# Reproductive Compatibility, Mating Behavior, and Random Amplified Polymorphic DNA Variability in Some *Aphelinus asychis* (Hymenoptera: Aphelinidae) Derived from the Old World

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**ABSTRACT** Reproductive compatibility, mating behavior, and random amplified polymorphic DNA (RAPD) variability were examined in samples of *Aphelinus asychis* obtained during foreign exploration for natural enemies of the Russian wheat aphid, *Diuraphis noxia* (Mordvilko). All specimens were reared initially from *Diuraphis* sp. collected at several Mediterranean basin localities and 2 localities in Central Asia. Using 7 laboratory cultures, we found 3 reciprocally and completely incompatible groups: the 1st group was represented by a culture from northcentral China, the 2nd group by a Kazakhstani culture, and a 3rd by 5 cultures from the Mediterranean basin (i.e., France, Greece, Morocco, Sicily, and Spain). One instance of partial, 1-way incompatibility was detected among the Mediterranean basin cultures. Behavioral experiments with 3 cultures showed that males respond to the sex pheromone of incompatible females and actively court them. However, females usually did not stop moving or elevate their abdomens in response to courtship by incompatible males. With 1 exception, genital contact and sperm transfer did not occur in incompatible pairings. Thus, rejection of incompatible males or failure to recognize incompatible males by females appeared to be 1 cause of incompatibility. In the exceptional case (Chinese males paired with Kazakhstani females), sperm transfer occurred but at significantly lower frequencies than in control pairings. Phenetic analysis of 61 RAPD loci in 6 laboratory cultures and 3 field samples from the Mediterranean basin delineated the same 3 groups as the compatibility experiment. Further work examining the geographic range, host range, and morphology of the incompatible groups is needed to clarify their taxonomic status.

**KEY WORDS** *Aphelinus asychis, Diuraphis noxia,* reproductive compatibility, random amplified polymorphic DNA, mating behavior, biological control

THE RUSSIAN WHEAT aphid, *Diuraphis noxia* (Mordvilko), has become a major pest of wheat in the United States since its appearance in Texas in 1986 (Stoetzel 1987, Brooks et al. 1994). Classical biological control is one of several strategies being pursued to control this exotic pest. During 1988–1994, foreign exploration for natural enemies of *D. noxia* resulted in the collection of at least 29 predator and parasitoid species and 6 species of fungal pathogen (Hopper et al. 1996).

One of the most commonly encountered parasitoids is *Aphelinus asychis* Walker, a solitary internal parasitoid of *D. noxia* and at least 38 other aphid species (Wilbert 1964, Kalina and Stary 1976). During exploration for *D. noxia* natural enemies, *A. asychis* has been found parasitizing *Diuraphis* spp. in Europe (France, Greece, Sicily, Spain), Asia (western (Xinjiang) China, northcentral (Ningxia) China, Kazakhstan, Pakistan), northern Africa (Morocco), and South America (Chile) (Gonzalez et al. 1994, Hopper et al. 1996). Other collection records show that *A. asychis* is distributed broadly in Europe and Asia, and that it now also occurs in South Africa, southern South America, and North America (Kurdjumov 1913, Ferrière 1965, Graham 1976, Kalina and Stary 1976, Jasnosh 1987, Aalbersberg et al. 1988, Hayat 1991, Reed and Pike 1991). The presence of *A. asychis* in the New World is probably the result of importation and release of Old World *A. asychis*. French *A. asychis* were imported and established to control *Metapolophium dirhodum* (Walker) and the English grain aphid, *Sitobion avenae* F., in Brazil and Chile (Zúñiga et al. 1986). Likewise, Old World *A. asychis* were imported and established against the yellow clover aphid, *Therioaphis trifolii* (Monel), in the western United States (van den Bosch et al. 1959). Also present in North America is *Aphelinus semiflavus* Howard, a species synon-

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**Table 1. Source of specimens used in the reproductive compatibility (RC), sperm transfer (ST), courtship (C), pheromone cross-reactivity (PC), and RAPD studies of Old World *A. asychis***

