



Molecular genetic analyses of species boundaries in the sea

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Abstract

The tools of molecular genetics have enormous potential for clarifying the nature and age of species boundaries in marine organisms. Below I summarize the genetic implications of various species concepts, and review the results of recent molecular genetic analyses of species boundaries in marine microbes, plants, invertebrates and vertebrates. Excessive lumping, rather than excessive splitting, characterizes the current systematic situation in many groups. Morphologically similar species are often quite distinct genetically, suggesting that conservative systematic traditions or morphological stasis may be involved. Some reproductively isolated taxa exhibit only small levels of genetic differentiation, however. In these cases, large population sizes, slow rates of molecular evolution, and relatively recent origins may contribute to the difficulty in finding fixed genetic markers associated with barriers to gene exchange. The extent to which hybridization blurs species boundaries of marine organisms remains a subject of real disagreement in some groups (e.g. corals). The ages of recently diverged species are largely unknown; many appear to be older than 3 million years, but snails and fishes provide several examples of more recent divergences. Increasingly sophisticated genetic analyses make it easier to distinguish allopatric taxa, but criteria for recognition at the species level are highly inconsistent across studies. Future molecular genetic analyses should help to resolve many of these issues, particularly if coupled with other biological and paleontological approaches.

Introduction

Taxonomists have described species for centuries without the aid of protein electrophoresis, RAPDs, RFLPs, AFLPs or DNA sequencing. Indeed, given the scarcity of taxonomists relative to the number of undescribed species, most species descriptions still do not involve molecular genetic analyses, and will not for the foreseeable future. Such analyses are expensive, and thus their application is most justified when other types of data yield equivocal results. Nevertheless, genetics and systematics are increasingly intertwined at all levels of the Linnean hierarchy. In this paper I will explore what molecular genetics can tell us about diversity at the species level in the sea. In particular, I will focus on the role of molecular genetics in recognizing cryptic or sibling species (i.e. species that are difficult to distinguish using more traditional techniques). Before doing so, however, a few

words are necessary concerning what is meant by a species.

A brief taxonomy of species concepts and how they apply to marine organisms

Genetics can contribute to our understanding of species as we define them, but does not itself provide the definition. Mayr (1963) developed a compelling argument for using reproductive criteria to define species, but the rise of cladistics has challenged the supremacy of his view. Many other species concepts have also been proposed, 22 according to a recent survey (Maiden, 1997). These different species concepts have distinct implications for the use and interpretation of genetic data, although some of the differences are more apparent than real (Avice & Wollenberg, 1997; De Queiroz, 1998).

Mayr's biological species concept defines species as groups of actually or potentially interbreeding individuals, with boundaries between species defined by intrinsic barriers to gene flow that have a genetic basis. These barriers may be expressed through their effects on: (1) key features of the mating system (e.g. by influencing mate choice, gamete attraction or fertilization, or the timing of reproduction); (2) other ecological characteristics, such as habitat preference, which influence the probability of mating; or (3) developmental pathways, so that inviability or sterility interfere with gene exchange in subsequent generations. Although the existence of hybridization is often viewed as a reason for discarding the biological species concept, this is not consistent with Mayr's recommendations (e.g. Mayr & Ashlock, 1991).

For the vast majority of marine organisms we do not know the genetic basis of reproductive barriers (Palumbi, 1994). The best studied examples concern the proteins involved in egg-sperm interactions in sea urchins (Metz & Palumbi, 1996; Metz et al., 1998a) and gastropods (Metz et al., 1998b; Swanson & Vacquier, 1998; Hellberg & Vacquier, 1999). Gastropods represent a particularly elegant model system because both the sperm protein and the egg receptor have been identified. However, this level of understanding is not a viable short-term goal for most marine organisms. Fortunately for systematists, reproductively isolated taxa will, with time, accumulate other fixed genetic differences that can be revealed using more standard and accessible genetic techniques. The concordance of several independent genetic characters provides particularly strong evidence for the existence of reproductive barriers in sympatry because other explanations for their concordance are generally less plausible (Avise & Ball, 1990).

Despite the intuitive appeal of the biological species concept, the phylogenetic species concept has become increasingly popular, in part associated with the success of cladistic methods within systematics as a whole. It has been presented under a variety of guises (De Queiroz, 1998) but, in its simplest and most extreme form, species are defined as minimum diagnosable units (Cracraft, 1989). Any type of diagnostic genetic difference can thus in principle be used to define a species, with the proviso that entities known to interbreed (e.g. males and females within a single population) do not qualify for separate species status. In sympatry, the biological and phylogenetic species concepts are equivalent, because fixed genetic differences imply barriers to gene exchange, and bar-

riers to gene exchange imply some sort of genetic difference (Knowlton & Weigt, 1997).

In allopatry, however, biological and phylogenetic species can be potentially quite different (Knowlton & Weigt, 1997). This is because the biological species concept requires that genetic differences be such that isolated populations are not potentially interbreeding, while phylogenetic species can be defined by any fixed difference, regardless of its triviality. Avise and Wollenberg (1997) argue that any approach based on one or a small number of diagnostic genetic characters is nonsensical because of the enormous resolving power of genetic techniques and the diversity of possible genealogical pathways for individual loci. They suggest, however, that a phylogenetic species concept based on multiple loci is compatible with the biological species concept, because reproductive barriers will emerge during the lengthy period of geographic isolation that is required for many loci to acquire fixed (diagnostic) differences.

