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Experimental Studies on Hybridization and Sexual Isolation Between Some Aphytis Species (Hymenoptera: Aphelinidae)

I. Experimental Hybridization and an Interpretation of Evolutionary Relationships Among the Species Sudha V. Rao and Paul DeBach II. Experiments on Sexual Isolation Sudha V. Rao and Paul DeBach



I. EXPERIMENTAL HYBRIDIZATION AND AN INTERPRETATION OF EVOLUTIONARY RELATIONSHIPS AMONG THE SPECIES

A biosystematic study was made of cultures of closely similar species of Aphytis (Hymenopters, Chalcidoidea, Aphelinidae) from various countries. Sexual isolation was an important barrier to interspecific hybridization. Laboratory manipulations were devised to partially overcome this. Some fertile interspecific hybrids were obtained and hybrid lines established. Fecundity and sex ratio of hybrids improved in successive laboratory generations. A mathematical index was developed, based upon extent of hybrid progeny production, sex ratio, and degree of fertility of F1 hybrids, which depicts the degree of reproductive isolation between a given pair of species. It was concluded that the described species, africanus, lepidosaphes, melinus, fisheri and boloxantbus, are valid species with respect to each other and with respect to all other species studied. Aphytis coheni and "khunti," A. lingnanensis and "2002," and "R-65-23" and "2002" are considered to be semispecies with respect to each other. Additionally, the following are considered to be valid species with respect to each other: lingnanensis and coheni; lingnanensis and "khunti"; "2002" and coheni; "2002" and "khunti"; lingnanensis and "R-65-23"; "khunti" and "R-65-23"; and cobeni and "R-65-23."

II. EXPERIMENTS ON SEXUAL ISOLATION

Biosystematic studies were made of various cultures of species of Appytis Howard (Hymenoptera: Aphelinidae) that were nearly or completely identical morphologically. The species were imported from different geographical regions of the world. Previous hybridization tests showed that reproductive isolation between cultures ranged from partial to complete. Those showing partial isolation are termed semispecies, but this term leaves certain nomenclatural and phylogenetic problems unsolved.

The present study was confined to members of the Lingnanensis and Melinus groups. No hybridization occurred between members of these groups. Within each group some degree of hybridization occurred among the various members. However, substantial sexual isolation was indicated, since in heterogamic crosses only a few, if any, females of the alien species were inseminated.

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II. Experiments on Sexual Isolation^{1,2}

ABSTRACT

Sexual isolation was studied in several species and semispecies of Aphytis, aphelinid parasites of diaspine scale insects. The multiple-choice technique was used in this study, and coefficients of isolation and joint isolation were derived by using previously developed formulas. These data, in combination with other information obtained from experimental hybridization, were used to clarify the taxonomic relationships among the different species and semispecies. We have attempted to define "semispecies" in more precise terms than those previously used. Our objective was to help determine what the degree of partial reproductive isolation between two populations must be in order that they may be called semispecies with respect to each other. To achieve this, we used the coefficient of joint isolation.

INTRODUCTION

IN BIOSYSTEMATICS, it is becoming increasingly common to recognize populations from different sources comprised of individuals that are morphologically similar, or even identical with each other, and yet reproductively isolated to a greater or lesser extent. Where reproductive isolation is complete, no theoretical problem in classification remains; we are dealing with good sibling species. Hybridization between good biological or morphological species is very rare in animals. On the other hand, broadranging species may have more or less widely separated populations that are partially isolated, reproductively. Gene exchange between these populations is restricted. They have not yet acquired species status, yet have evolved and differentiated considerably with regard to their sexual or biological responses, or both. Such populations create nomenclatural problems.

Mayr (1963) has apply designated such populations "semispecies." According to him, gene exchange occurs among semispecies, but not so freely as among conspecific populations. The term semispecies appears to be very useful, particularly since it stresses that evolution is a continuous process. Obviously, however, semispecies can exist in varying stages of sexual or reproductive isolation from the parental species. As in the case of two species that are completely isolated reproductively, the nature of the isolating mechanism between a pair of semispecies may be ecological, seasonal or temporal, sexual (ethological), mechanical, gametic, or may be due to hybrid inviability, sterility or breakdown (Dobzhansky, 1951). The effectiveness of the isolating mechanisms with semispecies is only partial, however. For example, individuals from one semispecies population may accept some

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mates from another semispecies population, but in a nonrandom fashion. This leads to restriction of gene flow, particularly if the subsequent steps necessary for successful hybridization are restricted or prevented. It can be conjectured that if two such semispecies populations were kept from hybridizing successfully over a period of time, such as by geographical isolation, the isolating mechanisms might become strengthened and eventually lead to complete reproductive isolation (see Mayr, 1963).

