Molecular Approaches in Crustacean Evolutionary Ecology

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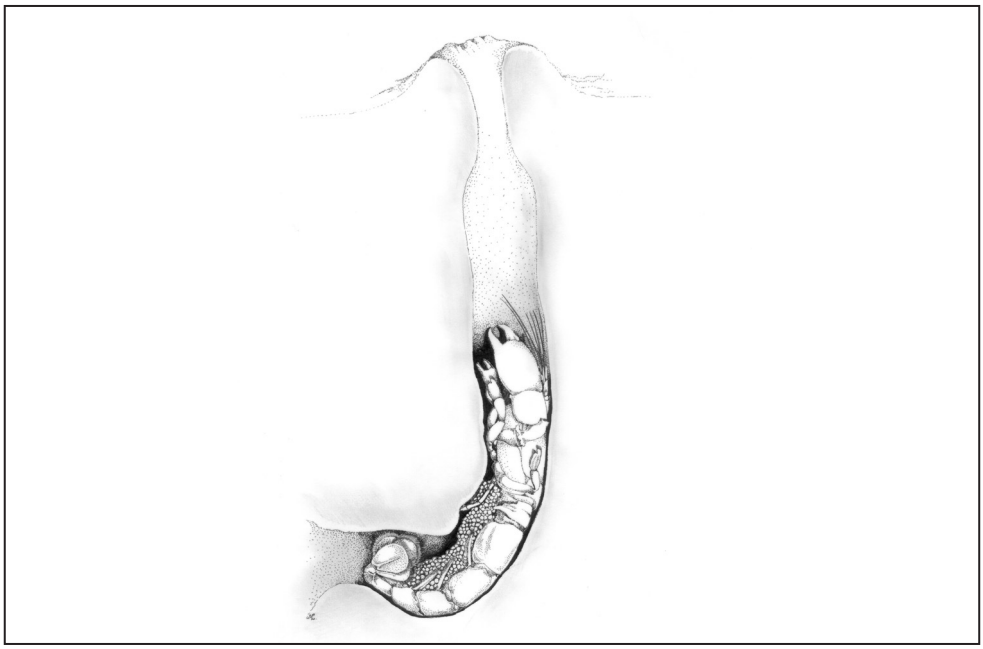

An ovigerous female ghost shrimp, Callichirus islagrande, *remains in her burrow while incubating her brood under her abdomen.*

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This chapter examines the uses of molecular markers to analyze relationships of descent among both individuals and taxa in the Crustacea. Molecular markers can be highly effective for these purposes but vary considerably in cost, ease of use, and suitability for specific applications (Avise 2004). The power of molecular markers to reveal parentage and kinship has revolutionized the study of social behavior in animals, producing such dramatic results as the discovery of widespread polyandry among animals (Zeh and Zeh 2003). Molecular markers have also allowed us to investigate mating systems even when it has not been practical to observe mating behavior. For example, it was possible to demonstrate multiple paternity in the thalassinidean ghost shrimp, *Callichirus islagrande* (Bilodeau et al. 2005), although mating of thalassinideans has never been observed because it occurs deep within their burrows. Molecular markers have also revolutionized phylogenetic analysis by providing a rich set of shared characters that enable powerful approaches to phylogenetic inference. This revolution in phylogenetics has in turn allowed the widespread adoption of phylogenetic comparative methods that use phylogenies to test hypotheses about the evolution of characters. This chapter is intended both as a critical assessment of these uses of molecular markers in crustacean evolutionary ecology and as an introduction for those considering their use.

Relationships Between Individuals

Genetic Markers for Individuals

Since the mid-1990s, microsatellite loci have become the preferred markers for inference of parentage and relatedness (A.G. Jones and Ardren 2003) and have become important for population genetics and genetic mapping. Another class of marker, the amplified fragment length polymorphism (AFLP) (Vos et al. 1995), has been used for many of the same applications, although they provide data that are fundamentally different. A comparison of these two classes of markers serves to illustrate some of the important considerations that arise in the selection of a genetic marker for studies of parentage and relatedness.

Microsatellites

Microsatellite sequences are short, tandemly repeated sequences that are scattered throughout the genomes of higher organisms (Tautz 1989). When a microsatellite sequence is replicated, either "replication slippage" or unequal crossovers can change the number of repeats, and successive changes can produce a large number of alleles that differ in length (Eisen 1999). Detection of microsatellite length variation is straightforward: polymerase chain reaction (PCR) is used to amplify the entire microsatellite sequence, and the length of the amplicon is determined by gel electrophoresis. Heterozygotes yield amplicons of two distinct sizes, while homozygotes yield a single amplicon. The mutation rate for replication slippage can be as high as 10^{-2} per replication (Cronn et al. 2002), and microsatellite loci tend to be much more polymorphic than most other genetic markers (Tautz 1989).

