NATURAL SELECTION FOR REPRODUCTIVE ISOLATION BETWEEN *DROSOPHILA PSEUDOOBSCURA* AND *DROSOPHILA PERSIMILIS* [1]

KARL F. KOOPMAN

Department of Zoology, Columbia University [2]

Received December 15, 1949

INTRODUCTION

One of the most important problems in the study of speciation has been that of the origin of reproductive isolating mechanisms, for it is by the building up of intrinsic barriers which prevent gene exchange between populations that we pass from the racial or subspecific to the specific level. Two theories have been proposed to explain how reproductive isolation might arise. The first, favored by Muller (1939, 1942), holds that reproductive isolation is a by-product of genetic divergence. As two subspecies differentiate in geographical isolation, genes which hamper free interbreeding with the other subspecies are incorporated into the genotype in the course of building up adaptive gene complexes. The reproductive isolation may be a pleiotropic effect, or perhaps be a result of change in gene function.

The other theory, advanced by Dobzhansky (1940), holds that provided the two subspecies have diverged from one another far enough so that hybrids between them are less well adapted for any available habitat than either parental type, natural selection will act to build up further reproductive isolation, thus preventing the formation of inadaptive hybrids with consequent wastage of reproductive potential and food resources. These two theories are by no means mutually exclusive, and indeed both mechanisms in all probability play an important part, perhaps being of greatest importance at different stages in the process. Actually, it seems very difficult to conceive of a situation in which the development of isolating mechanisms between two populations could proceed entirely by means of selection, since this would mean that at the outset, when selection is to begin its action, the hybrid and the two parental types would be equally viable and fertile and the former would be at no selective disadvantage. Under these circumstances, however, natural selection against the hybrid would be impossible. On the other hand, it is exceedingly probable that two populations which, during genetic divergence, had developed a considerable amount of reproductive isolation, would upon physical contact complete the process by means of natural selection.

Unfortunately, to date, the evidence, experimental or otherwise, showing either mechanism at work is virtually absent. The experiments herein described were made in order to determine whether, in artificial populations consisting of the two closely related species, *Drosophila pseudoobscura* and *D. persimilis* (the latter formerly known as *D. pseudoobscura,* race B), an increase in the reproductive isolating mechanisms could be detected if in each generation the hybrids between the two species were systematically eliminated. Under these conditions, if any hereditary variability for reproductive isolation was present, natural selection should act to prevent these hybrids from being formed.

MATERIALS AND METHODS

In order that not only representatives of the pure species, *D. pseudoobscura* and *D. persimilis,* but also both male and fe-

[1] Submitted in partial fulfillment of the degree of Doctor of Philosophy, Department of Zoology, Columbia University.

[2] Present address: Middletown Collegiate Center, Middletown, New York.

male hybrids could be easily recognized, mutant stocks of both species were used. For the *D. pseudoobscura* stock, males of the second chromosome mutant *glass* were outcrossed to a recently captured strain from Jacksonville, California, and the *glass* mutant reextracted. For the *D. persimilis* stock, males of the third chromosome recessive mutant *orange* were outcrossed to a recently captured strain from Porcupine Flat, California, and the *orange* mutant reextracted. This outcrossing and reextraction was done for two reasons. First, the viability of the strains was improved by heterosis, as well as by introduction of genes which had been under strong natural selection in the wild, as opposed to the relatively weak natural selection experienced by the mutant strains in the laboratory. Secondly, since mass cultures were employed, the store of genetic variability was increased, an important factor in the present experiment. By using these mutant stocks, all *D. pseudoobscura* individuals could be recognized by having *glass* eyes, all *D. persimilis* by being *orange*-eyed, while the only wild-type individuals were the hybrids, in which both mutant genes were covered by the wild-type alleles of the other species.

Since sexual isolation was the only isolating mechanism which could be readily measured, preliminary sexual isolation tests were made on the stocks at 16° C., using 10 females of each species and 10 males of either *D. pseudoobscura* or *D. persimilis*. The 30 flies used for each test were virgins and were kept together for a week in an ordinary shell vial with food, after which time the females were dissected and their seminal receptacles examined for sperm. The mating was done at 16° C. because at this temperature sexual isolation between *D. pseudoobscura* and *D. persimilis* is lowest (Mayr and Dobzhansky, 1945). For the population cage experiments, this temperature was desirable in order that changes in the sexual isolation could be most readily detected. For the later sexual isolation tests, the technique was similar, except

TABLE 1. *Main experiments, cage 2*

Generation	Number of parents		Number of offspring			Per cent hybrids
	pseudoobscura	*persimilis*	*pseudoobscura*	*persimilis*	hybrids	
1	320	680	82	313	227	36.5
2	60	60	561	473	322	23.7
3	300	300	246	995	665	34.9
4	120	120	406	336	126	14.5
5	250	250	162	86	292	54.1
6	80	80	273	415	38	5.2
7	180	180	617	478	17	1.5
10	150	150	820	257	72	6.3
11	180	180	653	729	42	2.9
12	440	440	2781	515	40	1.2
13	160	160	568	607	24	2.1
14	400	400	2217	712	259	8.1
15	300	300	2033	593	91	3.3
16	300	300	2613	453	89	2.8
17	300	300	2067	879	85	2.8
18	300	300	1925	1170	64	2.0
19	300	300	2808	1048	155	3.9
20	300	300	3372	1459	357	6.9
21	300	300	2109	1059	37	1.2
22	300	300	2117	1128	88	2.6

Equal numbers of males and females were used in all cases except in generation 6, in which 45 males and only 35 females of both *pseudoobscura* and *persimilis* were used.