Regarding nomenclature of semispecies, Mayr (personal communication) stated: "The International Code of Zoological Nomenclature recognizes only two categories at the species level, the species and the subspecies. The term 'semispecies' designates a stage which is somewhat intermediate between subspecies and species. What terminology to use for it depends on your evaluation of the particular situation. If you think it is closer to a species, you can use a binomen, if closer to a subspecies, a trinomen. In case of almost perfect intermediacy, one can place the middle name of the trinomen in parentheses."

In the first paper of this series (Rao and DeBach, 1969), the results of experiments on hybridization between some species and semispecies of Aphytis have been described. These tiny wasps $(\pm 1 \text{ mm in length})$ are hymenopterous parasites of diaspine scale insects. On the basis of crosses obtained and, partly, the morphology of the spermathecae, it was concluded that some of the species studied could be divided into a Lingnanensis group and a Melinus group. Within each group, at least some members hybridized to a small extent, either under normal or special conditions in the laboratory. No hybridization whatsoever could be accomplished between the two groups.

In the course of preliminary observations, we had noted that when females of one species were brought together with males of another, a rejection response was usually shown by the females, even if, on occasion, the males showed an interest in the females and courted them. This type of rejection response by the female was less frequent when the individuals involved belonged to semispecies within a group. It was especially true of the Lingnanensis group, in which, at least in one case, nearly random mating was indicated. The hybridization experiments indicated that, in most cases, only a fraction, if any, of the females was inseminated, among those placed with males of another semispecies or species. We were able to observe this, inasmuch as the "mated" females were allowed to oviposit in groups of five, and subsequent examination of the progeny of each group of females revealed that some yielded hybrid progeny, while others did not. This was a strong indication of the presence of sexual isolating mechanisms. We therefore decided to perform a series of multiple-choice experiments in order to obtain a quantitative measure of sexual isolation existing between the various species and semispecies and to learn to what extent sexual isolation determined reproductive isolation.

In the multiple-choice experiments the males of a species were given a choice between mating with homogamic or heterogamic females. The proportion of females of each category inseminated was then determined by examining the female spermatheca for the presence of sperm. (The terms homogamic and heterogamic are used in essentially the same sense as defined by Dobzhansky and Mayr (1944); matings between members of the same strains, races, semispecies or species are referred to as homogamic and those between different strains, races, semispecies or species as heterogamic.)

Since only one species of male was used, it might appear that the males were solely responsible for making a choice. However, in *Aphytis* (as in *Drosophila*), it is the female who makes the ultimate decision, even though the male may court her and attempt copulation. Therefore, in multiple-choice experiments, what is mainly determined is the degree to which the two species (or semispecies) of females repel or accept the males of a given species.

When equal proportions of homogamic and heterogamic females are inseminated, random mating has occurred and conspecificity is indicated (though not proved). If, on the other hand, significantly unequal proportions of homogamic and heterogamic females are inseminated, it shows that the two groups of females differ in their response to the males, and hence two species, or at least semispecies, are involved.

A similar technique was used by various authors in the study of sexual isolation in *Drosophila* (Stalker, 1942; Dobzhansky and Mayr, 1944; Dobzhansky and Streisinger, 1944). More recently, Elens and Wattiaux (1964) used a specially designed apparatus

For convenience, all the *Aphytis* cultures tested are referred to as species until their systematic status is dealt with in the discussion.

The following species of *Aphytis* were used:

Lingnanensis group: A. coheni, A. "khunti," A. lingnanensis, and A. "2002."

Melinus group: A. fisheri, A. holoxanthus, and A. melinus. (See first paper in this series, Rao and DeBach, 1969, for details.)

In order to obtain virgin females and males for the experiments, pupae were isolated as described in Rao and DeBach (1969). However, the vials containing female pupae of one species were pro-

for direct observation of sexual isolation in Drosophila. This method involves marking one strain of flies with a spot of plastic color dissolved in acetone, and introducing males and females of both strains into the observation chamber. Since copulating pairs of *Drosophila* do not move appreciably, observations could be made at time intervals less than the duration of copulation. This method is not practicable for studying sexual isolation in Aphytis, because acetone is lethal and these insects are so small and so active that the marks would be difficult to detect. Furthermore, although copulating pairs are not known to move during the copulation period of 4 to 5 seconds, there is not enough time to make observations and record data. Attempts to make multiple-choice mating observations on Aphytis by marking them with a daylight fluorescent pigment (Day-Glo) were unsuccessful because the insects cleaned the pigment off within a few minutes of being treated. during which time all normal activities were curbed.

