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Chloroplast and nuclear DNA variation among homozygous plants in a population of the autogamous annual *Microseris douglasii* (Asteraceae, Lactuceae)

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Key words: *Asteraceae*, *Microseris douglasii*. – Selfing, inbreeding, chloroplast capture, introgression, RAPDs, RFLPs.

Abstract: The autogamous diploid annual *Microseris douglasii* of California occurs in many isolated populations. The populations consist of one to many highly inbred biotypes. Morphological variation among populations usually is greater than within populations. In spite of the virtual absence of gene flow even within populations, genetically determined character differences are randomly distributed and associated throughout the range of the species. Recent evidence even suggests introgression of chloroplasts from the related *M. bigelovii*. Offspring families from 25 plants of a very variable population were raised and examined for segregation of morphological and molecular (RAPD) markers. All 25 original plants were completely homozygous for all markers, but each differed from all others at least in some markers. The population consisted of two genetically isolated groups of plants: a distinct inbred line (3 plants) and 22 plants with random associations of a common set of markers and characters, possibly recombinant inbreds from a past hybridization event. One of these 22 plants contained a chloroplast genome found in *M. bigelovii*, the other 24 plants a chloroplast genome found only in *M. douglasii*.

Self-fertilization is the typical reproductive mode in the annual species of *Microseris* (CHAMBERS 1955). Offspring of a plant collected in the field in most cases is morphologically uniform. This is true even for the most variable species in the group, *Microseris douglasii*. CHAMBERS (1955: 264) states that there may be a dozen or more “biotypes” within a population that are distinguishable mainly by characters of the mature achenes, while differences in size and growth habit are essentially due to environmental modification. The variation among plants in one population may be due to multiple colonizations of the site by achenes from other populations with or without subsequent hybridization. CHAMBERS (1955) illustrates variation within a population that looks like the result of random genetical segregation from a cross between two quite different initial colonizing biotypes. CHAMBERS (1955) has also raised 27 offspring families from plants of another, very variable population. Of these 27 families, 20 showed no segregation, 3 segregated an occasional variant, and 4 showed full segregation. This case, in which a natural

population has not yet reached full homozygosity, may be the exception rather than the rule in *M. douglasii*.

We can therefore sample the genetic variation within this species by selecting visibly different plants from each population in nature and maintaining these as inbred lines. These can be compared repeatedly and under various environmental conditions. Samples derived from one population in different years can be compared at one time under common conditions to follow genetic changes with time (PRICE & al. 1986, BACHMANN & BATTJES 1994), and the inbred lines can be crossed artificially to analyse their genetic differences in detail (MAUTHE & al. 1984 a, b, 1985; VLOT & BACHMANN 1990, 1991, 1993).

Recently, BACHMANN & BATTJES (1994) have surveyed the genetic variation for about 35 morphological characters in 41 inbred strains derived from 19 populations throughout the range of *M. douglasii*, and found that characters that are not obviously pleiotropic expressions of the same genes are freely recombined and randomly distributed throughout the range of the species and show no indication of adaptive association or distribution. The apparent absence of an adaptive significance of the genetically determined character differences is probably related to the very high phenotypic plasticity, especially of potentially adaptive characters such as the numbers of achenes per head (BACHMANN & BATTJES 1994, BACHMANN & ROELOFS 1995). Plastic responses to local environmental differences can completely swamp the expression of genetic variation in the field (BACHMANN & BATTJES 1994) and greatly reduce the impact of natural selection on the plastic characters.

The observation of extremely low rates of outbreeding (much less than one event per population per generation) together with an effectively random distribution and association of characters across the entire distribution range of the species is remarkable. It suggests that very rare outbreeding and very rare (long-distance) achene transport among populations are sufficient to maintain an overall allele distribution throughout the range of the species that does not differ from that in a panmictic population.

