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II. Isozyme Variations and Evolutionary Relationships in the *mesophragmatica* Species Group of *Drosophila*[1]

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Introduction

The *mesophragmatica* group represents a typical cluster of closely related species of *Drosophila* (Brncic and Koref-Santibañez, 1957). In the previous paper (Brncic, Nair and Wheeler, this Bulletin) an attempt has been made to establish the phylogenetic relationships within the group based mainly on cytological studies. A comparison of the different species in the group at the genic level could contribute much towards confirming these relationships and could provide information on the genetic divergence involved in their speciation. It would also help to reveal the correlation, if any, between cytological variations and genetic differences among closely related species.

The availability of techniques to detect isozyme variations by electrophoretic assay has made it possible to examine a previously inaccessible part of the genome and thus provide an estimate of the genetic similarity or dissimilarity between populations or species at a number of loci. The possibility of interpreting phylogenetic relationships by comparison of biochemical traits has been already explored in some species of *Drosophila*. For instance, Johnson *et al.*, (1966) studied esterase differences between taxonomically different species and found a close agreement in the patterns. Hubby and Throckmorton (1968) compared 7 isozymes, having about 19 systems, in "triads" of closely related species of *Drosophila* and found that sibling species shared isozymes with identical electrophoretic mobility about 50%, on the average, and lesser degrees of sharing occurred between more distantly related species. Kanapi and Wheeler (1970) did similar investigations in three species of the *Drosophila nasuta* complex and have found that the isozyme differences between species agreed well with the presumed degrees of relationship established by cytological and hybridization studies. It seems evident, therefore, that electrophoretic assay could be a useful tool in estimating genetic variability within and between species. In the present studies an attempt has been made, using these methods, to investigate a large

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TABLE 1

The enzymes assayed and the number and kind of variants observed in the whole group and in each species.

No.	Enzyme	Total enzyme variants in the group	Variants in each species					
			D. pavani	*D. gaucha*	*D. viracochi*	*D. mesophragmatica*	*D. gasici*	*D. brncici*
1	Esterase (EST B1)	1 to 2	1,2	1	1	1	1	1
2	Esterase (EST B2)	1 to 4	2,3	2,3	1,4	1	4	4
3	Esterase (EST R)	1 to 6	4,5,6	4,5,6	2	1,2	3	3
4	Amylase (AMY)	1 to 3	1	1	3	2	2	2
5	Alkaline phosphatase (APH 1)	1	1	1	1	1	1	1
6	Alkaline phosphatase (APH 2)	1 to 6	2,3,4,5	3,4,5	1	6	5	5
7	Octanol dehydrogenase (ODH)	1 to 3	2	2,3	2	2	1,2	2
8	Alcohol dehydrogenase (ADH)	1	1	1	1	1	1	1
9	Aldehyde oxidase (AO)	1 to 5	3	2	4,5	1,3	2	3
10	Choline esterase (COE)	1	1	1	1	1	1	1
11	Hexokinase (HK 1)	1 to 3	1,3	1,3	1,2	1	1	1
12	Hexokinase (HK 2)	1 to 2	1,2	1,2	1	1	1	1
13	Hexokinase (HK 3)	1 to 2	2	2	1	2	1	1
14	Isocitrate dehydrogenase (IDH)	1 to 3	1,2,3	2	1,3	3	3	3
15	∞ glycerophosphate dehydrogenase (GPD)	1 to 2	1,2	1,2	2	1,2	1,2	1,2
16	Malate dehydrogenase (MDH)	1 to 2	2	2	2	1	1	2
17	Malic enzyme (ME)	1 to 3	1,2	1	1,3	1	1	1
18	Fumarase (FUM)	1 to 3	2,3	2	2,3	1,3	1	2,3
19	Glucose-6-phosphate dehydrogenase (G6PD 1)	1	1	1	1	1	1	1
20	Glucose-6-phosphate dehydrogenase (G6PD 2)	1 to 2	1,2	2	2	1	1	2
21	Glucose Isomerase (GI)	1 to 3	1	2	3	2	2	2
22	6 phosphogluconate dehydrogenase (6PGD)	1 to 3	3	1	2	2	2	2
23	Aldolase (ALD)	1 to 2	2	2	2	2	1,2	2
24	Phosphoglucomutase (PGM)	1 to 2	1,2	1	1	1,2	1	1
	Total number of variants	65	40	33	30	29	27	26

number of loci in six species of the *Drosophila mesophragmatica* group namely: *D. pavani, D. gaucha, D. viracochi, D. mesophragmatica, D. gasici* and *D. brncici.*