| Culture | Collection site | Collection date | Original host | Collector(s) | Generation examined | Studies used for | Notes |
|---|---|---|---|---|---|---|---|
| ANT | Antibes, Alpes-Maritimes, France | 20 June 1989 | *D. noxia* | K. Hopper, G. Mercadier | $\approx F_{89}$ | RC, RAPD | Lab-rearing started from 14♀ and 5♂ |
| CHN | Pingluo, Ningxia, China | 21 June 1992 | *D. agropyronophaga* | K. Hopper | $\approx F_{35}$ | RC, ST, C, PC, RAPD | Lab-rearing started from 1 mated ♀ |
| GRC | Xino Nero, Greece | 28 May 1993 | *D. noxia* | D. Coutinot | $\approx F_{19}$ | RC, RAPD | Lab-rearing started from 2♀ and 2♂ |
| KAZ | Dmitrievka, Kazakstan | 16, 17 May 1991 | *D. noxia* | S. Halbert, T. Poprawski | $\approx F_{54}$ | RC, ST, C, PC, RAPD | Lab-rearing started from 18♀ and 18♂ |
| MOR | Annonceur, Settat, Morocco | 9–14 April 1992 | *D. noxia* | K. Chen, D. Coutinot | $\approx F_{38}$ | RC, RAPD | Lab-rearing started from 5♀ and 4♂ |
| MTP-1 | Montpellier, Herault, France | May–June 1992 | *D. noxia* | A. Farias | $\approx F_{35}$ | RC, ST, C, PC | Lab-rearing started from >100 ind. from 2 fields in Montpellier |
| MTP-2 | Montpellier, Herault, France | May–June 1993, 1994 | *D. noxia* | D. Kazmer, N. Ramualde | *P* | RAPD | 1 ♂ from each of 29 aphid colonies collected in 5 fields within a 10 km radius of Montpellier |
| SPN | Montmajor, Lleida, Spain | 16, 17 June 1993 | *D. noxia* | D. Coutinot | $\approx F_{17}$ | RC, RAPD | Lab-rearing started from 5 ♀ and 5 ♂ |
| APT | Apt, Vaucluse, France | 22 June 1993 | *D. noxia* | D. Kazmer | $F_1$ | RAPD | 1 son from each of 6 ♀ |
| SIC | Caltagirone, Sicily, Italy | 8, 9 May 1993 | *D. noxia* | D. Coutinot | $F_1$ | RAPD | 1 son from each of 6 ♀ |

ymized with *A. asychis* by Ferrière (1965). However, MacKauer and Finlayson (1967) argue that *A. semiflavus* should be retained as a separate species because of host range differences.

The initial purpose of this study was to determine if laboratory cultures of *A. asychis* established from *D. noxia* foreign exploration efforts are reproductively compatible. We had 2 reasons for asking this question. First, this material has been released or is being maintained in culture for potential release against *D. noxia*. One factor thought to impede the successful implementation of classical biological control programs is delayed recognition of sibling and cryptic species complexes (cf, Delucchi et al. 1976, Rosen 1986). Testing reproductive compatibility is a simple technique for uncovering such complexes. Second, we are conducting quantitative and population genetic studies of *A. asychis* populations found during *D. noxia* foreign exploration. Proper interpretation of these studies requires knowledge of barriers to gene flow.

The 1st experiment consisted of testing the reproductive compatibility of 7 laboratory cultures of *A. asychis* from the Old World. Because this experiment revealed the presence of 3 reciprocally incompatible groups, we performed additional experiments on sperm transfer, courtship behavior, and sex pheromone cross-reactivity to determine the basis of the incompatibility. Finally, to provide a 2nd source of data on the taxonomic relationships of Old World *A. asychis*, we surveyed variation in random amplified polymorphic DNA ([RAPD], Welsh and McClelland 1990, Williams et al. 1990). This survey was based on 6 of the laboratory cultures used in the compatibility experiment and field samples from 3 Mediterranean basin localities.

## Materials and Methods

**Parasitoids.** Laboratory cultures of *A. asychis* were routinely maintained in cages (42 by 34 by 45 cm) covered with 100 μm nylon screen at 20°C and a photoperiod of 14:10 (L:D) h. Unparasitized *D. noxia* reared on barley, *Hordeum vulgare* L., variety Dominique, were added to the cages each generation. Normally, 200–1,000 parasitoids were produced each generation.

Seven laboratory cultures were started from material collected from 5 Mediterranean basin localities (ANT, GRC, MOR, MTP-1, SPN), northcentral China (CHN), and Kazakhstan (KAZ, Table 1). For the RAPD study, we included the sons of field-collected females from southern France (APT) and Sicily (SIC, Table 1). Also for the RAPD study, we used field-collected males from the Montpellier area (MTP-2) in place of the Montpellier laboratory culture (MTP-1, Table 1).

All parasitoids except those from China were reared initially from *D. noxia* collected in cultivated wheat, *Triticum* spp., and barley fields. The Chinese material was reared from *D. agropyronophaga* Zhang found on an unidentified, wild grass species growing in a wheat and barley variety trial field.

Voucher specimens from all localities are on deposit at the Beneficial Insects Introduction Research Laboratory (Newark, DE).

**Reproductive Compatibility.** We examined all 49 possible crosses among the 7 laboratory cultures. Because of logistic limitations, the experiment was done in 2 parts separated by 2 wk. In part A, we examined the 25 possible crosses within and among the ANT, CHN, KAZ, MOR, and MTP cultures. In part B, we examined the 7 possible control crosses (i.e., crosses between males and females from the same culture) and the 22 experimental crosses (i.e., crosses between males and females from different cultures) that were not done in part A (i.e., all experimental crosses involving GRC or SPN). Between 6 and 12 replicates of each cross type were done.

