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GENETIC SYSTEM IN *RHYNCHOSIA MINIMA* AND *R. MEMNONIA* (LEGUMINOSEAE)[1]

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Local populations of *R. minima* and *R. memnonia* were studied from the point of view of genetic variability, biochemistry of proteins, free amino acids and chromosome number and behaviour in meiosis. Crosses were made between the two taxa. Morphologically each species is distinct from each other having continuous variations within itself. The biochemical results are highly correlated with morphological analysis.

In both, the chromosome number is 2n = 22 and under normal conditions crosses do not succeed. Success of a very low percentage was recorded with the application of certain growth regulating substances (IAA & kinetin). Seeds obtained were shrunken and abnormal. The incompatibilities in the two taxa seem to be both at the stigmatic as well as embryonic level.

Since hybridization between these two taxa is reported from Africa, the status of *R. memnonia* and *R. minima* as separate species or two subspecies belonging to one species should await investigation of the African population.

Introduction

The species *Rhynchosia memnonia* (Del.) DC. and *R. minima* (L) DC. are widely distributed in the tropics and subtropics of Africa and Asia, but the latter extends also to Australia and to the United States of America (Hooker 1879). Meikle (1954) reported that the two species interbreed in Africa. Since in West Pakistan both *R. memnonia* and *R. minima* are sympatric it was planned to check whether the species interbreed here as well. In order to evaluate the probable gene exchange between the two species, the populations were studied with respect to morphology, cytology as well as biochemistry.

Materials and Methods

A total of 80 plants of *R. minima* and *R. memnonia* was analysed morphologically. These plants were collected between Karachi and Pipri which is about 30 miles north-east. Seven characters were analysed morphologically and on this basis range and mean were calculated. In addition to these, six more characters were studied for the preparation of hybrid index (Anderson 1949). The characters used in hybrid index were chosen after the study of artificial hybrid seeds as well as certain contrasting characters easy to separate.

1. An unpublished Masters thesis of the Department of Botany, University of Karachi, Karachi.
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To estimate seed failure 1,000 pods of each species were collected randomly and the number of fertile and aborted seeds was counted. The estimation of seed weight of the two species was based on 1000 seeds, each lot collected randomly.

For studying germination of pollen of one species on the stigma of another, the buds were emasculated and then pollinated with another species. After three or four days of pollination the ovary was dissected out. It was stained with cotton blue and mounted in glycerine. The slide was then studied to observe pollen tube germination on the stigma.

Crosses were made reciprocally between the two species. Since over a thousand attempts ended in failure, pollinations were made after treatment of the stigma with 20 mg/l kinetin and 300 mg/l indoleacetic acid as described by Islam (1965).

Flower buds of suitable size were fixed in Carnoy's fixative, between 10 a.m. and 12 noon, and acetocarmine squash preparations were made for meiotic studies, as well as for testing pollen fertility (Table 6).

Paper chromatographic method was used for biochemical analysis of free amino acids. Fresh young leaves were ground with 80% ethanol and kept overnight at room temperature. After filteration the remaining debris was washed with fresh ethanol, and filtered. The two filtrates were pooled and 3 parts of chloroform were added to one part of ethanol extract. After thorough shaking the resulting upper aqueous layer was removed and its volume was reduced to 1 ml. This was used as amino acid extract which was loaded as single spot on Whatman paper no. 4. Chromatograms were run overnight by descending method in the solvent of n-butanol, glacial acetic acid and water in a ratio of 12:3:5. The solvent front was marked with pencil and the paper was dried at room temperature. For the detection of free amino acids this paper was sprayed with 0.2% ninhydrin and the chromatogram was placed in an oven at 100 C.

Proteins were estimated from the seeds with the help of standard graph of protein made from labritol solution. The reagents used were :—

Reagent I: 2 g sodium carbonate dissolved in 1N NaOH enough to make it 100 ml.

Reagent II : 1 g of sodium tartarate dissolved in 5% of $CuSO_4$ solution.

The total volume is made upto 100 ml.

Reagent III : 50 ml of reagent I and 1 ml of reagent II (50:1).

Reagent IV : Folin phenol reagent diluted with distilled water (1: 2).