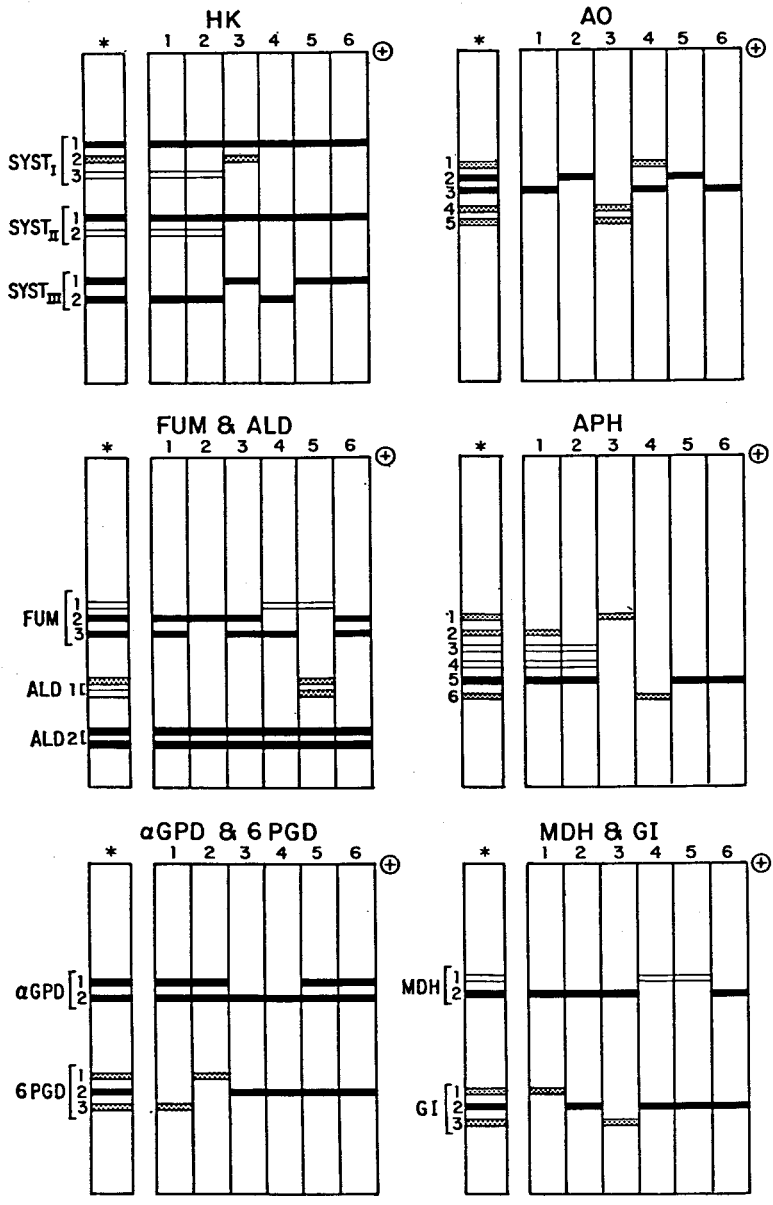
Materials and Methods

Nineteen different enzymes, comprising twenty-four loci, were investigated in the six species using electrophoretic assay. The enzymes analyzed, the abbreviations used for each, the number of scorable systems in each enzyme, and the variations observed with regard to each system in the whole group and in each species are shown in Table 1. For all enzymes except Amylase, starch gel electrophoresis was carried out using the single-fly assay technique as described by Johnson (1966) with slight modification. For Amylase, a modified horizontal acrylamide gel technique as described by McCune (1969) was employed. The buffers and staining procedures were modified from various sources (see Shaw and Koen, 1968, for details and references). In all cases flies of different species were assayed together in the same gel to eliminate differences due to gels, buffers, voltage or other variables. Whenever mobility differences were too small, flies of the two species concerned were assayed in a gel side by side along with a mixture of their homogenates to confirm the variations. In some systems of certain enzymes clear banding patterns were obtained in some species, but not in others. Whether absence of bands is due to null alleles or due to certain extraneous factors could not be determined, and hence such systems were not considered for the present comparisons. At least 25 flies of each species were assayed for each enzyme and, in most cases, the number of flies analyzed was much higher. About an equal