The very low rate of outcrossing also does not seem to have prevented introgression between *M. douglasii* and *M. bigelovii*, species that have hardly any overlap in their distribution ranges and show reduced fertility of the F1 hybrids when they are crossed artificially (CHAMBERS 1955: 262, PRICE & al. 1985, VLOT & al. 1992, HOMBERGEN & BACHMANN, unpubl.). CHAMBERS (1955: 232) has inferred introgression between the two species on the basis of morphology. Recently, WALLACE & JANSEN (1990) have determined the phylogeny of the species of *Microseris* with polymorphisms (RFLPs) in the chloroplast DNA. They have found very little variation among the various annual species, and this variation did not agree with the presumed phylogenetic relationships among the species. A detailed investigation of the distribution of the chloroplast variants (ROELOFS & BACHMANN, unpubl.) strongly suggests introgression of chloroplasts from *M. bigelovii* into *M. douglasii* even in populations not previously suspected to contain genetic material of *M. bigelovii*.

One of these is population E68 from Cortina Ridge. Three morphologically divergent plants from this population were examined, and one contained a chloroplast marker typical for *M. bigelovii*. All three differed from each other in their nuclear DNA, but all within the range of variation of *M. douglasii*. We have now

raised offspring of all 25 plants sampled from this population and determined their relationships using molecular markers for nuclear DNA (random amplified polymorphic DNAs, RAPDs: WILLIAMS & al. 1990, WELSH & McCLELLAND 1990) and restriction fragment length polymorphisms (RFLPs) for the chloroplast genome. This investigation shows for the first time at the DNA level the complete homozygosity of all plants from a natural population of *M. douglasii*. It allows us to recognize two genetically isolated groups of plants within the population, one a uniform biotype (an inbred line), the other probably derived from a past hybridization and segregation event. This second group contains the single plant with a chloroplast type typical of *M. bigelovii*. Since we found only one such plant, no correlation between any nuclear marker and the foreign chloroplast could be found that might help to reconstruct the introgression event.

Material and methods

Plant material. Mature capitula of *Microseris douglasii* were collected from a population (accession number E68) on Cortina Ridge, Colusa County, California, at the junction of Highway 20 with Highway 16. Single capitula were harvested from 25 plants (E68-0-01 to E68-0-25) on May 3, 1991, by JOHANNES BATTJES and KENTON L. CHAMBERS. Five offspring each of three plants representing the most diverse fruit types in the sample were raised in the greenhouse in 1991/1992 together with another 38 strains of *M. douglasii* from 18 populations covering the range of the species (BACHMANN & BATTJES 1994). Offspring of all 41 strains was raised in 1992/93 and examined for polymorphisms (RAPDs) in nuclear DNA and for markers for the four chloroplast DNA types found by WALLACE & JANSEN (1990) in the annual species of *Microseris* (ROELOFS & BACHMANN, unpubl.). One of the three plants of E68 contained a chloroplast type typical for *M. bigelovii* and suggested by its flower colour and leaf shape that it might contain nuclear genes of that species. We therefore raised offspring of all 25 plants from that population in 1993/94. These are third-generation greenhouse plants derived from E68-0-08, -14 and -25 and first generation offspring of the other 22 plants.

Plants were germinated in early October, 1993, and 15 offspring each were planted in flats (45 × 30 cm, 7 cm deep). Another two plants per family were planted individually in 10 cm clay pots and scored for the full set of morphological characters (Table 2 in BACHMANN & BATTJES 1994). A single leaf of 10 plants each from the flats was used to isolate DNA for RAPD analysis, leaves from all 15 plants were harvested and pooled for DNA isolation for Southern blots. Flower and fruit characteristics and leaf shape were scored from all 15 plants after they had regrown.

RAPD analysis. DNA from one leaf of 10 plants each per offspring family was isolated by a mini-preparation method described by HOMBERGEN & BACHMANN (unpubl.). PCR amplifications for RAPD analysis were performed in 25 µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM MgCl₂, 0.001% gelatine, 0.1% Triton X-100, 100 µM of each dNTP, 0.2 µM random decamer primer (Operon Technologies Inc.), 0.25 U Taq polymerase (HT Biotechnologies, UK) and 25 ng genomic DNA. We used primers OPA-1, -7, -11, -12, -17, and -18; OPB-5; OPC-2, -5, -6, -7, -8, -9, and -11 and OPH-2, -3, and -4 (from Operon Technologies primer kits A, B, C, and H). The reactions were overlaid with one drop of mineral oil. The PCR reaction was performed in a MJ Research PTC-100/96 thermal cycler programmed for 3 min at 94 °C, 35 cycles of 15 sec at 94 °C, 30 sec at 40 °C and 1 min at 72 °C followed by 5 min at 72 °C. Amplification products were resolved by agarose gel electrophoresis (1.5%) and visualised by ethidium bromide staining.