TABLE 2. *Main experiments, cage 3*

Generation	Number of parents		Number of offspring			Per cent hybrids
	pseudoobscura	*persimilis*	*pseudoobscura*	*persimilis*	hybrids	
1	450	450	660	1008	464	22.5
2	360	360	544	1050	102	6.0
5	200	200	164	1680	100	5.1
6	130	130	394	1013	644	31.4
7	260	260	512	1569	58	2.7
8	360	360	648	1709	67	2.8
9	300	300	733	2193	60	2.0
10	300	300	1149	657	59	3.2
11	300	300	886	835	106	5.8
12	300	300	473	813	66	4.9
13	290	290	997	1965	159	5.1
14	300	300	1712	1712	182	5.0
15	300	300	1008	2418	79	2.2
16	300	300	1427	1772	180	5.3

TABLE 3. *Main experiments, cage 4*

Generation	Number of parents		Number of offspring			Per cent hybrids
	pseudoobscura	*persimilis*	*pseudoobscura*	*persimilis*	hybrids	
1	220	220	275	133	400	49.5
2	90	90	48	795	180	17.6
3	300	300	600	1006	55	3.3
4	400	400	414	1777	22	1.0
5	340	340	564	1241	25	1.4
6	300	300	1037	964	70	3.4
7	300	300	1214	244	26	1.8
8	150	150	275	1010	32	2.4
9	200	200	1284	1993	554	14.5
10	300	300	1375	3264	29	.6
11	300	300	1356	982	16	.7
12	300	300	2145	2188	74	1.7

that the males and females were aged apart for 10 days at 16° C., then left together for four hours before dissection.

The population cage experiments were done, also at 16° C., using the modified L'Hereditier-Teissier apparatus described by Dobzhansky (1947) and Wright and Dobzhansky (1946). In the present tests, however, the stender jars containing food with larvae were removed from the cages at the end of approximately two weeks and these cups fitted under sections of glass tubing two inches in diameter and approximately five inches in length, the tubes being fastened at the bottom with cellulose tape to the cup and closed at the top with a large cotton plug. The old cage with its adult flies was meanwhile discarded. In this way, all matings took place at 16° C., insuring a maximum number of hybrids, yet, by removing the covered cups to room temperature or to 25°C., more rapid development could take place. In addition, by not having long-continuous occupancy of the cages by the flies, the danger of infection with mites was greatly cut down. Flies were collected from the cups at 24-hour intervals, and counted according to whether they were *D. pseudoobscura, D. persimilis,* or hybrids. Advantage was taken of the fact that the size of the testis in hybrid males

is determined by which species was the mother and which the father, since the testis of hybrid males is large in the cross *pseudoobscura* female × *persimilis* male, but small in hybrids from the cross *persimilis* female × *pseudoobscura* male (Lancefield, 1929) (Dobzhansky, 1935). In this way, by observing the testis size, some idea could be gained of the way in which the isolating mechanisms were built up. The *D. pseudoobscura* and *D. persimilis* were then separated, the males separated from the females, the hybrids discarded, and all flies of pure species stored at 16° C., usually up to three weeks. Then equal numbers of males and females of both species were put into a fresh cage and the cycle recommenced. The number of flies put into the cage varied considerably according to the number of individuals of each sex and species that could be collected in three weeks (see Tables 1, 2, and 3), but usually was between 200 and 800 flies. The optimum for obtaining large numbers of all types of flies seemed to be about 600. The only modifications of this procedure were for certain generations of cage 2. When making up this cage for the first generation, more *persimilis* than *peudoobscura* were put in because it was thought at that time that the *pseudoobscura* would be more vigorous than the *persimilis*. When the cage was being made up for generation 6, only small numbers of *persimilis* were available, especially of *persimilis* females. Hence more males than females were put in, so that the effective population size would be as large as possible, while at the same time the numbers of the two pure species would be kept equal.

Late in the experiments, some mixed-cage tests were made. These were carried out as in the others, except that *D. persimilis* from one cage and *D. pseudoobscura* from another were used. These were run for one generation only.

Main Population Cage Experiments

Between ordinary laboratory *D. persimilis* and *D. pseudoobscura,* several isolating mechanisms can be observed in operation (Dobzhansky and Epling, 1944). First of all, there is usually considerable sexual isolation, varying with the stocks used and with the temperature (Mayr and Dobzhansky, 1945). Hybrids seem to have the same viability as the pure species, but hybrid males are completely sterile. Females, when backcrossed to either parental species, lay the usual number of eggs, but the larvae arising from these eggs have such poor viability that in competition with larvae of the pure species, as in population cages, they never reach the adult stage. Hence, in a population cage with both species present, even if the hybrids were not removed each

Table 4. *Results of partial correlation tests*

	S	σ_S	p	Correlation (r)
Hybrids × total flies (2, 3)				
Cage 2	−70	30.8	.05>but>.01	−.368
Cage 3	−21	18.3	>.05	−.231
Cage 4	−34	14.6	.05>but>.01	−.515
Total flies × time (1, 2)				
Cage 2	122	30.8	<.01	.642
Cage 3	33	18.3	>.05	.363
Cage 4	38	14.6	<.01	.576
Hybrids × time (1, 3)				
Cage 2	−140	30.8	<.01	−.737
Cage 3	−19	18.3	>.05	−.209
Cage 4	−26	14.6	>.05	−.394

generation, as was done in the present experiments, no introgressive hybridization would be expected to occur. That such introgression is indeed lacking was established by Dobzhansky (1945).

