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ELECTROPHORETIC VARIABILITY IN TWO CLOSELY RELATED BRAZILIAN SPECIES OF THE *flavopilosa* SPECIES GROUP OF *Drosophila*.*

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ABSTRACT

D. incompta and *D. cestri* are two species of the *flavopilosa* group of the sub-genus *Drosophila*, morphologically undistinguishable except for the male and female genitalia. Both species are monophagous and live closely associated with *Cestrum* flowers (*Solanaceae*) inside which all the developmental stages of the two species can be oserved, apparently coexisting without harming one another. An analysis of the proteic variability by electrophoretic techniques within and between the two species revealed that the average frequency of polymorphic loci in *D. incompta* is about 47% and only 28% in *D. cestri*. The genetic similarity between the two species is about 33% despite their close phylogenetic relationships. The results are interpreted in terms of ecological displacement.

INTRODUCTION

Very recently, four species of the *flavopilosa* species group of the sub-

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-genus *Drosophila* have been discovered in Rio Grande do Sul: *D. flavopilosa* Frey 1918, *D. incompta* Wheeler and Takada (Wheeler et al., 1962), *D. cestri* Brncic and *D. cordeiroi* Brncic (Brncic, 1978). All four species have been found associated with the flowers of the *Solanaceae* shrubs, *Cestrum parqui* and *C. calycinum.* Two of them, *D. incompta* and *D. cestri*, seem to be the most abundant ones in certain localities near Porto Alegre. In Spring, during the plants'blooming period, a large amount of flowers contain pre-adult forms of both species, and adults may be seen fluttering over the shrubs.

The coexistence of the two species over the same breeding and feeding places seems to depend on certain behavioral and ecological differences. *D. cestri* females have an egg-laying behavioral pattern similar to that already described for *D. flavopilosa* (Brncic, 1966), that is, they lay the eggs inside the unopened flowers through minute holes made with their unusually strong spined ovipositor in the lower part of the corolla-tube. The females of *D. incompta* behave differently, by scarifying superficially the walls of the already opened flowers, especially the inner parts of the petals. These scarified areas serve as points for egg attachment. The very small larvae then move from these points and penetrate inside the corolla.

As a result of these facts, during the blooming period of *cestrum* plants the first adults to emerge are mostly *D. cestri,* i.e. the first species to colonize the flowers, and after a few days, the second species, *D. incompta,* begins to appear. There is very little overlapping between the two swarms. This has also been observed when *cestrum* flowers are brought to the laboratory and the flies that complete their development are recorded every day. To sum up, despite the fact that the two species apparently exploit the same basic resources, their ecological niches seem to differ in minor but clear-cut characteristics. But, no doubt, the existence of these morphologically cryptic species, utilizing resources that are not spatially or temporally separate, raises a lot of fascinating evolutionary and genetic problems that deserve analysis. The most obvious one is to know how distant the two species are regarding their genetic make-up.

The polytene salivary gland chromosomes of the larvae, picked directly from the flowers, are not good enough to permit an evaluation of the differences in banding patterns between the species.

Nevertheless, studies in progress by Nena Basilio Morales and the present Authors show that, at least in large populations of *D. cestri* and *D. incompta* obtained from Sapucaia do Sul, near Porto Alegre, no segregating inversions have as yet been observed.

The present paper reports the observed variations within and between the two species based on an analysis of electrophoretic variability.

MATERIALS AND METHODS

The material for the present study was obtained from several samples of flowers collected through October and November 1977.

It is not possible to maintain *D. cestri* and *D. incompta* on culture media commonly used in this laboratory. Because of this fact, the flowers of *Cestrum parqui* collected from Parque Zoológico (Sapucaia do Sul) were brought to the laboratory and placed into vials with a wet filter paper. The larvae completed their development inside the flowers. When the adults emerged, they were classified and later frozen at - 20° C until analysis.

Electrophoresis: Individual flies were homogenized in 5μl deionized water and absorbed by four wicks of Whatman No. 3 filter paper (0.4 x 0.2 cm). Each wick was applied to horizontal acrylamide (6%) gels combining two different buffer systems. An electric field of 10 v/cm was applied across the gels for 3 to 4 hours or until the migrating front was 9 cm from the sample slot line. After the bands appeared, the reaction was stopped by washing the gel with water and adding 100 ml of the fixing solution of methanol: water: acetic acid (5:5:1).

The following buffer systems were used in the study:

Buffer I: Discontinuous Tris-citrate (Poulik, 1957), for Esterase (Est), Leucine aminopeptidase (Lap) and Proteins (Pt).

Buffer II: Continuous Tris-borate EDTA, pH 9.0 (Ayala et al., 1972b), for Glutamate-oxaloacetate transaminase (Got), Aldehyde oxidase (Ao), Xanthine dehydrogenase (Xdh), and Malic dehydrogenase (Mdh).

The staining methods used are similar to those described by Ayala et al. (1972b, 1974b), with small modifications.

The allozymes were identified by their relative electrophoretic mobilities, the reference being the most common *D. cestri* allele.

RESULTS

Figure 1 shows the patterns of electrophoretic variants in *D. cestri* and *D. incompta* for the six enzyme systems and the proteins examined. The number of sites investigated per species was 18 for *D. cestri* and 15 for *D. incompta.* We found polymorphism at the following sites: Est-1, Est-2, Lap, Got-1, Ao, Pt-5 and Pt-6. The other sites were monomorphic: Est-3, Est-4, Pt-2, Pt-3, Pt-4, Pt-7, Pt-8, Pt-9, Mdh-1, Mdh-2, Xdh and Got-2.

The number of genes sampled, allelic frequencies, and the proportion of heterozygotes for the Est-1 and 2, Lap, and Got-1 loci are given in Tables I, II, III

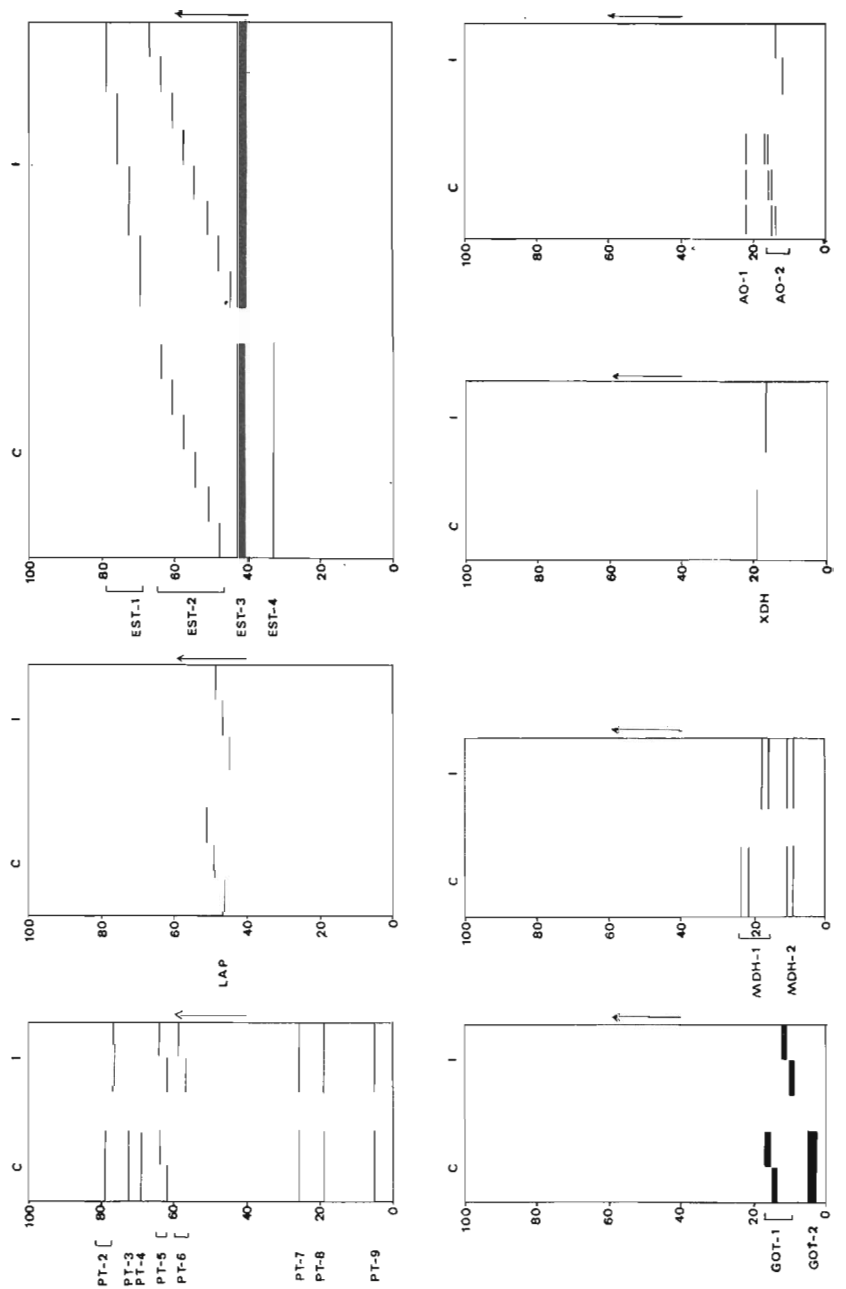

Figure 1. Diagrams showing electrophoretic variants in the two species of the *flavopilosa* group.
c. *D. cestri;* i. *D. incompta.*

and IV, respectively. It was not possible to study allelic frequencies for the other polymorphic sites because the bands sometimes were diffuse.

The expected frequencies of heterozygotes for the most common alleles of Est-1 and 2, Lap and Got-1 loci, calculated under the assumption of the Hardy-Weinberg equilibrium, were in all cases higher than those observed. The χ^2 homogeneity test indicated that these deviations were highly significant.

TABLE I. Allelic frequencies at the Est-1 locus

	Alleles						Heterozygosity	
Species	.91	.95	.97	1.00	1.03	Genes sampled	Obs.	Exp.
incompta	.030	.161	.208	.343	.258	236	.33	.80

Table I shows the allelic frequencies at the Est-1 locus, detected only in *D. incompta.* The Est-2 locus presents a similar pattern of allelic variation in both species (Table II). The Lap.$^{1.0}$ allele was predominant in *D. cestri* while *D. incompta* showed the Lap.$^{.95}$ allele as the most frequent one (Table III). Large differences between the two species were observed at the Got-2 locus (Table IV).

The percent of polymorphic loci was higher for *D. incompta* (0.47) than for *D. cestri* (0.28). The observed heterozygosity (number of heterozygous genotypes/total number of genotypes analyzed), calculated for each polymorphic locus studied, was higher in all cases in *D. incompta* (Tables I to IV).

An attempt has been made to estimate the genetic similarity between the two species based on isozyme data. The similarity between the two species was estimated as a fraction of identical sites out of the total number of sites present in both. When we compare closely related species, we may presume homology of sites with identical mobility (Nair et al., 1971) *D. cestri* and *D. incompta* shared 15 out of 45 bands, thus the genetics wimilarity between them was 0.333 or 33.33%.

DISCUSSION

Determinations of the extent of genetic variability estimatedby gel electrophoresis have been made in several species of the genus *Drosophila.* The average

TABLE II. Allelic frequencies at the Est-2 locus

	Alleles											Heterozygosity	
Species	.84	.89	.94	.96	1.00	1.04	1.07	1.09	1.13	1.16	Genes sampled	Obs.	Exp.
cestri	.007	.017	.047	.140	.338	.254	.150	.047	–	–	406	.22	.75
incompta	.010	.089	.133	.183	.209	.143	.087	.075	.050	.021	518	.31	.86

TABLE III. Allelic frequencies at the Lap locus

Species	Alleles						Genes sampled	Heterozygosity	
	.91	.93	.95	1.0	1.03	1.06		Obs.	Exp.
cestri	–	.005	.164	.817	.012	.002	426	.17	.30
incompta	.009	.065	.853	.071	.002	–	448	.21	.26

TABLE IV. Allelic frequencies at the Got-1 locus

Species	Alleles				Genes sampled	Heterozygosity	
	.75	.87	.90	1.0		Obs.	Exp.
cestri	–	–	.043	.957	512	.07	.08
incompta	.509	.491	–	–	220	.40	.50

frequency of polymorphic loci in *D. incompta* analyzed by us gene a value of 47%. This result indicates that *D. incompta* is more variable than its closely related species, *D. cestri* (28% of polymorphic loci). Nevertheless, the amount of polymorphism detected in these two Brazilian members of the *flavopilosa* group is far larger than that observed in other monophagous species such as the cactophilic species of *Drosophila* (Zouros, 1973; Barker and Mulley, 1976). The low variability found in those species was considered by the authors to be a function of the ecological restriction of the species. Our results are more comparable to those of the *willistoni* group species, which are widely ranging and ecologically versatile (Ayala and Powell, 1972a; Richmond, 1972).

Another interesting point is the low degree of genetic similarity between the species, about 33%. It has been postulated that there is a direct correlation between the evolutionary or taxonomic status of a species or subspecies, and the degree of genetic similarity (Hubby and Throckmorton, 1968). In the *mesophragmatica* group, it was found that sibling species like *D. pavani* and *D. gaucha* shared 67% of identical enzyme bands, and nonsibling species of the same

group, such as *D. pavani* and *D. gasici,* shared only 27% of enzymes (Nair et al., 1971). For the *willistoni* group, Ayala et al. (1974a) showed that local geographic populations of a species have about 90% genetic similarity; subspecies and semispecies about 80%, cryptic species about 50%, and nonsibling species about 35%. But, there are exceptions to this rule as reported by Carson et al. (1975) for the Hawaiian species *D. setosimentum* and *D. ochrobasis.* The two species are partly sympatric, but exhibit clear morphological, altitudinal and breeding-site differences. Nevertheless, they show a genetic similarity that resembles more that observed in the sub or semispecies studied by Ayala et al. (1974a) than that observed in unrelated species. Close interspecific electrophoretic similarity has been observed also between another couple of Hawaiian species, *D. silvestris* and *D. heteroneura,* by Sene and Carson (1977). The unusual aspect of these morphologically distinct species is that they are ecologically similar. Both have been reared from the same individual decaying stems of the host plant, *Clermontia.* According to Sene and Carson (1977) the genetic similarity between the two species (0.93 utilizing the Nei Index), may be due to the fact that species are newly formed, and allozymic differences may not have had time to accumulate. In addition, the biochemical similarity reflects the ecological closeness of the two species.

D. cestri and *D. incompta* represent a different kind of exception to the Ayala et al. rule. The two species are morphologically very close, practically cryptic, they are sympatric, and utilize a common resource that is not spatially or temporally separable. But, from the electrophoretic point of view, they are as distant as unrelated species.

There are two types of considerations that may be important to explain these apparent discrepancies. The first is that genetic differences are always historical products, and could reflect the antiquity of the evolutionary divergence and the degree of isolation. The second consideration is that the degree of difference depends mainly on the intensity of the evolutionary pressures. If two primarily allopatric species became sympatric, and this may be the case of *D. cestri* and *D. incompta,* character displacements, ecological specializations and a strengthening of sexual isolating mechanisms could occur.