Of the remaining species concepts, Templeton's (1989) cohesion species concept most explicitly addresses the limitations of the biological species concept, which he summarizes as being associated with either too much sex (e.g. extensive hybridization) or too little sex (e.g. asexual taxa or species with highly fragmented populations). In both cases, he recommends use of the concept of 'demographic exchangeability', which in essence involves defining species based on the sharing of comparable ecological niches. For example, when discrete forms exist despite extensive hybridization, he suggests recognizing those units that can be defined by shared ecological constraints. Similarly, for taxa with many isolated populations due to limited dispersal or clonal propagation, ecological distinctiveness provides the criterion for recognition at the species level. Although he does not explicitly address problems of the phylogenetic species concept, trivial genetic differences with no obvious ecological significance would presumably go unrecognized using the cohesion species concept.

Templeton's demographic exchangeability is not an explicitly genetic concept, although genetics may be invaluable for identifying situations where it is particularly appropriate (e.g. extensive hybridization, highly subdivided population structure, or clonal reproduction). However, in some groups, the level of genetic differentiation may itself serve as a proxy for estimating likely ecological divergence. For example, the sexual habits of many microbes do not lend themselves to species definitions based on reproduc-

tive barriers and, for a number of groups, our only information on these organisms are DNA sequences because they are unculturable at present. Thus Embley and Stackebrandt (1997) discuss a rule of thumb whereby prokaryote taxa be recognized at the species level when their 16S rDNA sequences are less than 98% similar. Implicit in this approach is the idea that substantial genetic divergence implies ecological differentiation of the type specified by the cohesion species concept, but the converse (small genetic differentiation) does not necessarily imply little ecological divergence (see below).

Among marine metazoans, strictly clonal reproduction is rare (Hughes, 1989), although the clam *Lasaea* provides a clear case of some niche diversification among ancient asexual lineages (Ó Foighil & Smith, 1996) for which the cohesion concept may be well suited. A much more widespread situation favoring application of the cohesion species concept is that of extremely limited dispersal (Knowlton & Jackson, 1993). When highly philopatric species live in discrete habitats separated by uninhabitable areas, large numbers of ecologically similar but genetically distinctive populations can result, some of which also exhibit reproductive incompatibilities. The copepod *Tigriopus californicus* (Burton et al., 1999) and the isopod *Excirrolana braziliensis* (Lessios & Weinberg, 1994; Lessios et al., 1994) provide compelling examples of the potential utility of the cohesion species concept for such situations, and there are undoubtedly many others.

The extent of hybridization in the sea remains a subject of controversy (Gardner, 1997), so that the extent to which it impinges on species concepts remains uncertain. Molecular genetic techniques are ideally suited for its detection (see Rieseberg & Linder, 1999 for a critical evaluation), and a number of marine examples are available (e.g. Liu et al., 1991; Bert & Arnold, 1995; Brown, 1995; Bert et al., 1996; Gardner, 1996; Lessios & Pearse, 1996; Foltz, 1997; Röhner et al., 1997; Quesada et al., 1998; Väinölä & Vainio, 1998; Comesaña et al., 1999). Although only 3 of the 108 cases summarized by Gardner (1997) employed genetic methods, he concludes that (1) rates of hybridization in the sea and on land are roughly comparable, and that (2) in the sea, sporadic hybridization is more common than either well-defined hybrid zones between distinct, allopatric taxa or hybrid swarms. The latter provide the biggest challenge for species concepts, a problem that has been explicitly discussed for corals (see below).

Examples of recent genetic analyses

Several recent reviews have listed cases where genetic analyses have played a critical role in the recognition of species boundaries (Knowlton, 1993; Thorpe & Solé-Cava, 1994). Rather than dwell on previously-covered examples, I will highlight others not covered in these reviews and, for a number of groups, attempt to summarize what genetic techniques have taught us to date. This summary is not intended to be comprehensive, but rather to point out interesting developments and give a feeling for the kinds of results and problems that have been encountered. I will focus primarily on marine invertebrates, with a few examples being drawn from other marine groups.

Bacteria and single-celled eukaryotes

Our understanding of the world of marine microbes has been transformed by the tools of molecular genetics. Major surprises include the discovery of entirely new major groups, never before cultured, based on rDNA sequences obtained from environmental samples (Embley & Stackebrandt, 1997; Potter et al., 1997; Rappé et al., 1998; Hinrichs et al., 1999). These findings are more akin to the discovery of new higher taxa than to recognition of diversity at the species level.

Nevertheless, in some cases, taxa formerly considered to be a single species have been shown to harbor enormous and unsuspected genetic diversity. One striking example concerns dinoflagellates associated with marine invertebrates, conventionally referred to as *Symbiodinium microadriaticum*. Only in the last decade has it been discovered that they exhibit genetic differences based on small subunit rDNA sequences comparable to those observed between different families or orders of free-living dinoflagellates. These genetically defined groups have characteristic differences in depth distribution (and other ecological attributes) as well (Rowan, 1998). Recently, taxa of cyanobacteria have also been shown to exhibit depth zonation (Moore et al., 1998; Ferris & Palenik, 1998), but in this case the genetic differences are so small that they might not normally be attributed to species level differentiation (*sensu* Embley & Stackebrandt, 1997). Cryptic species of planktonic foraminifera that differ in a variety of biological features have also been discovered through the use of genetic techniques (Huber et al., 1997). The cohesion species concept is in principle highly appropriate in many of these

cases. However, the relationship between genetic and ecological divergence at various levels of genetic differentiation is largely unknown for most microbial groups.

Multicellular plants

Marine angiosperms have been examined genetically, but recent studies have focused primarily on population genetics and clonal structure rather than alpha taxonomy (e.g. Kirsten et al., 1998). They will not be discussed further here. Marine algae, in contrast, provide a number of examples where genetic analyses have played a major role in clarifying species boundaries. Genetic techniques are particularly useful because recognizing species of marine algae is often complicated by their morphological simplicity (Van Oppen et al., 1996), morphological plasticity (e.g. Bakker et al., 1995a; Benzie et al., 1997; Blomster et al., 1998), and heteromorphic life histories.

