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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

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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* (Hymenoptera, 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 *boloxanthus*, are valid species with respect to each other and with respect to all other species studied. *Aphytis cobeni* and "khunti," *A. lingnanensis* and "2002," and "R-65-23" and "2002" are considered to be semi-species with respect to each other. Additionally, the following are considered to be valid species with respect to each other: *lingnanensis* and *cobeni*; *lingnanensis* and "khunti"; "2002" and *cobeni*; "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 *Aphytis* 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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THE AUTHORS:

Sudha V. Rao (now Mrs. Sudha Nagarkatti) is with the Commonwealth Institute of Biological Control, Indian Station, Bellary Road, Bangalore (6), India.

Paul DeBach is Professor of Biological Control, College of Biological and Agricultural Sciences, and Entomologist in the Experiment Station, Riverside.

I. Experimental Hybridization and an Interpretation of Evolutionary Relationships Among the Species^{1, 2}

INTRODUCTION

HYMENOPTEROUS PARASITES of the genus *Aphytis* Howard are known to be exclusively parasitic on the Diaspididae, and several are known to be effective in host population regulation. The Diaspididae, commonly referred to as armored scale insects, are small sucking insects that are hidden under a protective armor or scalelike covering. These scale insects cause injury to their host plants by sucking the plant fluids, which results in defoliation as well as dieback of twigs. The genus *Aphytis* contains several interesting species groups which include strains, semispecies, and sibling species that are morphologically identical or nearly so, but geographically separated, sometimes having preferred hosts and, as we now know, showing varying degrees of sexual isolation. Their morphological similarity has often made taxonomic separation difficult and, in the past, several species often masqueraded under one name (DeBach, 1960). To a lesser extent such is still the case. Added to this, *Aphytis* adults are extremely small (fig. 1), (approximately 1 mm long), thus making identification especially difficult.

It was obvious, therefore, that any successful attempts to classify these species and to elucidate their evolutionary relationships would necessarily

have to be made along biosystematical lines, which is the main object of this study. It was proposed to accomplish this by attempting to hybridize various strains, semispecies, and sibling species, as the case might be, and to study the degrees of reproductive isolation among them. For the sake of convenience, all the *Aphytis* cultures used are referred to as species until their systematic status is dealt with in the discussion.

There is undoubtedly a much greater number of closely related *Aphytis* species that attack different host insects occurring on various host plants in nature than is currently recognized. The present study, however, was necessarily restricted to those species that were being cultured in the Department of Biological Control, University of California, Riverside, where this work was conducted. These species, for the most part, were originally obtained abroad from the California red scale, *Aonidiella aurantii* (Maskell), on citrus, but a few other morphologically similar species obtained from other host insects were also included.

Although several different objectives were involved in this work, the present paper deals with:

(1) Experimental hybridization under ordinary laboratory conditions, as well as under special conditions,

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wherein mating was induced by various means.

(2) An interpretation of the taxonomic relationships among the various *Aphytis* species studied, based on the results of experimental hybridization.

Fig. 1. *Aphytis lingnanensis* adults. Left: Relative size as compared with pencil tip. Right: Female ovipositing in a host scale.

HISTORICAL CONSIDERATIONS

Most of the early history of the search for parasites of California red scale by California entomologists, for use in biological control, has been related in detail by Compere (1955, 1961). For many years nearly all the yellow *Aphytis* reared from the California red scale in various countries were assumed to be one species only, *A. chrysomphali* Mercet. In 1947-1948, S. E. Flanders at the University of California, Riverside, received shipments of diaspine scales from South China, which yielded an *Aphytis* species with a dark-pigmented pupa, unlike the yellow pupa of *Aphytis chrysomphali* already present in California. Although

Compere had found the former in collections from South China as early as 1932, he had made no attempt to introduce it into California, assuming it to be *A. chrysomphali*. However, slight biological and morphological differences, as well as the pupal differences, became evident, and he later described the new species as *A. lingnanensis*.

DeBach (1959) discovered two sibling species of *Aphytis* attacking the California red scale in the Orient, which he described as *Aphytis melinus* DeBach and *A. fisheri* DeBach. These are distinguishable from *A. chrysomphali* and *A. lingnanensis* only by careful microscopic examination. At the

same time, he discussed a species from the Oriental yellow scale, *Aonidiella orientalis* (Newstead), on rose at Khunti, India, referred to herein as *A.* "khunti," which in preliminary tests did not cross with the Chinese strain of *lingnanensis* with which it was morphologically identical, nor with any of the other known species. In 1960 DeBach collected yet another species identical in appearance to *lingnanensis* and to "khunti," but reared from the coconut scale, *Aspidiotus destructor* Signoret, in Puerto Rico. This species showed a high degree of reproductive isolation from *lingnanensis* and "khunti" in preliminary laboratory tests.

The importance to biological control of recognizing the presence of cryptic species became even more evident with the discovery, by DeBach (1960), of *A. holoxanthus* DeBach as a species distinct from *lingnanensis*, in collections of Florida red scale, *Chrysomphalus aonidum* (L.), originating from Hong Kong. In addition to slight differences in adult characters, he found that whereas *holoxanthus* attacked Florida red scale, *lingnanensis* preferred California red scale. Heretofore, *holoxanthus* had remained disguised under the name of *lingnanensis*.

About the same time, shipments of California red scale from Israel yielded a species very similar in appearance to *lingnanensis*, but reproductively isolated from it. It is unlikely that the native home of this species is Israel, inasmuch as it has not been found else-

where in the Mediterranean area. DeBach (1960) described this species as *A. coheni* DeBach.

Quednau (1964) reported that *A. africanus* Quednau, an African species, was formerly considered to be a biparental form of *A. chrysomphali* (which is uniparental), since the adult females were barely distinguishable. However, it was found in laboratory tests that *africanus* does not parasitize oleander scale, *Aspidiotus hederae* (Vallot), while *chrysomphali* does to some extent.

Compere (1955) mentioned that the Oriental species, *A. lepidosaphes* Compere, was earlier misidentified by him as *chrysomphali*. Subsequently, S. E. Flanders sorted it out from *chrysomphali* by means of biological methods. This species was found to be highly specific to *Lepidosaphes beckii* (Newman).

In March, 1965, a new *Aphytis* species ("R-65-23"), indistinguishable from *A. lingnanensis* and parasitizing citrus snow scale, *Unaspis citri* (Comstock), was received from Florida. Since very small numbers were obtained initially, there was not enough time to build up a sizeable culture for use in this study. However, limited crossing tests in the laboratory have given significant results.

The problem is becoming increasingly more complex as new species of *Aphytis* are acquired. Observations during the last four years are, however, being interpreted in the light of present information.

MATERIALS AND METHODS

The 10 different species of *Aphytis* involved in the present work are: *africanus*, *coheni*, *fisheri*, *holoxanthus*, "khunti," *lepidosaphes*, *lingnanensis*, *melinus*, "R-65-23," and "2002." Table 1 gives details regarding the collection sites of the different species, their hosts,

and some morphological and biological differences observed by Compere (1955), DeBach (1959, 1960), Quednau (1965), and a few by the senior author.

With regard to distribution of the *Aphytis* species, it should be recognized that citrus and other host plants and

TABLE 1

COLLECTION SITES* OF *APHYTIS* SPP. AND SOME MORPHOLOGICAL† AND BIOLOGICAL‡ DIFFERENCES AMONG THE SPECIES

<i>Aphytis</i> species	Collection sites	Host from which reared§	Adult characters	Pupal pigmentation	Egg to adult developmental period at 80° F	Average number progeny per female	Days to reach 50 per cent mortality at 50 per cent R.H. and:	
							50° F	80° F
<i>lingnanensis</i>	South China (Hong Kong)	<i>Aonidiella aurantii</i>	Thoracic sterna with dusky areas and a Y-shaped mark. Front wings basad of the speculum, usually with 40-50 setae; setae on sides of abdomen indistinct at 120X magnification; 10-12 paler setae on mesoscutum; large, overlapping, scale-like crenulae. Spermatheca large (.05 mm long, .04 mm wide) and elliptical.	Dark (shiny blackish), well-defined pigmentation on mid-thoracic sterna and on mid-ventral abdominal plates.	12	36.6	5	15
"khuntii"	N. W. India (near Delhi)	<i>Aonidiella orientalis</i>	Indistinguishable from <i>lingnanensis</i>	Same as <i>lingnanensis</i>	13	36.5	8	12
"2002"	Puerto Rico (San Juan)	<i>Aspidiotus destructor</i>	Indistinguishable from <i>lingnanensis</i> and "khuntii"	Same as <i>lingnanensis</i>	12	34.1	43	21
<i>coheni</i>	Israel (Ashkelon)	<i>A. aurantii</i>	Thoracic sterna with dusky areas darker than in <i>lingnanensis</i> ; front wings basad of the speculum, usually with 70 or more setae; setae on sides of abdomen very distinct and coarse at 120X magnification; 12-14 dark setae on mesoscutum.	Same as <i>lingnanensis</i>	13	28.7	8	14
<i>africanus</i>	South Africa (Rustenburg)	<i>A. aurantii</i>	Very similar to <i>lingnanensis</i> and <i>coheni</i> . Thoracic sterna dusky, but to a lesser extent than in <i>coheni</i> ; sometimes only a dark, Y-shaped mark between mesosternal plates is present; mesoscutum with 10-11 setae.	Similar to <i>coheni</i> and <i>lingnanensis</i> .	12	19.0	77	24
<i>lepidosaphes</i> . .	China (Lo-Kong-Tung, near Canton)	<i>Lepidosaphes beckeri</i>	Thoracic sterna with plain, dusky areas on prosternum; mesoscutum with 12 setae; 2-7 abdominal setae marginally on each of segments III-VII.	Pupae yellowish except for Y-shaped mark on mesosternum.	17	32.1	not known	not known
"R-65-23"	U.S.A. (Florida)	<i>Unaspis citri</i>	Indistinguishable from <i>lingnanensis</i>	Same as <i>lingnanensis</i>	13	19.2	not known	not known
<i>melinus</i>	N. W. India (New Delhi and Gurgaon) and West Pakistan (Lahore and Saidpur)	<i>A. aurantii</i> and <i>A. citrina</i>	No dusky areas or furcal pigmentation (Y-shaped mark absent); setae on mesoscutum relatively thin and pale; 6-8 pale spines seen on mesoscutum at 45X magnification; large, overlapping crenulae; spermatheca small (.03 mm in diameter) and spherical.	Dark (blackish) pigmentation on mid-thoracic sterna, less well-defined than in <i>lingnanensis</i> ; abdomen clear.	12	36.1	84	20
<i>fisheri</i>	Burma (Kalaw and Taungyi)	<i>A. aurantii</i>	Same as <i>melinus</i> , indistinguishable from it, except for 10 spines on mesoscutum.	Clear yellow without pigmentation on mid-thoracic sterna or abdomen.	13	18.7	98	24
<i>holozanthus</i> . . .	South China (Hong Kong)	<i>Chrysomphalus aonidum</i>	No dusky areas or furcal pigmentation; setae on mesoscutum dark and coarse; 10-12 dark setae on mesoscutum; overlapping large crenulae as in <i>melinus</i> and <i>fisheri</i> .	Dark-pigmented like those of <i>lingnanensis</i> , but a little less pigmentation at tip of abdomen.	13	32.3	94	24

* Collection sites listed represent those from which *Aphytis* cultures were obtained by the Department of Biological Control, University of California, Riverside.

† Morphological differences listed were taken from Compere (1955), DeBach (1959 and 1960), and Quednau (1964). Differences in morphology of the spermathecae were found by the senior author.

‡ Biological differences were observed by the senior author.

§ For additional laboratory and/or field hosts of some of the *Aphytis* spp., see Quednau (1964).

|| See table 6 for experimental and statistical information.

fruits have been moved around the world a great deal, and frequently in this process some of the scale insects and their parasites have also been transported. Therefore, one cannot be too definitive about the natural range of any one of the species dealt with here. The distributional data are based merely on present knowledge and it is not at all unlikely that a species found today in India may be collected in Israel, for example, a few years hence. Unless we know whether this species was intentionally or even accidentally imported or not, we would tend to assume that the range extends all the way from India to Israel. The reverse may also be true, so that a species recorded today only in Burma may actually have a somewhat wider range. Thus, we are limited by the amount of information available, and unless exhaustive surveys are made, we shall never know the actual situation. On the other hand, it is unlikely that the ranges of some of the species are appreciably more extensive than indicated here. Fairly extensive collections have been made in the past few years in the Orient, considered to be the native home of the California red scale.