In each replicate cross, we paired a virgin female with a virgin male in a glass tube (4 by 0.5 cm) containing a drop of honey-water. After 24 h, the male and female were transferred to a Plexiglas tube cage (25 by 4 cm) containing ≈150 *D. noxia* feeding on 7–12 barley plants. Fourteen days later, the aphid-infested barley stems were cut and subsequently held in plastic vials until all adult parasitoids had emerged. We then counted the numbers of mummies (i.e., mummified host aphids containing or that had contained pupal parasitoids), adult males, and adult females produced in each replicate. Because *A. asychis* is arrhenotokous (i.e., haploid males arise from unfertilized eggs and diploid females arise from fertilized eggs), only the presence of female offspring indicates that mating and fertilization were successful.

Four variables were analyzed: the number of mummies produced, the percentage of adult emergence from those mummies, the proportion of replicate crosses that produced 1 or more female offspring, and, in replicate crosses that produced female offspring, the sex ratio. For mummy production, adult emergence, and sex ratio, 1-way analyses of variance (ANOVA) were conducted using cross type as the classification variable. We tested for significant departures from complete compatibility by contrasting the mean value for each experimental cross to the mean of the 2 appropriate control crosses using the CONTRAST function in PROC GLM (SAS Institute 1989). To maintain the maximum experimentwise error rate at the 0.05 level, the comparisonwise error rate was adjusted using the Bonferroni inequality (i.e., comparisonwise error rate = $0.05/c$, where $c$ is the number of contrasts done in each analysis, Miller 1981).

Before analysis, the arcsin square-root transformation was applied to sex ratio and percentage of adult emergence. Each transformed observation was weighted by the reciprocal of its variance (i.e., variance = $1/(4N)$ where $N$ is sample size, Sokal and Rohlf 1981) using the WEIGHT function of PROC GLM (SAS Institute 1989). Confidence limits were constructed using the sum of the weights in place of the number of replicate crosses.

We used the Fisher exact test to compare the proportion of replicate crosses producing females in experimental crosses to the pooled value for the 2 appropriate control crosses. The comparisonwise error rate was adjusted using the Bonferroni inequality based on the number of comparisons done in each part of the experiment.

**Sperm Transfer.** The reproductive compatibility experiments identified 3 sets of cultures that were completely incompatible. We next sought to determine if sperm transfer occurred in completely incompatible crosses using 1 representative culture from each set (CHN, KAZ, and MTP-1). Virgin males and females from the 9 possible combinations of the 3 cultures were paired in glass tubes (4 by 0.5 cm). After 24 h confinement, we dissected the females in physiological saline and determined if sperm was present in the spermatheca using a compound microscope at 200× or more. Sperm transfer frequencies in experimental pairings were compared to the pooled value for the 2 appropriate control crosses using the Fisher exact test and a Bonferroni-adjusted comparisonwise error rate.

**Courtship Behavior.** Using the same 3 cultures of the sperm transfer experiment, we observed the courtship behavior of confined couples. Virgin males and females were paired in gelatin capsules (size 000) and observed for 5 min under a dissecting microscope. The presence or absence of the following behaviors was recorded: male wingfanning, mounting of the male on the back of the female, flagellation of the female head by a mounted male with his antennae, and contact between male and female genitalia. It was not possible to distinguish whether genital contact did or did not result in aedeagal intromission. Thus, if genital contact was observed, we dissected the females to determine if sperm transfer had occurred.

**Sex Pheromone Cross-Reactivity.** We used a bioassay developed by Fauvergue et al. (1995) to determine if males respond to the trail sex pheromone of incompatible females. The bioassay consisted of allowing 2 virgin females to traverse for 2 h a circular area (4 cm diameter) centered on the interior surface of a glass petri dish bottom (10 cm diameter). Females deposited the sex trail pheromone on the petri dish surface during this time. Immediately after that we placed 1 virgin male on the area traversed by the females and closed the test arena using the petri dish top. Every 2 min for the next 30 min, we scored the position of the male as 1 if he was in the area traversed by the females or 0 if he was not in this area. The sum of the 15 scores was used as the dependent variable. Males from the CHN, KAZ, and MTP-1 cultures were used in all possible combinations with CHN, KAZ, and MTP-1 females and a negative control (no female). The experiment was done in 5 complete, randomized blocks

with 2 replicates of each treatment combination in each block.

**Random Amplified Polymorphic DNA Variability.** We surveyed RAPD variability using 10 decamer primers (primers 2, 3, 6–12, and 16 from kit A, Operon Technologies, Alameda, CA). Techniques for sample DNA preparation, DNA amplification, and gel electrophoresis are given in Kazmer et al. (1995). Six males from each of the ANT, CHN, GRC, KAZ, MOR, and SPN laboratory cultures ($F_{17}$–$F_{89}$) were used. We also used 6 $F_1$ males from the APT and SIC populations and 29 males reared from field-collected mummies in the Montpellier region (MTP-2, Table 1).

