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Invited Review

The use of allozyme electrophoresis in invertebrate systematics

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The role of enzyme electrophoresis is discussed as it applies to taxonomy and systematics, particularly of invertebrates. Details are given of methods for distinguishing and identifying cryptic or sibling species and the different approaches to sympatric and allopatric populations are reviewed. The calculation and uses of genetic distance measures are outlined, as are the empirical relationship of such measures to different levels of taxonomic separation. Difficulties, drawbacks and limitations of the technique are explained together with the advantages. Evidence for molecular clocks is outlined briefly and their role in systematic studies is discussed, as are methods of analysing genetic divergence data for systematic purposes. References to studies covering a wide range of invertebrate taxa are tabulated.

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Introduction

One of the aims of taxonomists is to identify individual animals or plants as belonging to a particular nominate species. This process usually involves the use of diagnostic characters, gradually separating the individuals from all other possible groups of organisms apart from the one to which they belong (called species, however these might be defined; see, for example, Brandon & Burian 1984; Otte & Endler 1989). Another aim is to classify organisms, which involves joining groups of species into higher taxa and then grouping the groups into yet higher taxa and so on to give a series of hierarchical levels with each successively higher taxon encompassing a wider range of more diverse species. Since the binominal system of nomenclature was first instituted in the mid-eighteenth century, an aim of most taxonomic hierarchies has been for the systematic arrangement to reflect the perceived interrelationships of the organisms concerned. In the nineteenth century, with the growing acceptance of evolutionary theories, the intention of the systematists was for their taxonomic hierarchies to reflect their ideas of the evolutionary history of the group concerned. Traditional methodology has been based almost entirely on detailed studies of external morphology and internal anatomy, and from these inferences have been drawn as to the evolutionary relationships of individual organisms or groups or populations of organisms.

The usual method adopted by the systematist is to infer phylogenetic relationships from studies of phenotypic divergence. In many cases only particular pre-selected aspects of the phenotype (often those considered 'diagnostic' or 'important' in a taxon) are studied in detail, leading to conclusions that are partially subjective and therefore unstable because they can be subsequently reversed by other systematics who may place greater emphasis on alternative characters. Although the use of cladistic methodology has done much to standardise procedures and a variety of mathematical methods are available for the objective analysis of morphological data (Felsenstein 1981, 1988; Swofford 1985; Huelsenbeck & Hillis 1993), the choice of characters is inevitably subjective to a greater or lesser extent.

Systematics are so intimately linked to evolution that there is not—and should not— be any clear-cut boundary between them. The major distinction, if any, probably lies in the aims of various workers; the evolutionary geneticist studies organisms with a view to understanding the role and mechanisms of evolution, while the systematist is concerned with the evolution and interrelationships of particular animals or plants insofar as this information will enable populations, species, or other taxa to be

placed more accurately within an overall systematic arrangement.

Geneticists tend to think in terms of 'evolutionarily cohesive groups' or gene pools, which are groups of individuals that freely exchange genes among themselves. Gene pools can be composed of geographically separated groups of individuals as long as gene flow can occur between them. Gene flow under such circumstances can be achieved by, for example, the random transport of gametes or larvae between populations (gene dispersal), as well as by direct migration between them [reviews by Strathmann 1985 (larvae); Slatkin 1985, 1987; Maynard Smith 1989 (genes)]. Thus to a geneticist a gene pool could be considered the major operational unit at the level of groups of individuals, to a systematist it could correspond to, for example, a population, a species or a subspecies, and to a fisheries biologist it could be a unit stock (for discussions of the stock concept in fisheries see, for example, Berst & Simon 1981; Ryman & Utter 1987; Smith *et al.* 1990).

Obviously, a big problem for geneticists in the past was how to obtain information about gene frequencies from natural populations. Formal genetic analysis of morphological traits usually required extensive experimental work with the cross-breeding of numerous individuals under controlled conditions. Such requirements made it feasible only to apply these studies to very few organisms—generally only those which were easy to rear and had short generation times, most notable among them being the fruitfly *Drosophila*. At that time geneticists faced the dilemma of having produced many elaborate evolutionary theories, but having few ways of actually verifying them in nature. With the development of isozyme electrophoresis, through the coupling of protein electrophoresis and histochemical staining (Hunter & Markert 1957), it became possible to obtain quickly large amounts of gene frequency data from natural populations. The new data confirmed some old theories and refuted others, but, more importantly, produced new paradigms, with the discovery of much higher levels of genetic variation than originally expected, and the unveiling of the high evolutionary dynamics of natural populations.

For a considerable period practical studies of evolutionary genetics were dominated by the use of electrophoresis to investigate the biochemical evolution and time-dependent structural divergence of protein molecules (see Lewontin 1974; Nei 1987), and more recently other molecular methods of gathering evolutionary data have also become important [e.g. restriction fragment length polymorphism analysis (Avise & Lansman 1983; Dowling *et al.* 1990; Hoelzel 1992); DNA amplification and sequencing (Hillis & Moritz 1990; Hoelzel 1992)]. These techniques have had only a moderate impact upon systematics, although it is clear that if employed in conjunction with more traditional methods, they offer enormous potential. Here we intend to discuss some of the current and future possible applications of electrophoretic data in systematics.

One great advantage of enzyme electrophoresis over some alternative chemical or biochemical methods is that data are essentially objective. For example, interspecific relationships, especially in plants and also in some invertebrates, have been investigated by chemotaxonomy, using a variety of low molecular weight chemicals (e.g. Cronquist 1980; Bergquist & Wells 1983; Bergquist *et al.* 1986). Unfortunately, such chemicals are mostly of unknown genetic derivation, may undergo environmental modification, and therefore as taxonomic characters present many of the same problems as those more conventionally employed. However, biochemical studies using the electrophoresis of proteins that are primary gene products have the advantage that the molecule is of known genetic derivation, normally the direct product of a single gene locus, and that in almost all cases the molecular structure is solely genetically determined and likely to be free from environmental modification. Therefore, between related populations or species, differences in the structure of particular proteins are likely to be genetic.

Taxonomic uses of electrophoretic data

The practical basis of electrophoresis is the differential migration of water-soluble protein (e.g. an enzyme) molecules through a gel (for enzyme studies this is usually made of hydrolysed starch, cellulose acetate or polyacrylamide) under the influence of an electrical potential. Subsequently, chosen enzymes or other proteins are stained by specific solutions (Shaw & Prasad 1970; Harris & Hopkinson 1978; Richardson *et al.* 1986). The methodology of enzyme electrophoresis is covered in detail by numerous reviews (e.g. Richardson *et al.* 1986; Murphy *et al.* 1990; May 1992). In any given medium the rate of migration of a molecule depends upon several factors, including its size and electrical charge. If an amino acid substitution occurs in a protein molecule this may alter the net charge, or possibly cause conformational changes, and thus alter the rate of migration. Some of the mutations in regions of the DNA coding for one particular enzyme will, therefore, be expressed as enzyme molecules differing in their electrophoretic mobility (i.e. a new allozyme allele). This new allele will initially appear at very low frequencies in the population, so that some rare individuals analysed electrophoretically for the enzyme will be polymorphic (heterozygous) for that allele. The evolutionary fate of the new allele will depend on its relative physiological performance ('fitness', or its 'selection coefficient') and also on chance (stochastic) changes in frequency when passed on to the next generation (genetic drift). Populations evolving independently will, therefore, diverge, as new alleles appear randomly or disappear in each one. Studying the allele differences between populations thus allow us to estimate how much they have diverged.