The development of microsatellite markers can be tedious and expensive, and the equipment and expertise needed to perform the initial isolation of microsatellite loci

can exceed that found in laboratories primarily concerned with behavioral or organismal questions. After PCR primers for a microsatellite locus are developed, they must be tested to determine whether they amplify the correct sequence, whether the locus is polymorphic, and if possible, whether the locus follows Mendelian inheritance. It is not unusual for the development of microsatellite markers to stretch to a year, although after this process has become routine, a month or two is usually sufficient. An increasingly feasible alternative to in-house development is to contract commercial laboratories to perform many of the steps of microsatellite development (Selkoe and Toonen 2006). Brief descriptions of new microsatellites are regularly published in *Molecular Ecology Notes*. However, microsatellite primers designed for one species are not always useful for related species; they may fail to amplify microsatellite loci, or the loci can be less polymorphic.

Several types of artifacts can compromise the quality of microsatellite data. Null alleles fail to amplify because their flanking sequences do not match the primers (Callen et al. 1993). Primers might also amplify more than one microsatellite locus, especially if the loci are within repetitive sequence families (Harris and Crandall 2000). Allele "dropouts" can occur in PCR reactions when alleles with small numbers of repeats outcompete alleles with more repeats (Jensen and Bentzen 2004). Replication slippage during PCR can produce byproducts a few repeats shorter or longer than the original sequence (Shinde et al. 2003), which can make it difficult to judge the sequence's true size. Artifacts can often be detected as departures from Hardy-Weinberg proportions of genotype frequencies. For example, null alleles produce overall heterozygote deficiencies (e.g., Shaw et al. 1999), while allele dropouts result in heterozygotes deficiencies for specific combinations of alleles (Miller et al. 2002).

AFLPs

The AFLP technique is based on selective amplification of restriction fragments. The amplicons that result from this process, typically 50–100 in number, are resolved by gel electrophoresis as a pattern of bands. Alleles that produce bands are dominant, and so the exact genotype (homozygote or heterozygote) of individuals with a dominant phenotype cannot be determined (Vos et al. 1995). In comparison to microsatellites, AFLPs are relatively easy to develop. However, the use of AFLPs for some applications, such as population genetics, has been a subject of controversy (Sunnucks 2000). The black box nature of AFLP variation makes it difficult to generalize about underlying mutation processes or their rates. Errors in genotyping appear to be higher for AFLPs than for microsatellites (Bonin et al. 2004), and the dominance of AFLP bands makes it impossible to detect artifacts or bands produced by contaminating organisms as departures from Hardy-Weinberg proportions. These criticisms are valid, although some can be addressed by controls that demonstrate the reproducibility and Mendelian inheritance of AFLPs.

A little history might help to explain why the AFLP method has been criticized so strongly. Five years before the introduction of the AFLP method, the random amplified polymorphic DNA (RAPD) method was introduced (J.G.K. Williams et al. 1990). Like AFLPs, RAPD polymorphisms are dominant and scored as PCR products of specific sizes. When the RAPD method first appeared, it generated a great deal of enthusiasm because it was simple and quickly produced abundant data. However,

disappointment followed when it became clear that RAPD bands were often not reproducible (Riedy et al. 1992, Ellsworth et al. 1993, Ayliffe et al. 1994, Perez et al. 1998). The AFLP method is now unfavorably and perhaps unfairly compared with the RAPD method. An investigator considering the use of either RAPDs or AFLPs for population genetics or estimation of relatedness should be aware of the biases against these techniques by both funding agencies and journals.

Allozymes (Lewontin 1991, Avise 2004) remain a viable choice for analysis of parentage or relatedness when precision is not required and sufficient amounts of fresh or frozen tissue are available. Allozymes have less power to resolve relationships than do either microsatellites or AFLPs. However, for some purposes it is enough to estimate an average degree of relatedness for many pairs of individuals (Blouin 2003). For example, Duffy (1996; see also chapter 18) used allozymes to demonstrate that the degree of relatedness between individuals within colonies of the alpheid shrimp *Synalpheus* matched that expected for full siblings.

All types of genetic markers are prone to significant occurrences of artifacts and errors, which include both those inherent to the techniques themselves and those due to human errors. There is a growing consensus that studies based on genetic marker data should acknowledge the inevitability of artifacts and errors and incorporate strategies for reduction of errors, automation and blind controls to reduce subjectivity in the collection of data, and estimates of error rates in data analysis (Bonin et al. 2004).

Analysis of Relationships Between Individuals

Parentage Analysis

Parentage analysis can be used to address fundamental questions in behavioral and evolutionary ecology (Avise 2004). The Crustacea offer many interesting problems associated with mate choice, sperm competition, and mechanisms of paternity assurance that can now be approached by analysis of paternity (see Bauer and Martin 1991; see also chapters 7–12). A classic example of how this approach can be integrated with an understanding of a species' reproductive biology is provided by studies of mating in the majid crab *Chionoecetes opilio* (D.M. Taylor et al. 1985, Urbani et al. 1998, Sainte-Marie et al. 1999; see also chapter 9).