TABLE 6 : *Data representing the percentage of pollen fertility in different populations of* R. minima *and* R. memnonia

R. minima

Plant no.	Locality	Chromosome no.	% of fertility
1.	Hyd. Road	2n = 22	69.8
2.	University Campus	,,	72.5
3.	,,	,,	81.8
4.	,,	,,	73.8
5.	,,	,,	83.6
6.	,,	,,	55.8
7.	,,	,,	75.8
8.	,,	,,	72.8
9.	,,	,,	73.0
10.	,,	,,	79.1
11.	,,	,,	76.9
12.	,,	,,	81.5
13.	,,	,,	61.9
14.	,,	,,	69.0
15.	,,	,,	74.6
16.	,,	,,	72.0
17.	,,	,,	69.8
18.	,,	,,	65.0
19.	,,	,,	65.5
20.	,,	,,	85.4
21.	,,	,,	96.4
22.	,,	,,	65.5
23.	,,	,,	75.7
24.	,,	,,	81.2
25.	,,	,,	80.0
26.	,,	,,	80.3
27.	,,	,,	80.3
28.	,,	,,	70.3
29.	,,	,,	77.2
30.	,,	,,	83.0
31.	,,	,,	61.6
32.	,,	,,	74.6
33.	,,	,,	67.8
34.	Pipri	,,	82.1
35.	,,	,,	68.2
36.	,,	,,	79.2
37.	,,	,,	72.7
38.	,,	,,	82.8
39.	,,	,,	84.9
40.	,,	,,	81.8

R. memnonia

Plant no.	Locality	Chromosome no.	% of fertility
1.	University Campus	2n = 22	83.1
2.	,,	,,	82.7
3.	,,	,,	76.8
4.	,,	,,	80.7
5.	,,	,,	83.6
6.	,,	,,	83.3
7.	,,	,,	84.7
8.	,,	,,	81.4
9.	,,	,,	90.7
10.	,,	,,	76.7
11.	,,	,,	90.1
12.	,,	,,	79.7
13.	,,	,,	80.9
14.	,,	,,	88.0
15.	,,	,,	80.0
16.	,,	,,	85.9
17.	,,	,,	83.0
18.	,,	,,	70.1
19.	,,	,,	80.4
20.	,,	,,	70.9
21.	,,	,,	72.1
22.	,,	,,	79.6
23.	,,	,,	80.0
24.	,,	,,	81.8
25.	Hyd. Road	,,	40.6
26.	,,	,,	44.7
27.	,,	,,	27.1
28.	,,	,,	31.4
29.	,,	,,	66.6
30.	,,	,,	48.9
31.	University Campus	,,	78.1
32.	,,	,,	74.6
33.	,,	,,	85.6
34.	,,	,,	80.3
35.	,,	,,	84.3
36.	,,	,,	82.5
37.	,,	,,	78.5
38.	,,	,,	84.0
39.	,,	,,	82.5
40.	,,	,,	83.3

Dry seeds were ground in the macerator and 10 ml of trichloro acetic acid was added to the material. The mixture was left in the incubator for 4 hrs at 30 C, filtered and 10 ml of 0.5 N NaOH was added to the residue It was then left overnight in the incubator at 30 C in order to dissolve the proteins.

The next day 1 ml of protein sample and 5 ml of reagent II was taken in a test tube and allowed to stand for ten minutes. In this test tube 5 ml of diluted pholin phenol reagent was introduced, and it was shaken vigorously. A blue black colour was developed. Optical density was read after 30 min in an spectrophotometer at 750 millimicron wave length using red filter. These optical densities were then plotted on a standard graph to find out the concentration of proteins in micrograms

