EXTRANUCLEAR BARRIERS TO INTERSPECIFIC HYBRIDIZATION BETWEEN OENOTHERA HOOKERI AND OENOTHERA ARGILLICOLA

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THE extent to which heritable components outside the nucleus are involved in the various genetic events leading to species formation remains in question. The North American evening primroses of the subgenus *Euoenothera* offer an appropriate group of organisms to study some aspects of this problem. First, the cytogenetic behavior of *Oenotheras* provides a useful tool for the investigation of extranuclear inheritance, since it is relatively easy to obtain identical nuclei in combination with cytoplasmic elements from diverse origins. Exploiting this advantage, RENNER and his colleagues have focused special attention on the inheritance of a particular cytoplasmic inclusion—the chloroplast. From these studies has come convincing evidence that the chloroplast in *Oenothera* is an autonomous constituent of the genetic material, and further, that at least five genetically different plastid types exist in the strains of RENNER's collection (RENNER 1922, 1934, 1937; STUBBE 1959).

A second feature of the evening primroses that makes them suitable for analysis is the fact that the population structure and phylogenetic history of the North American *Euoenotheras* are known in considerable detail as a result of the studies of CLELAND and his students (see CLELAND 1956, 1958). There is thus available from each of the phylogenetic groups a representative sample of geographical races which have already been characterized cytogenetically.

Although some information has been collected on the nature of the plastid types present in the North American *Euoenotheras* (STINSON 1953), relatively little is known about the group as a whole, or about the bearing this information has on the phylogenetic history. Specifically, the question arises as to what part extranuclear elements, particularly the plastids, play in delimiting the various populations and species. One clue is perhaps provided by the phylogenetic studies mentioned above: it has been observed that the principal reason for failure to obtain viable hybrids between any two members of the North American population is chlorophyll deficiency in the F_1 offspring (see, for example, CLELAND 1950). This fact, coupled with the findings of RENNER's group on the inheritance of chloroplast characters in *Oenothera*, might suggest that intrinsic properties of the chloroplast contribute to the hybrid inviability. However, detailed genetic analyses are needed to ascertain the exact nature of the observed chlorophyll defects. The present paper reports such an analysis of interspecific crosses be-

tween *O. hookeri* and *O. argillicola*. An earlier, condensed report of certain of the results to be presented has been given elsewhere (STINSON 1958).

MATERIAL AND METHODS

Characteristics of the species: The two species *hookeri* and *argillicola* do not display, or do so in only minor degree, the cytogenetic aberrations characteristic of most members of the subgenus *Euoenothera*. Races of both species used in this investigation form seven pairs of chromosomes at meiosis, lack balanced lethals, and possess large, open pollinated flowers. The strains of the two species are further alike in having similar segmental arrangements, which are identical with or close to the arrangement taken to represent the putative ancestral chromosome condition for the North American *Euoenotheras*. In geographical distribution, however, the *hookeri*s and *argillicola*s are markedly different; *hookeri* is found in California and Northern Mexico, with an eastward extension into Colorado, whereas *argillicola* occupies a range restricted to certain shale barrens in the Appalachian Mountains from Virginia to Pennsylvania. The two species are also clearly distinguished by their very dissimilar appearance; they differ in almost all characteristics except flower size.

The three *hookeri* races chiefly used were the California strains Johansen, Devils Gate and franciscana deVries. These strains are easily separated by several visible characters. Two of them—Johansen and Devils Gate—have the same segmental arrangement, which is the most common one yet encountered in the *hookeri* population. The segmental arrangement of franciscana deVries differs from the Johansen arrangement by a single interchange, and is the third most frequent *hookeri* arrangement. The three *hookeri* races are not only complex-homozogotes, but also approach genic homozygosity as a result of self-pollination from 16 to 18 generations.

The species *argillicola* was represented by the strains Douthat 1, Douthat 2, Douthat 3, Douthat 4a and Douthat 4b, all collected in Virginia. They are different in appearance, and are slightly different in their end arrangements. All five races had been maintained by self-pollination for five or six generations prior to their use in these studies, and none showed any signs of segregation, at least for morphological characters.

A third species, *O. parviflora*, served as a source of alien cytoplasm to which the genomes of *hookeri* and *argillicola* were transferred. *O. parviflora* is found in northeastern United States and eastern Canada, and unlike the two previous species is composed of typical, complex-heterozygotes, which are self-pollinating and breed true in spite of their heterozygosity. Such plants have all 14 chromosomes in one circle at meiosis, and produce only two kinds of gametes, each with a different set, or complex, of seven chromosomes. One kind of complex is transmitted chiefly through the egg (alpha complex), whereas the other complex is transferred mainly by the pollen (beta complex). In addition to their differences in end arrangement, the alpha and beta complexes of *parviflora* also give rise to quite different phenotypic effects. All beta *parviflora* complexes produce charac-

ters which resemble closely those found in *O. argillicola*. Two kinds of alpha *parviflora* complexes are recognizable, those which give *biennis*-like effects, and those which yield *strigosa*-like characters. Phylogenetically, the *parvifloras* appear to have arisen through hybridization between the progenitors of the *argillicolas* and ancient *biennis* and *strigosa* populations (see CLELAND 1958).

Two *parviflora* races, unlike in segmental end arrangement, phenotype, and geographical origin, served as sources of cytoplasm. One race, *angustissima*, was first described by GATES (1913) from a collection near Ithaca, New York. The alpha complex of *angustissima* shows strong *biennis* tendencies. The second race, *Charlottesville*, stems from a collection in the vicinity of Charlottesville, Virginia. The alpha complex of *Charlottesville* is identical segmentally to a complex of wide distribution in the Southeast—the so-called *truncans* complex (STEINER 1952). The *truncans* complex of *Charlottesville*, however, is perhaps more *biennis*-like in phenotypic expression than are most other *truncans* complexes. It should be pointed out that *Charlottesville* is unique among the *parviflora* races in possessing an alpha complex with the *truncans* arrangement.

