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Comparison of chloroplast and nuclear phylogeny in the autogamous annual *Microseris douglasii* (*Asteraceae: Lactuceae*)

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Key words: *Asteraceae*, *Microseris*. – Chloroplast phylogeny, cpRFLP, cytoplasmic introgression, RAPD, selfing.

Abstract: Morphology suggests that the Californian annual *Microseris douglasii* is a monophyletic sister group to the other three diploid annuals of *Microseris*. Phylogenetic analysis of 44 inbred strains of *M. douglasii* derived from 23 populations with 72 RAPD markers in the nuclear DNA strongly supports this phylogeny. However, 13 chloroplast RFLPs divide *M. douglasii* into four distinct groups. Two of these each share one or more cpRFLPs with *M. bigelovii* and *M. pygmaea*. Several hypotheses can explain the incongruence between nuclear and chloroplast phylogeny: (1) random sorting out of chloroplasts during phylogeny from a polymorphic pool, (2) cytoplasmic introgression from the related annual *M. bigelovii* into *M. douglasii* after hybridization followed by elimination of the *M. bigelovii* nuclear genome. We suggest cytoplasmic introgression as the most likely origin. Possible remnants of nuclear introgression have been found in two populations of *M. douglasii* that are polymorphic for chloroplast types. In these populations *M. bigelovii* type chloroplast DNA seems to be accompanied by nuclear genes for flower color and leaf shape.

Recent studies about disagreement between nuclear and chloroplast-based phylogenies have provided potential evidence for reticulate evolution (RIESEBERG 1991). Allopolyploids are clear evidence of a reticulate pattern, and chloroplast data often reveal the maternal contribution to the original hybridization event. Reticulate evolution at the diploid level has also been documented using molecular data. In some cases this concerns re-examinations of species where introgression or hybrid speciation had already been proposed (RIESEBERG 1991: *Helianthus*, ARNOLD 1993: *Iris*, WHITTEMORE & SCHAAL 1991: *Quercus*). Molecular data in these studies provide additional evidence for hybridization, introgression or hybrid speciation. Other cases concern cytoplasmic introgression with little or no evidence of nuclear introgression (WENDEL & al. 1991: *Gossypium*, SMITH & SYTSMAN 1990: *Populus*). Different mechanisms have been proposed for cytoplasmic introgression, but finding the one responsible for a particular case is difficult (WENDEL & al. 1991). Moreover, in most studies it is not clear whether introgression contributed to the origin of a new taxon or whether it occurred afterwards. RIESEBERG & WEN-

Fig. 1. A Alternative phylogenies for the four diploid annual species of *Microseris* (doug *M. douglasii*, ele *M. elegans*, big *M. bigelovii*, pyg *M. pygmaea*). A Suggested relationship on the basis of morphology and hybrid fertility (CHAMBERS 1955). B Cladogram on the basis of chloroplast restriction site mutations after WALLACE & JANSEN (1990) doug refers both to diploids and to female component of allotetraploids; informative RFLPs are indicated as black bars

DEL (1993) stated that failure to consider this ambiguous scenario has frequently led to unjustified conclusions. Here, we describe the relationship among the four autogamous diploid annuals in the genus *Microseris*, where cpDNA data contradict earlier morphological and biosystematic data concerning species delimitation and relationship (CHAMBERS 1955).

Microseris douglasii (DC.) SCH.-BIP., *M. bigelovii* (GRAY) SCH.-BIP. and *M. elegans* GREECE ex GRAY occur in Western North America, while *M. pygmaea* D. DON presumably evolved after long distance dispersal to Chile. On the basis of morphology and crossing fertility CHAMBERS (1955) suggested the variable and widespread species *M. douglasii* to be a sister group to the three other species (Fig. 1 a). This was recently supported when the reduction of the number of microsporangia from four in most *Microseris* including *M. douglasii* to two in *M. elegans*, *M. pygmaea*, and *M. bigelovii* was described as a clear synapomorphy for these three species (BATTJES & al. 1994). A phylogenetic study by VAN HOUTEN & al. (1993) based on nuclear RFLPs grouped all four diploid annuals in one clade separated from the other species in the genus *Microseris* on the basis of four markers. Within this clade, *M. bigelovii* has been proposed as the closest relative of the Chilean *M. pygmaea*. Further phylogenetic resolution has not been obtained. WALLACE & JANSEN (1990) found 25 RFLPs in the cpDNA that support the monophyly of the four annuals together. They found only four cpDNA markers within the annuals, of which three are informative (one marker is intraspecific in *M. bigelovii*). These markers divide *M. douglasii* into three distinct groups, of which two each share one or two cpDNA markers with *M. pygmaea* and *M. bigelovii*. Figure 1 shows the discrepancy between the chloroplast and the nuclear (morphological) phylogeny of the four annuals.