Several recent reviews have provided important practical considerations for analysis of parentage with genetic markers (Gerber et al. 2000, Van de Casteele et al. 2001, A.G. Jones and Ardren 2003). The simplest approach is a process of elimination: Mendelian principles are used to exclude all potential parents except the two that are the true parents of the propositus, or focal individual. The probability that a nonparental individual can be excluded depends on the number of marker loci, the effective number of alleles at each locus, and whether alleles are dominant or codominant. Exclusion probabilities greater than 99% can be achieved with about five highly polymorphic microsatellite loci (see equation 4 in Gerber et al. 2000). In contrast, with dominant markers (AFLPs or RAPDs), it is not possible to exclude any individuals as potential parents. If the genotype of one parent is known, this further limits the possible genotypes of the other parent, and there is a greater probability that a nonparent can be excluded. Dominant markers can also be used to exclude individuals in this situation, although each locus has less discriminatory power than a locus with

codominant alleles. The most advantageous situation for reconstruction of parentage is when it is known which pairs of individuals mated so that potential parents can be considered as pairs rather than single individuals. In this case, a single microsatellite locus could be sufficient to exclude more than 99% of nonparental pairs.

Identification of parents by unambiguous exclusion of all nonparents is attractive in its simplicity but not always practical. In some cases, the available markers cannot exclude all nonparents. A second possibility is that the true parents will be incorrectly excluded as the result of errors or artifacts. In situations with multiple alternative hypotheses that cannot be rejected by tests of statistical significance, likelihood is a useful alternative (Edwards 1992). Likelihood methods for parentage analysis have been developed for both dominant (Meagher and Thompson 1986) and codominant markers (Gerber et al. 2000). Likelihood can be used simply to assign progeny to their most probable parents; however, for some purposes, it is more useful to assign "fractions of progeny" to each possible parent. For example, if the total reproductive success of an individual were to be estimated, fractional assignments provide less biased estimates than those from all-or-none assignments (Neff et al. 2001). Fractional assignments are generally based on Bayesian estimates of the probability of parentage.

Multiple Mating

Polyandry (mating with multiple males) is widespread among animals, although it is often unclear how it benefits polyandrous females (Reynolds 1996; but see chapter 9). Polyandry is typically defined at the level of a brood (Neff and Pitcher 2002). Detection of polyandry is straightforward with codominant genetic markers. If a female's genotype is known, nonmaternal alleles represented in her brood are assumed to be of paternal origin. If a female's genotype is unknown, it is assumed that there are at most two alleles per parent in a brood. For accurate estimates of the frequency of polyandry, two or more highly polymorphic codominant marker loci are needed, and for accurate estimation of the number of sires per brood, larger numbers of highly polymorphic loci are required. Microsatellites are the obvious choice for most investigations of polyandry (Neff and Pitcher 2002).

Genetic markers can also be used to detect polygyny (mating with multiple females). However, in the typical situation in which broods are found with females, the process is more complicated than detection of polyandry. This is because it must be shown that the same male sired multiple broods, which requires that the parentage of each brood be determined. Since the occurrence of polygyny itself is generally considered unremarkable, it is most often considered in the context of male reproductive success (e.g., Zamudio and Sinervo 2000).

Genetic markers have proved to be useful in the detection of polyandry in crustaceans. Within the order Decapoda, polyandry at the level of broods has been detected in the nephropid lobsters *Homarus americanus* (Nelson and Hedgecock 1977, M.W. Jones et al. 2003, Gosselin et al. 2005) and *Nephrops norvegicus* (Streiff et al. 2004), the cambarid crayfish *Orconectes placidus* (Walker et al. 2002), the porcellanid crab *Petrolisthes cinctipes* (Toonen 2004), the callianassid ghost shrimp *Callichirus islagrande* (Bilodeau et al. 2005), the cancrid crab *Cancer pagurus* (Burfitt 1980), and the palaemonid shrimp *Palaemonetes pugio* (Baragona et al. 2000). In the majid crab *Chionoecetes opilio*, females can carry stored sperm from multiple males,

but generally only one male sires each brood (Sevigny and Sainte-Marie 1996, Urbani et al. 1998, Sainte-Marie et al. 1999; see also chapter 9). The only nondecapod crustacean example of polyandry that we are aware of is the porcellionid isopod *Porcellio scaber* (Sassaman 1978).

