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EXPERIMENTAL STUDIES ON HYBRIDIZATION AND SEXUAL
ISOLATION BETWEEN SOME *APHYTIS* SPECIES (HYMENOPTERA:
APHELINIDAE). III. THE SIGNIFICANCE OF REPRODUCTIVE
ISOLATION BETWEEN INTERSPECIFIC HYBRIDS
AND PARENTAL SPECIES

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Interspecific hybridization is of rare occurrence in animals, especially in comparison to plants, obviously because of their very different modes of reproduction, ecology, and perhaps especially, of behavioral isolation (Mayr, 1963). Within the animal kingdom, hybridization frequency differs with the class, being more characteristic of animals which reproduce by means of external fertilization, e.g., fishes and amphibia. To illustrate this rarity of animal hybrids in nature, Mayr (op. cit.) states that perhaps one out of 60,000 wild birds is a hybrid.

In animals with internal fertilization, the rarity of hybrids may frequently be attributed to the presence of ethological isolating mechanisms. Where ethological isolating mechanisms are overcome, either under laboratory or natural conditions, and successful interspecific copulation occurs, hybrids may occasionally be produced. The steps in hybrid production subsequent to mating, such as fertilization of the egg by the foreign sperm, development of the embryo, etc., should proceed normally. These may be prevented by death of the sperm in the female genital tract, inability of the sperm to fertilize the egg, or by subsequent death of the zygote due to genetic imbalances.

Even when all these isolational barriers are circumvented and hybrids are produced, poor genetic fit (brought about as a result

of deleterious translocations, duplications, inversions, etc., in the chromosomes), resulting in hybrid sterility or inviability of the F_1 generation, usually prevents the successful establishment of a continuing hybrid line between two species (Mayr, 1963).

In rare cases in nature where reproductive isolation mechanisms are overcome and hybrids are produced, one of the following three phenomena usually operate eventually to eliminate the hybrids:

1) Total sterility of the F_1 hybrids prevents them from reproducing.

2) In the event that the F_1 hybrids are fertile, they usually have lower fitness than either parent species and may be eliminated due to competitive displacement. In other words, natural selection and survival of the fittest operates to eliminate them (see discussion in DeBach, 1966, p. 191). Frequently, although the F_1 hybrids may be fertile, fertility is reduced in subsequent generations and thus the hybrid line fails to become established. For example, see Patterson and Stone, 1952, p. 451.

3) The hybrids may back-cross with the parental species to produce inferior genotypes, which again are eliminated as a result of competitive displacement. Mayr (1963) says that introgressive hybridization is rare in animals because only a small fraction of hybrids will back-cross to either of the parental species (see also Ford, 1964, p. 38, 281). In none of these cases has hybridization caused the integrity of the species to be broken down completely although continuous gene exchange may occur.

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In the laboratory, however, the possibility of obtaining interspecific hybrids between animals is greater, since ecological, behavioral, and spatial barriers can be more easily overcome. Also, competition can be eliminated in the laboratory and thus greatly enhance the establishment of hybrid lines. Study of the behavior of the hybrid in the laboratory, with respect to its fitness in comparison to the parent species, its crossability with the parent species, and other characteristics, helps to determine whether or not the hybrid would be capable of establishing a successful, continuing line in the field.

We know of no previous demonstration of an interspecific animal hybrid that has been able to coexist with the two parental species and maintain its integrity in nature as a third biological species. In plants, interspecific hybridization is established as one of the methods of speciation, numerous allopolyploids being known, which, by virtue of being reproductively isolated from the parent species, qualify as a third species. In animals, however, such a phenomenon apparently never has been definitely established. In laboratory experiments, however, the possibility of obtaining a viable interspecific hybrid which would qualify technically as a biological species has at least been indicated.