Terminology and symbolism. The problem of terminology for extranuclear inheritance is often complicated by the fact that it is not always possible to associate the phenotypic character in question with a specific physical entity outside the nucleus. In the present investigation the initial transfers between genomes and cytoplasms presumably involved the *whole* cytoplasm, but the major interest is restricted to genetic systems that control chloroplast development. The question of whether the cytoplasmically inherited factors demonstrated to be part of these systems are located in the chloroplasts *per se*, or reside in other cytoplasmic elements is not only of theoretical interest, but is also pertinent to the practical problem of deciding on a terminology. This question will be considered later, but for the present it may be stated that available evidence appears sufficient to justify use of terms which refer to genetic properties of the chloroplasts themselves, as distinct from the remainder of the cytoplasm. Therefore, in dealing with the extranuclear components of inheritance the words *plastid* and *plastome* will be employed. The term *plastome*, as currently used by the German workers, designates the total genetic information localized in the chloroplast and does not have the same meaning as *plastidome* which refers to the total plastid complement of a cell in a morphological sense (see MICHAELIS 1957; SCHÖTZ 1958; STUBBE 1959).

For simplicity, the plastid types and genomes in the different species are abbreviated by upper and lower case letters respectively. Thus the genetic constitutions of major concern are symbolized as follows: *hookeri*, *H hh*; *argillicola*, *A aa*; *parviflora*, *P apßp*; *hookeri* genomes associated with *parviflora* plastids, *P hh*; *argillicola* genomes combined with *parviflora* plastids, *P aa*.

Cultural methods: Seeds were germinated on moist filter paper in petri dishes, and with the first signs of germination the desired number of seedlings were planted in soil in the greenhouse. The seedlings not planted were kept in the petri dishes under artificial illumination, where the plants remained alive in a cotyledon stage of development for 2–3 weeks. Since many of the chlorophyll

abnormalities are evident in the cotyledons, the petri dish populations provided relatively large numbers of plants (usually 100–200) for observation. Thus, in several instances observations of chlorophyll phenotypes were made primarily on the petri dish populations.

RESULTS AND INTERPRETATION OF CROSSES

1. *Crosses between hookeri* ($H hh$) and *argillicola* ($A aa$): Interspecific crosses between *hookeri* and *argillicola*, made over a number of years between various races of the two species, have always given rise to chlorophyll deficient offspring, which at best have survived only to a young rosette stage of growth. This result was obtained irrespective of the direction in which the cross was made. The following strains of the two species have thus far been tested in one or more reciprocal interspecific crosses (progenies consisted of 100–200 plants): *hookeri* races—Devils Gate, franciscana deVries, franciscana Shull, *hookeri* deVries and Johansen; *argillicola* races—Douthat 1, Douthat 2, Douthat 3, Douthat 4a, Douthat 4b. The expression of the chlorophyll abnormality is not the same in the reciprocal hybrids. The cross $H hh \text{♀} \times A aa \text{♂}$ produces seedlings whose chlorophyll defect is readily apparent in the cotyledons of both petri dish and greenhouse plants. Depending upon the parental strains used, the color of the seedlings ranges from yellow to almost white. The hybrids have failed to survive beyond the cotyledon stage in every instance except for the cross *hookeri* deVries $\times$ Douthat 4b, where the offspring approach a pale green color.

The chlorophyll deficiency in hybrids from the reciprocal cross, $A aa \text{♀} \times H hh \text{♂}$, is less drastic and appears somewhat later in development; plants from this cross are consequently longer lived. The cotyledons of seedlings grown in petri dishes are usually normal green, while the cotyledons of greenhouse plants are, as a rule, pale green. Green plants transferred from the petri dishes to the greenhouse soon become pale green, suggesting that the color difference between petri dish and greenhouse plants is probably largely a matter of light intensity. In any event, with continued development the plants become progressively yellow-green. Generally, the yellowing process begins before the cotyledons are full grown, and the first true leaves are almost always yellow-green. Many plants die by the time the second and third pair of rosette leaves are formed. The surviving plants remain yellow-green to pale green, and some of them, although slow growing, may live for 2–3 months in the greenhouse. No plants have survived more than a few weeks in the field however.

Thus, the cross $A aa \times H hh$, while producing less severe chlorophyll defects than the reciprocal cross, does, nevertheless, produce hybrids with chlorophyll defects that are lethal under ordinary conditions. It is possible that hybrids from the cross $A aa \times H hh$ could be grown to maturity if maintained under reduced light intensities. In this connection SCHÖTZ and REICHE (1957) have reported that certain chlorophyll deficient *Oenothera* hybrids which normally die in early growth stages may eventually become more or less green when grown under low light intensity for varying periods of time.

Clearly the chlorophyll abnormality present in hybrids from reciprocal crosses between *hookeri* and *argillicola* imposes a barrier to successful hybridization between the two species. In the sections that follow, the results of a successful attempt to overcome this barrier are presented.

2. *Transfer of hookeri and argillicola genomes to parviflora cytoplasm*: Underlying the operational procedure for the incorporation of a given genotype in a given cytoplasm is the assumption that the female parent contributes all the cytoplasm to the fertilized egg, or at least that any cytoplasmic contribution by the pollen parent is so small as to be discounted. It is well-known in *Oenothera* that this assumption is not always valid, since the pollen tube frequently transmits cytoplasmic elements in sufficient amounts to affect the phenotype and breeding behavior of the offspring. The problem is not always as serious as might be expected, however, especially for chlorophyll characters, since there are genetic means for detecting male plastids in the progeny. Thus, in the initial crosses aimed at the transfer of the genomes of *hookeri* and of *argillicola* to *parviflora* cytoplasm, it was assumed that cytoplasm was maternally inherited unless evidence to the contrary was found.

The procedure adopted for this transfer takes advantage of the segmental end arrangements of the particular races used, and of the absence of lethals in the two 7-paired species. Races of *parviflora* were crossed as female parents by the *hookeri* and *argillicola* strains to give a chromosome configuration of $\odot 14$ in each F_1 hybrid. As a consequence of regular alternate disjunction in the $\odot 14$ hybrids, the seven chromosomes of each complex separate as a unit at anaphase I. Since the *hookeri* and *argillicola* genomes are lethal free, 7-paired $^h\text{hookeri} \cdot ^h\text{hookeri}$ (*hh*) and $^a\text{argillicola} \cdot ^a\text{argillicola}$ (*aa*) segregates are recovered upon selfing the appropriate F_1 hybrids. The original female parent was *parviflora*, and, therefore, the 7-paired *hookeri*s and *argillicola*s which appear in F_2 possess alien cytoplasm and plastids from *parviflora*. Taking *hookeri* as an example, the method is outlined in Diagram 1. Substitution of an *argillicola* male plant for *hookeri* in the diagram yields *aa* genome combinations in *parviflora* cytoplasm. Identification of the 7-paired segregates in F_2 was made on the basis of phenotype, and confirmed by cytological examinations. For convenience, the *hookeri* and *argillicola* races with *parviflora* cytoplasm will be referred to as the recovered races of the two species.

