Electrophoretic techniques as a systematic research on Lepidoptera

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Introduction

Species is now often defined as a function of the genetic flow existing between groups of populations. Particularly in the case of 'feeble', allopatric taxa, this formulation, however, can reach a truly operational value only through the determination of the amount of genetic diversity existing between the populations involved. Many of the difficulties for achieving this task have been overcome with the development of electrophoretic techniques. Phenotype, in fact, is many steps apart from the genes and often may represent the action of several of them; proteins, on the contrary, are the main product of gene activity. The study of gene-enzyme systems can obviously match, therefore, the theoretical requirements needed to develop experimental research in this direction. Allozymes, i. e. each of the proteins that are codified by the different alleles of the same locus, have been extensively studied with electrophoretic techniques and are now known to segregate in crosses in a Mendelian manner; electrophoretically detectable enzyme variants are, therefore, strictly correlated to structural variations of the codifying genes.

Literature on this subject is now by far too rich to attempt here a general survey; some extensive reviews have been published, however, by Selander & Johnson (1973) for Vertebrate animals, by Ayala (1975) and by Throckmorton (1977) for *Drosophila,* etc.; the general argument has been treated by Johnson (1973), Gottlieb (1971), Lewontin (1974), Avise (1975), Powell (1975), Selander 1976) etc.

Lepidoptera, as a whole, figure among the less studied groups, by this point of view. The works of Jelnes (1975 a, b), on some *Aricia* sibling species, are, however to be recalled, as well as those of McKechnie et. al. (1975) on Californian *Euphydryas,* of Vawter and Brussard (1975) on *Phyciodes tharros,* of Johnson, & Burns (1966), Burns & Johnson (1967), Johnson (1971, 1976 a, b, c, d, 1977 a) on some species of the genus *Colias,* of Burns & Johnson (1971) on *Hemiargus isola,* of Handford (1973 a, 1973 b) on *Maniola jurtina,* of Lokki et. al. (1975) on *Solenobia,* of Jelnes (1971) on *Ephestia kueniella* of Felnes (1975) on *Thera,* of Eguchi *et al.* (1965), on *Bombyx*

mori, of Bullini et al. 1976) on some lepidopteran species, of Sbordoni et al. (1976) on *Amata* and of Bianco et. al. (1976), on the same genus.

Techniques

The general principle of electrophoretic separation of enzymes is rather simple: proteins, when placed in an electric field, migrate towards one of the poles, with a migration rate which is largely dependent upon the protein's net charge and to a lesser extent on the protein's molecular size und shape. This migration is accomplished in an electrophoretic medium soaked with an ionized buffer solution. The pH of the buffer and its ionic strength are chosen according to the molecular characteristics of the proteins to be studied, as their net charge will obviously depend on the proportion of charged (ionized) amino and carboxyl groups. After a suitable time, proteins will migrate different distances from the deposition point: the higher the charge, the farther a protein will move toward an electrode. The electrophoretic medium being generally represented by a gel (starch gel or acrylamide), whose pore size is rather similar to that of protein molecules, the migration rate (distance) will depend also upon 'molecular sieving'.

As enzymes are not, generally, purifield prior to performing electrophoretic separation, many proteins will migrate simultaneously in one or another direction when electric field is applied. In order to recognize allozymes of a particular protein, highly selective enzyme stainings are therefore employed, in the great majority of cases. This is not, however, the place for minutely describing electrophoretic and staining procedures; very detailed information may be found, for many enzyme systems, in the papers of Brewer (1970), Shaw & Prasad (1970) etc.

Quantification of results

Electrophoretic studies over a range of enzme systems may provide, in some instances, very reliable 'taxonomic characters' at the molecular level; as it was recently shown for *Amata* (Sbordoni et al., 1976), these characters may eventually provide sufficient ground also for the distinction of larval instars of sibling species, or for recognition of hybrid specimens.

In other instances, however, a statistical expression of genetic divergence may be preferable. A larger number of gene-enzyme systems is therefore to be chosen, in order to obtain an unbiased sample of the entire genome of the populations involved.

The analysis of the divergence degree, between closely related taxa, has received considerable attention in very recent years, particularly after the formulation of rather simple quantification methods.

Although a variety of coefficeints of this kind is available in the current literature, Nei's (1972) indexes of genetic identity I and genetic distance D are now among the most employed.

The normalized probability that two alleles of each population X and Y are identical at a given locus K expressed by the relation:

$$I_k = \frac{\Sigma x_i y_i}{(\Sigma x_i^2 \cdot \Sigma y_i^2)^{1/2}}$$

where x_i and y_i are the frequencies of the allele i respectively in population X and Y. This probability ranges, of course, from 1, in the case of identical frequencies of the allele i in the two populations, down to zero if the allele i (or y) is absent in one of the two populations.