Among the more intensive laboratory studies on interspecific hybridization in insects may be cited the work of Smith (1959, 1962, 1965) who has studied hybrids between species of *Chilocorus* (Coccinellidae) and other Coleoptera and has found that all native North American species of *Chilocorus* can be hybridized one way or another. In many cases, however, he has encountered a high degree of sterility; in others little is known of the fertility of the F_1 ; and in some others, where hybrids were readily produced, he believes that the normal geographic isolation between them in nature has "rendered the development of a sexual isolation barrier unnecessary" (see especially Smith, 1959).

More species hybrids of *Drosophila* have

been studied than of any other animal genus. Wharton (1944) reported many cases of cross-fertility in the Repleta group of *Drosophila* in laboratory experiments but says that "in nearly every case where the production of fertile hybrids is possible in the laboratory, potent isolating mechanisms operate to prevent such gene exchange in nature."

There are few cases reported involving animals wherein fertile F_1 hybrids were obtained that showed resistance to back-crossing. Foot and Strobell (1914), in reporting their studies involving hybridization between the stink bugs, *E. servus* and *E. variolarius*, stated that the hybrids were fertile among themselves but exhibited almost the same resistance to back-crossing as did the parent species to the original cross. Sears (1947) found that the cross *Drosophila munda* females \times *D. occidentalis* males produced hybrids that were fertile when inbred, but the hybrid males were sterile when back-crossed to either parent type. Hybrid males from the reciprocal cross were fertile when back-crossed to *munda* females but not to *occidentalis* females. Here again gene exchange between the hybrids and parental species was not entirely restricted.

The possibility of obtaining an interspecific hybrid, reproductively isolated from both parent species, but fertile when selfed, has been investigated by Sailer (1953, 1954) who concluded from work on hybridization of the stink bugs *Euschistus servus* (Say) and *E. variolarius* (P. de B.) as follows: "The possibility that an inbred hybrid population may eventually become genetically isolated from its parent species has not been disproved, and is in fact supported by evidence that eggs from back-cross matings show a lower fertility than do selfed matings. The objective of further research on this problem is the production of a synthetic species."

This idea was kept in mind during the present bio-systematical studies on some of the species of *Aphytis* which are parasitic on diaspine scale insects. The results of

these studies on experimental hybridization and sexual isolation have already been reported (Rao and DeBach, 1969a, 1969b). The purpose of the present paper is to discuss the nature of the hybrids that were obtained, with respect to the varying degrees of reproductive isolation that they exhibited from their parent species and to investigate the possibility of developing in the laboratory a synthetic biological "species."

MATERIALS AND METHODS

All species of *Aphytis* are obligatory external hymenopterous parasites of diaspine (or armored) scale insects, and are credited with being among the most effective entomophaga responsible for regulation of population densities of diaspine scale insects. A variety of broad biological, systematic and ecological studies have been carried on with them in this laboratory for over 15 years. In many ways they are ideal laboratory animals. They and their various diaspine scale hosts are easily cultured. The *Aphytis* laboratory life cycle is short—usually about 2 to 3 weeks. The principal disadvantage in working with *Aphytis* is their small size (usually 1 mm or less in length).

The following hybrids obtained in the *Aphytis* crossing experiments were studied with respect to back-crosses. The hybrids are denoted by using the first letter of the specific name or code number of the female parent, followed by that of the male parent as a subscript. The parents used in the original cross are shown below at right (for background information, see Rao and DeBach, 1969a and 1969b):

L_2 = female *A. lingnanensis* Compere \times male *A. "2002"*

L_k = female *A. lingnanensis* \times male *A. "khunti"*

2_k = female *A. "2002"* \times male *A. "khunti"*

C_2 = female *A. coheni* DeBach \times male *A. "2002"*

2_c = female *A. "2002"* \times male *A. coheni*

M_h = female *A. melinus* DeBach \times male *A. holoxanthus* DeBach

$H_{\text{"R-66-19"}}$ = female *A. holoxanthus* \times male *A. "R-66-19"*

The names given above in quotation marks

are code names first used to designate acquisitions (live cultures) of unknown taxonomic status. For reasons discussed by Rao and DeBach (1969a), *lingnanensis* and "2002", as well as "khunti" and *coheni*, are considered to be semispecies (not species) with respect to each other.

