

## **Is Molecular Biology the Magic Bullet for Tackling Benthic Marine Biodiversity?**

Dr Alex David Rogers, British Antarctic Survey, High Cross, Madingley Road, Cambridge, CB3 0ET, United Kingdom. Email: [Adr2@bas.ac.uk](mailto:Adr2@bas.ac.uk)<sup>1</sup>

Dr Robert D Ward, CSIRO Marine and Atmospheric Research, GPO Box 1538, Hobart, Tasmania 7001, Australia. Email: [Bob.Ward@csiro.au](mailto:Bob.Ward@csiro.au)

The seabed is home to an enormous diversity of animals that vary in size from large corals to tiny worms living amongst grains of sediment. Understanding how much diversity is present at the bottom of the oceans and how it is distributed is a great challenge. Overall estimates of diversity in the deep sea over the last 15 years have ranged from less than 1 million to 100 million species (e.g., Grassle & Maciolek, 1992; Lambshead, 1993). The realisation over the last two years that local diversity in the deep sea may reflect regional diversity and the view that many species collected in samples may represent “sink populations” suggest that the larger estimates of diversity previously suggested are unrealistic (Gage, 2004; Rex et al., 2005). However, global marine diversity is still a significant proportion of species on the planet Earth.

Scientists face a number of problems when tackling diversity, especially in little-explored parts of the world’s oceans, such as the deep sea. Firstly, for some of the most diverse and abundant groups of marine animals, there are very few taxonomists. This means that, for many groups, it is impossible that even the majority of common species will be described in the next several hundred years. Secondly, marine species often show a great deal of variation in their body shape and colour. This means that distinguishing species can be very difficult simply by looking at animals. Genetic methods have frequently demonstrated the occurrence of species complexes or cryptic species (e.g., Knowlton, 1993). These are groups of species that are difficult or impossible to separate by examination of body shape (morphology). This problem becomes especially acute when comparing specimens of animals from across huge geographic distances such as between the Pacific and Atlantic oceans.

Molecular biology offers many potential methods for improving our ability to distinguish species in the oceans. These rely on the fact that each species has a unique genome that differs from all other species.

### **Barcoding**

At the simplest, regions of genomic DNA that show differences between species can be amplified and sequenced to produce a species “barcode”. Ideally, such barcode regions should be easy to amplify in all the species of interest and they should be sufficiently long and variable in order to resolve each individual species. An international consortium has been established to foster and direct the development of DNA barcoding – the Consortium for the Barcode of Life (CBOL) ([www.barcoding.si.edu](http://www.barcoding.si.edu)). The intention is, wherever possible, to focus on a single gene,

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the mitochondrial gene cytochrome oxidase I (COI) (e.g., Hebert et al., 2003). The CBOL Web site has much useful information, including experimental protocols for barcoding. Another very useful site is the Barcode of Life Database (BoLD) at [www.barcodinglife.org](http://www.barcodinglife.org). This site contains background information on barcoding and links to barcode publications as well as the barcode database. Some of the barcode datasets have now been released to the public and can be readily accessed; these include the first batch of data from some of Australia's commercial and imported fishes (Ward et al., 2005). GenBank has recently inaugurated a special Barcode section for COI sequences aligned to voucher specimens.

The COI gene has already been demonstrated to separate species of most animal groups, although it does not appear to be suitable for a few groups, such as Cnidaria (sea anemones, jellyfish, corals, hydroids). This phylum has a DNA repair gene in the mitochondrial genome leading to low rates of mutation in mitochondrial genes and low species resolution of COI (France & Hoover, 2002; Hebert et al., 2003). This has led to some interest in also exploiting nuclear genes, especially those of the ribosomal RNA encoding multigene family (18S & 28S rRNA; e.g., Cook et al., 2005). It also appears that COI has low resolution in plant species, where the chloroplast genome is being explored to find a suitable barcoding gene.