Use of RAPD markers for genetic and phenotypic analyses has several problematic aspects (cf, Kazmer et al. 1995). We took the following steps to minimize or eliminate these problems. First, 2 replicate amplifications were done for each individual-primer combination to distinguish repeatable and unrepeatable bands. Second, dominance and nonparental bands are 2 problematic aspects of RAPDs that occur in diploid but not haploid individuals. Our use of haploid males avoided these problems. Third, different-sized bands generated by the same primer may represent allelic variants of fragment length polymorphic (FLP) loci or FLP-like loci (see Kazmer et al. 1995). Such bands do not represent independent loci (characters) and failure to recognize such instances results in the redundant presence of the same information in the data set. To eliminate this redundancy, we retained and analyzed only those bands that satisfied the following condition. When paired with each other band generated by the same primer, acceptable bands were those in which at least 1 individual had a presence–presence phenotype and at least 1 individual had either an absence–presence or presence–absence phenotype. Band pairs satisfying this condition for haploid males correspond to different, at least partially recombinant loci with presence and absence alleles. Our original data set consisted of 109 presence–absence polymorphisms. Use of this condition reduced the data set to 61 presence–absence loci.

We calculated Rogers genetic distance (Rogers 1972) for each pair of populations using the reduced data set and 200 bootstrap samples taken over loci from the reduced data set. Using the NEIGHBOR procedure of PHYLIP (Felsenstein 1993), additive trees were constructed for each of the 201 distance matrices by the neighbor-joining method (Saitou and Nei 1987). For each node of the tree generated by the original data set, the percentage of bootstrap samples that produced the same node was determined using the CONSENSE procedure of PHYLIP (Felsenstein 1993). This percentage was used as an index of support for monophyly of the group of populations defined by each node.

## Results

**Reproductive Compatibility.** Among the 7 laboratory cultures examined in this experiment, we found 3 groups of completely and reciprocally incompatible parasitoids. None of the experimental crosses between CHN, KAZ, and each of the Mediterranean basin cultures produced female offspring (Table 2). In contrast, at least some of the replicates in all of the control crosses and the experimental crosses between the Mediterranean basin cultures produced female offspring (Table 2). The percentage of replicate crosses producing females differed significantly from the pooled control cross value for 18 of the 22 experimental crosses that failed to produce female offspring (Table 2). Each of the 4 exceptional cases (CHN♂ × SPN♀, KAZ♂ × SPN♀, SPN♂ × CHN♀, SPN♂ × KAZ♀) involved SPN. The SPN control cross had a low percentage of replicate crosses that produced females (25%). Other experiments with the SPN culture show that it does not routinely have a low percentage of replicate crosses producing females (unpublished data).

We found 1 case of asymmetric partial incompatibility within the Mediterranean basin group. The percentage of replicate crosses between MTP-1♂ and MOR♀ that produced females (30%) was significantly less than the pooled control cross value (95%). The reciprocal of this cross (MOR♂ × MTP-1♀) did not differ significantly from the control cross mean (Table 2). We repeated this set of crosses 1 time (i.e., all 4 crosses involving MTP-1 and MOR) and obtained similarly significant results (unpublished data).

We found no other compelling cases of partial incompatibility. Sex ratio in 1 of the 20 experimental crosses that produced female offspring was significantly lower than the control cross mean (ANT♂ × GRC♀, Table 2). However, this result was based on only 1 replicate of the ANT♂ × GRC♀ cross. Other experiments with this cross showed that it does not routinely result in low frequencies of female offspring (unpublished data).

Postzygotic hybrid inviability did not appear to be associated with the incompatibilities we detected. Mean mummy production was not significantly different from the control cross mean in 40 of the 42 experimental crosses (Table 2). The basis of the significant difference in the 2 exceptional cases (KAZ♂ × SPN♀ and SPN♂ × KAZ♀) was a single replicate of the KAZ control cross in which the female produced 179 mummies. Removal of this outlier renders these 2 cases insignificant. Percentage of adult emergence differed significantly from the mean of control crosses in 1 case but was significantly greater in this instance (SPN♂ × GRC♀, Table 2).

**Sperm Transfer.** With 1 exception, reproductive incompatibility among the CHN, KAZ, and MTP-1 cultures was associated with the absence of sperm transfer. Sperm transfer occurred in most