The original collection sites of the parent cultures are listed below. These sites are not necessarily part of their indigenous areas, since the exact extent of their natural distribution is not known and accidental ecdysis is common in the genus: *A. lingnanensis*—South China (Hong Kong); *A. "2002"*—Puerto Rico (San Juan); *A. "khunti"*—Northwestern India (near New Delhi); *A. coheni*—Israel (Ashkelon); *A. melinus*—Northwestern India (New Delhi and Gurgaon) and West-Pakistan (Lahore and Saidpur); *A. holoxanthus*—South China (Hong Kong); *A. "R-66-19"*—Mexico (La Paz, Baja California).²

For details concerning field and laboratory hosts, morphological similarities and dissimilarities, methods for setting up the original species crosses, etc., the reader is referred to the earlier paper by Rao and DeBach (1969a).

For the back-cross tests, virgin females and males of the hybrids as well as of the parent species were needed. Pupae were, therefore, isolated during the green-eyed stage (which is the stage just prior to emergence of the adults) in individual vials and held at 80 F (± 2) and approximately 75% R.H. A high humidity is required for pupae removed from the host. On emergence, four to five virgin hybrid females were placed in a 3-dram vial together with three to four males of one of the parental species. Similarly, four or five females of the parental species were placed with three or four males of the hybrid, in the reciprocal cross. Thus, four different combinations were set up with each hybrid.

² This culture was acquired during the latter phases of this study in 1966 by the second author and was reared from cactus scale, *Diaspis echinocacti* (Bouche). The adults are morphologically very similar to the other species and the pupae differ only slightly in pigmentation.

Mating usually takes place immediately upon meeting of the sexes, but a 24-hour mating period was allowed during which the *Aphytis* individuals were left undisturbed in the vials. A streak of honey was provided in each vial for food.

At the end of the 24-hour period the females were anesthetized with carbon dioxide and carefully placed on a lemon bearing mature oleander scale, *Aspidiotus hederae* Vallot, 45–50 days old. This provided a good laboratory host. Each lemon was placed in a one-pint mason jar covered with a piece of muslin held tight by a screw-top lid. The *Aphytis* adults were transferred every 11th day until the female parasites died, in order to prevent development of a second generation on the same lemon. Progeny emerging from the parasitized scale on the lemons were collected every 4th day and the sex-ratio and total numbers recorded. Cactus scale, *Diaspis echinocacti*, was used only in experiments with “R-66-19” as the parasite females showed a pronounced host specificity.

CHARACTERISTICS OF REPRODUCTION IN *APHYTIS*

The *Aphytis* species used reproduce by arrhenotoky, which is the phenomenon whereby unfertilized (haploid) eggs give rise to males parthenogenetically, while fertilized (diploid) eggs give rise to females zygogenetically. This being the case, the production of males alone in any cross indicates the failure of the species to mate or at least of the egg to be fertilized, while the production of female progeny indicates successful fertilization, however limited such production may be. The ultimate success or failure of the “interspecific” (at least, heterogamic) cross is, of course, further determined by testing the fertility of the hybrid females over subsequent generations. Such a measure of the genetic fitness of the hybrid, therefore, involves determination of the ratio of females to males, as well as total progeny production in the F_2 and subsequent generations.

In interspecific crosses, therefore, only

the females in the F_1 generation are hybrids, while the males are not. In order to start a 50:50 hybrid culture, a few of the F_1 hybrid females were allowed to oviposit as virgins for 1 or 2 days and were then held in cold storage along with the others at 65 F, (with daily transfers to 80 F for 1 or 2 hours to enable feeding), until such time as their male hybrid progeny emerged after a period of 13–14 days. When the hybrid males emerged, they were placed for mating in vials at 80 F with hybrid females and the females then allowed to oviposit for production of 50:50 hybrid progeny.