Genealogical Relationships Other Than Parentage

Measurements of relatedness based on molecular markers can serve as the basis for estimates of heritability, the number of breeders in a population, variance in reproductive success, and tests of kin selection theory. Although there are few examples of measurements of relatedness for crustaceans, there are many for social insects. Microsatellite markers were used to demonstrate that males are produced by queens rather than workers in the apid bee *Schwarziana quadripunctata* (Tóth et al. 2003) and the vespid wasp *Brachygastra mellifica* (Hastings et al. 1998). For the primitively eusocial vespid wasp *Ropalidia revolutionalis*, microsatellite markers revealed that queens almost always mate singly, which is of interest because it creates the potential for conflicts over the production of males (Henshaw and Crozier 2004). In a study of the multiple-queen formicid ant *Leptothorax acervorum*, Bourke et al. 1997) used microsatellite markers to show that queens mated singly and were usually closely related to coexisting queens, and that because of a high turnover of queens, workers were usually not the offspring of the current queens. Other examples can be found in Ross (2001).

Many different statistical methods have been developed for estimation of relatedness from genetic markers (Milligan 2003). Methods exist for both codominant and dominant markers. Relatedness can be estimated as a continuous variable or by assignment to categories of relationship. Accurate estimation of relatedness for pairs of individuals generally requires a large number of loci; however, even a few loci can be sufficient to estimate average relatedness within groups (e.g., Duffy 1996). A good recent overview of these methods can be found in Blouin (2003).

Phylogenetic Relationships and the Comparative Method

Phylogenies as a Framework for Comparative Analysis

The parallel or convergent evolution of similar traits (i.e., character states) in unrelated species that are subject to similar environmental conditions is evidence that the traits are adaptations to those conditions. In contrast, traits shared by related species in similar environments cannot be considered independent instances of adaptation because they could have arisen once in a common ancestor and are now shared due to "phylogenetic inertia". Phylogenetic comparative methods (PCMs) are intended to separate shared history from independent evolution by analyzing the phylogenetic distributions of traits (Brooks and McLennan 1991, Harvey and Pagel 1991).

Phylogenies have been used to investigate whether similar behavioral traits have evolved independently in crustacean lineages. Kitaura et al. (1998) used a phylogeny based on mitochondrial DNA sequences to trace the evolution of mud-using territorial behavior in the semaphore crabs, genus *Ilyoplax*. Three behavioral traits—burrow plugging, barricade building, and fence building—were placed on this phylogeny.

Under the assumption that gains and losses of traits were equally likely, two most parsimonious reconstructions of ancestral traits were found. In one reconstruction, barricade building evolved three times, and in the other, it evolved twice and was lost once (Fig. 3.1a). Schubart et al. (1998) used a phylogeny based on mitochondrial sequences to examine the origins of adaptation to terrestrial life in endemic Jamaican land crabs. The phylogeny included related marine species from the Americas and Southeast Asia as well as those from Jamaica. The Jamaican species formed a single

