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## EXPERIMENTAL ANALYSIS OF REPRODUCTIVE INTERSPECIES ISOLATION OF *APIS MELLIFERA* L. AND *APIS CERANA* FABR.

Friedrich RUTTNER (1), Volprecht MAUL (2)

### SUMMARY

Virgin *cerana* queens, placed for mating in different locations in central Europe for several years, were unable to produce hybrid offspring. Mating behaviour being practically identical in both species, *mellifera* drones interfered in the mating process of *cerana* queens. In one case it was observed that interspecific copula resulted in severe injury to the *cerana* queen.

After instrumental insemination of both *mellifera* and *cerana* queens with heterospecific semen, normal fertilization and cleavage of the eggs were observed. During the blastula stage, however, development stopped and finally ended in a complete breakdown.

These observations are discussed from the point of view of the evolution of the two aforementioned species of *Apis*.

### INTRODUCTION

Of the four species of *Apis* which are generally recognized at the present stage of research\*\*, three are sympatric in southeast Asia, while the fourth species, *Apis mellifera*, occurs strictly allopatrically in the western half of the autochthonous *Apis* area. One of the Asiatic species, *A. cerana*, is so similar to *A. mellifera* both in morphology and in ethology that it has not always been clear whether these are distinct species. When v. BUTTEL-REEPEN (1906) attempted the first outline of a scientific classification of all taxa discriminated in the genus *Apis*, he placed *A. indica* (synonym of *cerana*) as one of the three subspecies within the species *A. mellifera*, together with the European subspecies « *mellifera* » and the African subspecies « *unicolor* ».

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\*\* An additional species, *A. laboriosa*, has been described from the Himalayan mountains by SAKAGAMI *et al.* (1980), its taxonomic position, however, needs further clarification.

Even though specific morphological and behavioural characteristics were found later in *A. cerana* (number of tomenta, venation of hind wing, male genitalia, structure of cappings of drone brood, position of bees while fanning, etc.), the situation has not been clarified definitively. In northern India « transitory types » *mellifera/cerana* as well as hybridization were reported (MUTTOO, 1951, VATS, 1953, SHARMA, 1960).

This question still remains unanswered : Is *A. mellifera* completely isolated from *A. cerana* genetically, regardless of the geographic isolation between the two species?

In the present theoretical concept, the species, as genetically isolated population, is regarded as the basic unit in evolution (DOBSHANSKY, 1951, MAYR, 1953). Reproductive isolation is the decisive step in speciation. The reproductive barrier between two more or less related populations may occur at different levels in the process of reproduction — in the phase of pre-mating, mating, post-mating and zygote —, which are of different selective significance. A pre-mating barrier means avoiding losses and less « troubles » in the life history of individuals than post-mating or zygotic ones. Thus the different steps of reproductive isolation are by themselves subject to natural selection. The detailed analysis of the whole system of the reproductive isolation of two related species may reveal a lot about their history, possible coexistence and their relative age in evolution.

For natural hybridization to occur between two species of honeybees, the whole sequence of the complex ritual of mating has to take its course. 1) Queens and drones of both species have to leave the hive at the same hour of day and 2) they have to meet at the same locations (drone congregation areas ZMARLICKY and MORSE, 1963, RUTTNER and RUTTNER, 1963, 1965, 1966, 1968, 1972). 3) They have to be attracted by the same specific sexual odours (GARY, 1962). 4) Copulation has to be feasible anatomically and 5) the physiological conditions of the semen and of the genital tract of the queen (including the spermatheca) have to be appropriate for motility, migration and survival of the spermatozoa to the moment of 6) fertilization of the egg. 7) Finally, the genetic and cytoplasmatic factors of the female and male have to be co-adapted to give a viable and fertile offspring.

Thus the two partners must meet many specific requirements for effective hybridization. It is the scope of this study to examine this problem in detail.

The first question is : Do hybrids between *mellifera* and *cerana* occur as result of free (open) mating? The information available from published data is contradictory. In the majority of cases, no hybrids were observed even when colonies of both types were kept at the same locality.

The second question concerns the analysis of the different sections in the sequence of mating behaviour of the two species. The results obtained in our

experiments including data published previously (no. 1-3) will be presented in the same order as listed above.

## RESULTS

### 1. *Time of mating flight\**

In one experiment conducted in the Bavarian Alps (RUTTNER *et al.*, 1972) with virgin queens and drones of *A. cerana indica* from Pakistan, the queen flight took place between 13.15 and 16.15 h (maximum between 14.15 and 15.45 h) and drone flight between 11.15 and 15.15 h (max. between 12.15 and 14.15 h). This coincides exactly with the flight time of *mellifera* queens and as far as the *cerana* drones are concerned, flight activity ends an hour earlier (for reviews on mating behaviour of *A. mellifera* see RUTTNER, 1956, G. KOENIGER, 1983).

The flight time of drones might possibly vary with the geographic origin of the *cerana* colonies observed. With *cerana* colonies originating from northern China, the drone flight in Germany terminated not earlier than after 16.15 h, exactly coinciding with the flight time of *mellifera* drones. In Sri Lanka with three *Apis* species flying at the same location, the flight time of *cerana* drones was determined to be restricted to the short period between 16.15-17.15 h (KOENIGER and WIJAYAGUNASEKERA, 1976).

In any case *cerana* drones from Pakistan and China, when kept in Europe, did show high flight activity at the same time of day as did queens and drones of *A. mellifera*. On one day, at the congregation area (see below, no. 2) the same proportion of drones of both species was captured before and after 14.45 h : *Mellifera* drones 1076, *cerana* drones 26 between 13.15 h and 14.45 h; *mellifera* 1136, *cerana* 26 between 14.45 and 16.15 h.

