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E. E. Schilling

Phylogenetic analysis of *Helianthus* (Asteraceae) based on chloroplast DNA restriction site data

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Abstract Chloroplast DNA (cpDNA) restriction site data were used to analyze phylogenetic relationships within *Helianthus*. Data from new samples were consistent with previous results and showed the genus to be a well-defined (11 site changes) lineage within subtribe Helianthinae. The affinities of 2 species, *Viguiera similis* and *V. phenax* (*V. ludens*) that have sometimes been included in *Helianthus* were shown to lie outside the genus. The two species of *Phoebanthus*, however, formed a phylogenetic ingroup to the perennial *Helianthus*. Within the genus, cpDNA data indicated that there are four distinct phylogenetic lineages. Two of the lineages each contained a single, annual species (*H. agrestis* and *H. porteri*, respectively). The remaining annual species collectively formed a third lineage (sect. *Helianthus*). The fourth lineage contained all of the perennial species, including those of *Phoebanthus*. Within the perennial lineage, there was little variation in restriction site characters. The 3 species of series *Pumili* formed a paraphyletic group at the base of the perennial lineage, and the 3 species of series *Ciliares* formed a group that was supported by a single restriction site character.

Key words Sunflowers · Helianthus · Asteraceae · Chloroplast DNA · Phylogenetic relationships

Introduction

There has been considerable interest in the relationships among species of *Helianthus* based in part on the agronomic importance of the common sunflower, *H. annuus*. The approach that produced a widely used classification involved morphological and crossing information (Heiser et al. 1969; Schilling and Heiser 1981). More recent work has employed detailed studies of inferred chromosome end arrangements (Chandler et al. 1986) and phytochemistry (Schilling and Mabry 1981; Schilling 1983; Schilling et al. 1987; Spring and Schilling 1989, 1990a, b, 1991). Some initial surveys based on molecular traits have also been presented (Choumane and Heizmann 1988; Gentzbittel et al. 1992; Heyraud et al. 1987).

The genus as circumscribed by Schilling and Heiser (1981) includes about 49 species of annual and perennial herbs native to North America. This generic circumscription is a modification of that of Heiser et al. (1969) by transfer of the South American species previously classified as part of *Helianthus* (Heiser 1957; Robinson 1979) to the Andean *Pappobolus* (Panero 1992) and by addition of *H. porteri*, which had previously been variously classified in *Heliomeris* or *Viguiera* (Blake 1918). Although it is distinctive morphologically, *Helianthus* is not defined within subtribe Helianthinae by any exclusive autapomorphies. Distinctive characters found widely in *Helianthus* include an herbaceous habit, a deciduous pappus of two awns (and sometimes additional scales), trifid pales, and a pronounced terminal style appendage.

The relationships and evolutionary history of the species of *H.* sect. *Helianthus* have been the topic of a recent series of detailed studies (Dorado et al. 1992; Rieseberg et al. 1988; Rieseberg 1991; Rieseberg et al. 1990, 1991; Rieseberg and Brunsfeld 1991; Rieseberg et al. 1993). These studies have provided a fairly comprehensive dataset for sect. *Helianthus* that suggests the likelihood that the section is monophyletic as currently circumscribed. They have also documented thoroughly that interspecific hybridization has played an important role in speciation and divergence within the section.

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E. E. Schilling (✉)
Department of Botany, University of Tennessee, Knoxville, TN 37996-1100, USA

The study presented here was undertaken primarily to focus on the relationships of the primarily perennial species classified as *H.* sect. *Atrorubens* and *H.* sect. *Ciliares*. In particular, species of sect. *Atrorubens* exhibit much of the diversity and taxonomic problems in *Helianthus*. The section includes diploids, tetraploids, and hexaploids. Studies of chloroplast (cp), DNA restriction site variation were initiated to attempt to characterize phylogenetic lineages within sect. *Atrorubens* and to seek information on relationships between diploid and polyploid species. The results showed a surprising lack of variation among species of sect. *Atrorubens* for restriction site features, but helped to clarify the circumscription of the section and its relationships to sects. *Helianthus*, *Agrestes*, and *Ciliares*. In particular, the presence of four major phylogenetic lineages within *Helianthus*, which correspond in part but not entirely to previously recognized taxa, was suggested by the restriction site studies.