The degree of host specificity of the *Aphytis* species and the range of hosts that they attack in the field and laboratory are not at present well known. Although Quednau (1964) has listed several hosts as being parasitized by the various *Aphytis* species, it is not clear which of these are attacked in the laboratory or in nature, or both. On the basis of our tests, *africanus* and *lepidosaphes* appear to be highly specific to *Aonidiella aurantii* and *Lepidosaphes beckii*, respectively, and do not breed in *Aspidiotus hederae*, which is a suitable laboratory host for all the remaining *Aphytis* species used in the present study. Also, *holoxanthus* is the only species that parasitizes both females and males of *Chrysomphalus*

aonidum. It did not attack *A. aurantii* in our tests. Although *lingnanensis* is known to attack males of *C. aonidum* in the field, it has not been found in females of this scale.

The morphological differences, especially within certain species-groups, are so few and so slight that biological differences must be used for recognition of some species. This latter method has been followed in part by Quednau (1965) for detecting species of *Aphytis* in field collections. At least a few of the biological differences among species are distinctive enough for this purpose and are helpful in differentiation when used in combination with morphological characteristics.

The laboratory host used for culturing most of the parasites was mature (about 50-day-old) oleander scale, *Aspidiotus hederae*, the uniparental strain of which was found by DeBach and White (1960) to be ideal for laboratory culture of *Aphytis* spp., since it remains suitable for oviposition by *Aphytis* females during the second and third instars, as well as throughout its maturing and producing stages. This contrasts with California red scale, suitable for oviposition by *Aphytis* only in the second and third instars, after which the body becomes attached to the scale cover, making it unacceptable to *Aphytis* females. However, in tests involving *africanus* and *lepidosaphes*, California red scale and purple scale, *Lepidosaphes beckii*, respectively, had to be used since *africanus* oviposits poorly on oleander scale and *lepidosaphes* is highly specific to purple scale. The latter species of *Aphytis* shows a distinct preference for third-instar female scales, which were therefore provided for oviposition.

Culture of scales and parasites was performed as described by DeBach and White (1960). Honey was provided as food for adult parasites in all tests.

Crossing experiments

Virgin individuals are required for hybridization studies. Therefore, males and females in the mature "green-eye" pupal stage were carefully removed from their host scales and isolated individually into $\frac{1}{4}$ -dram vials. Incubation at $80^{\circ} \pm 2^{\circ}$ F and 75 per cent rela-

tive humidity (RH) consistently gave about 95 per cent successful adult emergence.

In hybridization experiments, five females of one species were placed with males of another species. The "mated" females were then anesthetized with carbon dioxide and carefully transferred to a host-bearing lemon for

Fig. 2. Mason jar and scale-bearing lemon used as oviposition unit for *Aphytis* in crossing tests.

oviposition. The scale-bearing lemons were held on small wire stands in 1-pint mason jars with screw-top lids (fig. 2). After the females were added, each jar was covered with a piece of muslin held tight by means of the screw-top lid. Details regarding the method of setting up of the males and females for mating are discussed in a later section.

Rearing of hybrids

The progeny resulting from an attempted cross were isolated individually in the pupal stage and held for emergence in the same manner as previously described. Emerged adults were sexed for the presence of females, because in these arrhenotokous species

only the females of a cross are hybrids, since they are produced from fertilized eggs. The males are produced parthenogenetically from unfertilized eggs. When the cross was successful and hybrid females obtained, a few of the virgin females were allowed to oviposit individually on separate scale-bearing lemons in order to obtain hybrid males. The remaining virgin females were fed on honey and then held at 65° F (to increase longevity) until such time as hybrid males became available (usually 12 to 14 days) for fertilizing the F_1 females and thus providing a pure 50:50 hybrid stock. If this procedure is not followed, a 75:25 hybrid will be obtained that favors the parental female stock.

Holding the females at 65° F was necessary to prolong their life and delay physiological aging. The females held at 65° F were removed from the temperature cabinet once every two days, allowed to feed and move normally for a couple of hours at 80° F and then returned to the cool-temperature cabinet. Cultures of the hybrids were maintained as described for the parent species.

Fecundity and sex ratio of hybrids

Studies on the fecundity and sex ratio of the hybrids were made by using a modification of unpublished techniques previously developed by DeBach and associates. Newly-emerged (1 to 2 hours) hybrid females that had been isolated in the pupal stage and allowed to mate on emerging were allowed to oviposit, individually, on separate scale-bearing lemons in 1-pint mason jars in the same manner as the females from the original crosses. A surplus of hosts was made available. On the eleventh day, the female was transferred to a fresh, scale-bearing lemon, and such transfers were continued until the death of the female. This was necessary in order to obtain

an accurate record of her total progeny.

The period between transfers was limited to 11 days because the development of eggs into adults takes an average minimum of 12 days. Eggs are consistently laid on the first day the female is placed on the lemon; therefore, emergence of the F₁ adults would start on the twelfth day and they would produce second-generation progeny which would be confused with those of the parental female. Thus, by transferring the parental female to new hosts on the eleventh day, the occurrence of second-generation progeny is prevented.

After the parental female was transferred, the parasitized scales were examined for pupae and then reincubated. The total number and sex ratio of pupae were recorded. Sexes in the mature (green-eye) pupal stage can be distinguished with accuracy because the ovipositor of the females is apparent. Thereafter, the scales were checked every fourth day for additional green-eye pupae until it was certain that no more would be found. This was necessary since the female had oviposited over a period of 11 days and all stages (eggs, larvae, and pupae) were present at the end of that period.

GENERAL OBSERVATIONS ON MATING BEHAVIOR IN *APHYTIS* SPP.

One of the fortunate circumstances in working with *Aphytis* spp. is the fact that mating presents no problem whatsoever in the laboratory. Difficulties in this connection have been experienced by entomologists working with certain species of *Diptera* (Rao and Rao, 1964) and *Lepidoptera* (Shorey and Gaston, 1964) in which light, size of cage, time of day or night, even air currents, etc., have been critical. The existence of biological clocks that control many activities in insects

is well-known, but there is no evidence of such mechanisms controlling mating in *Aphytis*. Both females and males are sexually receptive immediately after emergence. Virgin females will mate with conspecific males at virtually any time of the day or night, and subsequent studies have shown that there is no evidence for a particular time of the day or night during which a peak number of homogamic inseminations occur. So far as is known, however, females will nearly always accept

a male only once. There is some evidence that the attractiveness of the female to the male is relatively reduced with age (DeBach, unpublished data). Males, on the other hand, are capable of inseminating females throughout their lifetime.

Courtship behavior and copulation

Males readily court and copulate with virgin conspecific females immediately after emergence. On approaching a conspecific female, the male appears excited, rapidly raises and lowers both pairs of wings once, proceeds towards her and mounts from the rear. A virgin female will usually remain quiet and allow the male to mount. The male then palpitates the antennae of the female with his own continuously until he is sufficiently stimulated to attempt copulation. During the process of palpitation, the vibrating wings of the male are repeatedly raised and lowered. The male then lowers his abdomen below that of the female while holding on to her by the forelegs, and copulates.

Courtship behavior of male *Aphytis* is relatively unelaborate as compared with some other insects. No observable differences in precopulatory behavior were detected among the various species of *Aphytis*.

The female, when successfully courted by a conspecific male, remains absolutely motionless, enabling the male to copulate. Occasionally a male will remount a female that he has already inseminated, but he will rarely show a second copulatory response; if he does, the female nearly always rejects him.

When a virgin female of one species and a male of another closely related species are placed together, the male, especially if newly emerged, will occasionally attempt to court the female. This usually results in a rejection response by the female, and even in rare

cases when the male is sufficiently stimulated and tries to copulate, the female kicks him off with her hind legs and moves away. She also depresses her abdomen, thereby preventing copulation. The latter rejection response in *Drosophila* was called "depressing" by Spieth (1952), and probably explains why so few heterogamic matings are successful.

A strong indication has been noted of the presence of a species-specific male pheromone which has a combined effect of quieting the female and making her receptive in homogamic matings. If this is true, the whole sequence of recognition, acceptance, and insemination may be controlled by a sensitive chemo-tactile mechanism. The relatively simple precopulatory behavior of both the male and the female further indicates that chemo-tactile rather than visual or "vibrational" stimuli are dominant. This was verified by the following series of experiments.

Evidence of a female sex attractant

An experiment was designed to investigate the presence of a sex attractant and to locate its source. Virgin, newly-emerged females and males of *A. lingnanensis* were selected. Glass cells, 1 inch in diameter, $\frac{1}{2}$ inch high, and open at both ends, were glued to a filter paper in a petri dish. Only one cell could be observed at a time but several such cells were kept ready. An anesthetized virgin female was placed in the cell, and the head, thorax, and abdomen were separated with a fine surgical scalpel. An anesthetized male was released into the cell. Next, a glass slide was placed over the observation cell to keep the male from escaping. Each section was tested individually for 15 minutes to determine whether it was attractive to the male. The males were thus observed reacting to the head, thorax, and abdomen in succes-

sion. Of the 10 males, six showed some interest in the head, while the other four did not even approach it. All 10 males showed great interest in the female thorax. They appeared highly excited, mounted the thorax and even attempted to copulate with it. They also seemed to feed on the body fluids exuding from the severed prothoracic end, and showed interest in the region adjoining the wing base. All these actions of the males strongly suggest the presence of an attractant in the female thorax. The attractiveness of the excised abdomen was more or less intermediate between that of the head and thorax, but rarely was any copulatory response shown.

As these observations were of a gross qualitative nature, another experiment was undertaken to obtain quantitative data on the relative attractiveness of the female body parts. The head, thorax, and abdomen of the female were placed a few millimeters apart in a single cell in order to observe the number of "visits" that the male made to each of the body parts and the kind of response that resulted. Virgin females, 1 to 24 hours old, were used. Observations were made on four males over 25-minute periods to determine the persistence of the attractiveness. As before, the female thorax proved to be the most attractive to the *Aphytis* male. Although the male made occasional attempts to copulate with the abdomen of the female, they were not so vigorous as in the case of the thorax. (This may even have been due to a slight masking effect caused by the presence of the thorax in the same cell.) The observations are presented in table 2.

The exact location of the source of the attractant was not determined, but the findings indicated that it might be in the region around the base of the wings. In order to test the hypothesis that pheromones, rather than visual or

other stimuli, were primarily involved, the following tests were conducted.

Visual stimuli

Tests were made to determine the presence of any light-induced or visual stimuli. Pupae of "khunti" in the mature, light-green-eyed stage were isolated into 1/4-dram vials so that each vial contained one female and one male pupa. It has been observed that light-green eyes are characteristic of those pupae producing adults within 10 to 12 hours. The vials were then held in a dark chamber for about three days to allow ample time for the adults to emerge and mate. After that period, the vials were taken out of the dark chamber, and the males were immediately removed. Only those vials in which normal adults of each sex had emerged were used for study. Females from such vials were dissected in normal saline, and their spermathecae were examined for sperm. In all 12 cases observed, normal insemination had taken place, indicating that the absence of light has no serious inhibiting effect on the excitation of the male, nor does it block his ability to court, copulate with, and inseminate a female. In the control vials placed in constant light the females also showed 100 per cent insemination. Simultaneously,

TABLE 2
RELATIVE ATTRACTIVENESS OF
FEMALE HEAD, THORAX, AND
ABDOMEN TO MALE *APHYTIS*
LINGNANENSIS OBSERVED OVER
25-MINUTE PERIODS WHEN
CHOICE WAS AVAILABLE

Male number	Number of "visits" to female		
	Head	Thorax	Abdomen
1.....	2	9	10
2.....	0	14	13
3.....	0	20	0
4.....	3	12	3

a series of vials, each containing a male pupa of "khunti" and a female pupa of "2002," was placed in a dark chamber; for comparison a similar series of control vials was kept in ordinary light. After three days the females from the two series were dissected and examined for sperm. None of the females had been inseminated in either series. This further supports the theory that visual or light-induced stimuli are not critical in the courtship and copulation pattern (table 3).

TABLE 3
HETEROGAMIC VS. HOMOGENIC
MATINGS IN *APHYTIS* SPECIES
IN LIGHT VS. DARKNESS*

Crosses	Females inseminated in darkness	Females inseminated in light
	per cent	per cent
"khunti" ♀ ♀ × "khunti" ♂ ♂ . . .	100	100
"2002" ♀ ♀ × "khunti" ♂ ♂	0	0

* The terms homogamic and heterogamic are used as defined by Dobzhansky and Mayr (1944). Matings among members of the same strains, races, or species are referred to as homogamic; those between different strains, races, or species, as heterogamic.

The role of wings in copulation

Since raising and lowering of wings by the male is an active process during the act of courting and copulation, the role that wings play was investigated. Newly-emerged virgin males and females of "khunti" and "2002" were used. These two strains were chosen because they showed a high degree of reproductive isolation and yet were morphologically identical. The wings of both males and females were delicately removed with a microsurgical scalpel and needles. Only those individuals showing no injury were used in the test. Normal individuals were used as controls. Twenty-four hours after the crosses were set up, the females were removed, dissected, and checked for the presence of sperm. The results of the test are given in table 4.