For these reasons we believe that there are no discrepancies between the situation observed in the Hawaiian species studied by Sene and Carson (1977) and that observed in the Brazilian members of the *flavopilosa* group. For *D. silvestris* and *D. heteroneura,* it may be advantageous to maintain an almost identical array of structural genes to exploit a common resource that may be abundant and not in such "short supply" as to determine competitive interactions. On the contrary, for *D. cestri* and *D. incompta,* the resource provided by *Cestrum* flowers (if they

are the only breeding site) could induce an ecological displacement, the genetic basis of which is reflected by the electrophoretic differences.

Undoubtedly, all the above considerations are mere hypotheses. We ignore the niche amplitude of the different species here considered, and what part of it is really shared and could represent a selective factor.

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REPRODUCTIVE ISOLATION IN *Drosophila prosaltans* (*saltans* GROUP)

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ABSTRACT

Reproductive isolation among 18 strains of *D. prosaltans* was studied in mass mating crosses. On the basis of the results, 3 isolation sets of strains were distinguished: set A, including strains from Central America; set B, including strains from South America, north of the Amazon River; and set C, including Brazilian strains, south of the Amazon River. Sexual isolation and hybrid sterility were isolating mechanisms found to operate between the 3 sets, mainly between A and B, and A and C. The results are to some extent comparable to those of *D. paulistorum* semispecies and in this way the 3 sets could also be classified as 3 semispecies. Analysis of chromosomal polymorphism of strains in every set indicated that sets B and C are ancestral to set A, thus suggesting that *D. prosaltans* originated in South America and dispersed to Central America.

INTRODUCTION

In spite of the many biological and environmental aspects involved in speciation, the whole process may be summarized in two basic events: adaptation and selection for reproductive isolation. The multiple aspects of these events and their interplay in speciation allow us to consider as probably unique the evolutionary history of each group of populations of a given species or the evolutionary history of each group of related species. Thus new lights on the knowledge of the speciation process may be expected from each of these newly studied groups.

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The purpose of the present work was to study the speciation process in *Drosophila prosaltans*. Since this species has a large distribution area (the largest among species in the saltans subgroup) which extends from Costa Rica to the south of Brazil and Paraguay, its populations are exposed to many different environments, among them geographic barriers such as mountains, rivers and forests. Under these conditions, genetic differences are expected to accumulate as the populations adapt to their environments. The development of reproductive isolation would then lead to the formation of independent gene pools.

Genetic differences in *Drosophila prosaltans* strains due to paracentric inversions have already been described (Cavalcanti, 1948; Bicudo, 1967, 1973; Bicudo, Hosaki, Machado and Marques, 1978). Data on hybridization tests are presented in this study. They show that a variable degree of reproductive isolation also exists between *D. prosaltans* strains. The evolutionary divergence within *D. prosaltans*, analyzed on the basis of both cytological and isolational pictures, is to some extent comparable to that of *D. paulistorum*, considered as a cluster species in "statu nascendi" (Dobzhansky and Spassky, 1959).

MATERIAL AND METHODS

Geographical origins and stock references of the strains used are in Table I. Eighteen strains from several localities including the northern and southern limits of the species distribution area were analyzed for reproductive isolation.

The experiments involved reciprocal mass crosses of ten pairs per vial (250 ml) using flies aged from 5 to 7 days. Two mass crosses were made in each direction and changed to new vials on the fifth day. The analysis was performed 25 days later. On the basis of the results, the crosses were classified into sterile, high productivity, or low productivity. This last class included crosses which yielded from just one larva up to about 20 adults. Females of sterile crosses were dissected and examined for the presence of spermatozoa in their reproductive tracts.

Depending on the number of available flies, the fertility of F_1 progeny was verified using pair mating or mass endocrosses which were classified, at the time of analysis, like the parental crosses. When F_1 endocrosses did not yield progeny, backcrosses were prepared in order to detect the sterile sex.

Banana culture medium was used and the stocks and tests were maintained at the temperature of 25^{o} C $\pm$ 1^{o} C.

Table I - Geographical origins and stock references of the strains used.

Origin		Reference
Costa Rica	Palmar	P_{1a}
	Piedras Blancas	P_{2a}
	San Isidro	P_2
Panamá		P_{26}
Trinidad	Sangre Grande	P_4
Colombia	Bucaramanga	P_5
Venezuela	Barquisimeto	P_{25}
Guiana	Apoteri	P_{22}
		P_{23}
Brazil	Macapá	P_{12}
	Belém (PA)	P_6
	Várzea, Belém (PA)	P_{13}
	(CE)	P_{10}
	Boa Viagem (CE)	P_{16}
	Salvador (BA)	P_{17}
	Campo Grande	P_{18}
	Leste (PA)	P_{20}
	(RS)	P_{21}

RESULTS

The results of intercrosses between 3 strains from Costa Rica (P_{1a}, P_{2a}, P_2), one strain from Panama (P_{26}), Trinidad (P_4), Colombia (P_5), Venezuela (P_{25}), and 2 strains from Guiana (P_{23} and P_{22}) are shown in Figure 1. This Figure is arranged in such a way that the large triangle above the diagonal (where the strain symbols are presented) contains the results of fertility and fecundity of the parental crosses, and the large triangle below the diagonal contains the results of F_1 endocrosses.

The 2 triangles in each small square are related to both directions of crosses of every comination: the triangle below the diagonal refers to the crosses between females from the left strain and males from the strain below (which are respectively above and right strains considering F_1 endocrosses); the triangle above the diagonal is indicative of the reciprocal crosses.

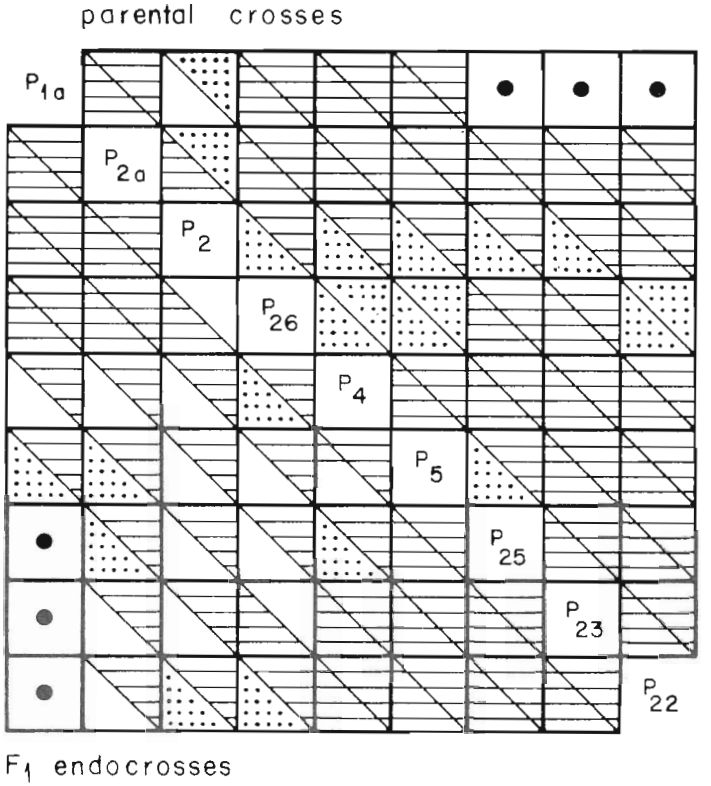

Figure 1. Fertility and Fecundity of the parental crosses and F_1 endocrosses of strains from Costa Rica (P_{1a}, P_{2a}, P_2), Panama (P_{26}), Trinidad (P_4), Colombia (P_5), Venezuela (P_{25}), and Guiana (P_{23}, P_{22}). Full lines = high fecundity; dotted lines = low fecundity; absence of lines = sterile crosses; a single dot = not prepared cross.

In both parental crosses and F_1 endocrosses fertility is indicated by the presence of lines (fertile crosses) or the absence of lines (sterile crosses); and fecundity is indicated by full lines (high productivity) or dotted lines (low productivity). Crosses which were not prepared because the strain P_{1a} was lost are indicated by a single dot in the center of the empty square.

Crosses in Figure 1 showed variable results. For example, the crosses of strains from Trinidad (P_4), Colombia (P_5). Venezuela (P_{25}), and Guiana (P_{23} and P_{22}) with each other were fertile and produced fertile progeny in both directions. In two cases, fecundity was low: in the parental crosses of P_5 females X P_{25} males and in F_1 endocrosses of P_4 females X P_{25} males. All the other combinations presented high fecundity.

The crosses of strains from Costa Rica (P_{1a}, P_{2a} and P_2) and Panama (P_{26}) with each other showed that, when P_{1a}, P_{2a} and P_{26} were involved, the fecundity of the parental crosses was high and their progeny was fully fertile However, in the tests involving any of these strains and P_2 (also from Costa Rica), a variable degree of isolation was detected. Productivity was low in all of the parental crosses in which P_2 were the females. In the crosses between P_{26} and P_2 the isolation affected also the fertility of F_1 progeny: their intercrosses were sterile in the direction P_{26} females X P_2 males.

Crosses of the strains from Costa Rica (P_{1a}, P_{2a} and P_2) and Panama (P_{26}) with the strains from Trinidad (P_4), Colombia (P_5), Venezuela (P_{25}) and Guiana (P_{23} and P_{22}) showed 3 situations concerning the parental crosses: (1) crosses in both directions were fully fertile; (2) crosses in one direction were fully fertile and the other exhibited a low productivity; and (3) crosses in both directions presented low productivities. The first situation was the most frequent. The second was detected in crosses in which the P_2 strain was used, and the third situation occurred in crosses which involved the P_{26} strain.

Most of the F_1 endocrosses in the 3 cases at hand exhibited one fully fertile direction while the reciprocal was sterile or yielded a low number of progeny, frequently half a dozen of pupae or adults at most.

The sterile or almost sterile F_1 endocrosses were produced in most cases by females from Central America and males from northern South America. Two exceptions were the combinations P_{2a} X P_{22} and P_{26} X P_{23}. In the first case, sterile F_1 endocrosses were produced in both directions of crosses, and, in the second, in the direction involving P_{23} females.

Figure 2 is arranged like Figure 1. Data included in it are concerned with the experiments involving the strain from Trinidad (P_4) and 9 strains from Brazil (P_{12}, P_6, P_{13}, P_{10}, P_{16}, P_{17}, P_{18}, P_{20} and P_{21}). Except for the crosses of P_{12} X P_6, P_{10}, P_{18} or P_{21}, and the crosses of P_{10} X P_{17}, all of the other combinations showed high productivity in both directions of the parental crosses and in both directions of the F_1 endocrosses.

The isolation degrees in crosses of P_{12} X P_6 and P_{12} X P_{18} were the strongest among Brazilian combinations: in both cases, a single direction was fertile and the small number of produced hybrids failed to yield F_2. In this direction females were P_{12}. In the intercrosses of P_{12} X P_{21} a single direction (also involving P_{12} females) was also fertile, but the progeny was numerous and fully fertile.

The isolation in the combinations P_{12} X P_{10} and P_{10} X P_{17} affected a single direction of the parental crosses, causing the production of a small number of progeny. This direction involved P_{10} females in the crosses with P_{17}, and P_{10}

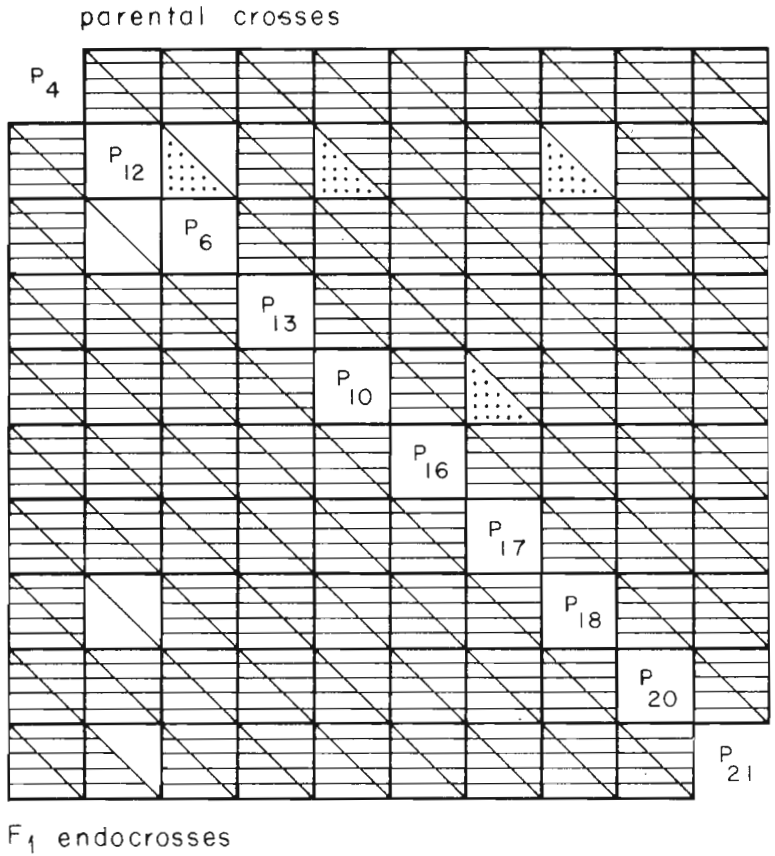

Figure 2. Fertility and fecundity of the parental crosses and F_1 endocrosses of strains from Trinidad (P_4) and Brazil P_{12}, P_6, P_{13}, P_{10}, P_{16}, P_{17}, P_{18}, P_{20} and P_{21}). For symbols, cf. Figure 1.

males in the crosses with P_{12}. The F_1 endocrosses were fully fertile in both directions of these combinations.

The results of crosses between strains from Colombia (P_5), Venezuela (P_{25}), and Guiana (P_{23} and P_{22}) and 4 Brazilian strains (P_{12}, P_6, P_{10}, and P_{21}) are given in Figure 3. In this Figure, data on parental crosses and F_1 endocrosses are presented separately, (3a and 3b, respectively). The triangles inside the small squares refer to both directions of crosses of every combination: those under the diagonal are concerned with crosses of females from strains included in the vertical column with males from the horizontal column; those above the diagonal

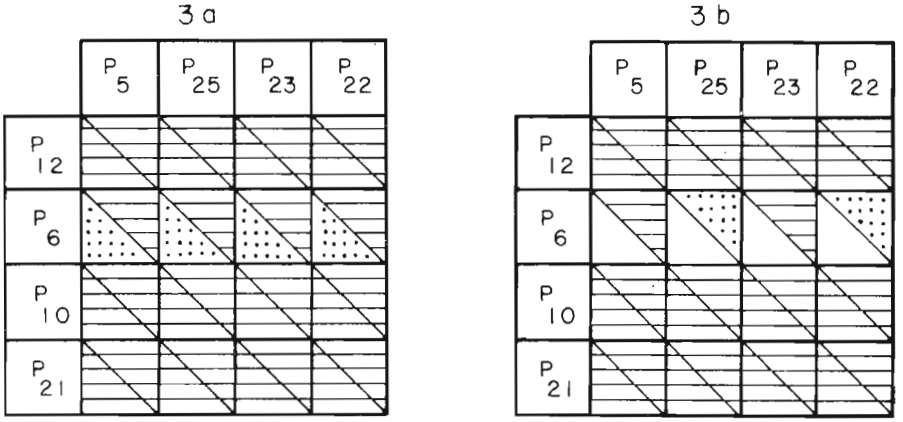

Figure 3. Fertility fecundity of the parental crosses (3a) and F_1 endocrosses (3b) of strains from Colombia (P_5), Venezuela (P_{25}) and Guiana (P_{23} and P_{22}) with Brazilian strains (P_{12}, P_6, P_{10} and P_{21}). For symbols, cf. Figure 1.

are concerned with the reciprocal crosses (females from horizontal column and males from vertical column).