The annuals reproduce nearly exclusively by selfing. They occur in isolated populations that consist of one to many highly homozygous "biotypes" (CHAMBERS 1955). ROELOFS & BACHMANN (1995) have used RAPDs to demonstrate the complete homozygosity of all plants collected from a natural population of *M. douglasii*. In spite of this lack of recombination, heritable variation in morphological

characters seems to be randomly distributed and associated throughout the range of *M. douglasii* and shows no indication of adaptive association or distribution (BACHMANN & BATTJES 1994). BACHMANN & ROELOFS (1995) suggest that this absence of an adaptive component of the genetic variants is probably related to a high degree of phenotypic plasticity which buffers them against effective selection. Apparently, very rare outbreeding and occasional achene transport among populations are sufficient to maintain random allele distributions within and among populations of *M. douglasii* (BACHMANN & BATTJES 1994, ROELOFS & BACHMANN 1995). Moreover, ROELOFS & BACHMANN (1995) suggested gene exchange between *M. douglasii* and the coastal species *M. bigelovii*. They identified one plant in a polymorphic population of *M. douglasii* that contained *M. bigelovii* type chloroplasts. Interestingly, this plant also seems to contain *M. bigelovii* alleles of the nuclear encoded characters, flower color and leaf shape, pointing towards introgressive hybridization between *M. douglasii* and *M. bigelovii*. CHAMBERS (1955) already inferred introgression between the two species based on morphological characters of the fruit head. He suggests the San Francisco Bay region as an area where *M. bigelovii* and *M. douglasii* have long been in genetic contact.

Here we reconstruct a nuclear phylogeny of the diploid annual *M. douglasii* based on nuclear RAPD markers and test the suggestion that *M. douglasii* is paraphyletic. The chloroplast phylogeny is reconstructed using the three informative cpRFLP markers of WALLACE & JANSEN (1990) together with new chloroplast data. The new data confirm the discrepancies between the nuclear and chloroplast based phylogenies. Of the different mechanisms that have been proposed to explain such discrepancies, our results thus far favor cytoplasmic introgression of *M. bigelovii* chloroplasts into *M. douglasii* through hybridization followed by the more or less complete elimination of the *M. bigelovii* nuclear genome.

Material and methods

Plant material. 44 inbred strains derived from 23 populations of *M. douglasii* have been selected to cover the complete range of the species (BACHMANN & BATTJES 1994). The RAPD variability within *M. bigelovii*, *M. pygmaea* and *M. elegans* has been thoroughly investigated by VAN HEUSDEN & BACHMANN (1992a, b, c). On the basis of these data, two strains of each species showing the greatest intraspecific differences in their RAPD patterns have been included in this study. The original sources of the strains are summarized in Table 1.

Plants were germinated in early October 1992, and 15 offspring per inbred strain were planted in flats (45 × 30 cm, 7 cm deep). Two plants per strain were planted individually in 10 cm clay pots for morphological analysis (BACHMANN & ROELOFS 1995). A single leaf from each strain was used to isolate DNA for RAPD analysis. Leaves from all 15 plants were harvested and pooled for DNA isolation for Southern blots.