TABLE 4
EFFECT OF REMOVAL OF WINGS
ON MATING IN HETEROGAMIC
AND HOMOGENIC CROSSES
IN *APHYTIS*

Crosses	Females inseminated
	per cent
10 dealated "khunti" ♀ ♀ × 10 dealated "2002" ♂ ♂	0
10 dealated "khunti" ♀ ♀ × 10 dealated "khunti" ♂ ♂	100
10 normal "khunti" ♀ ♀ × 10 normal "khunti" ♂ ♂	100

All the "khunti" females, both dealated and normal, were inseminated by their own males, but none of the "khunti" females were inseminated by the dealated "2002" males.

This finding indicates that the presence of wings in either the males or the females is not essential for successful courtship and copulation. It also indicates that females would not be able to discriminate between conspecific and alien males on the basis of wing-beat frequency, alone, but that some more subtle mechanism must be involved.

Waldron (1964) found that courting males of *Drosophila persimilis* Dobzhansky and Epling and *D. pseudoobscura* Frolova produce "pulsed vibration sound" with frequency of oscillation and pulse repetition rate that are markedly different in the two species; this, she believes, might contribute to reproductive isolation between the two species. Males with large parts of the wings removed were found to produce nearly normal vibration sounds and normal preliminary sounds. From her experiments on the effects of wing removal, Waldron thinks that the vibration sounds may be produced by thoracic muscle contraction transmitting vibrations to the substrate via the legs.

Although wing removal in *Aphytis*

did not seem to affect courtship or mating behavior, it remains possible that mechanisms such as those found in *Drosophila* by Waldron (1964) are present. Stridulatory mechanisms also could be involved.

The role of antennae in copulation

Since it is well known that the antennae of insects are major organs of sensory perception, no study such as this would be complete without an investigation of their role in the courtship and mating process. For this purpose, two closely related species, *Aphytis lingnanensis* and "2002," were selected. The antennae of 10 female "2002" were removed in the same manner as the wings. These females were placed in a 3-dram vial with five normal *lingnanensis* males. After a mating period of one-half hour, the males were removed and the females dissected and checked for sperm. None of the females were found to have been inseminated. A check was simultaneously set up with 10 normal "2002" females and five normal *lingnanensis* males, which were allowed to mate for one-half hour. Dissection of the females showed 75 per cent insemination. At the same time, 10 "2002" females with their antennae removed were set up for mating with five normal "2002" males. After the one-half-hour mating period, none of the females was found to be inseminated. A control in which normal "2002" females were paired with normal "2002" males gave 100 per cent insemination. This clearly proves the importance of the female antennae in carrying out normal mating activities. The results of these experiments are given in table 5.

Limited testing has also shown that *Aphytis* males, when deprived of their antennae, do not mate even with conspecific females.

Flügge (1934) found that removal of the third antennal segment deprives

TABLE 5
EFFECT OF REMOVAL OF ANTENNAE
ON MATING IN *APHYTIS*

Crosses		Females inseminated
♀ ♀	♂ ♂ (normal)	
		per cent
Antennae-less "2002"	<i>lingnanensis</i>	0
Normal "2002"	<i>lingnanensis</i>	75
Antennae-less "2002"	"2002"	0
Normal "2002"	"2002"	100

Drosophila of its sense of smell. Mayr (1950) showed that removal of antennae from females of *D. pseudoobscura* and *D. persimilis* reduced the sexual receptivity of the females. Further, he showed that sexual isolation between the two species almost disappeared when the females' antennae were removed.

In the present instance, however, sexual isolation seemed to be completed by removal of the females' antennae, especially since even conspecific matings were totally prevented by the operation. It is possible that without antennae the female is unable to perceive the male stimulus, being in a sense "immune" to the male pheromone. This is supported by the observation that females deprived of their antennae would not remain motionless as normal females do, even when conspecific males courted them, and instead moved away or "decamped" as Spieth (1952) termed this response.

To ascertain if the importance of the antennae is common to other species of *Aphytis*, a similar test was performed involving conspecific matings between females and males of *melinus*. It was found that 70 per cent of the females with antennae intact were inseminated, while none of the antennaeless females were inseminated. Mayr (1950) found a reduction in the number of inseminations after removal of antennae in *Drosophila melanogaster* Meigen, *D.*

persimilis, and *D. pseudoobscura*.

These observations were made for the primary purpose of finding out at what stages of courtship or copulation sexual (ethological) isolating mechanisms

could be interfered with or overcome and manipulated in order to increase interspecific hybridization. How this information was utilized in containing hybrids is described in the next section.

EXPERIMENTAL HYBRIDIZATION

Prior to this study, crossing tests had been made between various more or less closely related species or suspect species of *Aphytis* from time to time (DeBach, unpublished data) when new cultures were imported. In all except four cases, however, no hybrids had been obtained. (No manipulative tricks had been used to obtain interspecific matings.) The four exceptions that yielded a few hybrids were: *lingnanensis* × "2002"; *lingnanensis* × *coheni*; *lingnanensis* × "*khunti*"; and "*khunti*" × *coheni*. In these crosses, virgin females and males were merely set up together in vials and, after a 24-hour mating period, transferred into jars with scale-bearing lemons for oviposition. No detailed studies on the resulting hybrids were made.

It is again emphasized that biparental *Aphytis* spp. such as those used in the present experiments are arrhenotokous; that is, they exhibit haplo-diploidy, whereby unfertilized (haploid) eggs give rise to males parthenogenetically, while fertilized (diploid) eggs give rise to females. Therefore, the production of only male progeny in any cross invariably means a failure of mating, or at least of egg fertilization, while the production of female progeny indicates successful mating, however limited the production of females may be. The ultimate success or failure of the cross is, of course, further determined by testing the fertility of the hybrid females over subsequent generations.

From the observations described in the previous section on mating behavior in *Aphytis*, it is obvious that any at-

tempts to hybridize the strains or species would have to deal first with breaking down sexual (ethological) isolating mechanisms between them, which appeared to be very strong in some cases and moderate in others.

Since the female appears to make the ultimate decision regarding copulation by accepting or rejecting a courting male, it was desirable to develop means of inducing the female into accepting an alien male. This might be done by immobilizing the female in some way so that she would remain more or less quiet while an alien male mated with her and yet be able to recover after mating to a normal condition for oviposition. At the same time, the male also had to be induced to copulate with an alien female, since it was found that, particularly in interspecific crosses, copulatory response was rarely shown by a male, even if it courted the female.

In the present series of crosses, therefore, it was decided to use the indicated female sex attractant to stimulate the male, as well as some anesthetic, which might have an effect more or less comparable to that of the male pheromone in holding the female relatively quiet for a short period of time until insemination had been accomplished.

Attempts to extract the female sex attractant in various organic solvents proved unsuccessful, perhaps because of the practical difficulties involved with an insect as small as *Aphytis* (± 1 mm). However, it was found that males of a species could be induced to produce the copulatory response when placed with females of another species by crushing in the vial a few newly-

emerged females of the same species as the male. The males would thus become highly excited and immediately court and attempt to copulate with the alien females. In other words, the incidence of heterogamic matings could, at least in some cases, be considerably increased by using this method.

It has been found that in some insects the incidence of heterogamic matings can be enhanced by using an anesthetic such as ether or chloroform. Streisinger (1948) studied the behavior of males of certain species of *Drosophila* when given a choice of etherized females of their own and alien species. He obtained striking results in the case of *D. melanogaster* males. When the latter were given a choice between etherized females of their own kind and those of *D. persimilis*, no choice was discernible, whereas only conspecific copulations occurred with nonetherized females.

In *Aphytis*, it was found that ether and carbon dioxide did not subdue the females for a long enough time. Chloroform was tried and gave good results. Although chloroform is toxic to *Aphytis* species when they are exposed to it for several minutes, exposure for a short period of time (one-half minute) does not have a harmful effect. In addition, it subdues the females long enough so that the males can go through the copulatory act without being violently rejected. Although not always successful, at least some heterogamic matings were obtained in crosses where hybridization had never been accomplished before. For example, in the crosses "2002" × *coheni* and "2002" × "khunti," no hybrids whatsoever were obtained in the absence of chloroform anesthesia. On the other hand, when chloroform was used, the cross "2002" females × *coheni* males yielded 8.7 per cent hybrid female progeny, and 1.7 per cent resulted from the reciprocal cross. Similarly, in the case of "2002" females

× "khunti" males, 1.4 per cent hybrid female progeny were obtained when chloroform was used, compared with zero when it was not.

In view of these findings, the current series of hybridization trials were modified to include the use of crushed females and chloroform. This method was extremely useful as it gave information on whether the strains and species were merely sexually isolated or were genetically incompatible.

Three-dram vials were used to set up the crosses. In a cross such as *lingnanensis* females × "2002" males, 15 to 20 females of "2002" were crushed with a glass rod in the mating vial. Next, five *lingnanensis* females, anesthetized with chloroform for about 30 seconds in a separate vial, were transferred into the mating vial. Five males of "2002" were immediately introduced. The vials were provided with a streak of honey and left undisturbed for 24 hours. At the end of that period, the five females were transferred to jars containing single lemons bearing sufficient amounts of the appropriate host scale for oviposition.

After 10 days, when nearly all of their egg supply was exhausted, the females were anesthetized with carbon dioxide and removed from the jar. The scales were then examined for developing parasites. Any pupae found were isolated and held for emergence. On emergence, the F₁ progeny were sexed. The F₁ females, being hybrids, were retained; the nonhybrid males were discarded.

When hybrids were obtained in a cross, attempts were made to evaluate their average fecundity and also to determine the sex ratio of their progeny by methods described in the section on materials and methods.

In order to evaluate the results obtained from a given heterogamic cross with respect to the closeness of genetic relationship between two parental cultures, comparison has to be made with

standard values obtained from intra-specific or homogamic crosses. These intraspecific values were obtained by measuring sex ratio and number of female progeny per parental female produced under optimum conditions. The results are given in table 6. Additionally, of course, the ultimate success of a hybrid depends on its fertility in its F₁ and succeeding generations; thus F₁, F₂, F₃, etc., progeny production and sex ratios may provide additional information concerning the degree of genetic relationship between the original parental cultures.

With these standards established, a large series of interspecific crossing trials were conducted. Table 7 gives details of the number of parental females

and males used, the number of female and male progeny obtained in 84 different attempted crosses, and the conditions under which the crossing experiments were performed.

The following descriptions of tests and results apply only to the "successful" crosses. Although hybrid progeny were obtained only in one direction in some crosses, the reciprocal cross is presented for easy reference. The hybrids are denoted by using the first letter of the female parent species name, followed by that of the male parent as a subscript. For example, L₂ is a hybrid between a *lingnanensis* female and a "2002" male. Each cross is followed by its reciprocal, hence the text numbering of crosses used is 1a, 1b, 2a, 2b, etc.

1a. Female *lingnanensis* × male "2002"

(No morphological differences known)

Number of parental females and males used: 57

(No mating inducers were used in this cross because neither the presence of any female sex attractant nor the usefulness of an anesthetic, such as chloroform, was known at the time this particular cross was set up.)

Total F₁ progeny: 796

Total female progeny: 201 (25.3 per cent)

The percentage of female progeny was significantly lower than that in homogamic matings, which is approxi-

mately 64 to 66 per cent. This difference could be accounted for by one or more of the following reasons: (1) not all fe-

TABLE 6
STANDARD VALUES OF PERCENTAGE OF FEMALE PROGENY, AVERAGE TOTAL PROGENY PER PARENTAL FEMALE, AND AVERAGE FEMALE PROGENY PER PARENTAL FEMALE IN INTRASPECIFIC MATINGS

<i>Aphytis</i> species	Standard values, female progeny*	Average total progeny per parental female†	Average female progeny per parental female†
	per cent		
<i>africanus</i>	51.0	19.0 ± 1.03	10.8 ± 0.67
<i>coheni</i>	74.7	28.7 ± 1.00	19.9 ± 1.23
<i>fisheri</i>	57.6	18.7 ± 1.13	10.9 ± 0.73
<i>holozanthus</i>	63.6	32.3 ± 1.63	21.7 ± 1.35
"khunti".....	73.1	36.5 ± 1.94	25.6 ± 1.73
<i>lepidosaphes</i> ‡.....	52.9	32.1 ±	17.0 ±
<i>lingnanensis</i>	66.1	36.6 ± 1.6	25.2 ± 1.17
<i>melinus</i>	64.1	36.1 ± 1.74	24.0 ± 0.42
"2002".....	64.0	34.1 ± 1.2	23.0 ± 0.95
"R-65-23".....	60.0	19.2 ± 1.45	11.4 ± 0.89

* These values were obtained for each species by making counts from laboratory cultures (maintained at a constant temperature of 80° ± 2° F and 50 per cent R.H.) of the two sexes in 10 randomly collected samples of about 100 individuals each. Because the culture was not large enough, counts of "R-65-23" were confined to 200 individuals.