Materials and methods

Plant materials

Samples representing *Helianthus* sect. *Agrestes*, sect. *Ciliares*, 30 of the 32 species of sect. *Atrorubens* as well as the 2 species of *Phoebanthus* were analyzed (Table 1). Published information (Rieseberg 1991; Rieseberg et al. 1991) was used as the primary source of data for sect. *Helianthus*, although samples of 2 species were included for direct comparisons. Samples of 2 species that were included in *Helianthus* by Heiser et al. (1969) and Rogers et al. (1982) but not by Schilling and Heiser (1981) were also analyzed. Leaves were collected from naturally occurring populations and were stored in a cooler or in liquid N_2 before DNA extraction. Preparations of total DNA were made from fresh (0.5–2.0 g) or frozen (1–3 g) leaves by the procedure of Doyle and Doyle (1987).

Chloroplast DNA restriction site analysis

Methods for DNA restriction site analysis generally followed that of Jansen and Palmer (1988). Restriction endonuclease digestions (utilizing the following enzymes: *Ava*I, *Bam*HI, *Ban*I, *Ban*II, *Bcl*I, *Bgl*II, *Bst*NI, *Bst*XI, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*II, *Hinc*II, *Hin*dIII, *Nco*I, *Nsi*I, *Xba*I), agarose gel electrophoresis, and bidirectional transfer of DNA fragments from agarose gels to Zetabind (AMF CUNO), Hybond (Amersham), or Boehringer-Mannheim nylon filters were performed as described in Palmer (1986) and Jansen and Palmer (1987). For some sets of samples, the preparation of digoxigenin-labeled probes and filter hybridizations followed the manufacturer's instructions (Boehringer-Mannheim, "Genius Kit", Indianapolis, Ind.). For most samples, the preparation of $[^{32}\mathrm{p}]$-labeled probes and filter hybridizations followed Palmer (1986), except that random priming rather than nick-translation methodology was employed to label probes. The 22 cloned restriction fragments of lettuce cpDNA were combined into batches for the filter hybridization experiments. In general, the lower limit of detection of fragments was 0.4–0.5 kb. Mapping of fragments was done relative to data of Jansen and Palmer (1987, 1988), Schilling and Jansen (1989), K.-J. Kim (unpublished data) and J. L. Panero (unpublished data).

Table 1 List of samples of *Helianthus* and related species examined for DNA restriction site analyses

Helianthus		
Sect. *Agrestes*		
90-32	*H. agrestis*	Manatee County (Co.), Fla.
Sect. *Atrorubens*		
Ser. *Angustifolii*		
89-6	*H. angustifolius*	Morgan Co., Tenn.
48	*H. carnosus*	Flagler Co., Fla.
197	*H. floridanus*	Madison Co., Fla.
200	*H. heterophyllus*	Wakulla Co., Fla.
227	*H. longifolius*	DeKalb Co., Ala.
OS61	*H. radula*	Jackson Co., Fla.
OS54	*H. simulans*	Baton Rouge Par., La.
Ser. *Atrorubentes*		
150	*H. atrorubens*	Sevier Co., Tenn.
213	*H. occidentalis*	Wilson Co., Tenn.
H439	*H. pauciflorus*	(County not known), Ind.
1102	*H. silphioides*	Coffee Co., Tenn.
Ser. *Corona-solis*		
140	*H. decapetalus*	Polk Co., Tenn.
98	*H. divaricatus*	Hickman Co., Tenn.
90-16	*H. eggertii*	Lewis Co., Tenn.
171	*H. giganteus*	Stokes Co., N.C.
Y40	*H. grosseserratus*	Platt Co., Neb.
90-17	*H. hirsutus*	Lewis Co., Tenn.
238	*H. maximiliani*	Knox Co., Tenn.
90-8	*H. mollis*	Williamson Co., Tenn.
257	*H. nuttallii*	Boulder Co., Colo.
256	*H. resinosus*	Barbour Co., Ala.
IN-1	*H. salicifolius*	Origin not known
186c	*H. schweinitzii*	York Co., S.C.
133	*H. strumosus*	Knox Co., Tenn.
89-5	*H. tuberosus*	Morgan Co., Tenn.
106	*H.* × *multiflorus*	Cultivated
Ser. *Microcephali*		
138	*H. glaucophyllus*	Unicoi Co., Tenn.
125	*H. laevigatus*	Bedford Co., Va.
134	*H. microcephalus*	Pulaski Co., Va.
211	*H. smithii*	Randolph Co., Ala.
90-31	*H. porteri*	Alexander Co., N.C.
Sect. *Ciliares*		
Ser. *Ciliares*		
280	*H. arizonensis*	Navajo Co., Ariz.
RJ83	*H. ciliaris*	DeBaca Co., N.M.
2179	*H. laciniatus*	Coahuila, Mexico
Ser. *Pumili*		
420	*H. cusickii*	County unknown, Ore.
88-5	*H. gracilentus*	Los Angeles Co., Calif.
255	*H. pumilus*	Boulder Co., Colo.
Sect. *Helianthus*		
40	*H. debilis*	Cultivated, Italian White
2789	*H. niveus*	Baja California Norte
Phoebanthus		
195	*P. grandiflora*	Osceola Co., Fla.
204	*P. tenuifolia*	Franklin Co., Fla.
Viguiera		
2849	*V. similis* (*H. similis*)	Baja California Sur, Mexico
318	*V. phenax* (*H. ludens*)	Durango, Mexico