RAPD analysis. DNA from one leaf per strain was isolated by a mini preparation method described by HOMBERGEN & BACHMANN (1995). PCR amplifications for RAPD analysis ("Random Amplified Polymorphic DNA"; WILLIAMS & al. 1990, WELSH & MCCLELLAND 1990) were performed in 25 µl reaction mixture containing 0.25 U Supertaq polymerase (HT biotechnologies, UK), Supertaq buffer, 0.2 µM random decamer primer (Operon Technologies Inc. USA) and 25 ng genomic DNA, overlaid with one drop of oil. We used all 20 primers except OPA-4, 6, 13, and 16 from Operon Kit A; and OPB-1, 4, 5, and 6 from kit B. The amplifications were performed in an MJ Research PTC-100/96 thermal

Table 1. List of populations. All locations are in California, except as indicated. County, place of origin, year of collection, initials of the collectors and original accession numbers are listed. Collectors are KENTON L. CHAMBERS (CH), JÜRKE GRAU (JG), JAMES PRICE (JP), KONRAD BACHMANN (KB) and JOHANNES BATTJES (JB)

Microseris douglasii

- A26 Humboldt Co.: Garberville, 1969, CH 2926
 B13 Solano Co.: Rio Vista, 1973, CH 3676
 B14 Fresno Co.: Parkfield/Coalinga road, 1977, CH 4284
 B15/B21 San Francisco Co.: Presidio, 1973, CH 3727-8/3727-3
 B16 Monterey Co.: Jolon, 1973, CH 3723
 B18 San Luis Obispo Co.: 4 miles west of Atascadero, 1973, CH 3715
 B39 Monterey Co.: Tularcitos Creek, 1978, CH 4441
 B57 Fresno Co.: Panoche Road, 1978, KB and CH
 B77 San Mateo Co.: Woodside, 1973, CH 3726
 C57 San Luis Obispo Co.: Cayucos, 1980, JP and CH 4638
 D57 Santa Clara Co.: Jasper Ridge, 1982, JP and CH 4915
 D68 San Luis Obispo Co.: Cuesta Park, 1982, JP and CH (JP6)
 D81 Monterey Co.: Parkfield/Coalinga road, 1982, JP and CH (JP11)
 E34 Tehama Co.: Corning Corner, 1991, JB and CH
 E43 San Luis Obispo Co.: Sycamore Ridge, 1991, JB and CH
 E44 San Luis Obispo Co.: Cripple Creek, 1991, JB and CH
 E52 San Luis Obispo Co.: Cholame, 1991, JB and CH
 E55 Monterey Co.: Jolon, 1991, JB and CH
 E59 Alameda Co.: Midway, 1991, JB and CH
 E60 Solano Co.: Rio Vista, 1991, JB and CH
 E63 Solano Co.: Cook Lane, 1991, JB and CH
 E68 Colusa Co.: Cortina Ridge, 1991, JB and CH
 E73 Riverside Co.: Alberhill Mtn, 1991, CH

M. bigelovii

- C93 Santa Barbara Co.: Pt. Sal, 1973, CH 3719
 C94 Victoria: Uplands Park, British Columbia, Canada, 1967, CH (A-303)

M. pygmaea

- A92 origin unknown, Chile
 C96 Prov. de Choapa: El Teniente, Chile, 1980, JG

M. elegans

- D03: San Luis Obispo Co.: Cholame, 1980, CH 4608
 D04: San Luis Obispo Co.: Parkfield, 1980, CH 4609
-

cycler programmed for 3 min at 94 °C, 35 cycles of 15 sec 94 °C, 30 sec 40 °C, and 1 min of 72 °C followed by 5 min 72 °C. Amplification products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Hybridization of RAPD bands. RAPD patterns that included species specific bands have been blotted onto Qiabran plus membrane (Qiagen, Germany) using the vacuum blotting system of Biorad (U.S.). The relevant bands have been isolated from a 1.5% agarose gel. Re-amplification in the presence of ³²P labelled dATP yielded probes of these RAPD markers which were hybridized on the blot of the original RAPD pattern and on Southern blots of 0.8% agarose gels containing total genomic DNA cut with *Eco* RI.

Chloroplast RFLP analysis. DNA was directly isolated from 3 g of fresh leaf material per strain according to the maxi preparation method described by SAGHAI-MAROOF & al.