MATERIALS AND METHODS

vided with honey, colored with a vegetable dye,^{*} for the emerging adults to feed on. The color showed through the abdominal wall and permitted identification of species of females. Wings of Aphytis adults were not clipped (as is done with *Drosophila*) because these extremely delicate insects are invariably injured. Newly emerged males and females were sexed once again and held in isolation until experimentation.

Procedures in this series of experiments were relatively simple. Usually 10 virgin females of each of two species, *e.g., lingnanensis* and "2002," were placed together (total of 20) in an 8dram vial, and 10 males of one of the two species, either *lingnanensis* or

⁸ Schilling Food Colors, McCormick and Co., Inc.

"2002," were released into the vial. The females of one were marked with colored honey, as already described. As usual, carbon dioxide was used to anesthetize the insects for transfer. Following time for recovery from anesthesia, a 30-minute mating period was permitted. At the end of that period, the insects were anesthetized once again, and the males were discarded. The two sets of females, lingnanensis and "2002," were then separated into individual vials, after which they were dissected in normal saline and examined for the presence of sperm. The sperm were readily observable because of their vigorous undulating movements within the spermatheca. When placed under a cover slip in saline solution, the sperm continued to move for about 10 to 12 minutes, which gave sufficient time to make accurate observations and record data.

In the present experiments, the length of time that the females and males were kept together was adjusted to prevent the males from inseminating all of the females offered to them in the time given. This was necessary because if all of the females were inseminated (both heterogamic and homogamic), it would be difficult to determine whether or not homogamic matings occurred in preference to heterogamic ones. Furthermore, unless this limitation of time were imposed, it would not have been possible to calculate the coefficients of isolation and joint isolation, for reasons discussed in the next section.

Observations on sexual isolation, with the use of this technique, were made between members within the Lingnanensis group and between members within the Melinus group, but not between members of the two groups, since it was quite evident from previous hybridization experiments that no mating between the groups would occur. (R-65-23, an *Aphytis* species included in the studies on experimental hybridization, was not used in the studies on sexual isolation because not enough were available.)

Each of the multiple-choice experiments, involving a pair of species, was replicated five times for statistical reliability.

RESULTS

Table 1 shows the results of the multiple-choice experiments involving all possible combinations within each group. Of the eight combinations, four showed no heterogamic insemination whatsoever. Of the four that showed heterogamic insemination, only "khunti" and *coheni* approached the percentages of homogamic inseminations. The data from five replicates were pooled.

No significant differences were found among the replicates except in one experiment involving *coheni* and "khunti" females mated with "khunti" males $(X^2 = 31.2, P < .001)$. However, it is reasonable to expect one significant X^2 out of 16. Since the number of homogamic versus heterogamic matings were comparable even in this replicate, the ultimate result was not affected in any way.

The mating pattern may be said to be discriminatory in most of the crosses since, according to Schaffer (1968), the product of the frequencies of the homogamic inseminations does not equal the product of the frequencies of the heterogamic inseminations. An exception is the cross involving "khunti" and coheni, wherein the products are nearly equal and hence the mating pattern is nondiscriminatory. It seems unlikely that the mating patterns have been affected in any way by the numbers of females of the two species in each choice test, because very nearly equal numbers were used.

Using the data from table 1, we cal-

culated the coefficients of isolation $(K_{1,2})$ and $K_{2,1}$) and coefficients of joint isola-

tion
$$(K_{1 \text{ and } 2})$$
 employing the formula developed by Levene (1949):

$$K_{1, 2} = \frac{\log q_{1, 1} - \log q_{1, 2}}{\log q_{1, 1} + \log q_{1, 2}}$$
$$K_{2, 1} = \frac{\log q_{2, 2} - \log q_{2, 1}}{\log q_{2, 2} + \log q_{2, 1}}$$
$$K_{1 \text{ and } 2} = \frac{(K_{1, 2} + K_{2, 1})}{2}$$

number of strain 1 females not inseminated when placed with strain 1 where $q_{1,1} = \frac{\text{males and strain 2 females}}{\text{number of strain 1 females placed with strain 1 males,}}$

number of strain 2 females not inseminated when placed with strain 1 $q_{1, 2} = \frac{\text{males and strain 1 females}}{\text{number of strain 2 females placed with strain 1 males}}$ and

 $q_{2, 1}$ and $q_{2, 2}$ are similarly defined when strain 2 males are used.

If the length of time during which the females and males are kept together is increased indefinitely, ideally all strain 1 females will be inseminated; hence $q_{1,1}$

would become zero. Under similar conditions, $q_{2,2}$ would become zero. Therefore, $K_{1,2}$ and likewise $K_{2,1}$ would become zero.