† These values are based on 10 replicates for each species, in which single females were placed on scale-bearing lemons (at a constant temperature of 80° ± 2° F and 50 per cent R.H.), and allowed to oviposit until their death; total progeny and sex ratio were recorded.

‡ Values for *lepidosaphes* were taken from DeBach and Landi (1961). Raw data were not available for calculating standard errors.

TABLE 7
HYBRIDIZATION EXPERIMENTS: RESULTS IN THE F₁ OF CROSSES
BETWEEN *APHYTIS* SPP.

Text description code no.	Crosses		No. of parental ♀ ♀ and ♂ ♂*	Total progeny	No. of ♂ progeny	No. of ♀ progeny	Per cent ♀ progeny	Av. no. of ♀ progeny per parental ♀	Mating inducers used (+), not used (—)
	♀ ♀	♂ ♂							
1a.....	<i>lingnanensis</i>	"2002"	57	796	595	201	25.3	3.5	—
1b.....	"2002"	<i>lingnanensis</i>	63	670	577	93	13.9	1.5	—
2a.....	<i>lingnanensis</i>	"khunti"	35	549	477	37	6.7	1.1	+
2b.....	"khunti"	<i>lingnanensis</i>	45	894	894	0	0	0	+
3a.....	<i>coheni</i>	<i>lingnanensis</i>	43	411	406	5	1.2	0.1	+
3b.....	<i>lingnanensis</i>	<i>coheni</i>	40	514	470	44	8.6	1.1	+
4a.....	"R-65-23"	<i>lingnanensis</i>	3	43	36	7	16.3	2.3	—
4b.....	<i>lingnanensis</i>	"R-65-23"	5	130	130	0	0	0	—
5a.....	"2002"	"khunti"	28	523	517	6	1.14	0.2	+
5b.....	"khunti"	"2002"	30	434	434	0	0	0	+
6a.....	<i>coheni</i>	"2002"	48	471	463	8	1.7	0.2	+
6b.....	"2002"	<i>coheni</i>	29	334	305	29	8.7	1.0	+
7a.....	"2002"	"R-65-23"	3	80	43	37	46.3	12.3	—
7b.....	"R-65-23"	"2002"	3	23	17	6	26.0	2.0	—
8a.....	"khunti"	<i>coheni</i>	35	398	200	198	49.7	5.7	—
8b.....	<i>coheni</i>	"khunti"	35	404	197	207	51.2	5.9	—
	"khunti"	"R-65-23"	3	49	49	0	0	0	—
	"R-65-23"	"khunti"	5	41	41	0	0	0	—
9a.....	<i>coheni</i>	"R-65-23"	3	46	43	3	6.5	1.0	—
9b.....	"R-65-23"	<i>coheni</i>	5	45	45	0	0	0	—
	<i>lingnanensis</i>	<i>holozanthus</i>	25	294	294	0	0	0	+
	<i>holozanthus</i>	<i>lingnanensis</i>	30	144	144	0	0	0	+
	<i>africanus</i>	<i>lingnanensis</i>	35	265	265	0	0	0	+
	<i>lingnanensis</i>	<i>africanus</i>	25	556	556	0	0	0	+
	<i>lingnanensis</i>	<i>fisheri</i>	35	537	537	0	0	0	+
	<i>fisheri</i>	<i>lingnanensis</i>	36	504	504	0	0	0	+
	<i>melinus</i>	<i>lingnanensis</i>	30	185	185	0	0	0	+
	<i>lingnanensis</i>	<i>melinus</i>	45	506	506	0	0	0	+
	<i>lepidosaphes</i>	<i>lingnanensis</i>	25	216	216	0	0	0	+
	<i>lingnanensis</i>	<i>lepidosaphes</i>	28	90	90	0	0	0	+
	"2002"	<i>holozanthus</i>	35	411	411	0	0	0	+
	<i>holozanthus</i>	"2002"	25	314	314	0	0	0	+
	"2002"	<i>africanus</i>	25	308	308	0	0	0	+
	<i>africanus</i>	"2002"	20	290	290	0	0	0	+
	"2002"	<i>fisheri</i>	40	416	416	0	0	0	+
	<i>fisheri</i>	"2002"	25	341	341	0	0	0	+
	"2002"	<i>melinus</i>	25	305	305	0	0	0	+
	<i>melinus</i>	"2002"	45	530	530	0	0	0	+
	"2002"	<i>lepidosaphes</i>	20	162	162	0	0	0	+
	<i>lepidosaphes</i>	"2002"	20	182	182	0	0	0	+
	"khunti"	<i>holozanthus</i>	45	156	156	0	0	0	+
	<i>holozanthus</i>	"khunti"	51	321	321	0	0	0	+
	"khunti"	<i>africanus</i>	40	410	410	0	0	0	+
	<i>africanus</i>	"khunti"	28	430	430	0	0	0	+
	"khunti"	<i>fisheri</i>	34	118	118	0	0	0	+
	<i>fisheri</i>	"khunti"	26	114	114	0	0	0	+
	"khunti"	<i>melinus</i>	30	304	304	0	0	0	+
	<i>melinus</i>	"khunti"	25	240	240	0	0	0	+
	"khunti"	<i>lepidosaphes</i>	15	102	102	0	0	0	+
	<i>lepidosaphes</i>	"khunti"	22	196	196	0	0	0	+
	<i>coheni</i>	<i>holozanthus</i>	35	253	253	0	0	0	+
	<i>holozanthus</i>	<i>coheni</i>	35	218	218	0	0	0	+
	<i>coheni</i>	<i>africanus</i>	27	134	134	0	0	0	+
	<i>africanus</i>	<i>coheni</i>	45	124	124	0	0	0	+
	<i>coheni</i>	<i>fisheri</i>	45	212	212	0	0	0	+
	<i>fisheri</i>	<i>coheni</i>	40	288	288	0	0	0	+

TABLE 7—Continued

Text description code no.	Crosses		No. of parental ♀♀ and ♂♂*	Total progeny	No. of ♂ progeny	No. of ♀ progeny	Per cent ♀ progeny	Av. no. of ♀ progeny per parental ♀	Mating inducers used (+), not used (—)
	♀♀	♂♂							
	<i>coheni</i>	<i>melinus</i>	30	123	123	0	0	0	+
	<i>melinus</i>	<i>coheni</i>	35	207	207	0	0	0	+
	<i>coheni</i>	<i>lepidosaphes</i>	20	198	198	0	0	0	+
	<i>lepidosaphes</i>	<i>coheni</i>	15	103	103	0	0	0	+
	<i>holozanthus</i>	<i>africanus</i>	15	205	205	0	0	0	+
	<i>africanus</i>	<i>holozanthus</i>	20	312	312	0	0	0	+
	<i>holozanthus</i>	<i>fisheri</i>	30	174	174	0	0	0	+
	<i>fisheri</i>	<i>holozanthus</i>	30	202	202	0	0	0	+
10a.....	<i>fisheri</i>	<i>melinus</i>	32	371	369	2	0.54	0.06	+
10b.....	<i>melinus</i>	<i>fisheri</i>	56	373	373	0	0	0	+
	<i>africanus</i>	<i>fisheri</i>	26	389	389	0	0	0	+
	<i>fisheri</i>	<i>africanus</i>	28	218	218	0	0	0	+
	<i>africanus</i>	<i>lepidosaphes</i>	30	460	460	0	0	0	+
	<i>lepidosaphes</i>	<i>africanus</i>	18	197	197	0	0	0	+
	<i>africanus</i>	<i>melinus</i>	25	344	344	0	0	0	+
	<i>melinus</i>	<i>africanus</i>	36	492	492	0	0	0	+
11a.....	<i>melinus</i>	<i>holozanthus</i>	25	311	273	38	12.2	1.5	+
11b.....	<i>holozanthus</i>	<i>melinus</i>	30	228	228	0	0	0	+
	<i>holozanthus</i>	<i>lepidosaphes</i>	18	104	104	0	0	0	+
	<i>lepidosaphes</i>	<i>holozanthus</i>	27	221	221	0	0	0	+
	<i>holozanthus</i>	"R-65-23"	8	56	56	0	0	0	—
	"R-65-23"	<i>holozanthus</i>	6	45	45	0	0	0	—
	<i>fisheri</i>	<i>lepidosaphes</i>	16	115	115	0	0	0	+
	<i>lepidosaphes</i>	<i>fisheri</i>	21	198	198	0	0	0	+
	<i>melinus</i>	<i>lepidosaphes</i>	45	516	516	0	0	0	+
	<i>lepidosaphes</i>	<i>melinus</i>	26	168	168	0	0	0	+
	<i>fisheri</i>	"R-65-23"	4	43	43	0	0	0	—
	"R-65-23"	<i>fisheri</i>	5	69	69	0	0	0	—

* Equal numbers of females and males were used. Figure indicates number of each; i.e., for 1a, 57 females and 57 males were used.

males were inseminated, due to sexual (ethological) isolation; (2) fertilization of eggs was only partially successful due to poor viability, etc., of the alien sperm (gametic isolation); (3) not all fertilized eggs or subsequent developmental stages were viable (hybrid inviability).

In subsequent studies it was found that significant sexual isolation was present between *lingnanensis* females and "2002" males, which resulted in only a small proportion of females being inseminated in the interspecific cross. This, in itself, was sufficient to account for the smaller number of female progeny in crosses between these two species. The possibilities of gametic isolation or hybrid inviability, or both, being present were not investigated.

The hybrid females were found to be

less fertile than the parent species; however, there was an appreciable increase in progeny production per female and in percentage of female progeny in succeeding generations. This was probably the result of natural selection in the culture. Records of this improvement are presented in table 8.

Similar situations are known among plant hybrids, in which, despite a high degree of sterility in the early generations, subsequent generations show increased fertility (Grant, 1966). This author, in his studies on hybrids between *Gilia malior* and *G. modocensis*, found that the F_1 were highly sterile. Artificial selection for vigor and fertility in the generations F_2 to F_6 was very successful. By the F_8 and F_9 , full vigor and fertility, as well as normal chromosome pairing, had been recov-

TABLE 8

TOTAL PROGENY PRODUCTION AND FEMALE PROGENY PRODUCTION PER PARENTAL FEMALE IN THE FIRST FOUR GENERATIONS OF THE L₂ HYBRID

Generation	Total progeny of 10 females	Average total progeny per female	Per cent female progeny	Average female progeny per parental female
F ₁	179	17.9 ± 1.38	42.5	7.6 ± 0.58
F ₂	177	17.7 ± 0.85	40.1	7.1 ± 0.48
F ₃	250	25.0 ± 1.24	52.8	13.2 ± 0.68
F ₄	348	34.8 ± 2.21	60.1	20.9 ± 1.57

ered in the successful lines. Grant attributes this to the joint action of selection and drift.

Another such case is reported by Vaarama (1954), who observed increased general fertility beyond the F₁ generation in hybrids obtained between *Rubus idaeus* and *R. arcticus*. In this case, the number of drupelets per berry in the F₂ generation was about six times greater than in the F₁. This further increased in the F₃ but the increase was not so pronounced. The percentage of good pollen also increased similarly.

In the present case, also, selection and drift could have acted together to bring about the recovery of normal fertility. Selection also appears to have

played a role in favoring those individuals showing a higher propensity for mating.

Hybrids between *lingnanensis* and "2002" showed no morphological differences from the parents, which themselves are indistinguishable one from the other.

Finally, with reference to the cross between *lingnanensis* females and "2002" males, apparently no extremely serious genetical incompatibility factors were involved. Nevertheless, a substantial degree of either sexual or some other form of reproductive isolation was indicated. The extent to which this is present will be discussed and interpreted in a subsequent paper.

1b. Female "2002" × male *lingnanensis*

(No morphological differences known)

Number of parental females and males used: 63

(In this case, again, no mating inducers were used for the same reason as stated under cross 1a.)

Total F₁ progeny: 670

Total female progeny: 93 (13.9 per cent)

The reduced percentage of female progeny here again indicated that some reproductive isolating mechanism was operating.

Fertility of the hybrids was greatly reduced. Total progeny production by

the 2₁ in the F₁ generation averaged 1.2 per female. The few female hybrids obtained were used in an attempt to build up a hybrid culture, but eventually, because of the very low fertility of the hybrids, the culture was lost.