The data in this Figure show that, except for the intercrosses which used the Brazilian strain P_6, all others succeded very easily at the parental and at the F_1 endocrosses level. The P_6 strain showed a decrease in its ability to hybridize and to produce fertile hybrids with any of the strains from northern South America. In all of the intercrosses involving that strain, the direction of parental crosses which used P_6 females showed low productivity and failed to produce fertile F_1 endocrosses.

Figure 4 (a and b) is arranged like Figure 3. It includes the results of the intercrosses between the strains from Costa Rica (P_1a, P_2a, P_2) and Panama (P_{26}) and the Brazilian strains P_{12}, P_6, P_{10} and P_{21}.

The combinations in this Figure exhibited strong degrees of isolation. Most of the parental crosses (Fig. 4a) were fertile in a single direction and this direction either failed to produce fertile F_1 endocrosses or produced only poorly fertile F_1 endocrosses (Fig. 4b). The origin of males and females in this fertile direction was variable except when parental crosses involved the strain P_2a. In this case every combination was fertile when females were P_2a. In general, the

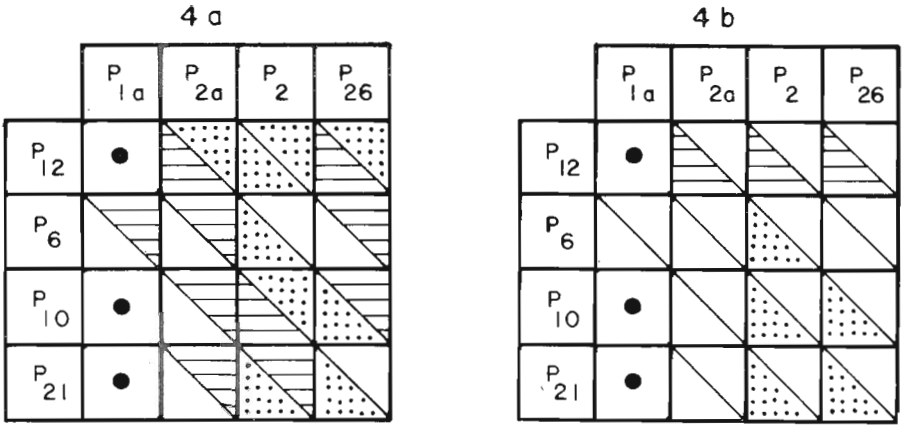

Figure 4. Fertility and fecundity of the parental crosses (4a) and F_1 endocrosses (4b) of strains from Costa Rica (P_{1a}, P_{2a}, P_2) and Panama (P_{26}) with Brazilian strains (P_{12}, P_6, P_{10} and P_{21}). For Symbols, cf. Figure 1.

sterility in the parental crosses was observed more often when females were from Brazil than when they were from Central America. The parental crosses of P_{12} were fertile in both directions, but one or both of these directions produced a small number of progeny. Their F_1 endocrosses did not yield any progeny when the males were P_{12}.

The other combinations which showed both fertile directions in the parental crosses (P_2 X P_{10}, P_2 X P_{21}, and P_{26} X P_{10}) produced fertile F_1 endocrosses only when Brazilian females were used, but even in these cases a small number of progeny was yielded.

Parental crosses involving P_6 showed a single fertile direction in every combination, but the F_1 endocrosses failed to produce progeny except in the crosses with P_2.

The number of females per sterile parental cross available for dissection at the time of analysis for any combination in this study varied from 6 to 20. No sperm was found in most of them. No inseminated female was detected in the sterile direction of the 3 combinations in Figure 2, and, among the 7 combinations which showed one sterile direction in Figure 4a, one inseminated female only was detected in the crosses of P_6 females X P_{26} males (1 inseminated: 6 dissected).

Females from several low productive parental crosses in Figures 1 to 4

were also dissected. From Figure 1: P_8 females X P_{28} males, P_2 females X P_{23} males, P_2 females X P_8 males, and P_{26} females X P_8 males. From Figure 2: P_{12} females X P_{18} males. From Figure 3: P_6 females X P_{22} males. From Figure 4: P_6 females X P_2 males. Most of the flies in every case had not been inseminated.

None of the dissected females from sterile or low productive crosses showed insemination reaction.

The backcrosses of females and males from sterile F_1 endocrosses showed that the males were sterile and the females were fertile in every case.

DISCUSSION

The genetic events leading to reproductive isolation and consequently to speciation are still unknown. Differences in gene regulation (King and Wilson, 1975, Wilson, 1975) or differences in structural genes not detected by current techniques (Ayala, 1976; Coyne, 1976) are hypotheses which still remain to be proved.

Species in "statu nascendi" (Dobzhansky and Spassky, 1959) constitute apparently the most promising material for the study of the genetic basis of speciation. Since evolution is a continuous process, many species are expected to present such borderline populations. However, examples of these species are relatively rare. The explanation advanced by Dobzhansky and his research group for this discrepancy is that the critical stages of the speciation process are passed rather rapidly (Dobzhansky, 1959, 1970; Richmond and Dobzhansky, 1976).

The most studied exemple of species in "statu nascendi" is *D. paulistorum*, considered as a superspecies evolving into 6 semispecies or incipient species (Dobzhansky and Spassky, 1959; Dobzhansky and Powell, 1975; Richmond and Dobzhansky, 1976; Ayala, 1976).

The present study indicated that *D. prosaltans* may constitute a species in the critical stages of the speciation process. Among the strains analyzed in this paper, those from Central America (Costa Rica and Panama) showed reproductive isolation in crosses with the strains from northern South America and from Brazil. The isolation degree was higher when Brazilian strains were used. In this case some combinations produced hybrids in a single direction of the parental crosses and the male hybrids were completely or almost completely sterile. Other combinations produced hybrids in both directions of crosses, but in many cases these hybrids were scarce and the males were completely sterile in one of the directions and almost completely sterile in the other.

The partial or complete absence of inseminated females among those

dissected from sterile parental crosses indicates that sexual isolation is an isolating mechanism which, with hybrid sterility, is acting to prevent laboratory crosses between strains from Central America and Brazil. In most cases, sexual isolation was stronger when the females were from Brazil than when they were from Central America, but when hybrids were produced in both directions of the crosses, male sterility was stronger in the direction involving females from Central America.

In crosses between strains from Central America and strains from northern South America, sexual isolation was less widespread than in crosses with Brazilian strains. This kind of isolating mechanism was only found in crosses involving strains P_{26} (Panama) and P_2 (Costa Rica). However, complete or almost complete hybrid sterility was detected in at least one direction of the crosses in every combination.

Despite the different behavior of strains from Brazil and northern South America in crosses with Central-American strains, most of the intercrosses of strains from northern South American and Brazilian strains were highly productive in both directions of the laboratory crosses and yielded fertile hybrids. However, one of the Brazilian strains, P_6, which intercrosses freely with the other Brazilian strains exhibited some sexual isolation and complete male sterility in the direction involving P_6 females and males from northern South America. The behavior of the Brazilian strain P_{12} should also be pointed out. This strain intercrossed easily with the strains from northern South America but showed a variable degree of isolation in crosses with other Brazilian strains. Thus, the behavior of P_{12} is closer to that of strains from northern South America than to that of other Brazilian strains. This fact was also observed in crosses with Central American strains: P_{12} showed a degree of isolation lower than that shown by other Brazilian strains and similar to that exhibited by strains from northern South America.

These results provided evidence for dividing the *D. prosaltans* strains into 3 isolation sets which obey the geographic origin of the strains on the basis of their ability to hybridize with each other. One of these sets is composed of strains from Central America, including Costa Rica and Panama; another is composed of strains from northern South America, including Trinidad, Colombia, Venezuela, Guiana and Brazil, north of the Amazon River; and the last is composed of the remaining Brazilian strains. In Figure 5 the 3 sets are respectively referred to as A, B, and C.

While the crosses of strains from different sets showed variable degrees of reproductive isolation, the results of tests within every set showed, with few exceptions, high productivity of parental crosses and fully fertile F_1 hybrids. The exceptions were strain P_2, in set A, and strain P_{28}, in set B. Both strains exhibited an incipient isolation in crosses with other strains from the same set.

Figure 5. Distribution area of the 3 isolation Sets. Set A = strains from Central America; Set B = strains from South America, north of the Amazon River; Set C = strains from Brazil, south of the Amazon River.

Strain P_4, from Trinidad, deserves a special mention. This strain barely intercrossed with a single strain from Set A (P_{26}), but intercrossed freely with the strains from Set B (except P_{28}) and with all of the strains from set C. The decision to include P_4 in set B and not in C was based mainly on geographical origin. In cases like this, it is possible that other methods may reveal lower degrees of isolation than those shown by mass mating crosses. On the other hand, we cannot discard the possibility that, if a greater number of mass crosses were prepared, few hybrids could be obtained in crosses which were completely sterile in these experiments. Aniway, the present data show very clearly the existence of a speciation process in progress in *D. prosaltans*.

In this study, laboratory strains were used. Many of them were kept as laboratory stocks for several years before these experiments were made. This situation must be mentioned because there is one well known case of origin of incipient isolation in the laboratory reported by Dobzhansky and Pavlowsky (1966, 1967, 1971) and Dobzhansky and Powell (1975). In *D. prosaltans,* however, the behavior of the strains consistent with their distribution area makes us believe that the isolation detected among the strains analyzed reflects the evolutionary stage of the natural populations from which they came, rather than an evolutionary response to laboratory conditions. Besides, the use of natural samples in studies of *D. prosaltans* is very difficult. This species, in spite of its large distribution area, is considered "rare". It has been collected, using different methods, in very low frequencies such as 1:10,000 (Cavalcanti, 1950), 1:2,700 (Pavan, 1959), and 1:200 to 1:2,000 (Mourão, 1966). A frequency of 1:50 was considered good (Bicudo *et al.,* 1978), but exceptionally good was a single collection of 28 *D. prosaltans* in 36 flies caught in the secretion of a *Campomanesia cagaiteira* Keaersk tree (Pavan, 1959). The impossibility of getting new samples using the same methods, and in the same places where the species was detected in previous collections, is another problem which has been mentioned by some authors (Cavalcanti, 1950; Pavan, 1959; this author's personal experience).

We still do not know if the "rarity" of flies in the collections really reflects the population size of *D. prosaltans* or is only the consequence of an almost complete absence of knowledge about feeding habits and their behavior in nature. Anyway, the "rarity" of the species makes the laboratory stocks very precious because they are the only available source of information on the species.

A discussion on the evolutionary status of *D. prosaltans* strains must involve concepts accepted by evolutionists. Ayala (1976) described 2 main stages in the geographic speciation. In the first, the allopatric populations (subspecies) exhibit incipient reproductive isolation in the form of partial hybrid sterility. In the second stage, the populations (semispecies) became sympatric and are

being submitted by natural selection to the development of more complete reproductive isolation mainly in the form of sexual isolation. Ayala (1976) mentioned, as examples of the first stage, 2 species from the willistoni group: *D. willistoni* and *D. equinoxialis* which are composed of 2 subspecies (*D. willistoni willistoni* and *D. w. quechua,* respectively, and *D. equinoxialis equinoxialis* and *D. e. caribbensis*). In both cases crosses of the subspecies in one direction produced sterile hybrid males, but no evidence of sexual isolation was obtained. The second stage of the speciation process was exemplified by *D. paulistorum* (also from the willistoni group), whose 6 semispecies show strong, although incomplete, ethological isolation and complete sterility of male F_1 hybrids. Some of the semispecies of *D. paulistorum* are sympatric, with apparently complete sexual isolation.

Although isofemale lines of *D. prosaltans* are not available at present for detection of sympatric reproductive isolation, the situation in *D. prosaltans* is to some extent comparable to the situation encountered in *D. paulistorum.* The presence of sexual isolation and male hybrid sterility operating between strains from Central America (set A) and Brazil (set C), and between Central America and northern South America (set B) is an argument to classify the 3 sets as 3 semispecies. The results of crosses between strains from sets B and C are similar to those of Transitional semispecies of *D. paulistorum* in crosses with Central American semispecies. Strains in set B are closely related to strains in set C, but, while some crosses of B x C succeeded easily and the hybrids were fertile, other crosses showed some sexual isolation and male progeny was sterile.

Accumulated data on chromosomal polymorphism of *D. prosaltans* revealed a total of 14 inversions distributed in the chromosome arms as follows: XL - 7 inversions; XR - 1 inversion; IIL - 1 inversion; IIR - 3 inversions; III - 2 inversions (Bicudo *et al.,* 1978). The gene arrangements of *D. prosaltans* strains used in the present study are shown in Table II. Among the 3 sets, set A is the most homogeneous as to chromosomal polymorphism: all of the strains in this set are homozygous for the inversion PXLa and for the standard arrangements in the other chromosomes (The P_{2a} strain has a homozygous and exclusive inversion overlapping PXLa: the PXLb inversion). However, the arrangements shared by the strains in set A are not exclusive of this set. The PXLa inversion was also found in the P_8 strain, included in set B and geographically close to set A; and the standard arrangements of chromosome arms XR, IIL, IIR and chromosome III were also found in some strains from sets B and C.

The distribution of the gene arrangements in the strains from sets B and C is as follows. Three inversions are shared by some strains from both sets (PIILa, PIIRa, PIIIb), while other inversions are exclusive of set B (PIIRb, PIIRc) or exclusive of set C (PXLd, PXRa). However, in both sets the exclusive inversions

Table II - Chromosomal arrangements of strains in every set. Data from Cavalcanti (1948); Bicudo (1967, 1973); Bicudo et al. (1978).

SET	STRAIN	Chromosomal arrangements				
		XL	XR	IIL	IIR	III
A	P_{1a}	a	+	+	+	+
	P_{2a}	ab	+	+	+	+
	P_{2}	a	+	+	+	+
	P_{26}	a	+	+	+	+
B	P_{4}	+	+	+	+	+
	P_{5}	ac	+	+	+ a	a
	P_{25}	+	+	+ a	+ b	+
	P_{22}	+	+	+ a	+	+
	P_{23}	+	+	+	+ bc	+
	P_{12}	+	+	+ a	+ a	+ b
C	P_{6}	+	+	+ a	+	+
	P_{13}	+	+	a	+ a	+
	P_{10}	+ d	+	a	+	+
	P_{16}	d	+	+	+ a	+ b
	P_{17}	d	+	a	+	+
	P_{18}	+ d	+	+ a	+	+ b
	P_{20}	+	a	+	+	+
	P_{21}	+	+	+	+	+

are only present in some of the strains.

The chromosomal 1 polymorphism in *D. prosaltans* strains of sets B and C resembles that of *D. paulistorum,* in which some inversions are shared by 2 or more semispecies, while other inversions are found in some species and not in others (Kastritsis, 1967, Dobzhansky and Powell, 1975). The monomorphism of *D. prosaltans* strains in set A is very interesting because it may be indicative of the origin and dispersion of the species.