Amplification products were listed as discrete character states per strain (present/absent)

and treated as cladistic data with PAUP 3.1. A bootstrap (FELSENSTEIN 1985) running 100 replicates of the heuristic search option, was carried out to generate an 80% majority rule consensus tree (SWOFFORD & OLSEN 1990). The related annual species *M. pygmaea* was taken as outgroup.

Chloroplast RFLP analysis. Leaves from 15 offspring obtained by selfing from a parent plant were pooled for DNA isolation. DNA was directly isolated from 3 g fresh leaf material according to the maxi preparation method of SAGHAI-MAROOF (1984) with small modifications. Tissues were ground to a fine powder in liquid nitrogen and incubated at 65 °C in 15 ml 2 × CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2.0% hexadecyltrimethylammonium bromide and 1.0% 2-mercaptoethanol) for 45 min. The lysate was extracted twice with 15 ml phenol/chloroform (1 : 1) and once with chloroform/isoamylalcohol (24 : 1). After isopropanol precipitation the DNA pellets were dissolved in 10 ml TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) followed by ethanol precipitation with 50 µl 7.5 M NH₄CH₃COOH and 20 ml of 96% ethanol. The final DNA pellets were washed with 70% ethanol and dissolved in 3 ml TE to a DNA concentration of about 0.1 µg/µl.

Approximately 2 µg DNA was digested with each of the restriction endonucleases *Eco* RI, *Eco* RV, and *Dra* I according to conditions recommended by the manufacturer (Eurogentec). Restricted DNA was separated on 0.8% agarose gels and transferred to nylon membranes (Qiabran plus) using a Biorad vacuum blotter. Blots were hybridized overnight with ³²P random primed labelled probe DNA (FEINBERG & VOGELSTEIN 1983) at 65 °C. Blots were washed twice for 20 min each with 2 × SSC at room temperature, twice for 30 min with 0.5X SSC/0.1% SDS at 65 °C, and 5 min with 0.5 × SSC at room temperature. 7.0 kb, 18.8 kb and 12.3 kb lettuce *Sac* I cpDNA clones described by JANSEN & PALMER (1987) were used as probes. DNA of *M. bigelovii*, *M. pygmaea* and *M. elegans* was used as control.

Results

Plants in the field are homozygous. The uniformity of the offspring families showed that each of the 25 plants sampled from population E68 was homozygous for all DNA polymorphisms and for all genes determining the morphological characters as far as that could be determined. At the same time, each plant differed genetically at least in some minor way from all others. This uniformity is most clearly seen in the roughly 150 amplification products obtained for each plant with 17 arbitrary primers. Figure 1 shows the uniformity of the amplification products obtained with primer OPA-7 in the offspring families of plants E68-0-21 and E68-0-22. Morphological characters with low phenotypic plasticity (high heritability) such as achene shape, flower colour, the percentage of peripheral hairy achenes or achene spotting also were uniform throughout families. Leaf shape is a character with intermediate heritability. Considering the great many genetically different leaf shapes in various strains, it is likely to be influenced by many genes. Figure 2 shows representative leaves from 10 offspring plants regrown after leaf harvest in four of the offspring families. Here, too, the uniformity within families is obvious in comparison with the differences among families.

Population E68 contains two separate gene pools. 17 primers produced 46 amplification products that were polymorphic among plants of population E68. We have subjected these to a cladistic analysis and have found two “monophyletic” groups supported by bootstrap values of 88% and 94% (Fig. 3). One of these comprises 3 plants (numbers 21, 24, and 25) which also agree in morphological char-