Genera recently examined using genetic techniques include *Porphyra* (Lindstrom & Cole, 1992; Stiller & Waaland, 1993; Brodie et al., 1996), *Phycodris* (Van Oppen et al., 1995a,b), *Gracilariopsis* (Goff et al., 1994), *Gracilaria* (Goff et al., 1994), *Pikea* (Maggs & Ward, 1996), *Cladophora* (Bakker et al., 1995a,b), *Caulerpa* (Benzie et al., 1997; Pillmann et al., 1997; Olsen et al., 1998) and *Enteromorpha* (Blomster et al., 1998). These taxa include economically important species (*Porphyra*, *Gracilaria*, *Gracilariopsis*), as well as bioindicators of pollution (*Enteromorpha*) and pest species (*Caulerpa*). Genetic techniques have been invaluable in evaluating the possibility of species introductions (Maggs et al., 1992; Van Oppen et al., 1995b; Maggs & Ward, 1996; Pillmann et al., 1997; Olsen et al., 1998), which are common among algae.

Although allozymes continue to be used in alpha systematics (e.g. Lindstrom & Cole, 1992; Van Oppen et al., 1995a; Benzie et al., 1997), DNA based techniques (especially sequencing of nuclear rRNA and chloroplast Rubisco genes) are becoming increasingly prominent. The ability to characterize dried herbarium material and compare it to other collections of uncertain status makes DNA-based techniques particularly attractive (e.g. Goff et al., 1994). On the other hand, in at least one case allozymes provided greater resolution than RAPDs (Van Oppen et al., 1995a). Diagnostic allozyme loci are often monomorphic within species, yielding low heterozygosities (e.g. Lindstrom & Cole, 1992; Van Oppen et al., 1995a; Benzie et al., 1997).

Many pairs of cryptic taxa are very distinct genetically (e.g. Olsen et al., 1987), and are not even necessarily sister species (Maggs et al., 1992; Goff et al., 1994; Bakker et al., 1995a; Blomster et al., 1998). In some of these cases, other kinds of differences had been noted prior to genetic studies (e.g. Brodie et al., 1996; Blomster et al., 1998), while, in other cases the genetic identification of cryptic species came as a complete surprise (Stiller & Waaland, 1993; Benzie et al. 1997; Pillman et al., 1997). Not all cryptic species are highly divergent genetically, however (Brodie et al., 1996), and genetic data have also been used to synonymize morphotaxa (Lindstrom & Cole, 1992; Blomster et al., 1998).

Porifera

Sponges are another group where morphological simplicity (Klautau et al., 1999) and morphological plasticity have led to uncertainties in systematics at the species level. Recent genetic studies include investigations of cryptic species in *Petrosia* (Bavestrello & Sarà, 1992), *Tethya* (Sarà et al., 1993), *Clathrina* (Klautau et al., 1994), *Cliona* (Barbieri et al., 1995), *Plakina* (Muricy et al., 1996), *Spirastrella* (Boury-Esnault et al., 1999), *Cinachyrella* (Lazoski et al., 1999), and *Chondrilla* (Klautau et al., 1999). Solé-Cava and Boury-Esnault (1999) review the results of these and earlier studies (63 in total) reporting levels of interspecific genetic divergence based on allozymes. As they note, almost all of these studies focused on taxonomically problematic groups where species boundaries were deemed unclear.

Most studies involved no more than 12 scorable loci, and substantial differences (usually one or more diagnostic loci) were generally detected. In some cases these differences were larger than those often reported between different genera of sponges. Genetic differences were typically associated with morphological differences that were also diagnostic, although relatively subtle and/or generally not recognized as 'significant' (e.g. color) in traditional approaches to sponge taxonomy. One sympatric pair (two *Suberites* species that differ in color) were, however, exceptionally closely related (Nei's *I* of 0.98).

The overwhelming message from these studies is that sponge biodiversity at the species level has been seriously underestimated. So-called cosmopolitan taxa probably consist of complexes of regionally more restricted forms (e.g. Klautau et al., 1999), and sympatric morphotypes defined by colony mor-

phology, color, habitat, or subtle skeletal differences are probably non-interbreeding. DNA analyses have not been applied to the problem of cryptic species in sponges, and some PCR-based techniques that are routinely used in other groups (e.g. RAPDs) are more difficult to use in sponges because of the symbionts embedded in the tissues. As technical difficulties are resolved, however, the extent of diversity may increase further, because the sometimes very large genetic differences observed with protein electrophoresis suggest that current approaches are touching only the tip of the iceberg.

Cnidaria

Several important anthozoan genera have been the subject of recent genetic studies of species boundaries: the anemones *Epiactis* (Edmands, 1996), *Anthopleura* (McFadden et al., 1997), and *Actinia* (Monteiro et al., 1997); the corals *Montipora* (Stobart & Benzie, 1994); *Montastraea* (Lopez & Knowlton, 1997; Lopez et al., 1999; Medina et al., 1999), and *Platygyra* (Miller & Benzie, 1997); the octocorals *Briareum* (Brazeau & Harvell, 1994) and *Plexaura* (Lasker et al., 1996); the soft coral *Alcyonium* (McFadden, 1999); and five genera of zoanthids (Burnett et al., 1997). Genetic analyses in other cnidarian classes are largely lacking, with the hydrozoan *Millepora* (Manchenko et al., 1993; Amaral et al., 1997) and the scyphozoan *Aurelia* (Greenberg et al., 1996) being the primary examples.