Preliminary sexual isolation tests (see table 4) showed, for the original stocks, high sexual isolation using *pseudoobscura* males, but little or no sexual isolation using *persimilis males*. After outcrossing to wild strains and reextracting, the sexual isolation with *pseudoobscura* males was lowered somewhat, but with *persimilis* males was somewhat raised. This rise in isolation index is, it should be noted, non-significant, essentially random mating still taking place when *persimilis* males are used. Hence, in a cage containing both species, a considerable number of hybrids would be expected in the first generation. This is borne out by the first generation counts from the three population cages which could be carried to completion, the percentages being 50, 36, and 22. In earlier experiments, using other stocks, from population cages which had to be discarded early because of mite infection, the hybrid percentages for the first generations were 60 and 27. In all population cages, however, which ran for more than four generations, a rather rapid decrease in the number of hybrids occurred. (See figs. 1–3 and tables 1, 2, and 3.) In all cases, within six generations, the percentage of hybrids had fallen to 5 per cent (though in some cases with some later temporary increase), and in certain cases reached as low as 1 per cent in later generations. This is in full agreement with Dobzhansky's work (1945), in which the percentage of hybrids in a population cage kept at 16° C. fell from 24 per cent on February 3 to 3.6 per cent on June 11.

A considerable heterogeneity may be noted in the percentage of hybrids after the first few generations; it may also be noted that in later generations a larger total number of flies was collected than earlier. It was therefore advisable to test the heterogeneity for significance and to determine whether the decrease in the percentage of hybrids could be explained by a correlation of percentages of hybrids

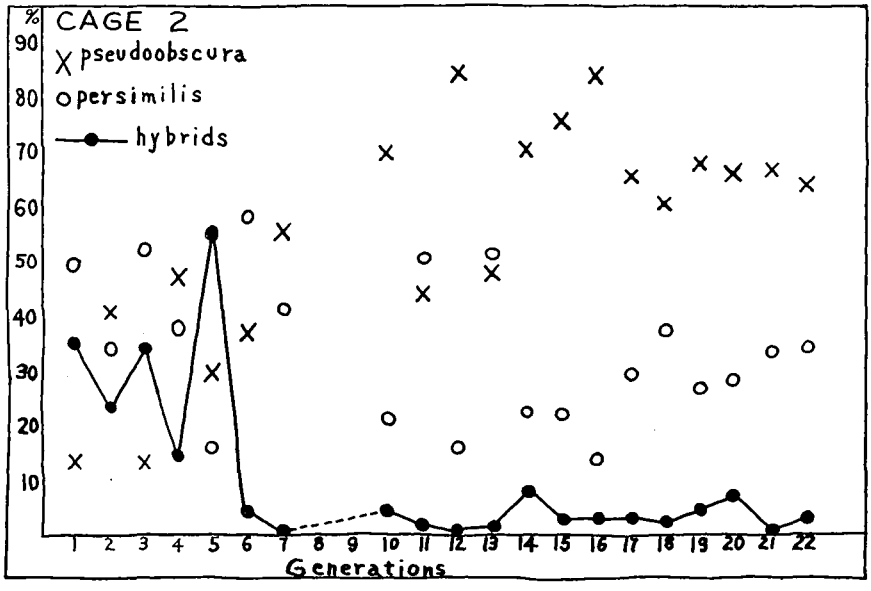

FIG. 1. Change in frequency of hybrids in cage 2, showing the percentage of the two pure species and the hybrids in each generation. (For additional data, see table 1.) No data for generations 8 and 9.

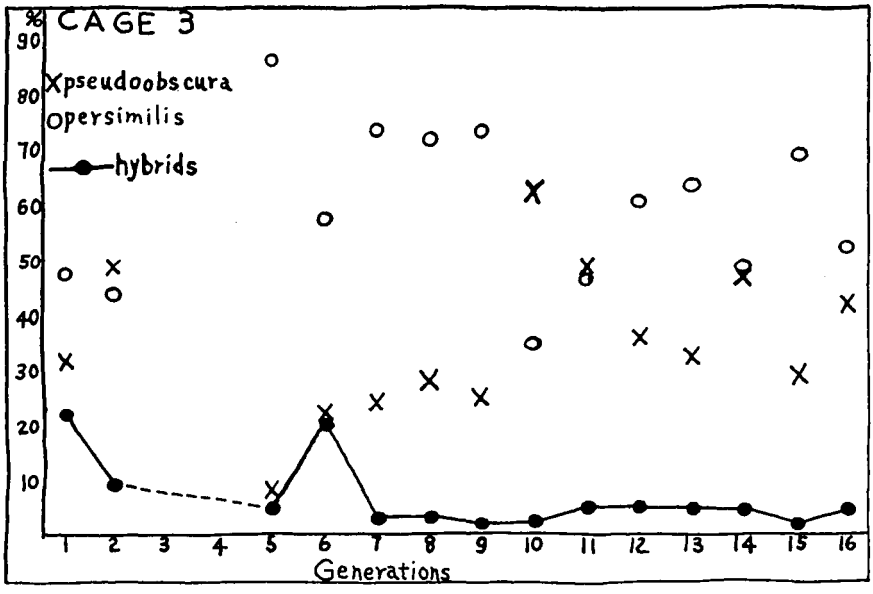

FIG. 2. Change in frequency of hybrids in cage 3, showing the percentage of the two pure species and their hybrids in each generation. (For additional data, see table 2.) No data for generations 3 and 4.

with the total numbers of flies. Such a correlation was possible, since the numbers of flies introduced each time the cage was reloaded were approximately the same, especially in later generations, and certainly showed no particular trend. The increase in total numbers of flies collected from generation to generation, therefore, was due to an increase in the percentage of eggs laid which later developed into adult flies. This meant a decrease in selection pressure for viability factors. Since the hybrids, being the only wild-type flies, were known to be superior in viability, reduced selection decreased their advantage over the mutant pure species. Hence their frequency would be reduced.

