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DNA analysis methods for recognizing species invasion: the example of *Codium*, and generally applicable methods for algae

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Abstract

Analysis of DNA can help to distinguish those morphological characters indicative of species difference from those representing retained traits or parallel evolution. This can be of great value in detecting recent invaders. The choice of which DNA characters to examine not only dictates the methodology to be used but must also be appropriate for the detection level sought. Restriction endonuclease fragment comparisons of plastid DNA have been used to assess *Codium* species; the results show *C. fragile* subsp. *tomentosoides* from east and west coast North America to be identical while sympatric endemic *Codium* species each display their own unique set of fragments. For species of other algae, plastid DNA fragment patterns are not necessarily identical across a morphological species, e.g. *Pandorina morum*. Such repetitive element probes as M13 and the use of RAPDs are more appropriate for analysis of populations within species. DNA base sequence comparisons of nuclear rDNA genes often yield too few variant bases between closely related species for reliable identifications. Analysis of the more variable Internal Transcribed Spacer (ITS) region, lying between the small and large ribosomal subunit genes in nuclear DNA, yields more extensive base pair variation between species and relatively little within species; it may be an alternative choice for endonuclease restriction fragment analysis or for sequencing.

Introduction

The notion of invader species is a peculiarly human concept since the entire history of life on earth is continual invasion and counter invasion. However, in the relatively short periods of time readily comprehensible to the human imagination, there are only two ways to explain any particular example of an extremely disjunct species distribution; either extinction has left two widely separated remnant populations, or some agency has newly introduced one species into a locale separate from the body of the species. In the absence of a perfect historical record, such cases are not always easy to distinguish. Nevertheless, numerous terrestrial examples of such invasion are known: the 'English' sparrow, Dutch elm disease, the Norwegian rat, the plague, African killer bees, and onward goes the list. Clearly there is an implication that it is the activities of humankind that sponsored these invasions, either directly or secondarily, and they belong to the second category, newly introduced species.

Aquatic examples of invading species have received much less publicity, except perhaps for the zebra mussel proliferating now in plumbing of Lake Erie. Yet in many ways, it may be easier for aquatic organisms to succeed in new environments than for terrestrial species, because aquatic environments are fundamentally less frequently stressed, more uniform and stable, than sites on dry land, and marine habitats should be even more welcoming to newcomers than freshwater bodies. In fact, it seems remarkable that physical barriers in the oceans of the world have kept the coastal flora subdivided by region, despite the great ocean currents, cyclonic storms and animal species that roam the oceans.

Some 150 foreign species of invertebrates and vertebrates have been recognized and identified so far in San Francisco Bay and in the Great Lakes of North

America (Barrat, 1994). Ballast water discharged by ships is the major suspected method of introduction. It is precisely because small geographic regions bear endemic species that invasions of foreigners are economically important. The invaders may compete directly with endemic congeners, they may display virulent characteristics that disrupt the species balance of a local environment, and they can be expected to bear disease organisms to which local species may be peculiarly susceptible. These threats present immediate problems for aquaculture industries and the potential for long-term disruptions of local fauna and flora. Often solution is sought in the introduction of natural enemies of invaders, but this requires first the identification of their source population, sometimes an onerous task.

The example of *Codium*

Among the algae of North America, one has stood out as the obvious invader of the east coast intertidal, a species of *Codium* called by the local children 'dead man's fingers' for the appearance of the limp, bleached thallus in the drift. I found none of this *Codium* in Woods Hole, MA, in 1954 and no record of its ever being found locally; there was an abundance of the invader by 1974, and there is a smaller but constant presence now. Where did it come from, and how? The immediate answer is fairly clear, because this *Codium* colonized in the Long Island area, and gradually spread northward and southward, an invasion described by Carlton & Scanlon (1985). The source of the initial invader is a much longer story, as yet incomplete, but the search for its ancestry suggests some of the molecular methods that might most usefully be applied to any attempt to reconstruct the origin of an invader.

In 1992, Goff et al. concluded that the same invasive species, *Codium* fragile subsp. tomentosoides (van Goor) Silva, had not only invaded up and down the east coast of North America but had also gained a foothold in San Francisco Bay on the west coast. This conclusion was based primarily on the pattern of restriction fragments produced by digesting plastid DNA with several different restriction endonucleases. The patterns from the DNA of the various populations of the invader were all alike, and were different from the other five species of North American *Codium* examined, each of which had its own unique pattern of such fragments. The uniformity of fragment pattern within a species, and quite different patterns between species, left little doubt that it was the same invader on both coasts, and not some 'sport' derived from a local *Codium* species. However, the original source of the invader has not yet been discovered among the nearly 100 species of *Codium* with a similar morphology, and there are two other 'weedy' species spreading in other parts of the world (Silva, 1955; 1957) still to be examined at the molecular level.