TABLE	1

MULTIPLE-CHOICE EXPERIMENTS: FREQUENCIES OF HOMOGAMIC AND HETEROGAMIC INSEMINATIONS BY MALES OF ONE SPECIES, ONLY, IN THE JOINT PRESENCE OF HOMOGAMIC AND HETEROGAMIC FEMALES

Crosses	Homogamic inseminations*			Heterogamic inseminations*			
çç	ೆ 7	n	s	per cent	n	s	per cent
``2002'' & lingnanensis lingnanensis & ``2002''	lingnanensis ''2002''	48 52	46 41	95.83 78.84	48 51	18 12	37.50 23.53
''khunti'' & lingnanensis lingnanensis & ''khunti''	lingnanensis ''khunti''	50 54	45 51	90.00 94.44	52 55	3 10	5.77 18.18
"2002" & "khunti" "khunti" & "2002"	"khunti" "2002"	52 48	43 46	82.69 95.83	52 50	0 0	0 0
coheni & lingnanensis lingnanensis & coheni	lingnanensis coheni	49 47	48 44	97.96 93.62	50 45	0 9	0 20.00
''2002'' & coheni coheni & ''2002''	coheni ''2002''	50 48	49 35	98.00 72.91	49 49	0	0
''khunti'' & coheni coheni & ''khunti''	coheni ''khunti''	48 52	47 41	97.92 78.85	48 50	42 41	87.50 82.00
melinus & holoxanthus holoxanthus & melinus	holoxanthus melinus	51 52	49 48	96.08 92.31	50 52	0	0
fisheri & melinus melinus & fisheri	melinus fisheri	50 51	48 47	96.00 92.16	49 50	0	0
		1	1	1		1	1

* n = number of females used (5 replicates of 10 females per "species," but occasionally 9 or 11); s = number of females inseminated; per cent = per cent females inseminated.

Crosses		Coefficients of	t	Р	Confidence	
φç	ೆ ೆ	$K_{1,2} \& K_{2,1}$			intervals	
``2002'' & lingnanensis lingnanensis & ``2002''	lingnanensis ''2002''	$0.74 \pm .07$ $0.71 \pm .06$	10.60 11.90	<.001 <.001	$.60 \leq \tilde{K}_{1,2} \leq .88$ $.59 \leq \tilde{K}_{1,2} \leq .83$	
''khunti'' & <i>lingnanensis</i> <i>lingnanensis</i> & ''khunti''	<i>lingnanensis</i> ''khu n ti''	$0.95 \pm .03$ $0.87 \pm .05$	31.60 17.40	<.001 <.001	$.89 \leq \tilde{K}_{1,2} \leq 1.0$ $.77 \leq \tilde{K}_{1,2} \leq .97$	
"2002" & "khunti" "khunti" & "2002"	"khunti" "2002"	$1.00 \pm 0^*$ $1.00 \pm 0^*$				
c oheni & lingnanensis lingnanensis & coheni	lingnanensis coheni	$1.00 \pm 0^{*}$ $0.85 \pm .01$	 85.00	<.001	$.83 \leq \tilde{K}_{1,2} \leq .87$	
"2002" & coheni coheni & "2002"	coheni ''2002''	$\begin{array}{rrrr} 1.00 \pm & 0^* \\ 1.00 \pm & 0^* \end{array}$				
''khunti'' & coheni coheni & ''khunti''	<i>coheni</i> ''khunti''	$\begin{array}{c} 0.30 \pm .14 \\ -0.05 \pm .12 \end{array}$	2.14 0.42	<.05 >.60	$.02 \leq \tilde{K}_{1,2} \leq .58$ 29 $\leq \tilde{K}_{1,2} \leq .19$	
melinus & holoxanthus holoxanthus & melinus	holoxanthus melinus	$1.00 \pm 0^*$ $1.00 \pm 0^*$		····		
fisheri & melinus melinus & fisheri	melinus fisheri	$\begin{array}{rrrr} 1.00 \pm & 0^{*} \\ 1.00 \pm & 0^{*} \end{array}$				

TABLE 2 COEFFICIENTS OF ISOLATION COMPUTED ON THE BASIS OF THE MULTIPLE-CHOICE EXPERIMENTS

* In these crosses, no heterogamic inseminations were obtained, and the standard error being zero, confidence intervals could not be determined.

According to Levene, the coefficient of isolation is an improvement over the Charles-Stalker isolation index (Stalker, 1942), since ideally the former is not affected by the duration of the experiment. The coefficient of isolation with standard errors and confidence intervals are given in table 2. Complete isolation is indicated by a value of 1.00 for the coefficient of isolation. Zero indicates random mating and hence absence of sexual isolation, while intermediate values are indicative of partial sexual isolation.