One of the advantages of electrophoresis is that allozymes are almost invariably codominant. This basically means that heterozygotes have different phenotypes from the homozygotes (the interpretation of gel banding patterns is discussed by several authors; for example, Richardson *et al.* 1986; Murphy *et al.* 1990). Identifying the heterozygotes and the homozygotes makes the calculation of gene frequencies very simple, and these frequencies can then be used for the comparison of

populations. Contrary to much of morphological, numerical or chemical systematics, molecular systematics is, thus, directly linked to the way genes evolve and their distribution in the species. Consequently, allozyme electrophoresis not only provides new characters for the study of species, but also brings with it a whole new set of revolutionary tools for their analysis and this is of great potential value in taxonomy and systematics. A few taxonomic studies have attempted to use electrophoretic patterns as taxonomic characters without any genetic interpretation; for example, by merely scoring bands as present or absent. Such approaches disregard the genetic information content and thus much of the value of the data and are also likely to be open to dispute on theoretical grounds (Murphy 1993). There are several earlier general or more specific reviews of the systematic uses of electrophoretic data (e.g. Avise 1974; Gottlieb 1977; Ferguson 1980; Thorpe 1982; Buth 1984; Solé-Cava & Thorpe 1987*a*; Hartl *et al.* 1990; Hillis & Moritz 1990).

In taxonomic or systematic studies the genetic data produced by electrophoresis can be used by systematists to answer two types of questions:
(1) Are the samples from which genes are being compared from the same freely interbreeding population (i.e. gene pool) or are they from different gene pools?
or
(2) How different (or, conversely, how closely related) are the gene pools which are being compared (or what genes do they share)?
Clearly the answer to question (1) is appropriate for distinguishing species (alpha systematics) and that to question (2) can be used to provide data on interrelationships of taxa (beta systematics or phylogenetics). However, data on levels of difference between gene pools can also be used to give an indication of whether these are likely to be conspecific.

In systematics, question (1) above would commonly become 'are these two biological entities (morphotypes, colour morphs, subspecies or whatever) the same or different species?' Enzyme electrophoresis is at its most powerful in providing answers to such problems, but only when the two entities are or can be considered to be sympatric.

From conventional definitions under the biological species concept (and also for the phylogenetic species concept; Cracraft 1983—see also Templeton 1989), two sympatric morphs should, if conspecific, be part of the same evolutionary unit. In other words, they should have (given certain assumptions and within the limits of expected and calculable sampling errors) the same gene frequencies at each gene locus. Clearly, samples of different morphs that are conspecific should be freely interbreeding and therefore be different only at the loci coding for their morphological difference and perhaps some closely linked loci. Even if these morphs were subject to some sort of assortative mating or strong selection pressure, the gene frequencies over all the other gene loci should, because of recombination, remain similar (Wright 1978).

Main assumptions are that gene frequencies are not altered by post-zygotic selection or by individuals with different gene frequencies migrating in from elsewhere. Given these assumptions significant variation at any locus, between two sympatric populations, demonstrates a barrier to gene flow and at least partial reproductive isolation. In sexually reproductive routinely outbreeding organisms this indicates the likelihood that two populations should be regarded as different species.

Available evidence suggests that selection is unlikely to cause major problems. Although many workers over the last 20 years or more have devoted a great deal of time and effort in trying to demonstrate the effects of natural selection on the frequencies of electrophoretically detectable alleles at enzyme loci, it is far from clear that these alleles are under natural conditions commonly influenced by selective forces. The extensive literature covering the putative effects of selection on allozyme loci (reviewed by, for example, Nei 1987) indicates that resultant differences are rarely, if ever, marked or even unambiguously detectable. It is thus improbable that any observed level of genetic differentiation of sympatric morphs will result from selection alone.

Migration of individuals to give mixed gene pools is generally more of a theoretical than a practical problem. Allopatric populations if reproductively isolated may be expected to diverge genetically over a period of time and eventually to accrue clear differences in gene frequencies at various loci. However, an almost inevitable feature of any model of allopatric genetic divergence is that (except with most improbably strong selective pressure) almost any degree of migration (as low as one individual per generation, irrespective of population size; Wright 1978; Nei 1987; Maynard Smith 1989) or gene flow between populations will preclude divergence. Herein lies the problem for the idea of the possible mixing of genetically diverged populations, because if they are close enough for individuals to coexist anywhere within their dispersive range, it would be extremely unlikely that at least a minimal amount of gene flow could not occur between them and thus genetic divergence would be most unlikely to have occurred in the first place (but see Palumbi 1992). This point is made by Todd *et al.* (1991) in relation to possible heterogeneous recruitment in the tropical opisthobranch mollusc *Stylocheilus longicaudus*.

It should always be borne in mind that allozyme data cannot prove that two morphs, subspecies, stocks or whatever are conspecific; it can only be concluded that no significant differences could be found. It is always a possibility, albeit unlikely in many cases, that small but genuine differences are concealed by sampling error or that differentiation may be present at loci which have not been examined.

Allozyme comparisons of sympatric species

Examples of real data to illustrate the sorts of differences that can be found between sympatric morphs are shown in Table I. The data are for two sympatric colour morphs (green and violet) of the Mediterranean sponge *Oscarella lobularis* and are taken from Boury-Esnault *et al.* (1992). Some gene loci may show no differences at all and these will usually be loci which are fixed for the same allele in all samples. Such a locus is that called *Me* (the locus coding for malic enzyme) in the two morphs of

Table I. *Allele frequencies for four enzyme loci in sympatric green and violet morphs of the sponge Oscarella cf. lobularis.* N= *number of sponges analysed for each locus. Data are from Boury-Esnault et al.* (1992)

Locus	Allele	Green	Violet
Lap	1	0.042	0.000
	2	0.875	1.000
	3	0.083	0.000
Me	1	1.000	1.000
Mpi	1	0.000	1.000
	2	1.000	0.000
	3	0.000	0.000
Pep.2	1	0.000	0.900
	2	0.083	0.100
	3	0.917	0.000
N		12	5

Oscarella. Clearly this shows no differences between the morphs, but neither does it demonstrate that they are the same species since (as discussed below), between related species gene frequencies at some loci are expected to be identical or very similar.