If study of the plastid DNA endonuclease restriction patterns was satisfactory for the study of *Codium*, will it be satisfactory for other algae, and is it the simplest and least costly method available? The answer lies first in posing the question more precisely: is A, a presumed invader, more closely related to B, a species growing in the same area, or to C, a distant species that however has perhaps the most similar morphology? The problem in such a question is the word species, the 'taxonomic' or morphological species, which we now know represents different levels of genetic diversity in different groups of algae (Coleman et al., 1994). Perhaps only when one knows the mating affinities of the groups in question, that which corresponds to the 'biological species' (Mayr, 1948; Manhart & McCourt, 1992), can one make highly probable predictions concerning which level of DNA analysis will be most suitable for distinguishing the entities involved. This is because organisms capable of mating with each other and producing viable progeny have so far been found to preserve several DNA characteristics useful for species identification, including nearly identical plastid DNA endonuclease restriction fragment patterns, and very low but characteristic levels of nucleotide variation in the internal transcribed spacer (ITS) regions of the nuclear rDNA repeats. By contrast, methods such as comparison of DNA fragment patterns using M13 or RAPDs are better for fingerprinting individuals or small localized populations since they reveal diversity between individuals even of a biological species, particularly a geographically widespread one (Coleman & Goff, 1991; Patwary et al., 1993; Coyer et al. 1994; van Oppen et al., 1994).

Examples of red algae

These are bold statements based on a bare minimum of data, all that are available on algae plus more extensive studies on flowering plant species (see Olmstead & Palmer, 1994), which tend to support the conclusions. For example, in the labyrinth of *Gracilaria* and *Gracilariopsis* species, most of which have not

been tested for sexual compatibility, nevertheless Goff and associates (Goff & Coleman,1988; Goff et al., 1994) found the west coast N American *Gracilariopsis lemaneiformis* (Bory) Dawson Acleto et Foldvik representatives to conform to the biological species expectation, showing uniform plastid DNA restriction fragment patterns. Two other methods applied to the same species similarly illustrated its extreme uniformity compared to nearest relatives. The first of these was sequencing of the internal transcribed spacer regions of the nuclear ribosomal repeats; <10% of ITS nucleotide positions were variable. The second method involved sequencing of the spacer lying between the large subunit and small subunit genes for ribulose-bis-phosphate carboxylase (RUBISCO) plus portions of the flanking gene sequences, a total of about 350 base pairs, in the plastid genome. Almost no variation (<1%) was found within the western N American representatives of the species, and >3% was present between sympatric species. Putative *G. lemaneiformis* from other continents were significantly different in both aspects; using these same characters, certain problematic entities could be identified unambiguously with their appropriate species within this massive group.

Characterization of plastid DNA restriction fragment patterns in additional species in the *Gracilaria/Gracilariopsis* complex (Bird et al., 1990; Rice & Bird, 1990) has helped to clarify their relationship and the results correlate with data on hybridization capability (Rice & Bird, 1990). Destombes & Douglas (1991) and Maggs et al. (1992) utilized sequencing of the RUBISCO spacer region to compare *Gracilaria verrucosa* (Hudson) Papenfuss populations and *Gymnogongrus* species, respectively. From the combined results it appears that this region is capable of defining species in at least some algal classes. However, it cannot be applied to algae of the green lineage where the SSU RUBISCO gene resides in the nuclear and not in the plastid genome. Sequencing of the plastid gene for RUBISCO large subunit itself has aided analysis of genera and species relationships of red algae, but variation within some species, where known, can sometimes exceed variation between species, a potentially perplexing problem for species identification until more is known (Hommersand et al., 1994).

Comparisons of methods using other algae

One other DNA region examined in these and other algae is the nuclear gene for the small subunit of ribosomal RNA. Bird et al. (1992, 1994) suggest that even sequencing of this gene fails to detect sufficient variability to sort isolates to species in the *Gracilaria/Gracilariopsis* complex – this gene is too conserved to serve as a species identifier. The same appears to be true for other algae, unicellular and colonial green algae (Jupe et al., 1988; Buchheim & Chapman, 1991, 1992; Larson et al. 1992; Lewis et al., 1992; Wilcox et al., 1992) and Dasycladaceae (Olsen et al., 1994), where <5% differences between species, and even sometimes between genera, were found.

Among the methods mentioned, this leaves two prospects for species identifications, plastid DNA restriction endonuclease fragment length polymorphism (RFLP) comparisons and nuclear rDNA ITS sequence comparisons. Plastid DNA RFLPs appear to differentiate species clearly in the examples mentioned, presumably the biological species in most cases, and are similarly informative in Vaucheria species (Kowallik, 1989), probably in the kelps (Fain et al., 1988; Fain, 1992), in *Ochromonas/Poteriochromonas* (Coleman et al., 1991), and in *Volvox* (Adams et al., 1990). Two exceptions are the finding of several RFLPs between physiological isolates of the diatom *Skeletomena costatum* (Grev.) Cleve, collected from one small area (Stabile et al., 1991, 1992), and some significant degree of RFLP among east coast North American isolates of *Chlamydomonas reinhardtii* Dangeard, isolates which will at least intercross sufficiently to form zygotes (Harris et al., 1991). An expected deviation is found in the taxonomic species *Pandorina morum* Bory, where unique RFLPs characterize each biological (sub)species (Moore, 1990).

