THE ORIGIN OF THE ALLOTETRAPLOID *CLARKIA GRACILIS*[1]

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The genus *Clarkia*, a close relative of *Oenothera*, is subdivided into ten sections of which *Primigenia* seems to be the oldest (Lewis and Lewis, 1955). This section includes six species grouped into three subsections. Subsections *Primigenia* and *Flexicaulis* contain five diploids, some of which are considered to be the most primitive members of the genus (Lewis and Lewis, 1955). Throughout the genus, the gene pools of the diploid species are without exception separated by strong barriers to gene exchange (Lewis, 1953). Lewis and Raven (1958) concluded that the accumulation of structural rearrangements, primarily reciprocal translocations, has undoubtedly played a major role in the formation of the well developed isolating mechanisms and hence to speciation at the diploid level in *Clarkia*. At the same time, such effective genetic isolation has apparently set the stage for the instantaneous speciation at the polyploid level through alloploidy (Lewis, 1953). Thus, it is no surprise that alloploids comprise one-third of all known *Clarkia* species and that natural polyploidy serves to knit the entire group together (Lewis and Lewis, 1955).

Such a knitting effect is evident in section *Primigenia*. *Clarkia gracilis* ($n = 14$) is the only polyploid species in the section and the sole representative of subsection *Jugales*. It combines morphological, ecological, and chromatographic characteristics, and also the genomes of the remaining two subsections, i.e., *Flexicaulis* and *Primigenia* (Abdel-Hameed, 1967). Lewis and Lewis (1955) considered this allotetraploid to combine genomes of *C. amoena* ($n = 7$) and *C. lassenensis* ($n = 7$). There are five recognized subspecies of *C. amoena* and for cytological, morphological, ecological, and geographical reasons it is certain that *C. amoena* ssp. *huntiana* was one parent of the tetraploid (Abdel-Hameed, 1967). The purpose of this paper is to investigate the origin of *C. gracilis* and its phylogenetic relationship to the diploid species of the section, with particular emphasis on *C. amoena* ssp. *huntiana* and *C. lassenensis*.

Clarkia gracilis is a polytypic species that was subdivided on the bases of morphological, ecogeographical, cytological, and chromatographic observations into four subspecies (Abdel-Hameed, 1967), *gracilis*, *albicaulis*, *tracyi*, and *sonomensis*.

MATERIALS AND METHODS

Seeds of two different sources, those representative of various natural populations and those secured from the greenhouse strains homozygous for certain chromosome end arrangements, were used. Bulk collections of seeds were made from wild populations by combining the seeds from a number of capsules collected at random, one from each plant. The chromosomally homozygous strains used were those studied by Snow (Snow, 1963*a*; Snow and Imam, 1964; Snow, unpublished).

The pollination procedure varied according to the breeding habit. Self-pollination did not require emasculation; cross-pollination, however, required emasculation of the female parent. Emasculation was practiced when the stigma lobes were still closed and the anthers had not

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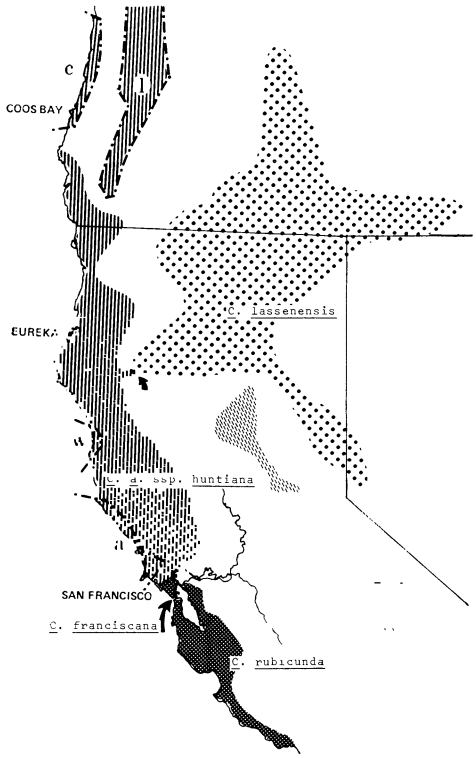

FIG. 1. Distribution in northern California and adjacent parts of Nevada and Oregon of the five diploids in the section *Primigenia*. The third subsection, *Jugales*, is composed of a single polytypic species whose four subspecies overlap the distribution of the five diploids. The small arrow points to the location of the single mixed population where sympatric association between *C. amoena* ssp. *huntiana* and *C. lassenensis* was recently discovered. a) *C. a.* ssp. *amoena*. w) *C. a.* ssp. *whitneyi*. c) *C. a.* ssp. *caurina*. l) *C. a.* ssp. *lindleyi*.