To test the heterogeneity, a χ^2 test was made for each cage, using the numbers of hybrids and pure species for each generation after a low value for the hybrids had been reached. The test, therefore involved generations 6, 7, and 10–22 for cage 2, generations 7–16 for cage 3, and generations 3–12 for cage 4. The χ^2 obtained was very high for all three cages, 550 for cage 2, 138 for cage 3, and 1,680 for cage 4. The exceedingly high χ^2 for cage 4 was chiefly caused by the very large number of hybrids in generation 9. All three χ^2 values are highly significant, with a probability far below .01. It is evident, therefore, that even after the early generations, the frequency of hybrids was undergoing significant changes. The causes of these changes are, however, not clear.

To determine how the percentage of hybrids was correlated with time and with total number of flies per generation, rank correlation tests were performed. Three of these tests were made for each cage, percentage hybrids × time, percentage hybrids × total flies, and total flies × time. Time is considered variable 1, total flies as variable 2, and percentage hybrids as variable 3. The method used was that of Kendall (1943). The results may be seen in table 4.

It is evident that the conditions in the three cages are somewhat different. In cage 2, the percentage of hybrids shows a very significant decrease, and the total number of flies a significant increase, but

the correlation of number of flies and hybrids is only on the borderline of significance. In cage 3, all three correlations are non-significant. In cage 4, conditions are essentially as in cage 2 except that the correlation of total flies × time is higher than the correlation of hybrids × time in cage 4, but not in cage 2.

In order to determine the partial correlation between percentage of hybrids and total, at the same time correcting for correlation of both with time, the formula

$$r_{23.1} = \frac{r_{23} - r_{13}\, r_{12}}{\sqrt{(1 - r_{12}^2)\,(1 - r_{13}^2)}}$$

(Kendall, 1942) was used. (Kendall gives no method for testing the significance of this partial rank correlation.) This formula gave a value of .20 for cage 2, − .17 for cage 3, and − .38 for cage 4. This shows that the partial correlation between total flies and percentage of hybrids may be either positive or negative and in two of the three cages is rather small. It therefore seems improbable that the correlation of percentage of hybrids with time can be explained by the correlation of total flies with time and of total flies with percentage of hybrids. It must also be remembered that these correlations apply to the entire period during which flies were being kept in each cage. It was only during the early generations, however, with a very few exceptions, that the percentage of hybrids was undergoing much change. The rank correlation test used disregards the magnitude of the changes and reflects only the rank of the values for total flies, per cent hybrids, and time. A real decrease in frequency of hybrids formed seems indicated. Natural selection would appear to be building up new reproductive isolation.

The detailed history of the three cages, of course, differed considerably. Cage 4 showed a very rapid steady drop in the percentage of hybrids, whereas cages 2 and 3 took a longer time and showed considerable fluctuations. In the two earlier cages, the percentage of hybrids for certain generations is unknown; generations 8 and 9 for cage 2, generations 3 and 4 for cage 3. This unfortunate state of affairs arose from widespread contamina-

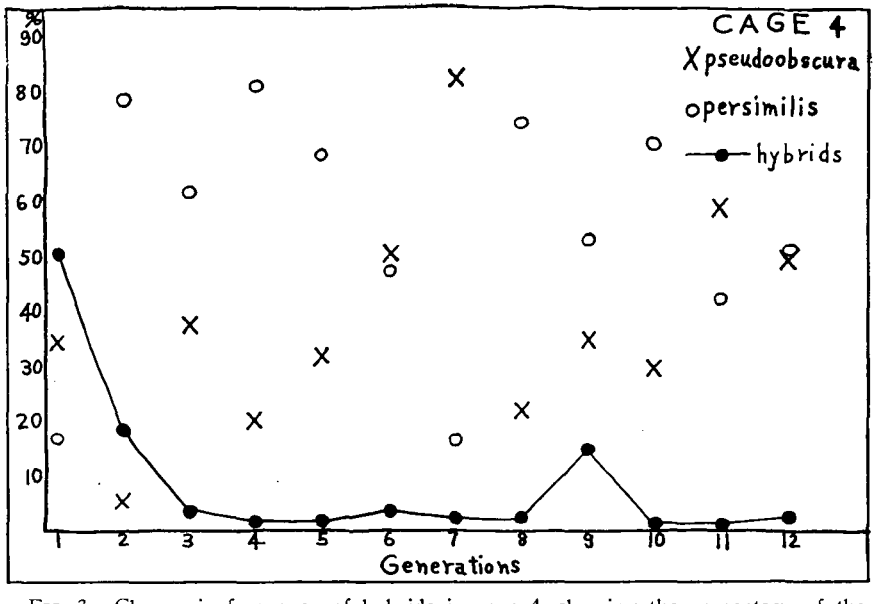

FIG. 3. Change in frequency of hybrids in cage 4, showing the percentage of the two pure species and their hybrids in each generation. (For additional data, see table 3.)

