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Nuclear DNA markers of the Australian tetraploid *Microseris scapigera* and its North American diploid relatives

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Abstract. The allotetraploid lactucean *Microseris scapigera* of Australia and New Zealand has presumably arisen in western North America by hybridization between an annual and a perennial diploid species followed by polyploidization and long-distance dispersal. A phylogenetic tree of various North American diploids, based on RFLPs in the nuclear DNA, confirmed the division of the genus into a clade containing the diploid annuals and a clade containing the diploid perennials. Four RFLP markers were shared among all accessions of *M. scapigera* and all the diploid accessions. Twelve markers found in the outgroup (*Uropappus lindleyi*) were absent in all *Microseris*. A cladogram of plants from six populations of *M. scapigera* based on eight RFLP markers shows a progressive specialization of three clades of two populations each. Two populations without any markers differentiating them from the North American diploids form the basic clade. These consist of plants with an apparently derived morphology that are self-compatible (or agamospermic) and thereby differ from most *M. scapigera*. Few markers in *M. scapigera* could be attributed to one or the other parental genome. As yet, we have found only one ITS 1 sequence of the nuclear ribosomal cistrons in *M. scapigera*. This sequence has features of both parental sequences.

Key words: *Microseris* – Long-distance dispersal – Nuclear RFLPs – ITS 1 sequence – Phylogeny

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Introduction

Microseris scapigera is a wide-spread and variable perennial lactucean of New Zealand and southeastern Australia. The species was first collected in New Zealand by Joseph Banks and D. C. Solander in 1769 during Captain Cook's first voyage and was included in the genus *Scorzonera* until J. D. Hooker in 1853 recognized that it was congeneric with *Microseris*, a genus established for the Chilean annual *M. pygmaea* D. Don. In 1866, C. H. Schultz-Bipontinus joined these two widely disjunct species with a group of annual and perennial species of western North America and thereby identified the natural clade. Chambers (1955) showed that *M. scapigera* is an allotetraploid (4x = 36) with 18 chromosomes as large as those of the perennial species of *Microseris* and 18 as small as those of the annuals. Morphologically, *M. scapigera* is intermediate between the Californian annuals and the isolated perennial *M. borealis* which grows on boggy meadows from Oregon to coastal Alaska. These observations suggest that *M. scapigera* has originated from an annual × perennial hybrid in western North America after chromosome doubling and long-distance dispersal to Australia or New Zealand. This scenario involves several very unlikely chance events. The North America-Australia disjunction seems to be unique for higher plants. Even so, diploid (annual and sterile) hybrids between *M. borealis* and Californian annuals have been obtained that resemble *M. borealis* morphologically, and triploid hybrids between *M. scapigera* and the diploid North American perennial, *M. laciniata*, are vigorous but sterile (Chambers and Bachmann, unpublished).

Molecular polymorphisms can provide independent evidence for the origin of *M. scapigera*. Wallace and

Jansen (1990) have analyzed the phylogeny of the chloroplast DNA (cpDNA) of all species of *Microseris*. They found that the cpDNA places *M. scapigera* in a strongly supported monophyletic group which includes all annual diploid species and all allotetraploids. The cpDNAs of all diploid perennials, including *M. borealis*, form another monophyletic group. These data support the speciation scenario suggested by Chambers (1955) and specifically indicate that the primary hybrid originated from the pollination of an annual plant by a perennial.

Here, we analyze restriction fragment length polymorphisms (RFLPs) in the nuclear DNA of *M. scapigera* and representative annual and perennial species. The aim of this investigation is to identify nuclear DNA markers shared by *M. scapigera* and (one or more of) the annual or perennial species. Such markers will allow a separate investigation of the two parental genomes and a determination of the relationship between these genomes and those of existing annual and perennial species.

Materials and methods

Plants

The strains used are listed in Table 1. Strains of the annual species of *Microseris* and of *Uropappus lindleyi* were maintained as inbred lines from individual field-collected plants. Each annual species is represented by two strains which previously have been identified by random amplified DNA polymorphisms (RAPDs; Williams et al. 1990) as the genetically most-divergent strains of the species in our collection (Van Heusden and Bachmann 1992a–c). Population samples of the perennials were raised from seed collected in nature and individual plants (two for each strain of *M. scapigera*) were selected at random from these populations. DNA was isolated from freeze-dried leaves as previously described (Vlot et al. 1992).