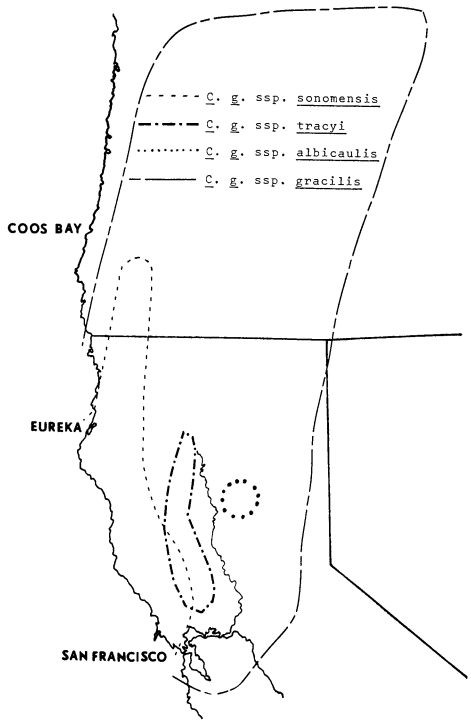

FIG. 2. Distribution of *C. gracilis*.

yet shed their pollen. Pollinations were done when the stigma became receptive. The flowers were protected from unwanted pollen by covering them with cellophane bags, which were removed after fertilization had occurred.

Buds for cytological study were fixed in 1:3 acetic-alcohol for at least 24 hours, then changed to 70% alcohol and stored in the refrigerator. They were stained in bulk using alcoholic-HCl carmine (Snow, 1963*b*), which gave the most satisfactory results. From these buds, slides were prepared by squashing 2–4 anthers after gentle heating in a drop of 45% acetic acid.

Pollen viability was estimated from counts of pollen grains which were stained with cotton blue in lactophenol. Only well stained, normally shaped grains were considered viable and calculations were based on a total of at least 200 pollen grains counted.

GEOGRAPHICAL DISTRIBUTION

Considerable differences in distribution area are found among the four subspecies of the tetraploid *C. gracilis*. One extreme is represented by *C. gracilis* ssp. *albicaulis*, as its colonies are concentrated in a limited area isolated geographically from the other tetraploid races as well as from the putative parental diploids. On the other hand, *C. gracilis* ssp. *gracilis* has a very wide distribution which surpasses any other sub-

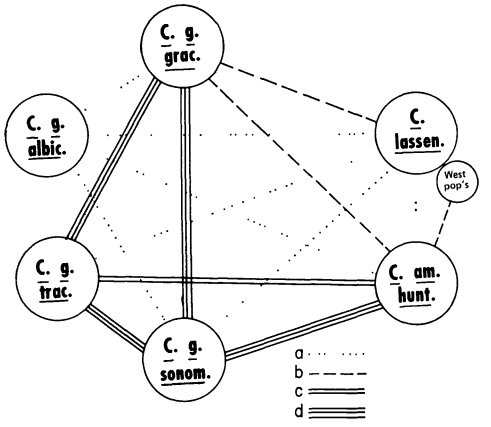

FIG. 3. Polygon showing inferred relationships from sympatric and allopatric associations in nature. a) No gene flow. b) Very little gene flow. c) Some gene flow occurs. d) Possibility for considerable gene flow.