number of males and females were used in all cases. There were male specific bands in some species, chiefly in esterases, but these have not been included in the present data. Adult flies were used for all enzyme assays with the exception of Alkaline phosphatase (APH). For APH, larvae were assayed since resolution of different bands was better in larvae as it is in *D. melanogaster.* However, in the *mesophragmatica* group, unlike in *D. melanogaster,* the larval APH bands are identical with the adult bands and presumably the larval and the adult APH locus is the same. The flies of the different species assayed were from the same populations as those in which cytological studies were done as detailed in the previous paper (Brncic, Nair and Wheeler, *op. cit.*). In *D. pavani* and *D. gaucha,* populations from different ecogeographical regions were examined while in the other 4 species only one population each was available. This could have contributed to the detection of a larger amount of variation in the first two species as shown in Table 1.

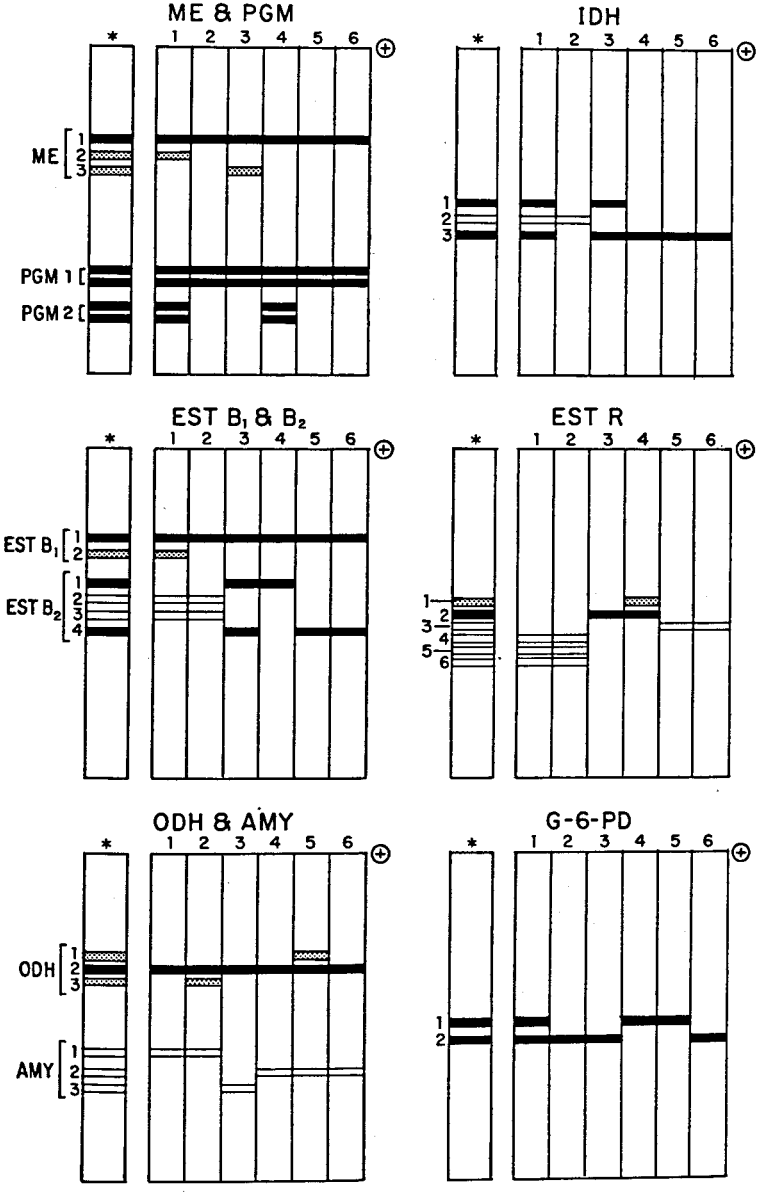
Results

The patterns of electrophoretic variants in the six species for the enzyme systems examined are shown in Figures 1 and 2. Enzymes with no variations in any of the species are not represented. Arbitrarily, the different bands have been numbered in each case progressively from the one showing maximum mobility towards the anode. In each diagram the first column represents the total number of bands seen in the whole group with regard to the particular enzyme.