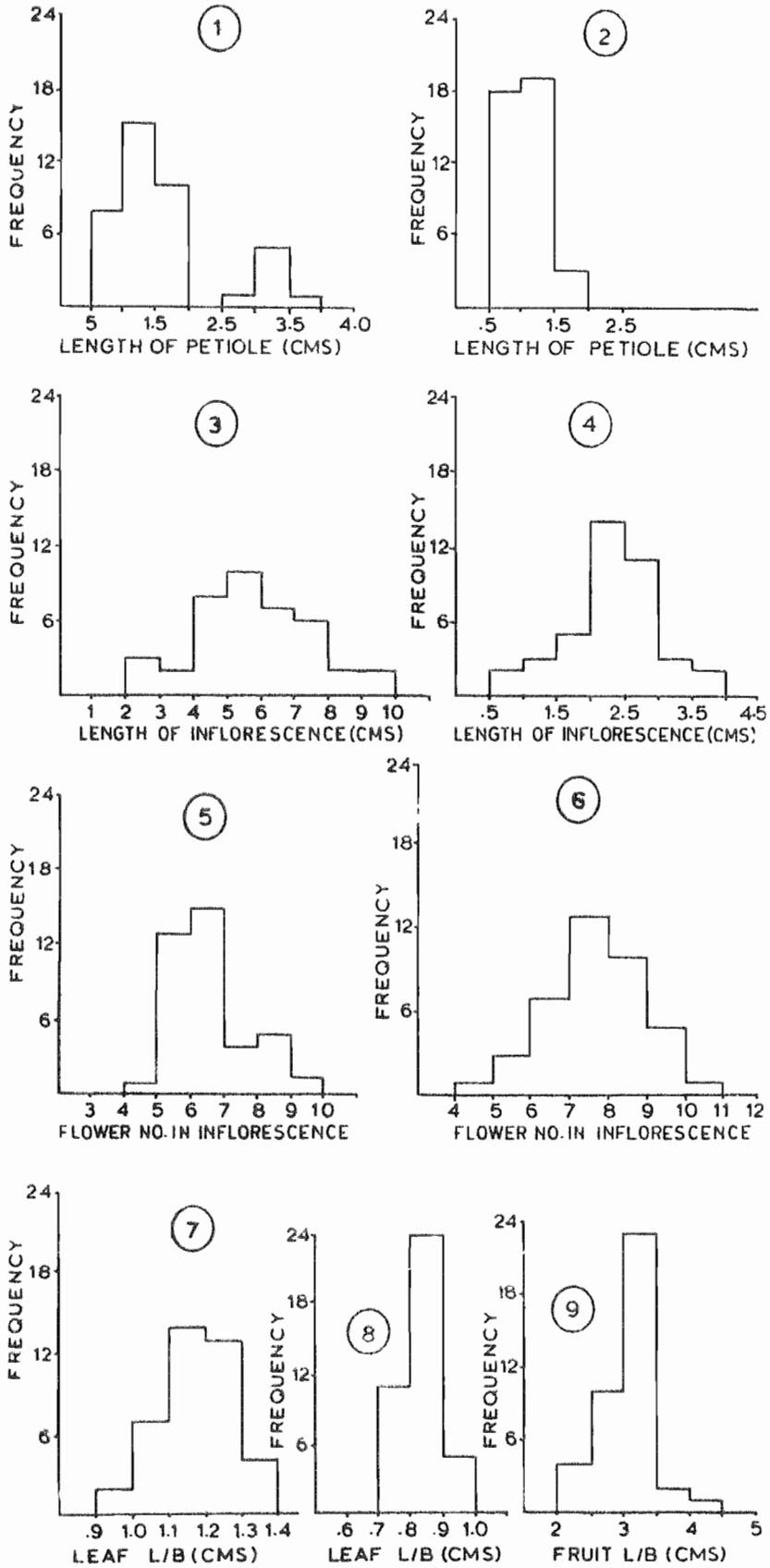
Results

Morphological study

The morphological analysis of the seven characters i.e. length of petiole, length of inflorescence, number of flowers in the inflorescence, length and breadth of leaf and length and breadth of fruit showed a normal sequence of distribution within the two populations, except the length of petiole of *R. minima* that showed discontinuity within its population. This is more clearly visible in the histograms (Figs. 1-9).

Hybrid index

Hybrid index was prepared using the characters in which the two species differ markedly. The characters studied were : seed pattern, seed colour, leaf hairs, presence or absence of leaf glands, fruit venation pattern, and fruit hairs.



Frequency histograms of Fig. 1. petiole length in *R. minima*. Fig. 2. petiole length in *R. memnonia*. Fig. 3. inflorescence length in *R. minima*. Fig. 4. inflorescence length in *R. memnonia*. Fig. 5. the number of flowers in *R. minima*. Fig. 6. the number of flowers in *R. memnonia*. Fig. 7. leaf index in *R. minima*. Fig. 8. leaf index in *R. memnonia*. Fig. 9. fruit index in *R. minima*.

The results obtained from the hybrid index demonstrate clearly an asbence of overlap between the two species (Table 1 and Fig. 10-11).