DIAGRAM 1.—Method of recovery of *hookeri* nucleus with *parviflora* cytoplasm and plastids.

To date the three *hookeri* races franciscana deV., Johansen, and Devils Gate have been recovered in the cytoplasm of the *parviflora* race Charlottesville. Only the latter two strains of *hookeri* have been adequately tested genetically, and consequently only their behavior will be considered. Three of the Douthat strains of *argillicola*—2, 3 and 4b—have likewise been recovered in Charlottesville cytoplasm, and Douthat 4b has in addition been isolated in the cytoplasm of the angustissima strain of *parviflora*.

In any F_2 population the recovered segregates are not absolutely uniform in phenotype, nor are they exactly identical to their original counterparts. With both *hookeri* and *argillicola*, however, the recovered strains are phenotypically very close to the original races, and there was never any doubt about the identification of the recovered types as to species and strain. This fact is even more striking when it is realized that most F_2 populations consisted of only 20 plants, and some of these were $\odot 14$ *aph* or *apa* individuals. It is quite clear that one is not dealing with the F_2 segregations that would be expected from hybrids between distinctly different species. In every case two phenotypic classes were immediately evident, and these corresponded to the two cytological classes, $\odot 14$ and seven pairs; closer inspection within each class revealed further segregations of a minor nature. The recovered plants selected for self-pollination and test crosses were judged most nearly to approach the original race in appearance.

The recovered strains of the two *hookeri* races, Devils Gate and Johansen, each differ from the original race in several minor respects, the most obvious and consistent differences being the slightly less spreading habit and smaller buds and flowers of the recovered strains. The recovered Devils Gate strain also tends to show a transient lighter green color on the tips of young branches, and again at maturity to display a somewhat pale green leaf color. Both recovered *hookeri* races are fully fertile. The third *hookeri* race, franciscana deV., only recently recovered in *parviflora* cytoplasm, is also very similar phenotypically to its original counterpart. There is a difference in color evident in the early stages of growth, the recovered strain being somewhat paler green than the original race. At maturity this difference disappears.

Hybrid progenies between either the Charlottesville or angustissima strains of *parviflora* and the *argillicola* races always contain solid green and green, yellow-green variegated plants. Genetic analysis of the variegation indicates that both the green plants and green branches on variegated plants possess cytoplasm, or more precisely, plastids from the female *parviflora* parent (STINSON 1957, and unpublished). Therefore, only green plants or green branches were used for purposes of isolating the *argillicola* genomes in *parviflora* cytoplasm.

The three recovered *argillicola* races, Douthat 2, Douthat 3 and Douthat 4b, are very similar to the original races for all characters. As with the *hookeri*s, some differences exist between the recovered and original strains, again most obvious in bud and flower size, and in growth habit. There has been no indication of chlorophyll differences between recovered and original strains. All three recovered *argillicolas* have exhibited normal fertility. In the case of Douthat 4b, several different lines have been established in each *parviflora* cytoplasm, and no two

lines are exactly alike. All, however, are unmistakably Douthat 4b-like, regardless of the source of *parviflora* cytoplasm.

3. *Breeding behavior of recovered races of hookeri* (*P hh*) and *argillicola* (*P aa*): (a) *P hh* ♀ × *A aa* ♂ —The results of this interspecific cross are markedly different from those obtained when the original *hookeri*s are crossed by *argillicola*. As previously noted, crosses of the type *H hh* × *A aa* yield chlorophyll deficient seedlings which die while still in the cotyledon stage of growth. In contrast, the cross *P hh* × *A aa*, with either the recovered Devils Gate or Johansen as *P hh* parent, produces green, viable hybrids. The contrast in the two types of crosses is clearly seen in petri dish plants (Figure 1). The cross *P hh* × *A aa* does not, however, give exclusively green plants; with the formation of the first and second true leaves it becomes apparent that some plants are green, yellow-green variegated (Table 1). Discussion of these and subsequent variegated plants is deferred until later. Thus, in spite of their close phenotypic similarity, the recovered and orig-

TABLE 1
Crosses involving recovered hookeri and argillicolas

Cross	F ₁			
	Total	Green	Variegated	Percent variegated
1) <i>P hh</i> females × <i>A aa</i> males				
Devils Gate × Douthat 1	60	49	11	18.3
Devils Gate × Douthat 3	110	105	5	4.5
Devils Gate × Douthat 4b	163	71	92	56.4
Johansen × Douthat 1	58	48	10	17.2
Johansen × Douthat 2	57	47	10	17.5
Johansen × Douthat 3	110	106	4	3.6
Johansen × Douthat 4a	30	18	12	40.0
Johansen × Douthat 4b	61	34	27	44.3
2) <i>P aa</i> females × <i>H hh</i> males				
Douthat 2 × Devils Gate	60	38	22	36.7
Douthat 2 × Johansen	59	15	44	74.6
Douthat 3 × Devils Gate	32	14	18	56.3
Douthat 4b* × Devils Gate	90	45	45	50.0
Douthat 4b* × franciscana deV.	124	85	39	31.5
Douthat 4b* × <i>hookeri</i> deV.	118	98	20	16.9
Douthat 4b* × Johansen	88	39	49	55.7
3) <i>P hh</i> females × <i>P aa</i> males				
Devils Gate × Douthat 2	75	75	0	0
Devils Gate × Douthat 4b*	289	289	0	0
Johansen × Douthat 2	111	111	0	0
Johansen × Douthat 4b*	179	179	0	0
4) <i>P aa</i> females × <i>P hh</i> males				
Douthat 2 × Devils Gate	59	59	0	0
Douthat 2 × Johansen	60	60	0	0
Douthat 4b* × Devils Gate	162	162	0	0
Douthat 4b* × Johansen	88	88	0	0

* Includes Douthat 4b strains with the two sources of *parviflora* plastids.