According to Carson (1959), new strains arise mostly from peripheral populations of parental species. The peripheral populations are usually monomorphic (da Cunha et al., 1959; Carson, 1959). In this way the polymorphic sets of *D. prosaltans* strains (B and C) may be considered ancestral to set A. This idea is reinforced by comparison of the present isolation data with Kaneshiro's (1976) data. This investigator, in a study of Hawaiian *Drosophila,* assumed that females of derived species mate randomly with males of ancestral species but females of ancestral species show strong sexual discrimination against males of the more derived species. The justification was that the courtship pattern of the derived species has elements in common with the ancestral population so that the females may recognize those elements in the courtship of males of ancestral species. The same does not happen between females of ancestral species and males of derived species since the courtship of these males contains only some elements of the pattern of ancestral species.

Crosses between *D. prosaltans* strains from Central America and strains from Brazil showed sexual isolation which in most combinations was stronger when females were from Brazil than when they were from Central America.

Thus, based on Kaneshiro's assumption, it is also possible to consider Central America strains of *D. prosaltans* to be derived from South American strains. These observations are also consistent with the ideas of Throckmorton (1974) on the origin and spreading of the saltans subgroup. On the basis of the geographic distribution of the species (Magalhães, 1962), this author suggested that the saltans subgroup originated in South America, and diffused northward into Central America, Mexico and the Caribbean.

The discovery of semispecies in *D. prosaltans* increases the opportunity of getting new information on the speciation process of the saltans group and may become useful in the approach to general problems such as the kind and the amount of genetic changes involved in speciation.

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GENES AND GENE INTERACTIONS AFFECTING PROTEIN AND LYSINE CONTENT IN THE ENDOSPERM OF MAIZE*

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ABSTRACT

Eleven near-isogenic lines of the maize inbred Oh-43 homozygous for each of the genes *se*, bt_1, bt_2, *du*, fl_1, fl_2, *h*, o_2, su_1, su_2, and *wx*, their respective double mutant combinations involving the o_2 gene, and the *normal* inbred were used in this study. The objective was to determine the effect of the mutant genes and of the interaction of the o_2 gene in double mutant combinations with those genes on protein and lysine content of the endosperm.

There was a wide variation in protein and lysine content among the endosperm mutants. The genes bt_1, bt_2, and su_1 were associated with high levels of protein while o_2 tended to decrease protein percent whether singly or in combination with each of the other genes. Protein yield per endosperm was reduced in high protein mutants due to reduced kernel weight. *Opaque-2* also reduced protein yield. Endosperms homozygous for *se*, bt_1, bt_2, o_2, and su_1 had significantly higher lysine percent of sample than *normal*. *Brittle-2*

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was associated with high lysine levels, similar to o_2. Lysine percent of sample and lysine percent of protein were increased by the incorporation of o_2 in double mutant combination with each of the other genes except fl_2. No significant enhanced effect was observed when fl_2 was combined with o_2. Because no synergistic effect on lysine concentration was obtained in the double mutant fl_2 o_2, different pathways leading to differential synthesis of the protein fraction of the maize endosperm may exist in fl_2 and o_2 as compared to starch-modifying mutants with high lysine concentrations.

INTRODUCTION

Maize (*Zea mays L.*) is one of the main sources of dietary protein for animals, including man. However, it has long been known (Osborne and Clapp, 1908) that the main protein of the maize kernel, zein, is deficient in the essential amino acids, lysine and tryptophan. Early attempts by geneticists and breeders at changing protein composition have emphasized total protein without regard to protein quality. This was due, in part, to unsuitable analytical procedures for amino acids. Further research (Frey, 1951) indicated that, as the protein content increased, zein made up an increasing proportion of the total protein.

The discovery by Mertz, Bates, and Nelson (1964) that the *opaque-2* (o_2) gene causes about 70% increase in lysine content in the endosperm of maize, has aroused the interest of researchers in several countries. The better protein quality of *opaque-2* as compared to normal maize has been demonstrated for rats (Mertz, Veron, Bates, and Nelson, 1965; Rosa, Forsyth, Glower, and Cline, 1977a, 1977b), pigs (Pickett, 1966; Rosa et al., 1977a, 1977b), and humans (Bressani, 1966; Clark, Allen, Meyers, Tuckett, and Yamamura, 1967; Clark, Glover, Betz, and Bailey, 1977), Protein fractionation studies carried out by Jiménez (1966), Mossé (1966), and Mossé, Baudet, Landry and Moureaux (1966) showed that the high lysine content of *opaque-2* maize was due to a pronounced reduction in the amount of zein and a large increase in the amounts of albumin, globulin, and glutelin, which are rich in lysine.

There are many mutants in maize which produce striking changes in texture, form, and amount of endosperm. Some affect the gelatinization temperature (Kramer, Pfahler and Whistler, 1958; Sandstedt, Hites, and Schroeder, 1968) and digestibility of starch (Sandstedt, Strahan, Ueda, and Abbot, 1962; Sandstedt et al., 1968), and the synthesis of amylose, amulopectin, water-soluble polysaccharides, and sugars in the endosperm (Creech, 1965; Kramer et al., 1958).

The possible interactions resulting from the combination in double mutant genotypes of o_2 with each of the several endosperm mutants should be explored for

the possibilities of providing a variety of high quality protein corns with specific starch characteristics. This paper reports the protein and lysine content in the endosperm of several single starch-modifying mutants and their respective double mutant combinations with the o_2 gene in the background of the dent maize inbred Oh43.

MATERIALS AND METHODS

Eleven near-isogenic lines established in the inbred Oh43 homozygous for each of the genes, *amylose-extender (ae), brittle-1 (bt_1), brittle-2 (bt_2), dull (du), floury-1 (fl_1), floury-2 (fl_2), soft-starch (h), o_2, sugary-1 (su_1), sugary-2 (su_2),* and *waxy (wx),* 10 double mutant lines involving each of these genes with o_2, and the *normal* Oh43 inbred line were used in this study. The double mutant genotypes were isolated as described by Barbosa and Glover (1978a, 1978b).

Seeds of each line were planted in rows 3 m long and 1 m wide. Spacing between plants was 33.3 cm and there were 10 plants in each row (plot). The experimental layout was a randomized complete-block design with two replications. All plants were self-pollinated.

Harvest was made at maturity and ears were forced-air dried. Well-filled ears from competitive plants were selected. A 30-kernel sample from one ear in a plot of each single mutant line was used. A 30-kernel sample was taken from each of the double mutant lines by compositing 10 kernels from each of three ears. All samples were degermed for analysis. In this paper degermed kernel is referred to as endosperm due to the negligible contribution of the pericarp to the protein and lysine content as compared to that of the endosperm (Barbosa and Glover, unpublished).

Endosperm samples were ground in a Wiley-mill to powder form prior to defatting. The defatted samples were further ground to a very fine powder in a Wig-L-Bug (Crescent Dental Mfg. Co.). Protein percent (N x 6.25) was determined by the micro-Kjeldahl procedure. Lysine analyses were made by the ion-exchange column chromatography method.

RESULTS AND DISCUSSION

Data on endosperm protein percent and protein yield per endosperm are presented in Table I. Of the single mutants, o_2 showed the lowest protein percent, significantly different from that of most genotypes including *normal.* This and

Table I. Protein percent and yield in defatted endosperm of single and double mutant genotypes.

Genotype	Protein			
	%		mg/endosperm	
	Single mutant	Double mutant with o_2	Single mutant	Double mutant with o_2
ae	11.65 bc[1/]	10.90 cd	24.02 abcd	17.15 a
bt_1	12.35 b	10.25 cd	21.47 bcd	11.55 d
bt_2	14.90 a	15.60 a	15.22 e	8.48 e
du	10.95 bcd	9.65 d	26.00 ab	14.41 b
fl_1	10.90 bcd	10.05 cd	24.06 abcd	13.75 bc
fl_2	11.45 bc	11.25 c	23.76 abcd	16.49 a
h	11.35 bc	9.85 cd	28.53 a	15.94 a
o_2	9.40 d	–	18.69 de	–
su_1	12.40 b	13.25 b	19.84 cde	12,30 cd
su_2	11.45 bc	10.70 cd	24.83 abc	16.14 a
wx	10.00 cd	9.60 d	25.00 abc	17.18 a
Normal	11.45 bc	–	27.75 a	–
C.V.	6.43	5.14	10.40	4.50
$s_{\bar{x}}$	0.52	0.40	1.71	0.46

1/ Means within columns followed by the same letter do not differ at the 0.05 level according to Duncan's Multiple-Range Test.

Table I. Protein percent and yield in defatted endosperm of single and double mutant genotypes.

Genotype	Protein			
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bt_1	12.35 b	10.25 cd	21.47 bcd	11.55 d
bt_2	14.90 a	15.60 a	15.22 e	8.48 e
du	10.95 bcd	9.65 d	26.00 ab	14.41 b
fl_1	10.90 bcd	10.05 cd	24.06 abcd	13.75 bc
fl_2	11.45 bc	11.25 c	23.76 abcd	16.49 a
h	11.35 bc	9.85 cd	28.53 a	15.94 a
o_2	9.40 d	–	18.69 de	–
su_1	12.40 b	13.25 b	19.84 cde	12,30 cd
su_2	11.45 bc	10.70 cd	24.83 abc	16.14 a
wx	10.00 cd	9.60 d	25.00 abc	17.18 a
Normal	11.45 bc	–	27.75 a	–
C.V.	6.43	5.14	10.40	4.50
$s_{\bar{x}}$	0.52	0.40	1.71	0.46

1/ Means within columns followed by the same letter do not differ at the 0.05 level according to Duncan's Multiple-Range Test.

Table II. Differences in protein percent and yield (mg/endosperm) among genotypes.

Single *vs.* double mutant			o_2 *vs.* double mutant		
Genotypes compared	Difference		Genotypes compared	Difference	
	Protein percent	Protein yield		Protein percent	Protein yield
ae - ae o_2	0.75	6.87**	o_2 - ae o_2	-1.50*	1.54
bt_1 - bt_1 o_2	2.10**	9,92**	o_2 - bt_1 o_2	-0,85	7.14**
bt_2 - bt_2 o_2	-0.70	6.74**	o_2 - bt_2 o_2	-6.20**	10,21**
du - du o_2	1.30	11.59**	o_2 - du o_2	-0.25	4.28*
fl_1 - fl_1 o_2	0,85	10.31**	o_2 - fl_1 o_2	-0.65	4.94*
fl_2 - fl_2 o_2	0.20	7.27**	o_2 - fl_2 o_2	-1.85*	2.20
h - h o_2	1.50*	12.59**	o_2 - h o_2	-0.45	2.75
su_1 - su_1 o_2	-0,85	7.54**	o_2 - su_1 o_2	-3.85**	6.39**
su_2 - su_2 o_2	0.75	8.69**	o_2 - su_2 o_2	-1.30	2.55
wx - wx o_2	0.40	7.82**	o_2 - wx o_2	-0.20	1.51

**and **indicate significant differences at the 0.05 and 0.01 levels, respectively, according to Least Significant Difference Test.*

other similar results (Glover, Crane, Misra, and Mertz, 1975; Nacif, Barbosa, Anderson, and Saraiva, 1974; Nelson, 1967) indicate that the reduction in protein content is an effect commonly associated with the *o*$_2$ gene. High protein levels were associated with *bt*$_1$, *bt*$_2$, and *su*$_1$. This is certainly related to the reduced starch synthesis which occurs in these mutants (Cameron, 1947; Cameron and Teas, 1954; Teas and Teas, 1953). In general, protein percent was lower in the double mutants as compared to the respective single mutants (Tables I and II). On the other hand, all double mutants had higher protein levels than o$_2$.

Protein yield per endosperm depends on endosperm weight and protein percent. Barbosa and Glover (1978c) have presented data on 100-kernel weight for the genotypes reported here. Bedides *o*$_2$, the single mutants *bt*$_1$, *bt*$_2$, and *su*$_1$, wich showed high protein percent, also yielded significantly less protein than *normal* (Table I). *Opaque-2* also reduced protein yield when combined with any of the other genes, the differences between double mutants and their respective single mutants being highly significant (Table II). When compared to the single mutant *o*$_2$, all double mutant genotypes had lower protein yield.

Data on endosperm lysine percent of sample, lysine yield per endosperm, and lysine percent of protein (L/P) are presented in Table III. Lysine percent of samples in the single mutants ranged from 0.17 (*wx*) to 0.46 (*bt*$_2$). The mutants *ae, bt*$_1$, *bt*$_2$, *o*$_2$, and *su*$_1$ showed significantly higher lysine levels than *normal. Brittle-2* significantly exceeded *o*$_2$ in lysine percent. As shown in Tables III and IV, with the exception of *fl*$_2$ *o*$_2$ (*fl*$_2$ *fl*$_2$; *o*$_2$ *o*$_2$) all double mutants had signifi cantly higher lysine percent of sample than their respective single mutants. Also, with the exception of *fl*$_2$ *o*$_2$, all double mutants showed higher lysine percent than the single mutant *o*$_2$. The effect of the *o*$_2$ gene in increasing lysine content is thus comulative with the effect of the other endosperm mutants except *fl*$_2$.

Lysine yield per endosperm (Table III) was higler in *o*$_2$ than in any other single mutant. However, only *wx* yielded significantly less lysine than *o*$_2$. Small variation in lysine percent of sample and weight per endosperm have a large effect on lysine yield per endosperm. Since lysine percent of sample tends to be higher in genotypes having lower 100-kernel weight (Barbosa and Glover, 1978c), lysine yield per endosperm tends to decrease the variation among genotypes. This, in addition to a large coefficient of variation, resulted in only one significant differen ce among single mutants. Among the double mutants, high values for lysine yield per endosperm were found for *ae, su*$_2$ *o*$_2$, and *wx o*$_2$. In general, the double mutants yielded more lysine than their respective single mutants, but less than *o*$_2$ (Table IV).

Data on lysine percent of protein (Table III) shows a range from 1.68 (*h*) to 3.31 (*o*$_2$). The single mutants *ae, bt*$_1$, *bt*$_2$, *du, fl*$_2$, *o*$_2$, and *su*$_1$ had significantly

Table III. Lysine percent of sample, lysine yield, and lysine percent of protein in defatted endosperm of single and double mutant genotypes.

Genotype	Lysine					
	Percent of sample		mg/endosperm		Percent of protein	
	Single mutant	Double mutant with o_2	Single mutant	Double mutant with o_2	Single mutant	Double mutant with o_2
ae	0.27 bc[1/]	0.42 cd	0.56 ab	0.66 a	2.34 bc	3.86 bc
bt_1	0.32 b	0.51 bc	0.56 ab	0.58 abc	2.58 b	4.99 a
bt_2	0.46 a	0.80 a	0.47 ab	0.43 e	3.09 a	5.10 a
du	0.25 bcd	0.35 d	0.60 ab	0.53 cde	2.27 bc	3.66 c
fl_1	0.22 cde	0.41 cd	0.49 ab	0.56 bcd	2.04 cd	4.05 bc
fl_2	0.26 bcd	0.32 d	0.54 ab	0.46 de	2.27 bc	2.80 d
h	0.19 e	0.36 d	0.48 ab	0.58 abc	1.68 d	3.61 c
o_2	0.31 b	–	0.62 a	–	3.31 a	–
su_1	0.28 bc	0.57 b	0.44 ab	0.53 cde	2.25 bc	4.25 b
su_2	0.21 cde	0.43 cd	0.46 ab	0.65 ab	1.87 cd	4.01 bc
wx	0.17 e	0.36 d	0.43 b	0.64 ab	1.71 d	3.72 c
Normal	0.20 de	–	0.48 ab	–	1.72 d	–
C.V. (%)	10.84	11.18	14.47	7.24	9.62	5.15
$s_{\bar{x}}$	0.02	0.04	0.05	0.03	0.15	0.15

1/ Means within columns followed by the letter do not differ at the 0.05 level according to Duncan's Multiple Range-Test.