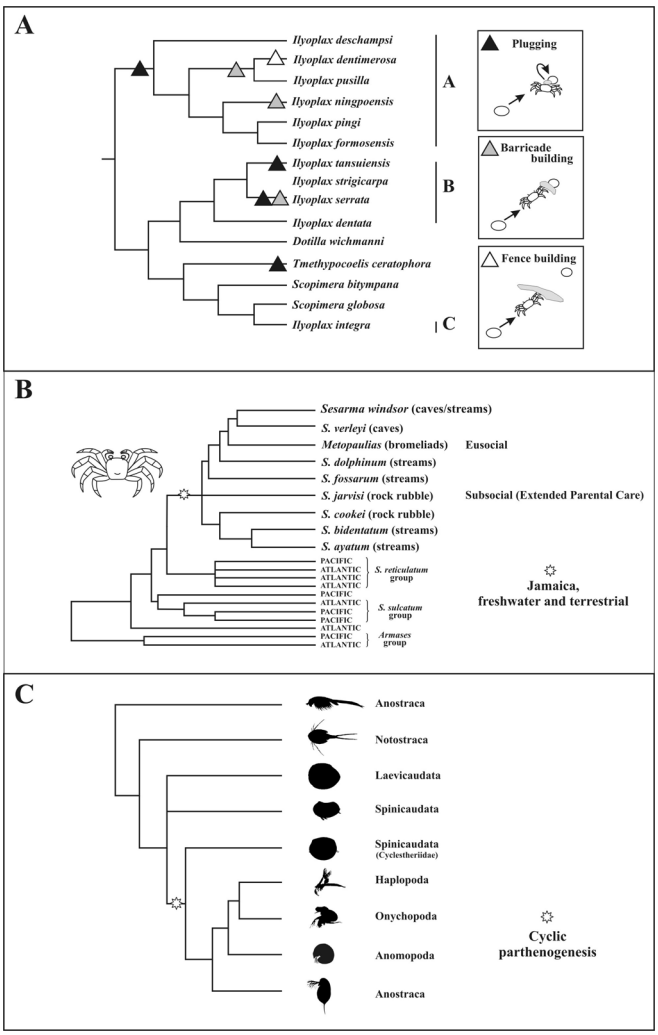

Figure 3.1 Phylograms of three crustacean taxa on which traits were mapped to infer their likely origins. (A) Intertidal crab species from the family Ocypodidae and three different territorial behaviors. (B) Crab species from the family Sesarmidae of different geographic origins, including the freshwater and terrestrial species of *Sesarma* from Jamaica. (C) Branchiopod crustacean taxa, including those taxa that exhibit cyclic parthenogenesis. Figures modified after Kitaura et al. (1998), Schubart et al. (1998), and D.J. Taylor et al. (1999).

monophyletic group, which is consistent with a single adaptive radiation from a marine ancestor (Fig. 3.1b). D.J. Taylor et al. (1999) used a phylogeny based on a combination of nuclear and mitochondrial DNA sequences and morphological characters to address questions about the origins of cyclic parthenogenesis in the shrimplike Branchiopoda. The five orders with cyclical parthenogens formed a monophyletic group, in support of the view that cyclical parthenogenesis arose once within the class (Fig. 3.1c).

The most powerful applications of PCMs are tests of hypotheses about causative relationships among traits or between traits and environmental factors. Duffy et al. (2000; see also chapter 18) used a phylogeny based on both mitochondrial DNA sequences and morphological characters to investigate the origins of eusociality in the sponge-dwelling alpheid shrimps (genus *Synalpheus*) and to test the hypothesis that eusociality has led to ecological dominance. Eusocial taxa were distributed among three distinct clades, each of which also contained noneusocial taxa. By the principle of parsimony, this provides evidence for three separate origins of eusociality within the genus. Phylogenetically independent contrasts (Felsenstein 1985) revealed a significant correlation between eusociality and the tendency for species to predominate within their host sponges. This uniquely marine example of the evolution of eusociality is reviewed by Duffy in chapter 18. Species of spiny lobsters (Palinuridae) are often characterized by gregarious behaviors such as shelter sharing and group migration. In chapter 13, Childress uses a phylogeny for Palinuridae based on mitochondrial DNA sequences to explore potential relationships between specific ecological and life history traits and the evolution of social behavior within this family.

There have been two forms of criticism against PCMs (Freckleton et al. 2002). The first is directed against the argument that traits shared by related species are not independent. The counterargument is that, irrespective of their origins, traits must be maintained by selection, and this occurs independently in every species. The second form of criticism concerns the assumptions of PCMs. All PCMs require assumptions about character evolution, typically that characters evolve randomly and are selectively neutral. Such assumptions are inconsistent with the use of PCMs to detect selective forces that result in directional evolution. If their assumptions are invalid, PCMs can have less power to detect significant patterns or can even generate incorrect results (Bjorklund 1997, Ackerly and Donoghue 1998, Cunningham et al. 1998). Not all PCMs are subject to the same criticisms. For example, Hansen (1997) developed a PCM that considers not only the independent origins of traits but also their maintenance by stabilizing selection. Although some PCMs assume that character evolution can be described by a random walk, others use models in which character evolution is directed or constrained by selection (Martins 2000). In general, most PCMs require that there be at least a correlation between phylogenetic relatedness and phenotypic similarity. Statistical tests can be used to determine if these correlations are significant (Cheverud et al. 1985, Ackerly and Donoghue 1998, Diniz et al. 1998), and the strength of the correlation can serve as a guide to how a PCM should be applied (Freckleton et al. 2002). In one simulation study (Martins et al. 2002), PCMs generally outperformed nonphylogenetic methods, even when the PCM's assumptions were violated.

There are several questions that should be answered before undertaking a phylogenetic comparative analysis. Are the traits best represented as discrete or continuous

characters? Are there enough species to provide the statistical power to detect patterns of character evolution? Is a suitable phylogeny already available, or will it be necessary to first construct one? The answers to these questions will determine the feasibility of the analysis and provide some indication of how to proceed. Before a PCM (or computer program to perform a PCM) is chosen, we recommend a fresh review of the most recent literature on this very dynamic subject rather than reliance on earlier studies as models.

Most PCMs assume that phylogenetic relationships are known with certainty, although this is never the case. Phylogenetic reconstructions can be treated as estimates of the true phylogeny, and as such, they are subject to error. There are two approaches to the problem of phylogenetic error in comparative analysis. The first is to attempt to minimize it by the use of reliable data and accurate methods of phylogenetic inference. The second is to allow for the occurrence of error with analyses that consider all plausible phylogenies rather than just a single "best" tree (Schultz and Churchill 1999, Huelsenbeck and Bollback 2001, Ronquist 2004). Both approaches imply that the phylogenies used for comparative analysis should meet high standards if we are to avoid erroneous or ambiguous conclusions. Since the support of future comparative analyses is a common justification for work in systematics, it is important to apply these standards as broadly as possible. Below we offer our suggestions on how this could be achieved for molecular systematics of crustaceans.