FIGURE 1.—Petri dish plants from crosses $H!h \times Aaa$ (left) and $P hh \times Aaa$ (right). FIGURE 2.—(a) green plant from cross $P hh \times Aaa$; (b) yellow-green plant from cross $Aaa \times P hh$; (c) yellow-green plant from cross $Aaa \times H hh$. FIGURE 3.—Yellow-green plant from cross $Aaa \times H hh$. FIGURE 4.—Green plant from cross $Paa \times H hh$. FIGURE 5.—Variegated plant from cross $P hh \times Aaa$. FIGURE 6.—Variegated plant from cross $Paa \times H hh$.

inal hookeris are revealed to be genetically different by their unlike maternal parent breeding behavior.

(b) $Aaa \text{ } \text{f} \times P hh \text{ } \text{m}$ —This cross, the reciprocal of the preceding one, provides the first indication that extranuclear factors account for the genetic differences between the original and recovered hookeris. For when the interspecific cross is

made using *P hh* individuals as male parents, the offspring are not green but yellow-green. This result has been obtained in each of the reciprocals of the crosses reported in the preceding section. Moreover, every cross of the type *A aa* $\times$ *P hh* was always compared with the appropriate cross of the type *A aa* $\times$ *H hh*. The yellow-green plants from the two kinds of crosses are indistinguishable. It is evident, therefore, (1) that the *P hh* strains breed differently in reciprocal crosses with *argillicola*, green plants being produced only when the recovered strain acts as maternal parent (Figure 2a, b); and (2) that as male parents *P hh* strains behave as the original *H hh* races (Figure 2b, c).

(c) *P aa* $\times$ *H hh* δ —The crosses of this type thus far investigated have had as female parents Douthat 2, Douthat 3, and Douthat 4b. The Douthat 4b strain, it will be recalled, has been recovered in two different sources of *parviflora* cytoplasm (Charlottesville and angustissima). The behavior of the recovered Douthat 4b strains in the two sources of *parviflora* cytoplasm has not been detectably different. Every cross of *P aa* $\times$ *H hh* has given progenies consisting of green and variegated plants, the latter with nongreen areas of yellow to yellow-white tissue (Table 1). Comparison between the cross *P aa* $\times$ *H hh*, which gives green (and variegated) plants, and the cross *A aa* $\times$ *H hh*, which yields chlorotic offspring, demonstrates that the recovered and original strains of *argillicola* do not breed alike as female parents. The difference in the maternal parent breeding behavior of original and recovered *argillicola* is illustrated in Figures 3 and 4 where two plants of the same age are compared. There is no doubt about the strikingly different outcome of the cross when a *P aa* plant replaces an *A aa* individual as female parent. (The chlorophyll deficient plant in Figure 3 died about two weeks after the photograph was taken.)

(d) *H hh* $\times$ *P aa* δ —Each of the reciprocals of the crosses described in the preceding section has been grown. All have given rise to yellow or yellow-white offspring virtually identical to those produced by the comparable *H hh* $\times$ *A aa* cross. The recovered *argillicolas*, therefore, behave differently in reciprocal crosses with *hookeri*, and as pollen parents are indistinguishable from the original race. This genetic behavior exactly parallels that demonstrated for the recovered *hookeris*, and is compelling evidence for an extrachromosomal basis for the unlike breeding behavior of *P aa* and *A aa* plants.

4. *Additional test for plastome differences between original and recovered races:* Insofar as genetic effects on chloroplast function are concerned, the reciprocally different breeding behavior of the recovered strains strongly indicates that the original and recovered races differ solely in their plastomes, and not in nuclear constitution. It is recognized, nevertheless, that the recovered strains are almost certainly genically different from the original races, as suggested by phenotypic appearances. In fact, the method utilized for the transfer of *hookeri* and *argillicola* nuclei to *parviflora* cytoplasm means that the recovered strains might be expected to carry certain genes from the α *parviflora* complex as a result of crossing over in the F_1 hybrid stage of the recovery process. In the case of the *hookeris* an additional test has been possible which offers further evidence that the presence of *parviflora* genes does not determine the observed breed-

ing behavior of the recovered *hookeri* races in crosses with *argillicola*. The test consisted of a comparison of the maternal parent breeding behavior of *hh* individuals recovered in *parviflora* cytoplasm and *hh* individuals recovered in *hookeri* cytoplasm, the *h* genomes in each case having been associated with the same α *parviflora* complex in F_1 .

The two kinds of recovered *hookeri*s were obtained by essentially the same method described earlier. This time, however, the *hookeri* races Johansen and Devils Gate were crossed reciprocally with a plant of the Charlottesville strain of *parviflora*. Since the alpha complex of Charlottesville is transmitted by the pollen as well as the egg, the reciprocal F_1 combinations $H \alpha ph$ and $P \alpha ph$ are possible. The former F_1 plants yield on self-pollination $H hh$ segregates (designated $H hh-rec$), while the latter F_1 combination produces $P hh$ segregates. The four F_2 populations derived from the four reciprocal F_1 's were grown in 1958. All $H hh-rec$ and $P hh$ segregates in the F_2 families were pollinated by the same *argillicola* race. Combining results from both *hookeri* strains, a total of 18 $H hh-rec$ and 15 $P hh$ plants were used in the testcrosses, and the progenies were grown in petri dishes in 1959. The $P hh$ plants gave in every case green seedlings, whereas all $H hh-rec$ plants gave yellow or yellow-white seedlings. These results are interpreted as follows: Provided that crossing over was similar in the reciprocal F_1 hybrids, and that the various crossover classes were equally viable in the two kinds of cytoplasm, the same crossover types should be represented among the *hookeri* genomes recovered in *hookeri* and in *parviflora* cytoplasm. Both *hookeri* recovered populations should be equally "contaminated" with α *parviflora* genes. If certain of these *parviflora* genes determined the outcome of crosses with *argillicola* males, one would expect to find plants among the two recovered types which breed alike in the testcrosses. In fact, however, all $P hh$ segregates behaved differently from all $H hh-rec$ segregates. Further, the behavior of $H hh-rec$ plants was not demonstrably different from that of original $H hh$ individuals. Even though the number of plants tested was not large, the fact that all $P hh$ types behaved identically, as did all $H hh-rec$ types, is difficult to reconcile with a strict genic interpretation. To explain the results on the basis of crossing over alone, it must be assumed that all 15 $P hh$ plants acquired the necessary *parviflora* genes, while none of the 18 $H hh-rec$ plants received the same *parviflora* genic contribution. This seems highly unlikely. A more reasonable conclusion is that the breeding behavior which $P hh$ individuals exhibit as female parents in crosses with *argillicola* depends upon the presence of *parviflora* plastids, and not *parviflora* genes.