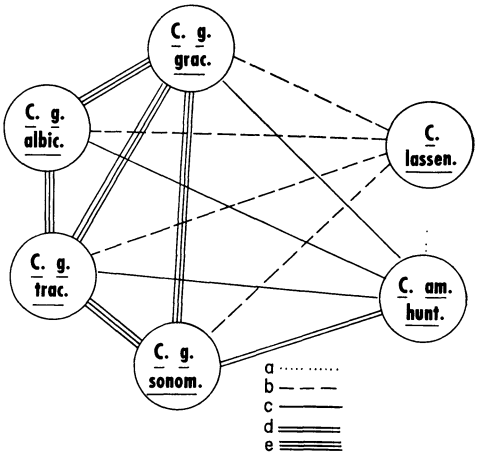

FIG. 4. Polygon showing inferred crossing relationships from greenhouse progenies. a) Rarely set seeds; developmental abnormalities in most progenies; few plants reach maturity and are of extremely low fertility. b) Rarely set seeds; developmental abnormalities in some progenies; low fertility. c) Relatively high seed set; normal progeny; almost all reach maturity, but show variable degrees of fertility. d) Relatively high seed set; normal progeny; progenies are relatively fertile. e) Abundant seed set; normal progenies with normal fertility.

species, as well as all diploids in the section (Figs. 1, 2).

The difference in distribution of *C. lassenensis* and *C. amoena* ssp. *huntiana* (Fig. 1) is correlated with the shorter life cycle of the former. *Clarkia gracilis*, which overlaps both diploids in its distribution (Fig. 2), seems to combine ecogeographical characteristics of each. The only sympatric association presently known between a diploid of subsection *Primigenia* and another of subsection *Flexicaulis* was found recently in a single mixed population of *C. amoena* ssp. *huntiana* and *C. lassenensis*. This sympatric association was discovered after an extensive search for such sympatry was conducted in the area where the two diploids come closest (Fig. 1). In this area and north of it, the contour of their distributions suggests that they might have been connected previously by a much wider area of sympatry.

In their natural populations, pairwise sympatric associations were observed between *C. amoena* ssp. *huntiana* and all sub-

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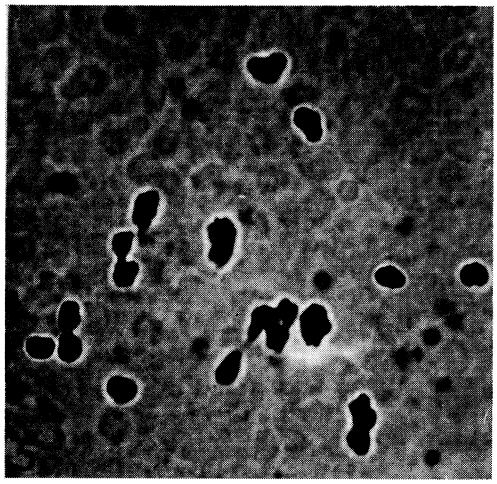
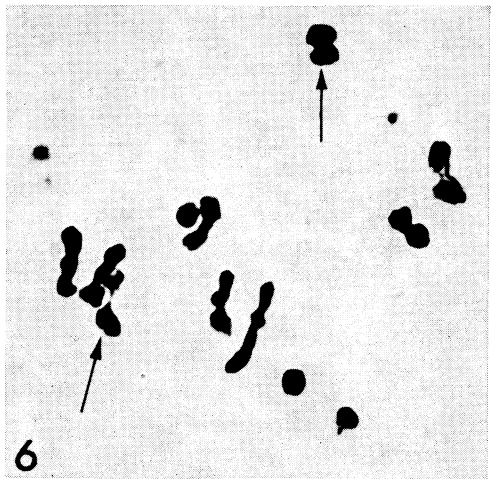
FIGS. 5–9. Fig. 5. Metaphase-I of a *C. gracilis* × *C. amoena* ssp. *huntiana* triploid showing 5 bivalents + a chain of 4 + 7 univalents. The chain of 4 indicate a single translocation difference between the genomes of the two parents (× 1,500). Fig. 6. Metaphase-I of a *C. gracilis* × *C. amoena* ssp. *huntiana* triploid showing 7 genuine bivalents + 2 pseudo-bivalents (arrows), each most likely resulting from non-homologous association between two univalents + 3 univalents (× 1,500). Fig. 7. Diakinesis of a *C. lassenensis* triploid showing 5 bivalents + 11 univalents, two of which are apparently associated non-homologously (arrow) (× 1,500). Fig. 8. Metaphase-I plate of a synthetic allotetraploid resulting from selfing an F_1 diploid hybrid. The total chromosome number is 27 appearing as 13(2) + 1. Bivalents are normal looking and most of them are ring bivalents (× 1,500). Fig. 9. Metaphase-I plate of F_1 × *C. gracilis* ssp. *gracilis* showing early anaphase-I disjunction of the majority of associated chromosomes. The chromosome configurations at this stage indicate their normal M_I associations and co-orientation as expected between two homologous sets of chromosomes (× 1,500).