**Table 2. Measures of reproductive compatibility among 7 Old World cultures of *A. asychis***

| Cross ♂ | Cross ♀ | Part[a] | No. replicate crosses | Mean no. mummies ± 95% CL | % adult emergence (lower, upper 95% CL) | % replicate crosses producing females | Sex ratio in replicate crosses producing females (lower, upper 95% CL)[b] |
|---|---|---|---|---|---|---|---|
| Control Crosses: | | | | | | | |
| ANT | ANT | A | 11 | 25.8 ± 10.5 | 85 (74, 93) | 64 | 27 (15, 40) |
| | | B | 10 | 21.6 ± 10.9 | 82 (69, 91) | 70 | 50 (36, 64) |
| CHN | CHN | A | 10 | 30.6 ± 11.0 | 74 (61, 85) | 60 | 46 (32, 61) |
| | | B | 7 | 22.3 ± 13.1 | 84 (70, 94) | 57 | 47 (31, 63) |
| GRC | GRC | B | 12 | 29.1 ± 10.0 | 82 (73, 90) | 67 | 50 (39, 61) |
| KAZ | KAZ | A | 9 | 26.1 ± 11.6 | 90 (79, 97) | 89 | 61 (48, 73) |
| | | B | 8 | 53.0 ± 12.2 | 89 (82, 95) | 63 | 52 (38, 66) |
| MOR | MOR | A | 10 | 26.0 ± 11.0 | 94 (85, 99) | 90 | 46 (34, 58) |
| | | B | 9 | 23.2 ± 11.5 | 86 (75, 95) | 44 | 34 (18, 51) |
| MTP-1 | MTP-1 | A | 10 | 22.6 ± 11.0 | 86 (74, 95) | 100 | 28 (17, 41) |
| | | B | 10 | 31.9 ± 10.9 | 81 (71, 90) | 40 | 43 (28, 59) |
| SPN | SPN | B | 8 | 26.4 ± 12.2 | 77 (64, 88) | 25 | 42 (17, 69) |
| Experimental Crosses: | | | | | | | |
| ANT | CHN | A | 8 | 16.8 ± 12.4 NS | 90 (75, 99) NS | 0 [c] | — |
| ANT | GRC | B | 6 | 13.8 ± 14.1 NS | 78 (57, 94) NS | 17 NS | 9 (0, 31) [c] |
| ANT | KAZ | A | 8 | 34.4 ± 12.4 NS | 80 (68, 90) NS | 0 [c] | — |
| ANT | MOR | A | 9 | 23.7 ± 11.6 NS | 79 (64, 90) NS | 56 NS | 37 (23, 53) NS |
| ANT | MTP-1 | A | 9 | 32.8 ± 11.6 NS | 85 (74, 93) NS | 89 NS | 29 (19, 40) NS |
| ANT | SPN | B | 10 | 16.7 ± 10.9 NS | 94 (84, 99) NS | 40 NS | 27 (11, 46) NS |
| CHN | ANT | A | 10 | 36.5 ± 11.0 NS | 90 (81, 96) NS | 0 [c] | — |
| CHN | GRC | B | 10 | 26.5 ± 10.9 NS | 81 (70, 90) NS | 0 [c] | — |
| CHN | KAZ | A | 10 | 28.8 ± 11.0 NS | 89 (79, 96) NS | 0 [c] | — |
| CHN | MOR | A | 9 | 35.4 ± 11.6 NS | 91 (83, 97) NS | 0 [c] | — |
| CHN | MTP-1 | A | 10 | 33.8 ± 11.0 NS | 94 (87, 99) NS | 0 [c] | — |
| CHN | SPN | B | 10 | 21.5 ± 10.9 NS | 77 (64, 88) NS | 0 NS | — |
| GRC | ANT | B | 10 | 22.6 ± 10.9 NS | 72 (58, 83) NS | 60 NS | 53 (34, 71) NS |
| GRC | CHN | B | 7 | 16.4 ± 13.1 NS | 83 (66, 95) NS | 0 [c] | — |
| GRC | KAZ | B | 10 | 31.8 ± 10.9 NS | 84 (74, 92) NS | 0 [c] | — |
| GRC | MOR | B | 9 | 36.3 ± 11.5 NS | 81 (71, 89) NS | 67 NS | 46 (33, 58) NS |
| GRC | MTP-1 | B | 10 | 26.6 ± 10.9 NS | 85 (75, 93) NS | 80 NS | 42 (30, 55) NS |
| GRC | SPN | B | 9 | 15.6 ± 11.5 NS | 93 (82, 99) NS | 67 NS | 44 (25, 64) NS |
| KAZ | ANT | A | 8 | 30.3 ± 12.4 NS | 72 (58, 85) NS | 0 [c] | — |
| KAZ | CHN | A | 8 | 14.3 ± 12.4 NS | 91 (75, 99) NS | 0 [c] | — |
| KAZ | GRC | B | 8 | 24.1 ± 12.2 NS | 90 (80, 97) NS | 0 [c] | — |
| KAZ | MOR | A | 10 | 21.7 ± 11.0 NS | 76 (62, 88) NS | 0 [c] | — |
| KAZ | MTP-1 | A | 10 | 30.6 ± 11.0 NS | 97 (91, 100) NS | 0 [c] | — |
| KAZ | SPN | B | 9 | 16.3 ± 11.5 [c] | 89 (76, 97) NS | 0 NS | — |
| MOR | ANT | A | 9 | 20.1 ± 11.6 NS | 84 (70, 95) NS | 89 NS | 39 (24, 54) NS |
| MOR | CHN | A | 7 | 15.6 ± 13.2 NS | 90 (73, 99) NS | 0 [c] | — |
| MOR | GRC | B | 11 | 12.7 ± 10.4 NS | 76 (60, 90) NS | 18 NS | 47 (11, 85) NS |
| MOR | KAZ | A | 10 | 32.9 ± 11.0 NS | 84 (73, 92) NS | 0 [c] | — |
| MOR | MTP-1 | A | 10 | 21.2 ± 11.0 NS | 82 (69, 93) NS | 80 NS | 28 (16, 41) NS |
| MOR | SPN | B | 6 | 11.7 ± 14.1 NS | 78 (55, 95) NS | 17 NS | 55 (10, 95) NS |
| MTP-1 | ANT | A | 10 | 27.1 ± 11.0 NS | 77 (64, 87) NS | 70 NS | 46 (33, 60) NS |
| MTP-1 | CHN | A | 8 | 24.0 ± 12.4 NS | 80 (65, 91) NS | 0 [c] | — |
| MTP-1 | GRC | B | 11 | 11.4 ± 10.4 NS | 92 (79, 99) NS | 36 NS | 46 (18, 76) NS |
| MTP-1 | KAZ | A | 9 | 43.3 ± 11.6 NS | 93 (86, 98) NS | 0 [c] | — |
| MTP-1 | MOR | A | 10 | 12.0 ± 11.0 NS | 87 (70, 98) NS | 30 [c] | 20 (6, 41) NS |
| MTP-1 | SPN | B | 9 | 22.7 ± 11.5 NS | 78 (65, 89) NS | 22 NS | 23 (3, 54) NS |
| SPN | ANT | B | 11 | 25.2 ± 10.4 NS | 89 (80, 96) NS | 55 NS | 63 (48, 77) NS |
| SPN | CHN | B | 7 | 37.0 ± 13.1 NS | 88 (78, 95) NS | 0 NS | – |
| SPN | GRC | B | 10 | 26.0 ± 10.9 NS | 96 (89, 99) [c] | 30 NS | 56 (33, 78) NS |
| SPN | KAZ | B | 10 | 16.9 ± 10.9 [c] | 76 (61, 88) NS | 0 NS | — |
| SPN | MOR | B | 10 | 35.5 ± 10.9 NS | 81 (71, 89) NS | 50 NS | 37 (23, 52) NS |
| SPN | MTP-1 | B | 10 | 28.3 ± 10.9 NS | 86 (76, 93) NS | 40 NS | 28 (15, 44) NS |