Sequencing of the ITS of the nuclear ribosomal repeats is the second possibility. In addition to the data on *Gracilariopsis lemaneiformis*, the nuclear ITS has been sequenced in the Volvocaceaen species *Gonium pectorale* Mueller, *G. sociale* Dujardin and *Pandorina morum* with results identical to the species delineations obtained from plastid DNA RFLPs (Coleman et al., 1994; Coleman, 1995), and has been valuable for recognizing species in the siphonaceous green algae *Cladophoropsis* spp. (Kooistra et al., 1992) and *Cladophora albida* (Huds.) Kuetz. (Bakker et al., 1992). Analysis of this region has begun in the diatoms (Zechman et al., 1994). Interestingly, computer analysis (Coleman, unpublished) of restriction endonuclease sites in the nuclear ribosomal repeat, subsequent to sequencing this region of the Volvocacean species, suggests that the same 'tree' could have been obtained by use of a reasonable number of restriction endonu-

cleases and analysis of the resultant fragment patterns. Thus, it might be possible to distinguish species patterns directly, without use of probes or sequencing, by doing a restriction endonuclease analysis of ITS DNA copies obtained by PCR.

Choice and basis of the analytic method

If analyses of either of these two types of DNA could lead to identification of an invader, which method should the investigator select? The choice obviously depends primarily on money, time and equipment available. For plastid DNA RFLPs, plastid DNA must either be isolated and purified in sufficient quantity to see on a gel, or total purified DNA can be digested with the enzymes and the bands visualized using probes on Southern blots. The very effective non-radioactive methods now available for labelling probes avoid safety problems, and a battery of probes derived from plant plastid genes or other methods described by Palmer et al.(1988) and Olmstead & Palmer (1994) should reveal sufficient bands. Whatever the method, DNA must be isolated in quantity and purified.

For nuclear rDNA ITS sequencing, crude DNA isolated very simply from even tiny amounts of algal material (Goff & Moon, 1993) suffices to serve as PCR template material; the same primer pair used by these authors has worked on all major eukaryote groups of algae (Coleman, unpublished), and these primers appear to ignore prokaryote DNA so that bacterial contamination is inconsequential. What is necessary is a PCR machine, and a sequencing apparatus along with necessary reagents, which are not inexpensive. Since PCR machines are relatively easy to share among laboratories, and single batches of the initial primer pair and of endonucleases can be utilized for many experiments, perhaps the least expensive initial survey might be to examine endonuclease restriction fragment patterns of this region using PCR-generated copies. Adachi et al. (1994) have taken exactly this approach. For those algae that combine hundreds of copies of the nuclear rDNA repeat with relatively small nuclear genomes, restriction of total DNA might suffice, for the bands derived from cutting the set of entire rDNA repeats are in such high molar concentration versus other gene regions that they are visible directly on the stained gel against the background of the many different fragment sizes generated from the remaining DNA e.g. in *Gracilaria chilensis* Bird, McLachlan et Oliveira (M. González, pers. com.). This eliminates even the need for a PCR machine.

In summary, two DNA methods seem most feasible for identifying the source of an invader, plastid DNA fragment analysis and nuclear rDNA ITS sequencing; use of both if possible is clearly the best. Where the two RUBISCO genes occur in the plastid, plastid DNA RUBISCO spacer sequencing may also suffice. The nuclear ribosomal genes themselves and the LSU gene of RUBISCO represent too coarse a sieve for species differentiation, while fragment size variation detected by such repetitive element probes as M13 and RAPDs represent too fine a sieve.

Obviously one should consider the genetic basis underlying these empirical results. For functional genes, it is generally observed among eukaryotes that evolutionary change is slow relative to speciation. On the other hand, with methods that rely on repeated nuclear elements, presumably scattered among non-coding regions, rates of sequence change are much greater; fragment length changes can occur theoretically at each meiosis when regions of variable numbers of repeats pair (see Coyer et al., 1994). The stability of plastid endonuclease restriction fragment patterns and of the nuclear rDNA ITS sequence within an algal species versus the significantly greater differences between species is not entirely expected since among angiosperms, plastid RFLPs and ITS sequences may be highly shared by even groups of species (Palmer et al., 1988; Baldwin, 1992). The explanation may partially lie in the relationship of taxonomic to biological species, for successful hybridization is possible between numbers of plant species. Alternatively or in addition, one might expect the situation to reflect the length of time since reproductive isolation occurred, something of which we know very little for the algae. Finally it should be pointed out that although the nuclear rDNA repeat is indeed a highly repeated sequence in the nuclear genome, it is subject to remarkable homogenization by genetic phenomena, not yet defined, which bring about concerted evolution, the tendency for repeats to be identical or nearly identical within an organism (Zimmer et al., 1980). This process must underlie the relative stability of the ITS versus other types of repeated sequences in nuclear genomes.

The first-time visitor is surprised at how few native plant species can be seen in Chilean towns and roadsides; instead these now display weed species from almost all continents of the earth, perhaps introduced with wheat seeds imported in the mid 1800's. It seems

inevitable that one day in the not so distant future, a common set of algal weed species will characterize far-flung shores.

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