### 2. *Meeting of queens and drones*

Two colonies of *A. cerana indica* (from Pakistan), each containing about 1,000 drones, were placed near a well-known drone congregation area in the region of Frankfurt-am-Main. During the time of drone flight (13-16 h) a total of 2,599 drones were caught while circling around a *mellifera* queen exposed at a height of about 8-10 m.

Fifty-three of them (2.12 %) were *cerana* drones. About 100 *mellifera* colonies were present at the same time within the flight range of the congregation area and it was concluded that *cerana* drones were visiting this *mellifera* congregation area

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\* The daytime is given as true local time.

with the same frequency as did *mellifera* drones — thus showing a comparable orientation scheme during mating flight (RUTTNER, 1973).

### 3. *Sexual attraction*

A living *cerana* queen, presented simultaneously at a *mellifera* congregation area generally attracts as many (*mellifera*) drones as does a *mellifera* queen. Only on days with generally low attraction (e.g. at the end of the season) is the *mellifera* queen preferred (RUTTNER and KAISSLING, 1968). Similar observations were made by BUTLER, CALAM and CALLOW, 1967. The stimulation of the receptors (pore plates) at the antennae of drones by secretions of the mandibular glands of queens of both species (or by synthetic 9-oxo-decenoic acid) in electrophysiological experiments was the same in *A. cerana* as in *A. mellifera*.

Summarizing nos. 1-3, no behavioural block was found between the two species to prevent hybrid matings. Yet to be studied is the second part of the events in the mating process — that is copulation, migration and storage of sperm and fertilization.

### 4. *Natural mating of cerana queens in Central Europe*

Young *cerana* queens were reared by the grafting method. Then small nuclei with at least one brood comb (to prevent absconding) were established as described by RUTTNER, WOYKE and KOENIGER (1972). The larvae for grafting were taken from colonies originating from China (Peking) and Pakistan (Peshawar). Considerable losses of queens (see table 1) occurred without causal connection with the mating flight (loss of colonies due to cold weather, absconding in consequence of initial absence of brood).

It must be emphasized that the experiments were not primarily to study hybridization problems, but to establish a stock of *cerana* colonies with young queens. For this reason, many *cerana* drones were reared in the parent colonies of the species. Thus, several thousand drones were present each year during the mating period. The queens and drones were placed together in locations of various distances from *mellifera* colonies. These distances were increased after each failure in matings during the years 1966-1973.

#### *Effects of the presence of mellifera drones*

No *cerana* queens that were fully fertile for more than four weeks were obtained when mated at distances from *mellifera* colonies up to three kilometers (table 1). Most of these queens were lost, some never started to lay eggs and some became drone layers. Some produced *cerana* worker brood during the first few weeks, and drone brood later in increasing amounts.

TABLE 1. — *Results of natural mating of cerana queens at different distances from mellifera colonies.*

Group	Distance between locations of <i>cerana</i> virgin queen and closest <i>mellifera</i> colonies (meters)	No. of queens reared	losses		Production of worker brood		drone brood	queens without eggs
			external factors	during mating flight	tempor.	perman.		
1	0	18	14	2	—	—	—	2
2	500	14	9	3	2	—	—	1
3	1,000	10	2	4	4	—	—	—
4	3,000	24	1	13	7	—	2	1
5	10,000 (1971)	44	10	14	3	11	—	5
5	10,000 (1973)	110	36	36	16	11	2	9

No hybrid bees (provided that these would be different morphologically from pure *cerana* workers) have ever been observed. Finally, in 1971 and 1973, experiments were made in the Bavarian Alps (valley of Graswang near Oberammergau), at least 10 km from the nearest *mellifera* hive. Periods of cold rainy weather in both years caused severe losses of the small nuclei (altitude about 1,000 m). However for the first time queens were obtained (fig. 1) with a good brood pattern and a long lasting production of *cerana* workers.

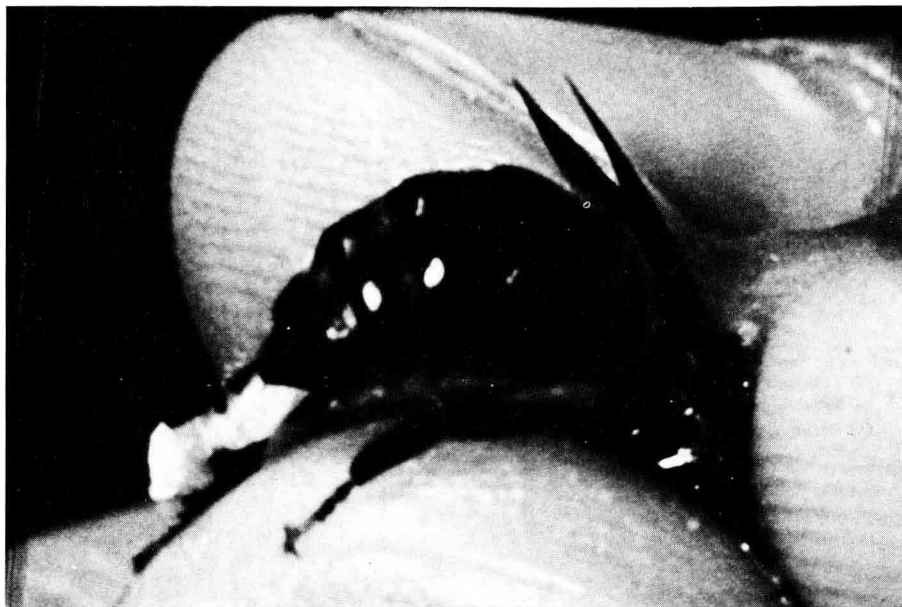


FIG. 1. — *A. cerana* queen, mated in Graswang, with *A. cerana* mating sign (RUTTNER *et al.* 1973).