The data in table 2 indicate that highly significant isolation exists in all cases except between *coheni* and "khunti." However, the results in one direction, "khunti" females \times *coheni* males (t = 2.14, P < .05 but > .02) show that this is a borderline case. The confidence intervals ($.02 \leq K_{1,2} \leq .58$) very nearly include zero, which would represent random mating. In the reciprocal cross, *coheni* females × "khunti" males, t = 0.42, so that P > .60, which represents random mating and hence the absence of sexual isolation. Here, the confidence intervals $(-.29 \leq K_{2,1} \leq .19)$ include zero.

These results are further supported by the coefficients of joint isolation (table 3). Levene (1949) stated that "...the coefficient of joint isolation measures the true reproductive isolation between the two strains under the (artificial) conditions of the experiment.

..." The coefficient of joint isolation differs from the simpler coefficient of isolation in that it takes into account results from reciprocal crosses. In all cases, except between "khunti" and *coheni*, highly significant sexual isolation is indicated. The confidence intervals for the coefficient of joint isolation between "khunti" and *coheni* include zero

Crosses		Coefficients of joint isolation	t	Р	Confidence	
φç	ೆರೆ	$K_{1,2}$			intervals	
^{(*2002''} & lingnanensis lingnanensis & ''2002''	lingnanensis ''2002''	0.72 ± .10	7.2	<.001	$.52 \leq \tilde{K}_{1,2} \leq .92$	
"khunti" & lingnanensis lingnanensis & "khunti"	lingnanensis ''khunti''	0.91 ± .03	30.3	<.001	$.85 \leq \tilde{K}_{1,2} \leq .97$	
"2002" & "khunti" "khunti" & "2002"	"khunti" "2002"	$1.00 \pm 0^*$	••••			
coheni & lingnanensis lingnanensis & coheni	lingnanensis coheni	0.93 ± .01	93.0	<.001	$.91 \leq \tilde{K}_{1,2} \leq .95$	
"2002" & coheni coheni & "2002"	coheni ''2002''	1.00 ± 0*				
''khunti'' & coheni coheni & ''khunti''	coheni ''khunti''	0.13 ± .10	1.3	≦.20	$07 \leq \tilde{K}_{1,2} \leq .33$	
melinus & holoxanthus holoxanthus & melinus	holoxanthus melinus	1.00 ± 0*				
fisheri & melinus melinus & fisheri	melinus fisheri	$1.00 \pm 0^*$				

TABLE 3 COEFFICIENTS OF JOINT ISOLATION COMPUTED ON THE BASIS OF THE MULTIPLE-CHOICE EXPERIMENTS

• In these crosses, no heterogamic inseminations were obtained, and the standard error being zero, confidence intervals could not be determined.

 $(-.07 \leq K_{1 \text{ and } 2} \leq .33)$, which again indicates virtual absence of sexual isolation.

Another index used by Levene (1949) is the coefficient of excess insemination $(m_{1,2})$ which, he stated, "... can serve as a measure of the extent to which the gene flow between the two strains is in one direction only." This index is calculated by using the following formula:

$$m_{1,2} = \frac{(K_{1,2} - K_{2,1})}{2}$$

Table 4, which gives the coefficients of excess insemination in one direction over the other, shows no statistically significant differences with the exception of coheni \times lingnanensis. In this case, matings between coheni males and lingnanensis females were highly favored over the reciprocal (P < .001). As a matter of fact, in the multiple-choice

experiments, no *coheni* females were inseminated by *lingnanensis* males. This has been supported by subsequent observations as well, in which only a very small number of *coheni* females (1.67 per cent) were inseminated by *lingnanensis* males (see table 5). However, the question of greater gene flow in one direction than in the other does not arise in this case since the hybrids obtained from both crosses have been found to be sterile (Rao and DeBach, 1969).

In order to determine to what degree, if any, the presence of homogamic females in the previous tests influenced the frequency of heterogamic inseminations in the multiple-choice experiments, a series of no-choice experiments was conducted. This involved only heterogamic crosses (except for the homogamic checks). The males were offered only a single species of female for mat-