Under standardized, controlled, and near-optimal laboratory conditions of 80 (± 2) F and 50% R.H., adults of all *Aphytis* species used in this study produce characteristic “standard” or “normal” sex-ratios. It was necessary to know the average ratio of females for each “species” in order to compare this with that obtained in interspecific crosses. These values were obtained for each culture from 10 randomly collected samples of about 100 individuals each from cultures held under optimal laboratory conditions. Age of females, host densities, etc., were the same for all tests. The “standard” per cent female progeny for each species is: *coheni*, 74.7; “khunti”, 73.1; *lingnanensis*, 66.1; *melinus*, 64.1; “2002”, 64.0; *holoxanthus*, 63.6; and “R-66-19”, 60.0.

EXPERIMENTAL RESULTS

A summarized version of the experimental results is presented pictorially in Figures 1 and 2. Each case is, however, discussed individually, since important differences occur between them. In the figures, the values in each square represent the proportion of female progeny produced in a particular cross, which as mentioned earlier, measures the relative success or failure of a cross.

The per cent female progeny when *lingnanensis* and “2002” were selfed was 66.1 and 64.0, respectively (Fig. 1a). When the L_2 hybrid was selfed, the per cent female progeny was 60.1. This value was determined in the F_4 generation, by which time

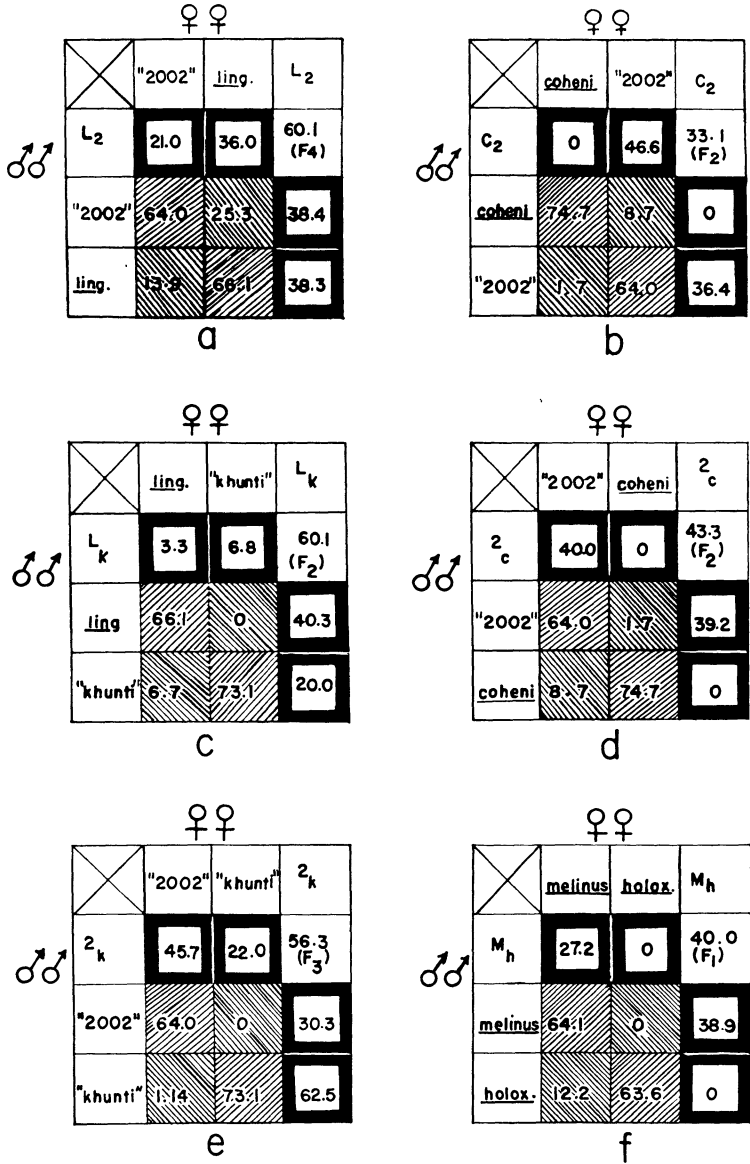


FIG. 1. Back-cross experiments using *Aphytis* hybrids. Numbers represent per cent female progeny. Dark bordered squares represent back-cross results and plain squares represent per cent female progeny in hybrid cultures. Striped squares represent results from "interspecific" and conspecific matings. Explanation of the code designations of the various crosses is given in "Materials and Methods."