(1984) and modified by ROELOFS & BACHMANN (1995). Approximately 2 µg of DNA was digested with each of the restriction endonucleases *Dra* I, *Eco* RI, *Eco* RV, and *Hinf* I according to conditions recommended by the manufacturer (Eurogentec sa, Belgium). Southern blotting was performed using the methodology described by ROELOFS & BACHMANN (1995) except for DNAs digested with *Hinf* I (4 basepair target site), which were blotted as follows. Restricted DNA was separated on 6% polyacrylamide gels and transferred to Hybond NTM membranes using the electroblotting methodology (GEBHARDT & al. 1989, VAN HOUTEN & al. 1993). Blots were hybridized overnight with ³²P random primed labelled DNA (FEINBERG & VOGELSTEIN 1983) at 65 °C, and washed twice for 20 min each with 2× SSC at room temperature, twice for 30 min with 0.5× SSC/0.1% SDS at 65 °C, and 5 min with 0.5× SSC at room temperature. Lettuce *Sac* I cpDNA clones described by JANSEN & PALMER (1987) were used as probes.

Data analysis. Polymorphic amplification products from the RAPD experiments were listed as discrete character states per strain (present/absent). A Nei’s distance matrix was generated and analysed using the neighbor joining method of PHYLIP, version 3.57c (FELSENSTEIN 1995). The same RAPD data were also analyzed cladistically with PAUP 3.1 (SWOFFORD & OLSEN 1990). A bootstrap (FELSENSTEIN 1985) of 100 replicates using the heuristic search option was carried out to generate a 50% majority rule consensus tree. Phylogenetic analysis of cpRFLPs has been obtained using the branch and bound option of PAUP 3.1.

Results

Nuclear RAPD data analysis. 20 primers revealed 82 scorable amplification products (two to five per primer). Of these, 10 were shared by all strains (Table 2), 72 were polymorphic in the strains of *M. douglasii* and the three related species studied by us. Of these 72 markers 19 were either autapomorphic or synapomorphic at the species level (Table 2).

Figure 2 shows the neighbor joining tree based on the Nei’s distance matrix derived from the RAPD data. *Microseris elegans*, *M. bigelovii* and *M. pygmaea* are clearly separated from *M. douglasii*, and the Chilean *M. pygmaea* is most similar to *M. bigelovii* among the extant Californian diploids. Within *M. douglasii*, strains derived from populations C57, D68 and E73 form a recognizable group. All other strains differ mainly by their individual characteristics (relatively long terminal branches), while there is no obvious grouping of similar strains (short basal branches).

Table 2. Groups detected with more than one RAPD amplification product. Note that all groups comprise all strains of one or more annual *Microseris* species included in this study

No.	Species				Frequen- cy
1.	doug	big	pyg	ele	10
2.	doug				6
3.		big	pyg		4
4.				ele	3
5.		big	pyg	ele	2
6.		big			2
7.			pyg		2

Fig. 2. Neighbor joining phenogram of 44 inbred strains derived from 23 *Microseris douglasii* populations with selected populations of the three other annuals of *Microseris* (listed in Table 1). A Nei's distance matrix of 72 discrete RAPD markers (absent/present) was used in a neighbour joining analysis of PHYLIP 3.57c. D Nei's genetic distance

A cladistic analysis of the entire data set yielded 15 shortest trees with a length of 362 steps. Since character state distributions resulting from recombination cause apparent homoplasies, the null hypothesis in a cladistic treatment of taxa connected by gene flow is the absence of structure in the resulting cladogram, and the occurrence of well-supported groups indicates limits to gene flow (VAN HEUSDEN & BACHMANN 1992a, b, c). Here, the 19 species-specific markers (Table 2) create the basic cladistic structure in the strict consensus tree. This tree could be

Fig. 3. 50% majority rule consensus tree of *Microseris* inbred strains used in the neighbor joining analysis (Fig. 4) and listed in Table 1. The RAPD markers were used as discrete characters (absence, presence) in a bootstrap analysis of PAUP 3.1 with 100 replicates of the heuristic search option. Bootstrap values are indicated as percentages. Branch lengths have no significance

Fig. 4. Original RAPD pattern obtained with primer OPA-15; a 500 bp band has been scored as autapomorphic for *Microseris bigelovii*. C Control, DNA replaced by H₂O in the PCR reaction; M marker, Lambda DNA cut with *Eco* RI and *Hind* III

Fig. 5. Autoradiogram of the hybridization experiment using the 500 bp amplification product as probe against a Southern blot of the original RAPD pattern (see Fig. 2). The probe hybridized with two RAPD bands of *Microseris bigelovii*

rooted with the data of VAN HOUTEN & al. (1993) and clearly shows *M. douglasii* to be a monophyletic sister group to the other three diploid annuals with a bootstrap value of 94% (Fig. 3). The close association between *M. bigelovii* and the Chilean *M. pygmaea* is supported by four synapomorphies.