TABLE 5. *Source of hybrid males*

Generation	Cage 2		Cage 3		Cage 4	
	per. ♂ × *pseudo.* ♀	*pseudo.* ♂ × *per.* ♀	*per.* ♂ × *pseudo.* ♀	*pseudo.* ♂ × *per.* ♀	*per.* ♂ × *pseudo.* ♀	*pseudo.* ♂ × *per.* ♀
1	?	?	189	10	136	66
2	122	21	37	29	48	21
3	192	10	?	?	4	21
4	52	0	?	?	1	8
5	92	6	3	60	8	4
6	20	1	0	103	14	16
7	?	?	1	39	8	5
8	?	?	9	26	6	4
9	?	?	11	29	169	103
10	12	20	16	7	2	16
11	17	3	44	5	0	8
12	15	1	21	8	3	35
13	4	3	7	88		
14	84	14	45	37		
15	11	34	16	18		
16	42	17	72	6		
17	17	13				

tion of both cages by wild type *D. pseudoobscura* or *D. persimilis* which occurred at this time. Even after the percentage of hybrids had reached a low level, there was a certain amount of fluctuation, the extreme being in cage 4, where the frequency of hybrids rose from 2 per cent in generation 8 to 14 per cent in generation 9 and back to 1 per cent in generation 10, thereafter remaining at a low level. The causes of these earlier and later fluctuations are unknown. They could not be caused by contamination since this was ruled out by the examination of wild type male testes. These dissections showed no sharp rises in percentage of flies with large testes corresponding to high frequencies of wild type flies. (Compare tables 1, 2, and 3 with table 5.) This would have been the case if any appreciable contamination had occurred at these times. Indeed, in those generations in which data are lacking because widespread contamination was known to occur, almost all wild type males examined had full-sized testes. It might be argued that some of the later rise might be due to the contamination earlier, resulting in the introduction of unselected genes, thereby reducing the reproductive isolation in the population. This, however, could only occur if appreciable numbers of the contaminants were *glass pseudoobscura* or *orange persimilis*. Otherwise, the F1 flies from the interbreeding of cage and contaminating flies would be wild type and would be discarded along with the species hybrids. The effects of contamination could therefore be confined to the generation immediately following the contamination. Reasons for the fluctuations must therefore be sought from other causes. Temperature was usually fairly constant, especially during the latter part of the experiments, but humidity was usually quite variable, and may have differentially affected the viability of the wild type hybrids, and even of the degree of reproductive isolation itself. These earlier and later fluctuations, however, do not in any way vitiate the clear demonstration that hybrids are in general much less frequent for the later generations than for the earlier ones in all three cages.

Another fluctuating variable was the relative number of pure *pseudoobscura* and *perisimilis* in each generation. Differences between generations, even in the

same cage, were often extreme. The relative numbers of the two species might even undergo a radical shift in one generation. This may be seen by comparing the counts of generations 7 and 8 in cage 4. These fluctuations in the number of pure species flies also point to considerable environmental fluctuations, which caused natural selection to favor now one, now the other species.

From the examination of the hybrid male testes, an interesting fact emerges (see table 5). In the first two generations, the number of hybrids coming from the cross *persimilis* male × *pseudoobscura* female outnumbered and usually greatly outnumbered those from the cross of *pseudoobscura* male × *persimilis* female. This is to be expected, since, in the preliminary sexual isolation tests, *pseudoobscura* males already showed a strong preference for their own females, whereas the *persimilis* males showed little or no such preference. In the later generations, however, the hybrids seemed to be much more randomly distributed between the two types, often varying rather widely among cages and from generation to generation within the same cage, now one, now the other kind of hybrid being more numerous. This was, to some extent, due to the small number of hybrid males which could usually be dissected in these later generations. In general, therefore, natural selection has acted mainly to reduce the number of fertilizations of *pseudoobscura* females by *persimilis* males, making their number roughly comparable to those of the reciprocal cross.

Multiple-Choice Experiments

When cage 2 had run for 10 generations and cage 3 for 5 generations, flies from each were subcultured in bottles and standard sexual isolation tests were made, using as a control the stocks from which the cages were originally made up. When the males were *persimilis,* the isolation index was not significantly different from zero when the original control stocks were used, but it was highly significant when the flies from cages 2 and 3 were used. As would be expected, the value of the isolation index for cage 2 (.52) was greater than the value (.30) for cage 3 (see table 6).