Problems and Solutions in Molecular Phylogenetics

Most molecular phylogenies of crustaceans have been based on single gene sequences or at best sequences of a few genes. Although such studies have been useful, trees based on single genes are unlikely to be entirely accurate. One reason is that gene trees are shaped not only by speciation events but also by genealogical processes within species (Neigel and Avise 1986, N.A. Rosenberg 2003). Thus, even if a gene tree is known with complete accuracy, it is unlikely to be an exact representation of the true species tree. Other sources of errors arise from the complexities of sequence evolution. Most commonly used phylogenetic methods assume that sequence evolution is represented by one of a limited set of models with parameters that can be estimated from the data (Felsenstein 1981, Huelsenbeck et al. 2001); use of an inappropriate model can strongly bias results (Lemmon and Moriarty 2004). Another source of error is the inadvertent use of paralogous sequences, such as duplicated genes or pseudogenes. Studies of crustacean mitochondrial gene sequences suggest this problem can be quite serious. A mitochondrial large-subunit ribosomal RNA (16S rRNA) nuclear pseudogene in the menippid crab *Menippe* was detected only because it coamplified with the functional mitochondrial gene; there were no telltale sequence characteristics that would have identified it as a pseudogene (Schneider-Broussard and Neigel 1997). Within the alpheid shrimp genus *Alpheus*, cytochrome oxidase I pseudogenes were common, often impossible to identify by sequence criteria alone, and sometimes amplified preferentially over the true mitochondrial sequence (S.T. Williams et al. 2001).

Theoretical considerations suggest that accurate phylogenetic reconstruction requires data from many independently segregating loci (Wu 1991). This requirement has been supported by empirical studies with varying numbers of loci. For example,

Table 3.1. Selected crustacean studies using DNA sequence data.

Sequence	Product	Reference	Group(s)
Mitochondrial Sequences			
COI	Protein	Folmer et al. 1994	Metazoa
COII	Protein	Perez-Losada et al. 2004	Aeglidae
ND5	Protein	Colbourne et al. 1998	Cladocera
12S	rRNA	Colbourne and Hebert 1996	Cladocera
16S	rRNA	Cunningham et al. 1992	Anomura
Nuclear Sequences			
EF-1α	Protein	Regier and Shultz 1997	Arthropoda
EF-2	Protein	Regier and Shultz 2001	Arthropoda
GPI	Protein	Williams et al. 2001	*Alpheus*
H3	Protein	Colgan et al. 1998	Arthropoda
Pol II	Protein	Shultz and Regier 2000	Arthropoda
18S	rRNA	Spears et al. 1992	Brachyura
28S	rRNA	Taylor et al. 1999	Branchiopoda
ITS1	rRNA	Schwenk et al. 2000	Cladocera
ITS2	rRNA	Schwenk et al. 2000	Cladocera

Abbreviations: COI, mitochondrial cytochrome C oxidase subunit I; COII, mitochondrial cytochrome C oxidase subunit II; ND5, mitochondrial NADH dehydrogenase; 12S, mitochondrial small-subunit ribosomal RNA; 16S, mitochondrial large-subunit RNA; EF-1α, nuclear elongation factor 1α; EF-2, nuclear elongation factor 2; GPI, nuclear glucose 6-phosphate isomerase; H3, nuclear histone H3; Pol II, nuclear RNA polymerase II; 18S, small-subunit nuclear RNA; 28S, large-subunit nuclear RNA; ITS1, first internal transcribed spacer of nuclear RNA; ITS2, second internal transcribed spacer of nuclear RNA.

a recent study with seven species of yeast demonstrated that, on average, 8–20 independently segregating loci were needed to achieve 100% bootstrap support of phylogenetic relationships (Rokas et al. 2003). At present, there are a limited number of loci in use for crustacean systematics. Although there are nominally 14 such loci (Table 3.1), the five mitochondrial genes represent only one segregating unit, as do the four nuclear ribosomal sequences; there are thus only seven independently segregating loci. The usefulness of some of these loci has been demonstrated only for some taxonomic levels or specific groups, for example, GPI and EF-1α in *Alpheus* (S.T. Williams et al. 2001) and EF1-α, EF-2, and POL II for higher taxonomic levels of the Arthropoda (Regier and Shultz 1997, 2001, Shultz and Regier 2000).