Data from *Viguiera* subg. *Bahiopsis* and *V.* sect. *Diplostichis* (Schilling et al. 1994) were used to polarize site changes within *Helianthus* and to evaluate the placement of *V. similis* and *V. phenax*. Wagner parsimony analysis was performed for restriction site data utilizing the PAUP program (Swofford 1993). Most parsimonious trees were sought by implementing the heuristic search option using the TBR branch-swapping option with MULPARS on.

Results

Chloroplast DNA restriction site variation

The approximate locations of cpDNA restriction sites in a typical member of *Helianthus* sect. *Atrorubens*, mapped relative to the lettuce *Sac*I probes, are shown in Fig. 1. The chloroplast genome of *Helianthus* is approximately 150 kb in length. Relative to the lettuce cpDNA genome there are deletions, each of about 0.4 kb in length, in the 7.7, 10.6, 12.3 and 15.4 probe regions. Especially notable is the 0.4-kb deletion in the inverted repeat region (in one of the inverted repeat areas, this is detected by the 12.3 lettuce *Sac*I probe; in the other, it occurs in the 15.4-kb region for which no lettuce cpDNA probe is available). Previous phylogenetic analysis has shown that the deletion is a derived trait within the subtribe Helianthinae, and is not observed in basal members of the subtribe, such as *Viguiera* subg. *Bahiopsis* (Schilling and Jansen 1989).

All samples of *Helianthus* shared a total of 20 restriction site changes relative to *Viguiera* subg. *Bahiopsis*, the most basal lineage of subtribe Helianthinae. Of these, 11 are synapomorphies for the genus (Table 2), including 10 that were previously reported (Schilling and Jansen 1989). The sample of *Viguiera similis* did not exhibit any of the site changes that are synapomorphic for *Helianthus*, but exhibited the 6 site changes (not shown) that have been reported to be characteristic of *V.* subg. *Bahiopsis* (Schilling and Jansen 1989; Schilling et al. 1994). The sample of *Viguiera phenax* (*Helianthus ludens*) did not exhibit any of the distinctive site changes for either *Helianthus* or for *V.* sect. *Diplostichis*. Instead, it exhibited site changes that have been observed in a relatively derived group of subtribe Helianthinae that includes *V.* subg. *Amphilepis* (Schilling and Jansen 1989; Schilling and Panero 1996a).

In all, 26 cpDNA site variants were observed among members of *Helianthus* and *Phoebanthus* (Table 2). These include some site changes that were reported previously (Schilling and Jansen 1989; Rieseberg et al. 1991), as well as newly observed ones.

Cladistic analysis of the cpDNA site data for *Helianthus* produced a single most parsimonious tree (Fig. 2). This tree had a length of 38 steps, a consistency index of 0.97 (0.92 with autapomorphies excluded), and a retention index of 0.97. One parallel restriction site change accounted for all of the homoplasy observed.

The cpDNA data suggested the presence of four major lineages within *Helianthus* (Fig. 2). Two of these each consisted of a single annual species, formed by *H. agrestis* and *H. porteri*, and supported by 5 and 3 site changes, respectively. The third lineage was formed by the remaining annual species and corresponded exactly to the group recognized as *H.* sect. *Helianthus*. This group was supported by three site changes among the enzymes that were used in this study; Rieseberg et al. (1991) report additional apomorphic site changes (as well as variability within the section) based on sampling with additional enzymes. The fourth lineage included all of the other perennial species of *Helianthus* as well as the two species of *Phoebanthus* (Fig. 2).

Within the lineage containing the perennial species, there was only limited variation between species. The species of ser. *Pumili* lacked 1 or more of the site changes exhibited by other perennials (Fig. 2). The species of *H.* ser. *Ciliares* shared 1 site change relative to most other perennials (Fig. 2). Except for a few, single, autapomorphic restriction site variants, the remaining perennial species, including the 2 of *Phoebanthus*, exhibited identical restriction site patterns.

Discussion

Data from cpDNA restriction site analysis help to confirm that *Helianthus* is monophyletic and to clarify its circumscription. All 40 samples of the genus that were examined shared 11 synapomorphic restriction site changes relative to other members of the subtribe Helianthinae. This is notable for *H. agrestis*, because Heiser et al. (1969) raised the question of whether or not it was properly placed in *Helianthus* because of its morphological distinctiveness.