Table IV. Differences in lysine percent of sample, lysine yield (mg/endosperm), and lysine percent of protein among several genotypes.

Single *vs.* double mutant				o_2 *vs.* double mutant			
Genotypes compared	Difference in lysine			Genotypes compared	Difference in lysine		
	% of sample	yield	% of protein		% of sample	yield	% of protein
ae - ae o_2	-0.15**	-0.10	-1.52**	o_2 - ae o_2	-0.11**	-0.05	-0.55*
bt_1 - bt_1 o_2	-0.19**	-0.02	-2.41**	o_2 - bt_1 o_2	-0.20**	0.04	-1.68**
bt_2 - bt_2 o_2	-0.34**	0.04	-2.01**	o_2 - bt_2 o_2	-0.49**	0.19**	-1.79**
du - du o_2	-0.10*	0.07	-1.39**	o_2 - du o_2	-0.04	0.09	-0.35
fl_1 - fl_1 o_2	-0.19**	-0.07	-2.01**	o_2 - fl_1 o_2	-0.10*	0.06	-0.74**
fl_2 - fl_2 o_2	-0.06	0.08	-0.53*	o_2 - fl_2 o_2	-0.01	0.16*	0.51*
h - h o_2	-0.17**	-0.10	-1.93**	o_2 - h o_2	-0.05	0.04	-0.30
su_1 - su_1 o_2	-0.29**	-0.09	-2.02**	o_2 - su_1 o_2	-0.26**	0.09	-0.96**
su_2 - su_2 o_2	-0.22**	-0.19**	-2.14**	o_2 - su_2 o_2	-0.12**	-0.03	-0.70**
wx - wx o_2	-0.19**	-0.21**	-2.01**	o_2 - wx o_2	-0.05	-0.02	-0.41

** and ** indicate significant differences at the 0.05 and 0.01 levels, respectively, according to Least Significant Difference Test.*

higher lysine percent of protein than *normal*. Lysine percent of protein in bt_2 (3.09) was exceptionally high, though not significantly different from o_2 (3.31). It may be suggested from this study and protein fractionation studies (Misra, Jambunathan, Mertz, Glover, Barbosa, and McWhirter, 1972; Glover et al., 1975; Misra, Mertz, and Glover, 1975) that an effect similar to that of o_2 in reducing zein synthesis while enhancing the synthesis of the lysine-rich fractions may be found in other starch-modifying endosperm mutants. A comparison between the lysine percent of protein values of the double mutants with their single mutant counterparts indicates an interesting enhanced effect of the starch-modifying endosperm mutants when combined with o_2, except in the case of fl_2. The double mutant fl_2 o_2 had a value for lysine percent of protein intermediate between that of o_2 and fl_2 (Table III). These data support the studies by Misra et al. (1972; 1975) which indicated that different pathways leading to reduced zein synthesis may exist in the floury and starch-modifying mutants with high lysine concentrations.

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UTILIZATION OF GAMMA RAYS IN THE SELECTION OF *Aspergillus niger* FOR ACID PRODUCTION

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ABSTRACT

Selection of *Aspergillus niger* for acid production was studied by the method of Foster and Davis with the use of gamma rays. Three selection cycles were carried out, and the acid production character of each population was analyzed quantitatively by the unitage acid factor. Isolates with high unitage values in relation to the paternal strain were assayed in a liquid fermentation medium. No correlation was found that would indicate unlimited use of Foster and Davis' method in the selection of more productive strains.

INTRODUCTION

The filamentous fungus *Aspergillus niger* is an important industrial microorganism employed in the production of various organic acids, especially citric acid (Lockwood, 1975). In view of its industrial importance, the fungus has been the object of several studies directed mainly at increasing citric acid production by genetic improvement (Yuill, 1951; Ciegler and Raper, 1957; Tavares and Azevedo, 1974; Bonatelli, Jr., 1977). The better acid producing strains have been frequently assayed by fermentation, although the methods described by

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Foster and Davis (1949) and by James, Rubbo and Gardner (1956) also seem to be efficient. These methods are based on an indicator medium for culturing the fungus, where acid production is estimated by the relationship between the diameter of the halo and that of the colony. The objective of the research presented here was to study selection of *A. niger* for acid production by the method of Foster and Davis (1949), using gamma rays as a mutagenic agent. The possible correlation with acid production in a liquid medium was also studied in order to evaluate the method in the selection of the best producers.

MATERIAL AND METHODS

Media. The Complete Medium (CM) was a complex medium containing yeast extract, hydrolyzed casein, nucleic acid hydrolysate, vitamins etc. (Pontecorvo, Roper, Hemmons, MacDonald and Bufton, 1953). Two percent agar was added to the solidified medium. The Foster liquid medium was composed of glucose, peptone, monobasic potassium phosphate, and heptahydrate magnesium sulfate. Bromocresol green solution and 2% agar were added to the solidified medium (Foster and Davis, 1949). The fermentation medium was the same as used by Doelger and Prescott (1934).

Organism. The 10V10 strain of *Aspergillus niger* was utilized. The strain is of industrial origin and is used in the production of citric acid by the surface fermentation method. This strain, and its derivatives, were stocked at 4° C on CM slopes.

GENERAL TECHNIQUES

Radiation. Five day cultures on CM slopes were exposed to doses of 20, 40, 60, 80, 100 and 120 Kr of gamma rays (^{60}Co source). A non irradiated culture was used as control. The survival curve showed that 1.6% survival was reached with 80 Kr, which was the dose chosen for later studies.

Selection. The selection technique for acid production has been described by Foster and Davis and is based on a pH indicating medium. In order to obtain isolated colonies of the strain first exposed to radiation and of the control, the conidia were transferred to a Tween 80 solution (0.1% v/v) and agitated. The number of conidia in the suspensions were counted with a hemocytometer and appropriate dilutions were carried out. Aliquots of 0.1 ml of each conidia suspension were seeded in plates of complete medium and incubated for 3 days

at 28° C. Each colony, isolated at random, was inoculated into the center of a plate with solid Foster medium and incubated at 28° C for 4 days. Acid production by the different colonies was determined by the relationship between the diameter of the halo and that of the colony, which gave the unitage acid for each colony. Approximately 5% of the colonies derived from the irradiated strain showing high unitage acid values in relation to the controls were selected and submitted to a second radiation. The strains thus obtained were also submitted to the selection technique, and a third selection was carried out for the strains submitted to the second radiation treatment. Fifty colonies were assayed for each strain.

Fermentation. Conidia from isolates with high unitage acid values were transferred to CM-containing plates and purified. Conidia were collected from each colony (10^8 conidia/ml) and inoculated into liquid medium. The surface fermentation method was used, with 250 ml flasks, each containing 25 ml medium. Two different media (Foster's liquid and fermentation medium) were used. The flasks were incubated at 28°C for 5-9 days. Each was done in duplicate and total acidity was obtained by titrating 10 ml filtered medium with aliquots of NaOH 0.1 N using phenolphthalein (1%) as an indicator.

Statistical Analysis. Each population was analyzed quantitatively in terms of the acid production character by the unitage acid factor. Correlation analyses between unitage acid and colony diameter, unitage acid and total acid production, average unitage acid for each irradiated population and selection cycle, were carried out using correlation coefficients. Comparison of the unitage acid of irradiated strains and of the controls was made by variance analysis (Snedecor, 1956).

RESULTS

Fig. 1 shows the unitage acid values (A) and the diameter of the colony (B) for the populations submitted to the first (1i), second (2i) and third (3i) gamma ray radiation, and for the controls. The correlation coefficients between unitage acid and colony diameter for the irradiated and control populations are shown in Table I. This table shows that all r values were significant, thus indicating the existence of a negative correlation between unitage acid and colony diameter. The five correlation coefficients obtained were compared by the B test (Piedrabuena and Baracho, 1976), which shows that the coefficients of the irradiated strains behave as if belonging to the same population, but are different from those of the controls, probably showing the effect(s) of gamma rays on the population. The effect of gamma rays, or lack of it, on the unitage acid was determined by variance analysis (Table II), which showed that the value of F is significant. The

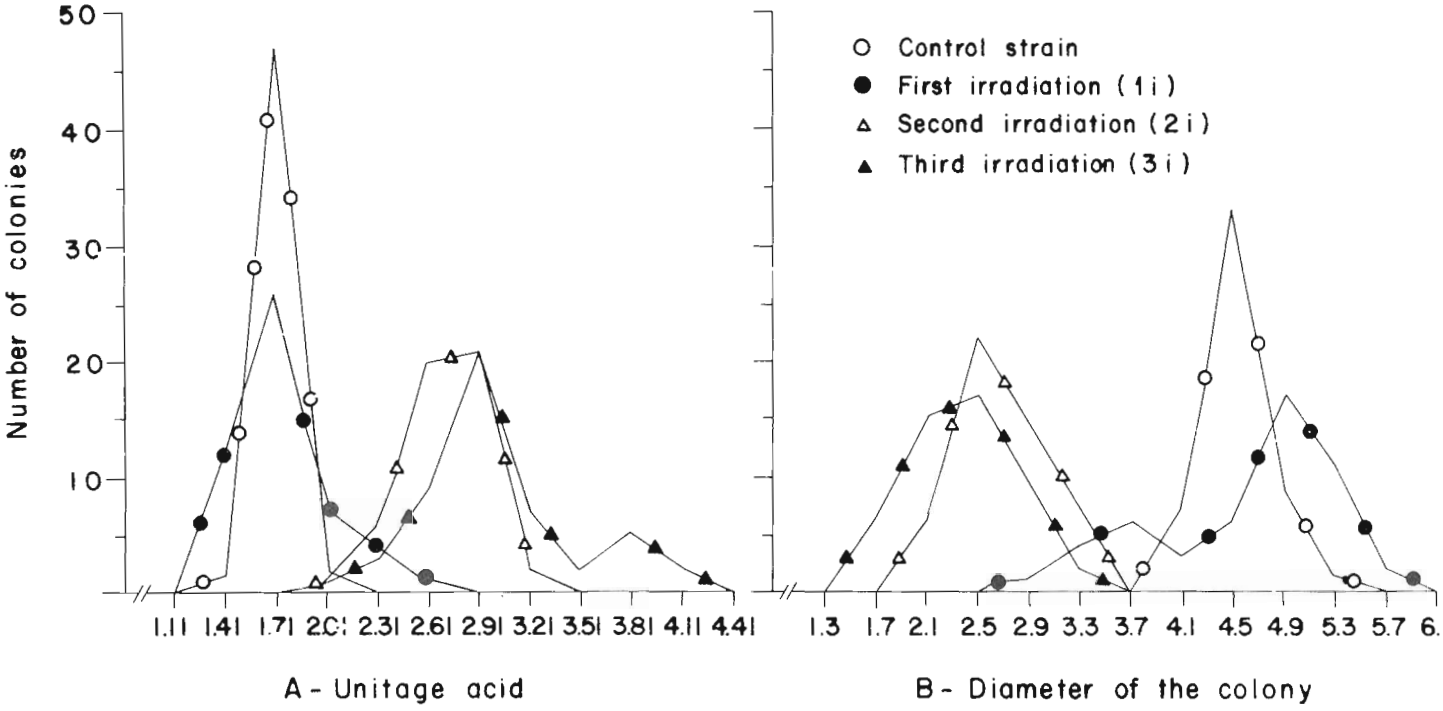

Figure 1. Distribution of unitage acid values (A) and diameter of the colony (B) for the strains submitted to the first (1i), second (2i) and third (3i) radiation with gamma rays (80 Kr) and controls.

Table I - Correlation coefficients between unitage acid and diameter of colony for the irradiated populations and control.

Strains	Correlation coefficient (r)
Control	r = - 0.48182**
(1i) First irradiation	r = - 0.88345
(2i) Second irradiation	r = - 0.87025**
(3i) Third irradiation	r = - 0.82218*

(**) Indicates significance at the 1% level.

Table II - Variance analysis for the effect of gamma ray treatment on unitage acid values.

Source of variation	D.F.	S.S.	M.S.	F
Treatment	3	63.367	21.122	227.118**
Error	196	18.348	0.093	
		C.V. = 13.2%		

(**) significant at the 1% level.

Table III - Comparison of the unitage acid averages among irradiated strains and control by the Tukey test.

Strains	Averages*
Control	1.70 a
(1i) First irradiation	1.77 a
(2i) Second irradiation	2.71 b
(3i) Third irradiation	3.00 c

* Averages with different letters are significant at the 1% level.

averages were compared by the Tukey test (Table III). The results indicate that the population belonging to the strain submitted to the third irradiation (3i) has the highest unitage acid average. Since three selection cycles were carried out, and each population had a unitage acid average, correlation was calculated, with a significant result (r = 0.95647), indicating a progression of the unitage acid character for each selection cycle, as shown in Fig. 2. Table IV shows the results of fermentation in two different media for isolates from populations submitted to radiation, the corresponding unitage acid and the most convenient fermentation time. The correlation coefficients between unitage acid and total acid production in the two culture media were calculated, and the *r* values were: *r* = - 0.0439 in the fermentation medium, and *r* = 0.1335 in the liquid Foster medium. Both *r* values are not significant, indicating no correlation between unitage acid and total acid production in either medium.

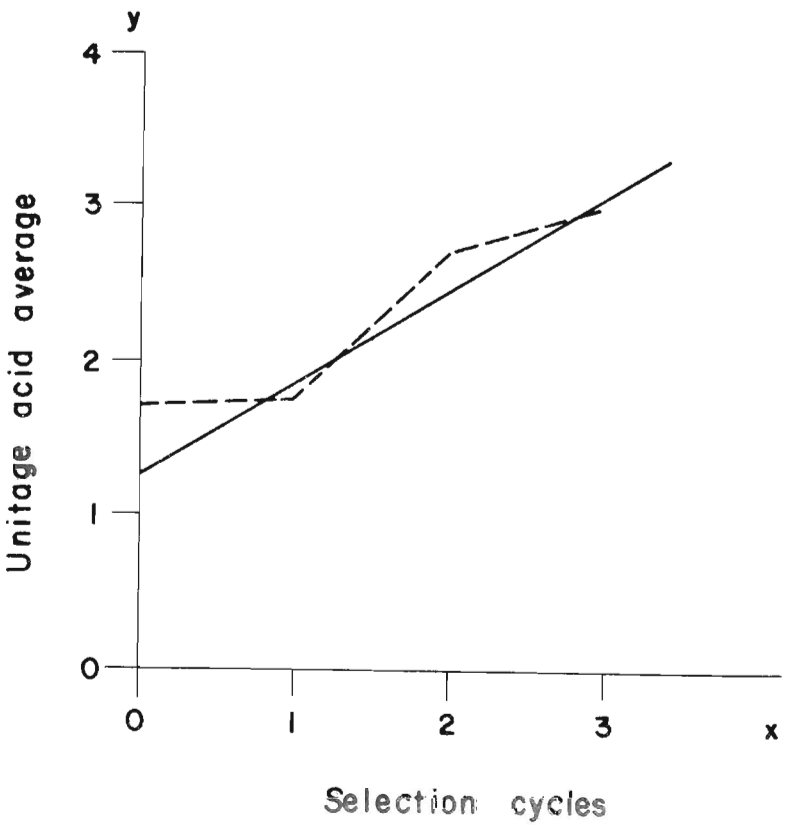

Figure 2. Calculated value Y = 1.26333 + 0.61500 x (————) and observed values (- - - - - -) for the unitage acid averages and selections cycles.