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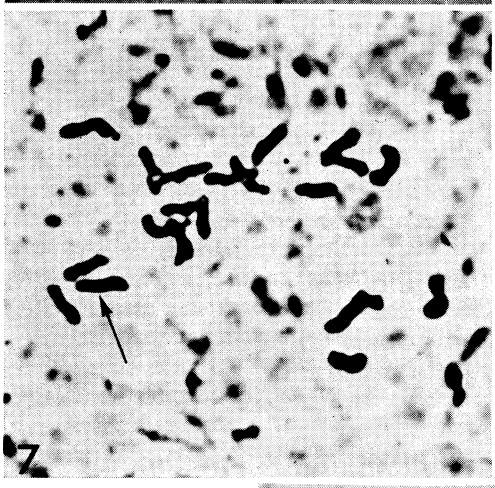
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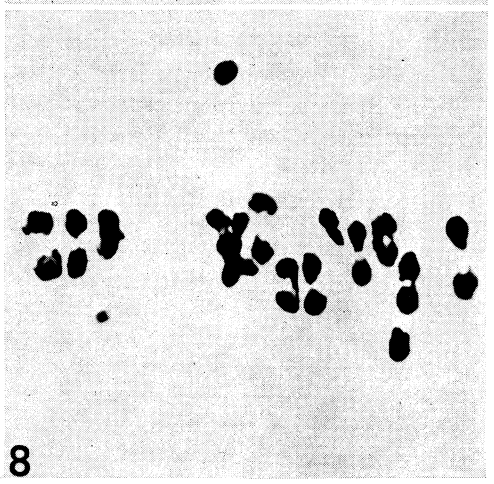
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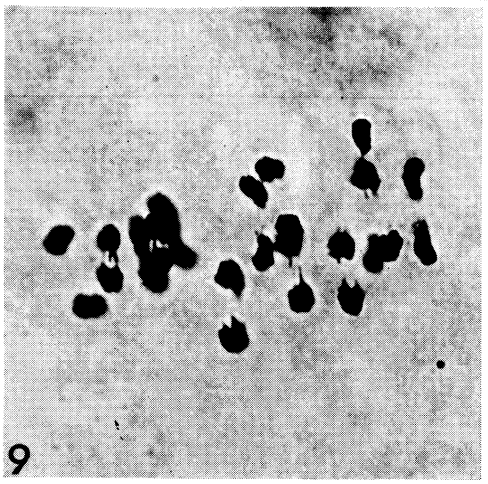
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TABLE 1. *Cytological analysis of tetraploid plants resulting from F_1 diploid hybrids between* C. amoena *ssp.* huntiana *and* C. lassenensis.

	2n	No. of cells	Bivalents/cell Average	Bivalents/cell Range	Multi./cell Max.	Multi./cell Lowest	Max. observed associations	Stainable pollen (%)
Unpollinated F_1s from:								
490, B_4B_4 × 949, AA	28	62	not analyzable, chromosomes sticky				–	13.8
490, B_4B_4 × 949, AA	28	75	9.5	3–13	4c	4c	13(2) + 2(1)	30.1
490, B_4B_4 × 949, AA	28	82	10.4	8–13	4c	4c	13(2) + 2(1)	23.0
490, B_4B_4 × 949, AA	?	105	massive chromosome fragmentation				–	0.5
949, AA × 485, AA	28	10	6.9	4–9	2(3c)	3c	9(2) + 10(1)	2.7
Self pollination of F_1 from:								
490, B_4B_4 × 949, AA	27	130	12.8	12–13	bivalents		13(2) + 1	70.0
F_1 × F_1 from:								
(949, AA × 485, AA) × (474, B_1B_1 × 942, AA)	28	49	12.2	10–14	4r	3c	12(2) + 4r	42.0

species of *C. gracilis* except *C. gracilis* ssp. *gracilis*. Repeated morphological observations on these mixed populations indicate that introgressive hybridization may be prevalent between certain taxa as shown in Figure 3.