In the analysis of the data, an attempt has been to estimate the genetic simi-



larities between the different species and also to postulate a phylogenetic relationship within the group based entirely on isozyme data. Based on cytological evidence it has been shown (Brncic, Nair and Wheeler, *op. cit.*) that the six species in the *mesophragmatica* group can be divided into three subgroups namely, 1) *D. pavani* and *D. gaucha*, 2) *D. viracochi* and 3) *D. mesophragmatica*, *D. gasici* and *D. brncici*. Based on this grouping, the genotype of a hypothetical stem line was postulated with regard to the 24 enzyme loci. When identical mobility bands were seen in at least two of the three subgroups, they were assumed to have had



FIGS. 1 and 2. Relative positions of bands representing electrophoretic variants in the six species. The first column, marked by an asterisk, represents the total number of variants found in the whole group for each enzyme. The numbers on top denote species as follows: 1) *D. pavani* 2) *D. gaucha* 3) *D. viracochi* 4) *D. mesophragmatica* 5) *D. gasici* 6) *D. brncici*. See text for other details.

a common origin from the stem line. Alleles responsible for electrophoretic variants which were observed only in one species or one subgroup were presumed to have arisen within the species or subgroup as the case may be. It is recognized

that the presumption of homology involved in this procedure may not be true. But in a closely related group such as *mesophragmatica* where the species are so similar cytologically, perhaps such a presumption may not be unwarranted. In Figures 1 and 2 the enzyme bands representing the hypothetical stem line are shown as solid, the group-specific bands are left blank and the species-specific variants are stippled. When a hypothetical ancestral genotype is postulated in the manner indicated, it is seen to be polymorphic in 11 out of 24 loci. This degree of polymorphism does not seem too high, especially since a contemporary species like *D. pavani* has 12 polymorphic loci out of 24.

The relationship between the different species of the group in terms of chromosomal and genic variations is shown in Table 2. The correlation between the chromosomal inversion differences and the genic differences does not mean that any of the alleles could be directly associated with any of the inversion sequences. This obviously requires mapping of the positions of the different enzyme loci. However, incorporation of a number of fixed inversions perhaps reflects the time distance in the evolutionary path and the genetic distance seems to have close agreement.

Genetic similarities between the different species of the group were estimated based on the number of identical enzyme bands they shared. This is given both as fractions and percentages in Table 3. The similarity between two species is expressed as the fraction or percentage of enzyme variants that are identical in the two species out of the total number of variants exhibited by the two species. For example, in a comparison between species A and B, if both species have 3 bands each for an enzyme of which only one band is identical, then the similarity between the two species would be $1/5 = 20\%$. This would explain the difference in denominators in Table 3, though the same number of enzyme systems have been examined in all cases.

In a second method of analysis of the isozyme data, the different species were compared using the coefficients of genetic similarity proposed by Rogers (1970)

TABLE 2

Inversions and alleles specific to subgroups and species

		Subgroups					
		I		II	III		
		D. pavani	*D. gaucha*	*D. viracochi*	*D. mesophragmatica*	*D. gasici*	*D. brncici*
Fixed inversions specific to:	a) subgroup		11	1		1*	
	b) species	2				1	
Alleles specific to:	a) subgroup		11	6		2	
	b) species	4	1		2	2	

* Common to *gasici* and *brncici* only

TABLE 3

Genetic similarity between species in fractions and percentages (see text)

	D. pavani	*D. gaucha*	*D. viracochi*	*D. mesophragmatic*	*D. gasici*	*D. brncici*
D. pavani	—	30/45 .666	17/52 .326	19/51 .372	14/52 .269	20/46 .434
D. gaucha		—	15/49 .306	15/48 .312	15/45 .333	18/44 .409
D. viracochi			—	17/42 .404	15/41 .365	20/36 .555
D. mesophragmatica				—	19/36 .527	19/36 .527
D. gasici					—	20/31 .645
D. brncici						—

and currently being used for analysis of data on biochemical polymorphisms in natural populations by members of the Genetics group at The University of Texas. The coefficients of genetic similarity obtained through computer analysis using the above method are given in Table 4. A dendrogram constructed on the basis of the above coefficients using the methods suggested by numerical taxonomists (Sokal and Sneath, 1963) is shown in Figure 3. The scale in the dendrogram indicates the level at which the stems come together, but obviously does not represent the relationships at the tips of the dendrogram.