2a. Female *lingnanensis* × male "khunti"

(No morphological differences known)

Number of parental females and males used: 35

(Mating inducers for both females and males were used in this cross, and hereafter, when use of mating inducers is indicated, they were used for both sexes.)

Total F₁ progeny: 549

Total female progeny: 37 (6.7 per cent)

The F₁ hybrid females in this cross were found to have higher fertility than the I₂ and 2₁ hybrids. The sex ratio was normal; that is, from 58 to 60 per cent females were obtained (as compared with the standard of 66 to 73 per cent),

and this has remained fairly steady in subsequent generations. Table 9 gives details of progeny production and female progeny production in the first two generations of the L_k hybrid.

2b. Female "khunti" × male *lingnanensis*

(No morphological differences known)
Number of parental females and males used: 45
(Mating inducers were used.)
Total F₁ progeny: 894
Total female progeny: 0

No hybrids were obtained in this case. From subsequent observations on sexual isolation alone, it was found that the probability of mating occurring in this

cross was extremely low, although it did occur. It is therefore possible that further attempts may yield hybrids.

3a. Female *coheni* × male *lingnanensis*

(Morphological differences very slight. See table 1.)
Number of parental females and males used: 43
(Mating inducers were used.)
Total F₁ progeny: 411
Total female progeny: 5 (1.2 per cent)

In this cross, the incidence of mating was not appreciably enhanced by the use of mating inducers.

Two of the virgin hybrid females were released for oviposition on a lemon bearing oleander scale, but they

failed to produce any progeny. Based on this small sample, sterility is indicated. The remaining three hybrid females had been withheld for subsequent anticipated mating with the hybrid males and hence their fertility was not determined.

3b. Female *lingnanensis* × male *coheni*

(Morphological differences very slight. See table 1.)
Number of parental females and males used: 40
(Mating inducers were used.)
Total F₁ progeny: 514
Total female progeny: 44 (8.6 per cent)

As in cross number 3a, 10 hybrid virgin females were placed with host scales for production of hybrid males,

but no progeny were obtained. Based on this sample, complete sterility is indicated. The condition of the remaining

TABLE 9
TOTAL PROGENY PRODUCTION AND FEMALE PROGENY PRODUCTION PER PARENTAL FEMALE IN THE FIRST TWO GENERATIONS OF THE L_k HYBRID

Generation	Total progeny of 10 females	Average total progeny per female	Per cent female progeny	Average female progeny per parental female
F ₁	104	10.4 ± 0.30	58.7	6.1 ± 0.43
F ₂	158	15.8 ± 0.61	60.1	9.5 ± 0.97

females, withheld for mating to the anticipated hybrid males, was not deter-

mined, but it seems unlikely that they would be fertile.

4a. Female "R-65-23" × male *lingnanensis*

(No morphological differences known.)

Number of parental females and males used: 3

Total F₁ progeny: 43

Total female progeny: 7 (16.3 per cent)

The percentage of female progeny produced was very low compared with the standard 60 to 66 per cent. The species "R-65-23" is a new one that was received only a short time before the present study was terminated. Since only a small culture was available, the number of individuals available for testing was less than usual. No mating inducers were used in crosses involving this new species in order to observe its crossability with the other species under ordinary laboratory conditions. The three parental "R-65-23" females were

confined individually on scale-bearing lemons; thus, it was possible to detect that only one of the three had been inseminated. This suggested the presence of sexual isolation.

Two of the hybrid females were confined with host scales for oviposition, but no progeny were obtained, indicating that the hybrids were sterile. From this it was evident that, in addition to sexual isolation between "R-65-23" females and *lingnanensis* males, hybrid sterility also acted as an isolating mechanism between the two species.

4b. Female *lingnanensis* × male "R-65-23"

(No morphological differences known.)

Number of parental females used: 5

(No mating inducers were used for the reason stated under cross 4a.)

Total F₁ progeny: 130

Total female progeny: 0

The possibilities of breaking down sexual isolation by artificial means were not tested for this cross.

5a. Female "2002" × male "khunti"

(No morphological differences known.)

Number of parental females and males used: 28

(Mating inducers were used.)

Total F₁ progeny: 523

Total female progeny: 6 (1.14 per cent)

This was one of the more difficult crosses to obtain. Earlier attempts without mating inducers had been completely unsuccessful. The hybrids that

were obtained, however, were found to be fertile; fertility and sex-ratio favorability increased markedly in subsequent generations as shown in table 10.

TABLE 10

TOTAL PROGENY PRODUCTION AND FEMALE PROGENY PRODUCTION PER PARENTAL FEMALE IN THE FIRST THREE GENERATIONS OF THE 2_k HYBRID

Generation	Total progeny of 5 females	Average total progeny per female	Per cent female progeny	Average female progeny per parental female
F ₁	27	5.4 ± 0.24	29.6	1.6 ± 0.51
F ₂	141	28.2 ± 1.93	47.5	13.4 ± 0.81
F ₃	206	41.2 ± 2.33	56.3	23.2 ± 1.19

5b. Female "khunti" × male "2002"

(No morphological differences known.)

Number of parental females and males used: 30

(Mating inducers were used.)

Total progeny: 434

Total female progeny: 0

Artificial breakdown of sexual isolating mechanisms in this case was unsuccessful.

6a. Female *coheni* × male "2002"

(Morphological differences very slight. See table 1.)

Number of parental females and males used: 48

(Mating inducers were used.)

Total F₁ progeny: 471

Total female progeny: 8 (1.7 per cent)

The F₁ hybrids were found to be fertile and, as in some of the other cases, fertility improved with successive generations, as shown in table 11.

Once the hybrid culture appeared to have become established, it was transferred from infested lemons to a banana squash bearing a large number of oleander scales. This was done with a view toward getting large numbers of the hybrids. Excessive host feeding by the

adult parasites killed many scales, and little or no oviposition occurred. The few progeny produced were males; thus the culture was lost. The poor results on banana squash as compared with lemons may indicate that the hybrid showed some host-plant preference, although this possibility requires further testing. Since the culture was lost, such testing obviously could not be done.

TABLE 11

TOTAL PROGENY PRODUCTION AND FEMALE PROGENY PRODUCTION PER PARENTAL FEMALE IN THE FIRST TWO GENERATIONS OF THE C₂ HYBRID

Generation	Total progeny of 10 females	Average total progeny per female	Per cent female progeny	Average female progeny per parental female
F ₁	114*	28.5 ± 1.19	29.8	8.5 ± 0.65
F ₂	461	46.1 ± 0.92	33.1	15.3 ± 0.59

* Total progeny of 4 females.

6b. Female "2002" × male *coheni*

(Morphological differences very slight. See table 1.)

Number of parental females used: 29

(Mating inducers were used.)

Total progeny: 334

Total female progeny: 29 (8.7 per cent)

The F₁ hybrids were found to be fertile.

Progeny production and percentage of female progeny increased with succeeding generations as shown in table 12.

No records were kept beyond the second generation. In the fifth generation, the adults were released on a banana squash bearing oleander scales, in

order to increase the culture. For some unexplained reason (as in the reciprocal cross 6a) there was extensive host feeding, but no oviposition occurred and the culture was lost. This strengthened the suspicion that hybrids between *coheni* and "2002" showed a change in their host-plant preference.

TABLE 12

TOTAL PROGENY PRODUCTION AND FEMALE PROGENY PRODUCTION PER PARENTAL FEMALE IN THE FIRST TWO GENERATIONS OF THE 2_c HYBRID

Generation	Total progeny of 10 females	Average total progeny per female	Per cent female progeny	Average female progeny per parental female
F ₁	103	10.3 ± 0.30	30.1	3.1 ± 0.23
F ₂	388	38.8 ± 0.77	43.3	16.8 ± 0.39

7a. Female "2002" × male "R-65-23"

(No morphological differences known.)

Number of parental females and males used: 3

The number of individuals used here was small, as only a few "R-65-23" were available, the culture being in its initial stages.

(No mating inducers were used.)

Total progeny: 80

Total female progeny: 37 (46.3 per cent)

This was one of the most successful crosses, with a high proportion of females (46.3 per cent, as compared with the standard of 60 to 64). Mating had been observed to take place very readily. The F₁ hybrids were highly fertile, producing an average of 32 progeny, per female, with 50 per cent females. Since

the progeny production and sex ratio were almost normal in the F₁, one might be led to believe that "2002" and "R-65-23" are conspecific. However, the fact that "2002" produced fertile hybrids with *lingnanensis*, but "R-65-23" did not, is indicative of significant genetical differences between the two.

7b. Female "R-65-23" × male "2002"

(No morphological differences known.)

Number of parental females used: 3

Here again the number of parental females used was small since the "R-65-23" culture was in its initial stages.

(No mating inducers were used.)

Total progeny: 23

Total female progeny: 6 (26 per cent)

In this cross, mating was not observed to occur as readily as it did in the reciprocal (cross 7a). The small number of total progeny was perhaps the result of "R-65-23" not favoring oleander scale as a host, and it is pos-

sible that this could have been responsible for the observed effect on production of female progeny. It is known that an unpreferred host does affect sex ratio in favor of male production.

8a. Female "khunti" $\times$ male *coheni*

(Morphological differences very slight. See table 1.)

Number of parental females and males used: 35

(No mating inducers were used.)

Total progeny: 398

Total female progeny: 198 (49.7 per cent)

This cross was also one of the more successful ones, the percentage of female progeny in the F_1 being relatively high (49.7), although not so high as the standard (73.1 to 74.7). The F_1 hybrids were fully fertile, and produced normal

numbers of progeny per female as well as a normal sex ratio with about 70 per cent females. This being the case, no records of fecundity or sex ratio were maintained beyond the F_1 generation.

8b. Female *coheni* $\times$ male "khunti"

(Morphological differences very slight. See table 1.)

Number of parental females and males used: 35

(No mating inducers were used.)

Total progeny: 404

Total female progeny: 207 (51.2 per cent)

The F_1 hybrids were fully fertile and produced a normal sex ratio with about 70 per cent female progeny. From crosses 8a and 8b it might seem that *coheni* and "khunti" are conspecific.

This possibility is also supported by studies on sexual isolation, wherein almost random mating was observed. To what extent this hypothesis is valid will be dealt with in the discussion.

9a. Female *coheni* $\times$ male "R-65-23"

(Morphological differences very slight. See table 1.)

Number of parental females used: 3

The number of individuals tested was necessarily small as the culture of "R-65-23" was in its initial stages.

(No mating inducers were used.)

Total progeny: 46

Total female progeny: 3 (6.5 per cent)

The hybrid females were found to be fertile to a low degree. In the F_1 they produced an average of 4.0 progeny per female. No subsequent record of progeny production or sex ratio was kept until the fifth generation in which only the sex ratio was recorded. In the fifth

generation, completed just before this report was written, counts showed the proportion of females to be only 17 per cent, which is much below normal. Whether this proportion will remain or improve in succeeding generations cannot be predicted.

9b. Female "R-65-23" $\times$ male *coheni*

(Morphological differences very slight. See table 1.)

Number of parental females used: 5

In this cross, again, few individuals were available because the culture of "R-65-23" was in its initial stages.

(No mating inducers were used.)

Total progeny: 45

Total female progeny: 0

10a. Female *fisheri* × male *melinus*

(Morphological differences in adults very slight, if any. Pupae differ in degree of pigmentation. See table 1.)

Number of parental females used: 32

(Mating inducers were used.)

Total progeny: 371

Total female progeny: 2 (0.54 per cent)

This pair of species was one of the most difficult to hybridize. No success whatsoever had been obtained in many earlier attempts. Even in the present trials, the number of female progeny obtained was extremely small. The two F₁ hybrid females were allowed to oviposit for two days and were then held at 65° F to enable mating with hybrid males when the latter emerged. However, no female progeny resulted from crosses between the hybrid females and males. On the basis of past experience with other crosses, the hybrids apparently do not mate at all.

The male pupae in the F₂ generation presented an interesting variety of pigmentation. DeBach (1959) found that mature pupae of *fisheri* are nonpigmented, while mature *melinus* pupae bear black pigmentation on the thoracic sternal plates, the pigmentation being uniform throughout the thoracic region. The F_m hybrids, on the other hand, showed a gradation of pigmentation. If it had been possible to follow this up through succeeding generations, some average stable form might have resulted. Since no female progeny were obtained, the culture could not be continued.

10b. Female *melinus* × male *fisheri*

(Morphological differences in adults very slight, if any. Pupae differ in degree of pigmentation. See table 1.)

Number of parental females and males used: 72

(Mating inducers were used.)

Total progeny: 439

Total female progeny: 0

11a. Female *melinus* × male *holoxanthus*

(Morphological differences in adults very slight. Pupae differ in degree of pigmentation. See table 1.)