[a] Experiment was done in 2 parts that were analyzed separately (see *Materials and Methods*).
[b] Percentage female.
[c], NS, Experimental cross values are and are not significantly different from the mean or pooled value of the appropriate control values, respectively.

**Table 3. Sperm transfer frequencies in control and experimental pairings of 3 Old World cultures of *A. asychis***

| Pair type ♂ | Pair type ♀ | No. pairs observed | % sperm transfer |
|---|---|---|---|
| Control | | | |
| CHN | CHN | 8 | 88 |
| KAZ | KAZ | 9 | 100 |
| MTP | MTP | 8 | 100 |
| Experimental | | | |
| CHN | KAZ | 8 | 38[a] |
| CHN | MTP | 8 | 0[a] |
| KAZ | CHN | 9 | 0[a] |
| KAZ | MTP | 8 | 0[a] |
| MTP | CHN | 8 | 0[a] |
| MTP | KAZ | 8 | 0[a] |

[a], Experimental pair value differs significantly from the pooled value of the appropriate control pairings.

**Table 4. Observations on the courtship behavior of confined couples within and among 3 Old World cultures of *A. asychis***

| Pair type ♂ | Pair type ♀ | No. pairs observed | % wing-fanning by male | % females mounted/ male antennal flagellation | % genital contact | % sperm transfer |
|---|---|---|---|---|---|---|
| Control | | | | | | |
| CHN | CHN | 6 | 100 | 0 | 0 | 0 |
| KAZ | KAZ | 6 | 100 | 67 | 67 | 67 |
| MTP-1 | MTP-1 | 6 | 100 | 50 | 50 | 50 |
| Experimental | | | | | | |
| CHN | KAZ | 6 | 100 | 67 | 17 | 17 |
| CHN | MTP-1 | 6 | 100 | 50 | 0 | 0 |
| KAZ | CHN | 6 | 100 | 33 | 0 | 0 |
| KAZ | MTP-1 | 6 | 100 | 33 | 17 | 0 |
| MTP-1 | CHN | 6 | 100 | 17 | 0 | 0 |
| MTP-1 | KAZ | 6 | 100 | 33 | 0 | 0 |

control pairings (88–100%) but did not occur in any of the experimental pairings except CHN♂ × KAZ♀ (Table 3). In the latter case, sperm transfer occurred but at a significantly lower frequency than in the control pairings (Table 3).