The cpDNA restriction site data supported the exclusion from the genus of 2 species that had been previously included in it (Heiser et al. 1969; Rogers et al. 1982), *Viguiera similis* and *V. phenax* (*V. ludens*). *Viguiera similis* shares the characteristic cpDNA restriction site pattern of *V.* subg. *Bahiopsis*, which is consistent with its morphological similarity to *V. tomentosa* for all diagnostic characters other than pappus type (Schilling 1990). The placement of *V. phenax* is somewhat more problematic. Reassessment of its morphology would suggest a placement with *V.* sect. *Diplostichis*, which has been shown by cpDNA data to be a relatively basal group within Helianthinae (Schilling et al. 1994; Schilling and Panero 1996a). In contrast, the cpDNA data placed *V. phenax* in a relatively derived clade in the subtribe, near members of *V.* subg. *Amphilepis*.

The cpDNA restriction site data indicated that *Phoebanthus* was an ingroup phylogenetically to the perennial members of *Helianthus* (Fig. 2). This was not entirely unexpected, because Heiser et al. (1969) questioned whether *Phoebanthus* should in fact be considered to be separate from *Helianthus*. There are, however, sufficient morphologically distinct characters in *Phoebanthus* to have led Robinson (1981) to classify it in a different subtribe of Helianthinae than *Helianthus*. Thus, the question of whether to submerge *Phoebanthus* taxonomically within *Helianthus* is still open.

The cpDNA restriction site data suggested that there are four major phylogenetic lineages within *Helianthus*,