In apparent contrast to the situation with sponges, where genetic differences between sibling species are usually high, there are a number of well-studied cases of sympatric taxa that show very low levels of genetic differentiation, including the absence of fixed allozyme differences (Solé-Cava et al., 1985; Solé-Cava & Thorpe, 1992; Brazeau & Harvell, 1994; Weil & Knowlton, 1994; Edmands, 1996; McFadden et al., 1997; Monteiro et al., 1997). The low level of differentiation observed between some sympatric species makes the interpretation of negative evidence in other groups (e.g. Burnett et al., 1997; Miller & Benzie, 1997) especially difficult. Moreover, in the *Montastraea annularis* species complex, the three taxa show no differentiation in ITS and COI sequences (Lopez and Knowlton, 1997; Medina et al., 1999), although one of these taxa has recently been clearly distinguished after screening of numerous AFLP primers (Lopez et al., 1999).

One possible reason for the small degree of genetic differentiation between what appear to be reproduc-

tively isolated taxa is that rates of molecular evolution in this group may be unusually slow (Romano & Palumbi, 1997). In corals, for example, rates of divergence of the mtDNA COI and cyt b genes appear to be less than 0.1% per million years (Medina et al., 1999; Van Oppen et al., 1999), compared to 2% per million years in many vertebrates (Avise et al., 1998), and 1.5–0.5% for crustaceans and fish separated by the Isthmus of Panama (Knowlton & Weigt, 1998; Schubart et al., 1998). Pont-Kingdon et al. (1998) report the existence of a homologue to the bacterial MutS gene (part of a mismatch repair pathway) in the mitochondria of an octocoral; although in principle this could contribute to low rates of molecular evolution, its absence in the sea anemone *Metridium* makes its general significance for Cnidaria unclear.

Alternatively, the lack of genetic differentiation could reflect the effects of regular hybridization. This perspective has been strongly argued by Veron (1995) for corals, many of which reproduce in mass spawning events at approximately the same time. Some genetic and chromosomal data have been interpreted from this point of view (Kenyon, 1997; Odorico & Miller, 1997).

This controversy is likely to persist for some time for several reasons. First, it is intrinsically difficult to distinguish between reproductively isolated taxa that have very few fixed genetic differences and taxa that are only imperfectly isolated. In both cases, most loci will show either no significant differences or only modest frequency differences. The weakness of negative evidence is particularly evident in this situation, as has been well illustrated by the effort required to find genetic differences between some plants (Howard et al., 1997). Second, the existence of puzzling patterns in existing data sets [e.g. highly divergent nuclear ITS-1 and mitochondrial 16S rDNA sequences within single individuals or single species (Odorico & Miller, 1997; Romano & Palumbi, 1997; Takabayashi et al., 1998)] suggests that conventional genetic assumptions may be inappropriate for cnidarians, further complicating interpretation of results.

Polychaeta

Most of the previously reviewed studies of polychaete sibling species did not include genetic analyses (Knowlton, 1993). The list of genera analyzed genetically is now longer, and additions include *Polydora* (Mustaquim, 1988; Manchenko & Radashevsky, 1993, 1994, 1998), *Capitella* (Baoling et al., 1991; Gamen-

ick et al., 1998), *Marenzelleria* (Bastrop et al., 1998), *Arenicola* (Cadman & Nelson-Smith, 1993), *Eulalia* (Bonse et al., 1996), *Petitita* (Van Soosten et al., 1998), *Nereis* (Rodriguez-Trelles et al., 1996), *Nerilla* (Schmidt & Westheide, 1997), and *Hediste* (Fong & Garthwaite, 1994; Abbiati & Maltagliati, 1996; Rohner et al., 1997; Sato & Masuda, 1997). Nevertheless, genetic information remains scanty, even for the genus *Capitella*, despite the highly influential early paper by Grassle and Grassle (1976) and the importance of this group for pollution studies.

Most genetic studies of polychaetes are based on protein electrophoresis, and very large genetic distances are not uncommon. *Petitita* and *Nerilla* have been analyzed by RAPDs and *Marenzelleria* by mitochondrial 16S rDNA sequencing. In many cases, morphological differences are very subtle, although sympatric forms often have clear differences in life history or ecology. Studies of allopatric forms often leave many unanswered questions, particularly when studied with techniques like RAPDs for which there is no established relationship between genetic divergence and reproductive isolation or ecological distinctiveness. However, the finding of reproductive incompatibility associated with very small levels of allozyme divergence between populations (e.g. Nei's $D < 0.01$; Rice & Simon, 1980) suggests that one cannot simply ignore subtle levels of differentiation. More breeding studies (e.g. as in Gamenick et al., 1998) would be particularly informative in this regard. The many small polychaetes that lack a dispersive phase may be genetically structured like the copepod *Tigriopus*, making the cohesion species concept particularly appropriate (see above).

Mollusca

Mollusks are arguably the best genetically studied phylum of marine invertebrates. Hence, the following summary of analyses of species boundaries is far from complete and highlights only the most recent work where multiple studies exist. Mollusks have also provided a superb model system for understanding the evolution of the gamete recognition proteins that may underlie the origin of reproductive barriers between some species (Metz et al., 1998b; Swanson and Vacquier, 1998; Hellberg & Vacquier, 1999). Finally, mollusks have an excellent fossil record, so that there is enormous potential for combining genetic and paleontological approaches (e.g. Collins et al., 1996; Reid et al., 1996).