Another locus, *Lap* (Leucine aminopeptidase), shows small differences between the samples of the two colour morphs; alleles 1 and 3 occur in the green but not in the violet morph. However, the data show that allele 1 occurred only once and allele 3 twice in the sample of 12 green sponges, whilst the common allele, allele 2, which was the only one found in the violet morph, occurred 21 times in the green morph (since the animals are diploid the number of alleles is twice the number of animals in the sample). The numbers of each allele found in each sample can be compared statistically and a probability calculated of obtaining the observed allele frequencies if the two samples were selected at random from a single freely interbreeding population. In this case whatever test is applied (for a brief discussion of suitable tests see below) there is clearly no significant difference at the *Lap* locus between these two sponge samples. Far greater difference is evident at the locus *Pep-2*, where only allele 2, which is at low frequency in each, is common to the two morphs. Here the statistical probability that the two samples are from the same gene pool is extremely small ($P \ll 0.001$).

The fourth locus shown in Table I is *Mpi* (Mannose phosphate isomerase), which shows a complete difference between the green and violet sponges sampled. Here statistical differences can be calculated by the usual range of tests, but in this special case where no allele is common to both morphs the probability can be calculated more simply (Carter & Thorpe 1981; Solé-Cava *et al.* 1985). In this case the sample sizes of the two colour morphs are 12 and 5 sponges, respectively. Since allele 1 has not been found in any of the 12 samples (24 alleles) of the green morph its empirical frequency in this morph is less than 1 in 24. Therefore, if from the same gene pool the chance of a violet sponge having both its alleles different is:

$$P < (1/24)^2.$$

The chances of all five violet sponges having different alleles from the green ones are given by:

$$P < \{(1/24)^2\}^5.$$

Hence

$$P < (1/24)^{10}$$

$$P < 2 \times 10^{-14}.$$

This last example also illustrates another point—the exceptional power of electrophoretic data to differentiate between populations, even on small sample sizes. The method used above to estimate probabilities for fixed allelic differences can be generalized with the formula:

$$P < (1/2N_1)^{2N_2}$$

where N_1 and N_2 are the numbers of animals in each sample. From this it can be seen that even very small samples can give highly significant differences. For example, a fixed allelic difference on samples of just three of each of two morphs will give:

$$P < (1/6)^6$$

$$P < 2.2 \times 10^{-5}.$$

There are many statistical tests for estimating probabilities of samples being from a single gene pool. Chi-square (X^2) tests, of which there are several variants ('correction factors') are commonly used, but most versions have restrictions as to minimum permissible sample sizes. An arguably preferable alternative, but more difficult to use and also subject to restrictions on sample size, is a *G* test (Sokal & Rohlf 1981). For small sample sizes, Fisher's Exact Test is often preferable, but is best calculated by computer. Frequencies of single alleles can often be compared using a Binomial Test which, like Fisher's Exact, gives an exact mathematical probability. The relative merits of some of these tests can be tested empirically (e.g. Todd *et al.* 1991). As an alternative to these conventional methods, Zaykin & Pudovkin (1993) advocate randomization tests as a different approach where suitable computational facilities are available.

This *Mpi* locus in *Oscarella lobularis* is what has been called, in the terminology of Ayala (1983), a diagnostic locus. Ayala (1983) uses two definitions of diagnostic, which are that any given individual can, on the basis of genotype at that locus, be assigned to one or other gene pool (i.e. species) with a certainty of either 99 or 99.9%. Clearly on the basis of *Mpi* genotypes these sponges can be differentiated to a much greater certainty than either of these criteria. Using the less stringent criterion, the *Pep*-2 locus, which also shows significant differences between these sponge colour morphs, can be described as diagnostic, because the probability of sampling an individual with an ambiguous gentoype is less than 1% (but above 0.1%). Methods for calculating the level at which loci are diagnostic are fairly simple and are explained in detail by Ayala (1983); see also Hillis & Moritz (1990).

Studies of enzymic loci have been used to discover or confirm the existence of cryptic or dubious species among sympatric morphotypes in a wide variety of organisms, many of them invertebrates. In many of these cases clear-cut diagnostic loci have been found (e.g. Grassle & Grassle 1976; Thorpe *et al.* 1978*a*; Haylor *et al.* 1984; Matsuoka & Hatanaka 1991; Solé-Cava *et al.* 1991*a*).

Table II. *Examples of studies using enzyme electrophoresis to examine cryptic speciation or systematic divergence in various invertebrate groups. Studies on parasites (mostly in specialist medical or veterinary journals) are omitted (reviews by Walliker, 1983; Miles 1983) and only a few examples are given of the vast literature on insects*

- Protists
  - Sonneborn 1975; Borden *et al.* 1977; Schlegel *et al.* 1988; Motta *et al.* 1991
- Sponges
  - Solé-Cava & Thorpe 1986; Stoddart 1990; Boury-Esnault *et al.* 1992; Solé-Cava *et al.*, 1990; 1991*a*, 1992.
- Cnidarians
  - Hydroids
    - Östman 1982; Thorpe *et al.* 1991
  - Sea anemonies
    - McCommas & Lester 1980; Carter & Thorpe 1981; Bucklin & Hedgecock 1982; McCommas 1982*a*, *b*; Haylor *et al.* 1984; Solé-Cava *et al.* 1985, 1994; Shaw *et al.* 1987; Solé-Cava & Thorpe 1987*b*, 1992; Shaw 1989; Russo *et al.* 1994
- Nematodes
  - Riley *et al.* 1988
- Nemerteans
  - Cantell & Gidholme 1977; Williams *et al.* 1983; Sundberg & Janson 1988; Rogers *et al.* 1992.
- Bryozoans
  - Gymnolaemates
    - Thorpe *et al.* 1978*a*, *b*, *c*, Maturo & Thorpe 1979; Thorpe & Ryland 1979
  - Phylactolaemates
    - Mundy & Thorpe 1979, 1980; Thorpe & Mundy 1980
- Echinoderms
  - Echinoids
    - Marcus 1977; Lessios 1979, 1981; Matsuoka 1989; Matsuoka & Suzuki 1989; Matsuoka *et al.* 1989; Matsuoka & Hatanaka 1991; Matsuoka & Nakamura 1991
  - Asteroids
    - Schopf & Murphy 1973; Kwast *et al.* 1980; Matsuoka 1981
- Polychaetes
  - Grasslé & Grasslé 1976; Nicklas & Hoffman 1979; Guerin & Kerambrun, 1983; Autem *et al.* 1985
- Arthropods
  - Arachnids
    - Lamy *et al.* 1970; Goyffon & Lamy 1973
  - Pycnogonids
    - King *et al.* 1986
  - Insects
    - Lepidoptera
      - Geiger & Scholl 1985
    - Diptera
      - Bullini 1983; Feder *et al.* 1989
    - *Drosophila*
      - Ayala *et al.* 1974; Lakovaara *et al.* 1976; Tsakas & Tsacas 1984
    - Ephemeroptera
      - Zurwerra *et al.* 1986; Hefti & Tomka 1989
    - Hymenoptera
      - Snyder 1977; Ward 1980; Halliday 1981; Heinze 1989
    - Homoptera
      - Guttman *et al.* 1981; Guldemond & Eggersschumaker 1989
    - Orthoptera
      - Harrison 1979; Sbordoni *et al.* 1981
    - Mallophaga
      - Nadler & Hafner 1989
    - Coleoptera
      - Krysan *et al.* 1989
    - Cheleutoptera
      - Bullini 1983
  - Crustaceans
    - Decapods
      - Muley & Latter 1980; Turner & Lyerla 1980; Sin & Jones 1983; Bert 1986; Knowlton 1986; Weber & Galleguillos 1991
    - Isopods
      - Steiner *et al.* 1977; Cariou 1981; Laulier 1989
    - Gammarids
      - Bulnheim & Scholl 1980, 1981; Bulnheim 1984; Scheepmaker 1988
    - Mysids
      - Vainola 1986; Minokawa *et al.* 1992
    - *Artemia*
      - Beardmore & Abreu-Grobois 1983; Abreu-Grobois & Beardmore 1991
    - Barnacles
      - Dando & Southward 1979; Flowerdew 1984, 1985.
    - Cladocerans
      - Benzie 1986
- Molluscs
  - Bivalves
    - Oysters
      - Buroker *et al.* 1979; Hedgecock & Okazaki 1984; Blanc *et al.* 1986
    - *Mytilus*
      - Skibinski *et al.* 1978, 1980; Johannesson *et al.* 1990
    - Pectinnids
      - Beaumond & Beveridge 1982; Macleod *et al.* 1985
    - Unionids
      - Davis 1983
  - Gastropods
    - Limpets
      - Berger 1977; Badino & Sella 1980; Hoagland 1984; Corté-Real *et al.* 1993
    - Abelonès
      - Fujino *et al.* 1980; Sasaki *et al.* 1980
    - Littorinids
      - Snyder & Gooch 1973; Moyse *et al.* 1982; Janson & Ward 1984; Johannesson & Johannesson 1990; Sundberg *et al.* 1990
    - *Hydrobia*
      - Davis *et al.* 1988
    - Pulmonates
      - Evans 1985; Emberton 1988
    - Opisthobranchs
      - Havenhand *et al.* 1986; Todd *et al.* 1991; Morrow *et al.* 1992
  - Cephalopods
    - Smith *et al.* 1981; Thorpe *et al.* 1986; Augustyn & Grant 1988; Levy *et al.* 1988; Brierley *et al.* 1993
- Ascidians
  - Aron & Solé-Cava 1991
  - Aron & Solé-Cava 1991