Southern blotting, hybridizations

DNA was digested with *Hin*fI according to conditions recommended by the manufacturer (BRL). Restricted DNA was separated on 6% polyacrylamide gels and transferred to Hybond N™ membranes (Kreitman and Aguadé 1986; Gebhardt et al. 1989a, b). Blots were hybridized overnight with ^{32}P random primed labelled DNA (Feinberg and Vogelstein 1983) at 65 °C. Blots were washed twice for 20 min each with 2 × SSC at room temperature, and twice with 2 × SSC at 65 °C. Depending on signal strength Kodak X-OMAT-AR films were exposed for 1–5 days.

Probes

Total genomic DNA was isolated from *Microseris pygmaea* strain C96. DNA was digested with *Pst*I (probe 12A) or *Eco*RI (all other probes), electrophoretically separated on 0.8% agarose gels (BRL), and fragments of the size range 0.5–2 kb were cut out. An additional NACS™ column (BRL) purification step was performed (Landry and Michelmore 1985). Fragments were ligated in dephosphorylated pUC18 plasmids, *E. coli* NM522 cells were transformed and plated on IPTG- X-gal plates. White colonies were picked and screened for chloroplast DNA sequences by colony hybridization. Clones that gave no signals were selected and DNA was isolated by miniprep procedures (Sambrook et al. 1989). Gel-purified fragments were used as probes for the detection of RFLPs.

PCR amplification, cloning and sequencing

PCR reactions were performed in buffer supplied by the manufacturer (Sphaero Q), together with 50 µM each of dATP, dTTP, dCTP and dGTP, 10 µM of each primer and 0.2 Units of Super *Taq* (Sphaero Q) in a final volume of 50 µl. Amplification temperatures were 60 s annealing at 42 °C, 90 s extension at 72 °C and 60 s denaturing at 94 °C; 45 cycles were performed in a

Table 1. List of *Microseris* species and strains used in this study. *Uropappus lindleyi* serves as outgroup. "Type" refers to ploidy (2n, 4n) and annual (an) or perennial (per) habit

Species	Abbreviation	Type	Strain	Origin
Uropappus lindleyi	lin	2n an	D59	California: Monterey Co.: Laureles Grade
M. bigelovii	big	2n an	C93	California: Santa Barbara Co.: Pt. Sal
M. bigelovii	big	2n an	C94	Canada: British Columbia: Victoria
M. borealis	bor	2n per	C91	Oregon: Clackamas Co.: Mt. Hood
M. douglasii	doug	2n an	B14	California: Fresno Co.: Parkfield
M. douglasii	doug	2n an	D40	California: San Luis Obispo Co.: Cholame
M. elegans	ele	2n an	D03	California: San Luis Obispo Co.: Cholame
M. elegans	ele	2n an	A24	California: Humboldt Co.: Garberville
M. laciniata	lac	2n per	A60	California: Mendocino Co.: Eel River
M. laciniata	lac	2n per	B85	Oregon: Josephine Co.: Grant's Pass
M. nutans	nut	2n per	C06	Oregon: Jefferson Co.: Camp Sherman
M. pygmaea	pyg	2n an	A92	Chile: Santiago Province
M. pygmaea	pyg	2n an	C96	Chile: Prov. de Choapa: El Teniente
M. scapigera	scap	4n per	F01	Australia: Victoria: Bannockburn
M. scapigera	scap	4n per	E02	Australia: Victoria: Beveridge
M. scapigera	scap	4n per	D91	Australia: Victoria: Blythvale
M. scapigera	scap	4n per	B94	Australia: New South Wales: Armidale
M. scapigera	scap	4n per	G02	Australia: Victoria: Dunmoochin
M. scapigera	scap	4n per	E74	Australia: Victoria: Streatham

programmable heat block (Gene Ataq Controller, Pharmacia). After amplification, the appropriate length fragment was gel-purified, blunt-ended-ligated in pUC18 plasmids and *E. coli* NM522 cells were transformed as described above. Positive clones were sequenced according to the dideoxy termination method using the Sequenase version 2.0 kit (USB) and an S2 sequencing apparatus model obtained from BRL.

Primers used for amplification and sequencing were Z-234 and Z-895R (Zurawski et al. 1984) for the chloroplast *rbcL* gene, ITS 2 and ITS 5 (White et al. 1990) for the internal transcribed spacer (ITS 1) of the nuclear ribosomal RNA cistrons, and Puc/M13 reverse (Q540A; Promega) and forward (Q539A; Promega) sequencing primers.