Sperm counts confirm the following results (table 2) : The greater the distance to the nearest *mellifera* drones, the greater the number of spermatozoa stored in the spermatheca. The majority of the queens had more than one million spermatozoa and one of them had more than three million. A similar situation was found in queens mated in the country of origin. However, the average number was distinctly lower due to the poor weather conditions in the Alps during both mating periods.

#### *Proof of hybrid mating*

At the mating place Graswang (distance 10 km) mating signs of *cerana* drones were observed adhering to queens returning from the mating flight (fig. 1; see RUTTNER *et al.* 1972).

TABLE 2. — *Number of spermatozoa in the spermatheca of cerana queens and distance to closest mellifera drones.*

Group	Distance between location of <i>cerana</i> virgin queens and closest <i>mellifera</i> colonies (meters)	No. of queens examined	No. of spermatozoa in the spermatheca (thousands)		No. of queens with more than 1 mill. sperm.
			Ø	range	
2	500	1	7	—	—
4	3,000	5	612	56-1,885	1
5	10,000	9	1,585	22-3,460	6
6	Country of origin	2	3,274	2,665-3,883	2

At the mating place in the Taunus mountains near Frankfurt (3 km from *mellifera* colonies) one queen was observed in her nucleus with a mating sign inserted in the sting chamber. Thirteen days later there were still no eggs in this nucleus and the queen still showed the mating sign. We removed and dissected the queen. The mating sign was tightly fixed to the queen. A closer examination revealed that it was a *mellifera* sign with chitinous plates (fig. 2) which penetrated the thin membrane of the bursa copulatrix and were glued to it by dried hemolymph. However there were spermatozoa in the spermatheca in low numbers (56,800). Both oviducts were filled with clumps of semen, a total of 1.7 µl. This corresponds to the amount of semen of two *mellifera* drones or of one *mellifera* plus five *cerana* drones.

### 5. *Heterospecific instrumental inseminations*

*Cerana* queens have been inseminated with *cerana* or with *mellifera* semen since 1966. In spite of the small volume of semen injected (1.0 - 4.0 µl) in all cases spermatozoa were stored in the spermatheca of the queen. In a number of cases their quantity was sufficient (up to 1.9 million) for satisfactory fertilization of the eggs laid by the queens. *Cerana* queens inseminated with *cerana* semen produced *cerana* workers and sometimes also drones. If inseminated with *mellifera* semen, only drone brood in a very scattered pattern was produced. The same occurred in the reciprocal cross (*mellifera* queens × *cerana* drones). In both crosses it was observed that many eggs laid by the queen did not develop and never produced larvae. A *cerana* queen inseminated with mixed semen (*cerana* and *mellifera*) produced only *cerana* workers.

One difference of *cerana* drones compared to *mellifera* drones was that they produced a very small quantity of semen. Moreover, only a small part of the semen from the drones can be collected even if fully mature drones are used. Thus a high