Examples of electrophoretic studies covering a range of invertebrate phyla are tabulated in Table II. There are also a few examples where electrophoretic data have been used to suppress nominate 'species' lacking a genetic basis (e.g. Maturo & Thorpe 1979; Graves & Rosenblatt 1980; Pierce *et al.* 1981; Moyse *et al.* 1982). Also omitted from Table II are many of the numerous publications on *Drosophila* spp. and the very large applied literature from work in parasitology and medical and veterinary entomology.

A potential area of suspicion for conventional taxonomists is the increasing number of new species being revealed by molecular studies. As many papers in molecular systematics report the finding of sibling species, it could be assumed that either molecular systematists are radical splitters, or, conversely, that classical taxonomy is over-conservative. Clearly, simply because of its resolving power, molecular taxonomy will always be likely to find cryptic species. Also, the nature of the data makes it difficult to conclude that two entities are the same (rather than merely not different), whereas differences are usually definitive. On the other hand, conventional systematics is heavily dependent on the number of characters to be used (to fully resolve a tree with n species, at least n-1 phylogenetically informative characters are needed). Many (mainly marine) lower invertebrates are morphologically very simple and often lack structures which can be readily quantified (counted or measured), so that the

number of morphological characters available for the classification of such species sometimes is simply inadequate (see Solé-Cava & Thorpe 1991*a*). Typological thinking also seems to persist in the minds of many taxonomists, with the result that much of the variation observed in many species is discarded as 'intraspecific polymorphism, with no taxonomic meaning'. There is, however, a third reason for the bias towards splitting in the literature on molecular systematics, which is the difficulty of publishing negative results. Molecular systematics can be used to test hypotheses generated by taxonomists, for example a debate as to whether taxa well-defined morphologically belong to the same or to different species. In these cases, whatever the conclusion of the work, it will have consequences for the taxonomy of the group, and, therefore, it is likely to be published. However, molecular systematics often involves the testing of hypotheses about the taxonomic status of 'morphs' detected through ecological work and field observation. These morphs would often be considered by classical taxonomists as part of the range of phenotypic variability of the species. In such cases, if clear genetic differences are found, the work has obvious interest for publication. However, if, after genetic analysis, no differences are found, the molecular systematist is left with the conclusion that classical taxonomy was right, and it becomes difficult to convince the editors that the work should be published. Hence negative results do not get published, and this is likely to bias the overall publication profile of molecular systematics. It may also lead to some duplication of effort by workers unaware that previous research found two morphs considered conspecific to be so.

Allopatric species

When populations are geographically separated there will often be little or no gene flow between them and consequently, even if they are of the same species, some genetic differentiation is to be expected. It should also be noted that even if populations were genetically identical small differences in gene frequency are likely to be observed because of sampling error; indeed, it would be remarkable if any polymorphic locus showed exactly the same allele frequencies in each of two populations, although of course this does sometimes occur by chance.

Therefore, if two morphs which may or may not be conspecific are collected allopatrically a test of whether or not they are from the same gene pool (within the limits of sampling error) will not be very informative because, of course, they are not necessarily expected to be from the same gene pool, even though they may be from the same nominate species. Hence the criterion which must be applied if electrophoretic data are to be used in such a situation is to assess the level of differentiation found between the morphs or populations.

When allozyme data started to be used for inter and intraspecific population comparisons, the main methodology employed in systematics was phenetic. The phenetic-ists' approach was to try to reduce all data into some form of 'overall similarity' between population pairs. Similarity matrices were then used to build dendrograms [i.e. trees that were intended to represent graphically the relationships between the taxa (or Operational Taxonomic Units)]. Thus it is not surprising that at an early stage molecular taxonomists derived various phenetic indices or 'genetic distance' measures from allozyme data (Nei 1972; Rogers 1972; Wright 1978; Thorpe 1979). The use of these indices quickly became a standard routine in molecular systematic work, and their use was aided by the appearance of computer programmes (e.g. Green 1979; Swofford & Selander 1981), which greatly simplified the production of trees from genotype frequency data.

Several statistically based measures of genetic similarity or genetic identity (measures of similarity) or of genetic distance (measures of dissimilarity) are available and may be used to reduce to a single figure levels of genetic differentiation between two gene pools (species or populations) over a range of enzyme loci (Cavalli-Sforza 1969; Workman & Niswander 1970; Hedrick 1971; Kimura & Ohta 1971; Latter 1972; Nei 1971, 1972, 1973, 1978; Nei & Chakraborty 1973; Rogers 1972; Thorpe 1979, 1982). These measures are mostly estimated from gene frequency data, although that of Hedrick (1971) is derived from genotype frequencies. All are time-consuming to calculate without suitable programmes and only those of Rogers (1972) and Nei (1972, 1978) have been extensively used. Nei's measures are now those in general use.