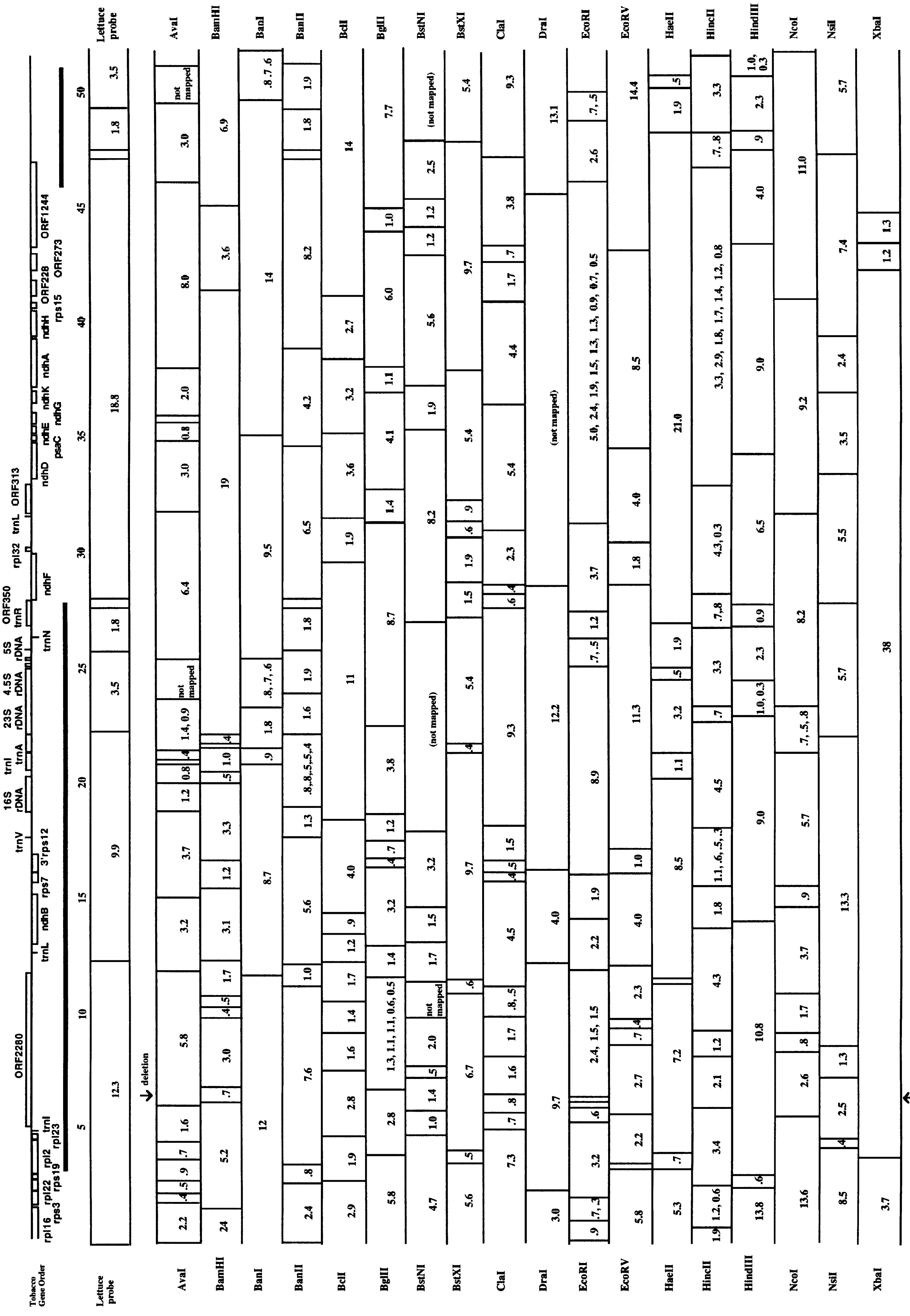
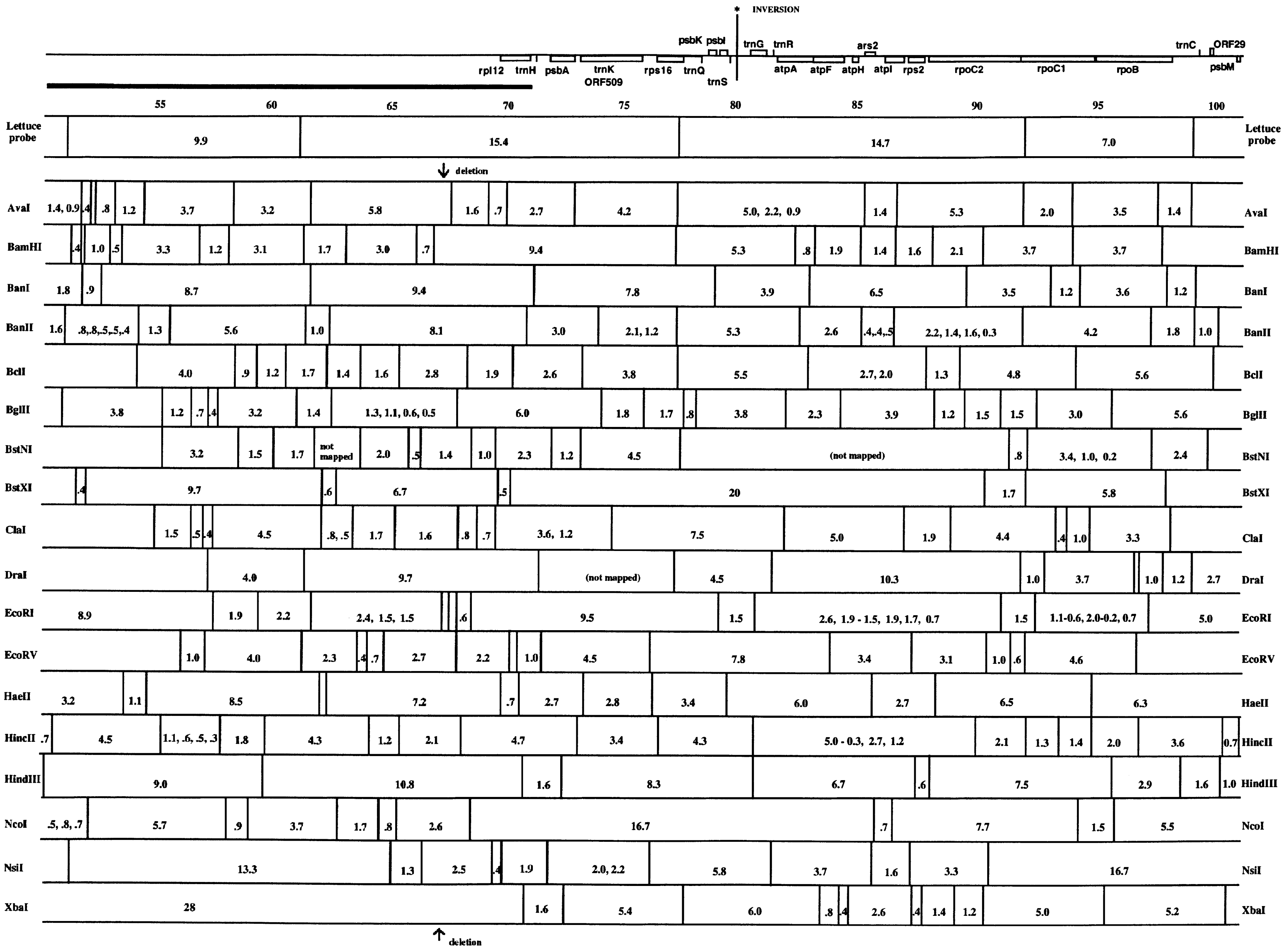