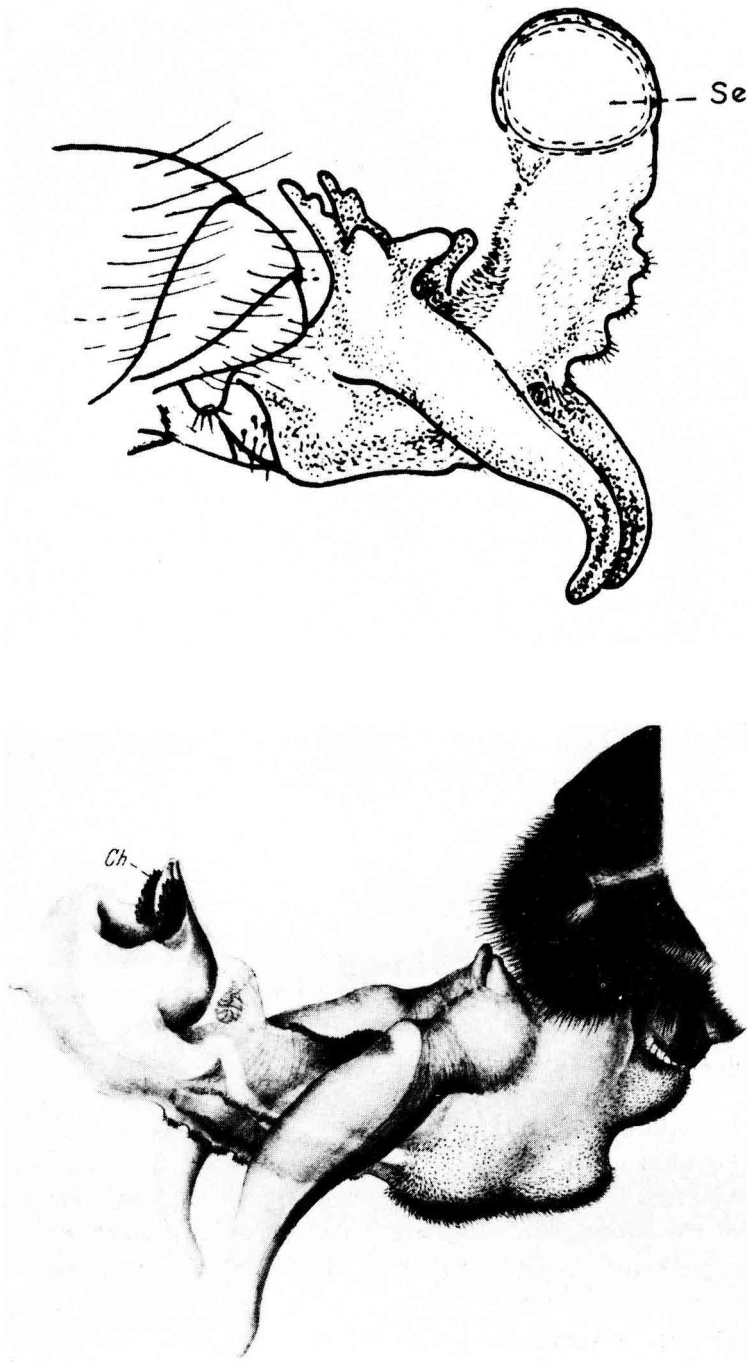


FIG. 2. — *Everted endophallus of A. cerana (above) and A. mellifera (below)*  
SE = semen, Ch = chitinous plates.

number of drones have to be killed to collect a sufficient amount of semen for an adequate filling of the spermatheca.

The studies on insemination in *A. cerana* were continued by WOYKE (1973). He found that 17 *cerana* drones were necessary (including those which ejaculate incorrectly or not at all) to collect 1  $\mu$ l of semen in the syringe. The transfer of *cerana* spermatozoa into the spermatheca is about equally efficient in *cerana* queens as in *mellifera* queens. Twice as many *mellifera* spermatozoa when injected into the oviducts of *cerana* queens were stored in the spermatheca than in the case of *cerana* spermatozoa. Thus heterospecific insemination is as efficient (at least in one direction) as homospecific.

#### 6. Hybrid (heterospecific) fertilization

The negative results of heterospecific insemination led to the question whether egg fertilization could be performed at all in eggs obtained from cross-inseminated *mellifera* queens. The presence of living spermatozoa in the newly deposited egg could be demonstrated, using an observation technique of V. SIEBOLD (1856). Eggs less than 1-hour old were lightly squashed between slide and coverglass under a microscope so that the yolk was forced out from the posterior end. Motile spermatozoa with rapidly undulating movements then became apparent close to the anterior pole.

For closer analysis, serial sections were prepared from eggs that were fixed using the technique of PETRUNKEWITSCH (1901) and modified later by MAUL (1967, 1970). Although all eggs were taken from worker cells, variably low rates of unfertilized eggs were found in the egg samples of most of the queens studied (average 8 %). The majority of the eggs that were fertilized exhibited normal formation of the pronuclei and fusion into the first mitotic spindle. Furthermore, the synchronous cleavage divisions occurred in the same pattern and time sequence as seen in homospecifically fertilized eggs of *Apis mellifera*.

Squash preparations from late cleavage stages, fixed and stained with dry ice and orcein-acetic acid (TRUCKENBRODT, 1964) showed a normal diploid chromosome set (fig. 3a).

Haploid eggs were determined by this technique at the same rates as unfertilized eggs mentioned previously (fig. 3b).

It can be concluded from these findings that heterospecific fertilization is possible between *Apis mellifera* and *Apis cerana* and that some developmental block must occur during the later stage.

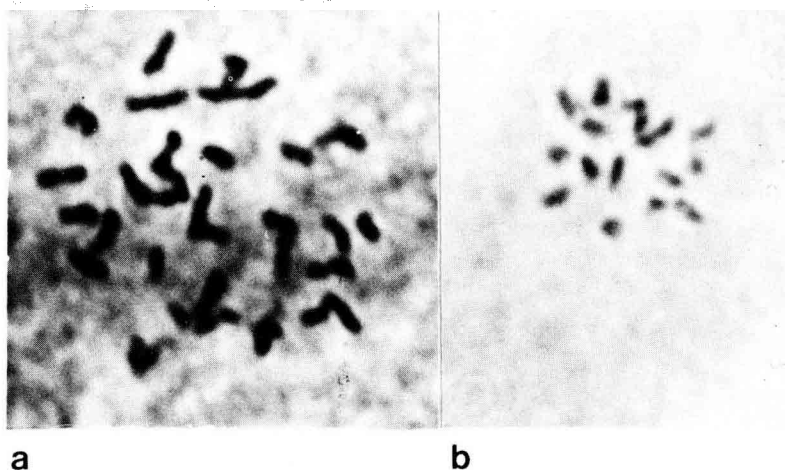


FIG. 3. — Metaphase plates from blastodermal stage in eggs from a cross mated queen *A. mellifera*  $\times$  *A. cerana*.  
*a* : diploid (32 chromosomes), *b* : haploid (16 chromosomes)  
 (squash preparation, orcein-acetic acid, phase contrast).

### 7. Development of the zygote

The embryonic development of reciprocal crosses between *Apis mellifera* and *Apis cerana* was studied morphologically by means of serial sections. Eggs deposited into combs within an excluder cage over short intervals were allowed to develop to obtain known-age eggs in an incubator and later fixed (technique : MAUL, 1970). Thus it was possible to obtain eggs whose ages were known within  $\pm 1$  hour. Approximately 400 eggs in total were examined from 3 queens *M*  $\times$  *C* and 3 queens *C*  $\times$  *M*. The *cerana* stocks originated from China (Peking) and Pakistan, *mellifera* queens were from Carniolan and Italian stocks.