Nei's (1972) genetic identity (I) for a single locus is given by:

$$I = jXY/\sqrt{(jX \cdot jY)}$$

where

$$jXY = \Sigma x_i y_i;$$

$$jX = \Sigma x_i^2 \quad \text{and}$$

$$jY = \Sigma y_i^2;$$

and x_i and y_i are the frequencies of the ith allele in populations X and Y, respectively. Over all loci the genetic identity, I is given by:

$$JXY/\sqrt{(JX \cdot JY)}$$

where JX, JY and JXY are the arithmetic means over all loci of jX, jY and jXY, respectively. Nei's genetic distance, D is given by:

$$D = -\log_e I.$$

I is on a scale of 0–1 and D is from 0 to infinity. Nei's genetic distance, D, is claimed to estimate the mean number of (electrophoretically detectable) substitutions per locus and, given certain assumptions, to be linearly proportional to evolutionary time.

For taxonomic use it is desirable to be able to place standard deviations or some other estimate of errors upon figures for genetic distance, since it may be important to be able to assess whether species relationships differ significantly. The sampling and other errors of all such measures are likely to be large (Nei & Roychoudhury 1974; Thorpe 1979, 1982; Nei 1987), but Nei's is the only measure for which detailed methods for the estimation of sampling and other errors are available (Nei & Roychoudhury 1974; Li & Nei 1975; Mitra 1975, 1976; Nei 1978, 1987).

The estimation of errors of Nei's *I* or *D* by published methods is complex and in practice is best carried out by computer. Other minor problems with Nei's measure are that methods are based on the assumption that populations are routinely outbreeding and in Hardy–Weinberg equilibrium. As Nei's *D* does not have the property of triangle inequality, it is not a metric, and this may cause potential difficulties if it is used in some tree-building algorithms (Swofford & Selander 1981; Rogers 1986).

If enzyme differentiation can be used to estimate genetic divergence between populations or species there should, within wide limits, be a general relationship between taxonomic separation and genetic divergence as estimated by Nei's or other measures. Several authors (e.g. Avise 1974; Thorpe 1979, 1982, 1983; Nei 1987) have suggested a broad relationship between the two, and it has been proposed (Thorpe 1979, 1982, 1983; Nei 1987) that protein variation between populations could be used as a guide to their taxonomic relationships. Such data could be used when, for example, it was unclear whether two allopatric populations should be regarded as conspecific (Solé-Cava *et al.* 1991), or to help solve disputes over whether two species should be considered to be congeneric.

There is a large volume of published information on enzyme differentiation between conspecific populations and congeneric species from a wide range of taxa (reviewed by Thorpe 1979, 1982, 1983). Although much of this work is on various classes of vertebrates or *Drosophila*, there are also various studies on other invertebrates and on plants. Intergeneric genetic distance data are relatively scarce and there are very few estimates for invertebrates (e.g. Skibinski *et al.* 1980; Havenhand *et al.* 1986; Matsuoka 1989; Matsuoka & Suzuki 1989; Matsuoka & Nakamura 1991; Solé-Cava *et al.* 1992, 1994; Brierley *et al.* 1993).

In general, allopatric conspecific populations tend to have mainly relatively small allele frequency differences at a few loci only. Congeneric species are often identical at some (usually monomorphic) loci, but completely different at others and have a few loci with overlap of gene frequencies. Between species in different genera most loci are usually entirely different (i.e. fixed for different alleles), but some may be identical. For single loci, whether between populations, between congeneric species or between species in different genera, *I* values tend to be at or very close to 1 or 0 with proportionately very few in between. This results in a 'U'-shaped frequency distribution for single locus *I* values (Avise 1983; Ayala 1983). Thus for many, particularly interspecific, comparisons, *I* values, taken by locus, tend to be very close to 1 or 0.

It was proposed by Thorpe (1979) that conspecific populations, congeneric species, and species of different genera should on average have been isolated for different lengths of time and therefore have different probabilities of having given values of genetic identity. Different theoretical distribution curves were suggested. Using data from numerous publications giving information on the frequency of different values of Nei's (1972) *I* for different levels of systematic divergence, Thorpe (1982) was able to plot empirical curves for probability against *I* values

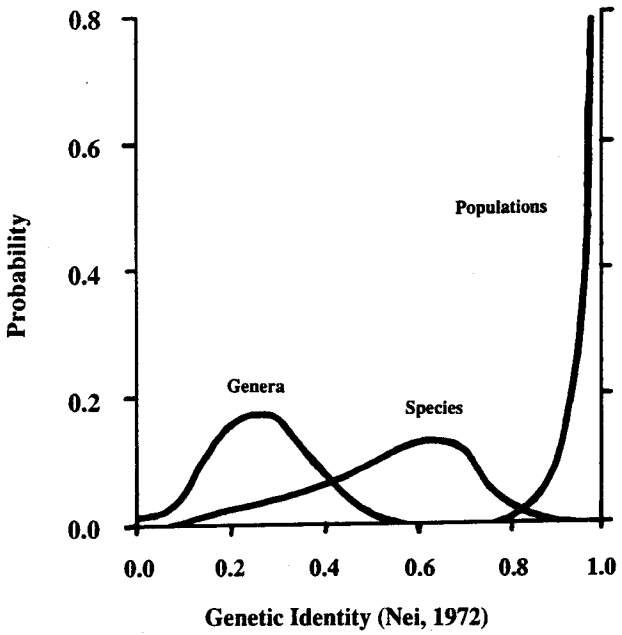

Fig. 1. Empirically derived frequency distributions for Nei's (1972) genetic identity, *I*, for genetic divergence estimates between conspecific populations, congeneric species and confamilial genera. Redrawn and simplified from Thorpe (1982).

between confamilial genera, congeneric species and conspecific populations. His results (shown in Fig. 1) cover data available for vertebrates, invertebrates and plants, but exclude birds, for which few studies were available. These empirical distributions closely approximate those previously suggested on theoretical grounds.

The results shown in Fig. 1 indicate that there is clearly a general relationship between taxonomic divergence and genetic distance. A notable feature of the many studies from which data were gathered is the overall generally similar relationship of *I* to systematic diversity in taxa ranging from cnidarians to mammals and plants. Thorpe (1982) concluded that genetic diversity within a species or genus appeared broadly comparable for most groups of vertebrates (except birds), invertebrates or plants. Predictably, there is considerable overlap of the range of *I* values between the lower part of the range for congeneric species and species of different genera, although that between species and conspecific populations is surprisingly small. Thorpe concluded that the critical level for *I* values distinguishing between species and genera appears to be around 0.35. About 85% of *I* values between congeneric species exceed 0.35 (76% above 0.4 and 90% above 0.3), while between genera 77% fall below 0.35 (87% below 0.4, 93% below 0.45). At the upper end of the range about 97% of *I* values between species are below 0.85 (95% below 0.8), while within species 98% exceed 0.85 (93% above 0.9). About 80% of conspecific *I* values are above 0.95.