While barcoding is still in its formative years, several large barcoding initiatives have begun. One is FISH-BOL, the Fish Barcode of Life initiative ([www.fishbol.org](http://www.fishbol.org)). This enterprise aims to coordinate the assembly of barcodes from all the fish species of the planet, and to deposit these sequences in a publicly available free-access database. The sequences will be derived from voucher specimens with authoritative taxonomic identifications. Once the library of sequences from identified specimens has been established, any unknown specimen (whether it is a whole fish, a fillet, a fin, an egg or a larva) will be able to be sequenced for the same gene and this sequence matched against the library to provide unambiguous species identification. Any DNA laboratory with access to the World Wide Web will be able to provide this service. The venture is in its very early stages of activation, but early results from pilot projects have demonstrated the power and the efficacy of the approach (Ward et al., 2005; see Figure 1 for example).

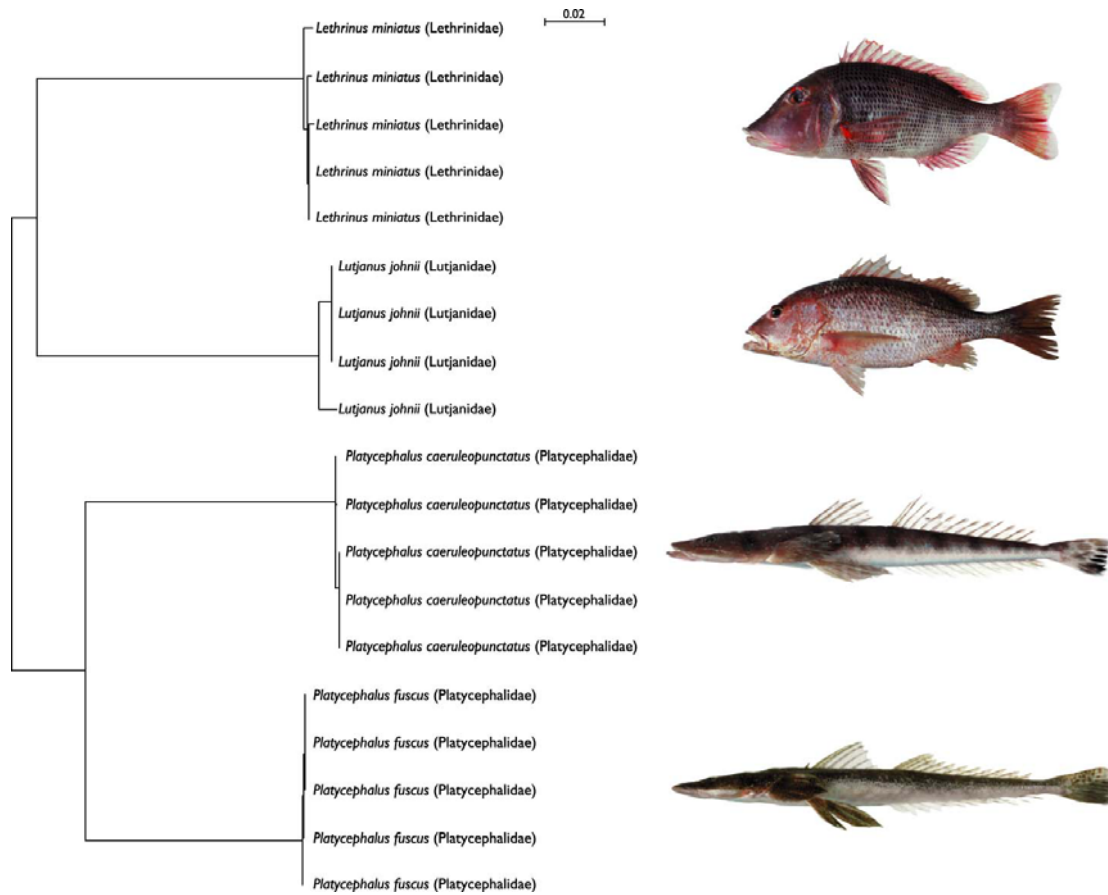


Figure 1. Nineteen individuals of four species of fish barcoded for COI. The top two species are in different families but look somewhat similar, the lower two species are from the same genus in a third family and morphologically can be easily confused. This neighbor joining tree of Kimura 2-parameter COI distance shows that the four species can be very easily discriminated by DNA barcodes, while there is little variation within a species. (Robert Ward)

The Census of Marine Life (CoML) is a major international study of the diversity and distribution of marine organisms, carried out through 14 field projects focused on different ecosystem types and taxonomic groups (see [www.coml.org](http://www.coml.org)). CoML is developing an activity to promote the use of barcoding across its field projects (see <http://www.coreocean.org/Dev2Go.web?id=255158?>).