During the first 24 hours of development normal superficial cleavage led to the formation of the so-called « preblastoderm » — a layer of low cells all over the egg surface with the residual « secondary periplasm » underneath.

The next step of blastodermal differentiation, the formation of cylindrical cells in the embryonic areas, was never performed by hybrid eggs of both types. Instead, the initial cell walls started to disintegrate again and the nuclei appeared to migrate back into the secondary periplasm (fig. 4, b-d). This disintegration proceeded from polar and dorsolateral areas, more or less to the ventral area. Not affected by this process was a special group of cells along the dorsal midline which corresponds to the extra-embryonic amnion.

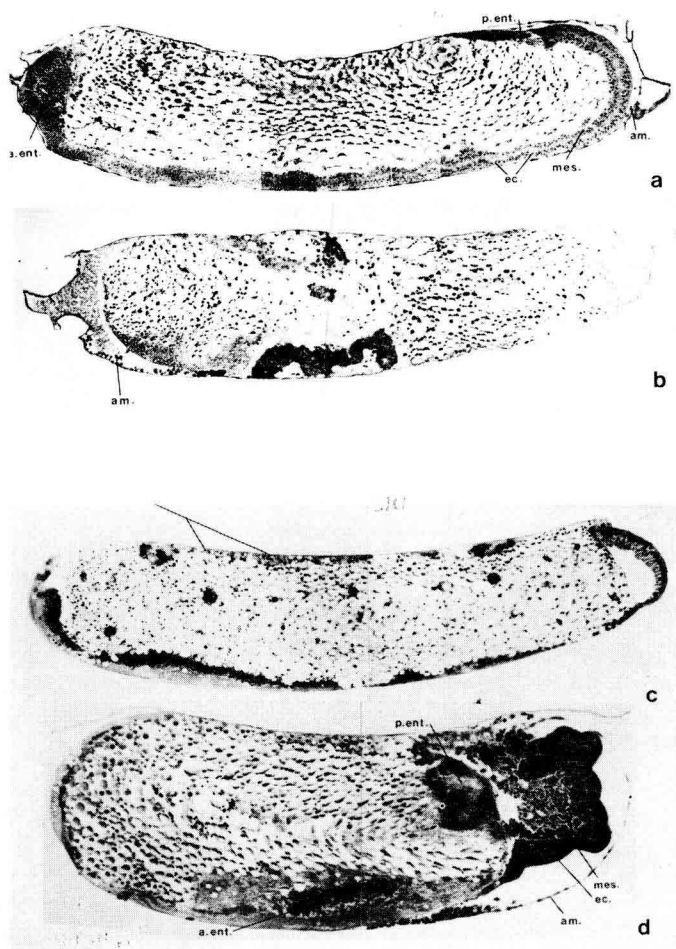


FIG. 4. — *Median sections of embryos* (about  $100\times$ ). Fixation Petrunkevitch, Staining Heidenhain, anterior pole points left.

*a* : Haploid embryo from unfertilized egg, 42-44 hours, with normal development.

*b-d* : Hybrid embryos *A. mellifera*  $\times$  *A. cerana* with defective development.

*b* : 55-72 hours; small cellular area in the center, isolated amnion cells anteriorly.

*c* : undated, small cellular rudiment posteriorly, plasmodium-like arrangement of disintegrating blastoderm with numerous nuclei.

*d* : Embryo *A. mellifera*  $\times$  *A. cerana* « Peking », showing the « best » developmental performance observed so far. Double layered embryonic area contracted toward the posterior pole and covered by a distinct amnion.

Two areas of disintegrated cells, resembling the anterior and posterior entodermal rudiments.

a. ent. = anterior entodermal rudiment; am. = amnion; ec. = ectodermal layer; mes. = mesodermal layer;

p. ent. = posterior entodermal rudiment.

Probably depending on yolk movements, the « embryonic » materials were shifted in irregular ways and separated into syncytial masses and masses with remaining but abnormal cell structures. Only the amnion cells maintained a normal appearance but were not arranged normally.

Apparently there was some variation in the degree of embryonic disintegration. Reciprocal hybrids with *Apis cerana* from Pakistan were all highly disintegrated by the third day of development. Hybrids  $M \times C$  (Peking), however, often showed patterns which at least were similar to the arrangement of an embryo (fig. 4d). The contracted cellular material was separated into an inner and an outer layer, sometimes even covered by a regular amnion. The syncytial masses formed two distinct blocks that could possibly correspond to the endodermal materials.

## DISCUSSION

In this detailed study of the consecutive steps in the process of sexual reproduction in the two species of honey bees, *A. cerana* and *A. mellifera*, an astonishing degree of congruence was found. This applied even to the rather complex behavioural and physiological situations, as the orientation during the mating flight or the storage of spermatozoa in the spermatheca.

Two effective blocks to hybridization exist, block no. 1 which occurs during the copula and block no. 2 during the zygotic development after successful fertilization of the egg.

Block no. 1 is the consequence of important morphological differences in the copulatory organs of the drones. The chitinous plates with their sharp edges of the *mellifera* endophallus seem to hurt *cerana* queens severely, at least in some cases.

Block no. 2 makes it impossible to determine whether *cerana* semen is transferred to *mellifera* queens and how frequently this event occurs. However, the *mellifera* mating sign found in the bursa copulatrix of a *cerana* queen is evidence that interspecific matings actually occur but that this may result in reproductive failure.