Among the bivalves, species in the genera *Crassostrea* (e.g. Boudry et al., 1998; Hare & Avise, 1998) and particularly *Mytilus* (e.g. Beynon & Skibinksi, 1996; Gardner, 1996; Quesada et al., 1998; Comesaña et al., 1999) have been especially well studied. Other recently analyzed groups of bivalves include *Geukensia* (Sarver et al., 1992), *Cardium* (Hummel et al., 1994), *Lithophaga* (Mokady et al., 1994), *Chamelea* (Backeljau et al., 1994), mytilids and three vesicomid genera from the deep sea (Cradock et al., 1995; Peek et al., 1997), and *Donax* (Adamkewicz & Harasewych, 1996). Species of gastropods have been even more extensively investigated. The genus *Littorina*, for example, has a long history of study [see recent works by Reid et al. (1996), Grahame et al. (1997), Tatarenkov & Johannesson (1998), and Kyle & Boulding (1998)]. Other genera that have been recently analyzed include *Hydrobia* (Ponder & Clark, 1988), *Stramonita* (Liu et al., 1991), *Alviniconcha* (Denis et al., 1993), *Trochus* (Borsa & Benzie, 1993), *Tectus* (Borsa & Benzie, 1993), *Austrocochlea* (Parsons & Ward, 1994), *Drupella* (Johnson & Cumming, 1995), *Columbella* (Oliverio, 1995), *Patella* (Côte-Real et al., 1996a,b; Ridgway et al., 1998), *Nassarius* (Sanjuan et al., 1997), *Nucella* (Kirby et al., 1997; Marko, 1998), and *Dendronotus* (Thollesson, 1998). Cephalopods are comparatively less studied, but genera where species boundaries have recently been revised or identified based on genetic analyses include *Photololigo* (Yeatman & Benzie, 1994), *Nautilus* (Wray et al., 1995), *Sepioteuthis* (Izuka et al., 1996) and *Illex* (Carvalho & Nigmatullin, 1998).

Molluscan alpha taxonomy has long attracted the attention of professional and amateurs alike because shells are readily collected and studied. However, differences in shell morphology are often the result of phenotypic plasticity, so that many past descriptions of species were unjustified. Consequently, the number of genetic studies that have resulted in the synonymization of species is probably higher for mollusks than most other marine invertebrate phyla. The above-mentioned studies of *Donax* (Adamkewicz & Harasewych, 1996) and *Nautilus* (Wray et al., 1995) are but two examples of numerous cases where distinctions between sympatric taxa based on shell morphology were not supported by genetic data. On the other hand, genetic data have also demonstrated that minor differences in shell morphology (or other characters) can sometimes be taxonomically important (e.g. Borsa & Benzie, 1993; Mokady et al., 1994; Parsons & Ward, 1994; Johnson & Cumming, 1995;

Oliverio, 1995; Izuka et al., 1996; Sanjuan et al., 1997; Thollessen, 1998). The unexpected discovery of highly divergent sympatric taxa within morphologically uniform groups is uncommon in mollusks, but it does occur; for example, in the deep sea (Craddock et al., 1995; Peek et al., 1997) and in cephalopods (Yeatman & Benzie, 1994).

The problem of the uncertain basis for shell variability is exacerbated in allopatry, where genetic and environmental differences are often confounded. Moreover, the same geographic comparison can reveal little or no genetic differentiation in some taxa and substantial genetic differentiation in others, sometimes even within the same genus (Adamkewicz & Harasewych, 1996; Côte-Real et al., 1996a,b). Along continuous coastlines, the existence of narrow zones of overlap with little evidence of hybridization provides definitive evidence for species boundaries (Liu et al., 1991; Ridgway et al., 1998), but far more complex patterns of hybridization are sometimes observed (e.g. *Mytilus*, see below), and in other cases no zone of overlap exists (see Discussion).

Several molluscan genera have been extensively studied using a variety of approaches, and the complexity of the resulting data sets is sobering. Studies of *Mytilus* have revealed a variety of patterns of hybridization depending on the taxa and the geographic locale, as well as a bizarre sex-linked mitochondrial transmission system (Beynon & Skibinksi, 1996; Gardner, 1996; Quesada et al., 1998; Comesaña et al., 1999). The multipronged attack on the population structure of *Crassostrea* across the Gulf of Mexico and Atlantic coast of North America (see most recently Hare & Avise, 1998) has shown that different techniques can give fundamentally different answers for reasons that still remain unclear. In *Littorina*, closely related pairs of taxa that occur in different habitats have in some studies been shown to exhibit reproductive barriers across environmental gradients within a site but, in other cases, samples from different environments within sites are more similar genetically than are those samples which are geographically isolated but environmentally similar (Kyle & Boulding, 1998; Tatarenkov & Johannesson, 1998). Some of this complexity is probably due to the proximity of the divergences being studied in all three genera (see Discussion), but is such complexity lying below the surface in the many other groups that have yet to be investigated so intensively?

Crustacea

Recent genetic analyses of species boundaries in decapod crustaceans include studies of the lobsters *Homarus* (Kornfield et al., 1995), *Jasus* (Ovenden et al., 1997) and *Panulirus* (Sarver et al., 1998); the crabs *Pinnotheres* (Stevens, 1990), *Liopetrolisthes* (Weber and Galleguillos, 1991), *Helice* (Irawan et al., 1993), *Sesarma* (Felder & Staton, 1994), *Uca* (Felder and Staton, 1994), *Emerita* (Tam et al., 1996), *Scylla* (Fuseya & Watanabe, 1996), *Carcinus* (Geller et al., 1997) and *Menippe* (Bert et al., 1996; Schneider-Broussard et al., 1998); and the shrimps *Alpheus* (McClure & Greenbaum, 1994; Knowlton & Weigt, 1997, 1998), *Synalpheus* (Duffy, 1996), and *Rimicaris* (Shank et al., 1998). Studies of other crustaceans include those of four genera of calanoid copepods (Bucklin et al., 1995, 1996, 1998); the isopods *Cyathura* (Brown et al., 1988), *Sphaeroma* (Laulier, 1989) and *Idotea* (Charfi-Cheikhrouha et al., 1998); the amphipods *Themisto* (Schneppenheim & Weigmann-Haass, 1986), *Abyssorchomene* (France, 1994) and *Eurythenes* (France & Kocher, 1996) and the mysid *Mysis* (Väinölä & Vainio, 1998).