When cage 2 had run for 14 generations, cage 3 for 9 generations, and cage

TABLE 6. *Sexual isolation tests*

Strains tested	No. of ♀ ♀ inseminated and not inseminated				Isolation index*	χ^2	Probability
	Homogamic		Heterogamic				
	+	−	+	−			
persimilis ♂ ♂—Preliminary test 1	46	37	55	36	−.04	.7	.4
persimilis ♂ ♂—Preliminary test 2	31	17	30	29	.12	1.5	.2
persimilis ♂ ♂—Control 1	82	130	80	125	−.07	.02	.9
persimilis ♂ ♂—Cage 2, 10th generation	129	66	40	152	.52	98.68	<.01
persimilis ♂ ♂—Cage 3, 5th generation	99	84	54	132	.30	22.77	<.01
persimilis ♂ ♂—Control 2	107	71	68	108	.21	15.48	<.01
persimilis ♂ ♂—Cage 2, 14th generation	97	19	25	91	.60	87.15	<.01
persimilis ♂ ♂—Cage 3, 9th generation	75	15	23	66	.52	57.01	<.01
persimilis ♂ ♂—Cage 4, 5th generation	106	41	26	128	.62	89.74	<.01
pseudoobscura ♂ ♂—Preliminary test 1	81	14	3	86	.93	120.9	<.01
pseudoobscura ♂ ♂—Preliminary test 2	42	13	6	45	.73	42.0	<.01
pseudoobscura ♂ ♂—Control 1	75	32	11	88	.73	71.16	<.01
pseudoobscura ♂ ♂—Cage 2, 10th generation	120	9	2	126	.96	211.9	<.01
pseudoobscura ♂ ♂—Cage 3, 5th generation	87	11	6	92	.87	131.0	<.01
pseudoobscura ♂ ♂—Control 2	59	6	17	50	.57	55.12	<.01
pseudobscura ♂ ♂—Cage 2, 14th generation	60	7	7	60	.80	79.53	<.01
pseudoobscura ♂ ♂—Cage 3, 9th generation	75	3	21	59	.57	78.55	<.01
pseudoobscura ♂ ♂—Cage 4, 5th generation	57	9	4	62	.87	77.68	<.01

* The isolation index is calculated from the percentage of homo- and heterogamic females inseminated (see Dobzhansky and Mayr, 1944), + 1.00 indicating only homogamic mating, 0.00 random mating, and −1.00 completely heterogamic mating.

4 for 5 generations, a second series of sexual isolation tests were performed, using the same technique and again with the original stocks subcultured generation after generation as controls. In this second set of tests, using *persimilis* males, the controls as well as the experimental cultures gave a value highly significantly different from random mating, though the isolation indices were much lower than those for the flies from the cages (.21 as opposed to .60. .52, and .62). In order to determine whether the sexual isolation for the flies taken from the cages was significantly different from those of the controls, the procedure used by Snedecor (1946) for three sets of attributes (Section 9.8) was applied. Due to the extremely laborious nature of the procedure, the comparison was made only between the control and the cage test most like the control in χ^2 and isolation index, namely cage 3. The χ^2 for these two sets of tests was highly significant ($\chi^2 = 15.81$, probability $< .01$ for 1 degree of freedom). Since the other cages had each given a χ^2 and isolation index even more different from that of the controls, it was evident that for the second set of tests, as well as for the first, the flies from cages which had run for several generations displayed a definitely greater interspecific sexual isolation than did the controls. As would be expected, both control and experimental tests using *pseudoobscura* males showed strong sexual isolation (see table 4). From these results, it would certainly appear that at least part of the reproductive isolation observed in the cages is sexual.

In this connection, it was thought that certain comparisons of sexual isolation between different cages might prove interesting using *persimilis* males, of course. This could also be done using the procedure for three sets of attributes from Snedecor. The first such comparison was made between the sexual isolation in cage 2 generation 10 and cage 3 generation 5. Both of these series of tests come from the first set. At the time the flies used in making up the stocks for this set of tests were taken from the cages, the percentage of hybrids in cage 2 stood at 6 per cent and had probably stayed at this point or lower for five generations. Never afterwards was the percentage of hybrids in this cage to go above 8 per cent. At the same time, however, cage 3, though it stood at 5 per cent, had probably never previously had such a small percentage of hybrids, and the fact that in the next generation it rose again to 21 per cent shows that the isolating mechanisms had not yet been stabilized. When the tests from these two cages, both from the first set, are compared statistically, we obtain a χ^2 of 7.67, which for one degree of freedom gives a probability of $< .01$.

The second comparison was made of the sexual isolation for cage 3 between generation 5 and generation 9. At generation 5, the isolating mechanisms had not yet been stabilized, as has already been mentioned, but by generation 9, the percentage of hybrids stood at 2 per cent and was never afterward to rise above 6 per cent. Hence, a difference might be expected. This comparison was, of course, not as valid as that between cage 2 and cage 3 from the first set of tests, since the two series of tests in this case were not done during the same period, and hence possibly not under strictly comparable conditions. Nevertheless, the results of this comparison might at least be suggestive. In this case, a χ^2 was obtained of 9.83, with a probability for one degree of freedom of $< .01$. These two comparisons would seem to give support to the idea that during the earlier generations, sexual isolation was increasing in all cages.

Mixed-Cage Experiments

In order to determine whether the isolation observed in the cages acts in general against all members of the other species, or is a more specific mechanism, isolating only the strains from the cage in which it is developed, mixed cage experiments were undertaken. In these

TABLE 7. *Mixed cage experiments*

(Equal numbers of each sex and species used as parents in each case)