The normalized (mean) genetic identity between the populations X and Y with respect to all studied loci is therefore defined as:

$$I = \frac{I_{xy}}{(I_x \cdot I_y)^{1/2}}$$

where Ix, I and Iy are the aritmetic means of Σx_i^2, Σy_i^2 and Σx_i respectively, over all studied loci, including monomorphic ones. The genetic distance between the two populations X and Y is finally defined as:

$$D = -\log_e I$$

The calculation of I and D values over a wide range of animal species (see Nei, 1975) demonstrated that a 50 % genetic distance (D = 0.5) is often representative of a species-rank divergence between two groups of populations. This statement, however, is only to be taken very prudently, because, particularly in some Insects, species level divergence may also imply, besides higher values (D = 0.9 or more), also very low genetic distances (D = 0.2). This fact, moreover, seems rather independent from how distinctive the investigated taxa may be from each other. Besides other considerations (see the next paragraph), a thorough knowledge of genetic-distance values which are to be expected for species or subspecies-rank divergence in the group studied, should therefore precede critical evaluations.

The possibility of calculating the time of divergency of closely related taxa, is also to be considered. Nei (1975) postulated that genetic distance values are linked to time by the relation:

$$t = \frac{D}{2a}$$

where t is the number of elapsed years and is a constant, whose value was assumed to be about 10^{-7}. Mutation (aminoacidic sobstitution) rate, however, may easily vary with time and a good deal of experimental work has also accumulated to demonstrate that evolution, in this respect, is often a rather saltational phenomenon (see Trockmorton, 1977 for *Drosophila).* As also other factors, as the eventual environmental fitness of mutant allozymes, may contribute to raise error in the calculation of t values, it is rather evident that this kind of evaluations should be considered only as row estimates. at the most.

It is, however, to be emphasized that Nei's I and D values cannot be considered as a measurement of true phyletic relations between studyed taxa, but only of some biochemical affinities, in the very sense of 'phenitical' relations of Sokal & Sneath (1963) (see also Sneath & Sokal, 1973). Species is, in this respect, a multidimensional entity and real distances from one another cannot be represented by a simple normalized mean. A multivariate statistical approach should, eventually, be more correct (see Kendall, 1966).

Operational difficulties

Although electrophoresis of proteins, by itself, is rather easily accomplished, some difficulties may arise at various steps of the procedure.

Specimens to be tested with electrophoretic techniques should be, possibly, be brought alive in the laboratory. Many enzyme systems, in fact, do not tolerate freezing which, for instance, may result in a very lowered activity of some LDH fractions.

Thermal shock, however, represents just one of the possible sources of error in electrophoretic procedures. Other enzymes, as FbPase (Fructose 1,6-Biphosphatase) must be essayed only on unstressed specimens, becaue hyperthermic conditions, unsufficient feeding etc., may cause a remarkable raise in 'free' proteolytic activity, which, in turn, results in the appearance of a second, slow moving, band of FbPase (Pontremoli et. al., 1973) Besides artifact production, however, other difficulties may arise, also when electrophoresis has been correctly performed.

The application of Nei's I and D indexes, for instance, requires an exact determination of the number of codifying loci that are responsible for the genetic variation observed, but in some instances the literal application of Nei's index could easily bring to misestimating the level of genetic diversity between two populations, particularly when cases of gene duplication are involved.

Apart from this kind of technical difficulties, there are other, more theoretical, aspects of the problem.

Electrophoretically undetectable enzyme variants, for instance, may represent a major problem: 'hidden' enzyme heterogeneity, in fact, was often demonstrated by both studies on protein thermal stability, isoelectric point determination with electrophocusing techniques and by simply varying the pore size of electrophoretic gels. Johnson (1977), working on *Colias meadii,* demonstrated that on 14 loci examined, only 40 variant enzyme classes should be observed with standard electrophoretic procedures, out of 103 variants detectable with more sophisticated techniques. The degree of undetected variation, moreover, may strongly affect similarity evaluations in between-specis, investigations. The genetic analysis of four species of the genus *Colias (C. meadii, C. alexandra, C. scudderi, C. philodice)* (Johnson, 1977) demonstrated that although the electrophoretic mobilities of the most common allozymes of the different species are very similar to each other, the physical characteristics of the same allozymes are very different and indicate that most of the alleles of one species do not occur in the others.

Conclusions

As it has been briefly outlined in this paper, electrophoretic techniques certainly represent a very important tool and are to be expected to bring, in the next future, a considerable amount of clarification in the taxonomy of several groups. It is, however, to be emphasized that, although this procedure does not imply, by itself, excessive operational difficulties, its incorrect application may easily be misleading.

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