TABLE 1

Characters and Index values of R. minima *and* R. memnonia

No.	*R. minima*	Index value	*R. memnonia*	Index value
1.	Leaf glabrous	0	Leaf pubescent	3
2.	Leaf glands present	0	Leaf glands absent	3
3.	Fruit glabrous	0	Fruit pubescent	3
4.	Fruit venation pattern present	0	Fruit venation Pattern absent	3
5.	Seed colour grey	0	Seed colour yellow	3
6.	Seed pattern present	0	Seed pattern absent	3
	Total Index value	0		18

Total index value of all the 39 individuals of *R. minima* is 0.

Total index value of all the 39 individuals of *R. memnonia* is 18.

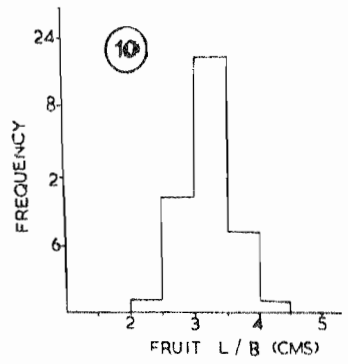


Fig. 10. Frequency histogram of fruit index in *R. memnonia*.

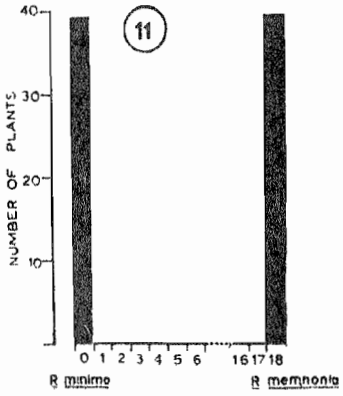


Fig. 11. Frequency distribution of total index values of 78 individuals in the populations of *R. minima* and *R. memnonia*.

Breeding system

Flowers found in the two species of *Rhynchosia* are in the form of an axillary raceme and anthesis takes place before the opening of flowers. Five hundred attempts to cross *R. minima* with *R. memnonia* failed. This failure was due to the non-germination of pollen of one species on the stigma of another. Similarly 120 attempts also failed when either IAA or only kinetin was applied to the stigma during pollination. Fruits were formed only when *R. minima* was used as female and the stigma was smeared with a mixture of IAA and kinetin. Fruit formation took place in 13.3% of the pollinated flowers (Table 2). The seeds obtained were wrinkled, small and yellow with black spots. It is significant to note that the seeds of *R. minima* (the mother plant) are greyish with black spots and the seeds of *R. memnonia* are yellow with no spots. The colour of the seeds, therefore had a combination of both the male and female parents.

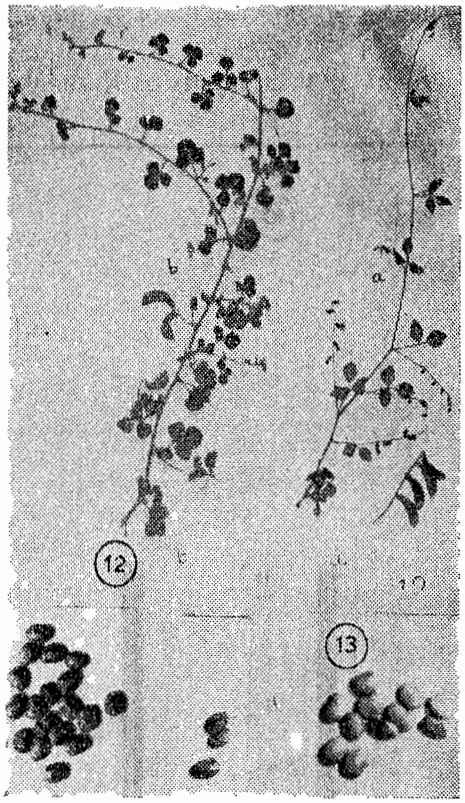


Fig. 12. Plants of members of *R. minima* (a) and *R. memnonia* (b).

Fig. 13 Seeds of *R. minima* (right), *R. memnonia* (left) and their hybrid (middle)

TABLE 2

Effect of growth promoting substances on fruit set

Parents	Hormones applied	No. of crosses	No. of fruit set	% of fruit set
R. mini x *R. mem*	No	200	0	0
R. mem x *R. mini*	No	300	0	0
R. mini x *R. mem*	IAA	30	0	0
R. mem x *R. mini*	IAA	30	0	0
R. mini x *R. mem*	Kinetin	30	0	0
R. mem x *R. mini*	Kinetin	30	0	0
R. mini x *R. mem*	IAA + Kinetin	30	4	13.3
R. mem x *R. mini*	IAA + Kinetin	30	0	0

Self-compatibility

Both the species of *Rhynchosia* were found to be self-compatible (Table 3). The results of seed failure and seed weight in naturally pollinated flowers of *R. memnonia* and *R. minima* are given in Tables 4 and 5.