the sex-ratio had definitely improved from that in the F₁ generation. The back-crosses, which were performed using the F₁ generation hybrids, showed definite reduction in the per cent female progeny. This represents a lack of compatibility be-

tween the hybrid and its parent species. A back-cross between L₂ females (F₁) and *lingnanensis* males yielded 38.3% female progeny and that between L₂ males (F₁) and *lingnanensis* females 36.0% female progeny. In back-crosses between L₂ fe-

males (F_1) and "2002" males, 38.4% female progeny resulted, while the reciprocal cross yielded 21.0% female progeny. However, there appears to be less reproductive isolation in general between the hybrids and the parents than between the parents themselves. Since the hybrids when selfed showed high fertility and sex-ratio more favorable than in the back-crosses, one might be prone to predict that, given sufficient time, reproductive isolation between the hybrids and the parents would reach completion and the hybrids would become a distinct third species. However, in the present case, back-crosses (using L_2 females and "2002" and *lingnanensis* males) made once again in the 52nd hybrid generation indicated that although reproductive isolation between L_2 and *lingnanensis* had strengthened, that between L_2 and "2002" had weakened. The per cent female progeny produced in these crosses as compared to the F_1 were as follows:

	Per cent female progeny	
	52nd gen.	1st gen.
$L_2 \text{ } \varnothing \varnothing \times \textit{lingnanensis} \text{ } \delta \delta$:	11.2	38.7
$L_2 \text{ } \varnothing \varnothing \times \text{"2002"} \text{ } \delta \delta$:	49.0	38.3

This indicates that L_2 may be approaching the genetic constitution of "2002" and diverging from *lingnanensis*. There is not enough evidence to predict whether a stage would be reached when L_2 and "2002" would mate freely and produce normal numbers of female progeny, while L_2 would become completely isolated from *lingnanensis*. This possibility, however, is at least indicated.

The situation is not very different in the case of the hybrids L_k and 2_k , with some differences in the per cent female progeny in back-crosses. Inasmuch as the L_k culture was discontinued after a few generations, no back-crosses could be attempted in subsequent generations. However, in the case of 2_k , the degree of reproductive isolation from both parents appeared to be relatively unchanged even after about 40 generations.

Three of the hybrids, i.e., C_2 , 2_c , and

M_h , showed another interesting feature. The hybrids C_2 and 2_c showed complete reproductive isolation from *coheni* but only partial isolation from "2002". Whether or not this degree of isolation was maintained as such or was modified in subsequent generations could not be tested since both the hybrid cultures were lost.³

In the case of the hybrid M_h , back-crosses to *holoxanthus* produced no female progeny whatsoever, indicating complete reproductive isolation. On the other hand, back-crosses to *melinus* indicated partial reproductive isolation. Back-crosses in subsequent generations showed no change.

The most interesting of all the hybrids was $H_{\text{"R-66-19"}}$ (see Fig. 2) despite the fact that investigations on this were incomplete. In the original interspecies cross, using 25 parental *holoxanthus* females and "R-66-19" males, a single hybrid female was obtained out of a total of 385 progeny, indicating very strong reproductive isolation between the parental species. This female was allowed to oviposit for 2 days and she laid 20 hybrid male eggs. She was then held in cold storage awaiting emergence of the hybrid male adults. Unfortunately, the female died on the 10th day, 2 to 3 days before any of her hybrid male progeny emerged. This prevented the development of a 50:50 hybrid line. Subsequent attempts to repeat the interspecific cross and get hybrid females were unsuccessful.