Table IV - Titration of the fermentation medium (F) and of the liquid Foster medium (L), days of fermentation and unitage acid values for isolates of irradiated strains derived from *Aspergillus niger*.

Isolates*	Unitage Acid	Culture Media	Days of Fermentation	Titration Values (ml of NaOH 0.1 N)
Control	1.70	F	9	74.3
		L	7	54.0
1 (1i)	2.50	F	8	36.7
		L	8	53.2
2 (1i)	2.42	F	8	71.4
		L	8	52.0
1 (2i)	2.92	F	9	152.4
		L	7	62.5
2 (2i)	2.50	F	9	17.7
		L	7	55.2
3 (2i)	2.42	F	7	100.0
		L	7	27.2
4 (2i)	2.34	F	8	108.6
		L	7	46.2
1 (3i)	3.04	F	9	32.0
		L	9	46.9
2 (3i)	3.26	F	9	54.0
		L	9	52.2
3 (3i)	3.34	F	9	22.0
		L	9	42.0
4 (3i)	3.75	F	9	102.0
		L	7	57.5

* 1i, 2i, 3i indicate the strains submitted to the first, second and third gamma ray radiation, respectively (80 Kr).

DISCUSSION

The results obtained for *r* (Table I) allow us to conclude that in the selection for acid production through high unitage acid slow-growing colonies can also be selected, as shown in Fig. 1 (B). The slow development of the colonies after treatment with physical mutagens has already been confirmed for *A. niger* (Hannan, 1972; Gardner, James and Rubbo, 1956), although the specific cause for this phenomenon remains unclear.

Tables II and III show that gamma rays provoked effect(s) of a probably genetic nature on the strain used. The irradiated populations showed greater variability than the non irradiated one in respect to unitage acid, indicating that radiation is an effective process for increasing variability, which is indispensable for further selection (Sermonti, 1969).

Fig. 2 shows the occurrence of positive correlation between the unitage acid average of the irradiated populations and the selection cycles, indicating a progress in selection. This fact allows us to conclude that it is possible to select strains with higher unitage acid values through gamma ray application, utilizing the solid Foster medium. It must be pointed out, however, that a continued unitage acid increase per each selection would result in stabilization of variability, even when it is stimulated by mutagenic agents, as verified in *Streptomyces* for streptomycin production (Alikhanian, 1962). This is probably the rule for any characteristic studied.

By the *r* values obtained through the analysis of the correlations coefficients between unitage acid and total acid production, it can be seen that no correlation exists between the two. On the basis of these results, it can be concluded that selection for unitage acid in solid Foster medium is not a *sine qua non* condition for the strain to have high productivity under industrial conditions. These data are in disagreement with those obtained by James *et al.* (1956), who found a positive correlation between metal-tolerant mutants with high unitage acid values and citric acid production. However, the results of James *et al.* (1956) were obtained only with mutant strains and not with prototrophic strains such as those used in the research presented here.

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CHROMOSOME NUMBER AND MEIOTIC BEHAVIOUR OF SOME WILD *MANIHOT* SPECIES NATIVE TO CENTRAL BRAZIL*

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ABSTRACT

Chromosome behaviour in meiosis was studied in seven wild *Manihot* (cassava, mandioca) species native to Central Brazil. These species are: *M. tripartita* Muell., *M. anomala* Poh), *M. zehntneri* Ule, *M. oligantha* Pax, *M. nana* Muell., *M. gracilis* Pax, *M. tomentosa* Pohl. The seven species had a regular meiosis with haploid number n = 18. No multi-associations, laggards or irregular distribution were observed. Pollen in the seven species has fair viability.

INTRODUCTION

The genus *Manihot* Adans is native to Tropical Central and South America. It contains about 98 species distributed from Mexico to South Brazil (Rogers and Appan 1973). Rogers and Appan divided the genus into 19 sections and considered *Manihot esculenta* Crantz (cassava, mandioca) as a complex hybrid wich occurs only by agricultural means. Taxonomic entities laid by Rogers and Appan are based purely on morphological characters. Although this genus includes cassava, the notable important staple food all over the tropics, it has received little cytological attention. Among the limited studies reported in the literature, Graner (1935) determined somatic chromosome number in *M. esculenta,* Cruz (1968) reported somatic chromosome number of 8 wild species. Magoon et al. (1969, 1970) studied

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meiotic behaviour in *M. esculenta* and *M. glaziovii.*

MATERIAL AND METHODS

Seven wild *Manihot* species were collected from different locations in the state of Goiás, Brazil. They are: *M. tripartita* Muell., *M. anomala* Pohl, *M. zehntneri* Ule, *M. oligantha* Paz, *M. gracilis* Pax, *M. nana* Muell., *M. tomentosa* Pohl (fig. 1). Seeds, or cuttings or whole plants were planted in the cassava germplasm collection at the "Instituto de Ciências Biológicas", Goiânia. When the plants flowered, inflorescences were fixed in a mixture of three parts of absolute alcohol and one part of propionic acid saturated with ferric acetate and kept in the refrigerator for 24 hours. The anthers were smeared with propionocarmine according to Swaminathan et al. (1954). Chromosome configurations in metaphase, chromosome distribution in anaphase I, and tetrad formation were studied . Pollen viability was determined by using acetocarmine and iodine stain. Five hundred pollen grains per species were examined.

RESULTS

Chromosome associations showed extreme regularity in metaphase I in all seven species. Formation of 18_{II} was seen in all the investigated species (Fig. 2). Anaphase I was usually normal with an equal distribution of 18 chromosomes to each pole. No laggards, delayed separation of bivalents, restitution nuclei or polyads were observed in any of the 50 pollen mother cells examined for each species. Pollen viability was found to be as follows: *M. tripartita* 90.6%, *M. anomala* 92,4%, *M. zehntneri* 91.3%, *M. oligantha* 90.1%, *M. gracilis* 94.7%, *M. nana* 92.8%, *M tomentosa* 90.4%.

DISCUSSION

Genetic number was found to be 18 in the seven wild species. Of these species, *M. anomala, M. zehntneri, M. oligantha, M. nana* and *M. tomentosa* had their chromosome number determined here for the first time. The chromosome number of *M. tripartita* and *M. gracilis* agrees with that reported by Cruz (1968) in somatic tissues. Cruz, studying somatic chromosome number in root tips of 8 wild species, found it to be 36 for all of them. Magoon et al. (1970), reported

the same number in *M. glaziovii.* The known chromosome numbers in wild *Manihot* species can be tabulated as follows:

Table I. Chromosome number in wild *Manihot* species.

Species	Habit of growth	n	2n	Author
M. handroana	Shrub	–	36	Cruz (1968)
M. jolyana	Shrub	–	36	Cruz (1968)
M. tripartita	Shrub	–	36	Cruz (1968)
M. tripartita	Shrub	18	–	Nassar (This paper)
M. tweedieana	Shrub	–	36	Cruz (1968)
M. pedicellaris	Shrub	–	36	Cruz (1968)
M. gracilis	Sub-shrub	–	36	Cruz (1968)
M. gracilis	Sub-shrub	18	–	Nassar (This paper)
M. dichotoma	Tree	–	36	Cruz (1968)
M. glaziovii	Tree	18	–	Magoon et al. (1970)
M. glaziovii	Tree	–	36	Cruz (1968)
M. anomala	Shrub	18	–	Nassar (This paper)
M. zehntneri	Shrub	18	–	Nassar (This paper)
M. oligantha	Sub-shrub	18	–	Nassar (This paper)
M. nana	Sub-shrub	18	–	Nassar (This paper)
M. tomentosa	Sub-shrub	18	–	Nassar (This paper)

Reports in the literature are in agreement that 2n = 36 for *M. esculenta*, cassava (Graner, 1935; Abraham, 1944; Cruz, 1968). Also there is agreement on regular 18 bivalent formation in different cultivars of cassava (Abraham, 1944; Magoon et al., 1969, Sohmer, 1968). Bolhuis (1953), Jennings (1959), Lanjouw (1939), and Magoon et al. (1970) reported easy and successful crossability between cassava and a number of wild *Manihot* species. Moreover, Jennings (1963), reported higher fertility for hybrids of cassava and some wild *Manihot* species. Rogers and Appan (1973), considering frequent hybridity between cassava and local wild relatives, assumed that natural hybridization must have played a large role in evolving different species in this genus. They were guided in this idea by the hypothesis of Harlan (1961) that a number of wild species may have developed as a results of change hybridization between crops cultivars and local wild species. Attempts at crossing cassava with the wild species mentioned here are under way. Preliminary results are in agreement with previous reports. A review of the literature, combined with regular meiosis seen in the investigated species, indicates that cytological barriers have not yet been established in this biological group.

The seven wild species showed fair pollen fertility compared to varying degrees of sterility in cassava cultivars reviewed in the literature (Cours 1951; Sohmer 1968; Magoon et al., 1968). Low fertility of cassava cultivars in comparison to the wild species may be attributed to maintenance of those cultivars for many centuries by means of vegetative reproduction. This would lead to accumulation of spontaneous mutations. As plants never passed through a sexual reproduction cycle, most of these mutations had no chance of being eliminated.

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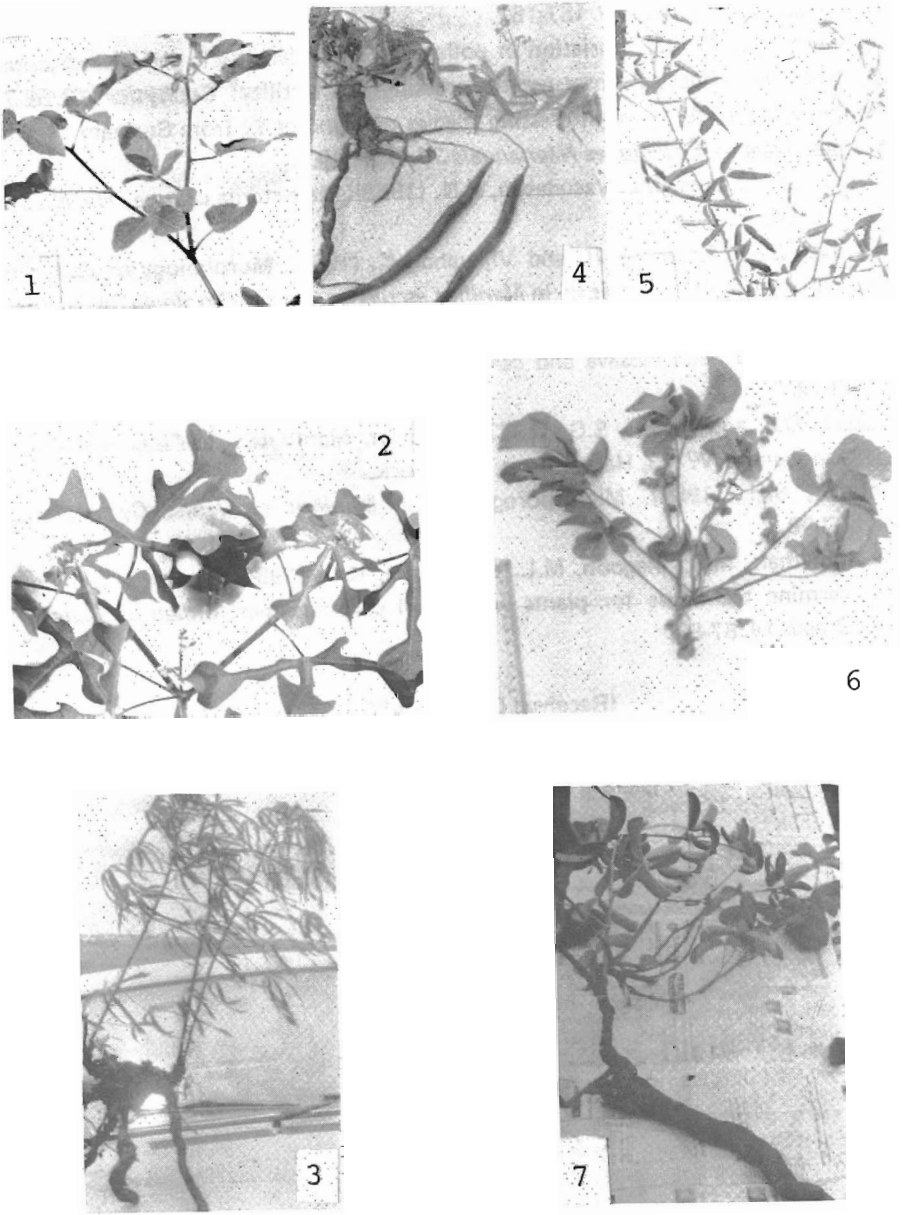

Figure 1. Growth habit and leaf shape of *M. tripartita* (1), *M. anomala* (2), *M. zehntneri* (3), *M. oligantha* (4), *M. gracilis* (5), *M. nana* (6), and *M. tomentosa* (7).

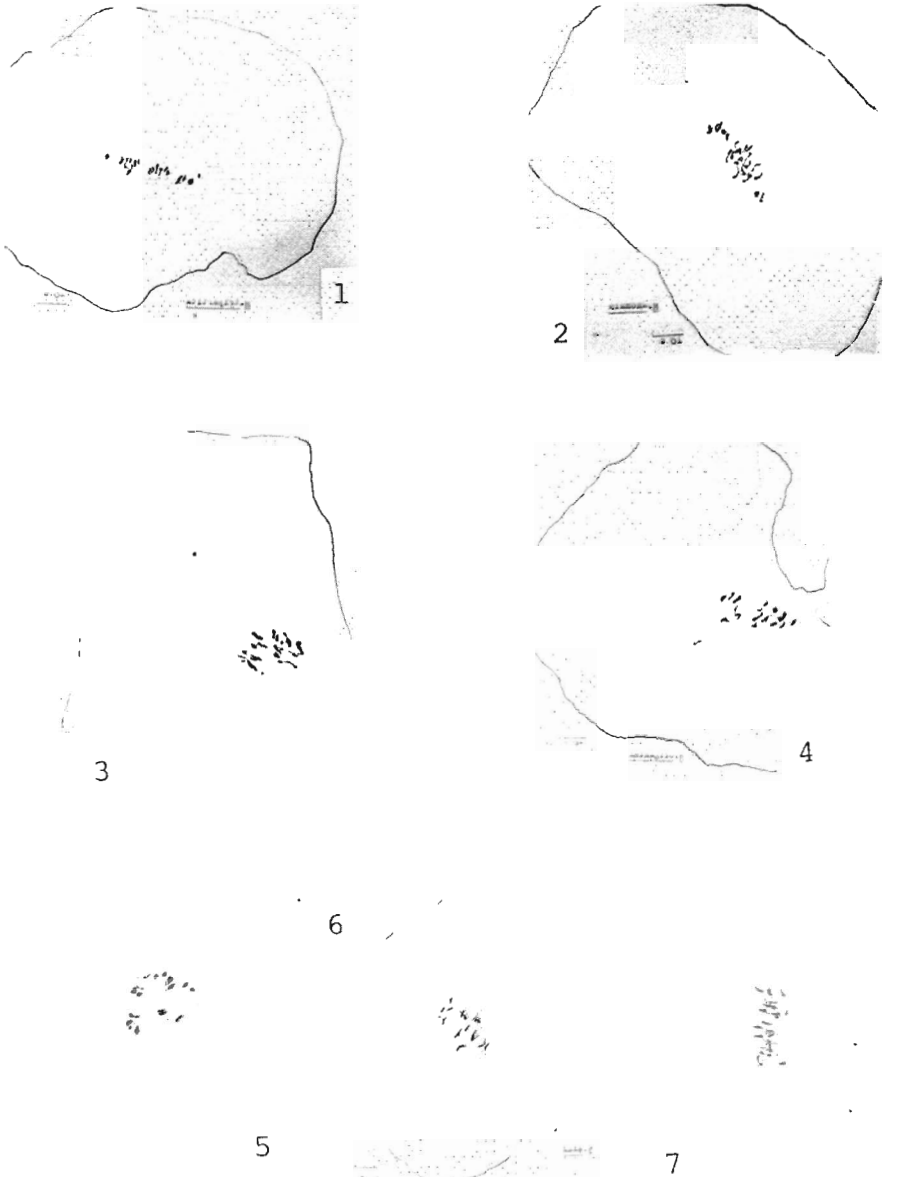

Figure 2. Camera lucida drawings of metaphase I in PMC's of *M. tripartita* (1), *M. anomala* (2), *M. zehntneri* (3), *M. oligantha* (4), *M. gracilis* (5), *M. nana* (6), and *M. tomentosa* (7), showing formation of 18 bivalents in these species.