TABLE 3

Data for self-compatibility

Plant	No. of buds bagged	No. of fruit set	% of fruit set
R. minima	112	26	23.2
R. memnonia	97	34	35.1

TABLE 4

Percentage of seed failure in naturally pollinated flowers of R. minima *and* R. memnonia

Plant	Total no. of seeds	No. of fertile seeds	No. of sterile seeds	% of fertile seeds	% of sterile seeds
R. minima	1883	1571	312	83.430	16.569
R. memnonia	1903	1416	487	74.400	25.600

TABLE 5

Data representing differences in the weight of the seeds of the two species

Plant	Weight of 1,000 seeds	Weight of one seed
R. minima	15.6200 g	0.015 g
R. memnonia	23.4474 g	0.023 g

The seeds of *R. memnonia* are heavier and bigger than those of *R. minima.*

Cytology

The chromosome number of *R. memnonia* and *R. minima* was found to be 2n=22 (Figs. 14 & 15). For these species the same chromosome number was reported by Senn (1938). The percentage of chromosomal irregularities was very low and a maximum of 3 micronuclei was observed in some telophase II.

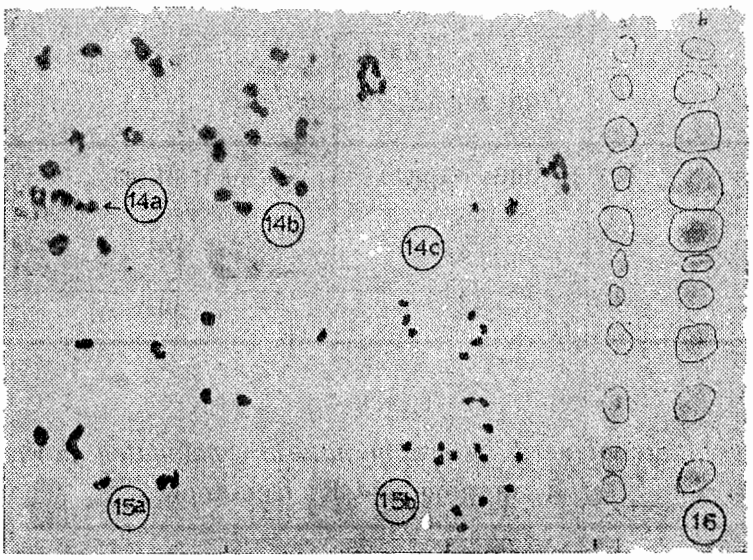


Fig. 14. Meiotic stages of *R. minima.* (a) Diakinesis (b) Metaphase and (c) lagging chromosomes at anaphase. x 800.

Fig. 15 Meiotic stages of *R. memnonia.* (a) Metaphase and (b) Anaphase. x 800.

Fig. 16. Chromatographic profile of *R. minima* (a) and *R. memnonia* (b).

Protein estimation

When an estimation of protein was made, it was found that in *R. minima* the amount of protein is 1.2 mg protein/g seed and in *R. memnonia* the amount of protein is 1.4 mg protein/g seed (Table 7). The proteins of the two species are not significantly different.

TABLE 7

Protein estimation of seeds of R. minima *and* R. memnonia

R. minima

Sample	Wt. of seed	Amount of NaOH in ml	Per cent transmittance	Optical density	mg protein/g in seed
1.	0.3	5	41.0	0.387	1.66
2.	0.5	5	33.0	0.482	1.40
3.	0.4	5	25.0	0.602	2.00
4.	0.3	2	38.0	0.420	0.83
5.	0.5	5	11.0	0.950	2.80
6.	0.3	2	52.5	0.276	0.50
7.	0.5	2	40.0	0.398	0.46
8.	0.3	5	48.5	0.310	1.33
9.	0.5	5	53.0	0.276	0.60
10.	0.5	5	49.0	0.310	0.80
				Mean	= 1.2 mg.
R. memnonia					
1.	0.3	5	51.5	0.284	1.33
2.	0.5	10	51.5	0.284	1.63
3.	0.5	5	40.0	0.398	1.16
4.	0.3	5	63.0	0.201	0.96
5	0.5	5	25.0	0.602	1.80
6.	0.5	5	47.0	0.328	0.94
7.	0.5	10	39.0	0.420	2.40
8.	0.5	5	55.0	0.260	0.76
9.	0.4	5	27.0	0.569	2.00
10.	0.4	5	39.0	0.409	1.40
				Mean	= 1.4 mg.