Number of parental females and males used: 25

(Mating inducers were used.)

Total progeny: 311

Total female progeny: 38 (12.2 per cent)

The M_n hybrids were fairly fertile. In the F₁ generation, the females produced an average of 9.0 offspring per female with 40 per cent female progeny. The average number of female progeny per parental female was 3.5.

11b. Female *holoxanthus* × male *melinus*

(Morphological differences in adults very slight. Pupae differ in degree of pigmentation. See table 1.)

Number of parental females used: 30

(Mating inducers were used.)

Total progeny: 228

Total female progeny: 0

Insemination in heterogamic crosses

From the preceding documentation of "successful" crosses, it is seen that, in a few cases, breakdown of sexual isolation could be accomplished in one direction, *e.g.*, "2002" females $\times$ "khunti" males, but not in the reciprocal direction, *i.e.*, "khunti" females $\times$ "2002"

males. It is not known whether insemination occurred at all in the latter cross, as the "khunti" females were not checked for the presence of sperm.

Laven (1959), working with the *Culex pipiens* complex, found situations in interstrain crosses wherein a full complement of offspring (72 to 98 per cent) was obtained in one direction but only a small number (2.3 to 7.7 per

TABLE 13
RELATIVE PROPORTION OF HYBRID FEMALE PROGENY TO MALES
OBTAINED IN RECIPROCAL CROSSES BETWEEN A
GIVEN PAIR OF SPECIES

Test no.	Reciprocal crosses	Total progeny (♀ ♀ and ♂ ♂)	No. of ♀ progeny	No. of ♂ progeny	χ^2 *	P
1.....	"2002" ♂ $\times$ "R-65-23" ♀ "R-65-23" ♂ $\times$ "2002" ♀	23 80	6 37	17 43	3.0	> .05
2.....	"2002" ♂ $\times$ <i>lingnanensis</i> ♀ <i>lingnanensis</i> ♂ $\times$ "2002" ♀	796 670	201 93	595 577	29.4	< .001
3.....	<i>lingnanensis</i> ♂ $\times$ <i>coheni</i> ♀ <i>coheni</i> ♂ $\times$ <i>lingnanensis</i> ♀	411 514	5 44	406 470	24.5	< .001
4.....	<i>coheni</i> ♂ $\times$ "2002" ♀ "2002" ♂ $\times$ <i>coheni</i> ♀	334 471	29 8	305 463	21.9	< .001
5.....	"khunti" ♂ $\times$ <i>coheni</i> ♀ <i>coheni</i> ♂ $\times$ "khunti" ♀	404 398	207 198	197 200	0.4	> .50

* In no. 1, for example, the χ^2 value is a measure of the difference between 6 ♀ progeny out of a total of 23 as compared with 37 ♀ progeny out of a total of 80.

cent) in the other direction. These figures refer to the hatching percentages in interstrain crosses, in which mating was not a problem. Laven attributed this unilateral incompatibility to cytoplasmic factors.

In the case of crosses between *Aphytis* spp., such as "khunti" females $\times$ "2002" males, and others in which unidirectional crossing was obtained, mating presented a problem. Since it was not known whether the females involved were even inseminated, the unidirectional crossability between these two species could not be attributed to cytoplasmic factors alone.

A comparison was made between those crosses in which varying proportions of hybrid female progeny were ob-

tained in the two reciprocal crosses between a given pair of species. It was found that three of the crosses, *lingnanensis* $\times$ "2002," *lingnanensis* $\times$ *coheni*, and "2002" $\times$ *coheni*, differed significantly ($P < .001$), while the other two, "2002" $\times$ "R-65-23" and "khunti" $\times$ *coheni*, did not. These data are given in table 13. Thus, there appeared to be stronger reproductive isolation between, for example, "2002" females and *lingnanensis* males, than between *lingnanensis* females and "2002" males. Cases in which no hybrids were obtained in either one or both directions could not be considered for want of information.

In none of the cases in which hybridization occurred was any "insemina-

tion reaction" observed, as has been found in species of *Drosophila* by Patterson (1947). There was no indication of a reaction mass being present in the vagina of females in either intraspecific or interspecific crosses, but this requires further study. In *Drosophila*, this reaction mass is known to remain soft in homogamic matings and is eventually expelled by the females. In heterogamic matings, however, the reaction mass is found to harden, thereby reducing the number of hybrids produced or even preventing the production of hybrids altogether, even though the female has been inseminated.

Leahy (1962) found that *Aedes aegypti* (Linn.) and *A. albopictus* Skuse are mechanically incompatible, and semen wasted in matings between the species was found externally on the abdomens of the females. Such a situation is not likely to occur in *Aphytis*, in which, unlike *Aedes*, the aedeagus is a deep, intromittent organ. However, since strong sexual isolation was demonstrated between most of the *Aphytis* species tested, it is possible that at the time of copulation any disturbance caused by the alien female results in fewer sperm being transferred into the spermatheca. To test this hypothesis and see if fewer sperm are transferred in heterogamic vs. homogamic matings, the following experiments were conducted.

Heterogamic matings were set up as follows:

lingnanensis females × "2002" males
lingnanensis females × "khunti" males
lingnanensis females × *coheni* males
 "khunti" females × *lingnanensis* males
 "khunti" females × "2002" males
 "khunti" females × *coheni* males
coheni females × *lingnanensis* males
coheni females × "khunti" males
coheni females × "2002" males
 "2002" females × *lingnanensis* males
 "2002" females × "khunti" males
 "2002" females × *coheni* males

Five or six females of each species were placed individually with males of another. Homogamic matings representing controls were set up simultaneously between conspecific females and males. The pairs were kept individually in small vials, with honey as food, for a period of 72 hours to ensure matings between the maximum number of pairs. At the end of the 72 hours, the females were released for oviposition on individual lemons with host scales, in 1-pint mason jars. After seven days, the parental females were anesthetized and removed from the jars. Some mortality occurred in this period, but some live females were dissected and examined for presence or absence of sperm in the spermathecae. A record was also kept of the progeny that resulted from the crosses. The results of these crosses are presented in tables 14, 15, 16, and 17.

It was found that the number of sperm delivered could vary considerably among females in homogamic as well as heterogamic crosses. For example, in the case of *lingnanensis* females × *lingnanensis* males (table 14), the first female listed was negative for sperm and produced only a single female offspring. Yet the total progeny production was quite normal, which means that the female was normal in every other respect. The other females in this series produced 25, 19, 18, 21, and 19 females, respectively, and were positive for sperm. Comparable numbers of sperm appeared to have been transferred in some of the heterogamic crosses, e.g., *lingnanensis* females × "2002" males and the single *lingnanensis* female × "khunti" male. A similar situation was observed in the case of "khunti" females × *coheni* males and "khunti" females × "khunti" males (table 15). The only inseminated female, in the cross "2002" females × *lingnanensis* males, died and was therefore not checked for presence of sperm (table 16). In the remaining hetero-

TABLE 14

PRESENCE OF SPERM IN SPERMATHECAE OF *LINGNANENSIS* FEMALES
AND SEX RATIO OF THE PROGENY IN HETEROGAMIC VS. HOMOGAMIC
CROSSES AFTER MATING AND ONE WEEK OF OVIPOSITION

♀ no.	<i>lingnanensis</i> ♀ × "2002" ♂			<i>lingnanensis</i> ♀ × "khunti" ♂			<i>lingnanensis</i> ♀ × <i>coheni</i> ♂			<i>lingnanensis</i> ♀ × <i>lingnanensis</i> ♂		
	Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny	
		♀	♂		♀	♂		♀	♂		♀	♂
1.....	+	12	4	—	14	14	—	0	23	—	1	28
2.....	—	16	9	†	0	39	—	0	22	+	25	12
3.....	—	17	43	†	0	9	—	0	9	+	19	7
4.....	+	3	4	†	0	22	†	0	0	+	18	8
5.....	—	0	27	†	0	13	—	0	1	+	21	7
6.....	—	0	21	†	0	8				+	19	4

* + = Live sperm found in spermatheca; — = live sperm not found in spermatheca.
† Female was dead; spermatheca not checked for sperm.

gamic crosses (tables 14–17), none of the females were inseminated.

It is possible that early death of alien sperm in the spermathecae of females, or hybrid inviability, or both, may be the cause of few female progeny in heterogamic crosses. However, the most striking result of these experiments was that the number of females inseminated in heterogamic crosses was small in comparison with that in homogamic crosses. This was presumably due to sexual isolation.

In the process of examining the in-

ternal reproductive systems in these species, it was observed that the spermathecae differ both in shape and in size. It was found that, based on the structure of the spermathecal capsule, *lingnanensis*, "2002," *coheni*, "khunti," and "R-65-23" could be placed in one group, which is termed the Lingnanensis group. In these species, the spermathecal capsule averages 0.05 mm in length and 0.04 mm in width (fig. 3). In contrast, the spermathecal capsules of *melinus*, *fisheri*, and *holoxanthus* are nearly spherical, and average 0.03 mm

TABLE 15

PRESENCE OF SPERM IN SPERMATHECAE OF "KHUNTI" FEMALES AND
SEX RATIO OF THE PROGENY IN HETEROGAMIC VS. HOMOGAMIC
CROSSES AFTER MATING AND ONE WEEK OF OVIPOSITION

♀ no.	"khunti" ♀ × <i>lingnanensis</i> ♂			"khunti" ♀ × "2002" ♂			"khunti" ♀ × <i>coheni</i> ♂			"khunti" ♀ × "khunti" ♂		
	Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny	
		♀	♂		♀	♂		♀	♂		♀	♂
1.....	—	0	5	†	0	17	—	20	7	+	36	14
2.....	—	0	29	†	0	13	†	12	24	†	29	9
3.....	—	0	40	—	0	37	†	41	31	+	34	18
4.....	—	0	46	—	0	16	†	26	38	—	37	20
5.....	—	0	29	—	0	19	†	10	21	—	42	10
6.....				—	0	22						

* + = Live sperm found in spermatheca; — = live sperm not found in spermatheca.
† = Female was dead; spermatheca not checked for sperm.

TABLE 16
 PRESENCE OF SPERM IN SPERMATHECAE OF "2002" FEMALES AND
 SEX RATIO OF THE PROGENY IN HETEROGAMIC VS. HOMOGAMIC
 CROSSES AFTER MATING AND ONE WEEK OF OVIPOSITION

♀ no.	"2002" ♀ × <i>lingnanensis</i> ♂			"2002" ♀ × <i>coheni</i> ♂			"2002" ♀ × "khunti" ♂			"2002" ♀ × "2002" ♂		
	Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny	
		♀	♂		♀	♂		♀	♂		♀	♂
1.....	—	0	37	—	0	11	†	0	15	—	14	8
2.....	†	5	6	—	0	16	—	0	41	—	21	11
3.....	†	0	11	—	0	17	—	0	17	+	19	7
4.....	—	0	16	†	0	11	—	0	17	†	17	8
5.....	—	0	56	—	0	10	—	0	26	—	18	6
6.....	—	0	24	—	0	22	—	0	25	+	13	9

* + = Live sperm found in spermatheca; — = live sperm not found in spermatheca.
 † = Female was dead; spermatheca not checked for sperm.

in diameter. These three species were therefore placed in another group, termed the *Melinus* group (fig. 4).

No differences have been observed as yet in other parts of the female or male genitalia. Whether differences in the structure of the spermathecae alone have any phylogenetic significance is a debatable point, but hybridization results also indicate that they do.

Analysis of results of experimental hybridization

The definite pattern, shown in the series of crosses in table 7, places *lingnanensis*, *coheni*, "khunti," "2002," and "R-65-23" in the *Lingnanensis* group. Besides being almost identical morphologically, these species show some degree of hybridization between various members within the group, either under

TABLE 17
 PRESENCE OF SPERM IN SPERMATHECAE OF *COHENI* FEMALES AND
 SEX RATIO OF THE PROGENY IN HETEROGAMIC VS. HOMOGAMIC
 CROSSES AFTER MATING AND ONE WEEK OF OVIPOSITION

♀ no.	<i>coheni</i> ♀ × <i>lingnanensis</i> ♂			<i>coheni</i> ♀ × "khunti" ♂			<i>coheni</i> ♀ × "2002" ♂			<i>coheni</i> ♀ × <i>coheni</i> ♂		
	Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny		Sperm (+/-)*	Progeny	
		♀	♂		♀	♂		♀	♂		♀	♂
1.....	†	0	11	*	15	9	*	0	14	*	14	3
2.....	—	0	15	*	5	14	—	0	35	+	17	10
3.....	†	0	18	*	4	13	—	0	29	+	12	2
4.....	†	0	12	*	9	17	—	0	18	*	14	6
5.....	†	0	22	*	3	16	—	0	27	*	12	3
6.....	—	0	15	*	8	11	—	0	39	+	12	4

* + = Live sperm found in spermatheca; — = live sperm not found in spermatheca.
 † = Female was dead; spermatheca not checked for sperm.