GENETICS OF DYGGVE-MELCHIOR-CLAUSEN SYNDROME

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ABSTRACT

Twenty-three kindreds including 37 patients with Dyggve-Melchior-Clausen (DMC) syndrome were analyzed by means of the "a priori method" by postulating complete (truncate) ascertainment, and through "sib method" by assuming single incomplete ascertainment, these being two opposite situations of sampling. The frequencies of affected subjects were 28.9% and 23.1% respectively, which do not significantly differ from 25%. These estimates support the recessive nature of the syndrome. The sex ratio of 20 females to 17 male patients reveals autosomal transmission of the disease. Data concerning parental consanguinity and reproductive performance of the patients indicate that prevalence of syndrome could be as low as 1:100,000, close to be expected mutation rate.

INTRODUCTION

Dyggve-Melchior-Clausen syndrome (DMC) is an osteochondrodysplasia usually included in the spondyloepiphyseal dysplasic group provided that mostly spinal and epiphyseal lesions are present. It should be a relatively rare disease, considering that, since its first description about fifteen years ago (Dyggve, Melchior and Clausen, 1962), only 23 sibships including 37 cases were reported in the

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literature.

Some clinical manifestations of the disease remain to be elucidated because some of the patients reported had symptoms resembling Morquio disease and/or Hurler syndrome (Dyggve *et al.*, 1962), while other investigators have claimed the presence of an abnormal mucopolysaccharide in the urine of affected subjects (cf. McKusick, 1972, pp. 527 and 610). Even the presence of mental retardation has recently been questioned by Spranger *et al.* (1976) who postulated the existence of two forms of the disease.

The following features should be considered as typical for DMC syndrome: (1) severe dwarfism caused by bone deformities, generalized platyspondily, irregularities of the iliac crest with lacy swollen appearance, probably due to slow and irregular ossification, i. e., to a deficiency in the formation of the cartilage-bone matrix (Rimoin *et al.*, 1974). (2) Absence of abnormal mucopolysacchariduria (MPS) and metachromasia in white blood and bone marrow cells, in contrast to MPS IV (Morquio) and MPS I (Hurler) diseases. (3) Mental retardation was not present in all typical cases described so far, but whether its etiology was endogenous or cultural, has not yet been clarified. Consequently, diagnosis of DMC syndrome is basically confirmed by radiological examination.

The authors have extensively investigated a typical kindred including three normal and two affected sibs. Clinical aspects of the investigation have been published elsewhere (Toledo *et al.*, 1978). The purpose of the present article is to evaluate genetical data reported to date for DMC syndrome, which was previously thought to be caused by recessive genes (McKusick, 1975, no. 22380).

DATA

Table I summarizes the relevant genetical data reported in the investigations published in the pertinent literature. Before Dyggve *et al.*, (1962) defined the clinical condition as a syndromic entity, three independent reports described detailed clinical aspects of DMC syndrome (Faucell et al., 1942, Smith & McCort, 1958, and Habaeck, 1961). Even Dyggve, Melchior and Clausen suggested that the disease could be an inborn error of metabolism, but investigations published afterwards (Table I) could not support this initial supposition (discussion in Toledo *et al.*, 1978).

No comprehensive genetical analysis was made with data published so far for DMC disease. In the investigations reported in the literature (Table I), no special emphasis was placed upon the genetical aspects of the disease. Therefore, no systematic ascertainment of the affected subjects was performed aiming at a

Table I - Previously reported genetical data concerning DMC patients.

no. of sibships	no. of patients	ratio of abnormal to normal sibs	sex ratio of affected sibs	parental consanguinity	references
1	2	2:4	1:1	1C	Faucell *et al.*, 1942
1	3	3:4	3:0	(na)	Smith and McCort, 1959
2	2	(na)	0:2	(na)	Hobaeck, 1961*
1	3	3:5	2:1	UN	Dyggve *et al.*, 1962
1	1	(na)	1:0	(na)	Gwinn and Barnes, 1968*
1	1	(1:0)	0:1	ab	Kauffmann *et al.*, 1971*
1	1	1:1	0:1	(na)	Barylak and Kozlowski, 1972
1	5	(na)	3:2	pnd	Afifi *et al.*, 1974*
1	2	2:8	1:1	ab	Naffah and Taleb, 1974
9	10	(na)	4:6	pnd (2) ab (7)	Spranger *et al.*, 1975*
1	3	3:0	2:1	2C	Spranger *et al.*, 1976
2	2	2:5	2:0	2C	Naffah, 1976
1	2	2:3	1:1	3C	Toledo *et al.*, 1978
23	37	18:30 (37.5%)	20:17 (1:18)	8:9 (47%)	

ab = absent; pnd = present but not determined; UN = uncle-niece; 1C = first cousin; 2C = second cousin; 3C = third cousin; na = data not available.

* not included in the segregation analysis; ( ) not included in the total.

genetical-statistical analysis of the families with affected children, and many papers do not report basic genetical data. At least ten families included recurrent cases but parental consanguinity was not clearly mentioned (Table I).

GENETICAL ANALYSIS

Family data were tentatively evaluated by two statistical approaches

Table II. Genetical analysis of Dyggve-Melchior-Clausen syndrome.

I. By the "a priori method" assuming complete ascertainement

Size of sibship (s)	Number of sibships (n)	Total no. of indiv. (sn)	Total no. of indiv. (corr)** (sn_c)	no. of affected indiv. observed (o)	no. of affected indiv. expected (e)	variance (σ^2)	% of affected indiv. (corr)** (f_c)
*1	1	1	4.0	1	1.00	0.	25.0
2	1	2	4.6	1	1.15	0.122	21.7
3	2	6	10.3	4	2.59	0.526	38.8
4	1	4	5.8	1	1.46	0.420	17.2
5	1	5	6.6	2	1.65	0.502	30.3
6	1	6	7.3	2	1.83	0.776	27.4
7	1	7	8.0	3	2.00	0.970	37.5
8	1	8	8.9	3	2.23	1.172	33.7
10	1	10	10.6	2	2.65	1.592	18.9
Total	10 (Σn)	48 (Σsn)	62.1 (Σsn_c)	18 (Σo)	15.56 (Σe)	6.08 (σ_t^2)	28.9 (f_{t_c})

* Not included in total because it does not give statistical information;

** corr. = corrected

$f_{t_c} = \Sigma o/\Sigma sn = 37.5\%$; $e = 1/4sn_c$; $f_c = o/sn_c$; $\sigma = \sqrt{6.08} = 2.47$; $d = \Sigma o - \Sigma c = 2.44$;

$d/\sigma = 0.99$; $P \simeq 0.30$

(Continued on page 63)

Table II. - (Continued)

II. By the "sib method" assuming single incomplete ascertainment

$$P = \frac{R - N}{T - N} = \frac{19 - 10}{49 - 10} = 0. \quad (23.1\%)$$

$$\sigma^2 = \frac{pq}{T - N} = 0.004; \quad \sigma = 0.068$$

$$d = 0.250 - 0.231 = 0.019; \quad d/\sigma = 0.28; \quad P \simeq 0.75$$

R = number of affected individuals in all sibships
T = total number of individuals in all sibships
N = number of sibships

Probable proportion of affected individuals between 23.1 and 28.9.

assuming both complete (truncate) and incomplete single ascertainments (Emery, 1976). By postulating a complete ascertainment, ten informative sibships presented in Table I were analyzed by means of the "a priori method" (Hogben, 1946). Observed frequency of affected sibs was 37.5%, which, when corrected, becomes 28.9% (Table II). This value does not depart significantly (P = 0.30) from the expected one (25%). Smallness of samples as well as lack of a complete survey of the normal sibs account for large (random) fluctuations in the frequencies of affected children belonging to sibships of different sizes.

In addition, judging by the nature of the data, mostly composed of families referred to clinicians, it is reasonable to presume that ascertainment was nearer

to the single incomplete type (cf. Fisher, 1934). By applying the "sib method" to family data (Table II), the proportion of affected children in the sibships (23.1 ± 6.8%) does not differ significantly ($p = 0.75$) from the theoretical value of 25%. Since both sexes are affected, autosomal transmission should be presumed. In fact, among 37 cases of DMC syndrome so far reported (Table I), sex-ratio is near unity (1.18). These results represent a strong indication that DMC syndrome follows an autosomal recessive pattern of inheritance. However, this conclusion must be confirmed by analyzing family data with more proper statistical techniques.

DISCUSSION

Genetical analysis of the reported cases leaves no room for doubt as to the recessive nature of the syndrome. However, pertinent information published so far has not stressed the genetical aspects of the disease, thus providing no data for a sound segregation analysis. Furthermore, on the basis of recessive inheritance, heterozygotic manifestations are likely to be discovered in the patients' parents under detailed clinical and laboratory examinations.

Another point deserves some attention. All types of parental consanguinity as indicated by kindreds with relevant information (Table I), reach 50%. By interpreting anamnestic data reported in the literature, the proportion of parents who are first-cousins is at least 10%, but it is more likely to be around 30%. The rate of first-cousin marriages prevailing in the populations from which the samples were taken is between 5 and 0.5%, the first value being more realistic (Saldanha, 1960, cf. Table I). According to Dahlberg's (1947) formula, these figures indicate that homozygote frequencies should be lower than:

$$q = \frac{c(1 - k)}{16k - 15c - ck} = 0.0072 \text{ and } q^2 = 0.00005^*,$$

a value in agreement with the rarity of the disease.

Furthermore, the absence of miscarriages, stillbirths and prenatal deaths in the sibships would suggest that the mutant gene is not under strong selective pressure during intra-uterine life. Otherwise, affected subjects are usually infertile so that fitness is expected to be null. Thus, mutation rates are likely to be of the

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* Where q is the gene frequency; c, the proportion of first-cousin marriages in the population; and k, the proportion of first-cousin parents bearing affected individuals.

same magnitude as homozygote prevalence in the population, by assuming balance between mutation and selective pressures.

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CLINICAL ANOPHTHALMIA AND TRANSLOCATION 7/15*

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ABSTRACT

A 20-month-old boy with apparent total absence of eyeballs and no evidence of rudimentary eyes on palpation of the orbital cavities, micrognathia, underdeveloped genitalia, neuropsychomotor retardation, generalized hypotonia and translocation 7/15 is presented. This is the first case reported in the literature of clinical anophthalmia associated with the structure of chromosome 7 or 15.

INTRODUCTION

Clinical anophthalmia has been described as associated to trisomy 13 and has been reported as occurring in other chromosomal aberrations by several authors (Table I). A different chromosome aberration was involved in each case, and only in two instances were banding techniques employed (Donoghue, 1976; Serville

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Table I. Clinical anophthalmia and chromosome aberrations.

Author	Age	Chromosome analysis	Malformations reported
Barbuta, Scripcaru and Harmanschi (1969)	16 months	46,XX/47,+G(?)	Mental deficiency, hypotonia
Chang, Perciaccante Miller, Rottino, Behanna and Bailey (1975)	death at 42 days	46,XY,-E,+r (17 or 18)	Absence of left optic nerve and eyeball, increased sagittal diameter of head, long thumbs, valgus deformity of feet.
Donoghue and Harvey (1976)	13 1/2 years	46,XX/47,XX+mar	Mental deficiency, retardation of growth, microcephaly, port wine stain on the neck, large great toes, gap between 1st and 2nd toe, valgus deformity of ankles.
Masket, Galioto and Best (1970)	30 months	46,XY/-60,XY	Mental deficiency, growth retardation, hypotonia, slight frontal bossing, patchy incomplete alopecia on back of head, shortened 5th finger, four toes bilaterally, minor club foot deformities, spina bifida, bilateral, dislocation of hips, crossed fused renal ectopia.
Serville, Broutest Peyeresblanques and Bouineau (1974)	26 months	t (4, 14)	Psychomotor retardation, lateral fissure of the face, chondrocutaneous appendices anterior to tragus, fusion of vertebral bodies, presence of hemi-vertebrae.
Wallace and Anderson (1964)	35 years	45,XX,t(B/D)	Mental retardation, cleft lip and palate, malformed low set ears, deafness, microcephaly, partial syndactily of fingers and toes.
Welter, Lewis, Scharf and Smith (1974)	20 years	47,XXY	Mental retardation, severe flexion contracture of lower extremities, rockerbottom, feet arachnodactylia, high arched palate, features of Klinefelter syndrome.
Present case	20 months	46,XY,t(7; 15)	Neuropsychomotor retardation, micrognathia, underdeveloped genitalia, microcephaly, hypotonia.

et al., 1958).

This report describes a case of clinical anophthalmia associated with severe neuropsychomotor retardation and other minor malformations, and with translocation involving chromosomes 7 and 15, resulting possibly in deletion of the terminal segment of the long arm of chromosome 15.