Source of parents		Number of offspring			Per cent hybrids
pseudoobscura	*persimilis*	*pseudoobscura*	*persimilis*	hybrids	
Cage 3—10th generation	Cage 4—6th generation	889	840	85	5
Cage 4—6th generation	Cage 3—10th generation	314	1436	95	5
Cage 2—19th generation	Cage 4—9th generation	1143	731	270	13
Cage 4—9th generation	Cage 2—19th generation	1186	818	39	2
Cage 2—19th generation	Cage 3—13th generation	2535	873	117	3
Cage 3—15th generation	Cage 2—21st generation	838	1091	64	3

tests, *peudoobscura* and *persimilis* were taken from different cages, each of which had run for several generations with a very low percentage of hybrids, and were put together in one cage, which was permitted to proceed for one generation and then the number of pure species and hybred flies were counted. All combinations of the three cages were made. The results may be seen in table 7. In all cases but one, the percentages of the hybrids were quite low and approximately the same as those appearing in the unmixed cages. The only exception was when *pseudoobscura* from cage 2 were put together from cage 4. The percentage of hybrids obtained in this case, while not quite as high as one later count from one of the unmixed cages (cage 4 generation 9), was considerably higher than was usual in these later counts. On the other hand, it was a good deal lower than even the lowest of the first generation counts from the unmixed cages (13 per cent as compared with 22 per cent). From this it appears that these isolating mechanisms selected out in each of the three cages were very similar, so much so that they could be readily interchanged and probably were mechanisms which would react towards all individuals of the other species or at least that segment sampled by the original stocks, rather than with only the particular strain against which they had been developed. This was perhaps to be expected, inasmuch as all three cages had been made up from the same stocks, so that all initially carried the same store of genetic variability for reproductive isolation, except for differences due to the sampling error involved in making up the cages. Under these circumstances, one might expect natural selection to fix essentialy the same gene complexes in each cage. An alternative hypothesis would be, of course, that different gene complexes having very similar phenotypic effects, or, at least, all acting effectively-against stock flies of the other species as a whole, were selected out in each cage. To me, at least, this would appear to be much less likely.

DISCUSSION AND CONCLUSIONS

Before attempting to evaluate these findings, it may be desirable to review briefly the effective isolating mechanisms between *Drosophila pseudoobscura* and *D. persimilis* existing in nature. Geographical isolation can hardly be said to exist, since the range of *D. persimilis* is entirely included in that of *D. pseudoobscura*. Ecological isolation, on the other hand, is considerable and is twofold. The two species have rather different macro-ecological or ecoclimatic preferences, *D. persimilis* being found, for the most part, at higher elevations in the mountains, *D. pseudoobscura,* to a greater extent, at lower elevations, including the lowlands (Dobzhansky and Epling, 1944). It is interesting to note that outside the range of *D. persimilis, D. pseudoobscura* is common in the mountains as well as in the lowlands, reaching the tree line, above 11,000 feet, on Pikes peak in Colorado.

Micro-ecological or ecotopic differences are also marked, *D. pseudoobscura* preferring warmer sunnier places. Whereas *D. persimilis* prefers the cooler shadier spots (Pittendrigh-unpublished). Sexual isolation seems to play an important part, newly captured strains having a rather high sexual isolation except at low temperatures. All these isolating mechanisms, however, are partial, and one would expect some hybrids to be formed in nature. This apparently does not occur, since of the thousands of salivary gland chromosomes which have been studied from flies caught in nature, not a single hybrid has been found, even from localities where both species occur together (Dobzhansky-unpublished). This total absence of hybrids is at present an unsolved problem. It may be due to other isolating mechanisms, not yet detected, which acting in conjunction with the known ones, completely prevent the appearance of hybrids. Those two isolating mechanisms, male hybrid sterility and backcross inviability, which are so important in keeping the species apart in the laboratory, apparently never have a chance to operate in nature, though of course they may have been important in preventing gene exchange in the past.

With these facts in mind, let us consider the central problem of whether natural selection can act to build up isolating mechanisms between distinct allopatric forms whose ranges meet. In the first place, there must already be some isolation before selection can act, both in a form which makes the hybrid less well adapted, and also as hereditary variability for further isolation. If the hybrid is as well adapted as either parental type for any availible environment, either parental or intermediate, the hybrids will not be discriminated against and the two original forms will be connected by a zone of subspecific intergradation. Hence it means that the first isolating mechanisms must arise as by-products of genetic divergence, but at least theoretically could be added to and built into a "gene-tight" isolating system by selection.

Up until now, however, there has been no direct evidence that selection can and does do this. To the knowledge of the writer, no experimental evidence other than that here cited has been brought forward to show that natural selection can create or strengthen an isolating mechanism. Direct evidence of this occurrence from natural populations is also very scanty and often capable of interpretation on other grounds. Perhaps the best established case concerns sexual isolation between *D. pseudoobscura* and *D. miranda* (Dobzhansky and Koller, 1939). Here, strains of *D. pseudoobscura* coming from localities within or close to the range of at least the northern populations of *D. miranda* show in general a greater sexual isolation with northern strains of *D. miranda* than do strains of *D. pseudoobscura* coming from more distant localities. It is probable that in populations of *D. pseudoobscura* living in close proximity to *D. miranda,* natural selection has acted to prevent hybridization by means of greater sexual isolation, whereas in populations which are geographically remote from the range of *D. miranda,* increased sexual isolation has had no such selective advantage. However, even here the situation is not so simple, since this relation fails to hold when southern rather than northern strains of *D. miranda* are tested against the geographical strains of *D. pseudoobscura.*

A more disputed situation occurs in the cases of the crows, *Corvus corone-cornix,* and the grackles, *Quiscalus quiscula-aeneus* discussed by Dobzhansky (1941). In both these cases, the hybrid zone between the ranges of the two incipient species is broad in northern regions in which the retreat of the glaciers has only recently permitted occupation by the parental forms, whereas the hybrid zone is narrow in the south where the two forms in each case have been able to occupy the habitat for a longer time. Dobzhansky interprets the facts as indicating

a spread, with retreat of the glaciers, from geographically isolated refuges in the far south. As the two forms spread north, they came in contact first in the more southern regions, later in the north. In the south, natural selection has been able to build up reproductive isolation, preventing much hybridization, whereas in the north, not enough time has elaspsed for this to occur. This hypothesis has not been favored by Mayr (1942), who attributes the narrow hybrid zone in the more southern localities to local ecological factors preventing the two forms from coming in contact to as great a degree as in the north. It now seems probable, however, that an extensive study of cases where incipient species have come together, in different places, at different times, after being geographically isolated, would reveal undoubted cases of natural selection for reproductive isolation.