In *R. minima* the amount of protein is 1.2 mg protein/g in seed

In *R. memnonia* the amount of protein is 1.4 mg protein/g in seed.

Free aminoacid analysis

For the analysis of free aminoacids 10 chromatograms were run with the extracts of both *R. memnonia* and *R. minima* on each chromatogram. The results showed 12 aminoacids in *R. minima* and 10 in *R. memnonia*, and these remained constant.

Data representing the rf values of aminoacids found in *R. minima* and *R. memnonia* are given in Table 8.

TABLE 8

Rf values of amino acids found in R. minima *and* R. memnonia

No. of amino-acids	*R. minima*	*R. memnonia*
1.	0.073	0.078
2.	0.178	0.175
3	0.248	0.239
4.	0.304	0.317
5.	0.366	0.375
6.	0.426	0.421
7.	0.473	0.465
8.	0.529	0.531
9.	0.617	0.604
10.	0.692	———
11.	0.729	0.719
12.	0.834	———

Discussion

Population variability is a good criterion to suspect a certain breeding system. Because genetical variability is usually reflected in the morphological diversity, therefore, the population structure of outbreeders, inbreeders, and apomicts is quite different from each other. In the outbreeders there is a free exchange of genes so that the whole genetical material is reshuffled and many old and new combinations appear in the subsequent generations, and the populations show a continuous variation pattern (Baker 1953b, 1959 ; Stebbins 1950). In habitual inbreeders and apomicts the situation is quite different ; here the populations show discontinuity because of the splitting of a taxon into numerous more or less pure lines (Stebbins 1950, 1957 ; Baker 1953b, 1959).

In *R. minima* and *R. memnonia* conclusion regarding breeding system could not be drawn from morphological evidences alone. Character analysis in both the species show more or less normal distribution except a bimodal distribution of only one character was observed in *R. minima* (Fig. 1). When inflorescences were bagged, fruit formation and seed setting took place in 23.2% cases in *R. minima* and 35.1% in *R. memnonia* indicating the presence of self breeding mechanism in both (Table 3). Presence of highly variable percentage of sterile pollen in the local populations of both the species also point out certain degree of gene exchange among them. Although both *R. minima* and *R. memnonia* are sympatric in Africa as well as in West Pakistan, and they are known to interbreed in Africa (Meikle 1954) yet morphological overlap of their diagnostic characters does not occur in the sympatric local populations of West Pakistan.

Similar to morphology biochemistry has been used extensively in showing gene exchange and phylogeny in plants (Alston and Turner 1959 ; Bate-Smith 1957 ; Hillis and Orman 1962 ; Turner and Mabry 1964 ; Gibbs 1954). Although the two species are not significantly different in the quantity of their seed proteins (Table 7) they are different in their free amino acids. Moreover this difference is constant and does not show a mingling of spots of one species with the other (Fig. 16). Thus biochemistry strengthens the evidence from morphology that the gene exchange between the two taxa is not plausible.

If a species is adapted for a stable environment alone then in such a case it is either obligatory inbreeding or apomictic. While a species which is adapted to a changing condition, heterozygosity has to be maintained through open pollinations. In a plant that is also widely distributed it is imperative that a mechanism to cross pollination through compulsion as self-incompatibility is not maintained. The ideal way through which both heterozygosity as well as wide distribution could be achieved is through the establishment of local populations from inbreeding, and production of heterozygosity through crossing between homozygotes bearing different genotypes. The experimental evidence demonstrates that the two species under consideration have this very compromising system in operation, and this seems to be the reason for the presence of micronuclei and lagging chromosomes. Moreover, a bimodal distribution in the petiole length of *R. minima* also supports the idea that some intraspecific population differences are present.