Crosses		Coefficients of excess	t	Р	Confidence	
Ç Ç	ೌರೌ	insemination $m_{1,2}$			intervals	
''2002'' & lingnanensis lingnanensis & ''2002''	lingnanensis ''2002''	$0.02 \pm .10$	0.20	>.80	$18 \leq \tilde{m}_{1,2} \leq .22$	
''khunti'' & <i>lingnanensis</i> <i>lingnanensis</i> & ''khunti''	lingnanensis ''khunti''	0.04 ± .03	1.33	≦.20	$02 \leq \tilde{m}_{1,2} \leq .10$	
''2002'' & ''khunti'' ''khunti'' & ''2002''	"khunti" "2002"	0*			•••••	
coheni & lingnanensis lingnanensis & coheni	coheni lingnanensis	0.08 ± .01	8.00	<.001	$.06 \leq \tilde{m}_{1,2} \leq .10$	
''2002'' & coheni coheni & ''2002''	coheni ''2002''	0*				
''khunti'' & coheni coheni & ''khunti''	<i>coheni</i> ''khunti''	0.18 ± .10	1.80	>.05	$02 \leq \tilde{m}_{1,2} \leq .38$	
melinus & holoxanthus holoxanthus & melinus	holoxanthus melinus	0*				
fisheri & melinus melinus & fisheri	melinus fisheri	0*				

COEFFICIENTS OF EXCESS INSEMINATION COMPUTED ON THE BASIS OF THE MULTIPLE-CHOICE EXPERIMENTS

TABLE 4

* In these crosses, since no heterogamic inseminations occurred, there is no excess insemination of one strain of females over the other.

ing. For purposes of comparison, it was necessary to conduct these tests with respect only to those previous multiplechoice experiments in which at least some heterogamic inseminations had occurred.

These tests were conducted in a manner almost identical with that of the multiple-choice experiments, except that all 20 females in each vial belonged to one species, and thus did not have to be marked. Ten alien males were placed with the 20 females.

As a control, or standard, a series of homogamic matings were made to determine what percentages of females were inseminated by homogamic males. Here again, the length of time during which the females and males were kept together was adjusted to prevent 100 per cent insemination, even in homogamic crosses. The results of these experiments are presented in table 5. Each of the

series of crossing experiments was replicated three times to obtain data that could be statistically compared with the results from the multiple-choice experiments. No significant differences were found among the replicates of the nochoice experiments, except in the case of "2002" males offered lingnanensis females, where one replicate gave a higher degree of inseminations than the others (P < .05). This still did not affect the final result obtained by comparing heterogamic inseminations in the multiplechoice and no-choice experiments. Furthermore, it is reasonable to expect one out of $12 x^2$ values to show significance.

It is obvious, from table 5, that the results of heterogamic crosses are quite similar to the results of the same crosses shown in table 1, with only the *coheni* \times "khunti" combinations approaching the homogamic results.

TABLE 5

NO-CHOICE EXPERIMENTS: COMPARISON OF FREQUENCIES OF HETEROGAMIC INSEMINATIONS IN THE ABSENCE OF HOMOGAMIC FEMALES WITH FREQUENCIES OF HOMOGAMIC INSEMINATIONS IN THE ABSENCE OF HETEROGAMIC FEMALES

Type of	Cro	sses	Number of	Number of	Number of	Per cent	
cross ç ç d'		ೆರ್	females used*	females inseminated	females not inseminated	females inseminated	
	lingnanensis	lingnanensis	58	54	4	93.10	
Homogamic	``2002` '	``2002` `	60	53	7	88.33	
crosses	coheni	coheni	56	50	6	89.29	
(control)	"khunti"	"khunti"	56	51	5	91.07	
Heterogamic crosses	lingnanensis ''2002'' coheni lingnanensis lingnanensis ''khunti'' coheni ''khunti''	"2002" lingnanensis lingnanensis coheni "khunti" lingnanensis "khunti" coheni	$\begin{array}{c} 56 \\ 60 \\ 60 \\ 55 \\ 60 \\ 60 \\ 56 \\ 58 \end{array}$	6 18 1 14 14 0 52 50	50 42 59 41 46 60 4 8	10.71 30.00 1.67 25.45 23.33 0 92.86 86.21	

* Three replicates of 20 females each (occasionally, individuals lost at time of transfer).

Multiple-choice and no-choice experiments

Statistical comparisons of the results of the multiple-choice (table 1) and nochoice (table 5) experiments indicated no significant differences between the number of heterogamic inseminations in the two types of experiments. These data are presented in table 6. It is quite evident that the percentage of heterogamic females inseminated in the multiplechoice experiments is uninfluenced by the presence of homogamic females. An interesting situation was apparent in the case of *lingnanensis* males offered *coheni* females, wherein a single female was inseminated in the no-choice experiment, but none was inseminated in the multiple-choice experiment, although the difference is not great enough to be significant (P > .30).