CYTOLOGICAL OBSERVATIONS

Interspecific Hybrids

Triploid crosses with *C. amoena* ssp. *huntiana* were highly successful, with the exception of a few crosses that failed to set seeds or produced hybrid progeny which showed pronounced developmental abnormalities (Fig. 4). These hybirds exhibited a rather regular meiotic association between the seven *C. amoena* chromosomes and one set of seven chromosomes from the tetraploid (Fig. 5). The other chromosomes were usually left unassociated as seven univalents although two, three, or more sometimes paired non-homologously (Fig. 6).

Triploid hybrids with *C. lassenensis* were much more difficult to obtain than with *C. amoena* ssp. *huntiana*, and only a few plants reached maturity (Fig. 4). Relatively poor pairing was found between the *C. lassenensis* genome and its counterpart from the tetraploid. Maximum multiple associations of a chain of five chromosomes (5c) were formed in only a few meiocytes. Some cells formed only 21 univalents. The largest number of bivalents observed was five (Fig. 7). It is clear that some of the *C. lassenensis* chromosomes can pair with their counterparts from the tetraploid *C. gracilis* but pairing is less than one would expect of homologous genomes. The existence of (3c) and (5c) associations indicates that there are at least two translocation differences between the *C. lassenensis* genome and one subgenome of the tetraploid.

Judged on the basis of ease in obtaining hybrids, their developmental abnormalities and their meiotic pairing behavior, the present forms of the polytypic allotetraploid *C. gracilis* are closer phylogenetically to the diploid *C. amoena* ssp. *huntiana* than to *C. lassenensis* (Fig. 4).

Greenhouse, cytological and other observations show beyond a doubt that the diploids, *C. lassenensis* and *C. amoena*, are well separated genetically by a multitude of strong pre- and post-zygotic isolating mechanisms (Abdel-Hameed, 1967) and are relatively distantly related.

Synthetic Allotetraploids

Amphidiploids of F_1 hybrids were produced by self-pollination, crosses between F_1s, and open-pollination (Table 1).

A thousand selfings of F_1 plants produced only a single capsule with three apparently normal seeds. Two of them ger-

minated, but one seedling was abnormal and died early. The other grew to a mature plant, which proved to have 27 chromosomes, indicating fertilization between gametes that contained 13 and 14 chromosomes. Over 90% of the M_1 plates showed the maximum expected association of 13 (2) + 1 (Fig. 8). Sixty-one per cent of A_1 cells showed no lagging chromosomes, 26% showed one and 1% showed three. Most T_{II} cells contained no micronuclei. However, a few showed 2–6 lagging chromatids while others had one or two micronuclei. Pollen viability was high (70%) and seed set was also rather high.

Several hundred crosses between F_1 plants were made, but only one seed was set which grew into a healthy plant. Cytological observations proved that this plant was produced by fertilization between two unreduced gametes, since the chromosome number was 28. Pachytene study revealed close synapsis of each pair of homologs. Diplotene and late diakinesis stages were difficult to analyze due to chromosome clumping. However, at least one diakinesis cell showed 11 ring bivalents, 2 rod bivalents, and 2 univalents.

Meiotic pairing at M_1 was excellent. In fact, a difference in bivalent size could be detected, seven bivalents being slightly larger than the other seven. The former were no doubt *C. amoena* ssp. *huntiana* chromosomes since this species has larger chromosomes than *C. lassenensis*. Seventy-nine per cent of M_1 plates showed associations of 12(2) + 4 or 14(2). The rest showed lower associations, the lowest being 10(2) + (3c) + 5(1). The majority of A_1 cells showed normal segregation of 14 chromosomes to each pole; however, some had 1 to 4 lagging chromosomes. Pollen stainability was 42%.