Thorpe (1983) compared ranges of *I* values at different levels of taxonomic divergence for species from a range of major taxa (mammals, birds, reptiles, amphibians, fish, invertebrates and plants—this selection of groups being mainly governed by the availability of data rather than systematic criteria). He concluded that over all these major taxa, except for birds, the ranges of *I* values

encompassed within a species or a genus were roughly similar within broad limits.

Thus, as previously discussed by Thorpe (1982, 1983; Nei 1987), it appears that *I* values can be taxonomically useful. If allopatric populations of uncertain status have genetic identities below about 0.85 it is improbable that they should be considered conspecific, while nominate species with *I* values above about 0.85 should be considered doubtful if there is no other evidence of their specific status. As Thorpe (1982) pointed out, 'there is a need to define species for ecological as well as systematic purposes; and for allopatric populations where status cannot be otherwise resolved, an estimate of genetic distance is probably the best criterion'. Nei (1987) reached a similar conclusion. However, it should be noted that *I* values are of limited relevance to taxonomic problems among sympatric populations, where gene frequency data have far greater resolving power. If gene pools are sympatric, significant differences should not be disregarded even if *I* values are unusually high (e.g. Avise & Ayala 1976; Solé-Cava *et al.* 1985). Whether genera should be defined by genetic identity values, or even whether it is generally desirable for genera to encompass roughly similar amounts of genetic diversity, is a matter for taxonomic debate (see Van Valen 1973). However, the possibility remains that, where morphological criteria provide no clear-cut evidence of the validity of a genus, genetic distance estimates could provide valuable and objective data (Thorpe 1982; Nei 1987).

Phylogenetic analysis

Genetic distance data are, of course, mainly used to estimate phylogenetic relationships between the various species studied. Numerous tree-building techniques are available (discussed by, for example, Avise, 1983; Hillis & Mortiz 1990; Miyamoto & Cracraft 1991; Allard & Miyamoto 1992; Kim 1993; Huelsenbeck & Hillis 1993), but the most frequently used method is unweighted pair group mean analysis (UPGMA), a procedure first detailed by Sokal & Sneath (1963). Such procedures can be incorporated into computer programmes for the calculation of genetic distance (e.g. Swofford & Selander 1981). The usual method is to construct a phenetic dendrogram by starting with the most genetically similar pair of populations or species and then work outwards. The main alternative methods are probably the distance-Wagner (Farris 1970; Swofford 1981), the Fitch–Margoliash (Fitch & Margoliash 1970) and the neighbor-joining (Saitou & Nei 1987) procedures, all of which are computationally more demanding. Alternative methodologies can often give dendrograms differing in detail or even substantially (Sundberg *et al.* 1990; Allard & Miyamoto 1992; Huelsenbeck & Hillis 1993). The distance-Wagner (Farris 1970; Swofford 1981) method is probably particularly unsuitable for use on genetic distance data since it is considered to be very susceptible to problems with measures which do not obey triangle inequality (Berlocher 1981), as is the case with Nei's *D*.

A particular problem with such phenetic approaches to estimating systematic relationships is that they depend on the assumption of similar divergence rates between branches of the 'tree' (Heulsenbeck & Hillis 1993). If one of the branches diverges much more quickly than the others, its overall distance from them will be so high that will invariably lead it to an external branch. For example (Fig. 2), suppose that from ancestral *a* two lineages were produced (*b* and *c*), that diverged from each other by two evolutionary steps (fixed for different alleles at 2 out of 20 loci studied; for this example it will be considered that the other loci were monomorphic and identical). Ancestral *b* then originated taxa *d* and *e*. Taxon *d* evolved with the same substitution rate as the others, and present one further evolutionary step from *b*. Taxon *e*, however, evolved much faster, and had changes at three other loci. A phenetic approach, using Nei's *I*, for example, would find that *d* was more related to *c* ($I = 17/20 = 0.85$) than to *e* ($I = 16/20 = 0.80$). The eventual UPGMA dendrogram produced from this hypothetical data set would wrongly cluster first the taxa *d* and *c*, and then those to *e*.

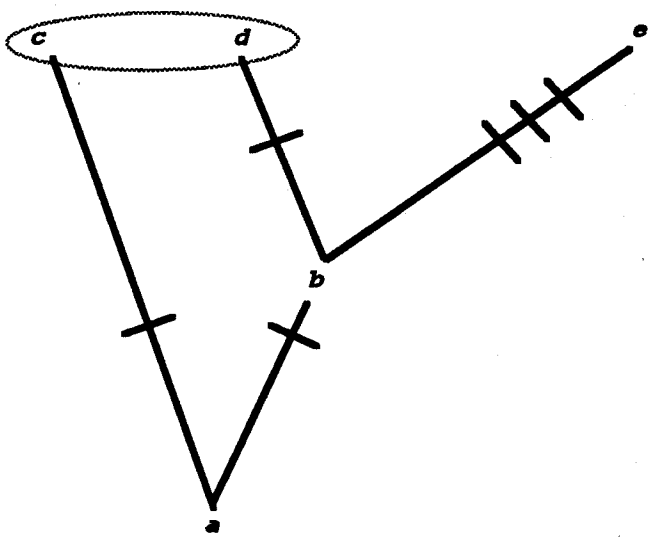

Fig. 2. Hypothetical relationship between species *c*, *d* and *e*. Marked along the branches are the derived states for each lineage. The circle around species *c* and *d* indicates their higher phenetic similarity.

A solution to this type of problem is offered by the technique of cladistic analysis, which is intended to discriminate between taxonomically informative and uninformative characters, using the concept of primitive (or plesiomorphic) and derived (or apomorphic) characters (Hennig 1966). For cladists, characters that have evolved prior to the separation of the studied group (plesiomorphies), or that are specific only to terminal nodes (autapomorphies), are of little phylogenetic interest. Characters are polarised by the use of some species that clearly do not belong to the group that is being studied. These belong to what is termed an 'outgroup', as opposed to the group of species studied which constitutes the 'ingroup'. Ideally only unique shared–derived characters (synapomorphies) should be used to produce phylogenetic hypotheses, and phylogenetic trees (or cladograms) built from different characters should be congruent. Naturally such idealised situations are rarely found, and the use of different characters often results in the production of conflicting trees. Cladists then need some means of choosing the 'best' trees, and usually the criterion used is that of parsimony. This assumes that the trees necessitating the

least number of evolutionary steps are considered closest to the true phylogeny of the group.

Cladistic analysis of conventional (morphological) characters has been used with much success in many taxonomic groups (reviewed by, for example, Wiley 1981), but the cladistic analysis of allozymes for phylogenetic purposes is more recent (reviewed by, for example, Patton & Avise 1983; Buth 1984; Hillis & Moritz 1990; Swofford & Olson 1990).