One difference was noted between the multiple-choice and no-choice experiments. In the multiple-choice experi-

Crosses		Multiple-choice experiments*		No-choice experiments*		X2	Р
çç	ೆರೆ	n	8	n	8		
linganensis	"2002"	51	12	56	6	3.07	>.0
"2002"	lingnanensis	48	18	60	18	0.68	>.3
coheni	lingnanensis	50	0	60	1	0.89	>.3
inonanensis	coheni	45	9	55	14	0.40	≧.5
ingnanensis	"khunti"	55	10	60	14	0.48	≧.5
"khunti"	lingnanensis	52	3	60	0	3.46	>.0
coheni	"khunti"	50	41	56	52	2.97	>.0
"khunti"	coheni	48	42	58	50	0.35	>.80

COMPARISON OF FREQUENCIES OF HETEROGAMIC INSEMINATIONS IN MULTIPLE-CHOICE VS. NO-CHOICE EXPERIMENTS TO TEST THE INFLUENCE OF THE PRESENCE OF CONSPECIFIC FEMALES IN THE

TABLE 6

*n = number of heterogamic females used; s = number of heterogamic females inseminated.

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ments, no statistically significant difference was observed between the following reciprocal crosses:

- lingnanensis females × "2002" males and "2002" females × lingnanensis males
- lingnanensis females × "khunti" males and "khunti" females × lingnanensis males.

However, in the multiple-choice reciprocal crosses, *lingnanensis* females \times *coheni* males and *coheni* females \times *lingnanensis* males, a significantly larger number of females were inseminated in the former than in the latter cross.

On the other hand, in the no-choice experiments, significant differences were observed between all the above-mentioned reciprocal crosses. A probable explanation for this is that in calculating the coefficient of excess insemination in the multiple-choice experiments, the values for heterogamic as well as homogamic insemination were taken into consideration, whereas in the no-choice experiments the reciprocal heterogamic crosses, alone, were compared. This can be appreciated if a comparison is made between the heterogamic inseminations, alone, in the multiple-choice experiments involving the reciprocal crosses between lingnanensis and "2002" and lingnanensis and "khunti"; the x² values were found to be 2.27 (P > .01) and 3.86 (P \simeq .05), respectively, which show significant differences in both the reciprocal crosses.

DISCUSSION

These studies on sexual isolation further corroborate the conclusions drawn from the experimental hybridization work reported in the first paper of this series (Rao and DeBach, 1969). The degree of sexual isolation ranged from complete (*e.g.*, between "2002" and "khunti," where the coefficient of joint isolation was 1.00) to nearly random mating (*e.g.*, between *coheni* and "khunti," where the coefficient of joint isolation was 0.13 \pm .10).

On the basis of indications in the present work, it appears desirable to attempt to define the term "semispecies" more precisely. Although the term has been meaningful to earlier students of evolution, no attempt has been made, to the authors' knowledge, to delimit the degree of reproductive isolation between two populations in order that they may be called semispecies with respect to each other. This situation is not too surprising, since the area is a very "gray" one. "Partial reproductive isolation" tells the story, but is a vague definition.

We have therefore attempted to en-

hance the usefulness of the term semispecies by indicating certain admittedly arbitrary upper and lower limits of reproductive isolation based on the coefficients of joint isolation. Populations having a coefficient of joint isolation that falls between those limits would be called semispecies; populations having a coefficient of joint isolation below the lower limit would be said to belong to the same species; and populations in which the coefficient of joint isolation lies beyond the upper limit would be said to belong to two different species. It must be pointed out that such a delimitation of semispecies cannot be based entirely on the calculated degrees of sexual isolation, since we must also take into consideration such factors as hybrid viability and hybrid fertility. Combining the various kinds of information, however, should enable one to evaluate what status two populations deserve to be given.

As a first approach, the limits suggested are:

COEFFICIENT OF	CATEGORY
JOINT ISOLATION	
0 -0.05	Same species
0.06 - 0.95	Semispecies
0.96 - 1.00	Different species

The lower limit appears to be logical, since mating even between individuals of the same population or of two populations of the same species may not always be 100 per cent successful. An upper limit, such as 0.96 to 1.00, also seems necessary, since we know that naturally occurring species may show occasional breakdown of sexual isolation and hybridize to a limited extent. The range 0.06 to 0.96 for the semispecies category is necessarily wide. Although these limits are arbitrary, they help to provide a useful quantitative expression of phylogenetic relationship, as well as to indicate the extent of divergence of a semispecies. We believe the limits suggested to be useful, at least for the genus under consideration.

Returning to the results of the present experiments, in the Melinus group an extremely high degree (virtually complete) of sexual isolation was found between the members, which in itself is sufficient justification for their being considered distinct species. Between holoxanthus and melinus and between melinus and fisheri, complete sexual isolation was indicated by the coefficient of joint isolation, which was 1.00, although with the aid of mating inducers it was possible to obtain rare hybrids between holoxanthus and melinus, and these hybrids were fertile (Rao and DeBach, 1969). The hybrids obtained in this manner between melinus and fisheri were sterile. No hybridization occurred between holoxanthus and fisheri; hence no multiple-choice experiments were conducted with these two species.