5. *Variegated plants from crosses involving recovered strains:* As previously mentioned, the two interspecific crosses employing the recovered strains of *hookeri* and *argillicola* as female parents— $P hh \times A aa$ and $P aa \times H hh$ —produce chlorophyll variegated plants as well as green plants (Table 1). In both crosses the variegation originally takes the form of irregular areas of green and nongreen tissue. With the development of subsequent rosette leaves the green and nongreen areas tend to become separated into discrete sectors, thereby giving rise to plants which show, usually, both a periclinal and sectorial chimeral ap-

pearance. Wholly green branches are almost always present on variegated plants, and rare solid colored nongreen branches are also found.

The variegated plants resulting from the two crosses are not identical, however, and it is this feature which distinguishes the two crosses. Variegation in plants from the cross $P hh \times A aa$ does not, as a rule, become evident until the first or second pair of rosette leaves are formed. The color of the nongreen areas is, from the beginning, yellow-green. In contrast, variegation in plants from the cross $P aa \times H hh$ is generally apparent in the cotyledons, and the nongreen areas are initially yellow-white. Thus, in the rosette stage, difference in color of the chlorophyll deficient tissue—yellow-green *versus* yellow-white—serves to differentiate the two types of variegated plants, as seen from comparison of Figures 5 and 6. This difference in the color of the nongreen tissues persists after the plants have broken rosette (as shown in Figure 6) but, interestingly, the yellow-white sectors eventually become yellow-green, and are indistinguishable from the yellow-green sectors on plants from the cross $P hh \times A aa$.

Chlorophyll variegation is common in *Oenothera* interspecific hybrids, and has been explained by RENNER (first in 1922) on the basis of BAUR's hypothesis of biparental inheritance of chloroplast primordia (1909). According to this hypothesis the transfer of plastid primordia by the pollen tube results in zygotes with a mixture of maternal and parental plastids. The two types of plastids are sorted out during somatic cell divisions, thereby establishing cell lineages that contain exclusively one kind of plastid. If the two kinds of plastids react differently with the hybrid nucleus, variegated plants containing green and nongreen sectors arise.

Two lines of evidence may be cited to support the conclusion that the BAUR-RENNER hypothesis accounts for the variegation encountered in progenies from the two crosses $P hh \times A aa$ and $P aa \times H hh$. First the color of the nongreen sectors (of rosettes) is in itself informative. If in the cross $P hh \times A aa$ the plastids in the yellow-green sectors are derived from the male ($A aa$) parent, the genetic constitution of these sectors is written $A ha$. This is the genetic constitution which also characterizes progeny from the cross $A aa \times H hh$, described above. The latter cross, it will be recalled, produces yellow-green offspring, and their color is very similar to the yellow-green sectors on variegated plants from $P hh \times A aa$ crosses. Tissues of the type $A ha$ in variegated plants and in ordinary hybrids, therefore, give rise to a yellow-green leaf color.

By a similar process of reasoning, the yellow-white sectors observed in variegated rosettes from the cross $P aa \times H hh$ are symbolized $H ha$. This genetic formula likewise describes offspring from the cross $H hh \times A aa$, a cross that produces yellow-white hybrids. Somatic tissues with the constitution $H ha$ are then identical phenotypically, irrespective of their origin.

This phenotypic similarity between chlorophyll deficient tissues in solid-colored hybrid plants and sectors on variegated plants applies not only to color, but also to the stage at which the deficiencies are first discernible. The chlorophyll defect in tissue of the genetic constitution $A ha$, both in hybrids from the cross $A aa \times H hh$ and in variegated plants from the cross $P hh \times A aa$, is apparent

only after the first or second pair of rosette leaves arise, and normally is not seen in the cotyledons. Likewise, the chlorophyll deficiency in *H ha* tissues, in hybrids from the cross *H hh* $\times$ *A aa* and in variegated plants from the cross *P aa* $\times$ *H hh*, is first manifest at the same stage of development, in this case, the cotyledon stage. Thus, tissues presumed on the hypothesis to have the same plastid type and complex combination are seen to show a near exact correspondence in chlorophyll phenotype.

A second support of the BAUR-RENNER hypothesis is derived from crosses in which the male parents possess the same kind of plastid as the recovered female parents, that is, crosses in which both parents contain *parviflora* plastids. This condition is satisfied by the crosses *P hh* $\times$ *P aa* and *P aa* $\times$ *P hh*. No variegated plants appeared in any of the progenies from such crosses, as seen in Table 1 (crosses 3 and 4). On the hypothesis variegated plants are not expected, since both parents have *parviflora* plastids which develop normally with the *ha* hybrid nucleus. Male transmission of plastid elements would not, therefore, give rise to variegated hybrids, and would not in fact be detected by phenotypic appearances of the offspring.

It will be noted from Table 1 that the proportion of variegated plants varies rather widely in the different *P hh* $\times$ *A aa* and *P aa* $\times$ *H hh* crosses. These differences may well be partly the result of plastid competition, as described by SCHÖTZ (1954). The results suggest that not all *hookeri* or all *argillicola* plastids have the same relative reproductive potential, but more exact measures of the variegation, including estimates of the extent of green and nongreen areas, are needed to establish this point.

6. *Characteristics of the viable hybrids between hookeri and argillicola*: Both the green and variegated plants from the various crosses involving the recovered versions of the two species are normal in all respects. The hybrids, phenotypically intermediate between the parents, grow more vigorously than either parent, with the exception of rare variegated F_1 individuals in which considerable nongreen tissue is present. All hybrid plants grown to maturity have been fully fertile. Wherever they have been made, cytological examinations disclosed no difficulties in chromosome pairing, and the meiotic chromosome configurations expected on the basis of known segmental end arrangements were found in every case.