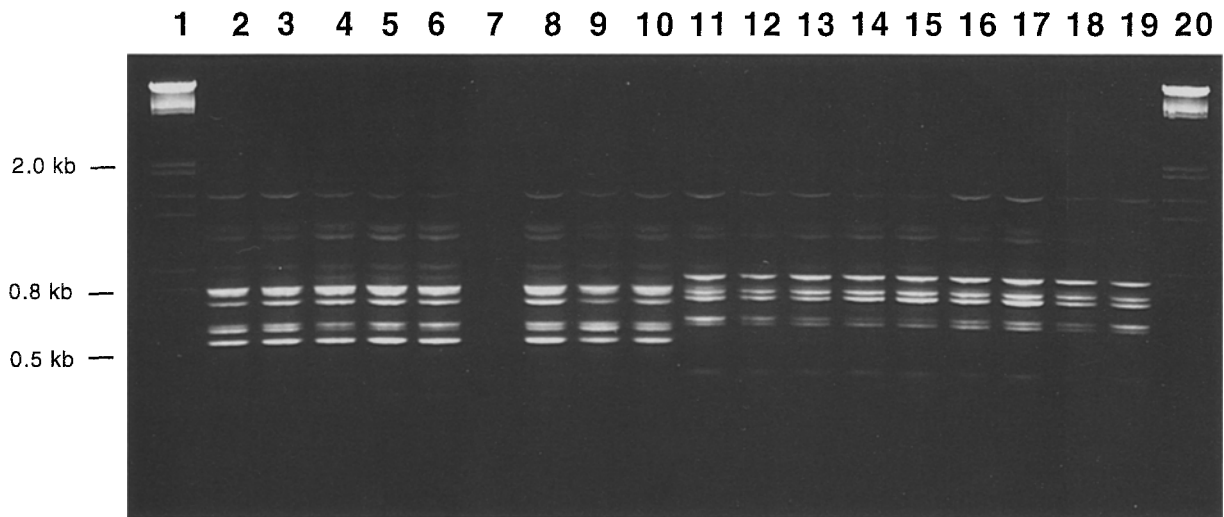


Fig. 1. *Microseris douglasii*. Amplification products of 9 first-generation offspring each of plant E68-0-21 (lane 2–10) and E68-0-22 (lane 11–19), PCR performed with primer OPA-7; Marker (lane 1 and 20); λ -DNA cut with *Eco* RI and *Hind* III. Lane 7: PCR reaction failed

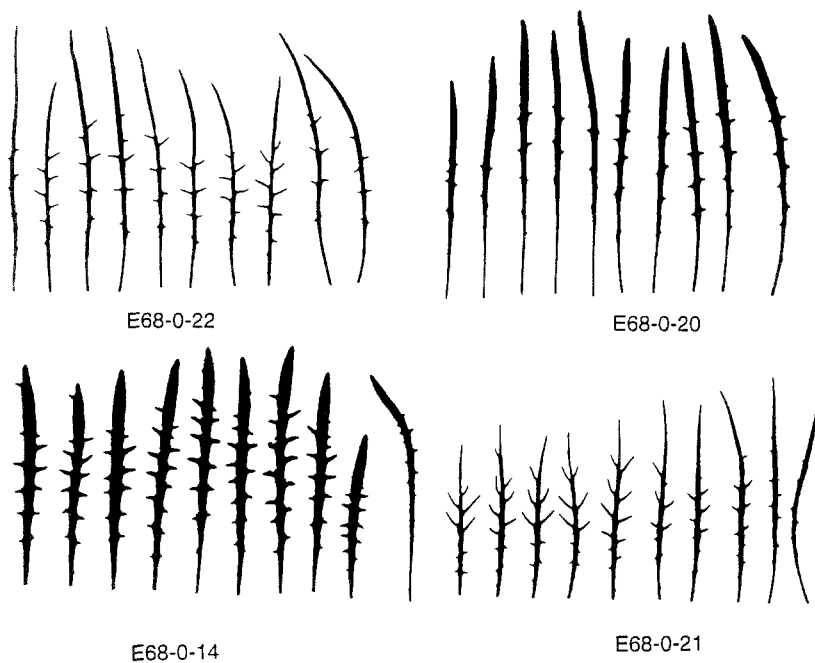


Fig. 2. *Microseris douglasii*. Representative leaves of 10 offspring each of first-generation offspring of plants E68-0-20, -21, and -22 and of third-generation offspring of plant E68-0-14