Fig. 3. Spermatheca of *Aphytis coheni* DeBach.

ordinary laboratory conditions or under the influence of mating inducers.

The same is true of *melinus*, *holoxanthus*, and *fisheri*, which fall into the *Melinus* group. These also are almost indistinguishable morphologically, and some degree of hybridization can be obtained between members within the group. The spermathecal characteristics, described previously, strengthen the placement of these species in the two aforementioned groups. No hybridization could be accomplished between the two groups.

An idea of the degree of reproductive isolation between the different species of *Aphytis* is given by figures 5 and 6, which are graphic representations of comparisons between percentages of fe-

male progeny in homogamic *vs.* heterogamic crosses. The smaller numbers of female progeny in heterogamic crosses indicate various levels of reproductive isolation. From figure 5, which includes members of the *Lingnanensis* group, it is seen that the highest degree of success was obtained in the cross between "khunti" and *coheni*. Percentages of female progeny in the two reciprocal crosses were 49.7 and 51.2, respectively, which is the closest of all crosses to the standard 73.1 to 74.7 per cent in homogamic crosses involving these two species. Other crosses produced fewer females, down to 1.14 per cent in the cross between "2002" and "khunti"; this is the farthest from the standard of 64 to 73.1 per cent obtained in homogamic

Fig. 4. Spermatheca of *Aphytis melinus* DeBach.

crosses. Still others, e.g., "R-65-23" × "khunti," produced no hybrid female progeny in either direction.

Figure 6 shows similar comparisons between *melinus*, *fisheri*, and *holoxanthus* of the *Melinus* group. When *melinus* females were crossed with *holoxanthus* males, 12.2 per cent hybrid female progeny resulted; this is quite significantly lower than the standard 63.6 to 64.1 per cent. The reciprocal cross was not successful, nor were crosses between *holoxanthus* and *fisheri*.

Sterile hybrids were obtained in the following crosses: *lingnanensis* female × *coheni* male and the reciprocal cross; "R-65-23" female × *lingnanensis* male; and *fisheri* female × *melinus* male. This is sufficient evidence to show that these

respective pairs of species are genetically incompatible. In all the remaining crosses in which hybrids were obtained, the fertility was initially somewhat low but, as indicated in the preceding section, increased progressively in subsequent generations.

The two species *africanus* and *lepidosaphes* did not hybridize with any of the other species. They seem to have diverged to the extent of becoming completely isolated reproductively. In all crosses involving these two species, none of the mating inducers were effective. Neither species showed any striking differences in the structure of the spermathecal capsules from that of members of the *Lingnanensis* group, nor in any of the other genitalic structures, so far as

Fig. 5. Percentages of female progeny in homogamic vs. heterogamic crosses of *Aphytis* belonging to the *Lingnanensis* group. Reciprocal heterogamic crosses are presented for comparison.

is known. It must therefore be concluded that strong sexual or other reproductive isolating mechanisms are operating in these cases.

All the remaining interspecific crosses

that failed to produce hybrid progeny are shown in table 7 (p. 529), which summarizes all successful and unsuccessful crossing trials.

In order to obtain a better and more

Fig. 6. Percentages of female progeny in homogamic vs. heterogamic crosses of *Aphytis* belonging to the *Melinus* group. Reciprocal heterogamic crosses are presented for comparison.

easily depicted measure of reproductive isolation than the mere percentage of female (hybrid) progeny produced in a cross (figs. 4 and 5), the P-D isolation index was developed by Dr. Timothy Prout and the second author of this paper. This was done after it was re-

alized that the *number* of female (hybrid) progeny per parental female may be just as important as the *percentage* of female (hybrid) progeny per parental female. They can be quite distinct. This index was calculated as follows:

Step 1:
$$\frac{\text{Per cent female progeny in heterogamic cross}}{\text{Standard per cent female progeny in homogamic cross}} = A$$

The standard value used in the denominator was that of the female parent in the heterogamic cross of the numerator. For example, if the heterogamic cross involved *coheni* females x "2002" males, then in the denominator, the standard values used were those for *coheni* derived from the homogamic cross.

Step 2:
$$\frac{\text{Average number of female progeny per parental female in heterogamic cross}}{\text{Standard average number of female progeny per parental female in homogamic cross}} = B$$

The standard value used in the denominator is again that of the female parent in the heterogamic cross of the numerator.

Step 3: $A \times B \times C = \text{P-D isolation index}$, where C is a "fertility factor," being unity when the F_1 hybrid progeny are all fertile and zero when they are all sterile.

As shown, this index takes into account the percentage of female progeny production, the average number of female progeny produced per parental female, and the fertility or sterility of

the F_1 hybrids. It also indirectly takes into account sexual isolation between the species. The values of the P-D isolation index may range from zero to +1, the lower and upper limits. Zero indi-

icates complete isolation or unquestionably distinct species, whereas unity indicates conspecificity. Intermediate values indicate varying degrees of reproductive isolation. This index applies to arrhenotokous Hymenoptera only.

With the use of this method, P-D isolation indexes between the different species of *Aphytis* were calculated. Although these figures tend to agree with the relationships shown in figures 4 and 5, there are important differences. These values are given in table 18, which

shows that the least degree of reproductive isolation occurs between "2002" females and "R-65-23" males (index of 0.387). On the basis of the P-D isolation index, "2002" females and "R-65-23" males show markedly less reproductive isolation than do either "khunti" males and *coheni* females or the reciprocal of the latter cross. This is a reversal of the results obtained from simply comparing the percentage of female progeny produced in the crosses as shown in figure 5.

DISCUSSION AND CONCLUSIONS

It may be appropriate to define what we mean by some of the taxonomic terms used in this paper. The biological species definition of Mayr, Linsley, and Usinger (1953) is acceptable to us, and the term "species" or "good species" would therefore refer to "actually (or potentially) interbreeding populations which are reproductively isolated from other such groups." The term "sibling species" is also used in the same sense

as that of Mayr, Linsley, and Usinger (1953). It refers to pairs or groups of species, nearly or completely identical, morphologically. "Strain" has often been used in a general sense, and in the present context merely refers to a population from a different geographical area or host, or a culture of as yet undetermined taxonomic status. The term "semispecies" is discussed on page 548.

From the hybridization studies it is evident that some of the morphologically identical but geographically separated species of *Aphytis* have acquired complete reproductive isolation and therefore readily conform to the definition of sibling species; others are less than completely isolated, reproductively, and hence present a nomenclatural problem. In considering data from these studies, we must remember that the hybridization studies were conducted under artificial conditions in which attempts were made to enhance mating in order to obtain hybrids and to determine if any genetical incompatibilities exist between a given pair of species. In our opinion, the chances of interspecific matings were greatly increased under the laboratory conditions employed, as compared with field conditions.

This discussion of experimental results and observations proceeds from

<div>♀ ♂</div>	<i>lingnanensis</i>	"2002"	"khunti"	<i>coheni</i>	"R-65-23"	<i>africanus</i>	<i>lepidosaphes</i>	<i>fisheri</i>	<i>holoxanthus</i>	<i>melinus</i>
<i>lingnanensis</i>	F 66.1	F 13.9	N	St 1.2	St 16.3	N	N	N	N	N
"2002"	F 25.3	F 64.0	N	F 1.7	F 26.0	N	N	N	N	N
"khunti"	F 6.7	F 1.4	F 73.1	F 51.2	N	N	N	N	N	N
<i>coheni</i>	St 8.6	F 8.7	F 49.7	F 74.7	N	N	N	N	N	N
"R-65-23"	N	F 46.3	N	F 6.5	F 60.0	—	—	N	N	—
<i>africanus</i>	N	N	N	N	—	F 51.0	N	N	N	N
<i>lepidosaphes</i>	N	N	N	N	—	N	F 52.0	N	N	N
<i>fisheri</i>	N	N	N	N	N	N	N	F 57.6	N	N
<i>holoxanthus</i>	N	N	N	N	N	N	N	N	F 63.6	F 12.2
<i>melinus</i>	N	N	N	N	—	N	N	St 0.54	N	F 64.1

Fig. 7. Hybridization experiments: outcome of crosses between the different species and semispecies of *Aphytis*. Dark-bordered squares represent homogamic crosses. Numbers represent percentage of female progeny. F = fertile hybrids; St = sterile hybrids; N = no hybrids; — = cross not attempted.

TABLE 18
P-D ISOLATION INDEXES* AMONG THE DIFFERENT SPECIES OF *APHYTIS*
THAT SHOWED ANY F₁ HYBRID PRODUCTION

Cross		Index factors			P-D isolation index (A × B × C)†
♀ ♀	♂ ♂	A	B	C	
<i>lingnanensis</i>	"2002"	0.383	0.139	1	0.053
"2002"	<i>lingnanensis</i>	0.217	0.652	1	0.141
<i>lingnanensis</i>	"khunti"	0.101	0.044	1	0.004
"2002"	"khunti"	0.178	0.087	1	0.015
<i>coheni</i>	<i>lingnanensis</i>	0.016	0.005	0	0‡
<i>lingnanensis</i>	<i>coheni</i>	0.130	0.044	0	0‡
"2002"	<i>coheni</i>	0.136	0.043	1	0.006
<i>coheni</i>	"2002"	0.023	0.010	1	0.0002
"khunti"	<i>coheni</i>	0.680	0.223	1	0.152
<i>coheni</i>	"khunti"	0.685	0.296	1	0.203
"R-65-23"	<i>lingnanensis</i>	0.271	0.201	0	0‡
"2002"	"R-65-23"	0.723	0.535	1	0.387
"R-65-23"	"2002"	0.433	0.175	1	0.076
<i>coheni</i>	"R-65-23"	0.087	0.050	1	0.004
<i>melinus</i>	<i>holoxanthus</i>	0.190	0.063	1	0.012
<i>fisheri</i>	<i>melinus</i>	0.009	0.006	0	0‡

* Method for calculating P-D isolation indexes is given on pages 545 and 546.
† Zero indicates complete reproductive isolation, while unity would indicate absence of reproductive isolation. Intermediate values indicate partial reproductive isolation.
‡ Hybrids in these crosses were sterile.

the simplest situations to the more complex. All the results of the hybridization experiments with regard to percentage of female F₁ progeny production in interspecific crosses and the fertility or sterility of the F₁ hybrids, as the case may be, are summarized in figure 7.

The only two species that show complete reproductive isolation from each other, as well as from all the others, are *africanus* and *lepidosaphes*. The morphological differences, however slight, and the high degree of host specificity also help separate them as good species.

In the *Melinus* group, which here includes *melinus*, *fisheri*, and *holoxanthus*, a high degree of reproductive isolation is present among the species, which in itself is sufficient justification for considering them to be good species. Complete reproductive isolation occurs between this group and the *Lingnanensis* group. With difficulty, some hybridization was obtained between the sibling species *fisheri* (females) and *melinus* (males) and between *melinus* (females) and *holoxanthus* (males). The F_m hy-

brids, however, are highly sterile, which means that *melinus* and *fisheri* are genetically incompatible. In nature, hybridization would therefore be unsuccessful.

The M_h hybrids, on the other hand, are fertile. However, there appears to be strong reproductive isolation between *melinus* and *holoxanthus*, so much so that even in the present case, mating inducers were necessary to effect mating between them. It is unlikely that they would be able to hybridize successfully in nature.

The *Lingnanensis* group is the most difficult to interpret from the standpoint of evolutionary relationships, since the members appear to be in a highly fluid state of evolutionary divergence.

Beginning with *coheni* and "khunti," an interesting situation is observed. They appear to mate nearly at random with each other, and hybridize substantially; yet somewhat less than the standard sex ratio and numbers of female progeny are obtained in crosses between them. Also, genetical differences (pos-

sibly structural differences in the chromosomes) appear to be present between the two, since crosses between "khunti" and *lingnanensis* yield fertile hybrids, while those between *coheni* and *lingnanensis* yield sterile hybrids.