Several F_2 populations have been grown from crosses involving the recovered and original strains of Devils Gate and Douthat 4b (F_1 configuration = $\odot 6$, four pairs). When derived from green F_1 plants or green branches on variegated plants, the F_2 families display great variability for all plant characters, suggesting that segregation for a relatively large number of genes must be taking place. The range of variability is more restricted in F_2 populations derived from nongreen branches. The latter F_2 families always contain some chlorophyll deficient individuals which die before reaching maturity. The surviving plants show a decided leaning toward the phenotype of the parent which contributed the plastids to the F_1 nongreen branch. That is, when the self-pollination is made on a yellow-green branch of the constitution *H ha*, the F_2 progeny are definitely *hookeri*-like,

while the F_2 progeny from a yellow-green branch of the constitution *A ha* show a closer resemblance to *argillicola*. This difference in breeding behavior is expected; evidently, the F_2 plants which fail to survive because of chlorophyll defects are noncompatible combinations of *hookeri* genes and *argillicola* plastids (from *A ha* branches), or noncompatible combinations of *argillicola* genes and *hookeri* plastids (from *H ha* branches).

DISCUSSION

The starting point for the investigation of the role of extranuclear factors in interspecific crosses between *hookeri* and *argillicola* was the recovery of the genomes of each species in combination with an alien *parviflora* plastome. Conclusions drawn from the comparative breeding behavior of the original and recovered races are based on the assumption that the two kinds of races have identical, or near identical, nuclei but different plastomes. It is important, at the outset, to establish the validity of this assumption. First, with regard to nuclear composition, it is evident from phenotypic appearances that no recovered strain is absolutely identical to its original counterpart. These slight deviations in phenotype could be due to the presence of certain *parviflora* genes, acquired through crossing over in one, or both, genomes of the recovered races. It is also possible that the phenotype of the recovered races is altered by alien *parviflora* cytoplasm, since it must be assumed that the recovered races possess not only *parviflora* plastids, but, in fact, an entire cytoplasmic complement from *parviflora*. Reciprocal backcrosses between the original and recovered strains should provide the evidence needed to determine which of these alternatives is correct. Such backcrosses, now in their early stages, already suggest that genic differences are largely responsible for the slight phenotypic modifications observed in the recovered stocks. Although this preliminary evidence indicates that the recovered races possess certain *parviflora* genes, it is clear that these genes are not responsible for the different maternal parent breeding behavior of the recovered races. This view is supported by the reciprocally different breeding behavior of recovered races, and by the reciprocal recovery experiments. In terms of their effects on chloroplast development, therefore, the original and recovered races may be considered to have identical nuclei.

With respect to the extranuclear composition of the original and recovered strains, one fact that needs to be taken into account is the tendency of *Oenothera* pollen to transmit cytoplasmic elements (presumably plastid primordia) which affect the chloroplast content of the offspring. This behavior could influence the outcome of the recovery process since the assumption of exclusive maternal transmission of cytoplasmic constituents does not necessarily hold. In the cross *parviflora* $\times$ *argillicola*, pollen transmission of plastids does occur, for progenies from such crosses contain variegated plants. However, since analysis of the variegation shows the green branches to contain *parviflora* plastids, self-pollination of these branches insures that the recovered *argillicola* races possess *parviflora* plastids.

The situation with the recovered *hookeri* is somewhat more complicated in that male transmission of *hookeri* plastids would be difficult (with the races Devils

Gate and franciscana deVries) or impossible (with Johansen) to detect by inspection of F_1 families from the cross *parviflora* $\times$ *hookeri*. This is so because *hookeri* plastids develop normally, or nearly so, in combination with the F_1 α *ph* nucleus, and their presence would not be revealed by sectors of nongreen tissue. In other words, the F_1 plants would be genetic chimeras without an accompanying chimerical phenotype. It is evident, nevertheless, that male transfer of *hookeri* plastids, if it occurred, did not interfere with the purpose of isolating *hookeri* nuclei in association with *parviflora* plastids. This is borne out by the fact that all recovered *hookeri*s tested to date have behaved alike in testcrosses, and have differed from the original race in maternal parent breeding behavior. A recovered race which had original plastids would be expected to breed as an original *hookeri*, particularly in view of the evidence from the reciprocal recovery experiments. The conclusion appears justified, therefore, that the recovered strains of *hookeri* used in testcrosses did actually possess plastids from *parviflora*.

The recovery of 7-paired *hookeri*s from $P \alpha ph$ F_1 plants is also interesting in light of STUBBE's findings (1959) of plastome effects on pollen viability. According to STUBBE, pollen grains which carry genomes of the *hookeri* or *strigosa* type are frequently nonfunctional when produced by plants with *parviflora* plastomes. Pollen inactivation obviously did not occur in the present case of the three different *hookeri* genomes combined with a *parviflora* plastome from the race Charlottesville; otherwise *hh* segregates would not have appeared in F_2 .

In summary, the evidence supports the assumption that from the standpoint of effects on chloroplast development the original and recovered races possess identical nuclei but different plastomes.

Still to be considered, however, is the further assumption that the extranuclear component of the genetic system which controls chloroplast development is localized in the plastid and not in some other cytoplasmic element. Evidence pertinent to this point comes particularly from the extensive studies conducted by the German workers on chlorophyll variegation in F_1 *Oenothera* hybrids. Recently STUBBE (1957) and SCHÖTZ (1958) have advanced strong arguments for the contention, long held by RENNER, that the chloroplast itself possesses genetic properties. These two authors stress the point that individual cells which contain two morphologically distinct plastid types can regularly be observed in variegated hybrids, provided the microscopic examination is made on appropriate areas of a young plant. Moreover, transitional stages between the two plastid types are not seen. The morphologically distinguishable plastids are taken to represent the parental plastids, contributed by the egg and pollen tube, and which, because of their unlike genetic constitutions, respond differently to the same nucleus. The presence of single cells with distinct plastid types—the so-called mixed cells—is clearly called for by the BAUR-RENNER hypothesis of biparental, non-Mendelian chloroplast inheritance.

The existence of mixed cells in variegated plants is somewhat difficult to reconcile with interpretations that locate the genetic properties in nonplastid cytoplasmic elements. The demonstration of mixed cells thus offers strong evidence that the chloroplast *per se* possesses and transmits genetic information.