A survey of the literature on the correlation between breeding system and seed weight in closely related amphimictic taxa shows that such correlation exits in widely divergent families (Ali 1968). This correlation exists also in the two taxa of *Rhynchosia*. The weight of seed is more in *R. memnonia* in which inbreed-

ing is more frequent under bagged conditions with 35.1 % fruit set as compared to *R. minima* where seed is lighter and percentage of fruit set is only 23.2.

Out of five hundred attempts made to cross *R. minima* and *R. memnonia* neither any fruit was formed nor any swollen ovary was observed. After pollination with another species the flowers would simply drop off within a few days indicating that pollen tube is not reaching the ovary. It was necessary, therefore, that a correct understanding of the isolating mechanism be made. After pollinating *R. minima* with *R. memnonia* and vice versa the germination of pollen tube was studied and it was found that the failure of crosses is because of the inability of pollen of one species to germinate over the stigma of the other. Since the difference between *R. minima* and *R. memnonia* was not involving either difference in chromosome number (Watkins 1932), or style length (Mangelsdorf and Reeves 1939 ; Stebbins 1950), it was thought logical that the crossing could be made successful through the use of growth factors that could overcome the stylar incompatibility as it has been demonstrated in the case of *Corchorus* (Islam 1965) and *Gossypium* (Zaid *et al.* 1966). In those species where such isolating barriers are present, crosses could become successful through the use of IAA and kinetin. Out of 180 crosses made through this method four fruits with one seed each were obtained when IAA and kinetin were applied in a mixture. Similar to male parent hybrid seeds were yellow but with black spots of the female parent (Fig. 13). Thus they exhibit the rare phenomenon of metaxenia. The hybrid seeds, however, were shrunken and unhealthy indicating the presence of some isolating mechanism at the embryonic level as well.

A common way in which interspecific hybridisation is prevented is through seed incompatibility (Davis and Heywood 1963). This phenomenon was studied intensively by Valentine (1961) in *Primula* Sect-Vernales. Here fertilization occurs but there is always some abnormality in the subsequent development of seed. This phenomenon is quite general in interspecific crosses in flowering plants and is regarded as the main barrier to crossing between allied species, but Grant (1963) pointed out that most of the interspecific barriers are multiple in nature, even then intersterility cannot be taken as indicative of a lack of relationship unless its cause is understood (Davis and Heywood 1963, Islam 1965).

It is, however, not a new phenomenon that species that are widely distributed may show different genetic behaviour. In large populations divergent evolution of segments of the same population due to isolation by distance is shown to be possible theoretically (Wright 1943). Clausen, Keck and Hiesey (1940) have demonstrated within *Potentilla glandulosa* all types of morphological and physiological differences that often separate good and distinct species. If some of the

extreme forms of this species such as subspecies *typica* and ssp. *nevadensis* become completely isolated from each other geographically, and develop genetic sterility barriers they would become amply distinct species. What keeps the species a single unit is the fact that every subspecies at some locality comes in contact and forms hybrid swarms. Terao and Midusima (1939) working with intervarietal hybrids of *Oryza sativa* found that the F_1 hybrids between certain varieties had pollen and seed sterility comparable to that of interspecific hybrids. Hiorth (1942) found that all the strain of *Godetia amoena* found in south of the Golden gate, in Central California were interfertile. On the other hand all the strains of *G. whitneyi* originating from North of the Golden gate, to British Columbia were partly interfertile. Both species have n=7 chromosome number and F_1 hybrid has good chromosome pairing. Pjatnitzky (1946 a, b) working on *Quercus* demonstrated that intersectional crosses such as between *Q. macrantha* × *Q. robur* and between *Q. macrantha* × *Q. alba* gave higher yields of acorn and seedlings than did the maternal parents when pollinated with their own species. In California *Q. douglasii* and *Q. lobata* are sympatric over a large part of their range but hybridize only in one small part of this area (Stebbins 1950). Snyder (1950) found that in *Elymus glaucus* only six out of twenty strains showed a high degree of interfertility although Snyder (1951) has also suggested that sterility barriers seem to have spread into these species through introgression with *Sitanion*. Praeger (1951) records frequent hybridization between *Senecio aquaticus* and *S. Jacobaca* in Ireland, whereas such hybridization is much less common in England. From all these examples it becomes clear that the species distributed over large areas tend to show different genetic behaviour and the two species discussed above are no exception to the rule.

The final answer, therefore, should emerge after the study of both *R. memnonia* and *R. minima* from their entire range of distribution.

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