In the Lingnanensis group, *coheni* and "khunti" exhibited nearly random mating. On the basis of the coefficient of joint isolation, however, they are classed as semispecies. Furthermore, we ob-

served from the hybridization experiments (see first paper) that fewer than the normal numbers of hybrid (female) progeny were obtained. (The Aphytis species used in these experiments are arrhenotokous, so that only the female progeny in the F_1 generation from a cross are hybrids, since they are produced from fertilized (diploid) eggs, while F_1 males are nonhybrid, being produced from unfertilized (haploid) eggs.) Some form of reproductive isolation other than sexual, therefore, was operating between these species. (The nature of the isolating mechanism has not been investigated.) This finding again necessitates designating coheni and "khunti" as semispecies rather than as strictly conspecific, but they are certainly very closely related.

It should be pointed out that a study of sexual isolation, alone, is inconclusive proof of genetic compatibility or incompatibility. Hence, attempts at experimental hybridization give additional information on the degree to which a pair of species is genetically compatible.

The coefficients of joint isolation between coheni and "2002" and between "khunti" and "2002" were both 1.00, indicating complete sexual isolation. Under the system suggested, "2002" would therefore be classed as a distinct. but sibling, species with respect to coheni and "khunti." Yet F_1 progeny, which were occasionally obtained in the two respective crosses with the aid of mating inducers, were at least partially fertile. Furthermore, fertility of the hybrids improved in subsequent generations. Since under ordinary conditions, complete sexual isolation was indicated, "2002" must still be considered a separates species with respect to coheni and "khunti." That sibling species hybridize in the laboratory was shown by Patterson and Stone (1952) and Carson (1954). Patterson and Stone presented data relating to hybridization between many species of Drosophila, and Carson showed that D. bocainensis and D. parabocainensis, which are sibling species, do not hybridize in nature (as seen from chromosomal analyses), but hybridize successfully in the laboratory to produce vigorous and abundant F_1 progeny. It is of interest that in all cases measured in our study, hybrids increased in fecundity and viability through the F_n , but in *Drosophila* the F_2 frequently showed reduced fecundity and viability as compared with the F_1 .

On the other hand, although "2002" and lingnanensis exhibited significant sexual isolation (P < .001), mating did take place occasionally under normal laboratory conditions, in the absence of mating inducers. Hybrids between them were fertile. The coefficient of joint isolation was 0.72. Hence, "2002" and lingnanensis can only be considered to be partially isolated, reproductively, and are, therefore, best referred to as semispecies.

Since lingnanensis showed significant sexual isolation (P < .001) from co-

heni, and also produced sterile hybrids with it, they can be considered to have acquired species status with respect to each other. The coefficient of joint isolation (0.93), being very close to the lower value of the arbitrary limits (0.96 to 1.00) set by the authors, also supports this opinion.

The situation with regard to *lingnan*ensis and "khunti" is more difficult to interpret. The coefficient of joint isolation (0.91) falls within the arbitrary limits set for semispecies, but again is very close to the limit set for species. Although mating in the laboratory is rare, it does occur between lingnanensis and "khunti," and the hybrids are fertile. Although this relationship somewhat resembles that between lingnanensis and "2002," it is clearly a borderline case. In view of this fact, and also that lingnanensis and "khunti" differ so vastly in their crossability with coheni, as well as with "2002," it seems justifiable to regard them as separate (sibling) species.

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A series of multiple-choice experiments was conducted to obtain a quantitative measure of sexual isolation between the species and semispecies within each group, and to study to what extent sexual isolation determined reproductive isolation.

Based on the number of homogamic vs. heterogamic females inseminated, coefficients of isolation, joint isolation, and excess insemination were calculated. In all except one case, significant sexual isolation was present.

To determine to what degree, if any, the presence of homogamic females in multiple-choice tests influenced the frequency of heterogamic insemination, a series of no-choice experiments was performed in which the males were offered only females of a single (alien) species or semispecies for mating.

Statistical comparisons of the multiple-choice and no-choice experiments indicated that no significant differences were present between the number of heterogamic inseminations in the two types of experiments. This finding shows that the presence of homogamic females did not influence heterogamic inseminations in the multiple-choice experiments.

An attempt to define the term "semispecies" more precisely, at least with respect to the genus *Apbytis*, was made by indicating certain arbitrary upper and lower limits of reproductive isolation based on the coefficients of joint isolation. With the use of these limits, the taxonomic status and phylogenetic relationships between members within each group were interpreted. The journal HILGARDIA is published at irregular intervals, in volumes of about 650 to 700 pages. The number of issues per volume varies.

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