by minimizing linkage disequilibrium within groups. Assignment tests can detect such groups even when there are no SNPs completely fixed (and therefore no monophyly of any gene genealogy) in each group or species.

Conclusion

Overall, we have argued that the important thing about species is not to choose the correct species "concept" but to have an appropriate way to infer species status, to delimit species. We have suggested that the *sine qua non* of delimitation methods, and the only one that does not depend on a specific species concept, is a form of genotypic clustering

implemented in assignment tests. However, these methods detect population structure via linkage disequilibrium. When the disequilibrium and clustering is due to factors other than those which one would wish to use to classify species, one may detect too many clusters. Important additional factors might include asexuality and lack of recombination, epidemic population growth and, perhaps less importantly, geographic population structure. In such cases, one is forced to make rather arbitrary decisions about what one wishes a species name to represent, and these decisions should normally be based on ecological or biological functions of interest. For example, if the organism is medically important, one might want to name each of the clusters within species, as is done for many virus or bacterial strains (e.g. H1N1, MRSA).

Darwin's conclusion about species was that the clusters are real, but that all life evolved from a single source. Reliance on sequences of single genes might be helpful if this is all that can be done, but multilocus data is much more powerful for detecting and clustering individuals into groups via assignment tests. Our ability to sequence whole genomes rapidly is improving very rapidly as technology advances. This makes multilocus information much easier to obtain today, even than two years ago. In an evolutionary world, we expect many intermediates between "good species" and variants, races, or genotypic clusters within species. With newer molecular tools, this is exactly what we are finding. The understanding of evolution means that there is no special reality of these species, compared to our expectation of clear differences when we all believed that species were created by divine fiat. As a result, all species delimitation must be somewhat arbitrary. This is particularly so in difficult cases, as Darwin pointed out. It is likely to be most difficult to decide on species status in taxa that have only recently diverged, and therefore share variation, or in taxa with low rates of recombination, high rates of asexuality, or strong geographic structure. Perhaps this seems a bleak and chaotic future for systematics. In any case, this is the actual situation, in protists as well as in macro-organisms. It is a fact of nature that we must face.

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