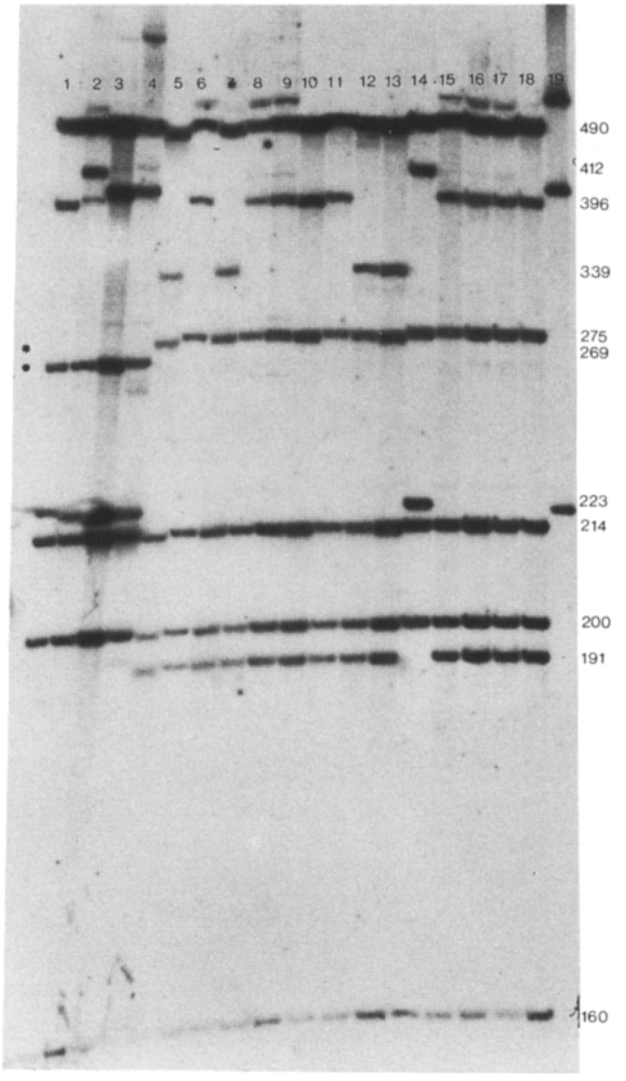

Fig. 1. DNA of various accessions of *Microseris* cut with *Hin*fI and hybridized with genomic probe N46. Fragment lengths in base pairs on the right. *Lanes 1 to 18*, DNAs from single plants of strains C06, A60, B85, C91, F01, D91, F01, D91, F01, B94, E02, G02, G02, E74, E74, D59, C93, D03, B14, A92 (see Table 1). *Lane 19*, size marker. Putative allelic fragments at 269 bp (perennials, *lanes 1 to 4*) and 275 bp (*M. scapigera, lanes 5 to 13*; *U. lindleyi, lane 14*; and annual *Microseris, lanes 15 to 18*) are marked with *asterisks*

Phylogenetic analysis

All phylogenetically informative bands detected with 14 probes were treated as equally-weighted characters and phylogenetic trees were calculated with PAUP version 3.0 (Swofford 1990). ITS 1 sequences from ribosomal cistrons were aligned by inspection.

Results

Eighty-one bands (identified by probe/band length) were detected with 14 probes in *Hin*fI-digested DNA of 19 selected individual plants (Fig. 1, Table 2). Some of the bands detected with a probe are probably alleles on the basis of their strain distribution (e.g., N212/228 and N212/230; N102/313 and N102/275; N46/275 and

Table 2. Distribution of the *Hin*fI restriction fragments detected with various nuclear probes among the examined strains. Two plants each of strains E74 and F01 differed by intrastrain polymorphisms. E74008 and F01007 refer to single plants in these strains. (NT, not tested)

Probe	Band length (basepairs)	Taxa
N169	1059	All
	661	All
N268	457	All
	347	All except lin, big, pyg
	245	bor
	234	All except bor
	209	pyg, big
	335	lin
N212	1175	Annuals
	804	Annuals
	228	All except lin
	230	lin
N246	891	Annuals
	221	Annuals
	204	All
N102	313	All except scap F01 and E74
	275	scap F01, E74
	223	All
N226	417	Perennials, scap F01, E74008
	204	All
	459	All except lin
	389	All except perennials, scap F01, E74008
		lin
	400	lin
	479	All except lin
	891	lin
N109	462	All except scap F01, B94, G02, E74
	417	scap F01, B94, G02, E74
	261	All except big, ele, pyg
	223	All except lin
	220	lin
	216	big, ele, pyg
	265	lin