Comparison of the numbers of female progeny obtained in the crosses (table 19) provides valuable information. The number of female progeny in the crosses "khunti" females $\times$ *lingnanensis* males and *coheni* males $\times$ *lingnanensis* females

TABLE 19
DIFFERENCES IN CROSSABILITY (HYBRID PRODUCTION) OF
APHYTIS SPECIES IN THE LINGNANENSIS GROUP*

Crosses	Total progeny (♀ ♀ and ♂ ♂)	No. of ♀ progeny	No. of ♂ progeny	χ^2 †	P
"khunti" ♂ $\times$ <i>lingnanensis</i> ♀	549	37	512	3.69	>.05
<i>coheni</i> ♂ $\times$ <i>lingnanensis</i> ♀	514	44	470		
"khunti" ♂ $\times$ "2002" ♀	523	6	517	29.61	<.001
<i>coheni</i> ♂ $\times$ "2002" ♀	334	29	305		
<i>lingnanensis</i> ♂ $\times$ "2002" ♀	670	93	577	8.11	<.01
<i>coheni</i> ♂ $\times$ "2002" ♀	334	29	305		
"2002" ♂ $\times$ <i>coheni</i> ♀	471	8	463	0.69	>.30
<i>lingnanensis</i> ♂ $\times$ <i>coheni</i> ♀	411	5	406		
"khunti" ♂ $\times$ <i>coheni</i> ♀	404	207	197	264.50	<.001
<i>lingnanensis</i> ♂ $\times$ <i>coheni</i> ♀	411	5	406		
"khunti" ♂ $\times$ <i>coheni</i> ♀	404	207	197	288.20	<.001
"2002" ♂ $\times$ <i>coheni</i> ♀	471	8	463		
"2002" ♂ $\times$ "R-65-23" ♀	23	6	17	0.95	>.30
<i>lingnanensis</i> ♂ $\times$ "R-65-23" ♀	43	7	36		
"R-65-23" ♂ $\times$ "2002" ♀	80	37	43	21.30	<.001
"R-65-23" ♂ $\times$ <i>coheni</i> ♀	46	3	43		

* Only crosses in which hybrids were obtained were compared.

† The χ^2 value is a measure of the difference in hybrid (female) progeny produced between a pair of crosses. For example, the χ^2 264.5 indicates that there is a highly significant difference between the numbers of female progeny produced in the crosses "khunti" ♂ $\times$ *coheni* ♀ and *lingnanensis* ♂ $\times$ *coheni* ♀.

do not show any significant difference ($P > .05$), but there is a highly significant difference between the crosses "khunti" males $\times$ "2002" females and *coheni* males $\times$ "2002" females ($P < .001$); this indicates that "khunti" and *coheni* cannot be essentially identical, genetically.

In crosses between "R-65-23" males and *coheni* females, 6.5 per cent hybrid female progeny were obtained. These hybrids were fertile. In contrast, no hybrids were obtained in crosses between "R-65-23" and "khunti," again indicating definite genetic differences between *coheni* and "khunti."

It is important to recognize the presence of these genetical dissimilarities between "khunti" and *coheni* in view of the somewhat lower than standard numbers of female progeny in crosses between them and, especially, because of the differences in their crossability with other species. Since they exhibit partial reproductive isolation, "khunti" and *coheni* may be called semispecies with respect to each other. This term was originally used by Mayr (1940) to designate the allopatric species of which a superspecies is composed. Subsequently, Mayr (1963) broadened the term to designate populations that have

nearly completed the process of speciation. He stated that gene exchange may still occur among semispecies but not so freely as among conspecific populations. It may be that continuous but limited gene exchange has been maintained between "khunti" and *coheni* through the bridging of populations in the Middle East; this would explain the absence of complete reproductive isolation.

Lingnanensis has a closer relationship with "2002" than with *coheni* or "khunti." Although these species show considerable reproductive isolation, hybrids between them are fertile. There is sufficient evidence, however, to indicate that "2002" and *lingnanensis* are indeed different taxonomic entities, since they (like *coheni* and "khunti") also show differences in their crossability with other species. For example, *lingnanensis* produces sterile hybrids with *coheni* and "R-65-23," while hybrids between "2002" and *coheni* and "2002" and "R-65-23" are fertile. There is no significant difference between the number of female progeny obtained in the crosses "2002" males $\times$ "R-65-23" females and *lingnanensis* males $\times$ "R-65-23" females ($P > .30$). The *lingnanensis* male $\times$ "R-65-23" female produced 16.3 per cent hybrid females, but the reciprocal cross produced no hybrids. Crosses between "R-65-23" and "2002" produced 46.3 per cent and 16.3 per cent hybrid female progeny in reciprocal crosses. However, the P-D isolation indexes between "2002" and "R-65-23" (0.387 and 0.076) indicate that these two species are isolated to a lesser degree than "R-65-23" and *lingnanensis* (P-D isolation index = zero in reciprocal crosses).

Partial but significant reproductive isolation exists between "2002" and *lingnanensis*. Because most *Aphytis* species are thought not to have originated in America, it seems likely that "2002" is a geographical isolate of *ling-*

nanensis that was probably transported to Puerto Rico, its present known home, where it perhaps occurs on a new host. Even though frequency of mating between "2002" and *lingnanensis* is relatively high compared with some of the other crosses, and fairly fertile hybrids are produced, "2002" and *lingnanensis* would best be called semispecies with respect to each other.

No definite taxonomic status can be assigned to "R-65-23" because of insufficient information. It is capable of exchanging genes with *coheni* to a limited extent, but apparently not with "khunti," and freely with "2002" but not at all with *lingnanensis*, with which it produces sterile hybrids. It appears from the hybridization experiments that there is strong sexual isolation between it and all the others, with the exception of "2002." This situation with "2002" might therefore be compared to the relationship between *coheni* and "khunti," so that "R-65-23" could at least be considered a semispecies with respect to "2002" and a species as compared with all the other members of the group.

Since *lingnanensis* produces only sterile hybrids with *coheni*, they should probably be considered to have acquired species status with respect to each other. On the other hand, with manipulation, "2002" produces fertile hybrids with *coheni* and "khunti," but normally shows complete reproductive isolation from them, which indicates that "2002" and *coheni* and "2002" and "khunti" should also be considered good species with respect to each other. The relationship between *lingnanensis* and "khunti" is somewhat difficult to interpret. They do produce few, but fertile, hybrids. However, their ability to cross with *coheni*, "2002" and "R-65-23" differs so greatly that it seems justifiable to regard them as different species. The possibility of the existence of bridging populations that maintain gene flow be-

tween members of the *Lingnanensis* group is discussed below.

All the intricate crossing relations among the species in the *Lingnanensis* group are diagrammed in figure 8, and

Fig. 8. Crossing relations within the *Lingnanensis* group of *Aphytis* showing percentages of F_1 progeny that are female and the nature of the F_1 hybrids. For example, *lingnanensis* crossed with "2002" produces between 15 and 30 per cent fertile hybrid female progeny, whereas crossed with *coheni* it produces from 1 to 5 per cent sterile hybrid female progeny. Normal intraspecies F_1 progeny production consists of 60 to 75 per cent females.

the degree of reproductive isolation between them is indicated. Briefly, figure 8 shows the percentages of hybrid female progeny obtained in different crosses, which are represented by arrows of varying widths. In those crosses wherein hybrids were obtained in both directions, the average of the two reciprocal crosses has been indicated. The solid and dashed arrows represent fer-

tile and sterile progeny, respectively, while dotted arrows represent absence of any hybrid progeny. Thus, for example, *lingnanensis* produces between 15 and 30 per cent fertile hybrid female progeny when crossed with "2002," while *lingnanensis* crossed with *coheni* produces between 1 and 5 per cent sterile hybrid female progeny.

It is not known whether the geographical distributions of the members of the *Lingnanensis* group show any degree of overlap or the presence of bridging populations. Assuming no overlap (on the basis of collections made so far), we can predict that as the populations diverge due to different selection pressures in the regions where they occur, they might develop even stronger isolating mechanisms, such as hybrid inviability (where it is not already present), and then proceed to total reproductive isolation.

Dobzhansky and Spassky (1959), in their study of sibling species of *Drosophila paulistorum* Dobzhansky and Pavan in Central and South America, found that bridging populations are present; these produce fertile hybrids with other populations that are reproductively isolated from each other. In some cases it was necessary to make as many as four consecutive crosses in order to connect two otherwise non-interbreeding populations without encountering sterility of at least the male hybrids. These authors, therefore, contend that all the sibling species should be considered as one whole (a single species) since gene exchange between them is possible through the bridging populations.

This view cannot be applied to the *Aphytis* species, at least at the present time, since no information is available on the existence of bridging populations. Unlike those of *Drosophila paulistorum*, the populations studied are widely separated. In order to determine if "khunti" from India might be such a

bridging population between *coheni* from Israel and *lingnanensis* from South China, hybrids between *coheni* and "khunti" were crossed with *lingnanensis*, but even insemination was not

successful. Future work should be directed toward searching for *Aphytis* populations in intervening geographical regions where such bridging populations might be found.

SUMMARY

Experiments were conducted that attempted to hybridize various strains, semispecies and sibling species of *Aphytis*, with a view toward studying the degrees of reproductive isolation between them and elucidating their evolutionary relationships. For the sake of convenience, each culture was referred to as a species until the study was completed. The species used were: *africanus*, *coheni*, *fisheri*, *holoxanthus*, "khunti," *lepidosaphes*, *lingnanensis*, *melinus*, "R-65-23" and "2002." (Undescribed species are referred to by code names or numbers.)

Interspecific mating between some species took place quite readily in the laboratory; between others it had to be accomplished by the use of mating inducers and, between still others, mating was not successful at all. Fertile hybrids were obtained in some cases and, although the fertility and sex ratio were abnormal initially, definite improvement and even a return to normal were observed in subsequent generations. Sterile hybrids were obtained in a few other crosses, proving the genetical incompatibility of the species involved.

From these observations it was concluded that the species of *Aphytis* used could be divided into four major groups: (1) the *Lingnanensis* group, comprising *lingnanensis*, "2002," *coheni*, "khunti," and "R-65-23"; (2) the *Melinus* group, comprising *melinus*, *fisheri*, and *holoxanthus*; (3) the *Africanus* group, consisting of *africanus*; and (4) the *Lepidosaphes* group, consisting of *lepidosaphes*. The last two species could not be included with either of the first two groups because they showed complete reproductive iso-

lation between each other, as well as from all the other species. Within each major group some degree of hybridization could be accomplished, but not between the groups.

An examination of the spermathecae of members of the *Lingnanensis* and *Melinus* groups also showed differences that justified the separation of these species into the two groups. The spermathecae of the members of the *Lingnanensis* group are elliptical, and larger than those of the *Melinus* group, which are nearly spherical, and small.

Since the *Aphytis* species used in this study are arrhenotokous, the degree of success or failure of a heterogamic cross is indicated by the proportion and relative number of female progeny resulting in the F_1 generation as well as the fertility of the F_1 individuals. An index of reproductive isolation was devised for comparing the percentage of female progeny in the F_1 in a heterogamic cross with the standard percentage of female progeny in a homogamic cross. In addition to percentage of female progeny production, this index also takes into account, indirectly, the sexual isolation between two species.

The following conclusions were made on the basis of this study:

- (1) *Aphytis africanus* and *A. lepidosaphes* are distinct species. Although morphologically only slightly different from the others, they show complete reproductive isolation from each other as well as from all the others.
- (2) *Aphytis melinus*, *fisheri*, and *holoxanthus* are considered distinct species. *A. melinus* and *fisheri* are

sibling species and *holoxanthus* is nearly so. Hybrids between *melinus* and *fisheri* are sterile, while those between *melinus* and *holoxanthus* are obtained in the laboratory only rarely.

- (3) In the *Lingnanensis* group, complex relationships are present. All species studied are siblings or nearly so. *Aphytis coheni* and "khunti" hybridize readily in the laboratory, yielding fertile hybrids. However, they show different crossing relations with "2002," *lingnanensis*, and "R-65-23," indicating that they are genetically rather distinct. They are, therefore, considered to be semispecies with respect to each other.

Since *lingnanensis* and "2002" hybridize fairly readily (although not so readily as *coheni* and "khunti"), but show great differences in their crossability with *coheni* and "R-65-23," they are considered to be semispecies.

The fact that *lingnanensis* produces sterile hybrids with *coheni* confirms the genetical incompatibility between the two, and thus it is concluded that they

have acquired species status with respect to each other.

When crossed with "khunti," *lingnanensis* produces few, but fertile, hybrids. However, the two show great differences in their crossability with *coheni*, "2002," and "R-65-23"; therefore, "khunti" and *lingnanensis* are considered distinct species with respect to each other.

Although "2002" does produce fertile hybrids with *coheni* and "khunti," it does so rarely and only with manipulation. Therefore, "2002" is considered a good species with respect to *coheni* and "khunti."

On the basis of available information, "R-65-23" is considered a semispecies in relation to "2002," with which it hybridizes fairly readily. With *lingnanensis*, it produces sterile hybrids, and it does not hybridize at all with "khunti"; therefore, with respect to both, "R-65-23" is considered a good species. Since "R-65-23" and *coheni* appear to exchange genes only to a very limited extent, hybridization in nature is highly questionable, and they, also, are considered good species with respect to each other.

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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 *Aphytis*, 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.

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