Most genetic analyses of species boundaries in Crustacea confirm or reveal the existence of cryptic species, some of which are distinguished by surprisingly large genetic differences given their morphological similarity (e.g. Palumbi & Benzie, 1991; Bucklin et al., 1995; Knowlton & Weigt, 1998; Sarver et al., 1998). However, some taxa have smaller genetic differences than expected (Kornfield et al., 1995), and others have been shown to be conspecific based on the absence of genetic differentiation between size classes (Shank et al., 1998) or spatially disjunct populations (Ovenden et al., 1997). Sympatric or parapatric taxa typically exhibit ecological differences, suggesting that species are less generalized than previously assumed; good recent examples of this include zonation by depth within the deep sea (France, 1994; France & Kocher, 1996) and the existence of species complexes of symbiotic crustaceans associated with different hosts (Stevens, 1990; Weber & Galleguillos, 1991; Duffy, 1996). Genetic analyses have been used to document species introductions (e.g. Geller et al., 1997) and can provide rapid methods for characterizing planktonic copepod samples (Bucklin et al., 1998).

Some groups of crustaceans are well studied taxonomically and genetically, and several genetic studies include explicit calibrations of rates of mo-

lecular evolution for the cytochrome oxidase I and rRNA mitochondrial genes (Cunningham et al., 1992; Sturmhuber et al., 1996; Knowlton & Weigt, 1998; Schubart et al., 1998). Thus there is considerable potential not only for the recognition of species boundaries but also for estimating the timing of their divergences (see Discussion). Genetic studies of crustaceans can also draw on a large base of genetic data available for other arthropods (e.g. *Drosophila*). Analyses of Crustacea are consequently more advanced than those of many other marine invertebrates in some respects. As with the molluscan examples discussed above, these more detailed analyses have brought an appreciation of potential complexities in the interpretation of genetic data. Examples include the problem of pseudogenes (Schneider-Broussard & Neigel, 1997), coadaptation between nuclear and mitochondrial genomes (Burton et al., 1999) and inconsistency of outcomes when different genes are compared (Bert et al., 1996; Tam et al., 1996; Schneider-Broussard et al., 1998).

Echinodermata

Sea urchins are a model system for developmental biologists, and have also been extensively studied using molecular genetic techniques from the perspectives of population genetics and dispersal (e.g. Palumbi et al., 1997; Lessios et al., 1998) and the nature of fertilization barriers (e.g. Metz & Palumbi, 1996; Metz et al., 1998a). Echinoderms have also figured prominently in attempts to calibrate rates of molecular evolution based on the Isthmus of Panama (Lessios, 1998), thereby facilitating estimates of the times of species divergences (see Discussion).

The use of genetic techniques to discriminate species is, however, comparatively limited; recent studies of potential species complexes not previously summarized include those of the starfish *Leptasterias* (Hrincevich & Foltz, 1996; Foltz, 1997) and the sea cucumber *Cucumaria* (Arndt et al., 1996). In the latter case molecular data were used to synonymize taxa as well as to separate them. Results from *Leptasterias*, as well as an earlier study of *Echinaster* (Tuttle & Lindahl, 1980), suggest that sibling species of starfish may often lack fixed differences in allozymes.

Other marine invertebrates

Cryptic species have been investigated genetically in only a few bryozoan genera, including studies of *Bugula* (Davidson & Haygood, 1999) and *Stylopoma*

(Jackson & Cheetham, 1994) not previously reviewed. *Stylopoma* is one of the few cases where genetic divergence and morphological divergence have been compared in detail; within this genus the correspondence is quite strong, even where differences are subtle, and lends support to the use of high-resolution morphometrics in recognizing species in the fossil record. The case of *Bugula* has applied importance: two cryptic taxa that differ by about 9% in the mtDNA COI gene harbor bacterial symbionts that differ in 4 of 1024 bp of the small subunit rRNA gene, and differ as well in the production of anticancer bryostatin compounds (Davidson & Haygood, 1999). The existence of cryptic species that differ in their bioactive compounds is likely to be a general phenomenon for which genetic analyses are ideally suited (King et al., 1995).

Genetic studies of species boundaries in ascidians are similarly limited, with only a few genera emerging from bibliographic searches: *Botryllus* (Aron & Solé-Cava, 1991), *Pyura* (Dalby, 1997) and *Herdmania* (Degnan & Lavin, 1995). In each of these cases, the species could be readily distinguished by color or other features, and genetic differences were substantial. This suggests that the alpha taxonomy of ascidians is plagued by excessive lumping, with many more cryptic taxa yet to be discovered.

Cryptic species of nemertean, often distinguishable by color, are also characterized by large genetic differences (taxa from Table 1 of Knowlton, 1993; Rogers et al., 1995; Manchenko & Kulikova, 1996a). However, genetic analyses of nemerteans have also shown that some forms that differ in color are in fact conspecific (Sundberg & Andersson, 1995; Manchenko & Kulikova, 1996b), indicating that color is a potentially useful cue, but one that demands genetic follow-up.

For most other groups, recent genetic analyses of species boundaries are even more limited (e.g. for hemichordates, see King et al., 1995; for gastrotrichs, see Todaro et al., 1996; for priapulids, see Schrieber et al., 1996; for vestimentiferans, see Kojima et al., 1997). General conclusions about the extent to which taxa are over- or under-split are impossible to draw from such a scattered record.