The genic similarities between the species calculated by percentage analysis as well as by Roger's method (Tables 3 and 4) show great resemblance and are in remarkably close agreement with the phylogenetic relationships postulated on the basis of cytological studies. Species that are very similar morphologically and cytologically also exhibit the highest degree of genic similarity. For example, among the subgroups, *D. pavani* and *D. gaucha* show the greatest morphological similarity, their chromosomal sequences are very much alike and they freely interbreed in the laboratory producing abundant, though sterile, hybrids (Brncic and Koref-Santibañez, 1962). Corresponding to this close relationship, these two

TABLE 4

Roger's coefficient of genetic similarity between species

	D. pavani	*D. gaucha*	*D. viracochi*	*D. meso-phragmatica*	*D. gasici*	*D. brncici*
D. pavani	1.00	.632	.395	.378	.259	.488
D. gaucha		1.00	.410	.382	.410	.494
D. viracochi			1.00	.513	.474	.617
D. mesophragmatica				1.00	.673	.626
D. gasici					1.00	.767
D. brncici						1.00

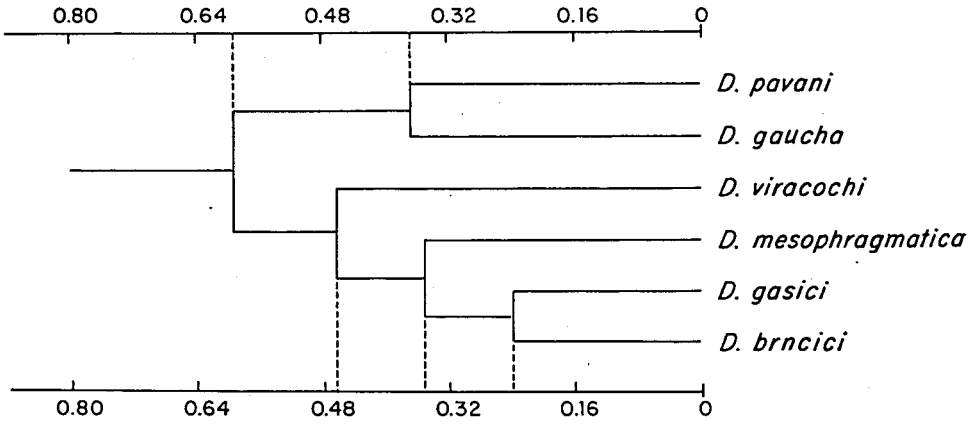


FIG. 3. The dendrogram showing relationship between species based on Roger's coefficient of genetic distance.

species show considerable genic similarity with regard to the enzyme loci examined. About 66% of the enzyme bands in the two species are identical and Roger's coefficient of similarity is .632 (Tables 3 and 4). This subgroup is far removed from other members of the group in having as many as 11 common fixed inversion differences. Paralleling this evolutionary distance as shown by chromosomal phylogeny, it is noticed that there are 11 new enzyme alleles specific to this subgroup and there are only two alleles in each species that are species-specific (Table 2).

The subgroup consisting of *D. mesophragmatica, D. gasici* and *D. brncici* represents a different evolutionary pathway but the three species are not too close. In fact *D. mesophragmatica* has been included in the subgroup only because it shares with the other two species the characteristic that the fifth chromosomes are represented by rods instead of dots. There are only two enzyme alleles specific to the subgroup while *D. mesophragmatica* itself has 4 species-specific alleles. However, *D. mesophragmatica* shows more than 50% genic similarity with both of the other two species and hence the genetic evidence supports the postulated relationship. *D. gasici* and *D. brncici* are indeed very close and this is reflected both in cytological and genetic similarities. They share about 65% of their enzyme bands and Roger's coefficient of genetic similarity is .767, the highest in the whole group.