CASE REPORT

The proband, E.S.D. (Fig. 1), a 20 month old boy, born in Nova Andradi-

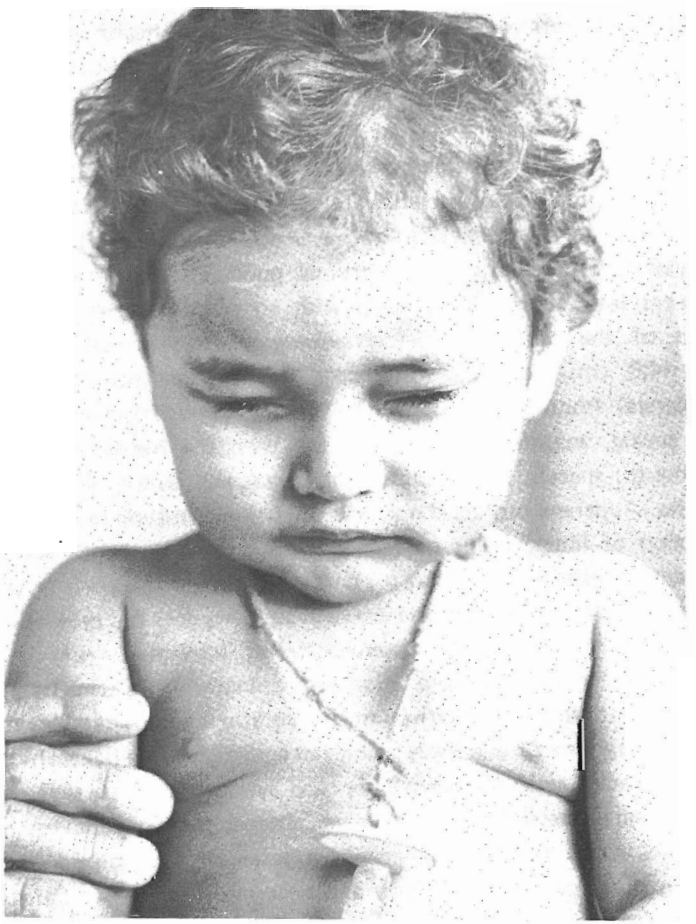


Figure 1. General aspect of the proband

na, Mato Grosso, Brazil, was examined for the first time on June 24, 1976 (RG. 1.160.437). Absence of eyes was detected at birth. A delay in his neuropsychomotor development was noted. *Antecedents:* first child, born of normal gestation (no bleeding, infections, fevers, drug ingestion or X-Ray exposure during gestation). Normal delivery with cephalic presentation, cried immediatly after delivery, no information available about birth weight or height, but was reported as being rather small; remained 5 days in hospital for maternal reasons; poor sucking during first few days; normal sucking afterwards; did not develop jaundice; could lift his head at 5 months; presented the first social smile at 5 months; maintained the sitting position without support at 7 months; has no locomotion, pronounces only 2 or 3 syllables, has no sphyncter control.

Mother: 22 years old; father: 24 years old; in good health and non consanguineous; brother: 6 month old boy, apparently normal; no history of similar or other genetic anomalies in the family. *Clinical examination:* Hypertrophy of submandibular and axillary ganglia; throat hyperemia; tachycardia with thythmical and normal murmurs; liver palpable on costal margin. *Somatoscopic examination:* normal, but thin, normally implanted hair; micrognathia, normal palate; short neck; underdeveloped genitalia. *Ophthalmologic examination:* presence of eyebrows, eyelashes, eyelids, upper and lower lachrymal punctae, conjuntivae with ample fornix; apparent total absence of eyeballs with no evidence of rudimentary eyes on palpation of the orbital cavities. *Neurological examination:* skull: cranial perimenter = 44.0 cm; biauricular distance = 26.5 cm; anteroposterior distance = 27 cm; bilateral frontal depressions. The child showed irritability at examination, strong crying, voluntary grasp of objects with the hands, recognition by touch and mouth; speech: understands simple requests poorly and pronounces 2 to 3 syllables. Apparent profound mental retardation. Attitude in dorsal position with semiflexion of limbs; holds objects when put into his hands and changes them from hand to hand; sits without support when left in this position; does not crawl or maintain orthostathic position even with support; does not present apparent deficiencies. Presents discontinuos antero-posterior movements of the trunk. Stereotyped movements of hands (pronation and supination) and arms (semiflexion and abduction), discontinuos and arrhythmic. Myotatic reflexes: patellar, adductor and bicipital were symmetric and lively; achillean and tricipital reflexes: hypoactive and symmetric. Facial axial reflexes: lively and symmetric. Superficial reflexes: cutaneous abdominal present and symmetric; cutaneo-plantar reflexes: bilateral sign of Babinski. Does not present primitive reflexes. Generalized hypotonia (Fig. 2), at palpation, passive movements and swinging. Cranial nerves from V to XII: normal. *Complementary examinations:* Skull - X rays: omega-shaped sella turcica; increased optic holes and symmetry of orbits. Electroencephalogram:

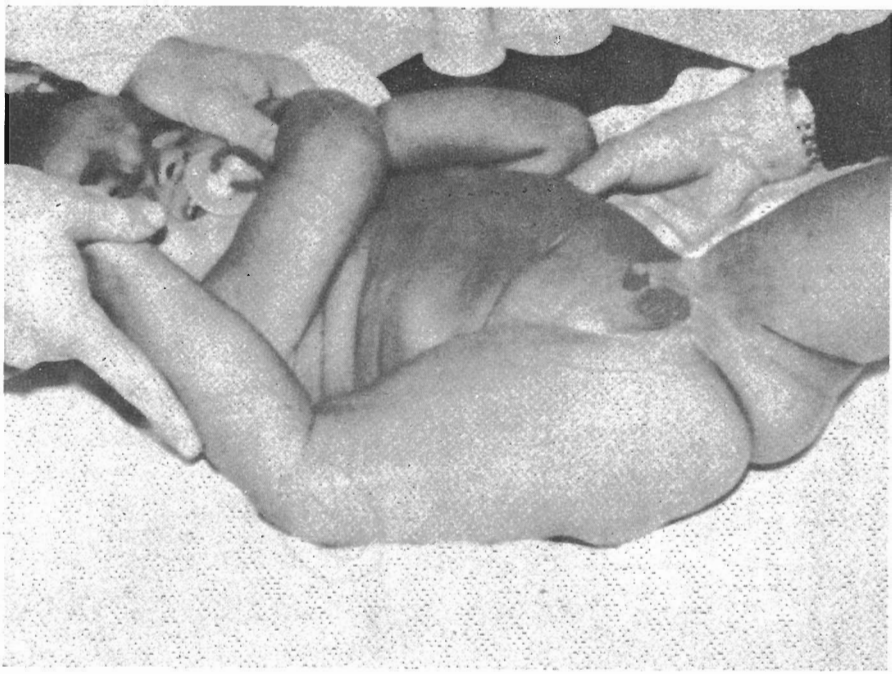


Figure 2. Hypotonia and hypogenitalism of the proband

normal. Cerebrospinal fluid: normal. Pneumoencephalogram: normal. Negative reactions for syphilis, rubella, toxoplasmosis and herpes virus. Reactive up to 1/32 for cytomegalovirus (mother is non reactive). *Dermatoglyphics:* general configuration with no abnormalities (Fig. 3); total ridge count: 144; atd angle: L = 40º , R = 42º ; Walker's relation: L = 21%; R = 35%; *ab* ridge count: L = 33; R = 35. *Cytogenetics:* Chromosome analysis in blood cultures disclosed an extra long group D chromosome as well as lack of a group C chromosome and extra group E chromosome in all cells, which could be interpreted as a C/D translocation. In order to ascertain the specific identity of the unusual chromosome, G-banding was performed. Banding-analysis (Fig. 4) demonstrated that the chromosome constitution of this infant was 46,XX,-7,-15 + der (7) + der (15) t(7;15) (7pter → 7q21; 15pter → 15q23:: 7q21 → 7qter), with consequent partial deletion of chromosome 15 (15q23 → 15qter). The parents had normal karyotypes.

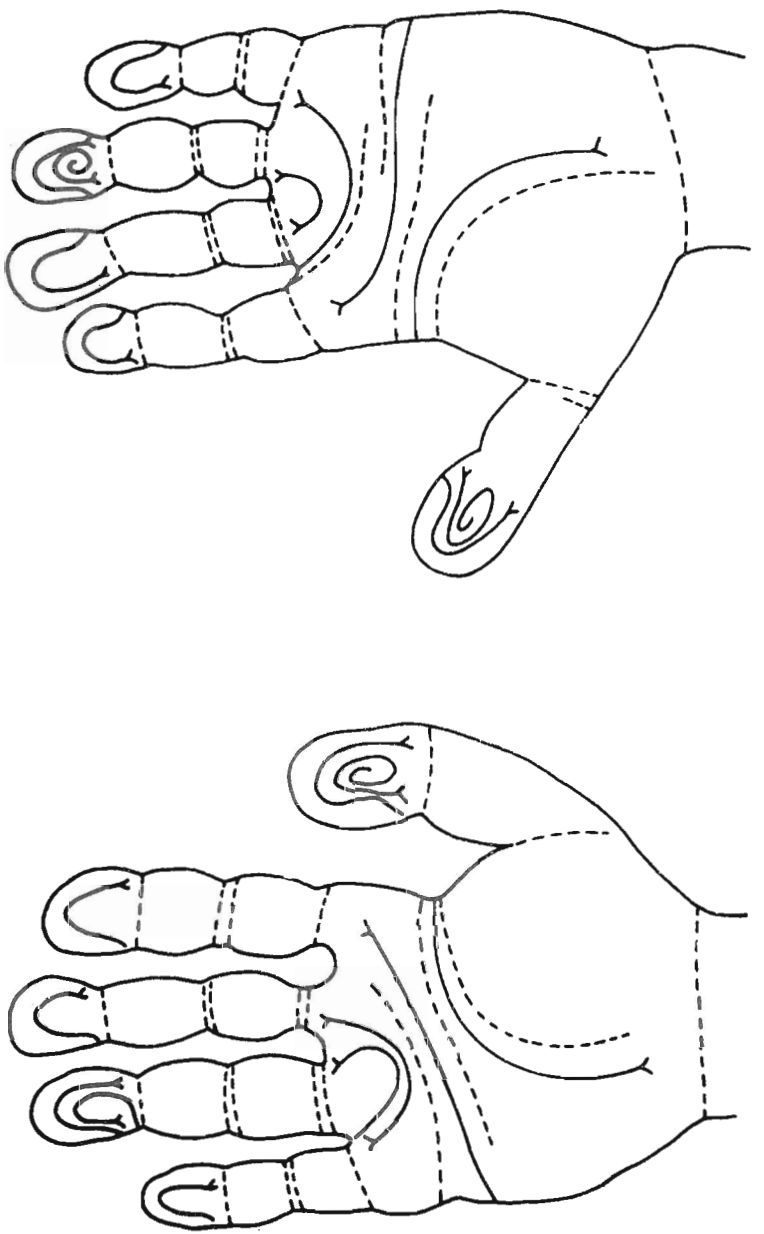


Figure 3. General aspect of the dermatologlyphic features

DISCUSSION

Clinical anophthalmia with chromosomal aberration has been frequently associated with trisomy 13 and occasionally associated with other chromosome aberrations (Table I). Among these, multiple malformations are described, with the exception of the case reported by Barbuta *et al.*, (1969), showing only clinical bilateral anophthalmia and encephalopathy.

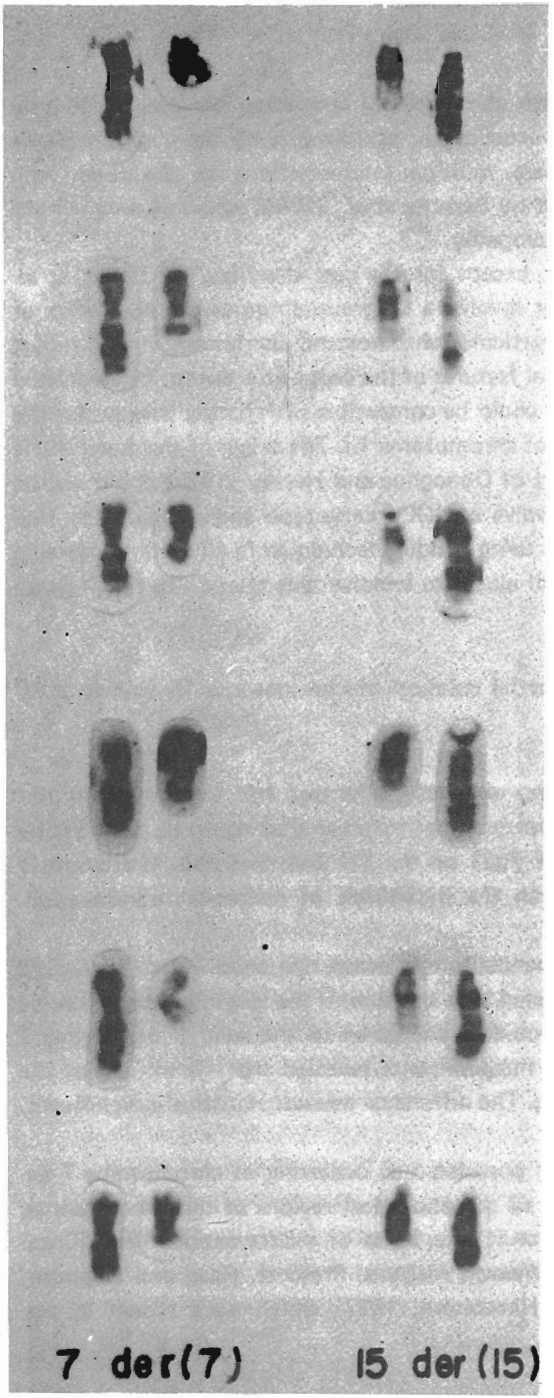
It seems significant that, except for the case described by Chang *et al.* (1975), the remaining cases either involve a D. group chromosome aberration or can be specifically related to a particular chromosome aberration. In the report of Barbuta *et al.* (1969) the clinical features of the propositus cannot be associated with either trisomy 21 or 22, but could be compatible with partial trisomy for the proximal segment of the long arm of chromosome 13. The origin of the metacentric chromosome present in the report of Donoghue and Hervey (1976) could not be determined. The other reports involve an XXY karyotype and a child with 10% polyploid mitoses. Further studies using banding techniques in all cases of sporadic and even familial anophthalmia will elucidate whether this relation to the D group chromosome holds true.

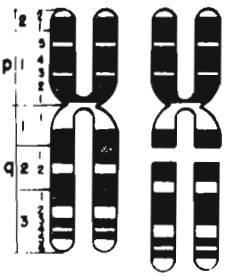
In the present report, partial deletion of chromosome 15 was assumed based on the following evidence:

a) Though reverse banding was not performed and the patient is not available for further study, the chromosomal material that could be detected by phase contrast microscopy beyond 7q21 on der (7) was negligible in relation to what would be expected based on the hypothesis of reciprocal translocation.

b) Measurements on 8 G-banded prints comparing pairs 7 der (7), and 15 der (15) within the same cell, revealed that the sum of the length of the derivative chromosomes is smaller than expected as compared to the sum of the normal 7 and 15 within the same cell. The measurements revealed that 10 to 15% of the length of chromosome 15 is missing. The difference was not statistically significant.

c) The deficiency was not postulated as occurring in chromosome 7 because, aside from the presence of all morphological regions of this chromosome, deletions affecting 7q21 → 7q32 result in a series of malformations (Bass, Crandall and Marcy, 1973; Higgirson, Weaver, Magenis, Prescott, Haag and Hepburn, 1976; Kaussett, Hsu, Paciuc and Hirschhorn, 1977) which were absent in our propositus.





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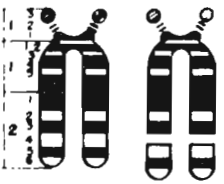

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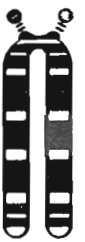
der(7)



der 15, t(7,15)

Figure 4. Chromosomes 7 and 15 with G-banding

To our knowledge, this is the first report in the literature of a patient with clinical bilateral anophthalmia and translocation 7/15, associated with possible partial deletion of the terminal segment of chromosome 15.

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