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Chloroplast DNA differences between cultivated hop, *Humulus lupulus* and the related species *H. japonicus*

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Abstract Chloroplast DNA (cpDNA) of *Humulus lupulus* and *H. japonicus* was examined by restriction endonuclease analysis with *Bam*HI, *Ban*I, *Bcl*I, *Bst*EII, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pae*R7I, *Pst*I, *Pvu*II, *Sal*I and *Xho*I. The restriction fragment patterns showed that the cpDNAs shared a large number of restriction sites. However, the chloroplast genomes of the two species could be distinguished by differences in restriction site and restriction fragment patterns in the *Pst*I, *Pvu*II, *Bcl*I, *Eco*RV, *Dra*I and *Hin*dIII digests. On the basis of the complexity of restriction enzyme patterns, the enzymes *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I were selected for mapping the chloroplast genomes. Single and double restriction enzyme digests of cpDNA from the two species were hybridized to cpDNA probes of barley and tobacco. The data obtained from molecular hybridization experiments were used to construct the cleavage site maps. Except for the *Pst*I digest, the arrangement of cpDNA restriction sites was found to be the same for both species. An extra *Pst*I site was present in *H. lupulus*. Three small insertions/deletions of about 0.8 kbp each were detected in the chloroplast genomes of the two species. Two of these insertions/deletions were present in the large and one in the small singlecopy region of the chloroplast genome. The cpDNA of *Humulus* was found to be a circular molecule of approximately 148 kbp that contains two inverted repeat regions of 23 kbp each, a small and a large single -copy region of approximately 20 kbp and 81 kbp, respectively. The chloroplast genome of hop has the same physical and structural organization as that found in most angiosperms.

Key words Chloroplast DNA · Restriction site map

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Introduction

The hop plant, *Humulus lupulus* L., is an economically important species cultivated for its female inflorescences (hops), which are used in the brewing industry. Hops impart bitterness and flavor to fermented malt beverages, mainly beer and ale. *Humulus* is one of two genera in the family *Cannabaceae*. There is no consensus on the number of recognized species in the genus *Humulus*. Currently, it is thought to be comprised of either two (Ehara 1955; Burgess 1964), three (Small 1978) or more (Rybacek 1991) species. The two well-recognized species are the common hop, *H. lupulus*, and the Japanese hop, *H. japonicus* (Sieb. et Zucc). *Humulus lupulus* is a climbing, dioecious, perennial plant that twines around any support in a clockwise direction (Neve 1991); *H. japonicus* is climbing and dioecious, but is an annual which produces very small female inflorescences that are of no value in the brewing industry (Neve 1991). However, *H. japonicus* may be of value in breeding for resistance to aphids in hop. It is often grown in gardens as an ornamental. A third, poorly characterized, hop species, *H. yunnanensis*, is recognized by some authors (Small 1978) and questioned by others (Neve 1991). Very little is known about this plant.

Humulus lupulus and *H. japonicus* are not cross-compatible (Small 1978). In addition to differences in gross morphology, *H. lupulus* and *H. japonicus* have different chromosome numbers, each with a well-developed sex-chromosome system. There are $2n = 20$ chromosomes in both male and female plants of *H. lupulus*, while *H. japonicus* has $2n = 16$ chromosomes in the female and $2n = 17$ in the male (Winge 1929; Skovsted 1953; Ono 1955). No information on the cytology of *H. yunnanensis* is available (Neve 1991).

Although *H. lupulus* is indigenous only to the Northern hemisphere, it is now grown commercially in about 30 countries between latitudes 30° and 50° (Haunold 1981). *Humulus japonicus* is widespread in much of China and Japan (Neve 1976). Although common hop

has been in cultivation in Central Europe for over a thousand years (Kohlmann and Kastner 1976), very little is known about its genetics. Hop research has primarily focused on breeding for production, disease and insect control, and brewing quality traits (Haunold 1981).