Fig. 1 See page 931 for legend



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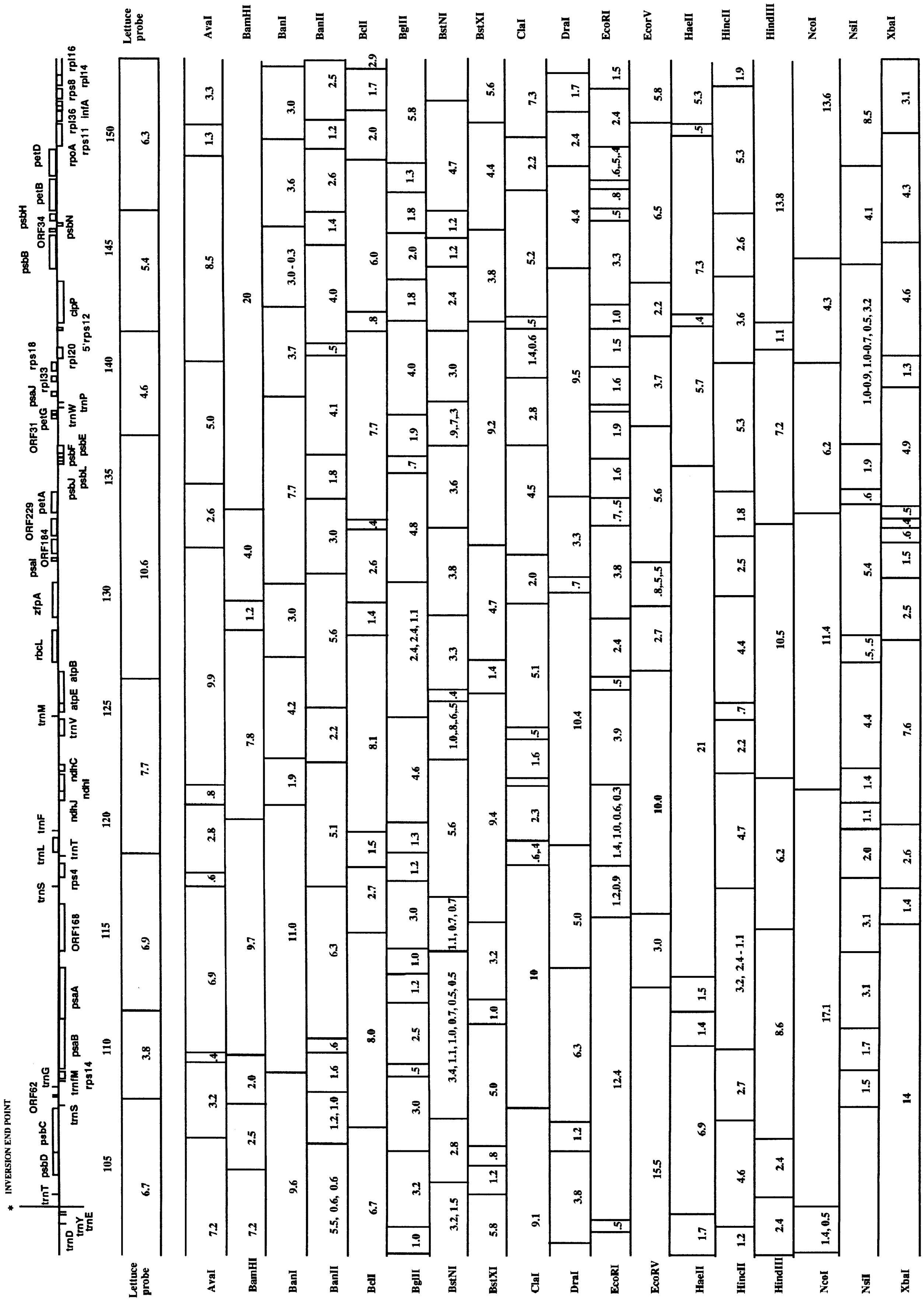

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Table 2 Distinctive chloroplast DNA restriction site variants for and within *Helianthus*. Variants 1–221 are numbered following Schilling and Jansen (1989), Schilling et al. (1994), and Schilling and Panero (1996b). Probe sizes from lettuce library are as listed by Jansen and Palmer (1987); variants in 15.4 region are as detected by 12.3 or 14.7 probes. Square brackets enclose fragments too small to be detected directly but are inferred from size change of larger fragments