In addition to a dependence on the number of loci, the accuracy of molecular phylogenetic reconstruction depends on the number of taxa sampled. The addition of taxa provides more information about the states of internal nodes in a phylogeny (Graybeal 1998) and better estimates of substitution rates at particular sites in DNA sequences (Pollock and Bruno 2000). There has been some debate over the relative merits of sampling more taxa versus more loci (Hillis et al. 2003, M.S. Rosenberg and Kumar 2003), but there are certainly examples in which well-supported but incorrect phylogenies were obtained when the number of loci was high but the number of sampled taxa was low (Soltis et al. 2004).

One of the central problems in molecular systematics is the high degree of homoplasy (parallel or convergent evolution of the same character state) in nucleotide substitutions and small insertions and deletions (indels) (Broughton et al. 2000). However, there are other types of molecular characters that are relatively free from homoplasy. Rokas and Holland (2000) reviewed the use of rare genomic changes that include indels of entire introns, unique indels in protein or RNA sequences (signature sequences), retroposon events (transposable elements), gene order rearrangements in organelle genomes, and variations in genetic codes. Mitochondrial gene rearrangements have proven useful for crustacean systematics. For example, C.L. Morrison et al. (2002) used them to construct a phylogeny in which it appears that carcinization evolved independently in the decapod lineages Brachyura, Porcellanidae, Lomisidae, Lithodidae, and the paguroid crab genus *Birgus*. Lavrov et al. (2004) used mitochondrial gene order to determine the close affiliation of the Pentastomida (tongue worms) with the Cephalocarida (horseshoe shrimps) and the Maxillopoda (ostracods, copepods, and barnacles).

Sequence alignment is a critical step in molecular phylogenetic analysis. Alignments represent assumptions about the homology of characters, and phylogenetic inference can be very sensitive to changes in alignments (D.A. Morrison and Ellis 1997). Alignment algorithms insert gaps only if they improve the overall alignment score by more than the value of the "gap penalty" (Setubal and Meidanis 1997). However, gap penalties are usually set arbitrarily and often do not accurately represent how sequences actually evolve (Gu 1995). Because of the complexity of the problem of multiple alignment (Bonizzoni and Vedova 2001), heuristic algorithms must be used that are not guaranteed to find alignments with the best scores. These limitations suggest that overly complex alignments are a dubious foundation for phylogenetic inference.

Alignments of noncoding sequences that are extremely variable in length (e.g., structural RNAs, introns, and intergenic sequences) are usually the most problematic. Unfortunately, such sequences represent nearly half of the gene sequences used in crustacean systematics (Table 3.1). These sequences tend to have numerous indels, runs of repeated nucleotides, and variable numbers of tandem repeats (e.g., microsatellite sequences). In such ambiguous cases, the conservative approach is to eliminate problematic regions from the data used for phylogenetic analysis, although this can also result in elimination of many otherwise informative sites (D.A. Morrison and Ellis 1997).

We hope that we have made a strong case for adding more loci, especially nuclear loci, to the current set available for crustacean molecular systematics. These might include both protein coding loci and conserved noncoding sequences. Many candidates can be found in public sequence databases, although considerable effort is needed to develop PCR primers that work reliably across a range of taxa. As new loci are identified for crustacean systematics, it will be important to demonstrate (to the extent that it is possible) the orthology of sequences from different taxa. The large set of loci that have been tested in phylogenetic studies of insects can serve as a guide to what is likely to be useful for the Crustacea. As of 2000, around 40 protein coding loci had been used for insect systematics as well as all of the major ribosomal RNA genes and numerous noncoding regions (Caterino et al. 2000).

Phylogenetic Analysis of Sequence Data

Overview

Considerable progress has been made over the past 20 years in the development of methods for phylogenetic analysis of DNA sequences (Swofford et al. 1996), although this has been accompanied by intense debate over these methods. There has been no final answer to the question of which method is most likely to produce the "true tree"; however, they can be objectively compared as statistical estimators that differ with respect to robustness, consistency, and efficiency. Here, we present a brief overview of methods for phylogenetic inference and consider application of these methods to comparative studies.

Distance Methods

Distance-based, or phenetic, methods use algorithms to cluster sequences into trees that reflect pairwise measures of distance between DNA sequences (Swofford et al. 1996). These distances are based on models of sequence evolution that correct for multiple substitutions at individual sites. The neighbor-joining algorithm has become the standard for building trees from distance data because it is robust and efficient in comparison to other distance methods (Saitou and Nei 1987). Distance methods are considerably faster than the others considered here, but simulation studies have shown that they are less likely to produce the correct tree (Huelsenbeck 1995a, 1995b).