Intraspecific variation among the strains of *M. douglasii* collapses essentially into a polytomy and this is responsible for the low consistency index (C.I. = 0.21) of the overall cladogram in Fig. 5. With two exceptions, monophyletic groups

Table 3. Chloroplast restriction site mutations used in phylogenetic analysis of the 4 diploid annuals in *Microseris*. Ancestral fragment(s) of the mutations are listed first. Mutations F, H, K, and L show an extra restriction fragment; mutations G and M show a fragment loss. Square brackets indicate instances in which the small fragments were not observed. For taxa abbreviations see Fig. 6. ^a Region corresponds to DNA probes constructed from Sac I fragments of *Lactuca* chloroplast DNA (JANSEN & PALMER 1987). ^b Chloroplast polymorphisms determined by WALLACE & JANSEN (1990)

Polymorphism	Enzyme	Region ^a	Mutation (kb)	Taxa
A ^b	DraI	12.3	2.0 = 1.2 + 0.8	big, pyg, doug-3, doug-4
B	Dra I	5.4	4.3 + [0.2] = 4.5	doug-2
C ^b	Eco RI	7.0	5.0 = 4.3 + 0.7	doug-1
D ^b	Eco RV	18.8	3.8 = 2.3 + 1.5	big, doug-4
E	Hinf I	18.8	0.6 + 0.6 = 1.2	big, pyg, doug-3, doug-4
F	Hinf I	18.8	+0.3	big, doug-4
G	Hinf I	14.7	-0.5	doug-2
H	Hinf I	14.7	+0.23	doug-1
I	Hinf I	14.7	0.62 = 0.35 + 0.27	ele
J	Hinf I	7.7	0.35 = 0.30 + 0.05	doug-1
K	Hinf I	7.7	+0.37	pyg
L	Hinf I	7.7	+0.24	doug-2
M	Hinf I	6.9	-0.36	big, pyg, doug-3, doug-4

within *M. douglasii* supported by bootstrap values above 50% are formed by lines derived from one population (B15/B21; C57, E73, E43, E59), and many populations are not recognizable as monophyletic units (E34, E44, E52, E55, E60, E63, E68). The two neighboring populations E60 and E63 share one genotype (represented by lines E60c, E63a, and E63b) and at the same time contain unrelated genotypes (E60a, E60b, and E63c). The only indication of subspecific structure within *M. douglasii* is the association of strains from the southern populations C57, D68, and E73 supported by a low bootstrap value of 57% but indicative of some genetic isolation relative to the other strains of *M. douglasii* (Figs. 4, 5).

We have investigated the nature of the species-specific RAPD markers by isolating the relevant amplification products from the gel, re-amplifying them in the presence of ³²P labelled dATP and using the radioactive amplification products as probes against a Southern blot of the original RAPD pattern. A 500 bp amplification product which was obtained with primer OPA-15 in *M. bigelovii* but not in *M. douglasii*, *M. elegans*, and *M. pygmaea* (Fig. 4) hybridized strongly and exclusively with amplification products from *M. bigelovii* (Fig. 5), both at the expected 500 bp position and with a shorter band that is hardly visible in the ethidium bromide stained gel (Fig. 4). Using the 500 bp amplification product as a probe against a Southern blot of *Eco* RI cut total genomic DNA, we obtained hybridization patterns in all of the annual species with restriction fragment length variation among them (data not shown). All species-specific RAPD markers have been tested in this manner. The nature of the amplification products is being investigated by sequencing.