Nevertheless, back-crosses were tried between the $H_{\text{"R-66-19"}}$ hybrid males which emerged after the hybrid female died and females of *holoxanthus* as well as females of "R-66-19". It was found that neither cross produced any female progeny whatsoever, thus indicating complete reproductive isolation from both parents. Since no hybrid females were available for back-crosses

³ The loss was due to their inability to breed on oleander scale reared on banana squash, a fact that was discovered too late to prevent loss of the culture. This indicates an interesting change in host preference or host suitability to the hybrids. A change in host-preference of the hybrids would be especially important.

	♀ ♀		
	<u>holox.</u>	"R-66-19"	^H "R-66-19"
♂ ♂	^H "R-66-19"	0	?
	<u>holox.</u>	63.6	?
	"R-66-19"	0.26	?

FIG. 2. Back-cross experiment using the hybrid H_h"R-66-19". Numbers represent per cent female progeny. Dark bordered squares represent back-cross results and shaded squares represent results of interspecific and conspecific matings. Explanation of the code designations is given in "Materials and Methods."

with the parental males, complete verification could not be obtained.

DISCUSSION

Problems of speciation in sexually reproducing higher animals have been subject to much discussion. Most arguments propose geographic isolation as a prerequisite for speciation to occur, but some consider that species can evolve sympatrically. Although no consensus has been reached, most evidence appears to favor the proponents of geographic isolation. Assuming that geographic isolation is necessary, the accumulation of significant gene differences between the two geographically separated populations is not likely to occur very rapidly. At the risk of appearing trite, it is generally agreed that the hypothesis of macrogenesis is not tenable in animals; in other words, that mutations would not be able to provide the necessary degree of genetic differences and hence give rise to a viable new species in one step. Speciation must therefore take place gradually, in a series of steps; and, of course, this involves natural selection acting on genetic variants in a population (see Patterson and Stone, 1952, p. 503).

In furtherance of the preceding general-

ization, instantaneous speciation through polyploidy is not known in sexually reproducing animals. It has only been proved in a few self-fertilizing or asexually reproducing lower animals. In a recent paper, Lewis (1966) has discussed speciation by "saltation" in plants. With the help of illustrations, he explains that adjacent populations, which are very similar in morphological and ecological adaptation, may differ greatly in chromosome arrangement and on occasion in basic chromosome number. The multiple chromosomal differences between such populations cause the hybrids between them to have such low fertility as to be generally considered sterile. According to Lewis, the relationship between two such species is that of parent and offspring. Although the best evidence of saltation is found in annuals, he believes that there is indirect evidence of it in woody plants.

Interspecific hybridization between two animal species leading to the formation of a distinct third "species" has apparently never been established, in view of the lack of knowledge of fertile and successful animal hybrids.

From the experimental results presented in this paper, one sees that at least in three of the cases the hybrids were completely reproductively isolated from one parent and partially from the other (e.g., the hybrids C₂, 2_c, and M_h). In the case of the hybrid M_h, successful back-crossing with *holoxanthus* did not occur even after 20 generations, although M_h did back-cross to a fair extent with *melinus*.

From the evolutionary or phylogenetic standpoint, since C₂, 2_c, and M_h show complete reproductive isolation from at least one parent, they could be considered to be semispecies. If complete reproductive isolation had appeared with respect to both parents, the hybrids could be considered artificially created biological species. Another unique feature observed in this case is that complete reproductive isolation between the hybrids and at least one of the parents appeared in a single step, i.e., in one generation as a result of hybridization,

whereas the development of isolating mechanisms is generally considered to be the result of long-term evolutionary processes.