Variant number	Enzyme	Probe	Fragments (kb)	Samples
Variants observed in all *Helianthus* and *Phoebanthus* samples:				
7	*Bgl*II	18.8	5.2 = 4.1 + 1.1	
22	*Bgl*II	14.7	3.3 + 0.6 = 3.9	
24	*Cla*I	14.7	1.3 + 3.1 = 4.4	
44	*Cla*I	15.4	4.8 = 1.6 + 3.2	
51	*Bcl*I	10.6	1.0 + 0.4 = 1.4	
66	*Bst*NI	7.7	5.3[+ 0.3] = 5.6	
91	*Eco*RI	6.9	2.1 = 0.9 + 1.2	
94	*Hin*dIII	6.9	4.4 + 4.2 = 8.6	
119	*Bst*NI	5.4	0.7 [+ 0.2] = 0.9	
147	*Nco*I	15.4	10.5 + 6.2 = 16.7	
222	*Cla*I	18.8	2.2 + 1.6 = 3.8	
Variants within *Helianthus* and *Phoebanthus*:				
25	*Eco*RI	14.7	9.4 = 5.6 + 3.8	*H. porteri*
43	*Cla*I	12.3	1.6 + 0.5 = 2.1	*H. angustifolius*
49	*Bam*HI	10.6	24 = 4.0 + 20	sect. *Atrorubens*, ser. *Ciliares*, *Phoebanthus*
54	*Eco*RI	10.6	3.7 = 2.1 + 1.6	*H. giganteus*
60	*Nco*I	10.6	4.7 + 6.7 = 11.4	sect. *Atrorubens*, ser. *Ciliares*, *Phoebanthus*, *H. pumilus*
76	*Bst*NI	7.0	2.0 + 0.4 = 2.4	sect. *Atrorubens*, ser. *Ciliares*, *Phoebanthus*, *H. pumilus*
92	*Eco*RV	6.9	10.0 + 3.0 = 13.0	*H. arizonensis*
97	*Bgl*II	7.0	5.6 + 0.9 = 6.5	*ser. Ciliares*
127	*Cla*I	4.6	2.0 = 1.4 + 0.6	sect. *Atrorubens*, ser. *Ciliares*, *Phoebanthus*, *H. cusickii*, *H. gracilentus*, *H. pumilus*
223	*Hinc*II	18.8	3.3 + 1.7 = 5.0	*H. agrestis*
224	*Nco*I	18.8	9.2 + 8.0 = 17.2	*H. glaucophyllus*
225	*Bam*HI	14.7	1.4 + 1.9 = 3.3	*Phoebanthus tenuifolius*
226	*Ban*II	12.3	2.4 + 0.8 = 3.2	*H. agrestis*
227	*Ava*I	10.6	4.9 = 2.5 + 2.4	*H. agrestis*
228	*Dra*I	10.6	2.3 + 1.0 = 3.3	*H. porteri*, *H. arizonensis*, *H. ciliaris*, *H. laciniatus*, *H. longifolius*
229	*Eco*RI	10.6	1.6 = 1.0 + 0.6	*H. ciliaris*, *H. laciniatus*
230	*Bst*NI	7.7	5.6 = 2.7 + 2.9	sect. *Helianthus*
231	*Dra*I	7.7	5.0 = 4.1 + 0.9	*H. agrestis*
232	*Bst*XI	7.0	5.8 + 1.7 = 7.5	sect. *Helianthus*
233	*Dra*I	6.9	11.3 = 6.3 + 5.0	sect. *Atrorubens*, ser *Ciliares*, *Phoebanthus*, *H. gracilentus*, *H. pumilus*
234	*Dra*I	6.9	5.0 = 3.2 + 1.8	*H. heterophyllus*
235	*Xba*I	6.9	14.0 = 12.3 + 1.7	*H. porteri*
236	*Bgl*II	5.4	2.0 = 1.3 + 0.7	*H. porteri*
237	*Dra*I	5.4	4.4 = 3.4 + 1.0	*H. agrestis*
238	*Dra*I	4.6	9.5 = 5.9 + 3.6	sect. *Helianthus*
239	*Eco*RI	4.6	3.3 + 0.7 = 4.0	*H. cusickii*

Fig. 1 Physical map of the chloroplast DNA genome of *Helianthus tuberosus* (*H.* sect. *Atrorubens*) showing the locations of recognition sites for 18 restriction enzymes. Restriction sites are designated by vertical lines and are mapped relative to the lettuce cpDNA probe library (Jansen and Palmer 1987). The circular maps have been linearized at the *Sac*I site separating the 6.3- and 12.3-kb lettuce fragments. Sizes of restriction fragments are shown in kilobases (kb). The *two horizontal, heavy lines* show the location of the inverted repeat (IR) regions. The gene order of the tobacco cpDNA genome is shown for reference on the *uppermost lines*, with the position of the approximately 22-kb inversion characteristic of most Asteraceae noted. *Arrows* show the location of the 0.4-kb deletion in Helianthinae

three of which are comprised of annual species. Two of these, sect. *Helianthus* and sect. *Agrestes* (*H. agrestis*), correspond to infrageneric taxa that have already been recognized (Schilling and Heiser 1981). The third, including only *H. porteri*, was not recognized as a separate taxonomic unit, although its distinctiveness is reflected in the fact that this species was only recently placed within *Helianthus*.