Table 2. (*Continued*)

Probe	Band length (basepairs)	Taxa
N61	468	All except lin, scap B94
	447	scap B94
	452	lin
	202	All
N111	219	nut, bor, scap
	215	lac
	230	pyg
	223	doug
	225	ele
	222	big
	204	All
	217	lin
N104	275	All except scap F01, E74
	245	scap F01, E74
	389	Annuals
	420	bor
	218	All
	412	nut, lac
	230	pyg, big, doug B14, scap
	182	lin, ele D03
	160	Annuals, scap
N7	282	big, doug, pyg
	468	All except lin, scap B94
	437	scap B94
	451	lin
	481	big, doug, pyg
N230	216	All
	180	pyg A92 (C96 NT), big C93 (C94 NT), scap
	407	All
	204	Perennials
	184	lin
	209	ele, doug
12A	330	nut, lac
	316	bor, scap
	355	Annuals
	295	scap B94
	288	scap F01007
	457	lin
N46	191	Annuals, scap
	200	All
	396	All except lin, scap F01, E74
	339	scap F01, E74
	412	lin
	275	All except perennials
	269	Perennials
	490	All
	223	Perennials, lin
	160	All
	214	All

N46/269: Fig. 1). The variability of the bands covers a wide spectrum. Fifteen of the bands were present in all samples, while we found two polymorphisms between specimens from one population of *M. scapigera* (N226/389 and N226/417; extra band 12A/288; Table 2). Twenty-two bands were considered phylogenetically informative for the diploid species (present or absent in the specimens of at least two species). The strict consensus of the two most-parsimonious trees for the diploid plants based on these 22 bands (27 steps, consistency index 0.815) is shown in Fig. 2. The two trees differ in the relative positions of *M. douglasii* and *M. elegans*. The position of *M. borealis* at the base of the perennial clade shown in Fig. 2 is not preserved in a bootstrap 80% majority-rule consensus tree of the data, which preserves the clade combining *M. bigelovii* with the Chilean *M. pygmaea*.

A tentative phylogeny of the strains of *M. scapigera* based on eight informative bands is shown in Fig. 3. A bootstrap analysis was performed to test the reliability of this intraspecific phylogeny. The morphologically-similar plants of populations F01 and E74 seem to belong to a monophyletic derived group, while plants of populations D91 and E02 show no synapomorphic or autapomorphic bands beyond the ones common to all *M. scapigera*.

Three bands unite all *Microseris* including *M. scapigera*. The data relevant to the position of *M. scapigera* within *Microseris* are summarized in Fig. 4. There are three bands common to *M. scapigera* and the annual species of *Microseris* (N46/191; N46/275; N46/160). Two more bands are shared between *M. scapigera* and

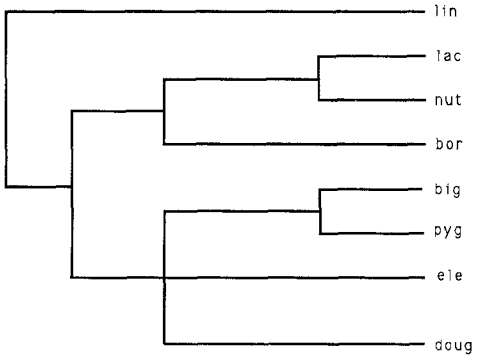

Fig. 2. Strict consensus of two minimal-length trees (27 steps) of diploid *Microseris* species (see Table 1). Branch lengths have no significance. Perennials (lac, nut, bor) and annuals (big, pyg, ele, doug) form separate clades

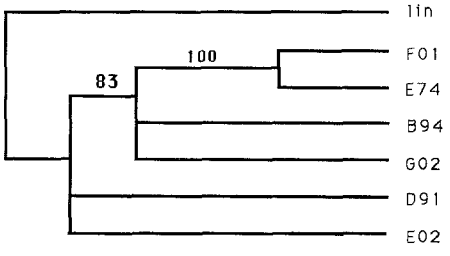

Fig. 3. Bootstrap 80% majority rule consensus tree of *Microseris scapigera* collected from six distinct populations (see Table 1). *Numbers* on branches refer to times that populations were grouped together in the bootstrap analysis (100 replicates)