Further possibilities for electrophoresis in taxonomy and systematics

Enzyme electrophoresis has now been used as an aid to taxonomy and systematics for a number of years, but its use has been essentially limited to the two main areas discussed above; distinguishing or confirming (usually sympatric) species, or measuring divergence between populations at any level from within species to between species from related genera. The estimation of genetic divergence between species at higher levels of systematic divergence has rarely been attempted because it has been assumed generally (e.g. Thorpe 1982) that above the level of related genera any apparent genetic similarity detected is likely to be coincidental. In many taxa this may be true, but a few more recent studies indicate that in some cases useful results can be obtained for comparisons between species from related families. Solé-Cava *et al.* (1994) used electrophoretic data on genetic diversity in the sea anemone family Actiniidae to assess the feasibility of performing cladistic analyses on data of this type. They used *Metridium senile* (family Metridiidae) as an out group and found, surprisingly, that this gave useful comparisons with species from the other family. Solé-Cava *et al.* (1992) also found measurable genetic similarity between species of the sponge families Plakinidae and Oscarellidae, but in this case the data cast grave doubts on the systematic status of the two families.

Another area where isozymes probably have a great role to play is in the identification of the different life cycle stages in various invertebrate species. For example, many marine invertebrates, particularly sessile species, have pelagic larvae which are thought to be very important for distribution and other purposes (Strathmann 1980, 1985; Todd 1985). There are a surprising number of larvae described from plankton samples where only a higher taxon can be identified and the precise identity of the parent species is unknown. There are also several groups of related species (e.g. patellid limpets) in which the larvae are considered inseparable. In such cases, if the technical difficulties of electrophoretic procedures for samples as small as a larva can be overcome, it should be possible to compare genes present in the larvae with those of adults of various species to establish the likely specific identity of the larva. This approach has been used recently by Hu *et al.* (1992) to separate larvae of three oyster species. To carry out such work, methods suitable for the analysis of very small quantities of material are necessary (see Easteal & Boussy 1987); to date, cellulose acetate gels, very thin layer starch gels and polyacrylamide gels have been used. The former are routinely used for very small organisms and have proved successful for analysis of the larvae of the barnacles *Chthamalus* (Dando 1987) and the ascidians *Botryllus* (Grossberg 1991), and *Dendrodoa* (J. D. D. Bishop pers. comm., 1993), although these studies were not for taxonomic purposes. The use of electrophoresis to identify larvae could save lengthy rearing studies and in some cases (e.g. many crab species) the rearing of larvae through to the adult stage is not a practicable proposition.

Molecular clocks

It is apparent that the level of difference between molecules can provide an indication of the level of genetic or systematic differentiation between populations. The proposal that the divergence of protein molecules is time-dependent and can be related to evolutionary time forms the basis of what has come to be known as the molecular clock hypothesis (see e.g. Wilson *et al.* 1977; Carlson *et al.* 1978; Thorpe 1982; Nei 1987; Hillis & Moritz 1990).

This hypothesis does not enjoy universal support (see Lessios 1979, 1981; Korey 1981; Li & Gojobori 1983; Gillespie 1984, 1986), and the accuracy and usefulness of such clocks are disputed (Smith & Coss 1984; Busack 1986), but there are now substantial data providing strong evidence that, following the genetic isolation of populations, the molecular structures of homologous protein molecules begin to diverge and continue to do so (see, for example, Wilson *et al.* 1977; Carlson *et al.* 1978; Vawter *et al.* 1980; Nei 1987). Such an idea is not new (Zuckerkandl & Pauling 1962). In its simplest form, the clock hypothesis predicts that the probability of the occurrence of an amino acid substitution in a given protein molecule is constant and thus the substitution is an approximately regular, but stochastic, process. From this it follows that the number of substitutions occurring between homologous proteins may be related to evolutionary time. Clearly such a clock can at best be stochastic since it is dependent upon the probability of a substitution being at least approximately constant and thus the accumulated number of substitutions being roughly proportional to time. It cannot therefore be regarded as a true clock with substitutions taking place at regular time intervals. Although the suggestion of random substitution is a consequence of the concept of selective neutrality, the existence of a molecular clock does not necessitate the assumption that all substitutions are selectively neutral (Fitch & Langley 1976; Sarich & Cronin 1976; Zuckerkandl 1976; Nei 1987).

For electrophoretic data the basis of the molecular clock is Nei's (1972) genetic distance measure, D, which, as mentioned above, is considered to be directly related to evolutionary time. While there is no doubt that a relationship of genetic divergence to evolutionary time makes genetic distance analysis a useful method in systematics, there are difficulties in using such data as an indication of taxonomic status. Taxonomic problems include a few instances of accelerated speciation. These have occurred very rapidly (possible as a result of great selective pressure) while the short time has permitted little biochemical evolution. Several studies provide support for the genetic distance clock with evidence (usually geological) for

recent speciation between species with high *I* values (e.g. Avise *et al.* 1975; Gorman *et al.* 1976; Gartside *et al.* 1977; Turner *et al.* 1979). There is also support for the clock from taxa where chromosomal changes may have caused rapid speciation (Nevo & Shaw 1972; Nevo *et al.* 1974). Similar problems appear in studies on passerine birds where most *I* values between species fall within the range typical of conspecific populations in other taxa. It would appear that in birds speciation and morphological differentiation occur at radically faster rates than in other taxa (Avise *et al.* 1980; Nei 1987). An alternative explanation is that the genetic distance molecular clock runs at a much reduced rate in passerine birds, but this is improbable since albumin and transferrin immunological distance clocks also suggest much accelerated speciation (Prager *et al.* 1974; Thorpe 1982). Thus, although not conforming to the norms of other groups, passerine bird data may be argued to lend support to an electrophoretic clock. As would be expected, in the now very large literature on genetic divergence there are various examples of unexpected results from genetic distance studies in systematics, but many of these cast doubt on the taxonomy rather than the molecular clock.

Other problems with the systematic uses of clocks mainly concern the shortcomings of electrophoresis itself. Clearly, distance data between taxa are only comparable if the techniques used are similar. In general, only a low proportion of allelic variation may be detectable by standard electrophoresis (perhaps 20–30%; Lewontin 1974; Nei 1987). However, different techniques, mainly using many gel and buffer combinations, heat denaturation or two-dimensional electrophoresis, can radically alter results, particularly estimates of numbers of alleles at some loci (e.g. Coyne 1976; Gill 1978; Sing 1979; Racine & Langley 1980*a*, *b*; Aquadro & Avise 1982; Ohnishi *et al.* 1983; Goldman *et al.* 1986).

Another potential problem is that substitution rates vary between loci. It has been known for some time that levels of polymorphism can differ among protein loci (Harris *et al.* 1977; Ward 1977, 1978; Koehn & Eanes 1978; Solé-Cava & Thorpe 1989; Da Silva *et al.* 1992). These differences can be linked to subunit size (Eanes & Koehn 1978; Ward 1977) and number (Harris *et al.* 1977; Ward 1977; Solé-Cava & Thorpe 1989), subcellular location of the protein (King & Wilson 1975; Wilson *et al.* 1977; Gottlieb & Weeden 1981), and locus duplication (Ferris & Whitt 1980). Heterozygosity can also be related to *I* values and therefore to rates of substitution (Skibinski & Ward 1981; Ward & Skibinski 1985). Sarich (1977) proposed that protein loci could be split into 'fast' and 'slow' evolving groups, which differ greatly in substitution rates. If this was so, differences in calibrations of *D* could be expected to result largely from different proportions of fast and slow loci (Sarich 1977; Maxson & Maxson 1979; Wyles & Gorman 1980). Empirical data (Skibinski & Ward 1981, 1982, 1983; Ward & Skibinski 1985) indicate that proteins differ widely in their mean rates of electrophoretic divergence, but, as pointed out by Skibinski & Ward (1981) and Thorpe (1982), this variation is not bimodal as would be predicted from the 'fast' and 'slow' locus hypothesis of Sarich (1977), but appears to approximate a normal distribution.