The barcoding approach is clearly an excellent one that complements conventional morphology-based taxonomy. However, when faced with a large number of environmental samples that may each contain thousands of individual animals, sequence-based identification of individuals is still relatively slow (although improved technology is increasing sample processing power and also reducing costs). Scientists are therefore exploring other ways to rapidly identify species from environmental samples. The way this is done depends on whether the scientific objective is to identify a single or few species, such as a harmful marine alga, or if it is to identify as many species as possible from a sample, as required in biomonitoring or biodiversity studies.

## **Species-specific primers, DNA fingerprinting and nanotechnology**

Identifying a single or a few species is technically relatively simple. This can be achieved by designing species-specific primers that will only amplify DNA from one or a few species (e.g., DeSalle & Birstein, 1996; Rocha-Olivares, 1998). These primers can be linked to fluorescent molecules. Alternatively, primers can be designed that amplify different sized products from several species of interest (e.g., Dixon et al., 1995). The DNA amplicons are then differentiated by molecular weight through electrophoresis. Another approach is to identify different species by digesting the polymerase chain reaction (PCR) products from individual specimens, using a restriction enzyme. The products from the digestion can produce a unique “fingerprint” for several species (e.g., Evans et al., 1998). Other methods can be used to produce species-specific fingerprints for species identification. The least complex of these is RAPD (Random Amplified Polymorphic DNA) Fingerprinting. This comprises of a PCR reaction using two short, random primers. Where the primers anneal to the genome sufficiently close to each other, a fragment of DNA is amplified. These fragments are then visualised using electrophoresis (e.g., Coffroth & Mulawka, 1995).

The emerging science of nanotechnology<sup>2</sup> is offering new methods for detecting individual biomolecules at unprecedented levels of sensitivity. For example, Quantum Dots (QDs), minute (2-10nm) nanocrystals that emit light of a specific wavelength following laser excitation, can be used to detect specific DNA sequences for identification of organisms. The QDs are coupled to streptavidin that has a high affinity to biotin. Two biotinylated single stranded DNA probes designed to hybridise with different parts of a target DNA sequence can be coupled to two different QDs with specific emission spectra. In the presence of target DNA, the QDs crosslink by hybridising onto the complementary DNA sequences of the target producing an emission signal on laser excitation detected by a confocal microscope when the sample is driven through a microcapillary (Yeh et al., 2005). Alternatively, a single QD may be used and two single-stranded DNA probes—one biotinylated and the other conjugated to a fluorophore—are designed to hybridise with different binding sites of target DNA. The two probes are mixed with the sample and in the presence of target DNA sequences hybridise to form a molecular sandwich that then bonds to the QD again producing an emission when excited by a laser that is detected in a confocal microscope (Yeh et al., 2005). This approach offers the potential to detect specific DNA sequences from organisms such as picoplankton and virus particles. It is also potentially useful for accurate quantification of single species or groups of microorganisms from environmental samples as it does not require PCR amplification of target DNA to provide enough material for detection.

### **Identifying multiple species**

Identifying multiple species from environmental samples is a much more complex problem. A recent study has employed methods usually applied to analysis of microbial communities, whereby DNA is extracted from environmental samples and amplified using PCR for 18S rRNA. The amplification products, which are a mixture from different species in the sample, are then separated using denaturing gradient gel electrophoresis (DGGE) (e.g., Cook et al., 2005). This separates PCR products according to their sequence as the concentration of chemical denaturant

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<sup>2</sup> Nanotechnology is engineering at the atomic or molecular scale, typically at scales of 1 – 100 nanometres.

required to disassociate double-stranded DNA is determined by the strength of chemical bonds between each nucleotide pair. However, this method was found to only detect the common species in marine environmental samples. Therefore, it may be suitable for biomonitoring applications, where the aim is to detect changes in communities, but is unsuitable for estimating species richness.