**Courtship Behavior.** The absence of sperm transfer in most experimental pairings among the CHN, KAZ, and MTP-1 cultures was associated with a breakdown in the normal courtship sequence. Successful, control courtships consisted of the following sequence of behaviors: male wing-fanning, arrestment of the female, mounting of the male on the female dorsum, male antennal flagellation of the female head, elevation of the female abdomen, and genital contact/intromission. In experimental pairings, we always observed male wingfanning and at least 1 male in each type of pairing was observed to dorsally mount and flagellate the female with his antennae (Table 4). Unlike successful control pairings however, the female did not stop walking in unsuccessful experimental pairings. Furthermore, male mounting and antennal flagellation always resulted in genital contact and sperm transfer in the control pairings. Genital contact was observed in only 2 of the 14 cases of male mounting in experimental pairings. In one case (a CHN♂ × KAZ♀ pairing), the female elevated her abdomen and sperm transfer occurred. A reduced, but nonzero, frequency of sperm transfer was observed for this type of pairing in the sperm transfer experiment. In the other case (a KAZ♂ × MTP-1♀ pairing), the female did not elevate her abdomen and sperm transfer did not occur.

Sperm transfer frequencies in control pairings were lower in this experiment (Table 4) than in the sperm transfer experiment (Table 3), including a zero value for the CHN♂ × CHN♀ pairings. This is most likely due to differences in the amount of time that pairings were maintained. In the sperm transfer experiment, pairings were maintained for 24 h before dissection, whereas we observed pairings for a maximum of 5 min in the courtship experiment.

**Sex Pheromone Cross-Reactivity.** Reproductive incompatibility among the CHN, KAZ, and MTP-1 cultures was not associated with differential pheromone cross-reactivity. Male responsiveness was independent of all interactions between block, female type, and male type and the block and male type main effects (Table 5). Female type had a significant effect on male responsiveness. As expected, male responsiveness in the negative control was significantly less than in the other treatments (Fig. 1). However, there was significant heterogeneity among the CHN, KAZ, and MTP-1 females in the magnitude of male responsiveness (Fig. 1).

**Random Amplified Polymorphic DNA Variability.** RAPD data for 61 polymorphic loci were concordant with the reproductive compatibility data. The most interior node of the neighbor-joining tree based on Rogers genetic distances was a trifurcation delineating the CHN, KAZ, and Mediterranean basin groups (Fig. 2). Branch lengths leading to the CHN and KAZ cultures were rela-

**Table 5. ANOVA results for pheromone cross-reactivity among 3 reproductively incompatible cultures of *A. asychis***

| Source | df | Mean square | *F* | (df1, df2) |
|---|---|---|---|---|
| Female type (FT) | 3 | 5.35 | 21.8 | (3, 12) * |
| Male type (MT) | 2 | 1.01 | 1.5 | (2, 8) NS |
| Block (B) | 4 | 0.46 | 1.1 | (4, 2.72) NS |
| FT × MT | 6 | 0.42 | 0.9 | (6, 24) NS |
| FT × B | 12 | 0.25 | 0.5 | (12, 24) NS |
| MT × B | 8 | 0.67 | 1.4 | (8, 24) NS |
| FT × MT × B | 24 | 0.48 | 1.4 | (24, 60) NS |
| Error | 60 | 0.35 | | |

A mixed-model ANOVA was used with block as a random effect. *, $P < 0.0001$; NS, $P > 0.05$.

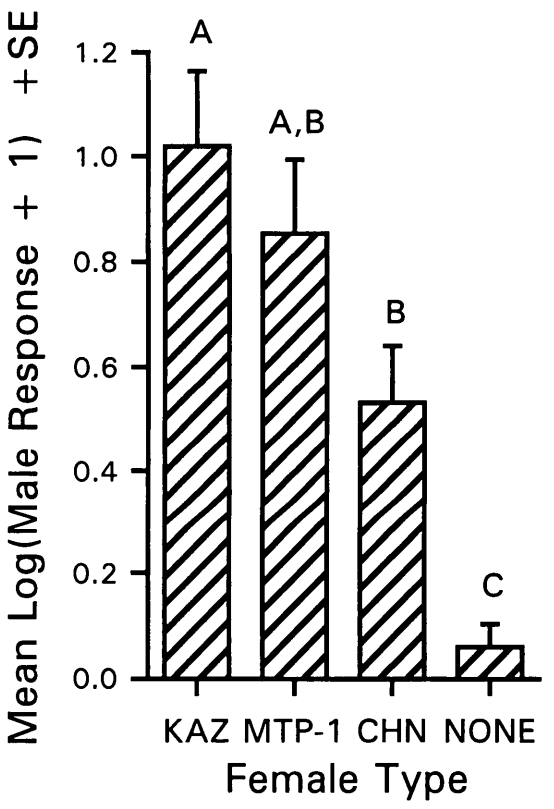


**Fig. 1.** Mean male responsiveness to traces left by KAZ, MTP-1, and CHN females and a negative control in the sex trail pheromone bioassay. Means designated by the same letter do not differ at the 5% level using the Ryan–Einot–Gabriel–Welsch *F* statistic test (SAS Institute 1989).