Fish

Sharks and bony fishes have been extensively studied genetically, largely from the perspective of elucidating stock structure, but also for recognizing species boundaries; Ward & Grewe (1994) and Shaklee &

Bentzen (1998) provide general reviews of this literature. Additional analyses of species boundaries not cited by these authors include studies of *Myliobatis* (Levy & Conceição, 1989), *Siganus* (Lacson & Nelson, 1993), *Naso* (Dayton et al., 1994), *Pleuronichthys* (Watanabe et al., 1994), *Osmerus* (Taylor & Dodson, 1994), several damselfish genera (Lacson, 1994; Lacson & Clark, 1995; Lessios et al., 1995), *Lutjanus* (Lee & Cheng, 1996), *Trematomus* (Bernardi & Goswami, 1997), *Cyclothone* (Miya & Nishida, 1997), *Mugil* (Rossi et al., 1998), and *Chaetodon* (McMillan et al., 1999).

Many of these studies exhibit the same kinds of pattern as those shown by marine invertebrates, namely the recognition that similar forms in either sympatry or allopatry are clearly distinct on a genetic basis. What is somewhat surprising, however, is the considerable inconsistency among authors in their assignment of species level status to such forms. For example, allopatric populations of damselfish species belonging to several genera and *Mugil cephalus* are in each case distinguished by several fixed allozyme differences (Lacson, 1994; Lacson & Clark, 1995; Lessios et al., 1995; Rossi et al., 1998), but only in one publication is species level distinction recommended (Lessios et al., 1995). At the opposite end of the spectrum, allopatric butterflyfishes that can only be distinguished genetically by diagnostic sequences in the rapidly evolving control region of mtDNA are nevertheless recognized as distinct at the species level (McMillan et al., 1999).

Many fishes are brightly colored, and fish systematists have often drawn on these color patterns to distinguish closely related species. Several recent studies (Planes & Doherty, 1997; McMillan et al., 1999) show how complex the relationship can be between color pattern and genetic divergence. In the tropical damselfish and butterflyfish studied by these authors, some evidence for assortative mating was found, but patterns were often inconsistent across regions. In both cases partial reproductive boundaries associated with color pattern appear to have been established very recently (see Discussion).

Marine turtles and mammals

Slow rates of molecular evolution have been suggested for both marine turtles (Bowen et al., 1993) and marine mammals (Garcia-Rodriguez et al., 1998). As a consequence, even sympatric sibling species in these groups may be hard to distinguish genetically, and

the interpretation of small differences in allopatry is problematic. Nevertheless, genetic analyses have confirmed or suggested cryptic species in a number of groups. In the turtles, genetic data were critical for the recognition of the distinctiveness of the Kemp's and olive ridley turtles (*Lepidochelys*) and for showing that Atlantic and eastern Pacific green turtles (*Chelonia mydas*) were more distinct than the eastern Pacific green turtle and the so-called black turtle from the western Pacific (*C. agassizi*) (Bowen et al., 1993; Dutton et al., 1996). In marine mammals, examples of sibling species that have been recognized or confirmed using molecular approaches include spotted vs. harbor seals (*Phoca*), cryptic species within both Bryde's and minke whales (*Balaenoptera*), the shortbeaked vs. longbeaked common dolphin (*Delphinus*), and several taxa of bottlenose dolphins (*Tursiops*) (Rice, 1998). Killer whales (*Orcinus*) exhibit slight but fixed mtDNA differences between northern 'residents', southern 'residents' and 'transients' (Hoelzel et al., 1998); transients and residents differ ecologically as well as genetically, but the role of cultural transmission of behaviors coupled with very limited dispersal between social groups complicates the analysis of these data. Recent mitochondrial DNA analyses of salt-water manatees (*Trichechus manatus*) have revealed three deep, allopatric clades that are as genetically distinct from each other as they are from the freshwater manatee (*T. inunguis*) and comparable to the differences observed between genera of whales (Garcia-Rodriguez et al., 1998).

Many marine mammals have several geographically defined subspecies (Rice, 1998). Genetic techniques may lead to the discovery of unique genetic features that would permit their recognition on the grounds of the phylogenetic species concept. Regardless of one's opinion of the various species concepts, some form of recognition of such taxa may facilitate conservation of these often endangered groups.

Common themes and problems

Many of the patterns that emerged in this review, based primarily on the more recent literature, are similar to those I outlined before (Knowlton, 1993): many sibling species continue to be discovered, and in sympatry they often have characteristic differences in ecology or life history, and can, in retrospect, be identified by subtle differences in morphology or color pattern. The importance of identifying species bound-

aries for both basic and applied science is widely acknowledged.

The biggest change in the time between these two reviews is the increasing prominence of DNA analyses. For the 91 marine invertebrate genera listed in Knowlton (1993, Table 1), protein data had been published for 68 (75%) while DNA analyses were available for only 6 (7%). In contrast, in the papers surveyed for this review, analyses of species boundaries used DNA-based techniques in 36 of 102 genera.

DNA and allozyme data sets have different strengths and weaknesses which have been well reviewed by others (e.g. see Shaklee & Bentzen, 1998); my purpose in focusing on the former is not to disparage the latter, but rather to consider where newly available DNA studies have important contributions or implications for studies of species boundaries. Two areas merit special attention: (1) the greater resolving power of DNA analyses and the consequent implications for analyses of allopatric taxa, and (2) the ability to estimate the time of origin of divergent taxa using the molecular 'clock'.