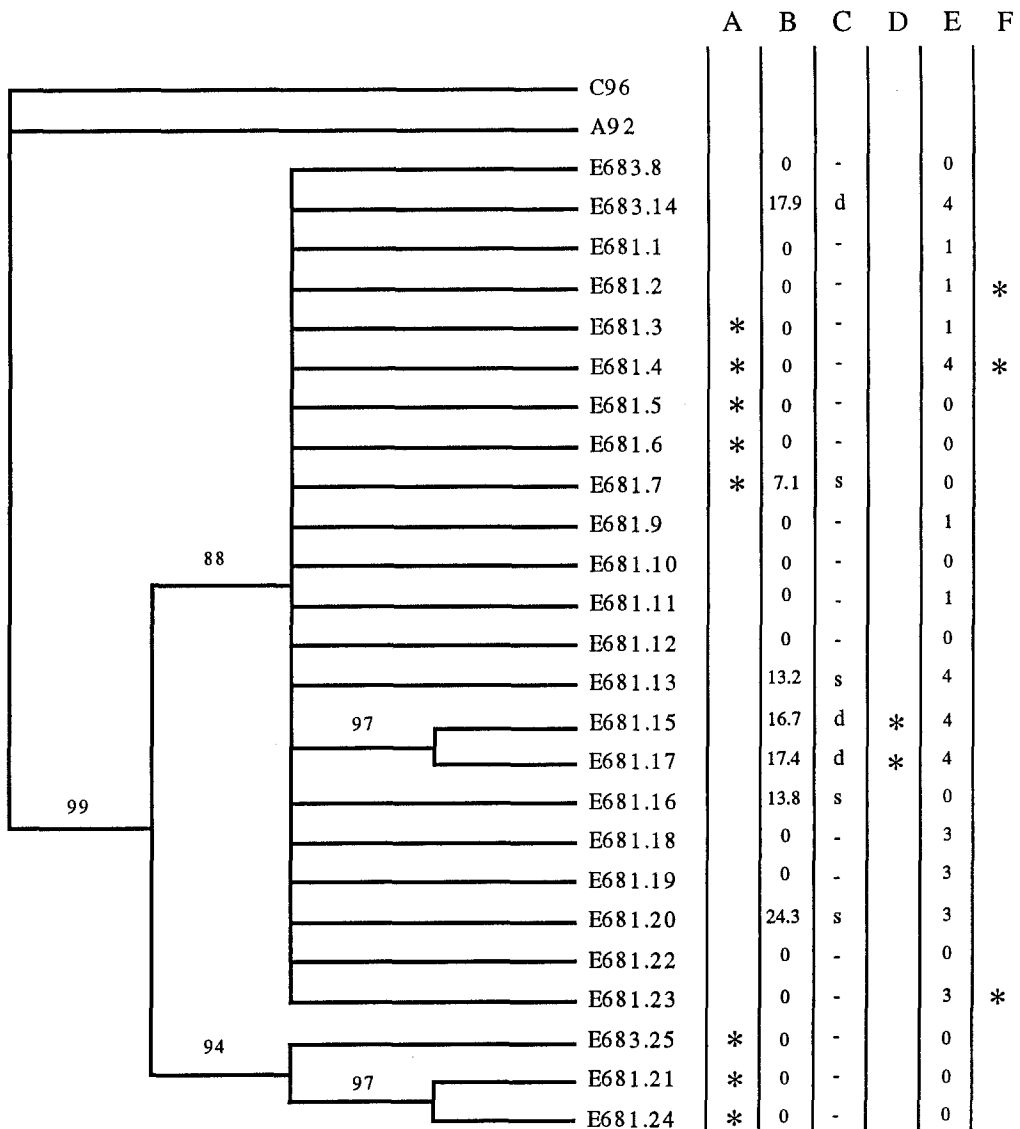


Fig. 3. *Microseris douglasii*. 80% majority rule consensus tree (generated by bootstrap analysis using PAUP) of the homozygous genotypes of 25 plants sampled from population E68 based on 46 polymorphic nuclear DNA markers (RAPDs). Numbers on the branches indicate the percentage the group was found. A–F Character states for polymorphic morphological characters with high heritability. A Spots on achenes, B % hairy peripheral achenes, C hairy achenes, when present, either dense (d) or scarcely hairy (s), D red stripe on abaxial side of peripheral florets, unmarked plants have a light grey stripe; E flower colour: white (0) to dark yellow (4), F white achenes, other plants have brown achenes

acters such as thin spotted achenes with no hairs on the peripheral ones, white ligule colour with a gray stripe on the peripheral ligules and sharply pointed leaves with prominent teeth that bend upwards (plant 21 in Fig. 2). In leaf shape and RAPDs, plants 21 and 24 are nearly indistinguishable, while plant 25 differs only slightly (data not shown). There is considerable variation in RAPDs and morpho-