Among the six species, *D. brncici* seems to show the closest agreement with the postulated hypothetical stem line with regard to the enzyme alleles and it has no new alleles specific to itself. It cannot, however, be considered as in the direct ancestral line because of its characteristic rod-shaped fifth chromosome which it shares with the other two members of the subgroup and the unique chromosomal inversion it shares with *D. gasici* (Brncic, Nair and Wheeler, *op.cit.*). *D. viracochi* forms a subgroup by itself. In chromosome sequence it is closest to *D. mesophragmatica,* differing in only one fixed inversion. Genetically, too, it is closer to the subgroup comprising *D. mesophragmatica, D. gasici* and *D. brncici* as is shown in Tables 3 and 4 and the dendrogram (Fig. 3). However, its fifth chromosomes are represented by dots as in *D. pavani* and *D. gaucha.*

DISCUSSION

There are two questions to which one could seek answers through the study of allelic variations at a number of loci in a group of related species. First, whether these variations are useful as diagnostic aids in establishing phylogenetic relationships; and second, whether the differences at the genic level could throw more light on the kind and amount of genetic reorganization involved in the process of speciation. Before attempting to answer these questions, some difficulties involved in interpreting isozyme variations should be pointed out:

1) Homology of alleles responsible for enzymes of identical electrophoretic mobility in different species remains in doubt. As Zuckerkandl and Pauling (1965) have pointed out, when the common ancestor of contemporary forms is too remote and a thorough reshuffling of hereditary material has taken place, then the notion of homology becomes meaningless.

2) Isozyme variations do not provide any evidence as to the "mutation distance" between species. It is conceivable that two or more gene loci are involved in providing different polypeptide subunits to a single enzyme entity and hence it is hard to estimate the number of mutations that are involved in one mobility change.

3) Isozyme variations do not give any clue regarding the sequence of changes. There is no positive way one can determine which variant or allele is more ancient, except in relation to other known facts of evolutionary sequence.

4) Isozyme variations reflect changes only in a restricted part of the genome. Hence the estimation of genic similarities based on isozyme variations alone need not necessarily represent the correct situation with regard to the whole genome. For instance, Gillespie and Kojima (1968) have shown that the degree of variation in enzymes involved in glucose metabolism is much less than in some others. Though a significant difference in this respect is not seen in the present studies, it seems reasonable to expect lesser variation in those genes that are concerned with the essential, basic manifestations of life.

5) There is very little information with regard to the functional differences between the different electrophoretic variants of enzymes. It is quite likely that these variations represent amino acid substitutions without any serious impairment of functional specificity of the enzymes. Until more is known about the physiological relevancy of these variants, it is difficult to assess whether they have, by themselves, any evolutionary significance.

In spite of these handicaps, the present studies and various earlier studies indicate that isozyme analysis can indeed be very useful in answering the questions raised at the beginning. With regard to phylogenetic relationship between species, data from enzyme analysis can provide much meaningful information independently as well as in conjunction with other known evidence. Even if homologies are questioned, there is no doubt that these endophenotypic characters have as much relevance as other morphological characters in determining taxonomic affinities. The close parallelism shown in these studies between isozyme variations and other characters confirms this.

The second question is that of genic divergence involved in speciation as revealed by isozyme study. Hubby and Throckmorton (1968) from their studies

already referred to, have concluded that speciation does not *require* change in a large number of loci. They also find that there is greater genic similarity between sibling species than between more distantly related species. The present studies confirm their findings with regard to the enzyme loci. However, these conclusions involve equating the variations at the enzyme loci with the rest of the genome. This type of comparison of the structural genes does not take into account the possible and perhaps more important changes in the regulatory mechanisms.