The evidence here presented shows, not only that natural selection can act to strengthen isolation between species, but it also brings out an important aspect of the difference between species and subspecies. When subspecies are involved, gene exchange between the two populations is always possible, the hybrid types are fitted for various intermediate habitats, geographical or ecological, between those of the two parental types, and fit into their population structure. Once the threshold has been passed, however, and the two populations have reached the status of full species, the relation between them becomes quite different. The hybrids are relatively ill-adapted for any available habitat. There are usually several isolating mechanisms which to a greater or lesser degree prevent gene exchange, and these are likely to be strengthened and added to by natural selection. Furthermore, if, because of a change in the environment, some of these isolating mechanisms are broken down, others are likely to take their place, due simply to the advantage of preventing the wastage of gametes which would otherwise result. *D. pseudoobscura* and *D. persimilis* have reached this stage of genetic divergence. In the experiments here described, ecological isolation was completely eliminated by keeping all flies in a small cage with only one type of culture medium. Existing sexual isolation was greatly weakened by keeping the cage at a low temperature. In this way, what are probably the two most important isolating mechanisms keeping the two species apart in nature were largely eliminated, yet in a surprisingly short time new isolation (in this case at least partly sexual) was built up, which brought the number of hybrids again to a low level. This change, of course, was aided by the practice of removing the hybrids entirely each generation, in this way simulating complete hybrid inviability. This, however, could hardly have had much effect, because of complete male hybrid sterility and strong backcross inviability under cage conditions. This seems to me to typify the true genetic relation of a good species to related forms. By virtue of its isolating mechanisms, in part at least maintained by selection, it is genetically independent of other organisms. In its further evolution, it is on its own, dependent entirely on its own mutations, since it is unable, as is a subspecies, ever to acquire advantageous genes from other populations.

Summary

Using artificial mixed populations of *Drosophila pseudoobscura* and *D. persimilis,* it has been possible to show, over a period of several generations, a very rapid increase in the amount of reproductive isolation between the two species as a result of natural selection. This isolation has been shown to be at least partly sexual. The implications of these findings for theories of speciation are discussed.

Acknowledgments

The author wishes to express his deep indebtedness to Dr. Th. Dobzhansky for suggesting the problem, for continual guidance and inspiration during the course of the work, and for many helpful techni-

cal suggestions throughout the experiments. The help very kindly given by Dr. Howard Levene in the statistical analysis of the data is also gratefully acknowledged.

Bibliography

Dobzhansky, Th. 1935. Maternal effect as a cause of the difference between the reciprocal crosses in *Drosophila pseudoobscura*. Proceedings of the National Academy of Sciences, **31**: 443–446.

Dobzhansky, Th. 1940. Speciation as a stage in genetic divergence. American Naturalist, **74**, 312–321.

Dobzhansky, Th. 1941. Genetics and the origin of species. 2nd edition, New York, Columbia University Press.

Dobzhansky, Th. 1945. Genetic structure of natural populations. Carnegie Institution of Washington Year Book, **44**: 127–134.

Dobzhansky, Th. 1947. Adaptive changes induced by natural selection in wild populations of *Drosophila*. Evolution, **1**: 1–16.

Dobzhansky, Th., and Koller, P. C. 1939. Sexual isolation between two species of *Drosophila*—A study in the origin of an isolating mechanism. Genetics, **24**: 97–98.

Dobzhansky, Th. and Mayr, E. 1944. Experiments on sexual isolation in *Drosophila*. I. Geographic strains of *Drosophila willistoni*. Proc. Nat. Acad. Sci., **30**: 238–244.

Kendall, M. G. 1942. Partial rank correlation. Biometrika, **32**: 277–283.

Kendall, M. G. 1943. The advanced theory of statistics. London, C. G. Griffen and Co.

Lancefield, D. E. 1929. A genetic study of crosses of two races or physiological species of *Drosophila obscura*. Zeitscrift für induktives Abstammungs- und Vererbungslehre, **52**: 287–317.

Mayr, E. 1942. Systematics and the origin of species. New York, Columbia University Press.

Mayr, E., and Dobzhansky, Th. 1945. Experiments on sexual isolation in *Drosophila*. IV. Modification of the degree of isolation between *Drosophila pseudoobscura* and *Drosophila persimilis* and of sexual preferences in *Drosophila prosaltans*. Proc. Nat. Acad. Sci., **31**: 75–82.

Muller, H. J. 1939. Reversibility in evolution considered from the standpoint of genetics. Biol. Rev., **14**: 261–280.

——. 1942. Isolating mechanisms, evolution, and temperature. Biol. Symposia, **6**: 71–125.

Snedecor, G. W. 1946. Statistical methods. 4th edition.

Wright, S., and Dobzhansky, Th. 1946. Genetics of natural populations. XII. Experimental reproduction of some of the changes caused by natural selection in certain populations of *Drosophila pseudoobscura*. Genetics, **31**: 125–156.