The observed (4r) and (3c) associations in a sizable portion of M_1 plates point to the existence of a translocation in the complement. Since the standard and B_1 end arrangements were known to have entered into the parentage of this plant, heterozygosity for the translocation is satisfactorily explained.

Diploid F_1 × C. gracilis *Crosses*

To complete this study of the origin of *C. gracilis*, it was most desirable to obtain hybrids between the synthetic and natural allotetraploids. Because colchicine treatments of the diploid F_1s failed to produce tetraploids, such crosses could not be made at the tetraploid level. However, the desired hybrids were obtained by pollinating several F_1 hybrids by representatives from populations of the four subspecies of *C. gracilis*. These crosses proved much more successful than crosses between F_1 hybrids. Forty-six good seeds were obtained, of which 38 produced mature plants. Seven were examined cytologically (Table 2).

From crosses with *C. gracilis* ssp. *gracilis*, one plant was produced. It had 26 chromosomes (Fig. 9), the maximum association at M_1 being 3(2) + (4r) + (6c) + 10(1). The natural tetraploid parent of this hybrid came from the Copernicus Peak I (904) population, and should have had the standard arrangement of *C. gracilis*, whereas the maternal genome represented the combination between the *C. amoena* ssp. *huntiana* B_4 and the *C. lassenensis* A arrangements. Therefore, the expected association between chromosomes of the two *C. amoena* complements would be (4r). As noted in a previous section, the second subgenome in the standard AA,AA arrangement of *C. gracilis* differs by at least two translocations from the AA standard arrangement of *C. lassenensis*. This accounts for the (6c).

Four plants from F_1 × *C. gracilis* ssp. *sonomensis* crosses were examined cytologically. All had 28 chromosomes. Two of them combined the B_1 and B_4 end arrangements of *C. amoena* ssp. *huntiana* in their genomes, and the maximum expected association of (6r) was realized in some of their meiocytes. The other two contained the standard AA,AA arrangement of *C. gracilis* and the standard arrangements of both *C. amoena* ssp. *huntiana* and *C. las-*

TABLE 2. *Cytological analysis of a sample of plants derived from F_1 diploids* × C. gracilis, *chosen for high pollen stainability.*

	2n	No. of cells	Bivalents/cell		Multi./cell		Max. observed associations	Stainable pollen (%)
			Average	Range	Max.	Lowest		
F_1 × *C. g.* ssp. *gracilis*:								
(490, B_4B_4 × 949, AA) × 904, AA,AA	26	13	4.4	3–6	4r + 6c	3c	3(2) + 4r + 6c + 10(1)	1.5
F_1 × *C. g.* ssp. *sonomensis*:								
(490, B_4B_4 × 949, AA) × 599, B_1B_1, AA	28	86	4.7	4–6	6r	3c	5(2) + 6r + 12(1)	1.6
(490, B_4B_4 × 949, AA) × 599, B_1B_1, AA	28	27	4.8	4–6	6c	3c	6(2) + 6c + 10(1)	3.8
(949, AA × 485, AA) × 469, AA,AA	28	177	7.1	6–12	3c	3c	12(2) + 4(1)	2.5
(949, AA × 485, AA) × 469, AA,AA	28	105	7.3	6–12	3c	3c	12(2) + 4(1)	6.0
F_1 × *C. g.* ssp. *albicaulis*:								
(490, B_4B_4 × 949, AA) × 608, B_2B_2 + B_6B_6, AA	28	40	5.7	2–8	3c	3c	6(2) + 3c + 4c + 9(1)	1.0–78.6
(949, AA × 485, AA) × 608, B_2B_2 + B_6B_6, AA	42	24	12.8	11–14	3c	3c	11(2) + 3(3c) + 11(1)	19.7

senensis. These plants were the only ones obtained which combined the standard arrangements of all three species. The maximum observed bivalent association was 12(2), which shows that at least five of the standard *C. lassenensis* chromosomes could form bivalents with chromosomes of one of the subgenomes in the *C. gracilis* complement, in agreement with above-mentioned observations. However, in both a (3c) was observed in a few cells, which is not unexpected since two translocations are known to exist between the standard arrangement of *C. lassenensis* and its counterpart in the standard arrangement of *C. gracilis*.