In any case, the amount of genic divergence need not necessarily have too much relevance as far as speciation is concerned. If we accept reproductive isolation as the criterion for differentiation of biological species, it seems evident that such isolation could occur without reference to morphological or genic variations. Populations of the same species which are physically isolated from one another for a long period of time may build up a considerable amount of genetic variance depending on differences in selective forces. Between many such populations no reproductive barrier may exist (Dobzhansky, 1964; Grant, 1966). But in some cases, it is equally likely that a reproductive barrier is built up as a by-product of other genetic changes (Dobzhansky, 1937; Muller, 1939). The formation of distinct biological species in this manner must be considered as an accidental evolutionary phenomenon. That reproductive isolation can occur by chance has been confirmed by several laboratory observations (Koref-Santibañez and Waddington, 1958; Rick, 1963). When populations become distinct biological species in this manner, the genic divergence between these species will depend upon the elapsed time and difference in selective pressures on the populations after isolation, rather than on when the actual reproductive barrier occurred. Two kinds of situations may exist in this regard: 1) Different populations of the species may show a considerable degree of genic divergence without being reproductively isolated; 2) Populations may develop reproductive barriers and become biological species before building up any great degree of genetic change.

The present studies indicate that both of these situations may exist in the *mesophragmatica* group. For instance, *D. gasici* and *D. brncici* are two species which are very close cytologically and genetically, and yet there is complete reproductive isolation between them. The same situation exists between *D. pavani* and *D. gaucha*. Perhaps these may be cases where reproductive isolation has occurred before much genetic variance had been incorporated. On the other hand there are two populations of *D. gasici*, one from Bolivia and the other from east of the Andes in Colombia, which show three fixed inversion differences (Brncic and Koref-Santibañez, 1965) and between which preliminary studies indicate considerable genetic difference in the enzyme loci. But they freely interbreed producing fertile progeny. Perhaps these populations represent the first type of situation where genetic divergence in isolated populations has not been accompanied by accidental reproductive isolation. It seems likely that as more and more populations are studied in this regard, similar situations will be seen in other species.

In this context, the kind of reproductive isolation between two closely related species may itself provide some clue to the type of speciation that has occurred. Admittedly, hybrid sterility is not an economical method of preserving genetic integrity. Hence, as has been pointed out (Grant, 1963; Rick, 1963) selection

for reproductive barriers can have reference only to isolating mechanisms in the parental generations. Such selection would lead towards establishment of some premating mechanism of reproductive isolation. This selection has been designated as the "Wallace effect" by Grant (1966). The fact that such selection is common in nature is demonstrated by studies on some *Drosophila* groups (King, 1947; Dobzhansky, Ehrman and Pavlovsky, 1957) where it was shown that sympatric species are separated from one another by stronger ethological barriers than are geographically isolated species. The speed of this selection in nature would depend upon the frequency of hydridization. Under laboratory conditions the effect of such selection sometimes becomes evident within a few generations (Koopman, 1950; Paterniani, 1969).

The absence of an effective premating reproductive isolation in two species with overlapping distribution could be due to two reasons: 1) the seasonal or ecological divergence between the species is so complete that there is no hybridizing in nature, or 2) the geographical overlap is so recent that natural selection has not had enough time to bring about the change. The latter situation seems to exist with regard to *D. pavani* and *D. gaucha* whose distributions overlap in the eastern slopes of the Andes in Argentina, and between which, apparently, there is no significant seasonal or ecological isolation. It is possible that *D. pavani* is a recent immigrant to this area from its normal distribution range in Chile and perhaps these two species have not been existing together in the same area for a sufficiently long time for natural selection to have brought about any effective sexual isolation. A significant observation in this context is that of Koref-Santibañez and Del Solar (1961) who found that *D. pavani* males from this area of overlap show a greater degree of discrimination against *D. gaucha* than do males from areas in Chile. This suggests that selection against hybridization is in progress.

Summary

Twenty-four loci controlling 19 enzymes were examined in six members of the *mesophragmatica* species group of *Drosophila* using electrophoretic assay. Genetic variation as revealed by this study shows a close resemblance to the morphological and cytological variations between the species. The phylogenetic relationships of the species, based on isozyme data, parallel the postulated cytotaxonomic relationships, suggesting that isozyme variations could be used, with confidence, as diagnostic aids in taxonomy.

The estimation of genic divergence as revealed by isozyme variation between the different species, shows that speciation does not require change in a large number of loci. However, it is argued that critical changes in the speciation phenomena do not depend on changes in the majority of loci (or on drastic modifications in the chromosomal sequences) but rather on changes at the level of genetic regulatory mechanisms and on coadapted polygenetic systems.

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