In this paper, we present the physical maps of the chloroplast genomes of *H. lupulus* and *H. japonicus*. The basic differences between the chloroplast genomes of the two hop species are described. The general features of the chloroplast genomes of *H. lupulus* and *H. japonicus* are compared with those described for other flowering plants. Chloroplast DNA restriction site maps not only provide information about genomic evolution, they are also useful to systematists interested in organismic phylogenies (Kellogg 1992).

Materials and methods

Plant materials

The leaf material used for cpDNA extraction was obtained from greenhouse-grown plants of *H. lupulus* (cv. 'Cluster') and *H. japonicus*. Young leaves were cut from the plants and the petioles placed in tubes containing water. The tubes were placed in a large plastic bag to maintain a high level of humidity and placed in a dark chamber for 2 days to destarch the leaves. The leaves were then frozen in liquid nitrogen and stored at −70 °C in a freezer until use.

cpDNA isolation, restriction analysis and gel electrophoresis

Isolation of pure cpDNA from the two hop species, single and double restriction endonuclease digestions and agarose gel electrophoresis were carried out as described in Pillay (1993). Single digestions of the cpDNA were carried out with the enzymes *Bam*HI, *Ban*I, *Bcl*I, *Bst*EII, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pae*R7I, *Pst*I, *Pvu*II, *Sal*I and *Xho*I. Five of these enzymes, *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I, were selected for constructing a physical map of the chloroplast genomes of *H. lupulus* and *H. japonicus*. These enzymes were chosen because they produce relatively simple restriction patterns in which all of the fragments are well resolved by agarose gel electrophoresis. Such enzymes are appropriate for constructing a complete restriction endonuclease cleavage site map of the chloroplast genome. For construction of the cleavage site maps, the cpDNAs were digested with *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I individually (single digestion) or in combination (double digestion).

Southern transfer and DNA hybridizations

Southern transfer of digested DNA fragments, nick translation of cloned cpDNA fragments and DNA-DNA hybridizations were carried out as described in Pillay (1993) with the following modifications in post-hybridization washes. Following hybridization, the membranes were washed 2 times in 2 × SSC, 0.5% SDS at room temperature for 15 min each. The membranes were then washed 3 times in 2 × SSC, 0.1% SDS for 15 min each at 65 °C.

The probes used in this study were a set of *Pst*I clones of barley cpDNA (Day and Ellis 1985) and selected tobacco cpDNA clones (Fluhr et al. 1983). In this study the barley and tobacco clones will be designated as P (number of probe) B and P (number of probe) N, respectively. The tobacco cpDNA clones were provided by Dr. M. Edelman, the Weizmann Institute of Science, Israel.

Analysis of data and map construction

The migration distance of each fragment produced from the single and double digests were obtained from photographs of ethidium bromide-stained gels. The molecular size of each fragment was estimated by comparison with the migration of known molecular weight standards using linear regression. The molecular weight standards were *Hin*dIII-digested lambda DNA and a 1-kb DNA ladder marker.

The autoradiograms were examined and the various hop cpDNA fragments hybridizing to the different clones were recorded. This information was used to construct the physical maps of the hop chloroplast genomes by the overlap filter hybridization technique (Palmer 1986). The single digests were used to sequentially align the various fragments into a map. The double digestions were used to map the restriction sites in correct orientation relative to those of the *Pst*I map.