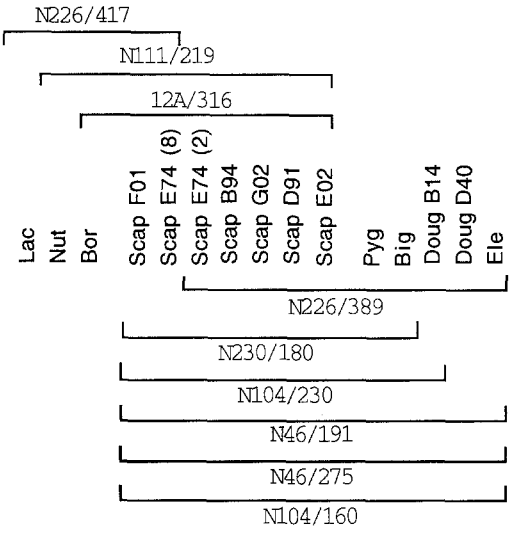

Fig. 4. Distribution of restriction fragments (probe/fragment length) shared among perennial (lac, nut, bor: *brackets above*) or annual diploids (pyg, big, doug, ele: *brackets below*) and *Microseris scapigera*

some of the annual strains (N104/230; N230/180). *M. scapigera* shares one band (12A/316) with *M. borealis*, and another band (N111/219) with *M. borealis* and *M. nutans*. A third band (N226/417) is shared by the perennials and some plants of *M. scapigera*. The same probe detects another band (N226/389) which is present in all plants not showing N226/417, i.e., all annuals and some *M. scapigera*. It is striking that of two individuals from the (derived) population E74, one shows the "perennial" and one the "annual" marker for probe N226.

We have amplified and cloned a region between primers Z-234 and Z-895R of the chloroplast *rbcL* gene from *M. scapigera* and *M. pygmaea* (600 bp) and found no difference in the nucleotide sequence between the two species (data not shown). We have amplified and cloned the internal transcribed spacer, ITS 1, of the nuclear ribosomal RNA cistrons from *M. scapigera* D91, from the annuals *M. douglasii* D40 and *M. pygmaea* C96, from the perennials *M. laciniata* A60 and *M. borealis* C91, and from *U. lindleyi* D59. The sequences are shown in Fig. 5. The ITS 1 consensus sequence consists of 256 nucleotide pairs. We found 47 variable positions within ITS 1 and no variable positions in the sequenced parts of the 18*s* and 5.8*s* regions (together 65 bp). Twenty (43%) of the variable positions separate *U. lindleyi* from *Microseris*; another 19 positions are not phylogenetically informative. At the remaining eight positions, the ITS 1 sequence of *M. scapigera* shares nucleotides with the annuals at three (nucleotides 101, 102, 113), and with the diploid perennials at five (140, 238, 243, 259, 294), positions. Two of these diagnostic positions define restriction sites, an annual-specific *Nar*I site (GGCGCC) including position 140 and a perennial-specific *Ssp*I site (AATATT) including position 238. The *Nar*I site is absent, and the *Ssp*I site present, in the *M. scapigera* sequence. We have digested six independent ITS 1 clones from *M. scapigera* with *Nar*I and have hybridized an *Ssp*I digest of total genomic DNA of *M. scapigera* with an ITS 1 probe. None of the six clones was cut by *Nar*I, while the genomic restriction pattern indicates the presence of an *Ssp*I site in all ITS 1 sequences of *M. scapigera*.

Discussion

RFLP analysis of nuclear DNA of *Microseris* and the comparison of the ITS 1 base sequences have confirmed the results of Wallace and Jansen (1990) on chloroplast DNA. The annual *Uropappus lindleyi* is clearly separate from the rest of *Microseris* in which it had been included until its close molecular association with *Nothocalais* and *Agoseris* led to its separation (Jansen et al. 1991). The diploid species of *Microseris* form two monophyletic groups, one comprising the annual, the other the perennial species. Within these groups, the species relationships are uncertain on the basis of the cpDNA markers (Wallace and Jansen 1990). Even a large number of RAPD markers was unable to resolve the relationship between the Chilean annual, *M. pygmaea*, and two closely-related North American diploids, *M. bigelovii* and *M. elegans* (Van Heusden and Bachmann 1992c). Here, we have found an association between *M. pygmaea* and *M. bigelovii* which is supported by probes N230, N268, N109 (together with *M. elegans*) and N7 (together with *M. douglasii*). In order to resolve the phylogenetic relationship among the diploid annuals, we still need a thorough analysis of the intraspecific variation in *M. douglasii*.