This view of the chloroplast has not gone unquestioned, however. On the basis of his electron microscope studies of chloroplast structure and development WETTSTEIN states (1957, 1959) that while there is uncertainty regarding the origin of chloroplasts, his studies indicate that "the complicated lamellate structure of the chloroplast can be built up from structural elements not notably different from those found in the ground cytoplasm." Thus, for higher plants, WETTSTEIN does not regard the concept of plastids as autonomous, self-duplicating bodies to be firmly established. Further, he believes it difficult to identify mixed cells with assurance, since cells with only one kind of plastid, either normal or defective, may show transitional stages of plastid development, and hence give the appearance of being mixed cells. Finally WETTSTEIN points out that a study (STUBBE and WETTSTEIN 1955) of certain mutant *Oenothera* plastids, inherited in a non-Mendelian fashion, demonstrated that the mutant plastids first reach a normal stage of development and then degenerate. This suggests to him that development of the plastid is not under plastome control. These criticisms of WETTSTEIN have been considered in detail by SCHÖTZ (1958), who does not believe them sufficiently compelling to abandon the idea of plastid-localized hereditary properties.

The problem of determining the location of the extranuclear components of the chloroplast system in *Oenothera* is perhaps still not finally resolved. However, genetic data from many investigations in *Oenothera* leave little doubt of the genetic continuity (as opposed to cytological continuity) of the extranuclear elements, which, together with the nucleus, determine plastid phenotypes. The ultimate decision as to the physical basis of these elements will certainly be determined in part by electron microscope studies of chloroplast origin, development, and multiplication. For the time being, the concept of a plastome, as previously defined, appears to be a useful and convenient working hypothesis.

The crosses involving the recovered and original races show without doubt that extranuclear factors play a critical part in determining the outcome of hybridization between *hookeri* and *argillicola*. A summary of the most important crosses is given in Table 2. Regardless of the plastome involved, all crosses between *hookeri* and *argillicola* produce the F_1 genome combination *ha*. The manner in which this nucleus expresses itself on chlorophyll formation depends on the kind of chloroplast present. With either *hookeri* or *argillicola* plastids the result is a lethal chlorophyll deficiency. When *parviflora* plastids are associated with *ha* nuclei, however, chlorophyll formation is normal. The chlorophyll abnormality in the *H ha* and *A ha* hybrids cannot, therefore, be solely attributed to properties of the nucleus. These observations lead to the view that the processes involved in chlorophyll formation are controlled by a genetic system which consists of nuclear and extranuclear components. The chlorophyll defect in reciprocal hybrids between *hookeri* and *argillicola* is explained as the consequence of a functional disharmony between the two components. The disruption of the chlorophyll apparatus thus sets up an effective barrier to gene exchange between the two species by causing death of hybrids at early stages of growth. Once the barrier is overcome by changing the plastome, the hybrid nucleus functions

TABLE 2

Summary of various interspecific crosses

Cross	Chlorophyll phenotype	Genetic constitution
<i>hookeri</i> females $\times$ <i>argillicola</i> males		
$H\ hh \times A\ aa$	all yellow-white, inviable	$H\ ha$
$P\ hh \times A\ aa$	1. green plants	$P\ ha$
	2. variegated plants	
	green sectors	$P\ ha$
	yellow-green sectors	$A\ ha$
$P\ hh \times P\ aa$	all green	$P\ ha$
<i>argillicola</i> females $\times$ <i>hookeri</i> males		
$A\ aa \times H\ hh$	all yellow-green, inviable	$A\ ha$
$P\ aa \times H\ hh$	1. green plants	$P\ ha$
	2. variegated plants	
	green sectors	$P\ ha$
	yellow-white sectors	$H\ ha$
$P\ aa \times P\ hh$	all green	$P\ ha$

in such a way that the vegetative and reproductive development of the species hybrids is fully normal.

The fact that *parviflora* plastids are compatible with the *ha* nucleus, whereas *hookeri* and *argillicola* plastids are not, must mean that *parviflora* plastids are inherently different from those in both *hookeri* and *argillicola*. But these differences in plastomes are not always apparent, even when the necessary genetic condition of identical nuclei is realized. This is illustrated in the present case by the recovery of *hookeri* and *argillicola* nuclei in association with the plastome of *parviflora*. The chlorophyll phenotypes of the recovered and original strains are, with the exceptions of the slight and transient differences in the *hookeri* races Devils Gate and franciscana deVries, identical. If observations were restricted to the recovered and original races alone, the conclusion could be drawn that *parviflora* plastids are virtually indistinguishable from those in *hookeri* and *argillicola*. The *ha* nucleus does, however, serve to differentiate the *parviflora* plastome from that of the other two species. Thus, as MICHAELIS (1954), among others, has emphasized, particular genomes are often needed to uncover cytoplasmic differences, and failure to recognize this undoubtedly allows hereditary variations in the cytoplasm to go undetected.

It is also possible to arrive at certain conclusions regarding the comparative properties of the chlorophyll-determining systems in *hookeri* and *argillicola*. Direct evidence bearing on the plastomes of the two species is furnished by the nature of the chlorophyll deficiency in the reciprocal hybrids $H\ ha$ and $A\ ha$. Both hybrids show a chlorophyll defect, but as pointed out in the earlier description, with respect to intensity and time of onset the defect is manifest differently in *hookeri* and *argillicola* plastids (Table 2 and Figures 5 and 6). Thus, development of both *hookeri* and *argillicola* plastids is adversely affected by the *ha* genome combination, but the two types of plastids react in visibly different ways, and hence must be inherently different.

It is also readily apparent that the genic components which determine chlorophyll characters are unlike in *hookeri* and *argillicola*. This may be demonstrated by comparisons between the original species and the interspecific hybrids. Comparing *hookeri*, *H hh*, with the hybrid *H ha*, it is seen that replacement of an *h* by an *a* genome leads to defective *hookeri* plastids. Although this result does not necessarily mean that *a* genomes cause defects in *hookeri* plastids by direct action on the plastids, it does show that the *a* genome is different from the *h* genome in terms of genic effects on chlorophyll formation. Similarly, comparison of *argillicola*, *A aa*, with the hybrid *A ha* reveals that substitution of an *a* by an *h* genome results in abnormal *argillicola* plastids, again indicating that *hookeri* and *argillicola* genomes are different in their content of chlorophyll genes.