The fourth major lineage supported by cpDNA data within *Helianthus* included all of the perennials, which had been classified taxonomically as two different

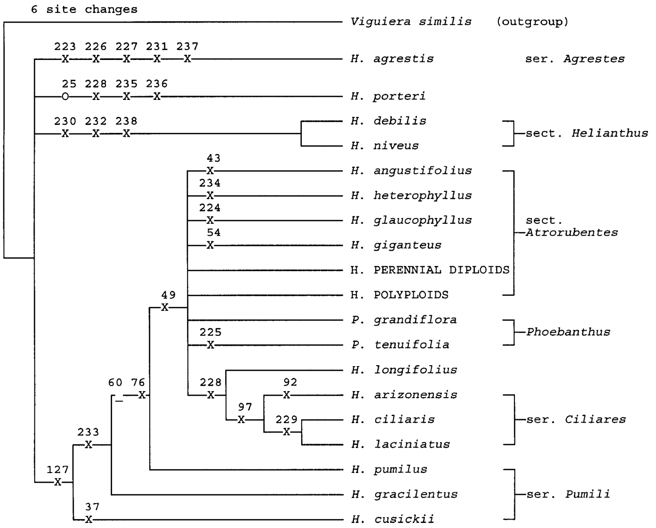

Fig. 2 Single most parsimonious cladogram showing relationships among samples of *Helianthus* and *Phoebanthus* based on cpDNA restriction site data. Site changes numbered as in Table 2. *X* Unambiguous change, *O*, homoplasious change. H. PERENNIAL DIPLOIDS includes: *H. atrorubens*, *H. carnosus*, *H. decapetalus*, *H. divaricatus*, *H. floridanus*, *H. grosseserratus*, *H. maximilianii*, *H. microcephalus*, *H. mollis*, *H. nuttallii*, *H. occidentalis*, *H. pauciflorus*, *H. radula*, *H. salicifolius*, *H. silphioides*, *H. simulans*. H. POLYPLOIDS includes: *H. eggertii*, *H. hirsutus*, *H. laevigatus*, *H. multiflorus*, *H. resinosus*, *H. schweinitzii*, *H. smithii*, *H. strumosus*, *H. tuberosus*

sections (Schilling and Heiser 1981). The cpDNA data were incongruent with the recognition of sect. *Ciliares* as a unified and monophyletic group. The 3 species of ser. *Pumili* formed a relatively basal, paraphyletic lineage within the perennials (Fig. 2). The 3 species of ser. *Ciliares* formed a coherent group, supported by a single apomorphy. Within ser. *Ciliares*, the diploid *H. laciniatus* shared a further apomorphic site change with the polyploid *H. ciliaris*. This provides weak evidence that *H. laciniatus* and *H. ciliaris* share a common maternal ancestor. Within the other section that includes perennial species, sect. *Atrorubens* (sect. *Divaricati*), there was little or no variation between species in either cpDNA or rDNA restriction site characters, and thus no basis for evaluating the division of this section into series.

The overall amounts of cpDNA divergence would point to a relatively recent origin for the genus and particularly for lineages within the genus. The use of cpDNA data as a molecular clock is not without difficulty (Wendel and Albert 1992) and is further complicated by the poorly substantiated hypotheses necessary to make use of restriction site data. On the basis of the 16 enzyme datasets used for the broad survey of Helianthinae (Schilling and Jansen 1989; Schilling et al. 1994) members of *Helianthus* differ from other perennial sister species of the subtribe by between 25 and 36 restriction site changes of the 525 sites sampled. Estimates of rates of evolutionary change for cpDNA that have been proposed fall in the range of 0.03%–0.1% per million years. Using this range, we estimated that the age of the genus was approximately 4.75–22.7 million years. Within the genus, species within lineages differ by 9–13 site changes. This suggests that the extant lineages arose between 1.7 and 8.2 million years ago. Within the perennial lineage, the majority of species differ by at most 1 site change. This puts an upper bound on the time of divergence among these species of 0.2 million years ago and leaves open the possibility that divergence has been even more recent.

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