Two F_1 × *C. gracilis* ssp. *albicaulis* tetraploid hybrids were selected for study because they showed the highest pollen viability in this group. One proved to have 28 chromosomes and showed a wide range of variable associations at M_1, which was reflected in an extremely wide range of pollen fertility (between 1.0 and 79%). The chromosome count of the other plant was 42, indicating fertilization between two unreduced gametes, one from the F_1 maternal parent with 14 chromosomes, the other from the tetraploid parent with 28 chromosomes. As many as 3(3c) were observed in a few cells, but the majority showed 14(2) + 14(1). The maximum association found was 11(2) + 3(3c) + 11(1) in a few M_1 plates.

DISCUSSION

In search for the parental diploids of *C. gracilis*, Håkansson (1942) concluded that *C. gracilis* is an allotetraploid species which gained one of its two subgenomes from *C. amoena*. Cytological data presented here confirm Håkansson's findings. Hiorth (1941) suggested on morphological grounds that *C. arcuata* might be the other parent of *C. gracilis*. Later, Håkansson (1946) was able to exclude the possibility that *C. arcuata* might be the second parent of *C. gracilis* with cytological evidence. Lewis and Lewis (1955) on morphological grounds assumed that *C. lassenensis* was the donor of the second subgenome. The present study offers a critical test of this assumption.

The lack of M_1 pairing between the

chromosomes of *C. amoena* ssp. *huntiana* and *C. lassenensis* in the diploid hybrid, in addition to the irregularities in meiotic behavior exemplified by univalent pseudoassociation, precocious division, and fragmentation prior to M_1, indicates that there is little homology between the two sets of chromosomes. Furthermore, the difficulty with which these F_1 diploid hybrids were obtained reflects the effect of a number of strong pre- and post-zygotic isolating mechanisms observed between the two species. The F_1s were highly sterile and were morphologically intermediate between both parental diploids.

Although as many as five of the seven *C. lassenensis* chromosomes were able to pair with their counterparts in *C. gracilis*, such associations were observed in only a few meiocytes. The majority showed relatively poor pairing between the two sets of chromosomes. In addition, the standard arrangement, which is the most prevalent one in *C. lassenensis* populations, differed from its counterpart set in the standard strain of *C. gracilis* by at least two translocations. The two genomes seem to have undergone some chromosomal repatterning. Although these triploid hybrids did not show the expected meiotic pairing of 7(2) + 7(1), that was frequently observed in the case of *C. amoena* ssp. *huntiana* triploids, they showed better pairing than observed in *C. arcuata* triploids by Håkansson (1946). There is no doubt that the allotetraploid *C. gracilis* combines two subgenomes, one from *C. amoena* ssp. *huntiana* in subsection *Primigenia*, the other donated by a diploid species from subsection *Flexicaulis*.

There are two major hypotheses to consider. The first is that *C. lassenensis* is the actual donor of the second subgenome in *C. gracilis*. Here we face several possibilities. One is that *C. lassenensis* chromosomes have undergone chromosomal repatterning since the formation of *C. gracilis*. Such a postulate does not seem likely since the standard arrangement in this species is the most widespread end arrangement in natural populations and hence is probably the primitive one (Snow and Imam, 1964). In addition, *C. lassenensis* exhibits a high degree of cytological uniformity in nature. Thus, it is unlikely that extensive structural changes have occurred in its populations. Another possibility is that the *C. lassenensis* chromosomes in *C. gracilis* have undergone chromosomal repatterning since the formation of the tetraploid. These structural changes would have been in the form of intergenome or intragenome translocations. If intragenome interchanges occurred, we might expect such interchanges to occur in about equal frequency within each of the two subgenomes in *C. gracilis*. The fact that the seven chromosomes of standard *C. amoena* usually formed 7(2) in the triploids with standard arrangement of *C. gracilis* excludes the possibility of both intragenome and intergenome interchanges, since such interchanges would result in multiple associations. One might argue that gene flow between *C. amoena* and *C. gracilis*, through sympatric association, has resulted in a closer structural relationship between the *C. amoena* genomes. If this was the case, one might expect that the genomes of the highly inbred *C. g.* ssp. *gracilis* and the geographically isolated *C. g.* ssp. *albicaulis* would show less homology with the *C. amoena* genome and more homology with the *C. lassenensis* genome than observed in triploids with *C. gracilis* sspp. *sonomensis* and *tracyi*. Since these expectations were not fulfilled, the hypothesis that *C. lassenensis* was the actual donor of the second subgenome of *C. gracilis* was rejected.