Even though the semen in the spermatheca of *cerana* queens does not indicate its source, there is sufficient indirect evidence accumulated during our experiments over several years, to try to answer this question. *Cerana* queens placed for mating at different distances from *mellifera* drones and provided with *cerana* drones do not behave as normal queens. An extremely high proportion of the queens were lost in spite of the presence of drones of their own species and the number of queens that finally became drone layers was low. The high losses were most likely the result of the interference of *mellifera* drones, which disturbed the homospecific matings and were themselves incapable of mating with *cerana* queens. The increasing proportion of *cerana* matings with increasing distance from *mellifera* drones can best

be explained by this assumption. The observation of the possible consequence of such a mismating proofed that this assumption is not completely wrong.

It is astonishing that *mellifera* spermatozoa instrumentally injected in the oviducts of *cerana* queens — or vice versa — are transferred into the spermatheca, survive there for a yet undetermined period, and migrate through the narrow ductus spermaticus to finally fertilize the heterospecific eggs. This is exactly the same with homospecific spermatozoa, even to the fusion of the sperm nucleus with the pronucleus of the egg and the start of the development of a hybrid embryo.

The second block (abnormal development), as observed in our hybridization experiments, started with the formation of the blastoderm. Although there was apparently some variation in the degree of disturbance of the embryonic development after cleavage, the basic phenomenon was identical in both reciprocal hybrids. These findings are congruent with the results of species hybridization experiments in many other animals where the development proceeds normally to the blastula stage (which corresponds to the blastoderm of insect eggs), but is blocked or disturbed from the gastrula stage onward (review by STEBBINS, 1958). Such early findings have supported the hypothesis that cleavage is controlled by the cytoplasmic system of the egg rather than by the genome (DUSPIVA, 1969).

The development up to the blastula or a corresponding stage is controlled by « maternal messengers ». Differential gene expression, followed by differential protein synthesis, usually does not start earlier than at blastula-like stages (DUSPIVA, 1969; DAVIDSON, 1967; EDE, 1981). Biochemical studies of the early development of lethal hybrids were reviewed by BRACHET and MALPOIX (1971). From data on sea urchin hybrids they assumed that certain control mechanisms were deficient in the hybrid system, leading to the accumulation of excessive paternal m-RNA in the nuclei. In their opinion, the nuclear membrane might have played a selective role allowing only some m-RNAs to move out towards the cytoplasm. Generally, KOROCHKIN (1981) supported the idea that after a low-level activity of almost all the structural genes during early development, the onset of cellular differentiation was controlled by means of differential repression of structural genes.

This could mean in our case, that the hybrid genome was unable to react properly to the signals from the cytoplasmic system of the egg. These observations prove that in the hybrids, paternal genes too were involved in the developmental process — in contrast to the hypothesis of OHNO (1969), that in interspecies hybrids only maternal regulatory genes are active in later stages of development. In the honey bee, the maternal genome would guarantee a completely normal development to a haploid male larva.

Morphologically similar anomalies of embryonic development are sometimes observed in the honeybee as a result of inbreeding (PAULINO, unpubl. data). However, the defective development of inbred embryos is highly variable,

affecting sometimes also the earliest steps of development. These developmental blocks are understood as maternal effects. The inbred female is unable to produce the proper ooplasmic system during oogenesis.

On the basis of differences in morphology and behaviour between *A. mellifera* and *A. cerana*, two more arguments can be added to support the fact that they are indeed separate species. However, these two hybridization barriers do not contribute anything to the advancement of speciation in these taxa. They are strict allopatric, vicariant species, with a similar spectrum of adaptability to environment, and they are not observed in any place sympatrically (fig. 5). Furthermore, it can be stated that the strains examined never did coexist in the same location in the past. Though *A. mellifera* and *A. cerana* were prevented from hybridization, no means of coexistence was opened by these two barriers to interspecific reproduction. One species interferes with the mating of the other and the result is infertile or lost females. The end of artificially created coexistence is usually the nearly complete replacement of one species by the other.