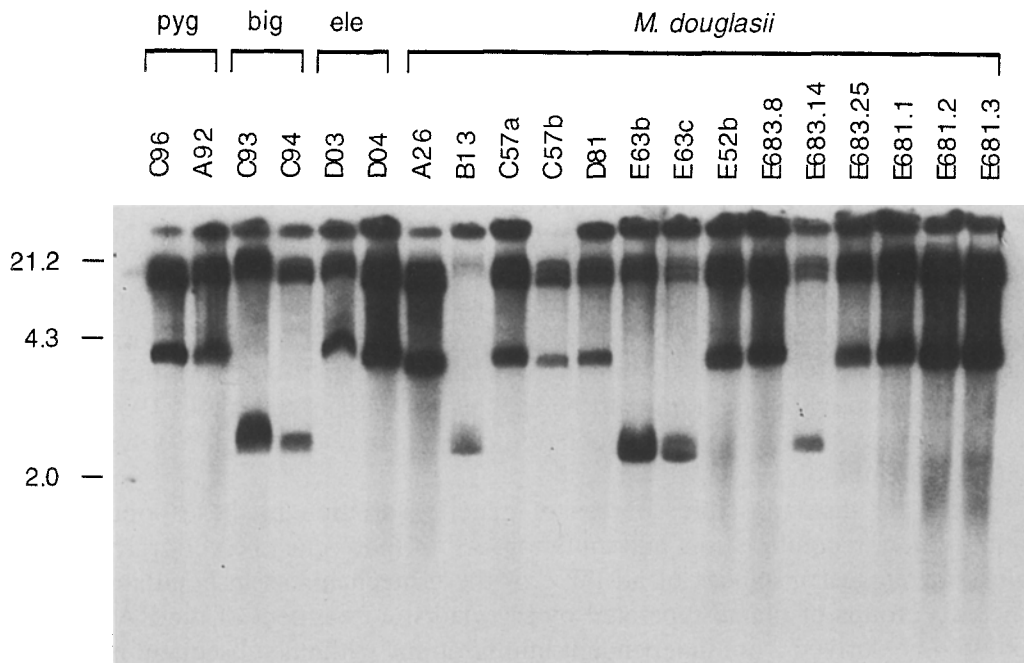


Fig. 4. DNA of annual *Microseris* cut with *Eco* RV and hybridized with lettuce 18.8 kb cpDNA clone (JANSEN & PALMER 1987), detecting a site gain (3.8 kb to 2.3 kb + 1.5 kb; WALLACE & JANSEN 1990, "mutation 11") typical for *M. bigelovii* cpDNA and shared by a few strains of *M. douglasii* including third-generation offspring of E68-0-14 (E683.14). *pyg* *M. pygmaea*, *big* *M. bigelovii*, *ele* *M. elegans*. Fragment length of the marker in kb at the left

logical characters among the remaining 22 plants. These plants are grouped together in the cladistic treatment because the various polymorphic characters are randomly associated among these plants and do not suggest any further association, with the exception of plants 15 and 17 which are nearly identical.

Only one plant contains "foreign" chloroplasts. Among the 3 plants from population E68 included in the species-wide survey (ROELOFS & BACHMANN, unpubl.), two (numbers 8 and 25) contained a chloroplast type found only in *M. douglasii*, while E68-0-14 contained a chloroplast type shared with *M. bigelovii*. The present survey has shown that E68-0-14 is the only plant among the 25 sampled from the population that contains the "foreign" chloroplast genome (Fig. 4). That genome differs by more than the diagnostic mutation shown in Fig. 4 from a typical *M. douglasii* chloroplast DNA. An origin by back mutation within population E68 can therefore be excluded.

Discussion

The object of this investigation was three-fold: (1) The availability of many simple molecular markers makes it possible to check in detail the suggestion that plants of the annual species of *Microseris* in the field are highly homozygous; (2) to try to infer from the variation among plants how intrapopulation variation arises and is maintained in the virtual absence of outbreeding; and (3) to look at the dis-

tribution of the two chloroplast types present in population E68 and to try to reconstruct the origin of this polymorphism.