Chloroplast phylogeny. The 13 cpRFLPs informative for the four diploid annuals are listed in Table 3. The chloroplast data of *M. douglasii* revealed one

Fig. 6. Single most parsimonious tree of the chloroplast genomes in the four diploid annuals of *Microseris*. The branch and bound option of PAUP 3.1 has been used. For strain abbreviations see Table 1, for RFLPs A to M see Table 3. The six different chloroplast genomes are designated doug-1, doug-2, ele, pyg, doug-3, doug-4 = big. Black bars indicate derived mutations as listed in Table 3

most parsimonious tree of 13 steps with the branch and bound algorithm of PAUP version 3.1 (Fig. 6). This tree shows no homoplasy (C.I. = 1.0) and was rooted using the data of WALLACE & JANSEN (1990). *Microseris elegans*, *M. pygmaea* and *M. bigelovii* are each identified as monophyletic groups with *M. bigelovii* sharing a common ancestor with the Chilean annual *M. pygmaea* on the basis of three synapomorphies. Chloroplasts of *M. douglasii* are distributed over four distinct groups, one more than in the chloroplast cladogram of WALLACE & JANSEN (1990). Group 1 (doug-1) seems to represent the core type for typical *M. douglasii* cpDNA and is characterized by three cpRFLPs; group 2 (doug-2), also restricted to *M. douglasii*, is characterized by three autapomorphies; group 3 (doug-3) shares three markers with the related annuals *M. pygmaea* and *M. bigelovii*; cpDNA of group 4 (doug-4) is identical to that of *M. bigelovii*. Furthermore, populations E60, E68 and C57 are polymorphic for chloroplast type: strains E60b, E60c, and E68b contain *M. bigelovii* type (doug-4) chloroplasts, whereas E60, E68a, and E68c contain typical *M. douglasii* cpDNA (doug-1). Above, we have noted the close relationship of the nuclear DNA of E60c with E63a and b (Figs. 2, 3). This close relationship is supported by the common chloroplast genome.

Fig. 7. Comparison of chloroplast and nuclear-based phylogenies for the four diploid annuals in *Microseris*. Chloroplast types designated as in Fig. 6. Dashed lines connect incongruent chloroplast types with the corresponding branches on the nuclear RAPD tree

Comparing the chloroplast based cladogram with the nuclear based cladogram (Fig. 7) similarities and conflicts are observed. Both trees support the close relationship between *M. bigelovii* and *M. pygmaea*. Chloroplasts of type doug-2 are limited to populations E73 and C57 which are related in the nuclear phylogeny. C57, however, is polymorphic for chloroplast types doug-2 and doug-4 and population D68, which also belongs to this group on the basis of nuclear characters, contains chloroplasts of the type doug-3.

Figure 8 shows the geographic distribution of the different *M. douglasii* populations with their chloroplast types. *Microseris douglasii* and *M. bigelovii* occur side by side in the area between San Luis Obispo and Morro Bay, and until recently occurred in the San Francisco Bay region including the lower Sacramento River area. *Microseris douglasii* accessions B13, B15/21, B77, C57a, E60b, c, and E63a, b, c which contain the doug-4 (*M. bigelovii*) type cpDNA occur in this area. However, *M. douglasii* accessions E68b (Cortina Ridge) and B39 (Tularcitos) contain doug-4 (*M. bigelovii*) chloroplasts and occur in regions where there is no indication that the two species ever were in contact. *Microseris douglasii* population D68 which contains doug-3 chloroplasts (possibly derived from the common ancestor of *M. bigelovii* and *M. pygmaea*) also occurs outside of the distribution area of *M. bigelovii*.

Discussion

Our data show that the discordance between the chloroplast and nuclear phylogenies of the annual species of *Microseris*, first suggested by the cpDNA data of WALLACE & JANSEN (1990), is real. Additional cpDNA markers clearly support a phylogenetic hypothesis for the chloroplast genomes that does not parallel the phylogeny based on nuclear markers.

Fig. 8. Geographic distribution of various chloroplast types in *Microseris douglasii* populations. Polymorphic populations: dot in open triangle, doug-1 and doug-4 chloroplasts; dot in open square, doug-2 and doug-4 chloroplasts. Arrows point towards areas where *M. douglasii* occurs side by side with *M. bigelovii*

The use of RAPDs for the construction of phylogenetic relationships may require some justification. Most RAPD variation is intraspecific allelic variation, and genetic similarity based on RAPDs may be due more to gene flow and recombination than indicate common ancestry (CLARK & LANIGAN 1993, WHITKUS & al. 1994). We have therefore also performed a cladistic analysis of the RAPD variation and have shown that this reveals some species-specific autapomorphic bands and some synapomorphies. These have been confirmed by hybridization, and the nature of amplification products that are informative about the phylogeny of the species is under study.