Lumping vs. splitting of allopatric taxa

Can the degree of genetic differentiation be used to determine whether allopatric taxa merit recognition at the species level? For Cracraft's (1989) version of the phylogenetic species concept the answer is simple: any fixed difference will do. When genetic research was dominated by allozyme analysis, this was a reasonable and often used approach, since fixed allozyme differences do not rapidly evolve. Thorpe's (1983) summary of levels of genetic divergence (e.g. Nei's D) associated with clearly distinct species provided a related criterion that has also been widely adopted by systematists attempting to make taxonomic decisions for allopatric taxa.

As Avise & Wollenberg (1997) point out, however, in an age of ready DNA analysis, finding fixed differences becomes far simpler, with the real possibility of needing to recognize trivially divergent taxa at the species level if Cracraft's (1989) version of the phylogenetic species concept is applied. They persuasively argue that multiple concordant differences are a far better criterion for recognizing species boundaries and, that moreover, much of the conflict between the biological and phylogenetic species concepts evaporates when this approach is used.

An alternative, but not unrelated, approach would be to use estimates of time since divergence to make

assessments of likelihood of interbreeding. Studies of the Isthmus of Panama, for example, suggest that 3 million years is long enough for reproductive barriers to form in a variety of marine taxa (Knowlton et al., 1993; Lessios, 1998), although additional taxa should be studied to determine how general is this result. Data from the Isthmus are of particular relevance because they are based on allopatric taxa that have not been influenced evolutionarily by secondary contact since the connection between the Caribbean and eastern Pacific closed. This absence of secondary contact is critical, because currently sympatric and allopatric species do not exhibit the same relationships between reproductive isolation and genetic differentiation. In *Drosophila*, the only group for which we have extensive data, prezygotic barriers are three times stronger in sympatric taxa than in allopatric taxa for closely related species of similar age, presumably due to the effects of reinforcement (Coyne & Orr, 1997). Thus it is appropriate, even within a single genus, that some sympatric species are more similar to each other genetically than are allopatric populations of other species (e.g. Russo et al., 1994).

The age of recently diverged species

The ability to estimate the divergence times of species by comparing DNA sequences, even if only approximate, offers the opportunity to examine the relationship between speciation and the broader paleontological record of climatic change. In particular, did many of the closely related taxa we are discovering with genetic techniques emerge during the enormous and cyclical changes in temperature and sea level associated with the waxing and waning of the ice ages over the last 2.5 million years? Alternatively, are they older or much younger?

Few attempts have been made to answer this question for marine organisms generally, and indeed the same question has generated debate for the much better studied terrestrial realm (Avise & Walker, 1998; Avise et al., 1998). Benzie (1999) provides the only general summary of which I am aware which is based on genetic data for marine organisms, and he focuses on coral reef taxa. He argues that both intraspecific population structure and interspecific species divergences often date to the Pleistocene (less than 2 mya). The importance of lowered sea level in generating divergences between conventionally conspecific populations in the Indian Ocean and western Pacific seems robust as it is based on a number of analyses; the pat-

terns observed are similar to those summarized on land by Avise et al. However, species-level divergences calibrated by mtDNA clocks are based on a very limited sample in Benzie's (1999) analysis and are dominated by Indo-West Pacific butterflyfishes, and by taxa separated by the Isthmus of Panama (which cannot have diverged more recently than 3 *mya*, see below).

Taxa separated by the Isthmus of Panama provide other important data on this subject, however. If the most similar transisthmian sister species taxa are assigned a divergence time of 3 million years ago, one can then ask: how many non-transisthmian taxa are younger? For snapping shrimp, the answer is none: all other sibling species analyzed to date exhibit divergences suggesting times of origin that precede the closure of the Isthmus (Knowlton et al., 1993). A number of other genetic studies also suggest older divergences between sibling species (e.g. Bakker et al., 1995a,b; Bucklin et al., 1995; Lessios et al., 1995).

There do, however, seem to be situations where more recent divergences are not uncommon. Marine snails and fish provide some of the best documented examples of speciation events which are almost certainly comparatively recent (Reid et al., 1996; Marko, 1998; Metz et al., 1998b; McMillan et al., 1999). Interestingly, many snails do not seem to conform to the classical allopatric model, in that sister species often come from the same biogeographic province (Hellberg & Vacquier, 1999). It is difficult to know whether these cases of speciation during the past 2 *my* reflect some special attribute of the groups involved [e.g. sexual selection acting on color patterns in fishes (see Galis & Metz, 1998 for freshwater counterparts) and divergent selection in gamete recognition proteins in gastropods (Swanson & Vacquier, 1998; Hellberg & Vacquier, 1999)], or whether similar situations will emerge elsewhere when other groups are more intensively studied. However, marine taxa provide no good cases of instant speciation or speciation in historical times; the only proposed example (a founder event in a laboratory population of polychaetes) has since been withdrawn based on new genetic data (Rodríguez-Trelles et al., 1996).

Final comments

As part of a symposium on the genetics of marine organisms, this paper has focused almost exclusively on the application of molecular analyses to the problem of understanding species in the sea. There is no denying

the power of these techniques, but they have far more potential when combined with insights based on other approaches. Many have made this point, but perhaps none so eloquently as Evelyn Hutchinson (1975). His tribute to Robert MacArthur (best known today for his mathematical theory) closed with the following: "... a wide and quite deep understanding of organisms, past and present, is as basic a requirement as anything else ... it may best be self-taught, but how often is this difficult process made harder by a misplaced emphasis on a quite specious modernity. Robert MacArthur really knew his warblers."

Unfortunately, we simply do not know our squids, starfish and shrimp as well as ornithologists know their birds, in part because of long-established taxonomic traditions that depend on preserved material. If in our enthusiasm for molecular genetics we simply replace formalin with liquid nitrogen or DMSO, we will make only limited progress in elucidating the pattern and process of speciation in the sea.

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