The second major hypothesis is that the *Flexicaulis* subgenome was donated by a diploid species that is now extinct, but from which both *C. lassenensis* and *C. arcuata* have differentiated. This ancestral *Flexicaulis* genome had seven chromosomes and presumably combined the general morphological and ecological features of subsection *Flexicaulis*. Despite the lack of direct evidence, this hypothesis is favored in view of the following observations: 1)

The absence of strong chromosomal homology between the existing *Flexicaulis* genomes and the second subgenome of *C. gracilis*; 2) The geographical relationship of *C. gracilis* and species of *Flexicaulis*; 3) The ecological preference for serpentine soil that was observed in *C. gracilis* ssp. *tracyi* and the ability of *C. gracilis* ssp. *sonomensis* to grow on serpentine, a characteristic edaphic feature of *C. arcuata*; 4) The morphological resemblances of *C. gracilis* ssp. *albicaulis* and *C. arcuata*.

In view of this hypothesis, the fact that the *C. lassenensis* genomes showed relatively better pairing with one set of chromosomes in *C. gracilis* triploids than that observed in triploids with *C. arcuata* can be interpreted if we assume that the inbreeding *C. lassenensis* had undergone less chromosomal repatterning than the outcrossing *C. arcuata*. Triploids with *C. arcuata* involved two races of *C. gracilis* (Håkansson, 1946). Triploids with *C. g.* ssp. *sonomensis* have weak pairing, mostly of 2(2) + 17(1). Those with *C. g.* ssp. *albicaulis* showed better pairing, with 4(2) + (3c) + 10(1) as the prevalent configuration. The improved pairing is not surprising since both taxa at present grow in the area of Butte Canyon, to which *C. gracilis* ssp. *albicaulis* is restricted, and these two outbreeding species may have enjoyed some degree of introgressive hybridization.

Data from paper chromatography also support the second hypothesis (Abdel-Hameed, 1967). Cytological and geographical data indicate that the polytypic allotetraploid, *C. gracilis*, may have originated more than once during late Pleistocene when the two parental diploids came together after being earlier well separated geographically and genetically.

Summary

Various lines of evidence indicate that the putative parental diploids of *C. gracilis* are most likely to be *C. amoena* ssp. *huntiana* (subsection *Primigenia*) and *C. lassenensis* (subsection *Flexicaulis*).

Triploid hybrids between *C. amoena* ssp. *huntiana* and *C. gracilis* showed that *C. gracilis* contains one subgenome from *C. amoena* ssp. *huntiana*. In the standard strain of the tetraploid this subgenome is homologous to that of the standard strain of *C. amoena*.

Clarkia lassenensis × *C. gracilis* triploid hybrids showed a lower homology between the diploid genome and the second subgenome of *C. gracilis*. At least two translocations were detected in triploid hybrids that involved the standard strain of each, and meiotic pairing was relatively poor.

The extremely low meiotic pairing and the large number of pre- and post-zygotic isolating mechanisms observed in F_1 diploid hybrids between *C. amoena* ssp. *huntiana* and *C. lassenensis* indicate that these two species are rather distantly related genetically.

The synthetic allotetraploids showed normal chromosome pairing and regular meiosis. They resemble natural tetraploids morphologically.

It is concluded that the *Primigenia* subgenome in *C. gracilis* was certainly donated by *C. amoena* ssp. *huntiana*, while the other was donated by a *Flexicaulis* diploid species. The *Flexicaulis* subgenome was probably donated by an extinct primitive diploid, which combined features now prevalent in *C. lassenensis* and *C. arcuata*, and perhaps was an ancestor of these two diploids. The allotetraploid *C. gracilis* may have originated more than once in the late Pleistocene when the two parental diploids came together after earlier being well separated geographically and genetically.

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