Intraspecific variation for morphological characters and the degree of self-incompatibility in *M. scapigera* throughout its distribution range in Australia and New Zealand has been extensively studied by Sneddon (1977), who recognized the Australian plants as a separate species, *M. lanceolata* (Walp.) Sch.-Bip. and provisionally described three "races" within this species. The few specimens of *M. scapigera* included in this study represent morphologically and ecologically distinct biotypes of the Australian taxon. Since self-incompatibility is typical for the Australian *Microseris* (Chambers 1955; Sneddon 1977) and since there is considerable morphological variation within each population, it is remarkable that our molecular markers separate the Australian plants into three distinct groups (Fig. 2) which agree with morphological differences. The phylogenetic sequence of these groups is defined by the alleles N109/417 which is a

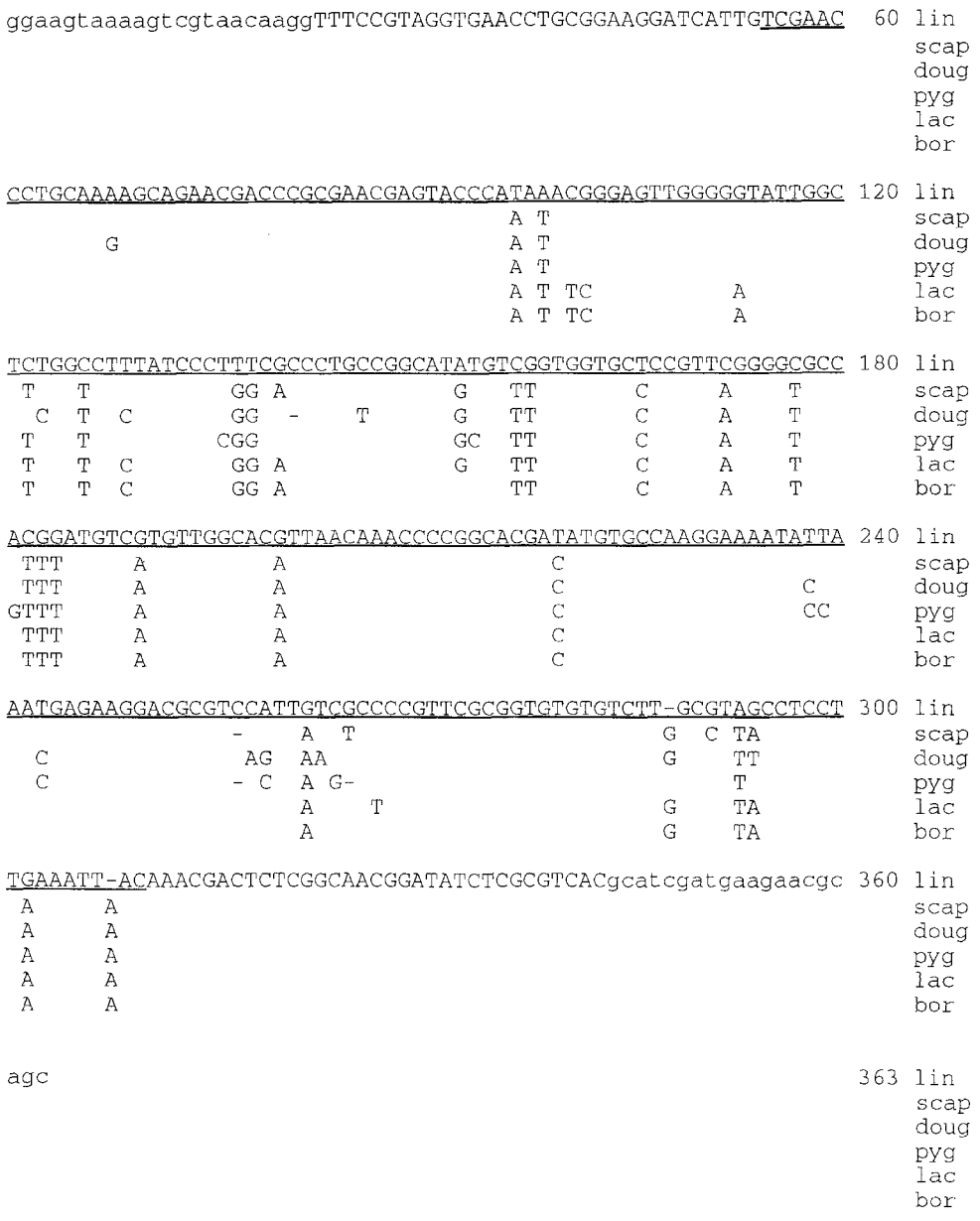

Fig. 5. Nucleotide sequence of the nuclear ribosomal ITS 1 spacer region and 18*S* and 5.8*S* flanking regions of several *Microseris* species (see Table 1) relative to *Uropappus lindleyi* (above). Primer sequences are shown in *lower case*, the ITS 1 sequence is *underlined*

synapomorphy for plants from populations E74, F01, G02 and B94, and alleles N46/339, N102/275, and N104/245 which is a synapomorphy for E74 and F01. Plants from populations D91 and E02 have no apomorphic markers beyond those common to all *M. scapigera* and are therefore presumably the most primitive accessions in our collection. This result is very surprising since these two populations deviate strikingly from others in the species. Morphologically they fit the definition of "*M. lanceolata* race 3" (Sneddon 1977), which Sneddon knew only from herbarium material. In contrast with most plants of *M. scapigera*, plants from populations D91 and E02 readily set seed in isolation. DNA fingerprint patterns using the oligonucleotide $(GATA)_4$ as a probe revealed very little variation among plants of these populations (data not shown) which indicates extreme inbreeding or even apomictic reproduction. While their morphology does not correspond with our expectations for the hypothetical ancestor of all *M. scapigera* that arrived by long-distance dispersal from North America, their reproductive mode would resolve one of the improbable features of our speciation scenario: establishment after long-distance dispersal would be very unlikely for an