More recently, Thorpe (1989) extrapolated from the data of Skibinski & Ward to examine some possible theoretical effects of the observed empirical variation in rates of electrophoretic divergence of different proteins on the putative linear relationship of Nei's (1972) Genetic Distance measure, *D*, with time since evolutionary divergence. He concluded that, although very high *D* values can be expected to deviate significantly from stochastic linearity with time, for the sort of time scale over which *D* values are normally estimated deviation is slight and likely to be insignificant when compared to other sources of error. Thus from this study it appears that for most practical purposes interprotein differences in mean rate of amino acid substitution need not be taken into account when calibrating genetic distance estimates against evolutionary time.

Another reason to doubt the linearity of higher values of Nei's *D* is that the measure appears to suffer from a 'saturation' effect (King 1973; Maxson & Maxson 1979). Problems are (a) that a maximum of one substitution per locus can be detected, and (b) that a finite electrophoretic similarity may be detected; even between quite unrelated species as a result of the limited number of bands that can be resolved by electrophoresis. It has been suggested that although there may be no similarities in molecular structure, coincidental genetic identity may be expected at about 10% of loci (Maxson & Maxson 1979). Using a total of about 100 loci between various species pairs from different major taxa, Thorpe (1982) found only about 5% (presumably coincidental) identity. This suggests a limiting value on Nei's *D* of about 3 rather than infinity, although this could be corrected by using (for example) $D = -\log_e[I + 0.051(1 - I)]$ for the calibration of higher values of *D*.

The putative stochastic linearity with evolutionary time of Nei's (1972, 1978) genetic distance measure, *D*, has been supported by many authors (reviewed by Nei 1987) and there is also evidence from several taxa of good correlations between genetic distance and other molecular clocks (e.g. albumin immunological distance, DNA hybridisation or sequencing; Sarich 1977; Highton & Larson 1979; O'Brien *et al.* 1985; Nei 1987; Stepien *et al.* 1993). For various groups approximate calibrations have been made of genetic distance against estimated divergence time. The precise calibration of *D* values is debated and these may be best calibrated separately for each taxon. Calibrations vary and are discussed elsewhere (Thorpe 1982, Nei 1987), but generally give a result that one *D* unit indicates several million years of evolutionary time. Overall it is doubtful whether the genetic distance molecular clock is precise, and probably, as Nei (1987) has suggested, '(the clock) is quite 'sloppy' but useful for obtaining a rough idea of evolutionary time when fossil records are absent or unreliable'. Unfortunately, few invertebrate taxa have good fossil records.

For most taxonomic purposes the possibility of Nei's *D* being nonlinear with time is unimportant since only relative values are needed. As long as the 'mix' of loci contributing to *I* or *D* values between taxa is roughly similar, the values should be approximately comparable. However, distance values should only be used in conjunction with conventional methods or where other methods

lead to ambiguous conclusions. There is an unfortunate tendency for (particularly) electrophoretic data to be subjected to far more exact analysis than is justified. The literature concerning methods for the analysis of such data is large and growing rapidly. Most genetic divergence estimates based on practicable numbers of loci will have very large errors and, as pointed out by Thorpe (1982), for many studies of congeneric species, few if any *I* values will differ significantly, and frequently the data could not refute the hypothesis that all the species diverged simultaneously from one common ancestor. Nevertheless, *I* or *D* values are often quoted to spurious degrees of accuracy and systematic distinctions made using differences substantially smaller than the errors.

Conclusions

The genetic interpretation of isozyme patterns has proved to be extremely valuable for systematics (Table II). From the data available, four main conclusions can be drawn:

Scope. Allozymes are at their most powerful in discriminating and identifying sympatric species, since sympatric populations that do not exchange genes are considered different species under any of the current species concepts. Allozyme analysis can also be very useful for comparing allopatric populations, but in this case its power will depend on the species concept used. Under the most commonly used phylogenetic or biological species concepts the discovery of fixed allele differences demonstrate that the populations are probably evolving independently, and therefore warrant specific status. For other species concepts the amount of divergence may have to be taken into account.

Allozyme electrophoresis can also be used to estimate divergence between congeneric species or confamilial genera. It has been suggested that allozyme analysis can be useful to compare groups with divergence times up to roughly 50 million years (Hillis & Moritz 1990).

Limitations. Because most enzymes are quickly degraded after cell death, allozyme analysis depends on the availability of fresh or frozen tissues. This limits the application of the technique to extant species, and cannot be used for the normal taxonomic procedure of comparing individuals with type specimens (usually fixed in ethanol or formalin). Also, electrophoretic data are difficult to compare between laboratories, because of the wide variation in migration rates of the molecules with different buffer systems, in extraction methods and in electrophoretic procedures. This is not a problem for studies conducted completely in one laboratory, since the hypotheses are tested in more or less self-contained projects. However, it is unfortunate that allozyme data for different organisms cannot accumulate with time in the same way as DNA sequence data.

Splitting and lumping. Although there is a definite publication bias in molecular systematics studies towards reporting differentiation (see above), it still remains very clear that the amount of zoological diversity among invertebrates is much higher than the usual estimation from classical taxonomy. In the same way that genetic studies have demonstrated that dispersal of benthic marine invertebrates is often much more limited than that originally predicted from length of larval life (Knowlton & Keller 1986; Todd *et al.* 1988, 1993), in systematics there seems to be a much finer differentiation between species than many taxonomists would be willing to recognise. In fact, since dispersal is related to genetic differentiation (Palumbi 1992), it is possible that the two facts are related, and these findings could indicate a current shift from more conservative typological concepts (in systematics and ecology) to a population approach to ecology and evolution.

Future trends. In spite of the new wave of molecular techniques that are becoming more and more useful, principally for phylogenetic comparisons of higher groups (Hillis & Moritz 1990), allozyme analysis is probably going to remain a basic method for systematics, principally at the species level. It is significantly cheaper than other molecular methods, and is, at the moment much more robust and easier to perform, so that the emphasis of the work can be on the systematic problem itself rather than on the techniques involved. However, a closer collaboration between systematists and geneticists is essential for the success of this field. Population geneticists have the knowledge of the methods to be used and their limitations, and can interpret the evolutionary implications of the results of the genetic analysis. Systematics are much better able to identify the problems which need to be tackled and only they are really able to assess the taxonomic implications of the results.

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