In the case of the hybrid H^{"R-66-19"}, the chances are that the hybrid would have been completely reproductively isolated from both parents, since this was at least partly indicated by the back-crosses in which males of the hybrid and females of the parent species were used; unless, of course, the males were sterile, of which we have no evidence. Because sterility of one of the sexes among *Aphytis* hybrids was not observed in previous crosses, it would seem safe to assume that the hybrid males were fertile. Since the single hybrid female showed good fecundity, it is very likely that a successful perpetuating hybrid line would have been established, if only the female had survived a little longer. Such a hybrid then would have represented a new biological species.

It seems unlikely that hybrids, such as those obtained in the laboratory, would develop in nature, since the laboratory conditions were made to favor hybridization but much more especially to favor survival of the hybrids. All hybrid cultures were initially poorly viable and it is questionable that they would persist in nature in competition with the parental species. Introgressive hybridization is therefore unlikely.

However, suppose that the hybrids had been produced in nature. The fact, for example, that mating between C₂ and *coheni* and C₂ and "2002" occurs less readily and fewer female progeny result in such crosses than when C₂ hybrids are selfed, would indicate one advantage in favor of the C₂ hybrid for natural selection to work on. This means that reproductive isolation between C₂ and *coheni* and between C₂ and "2002" gradually would be strengthened. In other words, C₂ individuals showing a higher propensity for mating with their own kind would be selected for and hence might increase in numbers, particularly if they happened to have changed host preferences or other favorable essential differences from the parental species. This could conceiv-

ably lead eventually to complete reproductive isolation between C₂ and the parent species. Hence, a pure C₂ stock would be left with a species status, unless it was eliminated through competition with the parental *coheni* and/or "2002". Such a possibility seems even more likely if we are to accept the theory of sympatric speciation in Chalcidoidea proposed by Askew (1968). According to him, an unusually high degree of inbreeding results from sib-matings in isolated populations. This, coupled with the haplo-diploid method of reproduction, which enables more rapid selection, seems to provide chalcids with features promoting a fast rate of evolution. Also, if parts of a population within the range of the whole population utilize different hosts or the same host on different host plants, sympatric speciation would be further facilitated.

The field liberation of such laboratory reared hybrids in an entirely new area where neither parent species occurs would be tantamount to releasing a third species altogether. The results of our hybridization studies are therefore significant from an applied standpoint. *Aphytis* species, as mentioned earlier, are parasitic on diaspine scale insects which are serious plant pests worldwide. All, except two of the species used herein, primarily attack the California red scale, *Aonidiella aurantii* Maskell. *A. holoxanthus* attacks the Florida red scale, *Chrysomphalus aonidum* (L.), while "R-66-19" attacks cactus scale, *Diaspis echinocacti*. Since the hybrids show resistance to back-crossing and have at least in one case proven to be biologically different (having higher heat tolerance), their use in biological control might be almost equivalent to introducing new exotic species. It will be especially interesting to study the field host-preferences of the M_h hybrid since one of its parents, *A. melinus*, attacks the California scale and the other parent attacks the Florida red scale. From laboratory observations it was found that the hybrid would attack the California red scale quite readily, but no tests have been made

using the Florida red scale because this species does not occur in California.

SUMMARY

It is well known that successful interspecific hybridization in animals is an extremely rare phenomenon. In most cases in nature, where it has been observed in animals, it takes the form of introgressive hybridization wherein the two species involved maintain their integrity while continuously exchanging genes. In the course of hybridization experiments using *Aphytis* spp. (Hymenoptera: Aphelinidae) which are parasites of diaspine scale insects, the authors found that in some cases the hybrids were partially reproductively isolated from both parent species, while in others the hybrids were partially reproductively isolated from one parent and completely isolated from the other parent species. Even more interesting was one interspecific hybrid in which the hybrid males probably showed reproductive isolation from females of both parent species. Crosses involving the hybrid females in this particular case could not be carried out as the culture was lost.

These observations point out the possibility of production of new biological species through interspecific hybridization in a single step, which to our knowledge is a phenomenon hitherto unobserved in animals. From an applied standpoint, such hybrids would conceivably represent the equivalent of new exotic species for introduction against a given pest in a biological control program.

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