DNA amplification from arbitrary primers has provided strong support to the interpretation by CHAMBERS (1955) of the genetic structure of these populations. All plants in population E68 appear to be completely homozygous. This confirms our impression based on raising offspring of hundreds of field-collected plants of the annual species of *Microseris*. Segregation is occasionally observed, but seems to be the exception. Most populations must go for many generations without cross-fertilization in order to reach complete homozygosity. During this time, there seems to be virtually no selection on the characters that are polymorphic. We have previously found virtual random association and distribution of the variable characters throughout the range of the species (BACHMANN & BATTJES 1994). Here we find an essentially random association of the characters that are polymorphic within a population.

There are three possible sources of genetic variation in these populations: immigration, recombination, and mutation. All of these must be very rare events. Our data suggest instances of all three of these mechanisms in population E68. The two groups of plants separated by the cladistic treatment of the RAPDs are likely to be derived from independent immigrations without subsequent recombination between the two gene pools. The random association of very distinctive alternate character states within the larger group of 22 plants suggests that this group is the result of recombination involving at least two genetically different immigrants followed by inbreeding and the fixation of many "recombinant homozygous" lines. Each of these lines maintains itself by selfing, and we should expect to find more than one plant of each inbred line in the population. Only the genotypes of plants 15 and 17 seem to belong to one inbred line but even these are not completely identical. The same is true for plants E68-0-21, -24, and -25, where the data suggest that plants 21 and 24 share mutations that have arisen within the inbred line.

The genotype diversity in this population is astonishingly high. It is not impossible that of the three factors creating genetic diversity, mutation is the most effective at least for sequences detected as RAPDs. The 21 separate lines among the 22 "recombinant inbreds" may not derive from 21 different selfing products of the putative original hybrid, they may have accumulated enough RAPD mutations during inbreeding to disguise fewer origins.

The population structure revealed by the RAPD data supports previous ideas about the genetic structure of these autogamous annuals (CHAMBERS 1955, BACHMANN & BATTJES 1994) in their most extreme form. They show the peculiar combination between the virtual absence of outbreeding with the rather complete preservation of the products of occasional hybridizations in the form of inbred lines.

The unexpected result of this study concerns the distribution of the two chloroplast types within the population. Comparisons of chloroplast and nuclear phylogenies have recently revealed surprisingly many cases in which the two are not concordant (RIESEBERG & BRUNSFELD 1992). WENDEL & al. (1991) list various mechanisms that may lead to "chloroplast capture" (or "cytoplasmic introgression"). None of these are very likely explanations of the present case. Chloroplasts are maternally inherited in *Microseris*, and we would expect two types in one popula-

tion to be associated with two genetically isolated lines. However, the nuclear markers suggest that the single plant, E68-0-14, containing the “foreign” chloroplast type has segregated from the same gene pool as 21 other plants in the population. If this is segregation from a single hybrid, we would expect only one chloroplast type in this group of plants. If the “foreign” chloroplast has been introduced by a hybridization with *M. bigelovii* as maternal parent and afterwards has been eliminated by backcrossing to *M. douglasii*, a complex repeated crossing scheme would be involved that does not agree with the virtual absence of outcrossing in both putative parental species. The fact that the “foreign” chloroplast type also occurs in at least three other populations of *M. douglasii* (ROELOFS & BACHMANN, unpubl.) shows that resolving this puzzle is essential for an understanding of the population genetics of this species.

White flowers and pointed leaves are typical for *M. douglasii*, while orange flower colour and spatulate leaf shape are restricted to some biotypes of *M. bigelovii* (BACHMANN & al. 1982, 1984). Plant E68-0-14 contains the foreign chloroplast type. It is also one of 5 plants with dark yellow flowers (Fig. 3) and the plant with the most strongly rounded leaves (Fig. 2) in the population. This had suggested to us that plants in population E68 containing the *M. bigelovii* chloroplast type also might contain nuclear markers of that species, and that these could be identified and their distribution within the population be determined. Finding only a single plant with the foreign chloroplast has prohibited this approach in population E68. We are now planning to develop a PCR based screening method for the diagnostic chloroplast mutant in order to be able to screen much greater numbers of plants without having to raise offspring families of each plant.

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