Most of the RAPD variation is indeed intraspecific, and there is less evidence for genetically isolated subspecific units in *M. douglasii* than there has been found in *M. elegans* (VAN HEUSDEN & BACHMANN 1992a), *M. bigelovii* (VAN HEUSDEN & BACHMANN 1992b), or *M. pygmaea* (VAN HEUSDEN & BACHMANN 1992c). This agrees

with the intraspecific morphological variation in *M. douglasii* (BACHMANN & BATTJES 1994). Considering the extreme degree of inbreeding (CHAMBERS 1955, BACHMANN & ROELOFS 1995), and the scattered distribution in isolated populations (CHAMBERS 1955), the random association and distribution of morphological and RAPD characters suggests an imperceptibly slow but highly effective gene flow and the selective neutrality of many of the multilocus genotypes.

The nuclear phylogeny based on RAPDs agrees down to the details with that based on morphological markers, especially the reduction of microsporangium numbers from four to two which separates *M. elegans*, *M. bigelovii* and *M. pygmaea* from *M. douglasii* (BATTJES & al. 1994). It also confirms the close association of the Chilean *M. pygmaea* with the North American coastal species, *M. bigelovii* (VAN HOUTEN & al. 1993) and thereby provides additional information about this case of speciation after long-distance dispersal.

This association is also supported by three synapomorphic RFLPs in the chloroplast data (Fig. 6 A, E, M), and the discrepancies between chloroplast and nuclear DNA can be described as the occurrence of chloroplasts from the *M. pygmaea/bigelovii* lineage ("doug-3"), or specifically from *M. bigelovii* ("doug-4"), in *M. douglasii* (Fig. 7). Such discrepancies could only be discovered once molecular markers for chloroplast phylogenies became available. Since then they have been found with a unsuspected frequency. RIESEBERG & WENDEL (1993) reviewed 37 examples of "chloroplast capture", SOLTIS & KUZOFF (1995) put the number at "well above 50".

Various explanations have been suggested for discrepancies between chloroplast and nuclear phylogenies (reviewed by SOLTIS & KUZOFF 1995). At the taxonomic level that we are dealing with here, and excluding artefacts of tree construction, only two are likely: lineage sorting from an ancestor containing a mixture of chloroplast types (RIESEBERG & WENDEL 1993, WENDEL & al. 1993) and hybridization followed by the exclusion of the maternal nuclear genome. Since we have found populations of *M. douglasii* polymorphic for chloroplast types (but never individuals with mixed chloroplast populations), lineage sorting could still be going on in *M. douglasii*, but the horizontal transfer of chloroplasts through hybridization seems more likely.

This is supported by the observation that the polymorphism for chloroplast types doug-1 and doug-4 in population E68 of *M. douglasii* is paralleled by a polymorphism for flower color and leaf shape that suggests possible remnants of nuclear genes from *M. bigelovii* in the *M. douglasii* nuclear genomes (ROELOFS & BACHMANN 1995). A similar correlation is seen in population C57, but not in other populations polymorphic for chloroplast type. The introgression of small amounts of nuclear material from one species into another is difficult to prove, but RAPD polymorphisms between *M. douglasii* and *M. bigelovii* have been mapped (BACHMANN & HOMBERGEN 1996) relative to diagnostic characters such as leaf shape (BACHMANN & al. 1984) and flower color, and this, together with the molecular hybridization experiments cited above may permit the demonstration of remnants of nuclear introgression. It still remains to be shown, how and why the maternal nuclear genome was eliminated.

The geographic distribution of the chloroplast types within *M. douglasii* suggests that once they have entered the species in areas of sympatry, they can be

spread throughout *M. douglasii* even in areas inaccessible to *M. bigelovii*, such as Cortina Ridge (E68) or Tularcitos (B39).

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