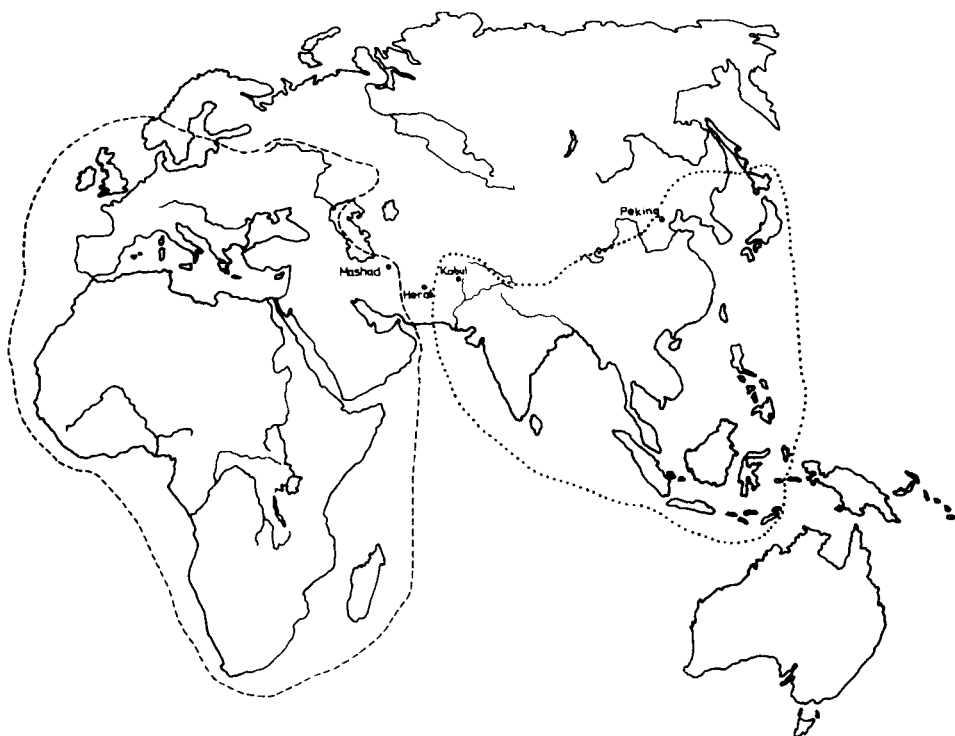


FIG. 5. — Distribution of *A. mellifera* (Eurafrica) and *A. cerana* (Southeast Asia).

The simple geographic isolation has the same effect of genetic isolation of the two species. Thus the reproductive barriers during mating and early embryonic development have no meaning in their history and they must be regarded as an accidental by-product of evolutive divergence.

This evolutive divergence is a function of time and it may be concluded that geographic separation of *mellifera-cerana* occurred much earlier than the separation of different races of *A. mellifera* which are fully fertile in hybridization in spite of complete geographic isolation. On the other hand, the process of speciation is distinctly more advanced in the other *Apis* species (*floreana*, *dorsata*).

Mature species in the final stage of speciation have developed a pre-mating block that even prevents attempts to copulate.

In most cases they have « forgotten » the signals derived from the common ancestor and they do not respond to each other. This behavioural pre-mating barrier is subject to natural selection, the so-called WALLACE effect (FISHER, 1930). This was demonstrated by KOOPMAN (1950) in experimental populations of two related *Drosophila* species. The initial proportion of hybrids in the population was reduced after several generations from 36,5 % to 2 %. Similar observations were made with wild populations of *Drosophila* (AHEARN *et al.*, 1974). Two sympatric *Drosophila* species on the island of Hawaii were sexually isolated, while the two same species mated freely with a third, allopatric, species on a nearby island, Maui.

In honeybees, the simplest way to establish reproductive isolation is to shift the time of mating flight. The three sympatric *Apis* species of Sri Lanka show three distinctly different daily periods for drone flights (KOENIGER and WIJAYAGUNASEKERA, 1976). As different flight times were recorded for drones of *A. florea* from India (MEHTA, 1947) and from Sri Lanka, this characteristic seems to be of considerable elasticity and it seems to respond quickly to selection.

It is likely that during undisturbed coexistence the two species, *A. mellifera* and *A. cerana* could finally develop a similar kind of pre-mating isolating barrier. An indication of the existing variability in the drone flight period in *A. cerana* was observed during these experiments.

We can conclude from these observations that both species did remain completely isolated geographically from the time of initial separation to our present century.

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## RÉSUMÉ

ÉTUDE EXPÉRIMENTALE DE L'ISOLEMENT REPRODUCTIF INTERSPÉCIFIQUE  
D'*APIS MELLIFICA* L. ET D'*APIS CERANA* FABR.

On a cherché à savoir si les deux espèces d'abeilles *Apis mellifica* et *Apis cerana* étaient totalement isolées l'une de l'autre du point de vue génétique et à quel moment du processus de reproduction existait un éventuel obstacle au croisement. On a étudié systématiquement toutes les phases du processus de reproduction : comportement d'accouplement, copulation, rétention du sperme, fécondation de l'œuf et développement de l'embryon. On s'est également reporté à des travaux plus anciens, déjà publiés.

*Comportement d'accouplement*

Les mâles d'*Apis cerana* du Pakistan et de Chine volent en Europe centrale à la même période que les mâles autochtones *mellifica* et ils fréquentent les mêmes lieux de rassemblement. Les reines des deux espèces sont attractives pour les mâles de l'autre espèce — bien qu'un peu moins que pour les mâles de leur propre espèce.

*Copulation*

En République fédérale allemande on n'a réussi à obtenir des reines *cerana* fécondes que loin des lieux de rassemblement de mâles *mellifica* (à plus de 3 km). L'observation d'une reine *cerana*, dans le vagin de laquelle les signes d'accouplement d'un mâle *mellifera* étaient fixés, prouve qu'il existe un obstacle mécanique à l'accouplement.

Le nombre de spermatozoïdes dans la spermathèque de reines *cerana*, qui se sont accouplées librement, augmente durant la période d'accouplement au fur et à mesure que l'on s'éloigne des mâles *mellifica*. Le résultat d'expériences d'accouplement de plusieurs années avec des reines *cerana* était soit du couvain d'ouvrières *cerana*, soit du couvain de mâles, mais on n'a jamais observé d'hybrides.

*Rétention du sperme*

Après insémination artificielle on a trouvé jusqu'à 1,9 million de spermatozoïdes vivants dans la spermathèque.

*Fécondation et développement*

On a trouvé dans des œufs fraîchement pondus des spermatozoïdes qui présentaient des mouvements en spirale. Les coupes effectuées ont établi qu'après la fécondation une segmentation normale a tout d'abord lieu; les nuclei ont un nombre diploïde de chromosomes (fig. 3), mais 24 heures après la ponte de l'œuf le développement s'arrête et finalement le germe disparaît sous la formation d'amas cellulaires (fig. 4). Le moment où se produit le dépérissement du germe hybride est tout à fait typique des hybrides interspécifiques : tandis que le développement est dirigé dans un premier temps par des enzymes maternelles, le génome hybride de l'embryon entre en action à la fin du stade blastoderme — et c'est durant cette phase que surviennent les perturbations du développement comme conséquence de l'absence d'accord entre le cytoplasme et le nucleus.