Maximum Parsimony

Maximum parsimony (MP) represents an optimality criterion to compare trees rather than an algorithm to build trees (Swofford et al. 1996). Phylogenetic trees are considered as hypotheses; the tree that requires the fewest character state changes is the preferred hypothesis. MP has been justified on the basis of William of Occam's famous dictim, "Entities should not be multiplied unnecessarily," as well as the fact that it does fairly well at finding the true tree in tests with simulated sequence data (Huelsenbeck 1995a, 1995b). MP also works with any type of sequence characters, including indels, and allows molecular and morphological data to be combined. Although MP is still a respected and widely used approach, it suffers from several disadvantages that limit its range of application. For more than about 10 taxa, a prohibitively large number of trees must be examined to guarantee that the most parsimonious tree is found. This necessitates the use of "heuristic" methods that are not guaranteed to find the best tree. Under some circumstances, MP is statistically inconsistent; with more data, it will converge on an incorrect estimate of phylogeny in which rapidly evolving taxa are grouped together even if they are unrelated (Felsenstein 1978). A more fundamental limitation for many PCMs is that MP does not provide estimates of branch lengths.

Maximum Likelihood

Maximum likelihood (ML) is another criterion to compare trees, but unlike parsimony, it is based on statistical theory. The tree with the ML can be considered to be

an estimate of the true tree, rather than simply a preferred hypothesis (Felsenstein 1981). Likelihood provides a measure of how much the data support an estimate or hypothesis and can be used to construct confidence limits (Edwards 1992). As in the case of distance methods, the calculation of likelihoods must be based on models of sequence evolution. With the correct model, ML tends to outperform most other methods at finding the true tree, but with the wrong model, it can perform poorly (Huelsenbeck 1995a, 1995b). Generally, the simplest model that can explain the data according to one or more criteria is preferred (Posada and Crandall 1998). As with MP, a prohibitively large number of trees would need to be examined to guarantee that the ML tree is found, but in addition, the parameters of the model of sequence evolution must also be estimated. For this reason, ML analyses are computationally the most demanding.

Bayesian Inference

Bayesian methods of phylogenetic inference use the same models of sequence evolution as distance methods and ML but are unique in estimating the probabilities of phylogenetic trees (Huelsenbeck et al. 2001). Although it is generally impossible to evaluate the probabilities of all possible trees, the distribution can be approximated with the Markov chain Monte Carlo (MCMC) method. From this distribution, a consensus tree with the probability of each branch evaluated can be constructed (Larget and Simon 1999). This use of the MCMC method makes the Bayesian approach less computationally demanding than ML (Huelsenbeck et al. 2001).

Bayesian methods offer an attractive solution to the problem of phylogenetic uncertainty in comparative analysis. Trees can be sampled according to their posterior probabilities so that the results of the analysis are not based on a single tree but are weighted to reflect the most probable trees (Huelsenbeck and Bollback 2001). Bayesian approaches have also been developed for the reconstruction of ancestral character states (Schultz and Churchill 1999) and can accommodate both phylogenetic uncertainty and uncertainty in the reconstruction of ancestral character states (Huelsenbeck and Bollback 2001).

Prospects for the Future

Within the Crustacea, diverse and often puzzling reproductive adaptations have evolved that are apparent in morphology and behavior (Bauer and Martin 1991; see also chapters 7–12). Molecular markers are tools that we can use to examine the effects of these adaptations on individual fitness and the factors that have influenced their evolution. However, the power and promise of molecular markers also represent a new set of challenges. The effort, expertise, and expense required to collect specimens that are suitable for DNA analysis, perform the necessary laboratory work, and perform sophisticated data analyses are often beyond the means of an individual investigator. Collaboration has become essential. Some forms of collaboration are already well established, such as collaborations between classically trained morphologists and molecular biologists. However, broader forms of collaboration will be needed to establish community resources that will allow us to reap the full potential of our

efforts. Repositories of information and knowledge are needed, as are physical repositories of voucher specimens, DNA samples, and PCR primers. Small aliquots of genomic DNA from important specimens should be made available as a matter of course, as should novel PCR primers. Efforts should be made to coordinate the development of new loci for systematics to avoid the "Tower of Babel" situation that has developed in insect systematics (Caterino et al. 2000). Along with these shared resources, there is a need for recognized standards of quality that reflect both immediate needs and the long-term utility of our work.

Summary

Molecular markers provide powerful means to analyze relationships of descent both among individuals and taxa. Microsatellite loci have become the standard for studies of paternity and kinship because they are highly polymorphic and codominant, properties that provide statistical power and facilitate the detection of artifacts. They have proven to be useful for the analysis of crustacean mating systems, although their full potential has yet to be realized.

Phylogenetic comparative methods are intended to separate instances of convergent or parallel evolution from shared evolutionary history by analyzing the phylogenetic distributions of traits. They have been criticized for the assumptions they make about how traits evolve, although not all of these methods make the same assumptions. Most assume an accurate phylogeny is known, which implies that a high standard should be required of phylogenies that will be used for comparative analysis. Crustacean phylogenies have mostly been based on small number of sequences that do not have the most desirable properties for phylogenetic inference. This situation is likely to be remedied by the development of PCR primers that amplify additional independently segregating nuclear loci.

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