## Microarrays

A new genomic technology that may be applicable to identification of species diversity in samples is the microarray. Microarrays generally consist of glass slides onto which have been printed hundreds or thousands of spots of oligonucleotide probes. The probes are complementary to specific DNA targets, usually genes, as microarrays are generally used to study changes in the expression of multiple genes (see Figure 2). However, this technology can be used to detect the presence of species. Species-specific microarrays entail designing probes specific for all the potential species in a community or region, a process that involves a considerable effort comparable to the development of a library of DNA barcodes (see above). Note that for DNA identification, species do not have to be known (classified with an official name), but will be identifiable through DNA sequence information in a consistent way. Environmental samples are then amplified, using PCR, and the amplification product is simultaneously labelled with a fluorescent marker. Species presence is detected by fluorescence of a specific probe spot. Such methods have already been used to identify viruses (e.g., Wang et al., 2002), and are currently being explored for use in marine ecology. An alternative approach with microarrays is to array a large number of short oligonucleotides and to attempt to identify species by patterns of spot fluorescence. Such applications are largely in the concept stage at present but one is commercially available. This is The FoodExpert-ID chip from Biomerieux, which detects 25 commercially important animal species.

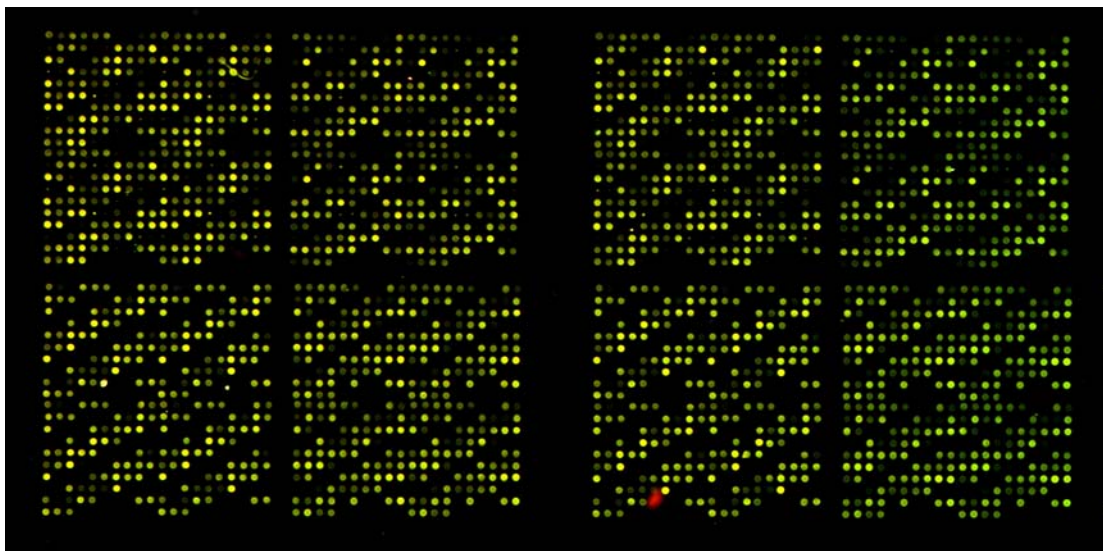


Figure 2. Microarray image from test slide for plunderfish gene expression studies (Photo: British Antarctic Survey; Gavin Burns).



One of the main disadvantages of microarrays is that development of facilities for printing is expensive and requires considerable investment in laboratory infrastructure (Figure 3), although this is now a service offered by many companies. Microarray technology is developing further, with new ways of applying probes to slides and new slide-coating chemicals that improve the performance of arrays.



Figure 3. Microarray spotting robot at British Antarctic Survey (photo: AD Rogers)

In some cases, microarray technology has been supplemented by combining chips with microelectronics and microfluidics. It is possible to add samples directly to a chip, and for PCR and array hybridisation to all take place within it (e.g., Cheng et al., 1998). These bioelectronic chips may allow miniaturisation of such technology, particularly for the identification of specific organisms, to a point where the technology is portable.

Presently, marine systematics and biodiversity studies are undergoing a resurgence of interest. Molecular biology and its associated technology now has a firm role within marine biodiversity studies as a means to aid the taxonomist and to help the marine ecologist to identify animals in a consistent way. Whilst some of this technology is expensive and bulky, the fact that biomedical science is driving the further development of sequencing, array and nano- technology forward is making it more accessible to marine scientists. Portable hand-held or moored sensors for identification of marine animals or under-way near-real time analysis of biodiversity may soon no longer be a dream but reality.....

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