Results

The restriction enzyme patterns observed with *Bam*HI, *Ban*I, *Bcl*I, *Bst*EII, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Pae*R7I were strikingly similar between the cpDNA of *H. lupulus* and *H. japonicus* with many common sites. However, some mutational differences were observed between the cpDNAs of the two species. These enzymes were, however, not suitable for initiating mapping because they produced a large number of fragments. The restriction fragment patterns produced with *Pst*I (Fig. 1) were strikingly different in *H. lupulus* and *H. japonicus* and could be used to distinguish the cpDNA of the two species. *Pst*I produced 11 cuts in *H. lupulus* and only 10 cuts in *H. japonicus*. Digestion of DNA from both species with the enzymes *Kpn*I, *Pvu*II, *Sal*I and *Xho*I produced 11, 12, 10 and 15 cleavage sites per enzyme, respectively. The fragment patterns observed with *Kpn*I, *Sal*I and *Xho*I showed very small size differences among some fragments of the two hop species. These differences were only observed when the cpDNA digests of the two species were run in adjacent lanes on a gel (Fig. 2). A distinct difference was evident in the electrophoretic mobility of a single *Pvu*II fragment between the two species (Fig. 1). Careful analysis revealed the presence of three small insertions/deletions of approximately 0.8 kbp each located in a different region of the cpDNAs. The insertion/deletion in the small single-copy region was clearly visible in the *Bcl*I, *Eco*RV and *Hin*dIII digests. Two insertion/deletions were present in the large single-copy region: one was clearly observed in the *Pvu*II digest, while the other was visible in the *Pst*I and *Dra*I digests. In Fig. 3 the single and double restriction enzyme digests used for mapping are shown for *H. lupulus*.

Some of restriction fragments contained more than one dose of a restriction sequence as observed from the intensity of the ethidium bromide-stained bands. In some instances, these bands were representative of the inverted repeat region; in other cases, the darkly stained bands represented two non-identical sequences of DNA. For example, two 14.1-kbp non-identical DNA fragments are present in the *Sal*I digests. The presence of

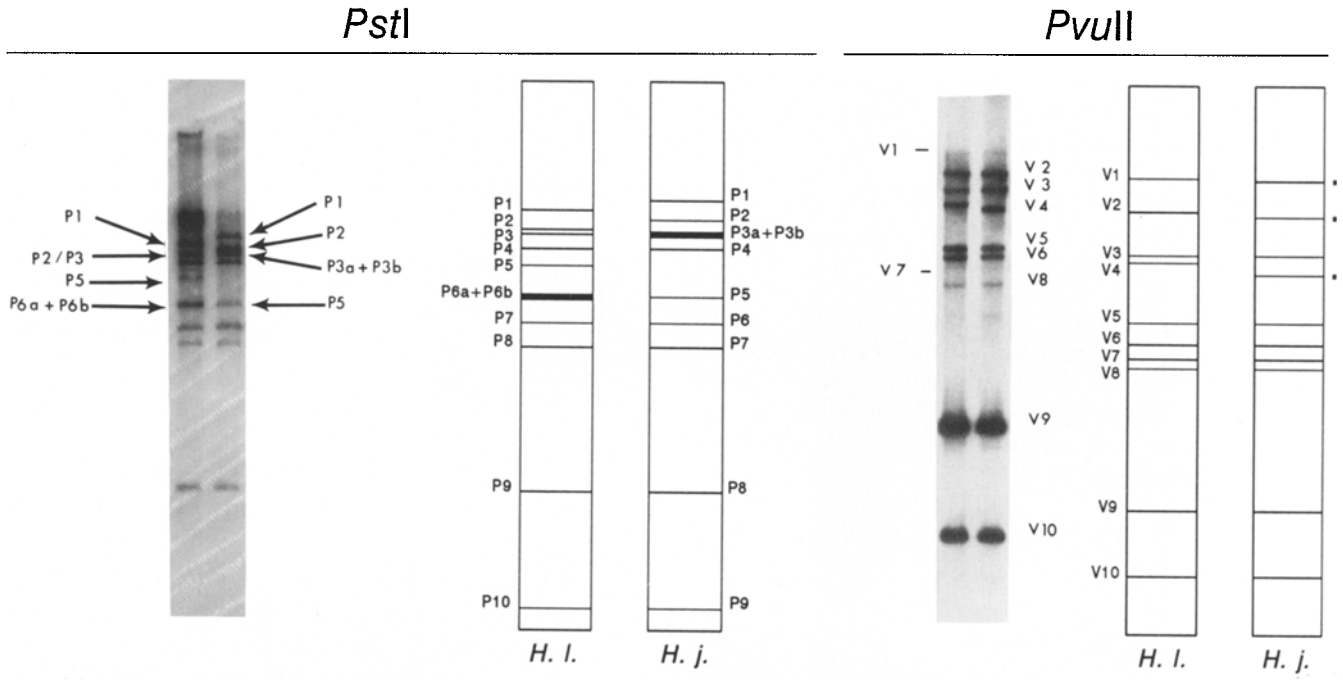
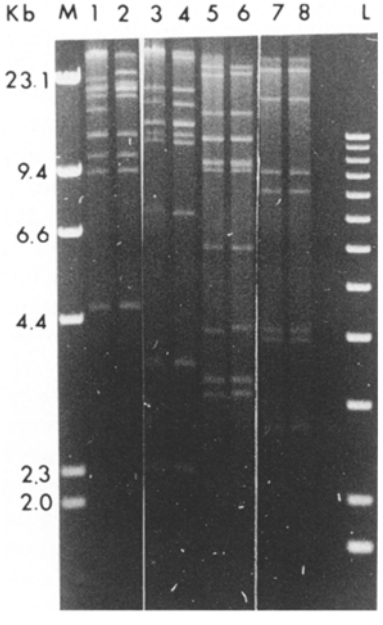