Il existe par conséquent chez ces deux espèces deux obstacles au croisement, l'un mécanique lors de la copulation, l'autre génétique au début du développement embryonnaire. Les tentatives d'accouplement avec des mâles d'une autre espèce, qui ont eu lieu dans des conditions d'accouplement libre avec un comportement d'accouplement semblable et des phéromones sexuelles identiques, ont conduit tôt ou tard à la mort de la reine. C'est pourquoi il est difficile de maintenir sur un même territoire *A. cerana* et *A. mellifica*.

On explique l'interruption du développement embryonnaire des hybrides comme l'effet secondaire d'un développement séparé par une longue période en isolement géographique.

*A. mellifica* et *A. cerana* sont décrites comme deux espèces séparées géographiquement et génétiquement, mais chez lesquelles la dernière étape de la naissance d'un obstacle dans le comportement de l'accouplement et donc possibilité d'une existence sympatrique ne s'est pas encore effectuée.

## ZUSAMMENFASSUNG

EXPERIMENTELLE ANALYSE DER REPRODUKTIVEN INTERSPEZIFISCHEN  
ISOLATION VON *APIS MELLIFERA* L. UND *APIS CERANA* FABR.

Es wurde die Frage untersucht, ob die beiden Bienenarten *Apis mellifera* und *Apis cerana* genetisch voneinander vollständig isoliert sind und an welcher Stelle des Reproduktionsvorganges eine eventuelle Kreuzungsbarriere vorhanden ist. Es wurden systematisch sämtliche Phasen des Fortpflanzungsvorganges untersucht — Paarungsverhalten, Copula, Speicherung des Spermas, Eibefruchtung und Entwicklung des Embryos. Dabei wurde auch auf ältere, schon publizierte Arbeiten der Autoren zurückgegriffen.

*Paarungsverhalten*

Drohnen von *A. cerana* aus Pakistan und China fliegen in Mitteleuropa zur selben Zeit wie heimische *mellifera*-Drohnen und sie besuchen dieselben Drohnensammelpplätze. Königinnen beider Arten sind für Drohnen der jeweils anderen Art attraktiv — wenn auch nicht ganz so stark wie die eigenen.

*Copula*

Im Gebiet der Bundesrepublik ist es nur bei großer Entfernung des Paarungsplatzes von *mellifera*-Drohnen (mehr als 3 km) gelungen, fruchtbare *cerana*-Königinnen zu erzielen. Die Beobachtung einer *cerana*-Königin, in deren Vagina sich das Begattungszeichen eines *mellifera*-Drohnen verspießt hatte, weist darauf hin, daß ein mechanisches Paarungshindernis vorliegt.

Die Spermienzahl in der Spermatheka von frei gepaarten *cerana*-Königinnen wächst mit steigender Entfernung zu *mellifera*-Drohnen während der Paarungszeit. Das Ergebnis mehrjähriger Paarungsversuche mit *cerana*-Königinnen war entweder *cerana*-Arbeiterbrut oder Drohnenbrut; Hybriden wurden nie beobachtet.

*Speicherung des Samens*

Nach instrumenteller Besamung wurden in der Spermatheka lebende Spermien gefunden (bis zu 1,9 Mill.).

*Eibefruchtung und Entwicklung*

Im frisch abgelegten Ei wurden Samenfäden mit lebhaften Schlängelbewegungen gefunden. Auf Schnitten wurde festgestellt, daß nach der Befruchtung zunächst eine normale Furchung einsetzte; die Zellkerne hatten die diploide Chromosomenzahl (Abb. 3). Vierundzwanzig Stunden nach Ablage des Eis kam jedoch die Entwicklung zum Stillstand und schließlich ging der Keim unter Bildung von Zellklumpen zugrunde (Abb. 4). Der Zeitpunkt des Absterbens des Hybridkeimes ist ganz typisch für Artbastarde: Während zunächst die Entwicklung durch maternale Enzyme gesteuert wird, tritt am Ende des Blastodermstadiums das Hybridgenom des Embryos in Aktion — und in dieser Phase kommt es zu Entwicklungsstörungen als Folge der fehlenden Abstimmung zwischen Cytoplasma und Kern.

Demnach gibt es bei diesen beiden Arten zwei Kreuzungsbarrieren, eine mechanische bei der Copula und eine genetische in der frühen Embryonalentwicklung. Bei noch gleichartigem Paarungsverhalten und gleichen Sexuallockstoffen kommt es unter den Bedingungen der freien Paarung zu Copulationsversuchen mit Drohnen der anderen Art, die früher oder später zum Tode der Königin führen. Es ist deshalb schwierig, *A. cerana* und *A. mellifera* in derselben Gegend zu halten.

Die Störung der Embryonalentwicklung der Hybriden wird als Nebeneffekt einer durch längere Zeit getrennten Entwicklung in geographischer Isolation erklärt.

*A. mellifera* und *A. cerana* werden als geographisch wie genetisch getrennte Arten beschrieben, bei denen aber der letzte Schritt in der Artbildung (Entstehung eines Blocks im Paarungsverhalten und damit Möglichkeit zur sympatrischen Existenz) noch nicht vollzogen ist.

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