obligate outcrosser. The availability of these plants certainly is crucial for a further analysis of the evolution of *M. scapigera*. A corollary of this phylogenetic pattern is the prediction that the plants from New Zealand should be derived from Australian plants.

Both the cpDNA data of Wallace and Jansen (1990) and our nuclear DNA data leave no doubt about the very close relationship of *M. scapigera* with its North American relatives. We have found three markers for all *Microseris* and three synapomorphic markers for the diploid annuals together with *M. scapigera*. We have found no synapomorphy for all perennials and *M. scapigera*. All three potential "perennial markers" (Fig. 3) are found in *M. borealis*, which agrees with Chambers' morphological analysis. A similar overlap between *M. scapigera* and *M. bigelovii* and *M. pygmaea* (and one of two strains of *M. douglasii*) among the annuals does not agree with the derived position of these two species. The most parsimonious interpretation of the data is a primitive position of the annual ancestor of *M. scapigera* among the annuals.

Nuclear RFLPs have been used previously to examine diploid/tetraploid relationships. Song et al. (1988) have studied the classical three amphidiploid combinations of three diploid *Brassica* species. They found RFLP markers specific for the A, B, and C genomes, but combined all accessions of both ploidy levels in their cladogram. Kochert et al. (1991) compared eight cultivars and 14 species of peanuts (*Arachis*) and found that they could recognize specific restriction fragments of the diploid parental species in the banding patterns of the tetraploids. Both cytotypes were combined in a dendrogram based on band sharing among the various species. Brummer et al. (1991) prepared independent cladograms for the diploid and the tetraploid accessions of alfalfa (*Medicago sativa*).

Here, we analyzed the relationship of the diploid species and tried to locate the two ancestors of *M. scapigera* within this tree. Two observations suggest that the "annual" and the "perennial" genomes combined in *M. scapigera* may not have evolved completely independently. One of these is the strange distribution of the "annual" and "perennial" markers detected with probe N226 (Fig. 3) which seem to be mutually exclusive. The other is the single ITS 1 sequence that we have found up to now in *M. scapigera*. Three nucleotide positions in its 5′ half suggest that this sequence comes from the annual ancestor, five positions in the 3′ half link it with the perennials. If there is recombination between the two parental genomes, the repetitive ribosomal cistrons are a likely place for its occurrence.

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