The differences which have been noted above in plastomes and genomes have also been found in the strains of RENNER's collection (STUBBE 1959).

Phylogenetic implications: Evidence from the studies on *Oenothera* phylogeny points to both similarities and differences between *hookeri* and *argillicola*. Both species have retained in common certain primitive characteristics which must have been present in the hypothetical ancestral *Euoenothera* population. These characteristics are: (1) mostly paired chromosomes; (2) alethal complexes; (3) open-pollinated flowers; (4) segmental end arrangements which are identical with or close to the putative ancestral arrangement. One difference between the *hookeri*s and *argillicola*s is found in their unlike morphology. The lack of almost any phenotypic resemblance between the groups, as well as the wide range of variability observed in F_2 populations indicates that numerous genic differences exist between the two species. A second feature which distinguishes the two species is their geographical distribution, for they are currently separated by the width of the North American continent. Third, the *hookeri*s and *argillicola*s are differentiated by their relationships with other phylogenetic groups. The closest relatives of the *hookeri*s are the species *O. elata* and *O. strigosa*. The *argillicola*s, on the other hand, display little or no affinity with either the *elatas* or *strigosas*, and show instead a close connection with the *parvifloras*.

Taking into account both the similarities and differences between the *hookeri*s and *argillicola*s, it has been possible to arrive at a reasonable conclusion concerning their positions in the phylogenetic scheme (STINSON 1953; CLELAND 1956, 1958). The *argillicola*s are regarded as relics of the first *Euoenothera* population to emerge, a population probably not far removed from the related subgenus *Raimmania*, and one which, in all likelihood, once occupied an extensive eastern range. In contrast, the *hookeri*s have remained nearer the presumed center of distribution in Mexico or Central America. CLELAND (1958) has stated that the *hookeri*s may be considered as either an ancient group, representing the original population, or alternatively as the most recent group to develop from the center of origin. No matter which of these alternatives is accepted, the conclusion is inescapable that the *hookeri*s and *argillicola*s have been isolated geographically over a long evolutionary span. In summary, the common possession of primitive cytogenetic properties must mean that both groups ultimately trace their ancestry to a common origin; but the facts from phenotypic appearance, geographi-

cal distribution and species relationships argue that the two populations diverged early in their evolutionary history. Their common ancestry is thus a remote one.

How may the findings of the present study be applied to this picture of a remote common ancestry? First, it is clear that because of the nature of the genetic systems controlling chloroplast function in the two species, they are not only geographically, but also reproductively isolated. The analysis of these systems led to the conclusion that both the nuclear and extranuclear components are so different in the two species that the plastids of one species cannot function properly with even a single genome of the opposite species. Considering the conclusions drawn above relative to the phylogenetic histories of *hookeri* and *argillicola*, it is probable that this divergence in genomes and plastomes arose during the period of spatial isolation where the two populations were exposed to different environments and selective pressures. The differences between *hookeri* and *argillicola* revealed by the comparative genetics of chloroplast development are thus additional aspects of the total genetic divergence between the two species. But the differences in genomes and plastomes are of special biological interest because they alone, among the genetic properties of the two species, constitute the mechanism responsible for hybrid inviability.

In this connection it should be emphasized that the barrier to interspecific hybridization imposed by the interaction of genome and plastome operates in reciprocal crosses. STEBBINS (1958) has commented that gene cytoplasmic interactions are perhaps not likely to be effective genetic isolating mechanisms, since crosses which fail in one direction can frequently be made in the opposite direction, and hence gene exchange is not precluded. This situation does not hold in the present case; the barrier is complete, and the final criterion for species delimitation—reproductive isolation—is realized.

If the results of this study, based on analysis of a relatively few strains of each species, are taken to be representative of interspecific hybridization between *hookeri* and *argillicola*, it may be concluded that in *Oenothera* at least cytoplasmic factors can play a part in speciation by their involvement in genetic systems which act as isolating mechanisms. It is interesting to note that CASPARI (1948), after reviewing cytoplasmic inheritance in many organisms, came to the conclusion that the significance of cytoplasm in evolution would most likely be found in its ability to act as a barrier to interspecific hybridization. JONES (1951), who noted that the fundamental properties of pollen fertility and chlorophyll development are often partly determined by cytoplasmic elements, came to the same conclusion.

SUMMARY

Reciprocal crosses between the two 7-paired lethal-free species, *O. hookeri* and *O. argillicola*, produce chlorophyll deficient offspring which die in the early seedling or rosette stage. These chlorophyll defects were demonstrated to arise from an incompatibility between the chloroplasts of either species and the hybrid nucleus. Proof of this contention was the discovery of a plastid type in which the

hybrid nucleus failed to produce the chlorophyll disturbance. This was accomplished by recovery of the nuclei of three races of both *hookeri* and *argillicola* in association with plastids derived from a third species, *O. parviflora*. The recovered races are green and fertile. When made using either kind of recovered race as female parent, the interspecific crosses gave green and variegated offspring, both classes being fully viable. Evidence is cited which supports the view that the variegation results from the transmission of plastids through the pollen. Crosses in which both parents were recovered strains yielded only green plants. The behavior of the recovered races in reciprocal crosses, and testcrosses involving reciprocally recovered *hookeri* stocks showed that the difference in maternal parent breeding behavior of original and recovered races was not due to contamination of the latter races with *parviflora* genes, but instead depended upon the presence of *parviflora* plastids in the recovered strains. It has been possible, therefore, by genetic manipulation of extranuclear elements to overcome the barrier to interspecific hybridization between *hookeri* and *argillicola*. The F_1 hybrids thus obtained are vegetatively vigorous and reproductively normal, as are the F_2 populations which have been grown.

Reasons are given for the tentative assumption that the extrachromosomally inherited component of the genetic system controlling chloroplast function is the chloroplast itself rather than some other cytoplasmic element. On this basis, it was pointed out that the plastids of *hookeri* and *argillicola* are not only different from those in *parviflora*, but also from each other. In terms of their effects on chloroplast development, the genomes of *hookeri* and *argillicola* are likewise dissimilar. These plastid and genome differences, which account for the reproductive isolation between *hookeri* and *argillicola*, are taken to support the conclusion that, in spite of their similar cytogenetic properties, the two species are only remotely related.

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