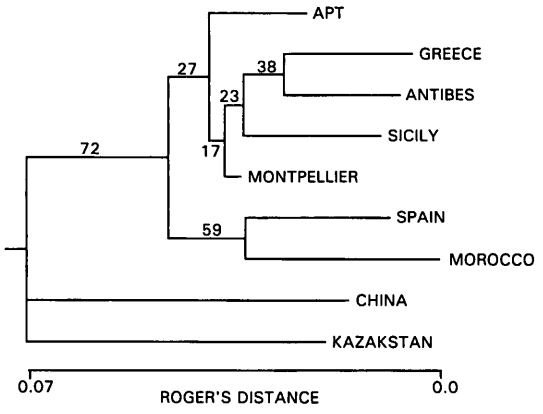


**Fig. 2.** Neighbor-joining phenogram based on Rogers distance for 9 Old World samples of *A. asychis*. Numbers next to each node indicate the percentage of bootstrap samples ($M$ = 200 ) in which a node identifying the same set of samples appeared.

tively long and the cumulative branch lengths leading to each Mediterranean basin sample were similarly long. Note that the Mediterranean basin group includes 2 field samples not tested in the compatibility experiment (APT and SIC) and that a field sample from Montpellier (MTP-2) was used in place of the laboratory culture from this region (MTP-1). Only 2 nodes appeared in ≥50% of the bootstrap samples: 1 delineating the Mediterranean basin populations (72%) and 1 delineating the SPN and MOR cultures (59%).

## Discussion

One cause of the observed reciprocal incompatibilities appears to be rejection of incompatible males or failure to recognize incompatible males by females. Males responded positively to the sex trail pheromone of incompatible females and male wingfanning was always observed in confined couples. Also, males were observed to dorsally mount and antennally flagellate incompatible females. In contrast, females did not stop walking nor elevate their abdomens when courted by incompatible males in unsuccessful courtships. This was not true of females courted by compatible males in successful courtships.

Reduced, but nonzero, sperm transfer frequencies were observed in 1 incompatible cross (i.e., CHN♂ × KAZ♀). This suggests the presence of a discrete polymorphism in the trait(s) governing successful courtship among Chinese males or Kazakhstani females. Alternatively, quantitative variation about a threshold for successful courtship may be present among these cultures, with Chinese males or Kazakhstani females having trait values near this threshold. The basis of incompatibility when sperm transfer does occur in CHN♂ × KAZ♀ needs to be investigated.

One partial, 1-way incompatibility was detected among the Mediterranean basin cultures. Relative to the control crosses, a significantly lower percentage of replicate crosses produced female offspring when Montpellier males were paired with Moroccan females. The basis of this incompatibility is not known. Like the CHN♂ × KAZ♀ pairings, courtship success and sperm transfer may be less frequent but in this instance hybrid female offspring are produced. Other cases of partial compatibility may be present among the Mediterranean basin cultures. Our analyses were based on the presence and frequency of $F_1$ females. Experiments examining the fitness of $F_1$ females may reveal additional cases of incompatibility.

Our results indicate the presence of 3 reproductively disjunct and genetically differentiated groups among the *A. asychis* cultures examined. These cultures represent the majority of the *A. asychis* cultures established during *D. noxia* foreign exploration and the same cultures, or descendants thereof, are being maintained at several facilities in the United States. Recognition of the 3 groups provides a basis for prioritizing culture mainte-

nance, releases, and foreign exploration in the *D. noxia* biological control program.

Further work is necessary to determine the taxonomic status of these groups. First, although we have at least partially defined the geographic extent of the Mediterranean basin group, the geographic extent of the Chinese and Kazakhstani groups is unknown because each of these groups is represented by a single culture/locality. Additional material from Central Asia is necessary to address this point. Second, the host ranges of these groups are unknown. All of the material used in our study was initially reared from 1 of 2 *Diuraphis* sp. yet *A. asychis* has been reared from at least 39 aphid species (Wilbert 1964, Kalina and Stary 1976). Third, our results are based on long-term laboratory cultures started from few individuals and it is possible that the incompatiblities and genetic differentiation are artifacts that arose during laboratory rearing. One argument against this possibility is the cohesiveness of the Mediterranean basin group. If incompatibility developed randomly among laboratory cultures we might have observed incompatibility among some of the Mediterranean basin cultures but we did not. Also, this group remained intact in the RAPD experiment where 2 additional field samples from the Mediteranean basin were included and a Montpellier field sample was used in place of the Montpellier laboratory culture. Furthermore, we do not know of any published examples of nonmicrobial, laboratory-acquired incompatiblity in insect parasitoids. Finally, morphological analysis of our material and museum material should be done.

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