Fig. 1 Southern hybridization patterns and interpretive drawings of cpDNA restriction fragment patterns of *Pst*I and *Pvu*II digests of *H. lupulus* (*H.I.*) and *H. japonicus* (*H.j.*). The *Pst*I digest was hybridized to total cpDNA; the *Pvu*II digest was hybridized to selected cpDNA fragments. The approximate location of fragments V1 and V7 are indicated. Differences in fragmentation patterns of *Pst*I and *Pvu*II digests are illustrated. *P* and *V* designates the *Pst*I and *Pvu*II digests, respectively

non-identical double fragments tends to complicate the mapping procedure, especially when these fragments are not part of the inverted repeat region. The number and molecular size of each fragment from the single enzyme digests are listed in Table 1. The fragments of each enzyme digest are arranged in order of decreasing size (kbp) and designated with the initial letter of the enzyme used; fragments from *Pvu*II are designated with "V". The total chloroplast genome size of hop calculated by addition of the digested fragment lengths of the *Pst*I digest is approximately 148 kbp.

Fig. 2 Representative restriction digest patterns of cpDNA of *H. lupulus* and *H. japonicus* digested with *Pst*I (*lanes 1* and *2*), *Sal*I (*lanes 3* and *4*), *Kpn*I (*lanes 5* and *6*) and *Xho*I (*lanes 7* and *8*) with the *H. lupulus* digest being the first in each pair. Fragment sizes of lambda DNA-*Hin*dIII digest (*M*) are shown in the *left margin*. The 1-kb ladder marker (*L*) is shown on the *right*



The cpDNA clones of barley and tobacco generally had a high homology with hop cpDNA, producing strong, specific hybridization. However, neither the P2B nor the P3B clone hybridized to the 3.3-kbp *Xho*I fragment of hop cpDNA. This 3.3-kbp fragment hybridized strongly to the P5N clone. Similarly, the P7B clone did not hybridize to the 4.2-*Xho*I fragment. The position of this fragment was determined by the P2AN clone. A typical hybridization pattern produced with a barley cpDNA clone is represented in Fig. 4. The physical maps of *H. lupulus* and *H. japonicus* are shown in Fig. 5.

Discussion

One of the striking features of this study is the high degree of similarity of cpDNA restriction sites in *H. lupulus* and *H. japonicus* in contrast with differences found in the chromosome number and gross morphology of the two species. The cpDNA of the two hop species differs only in size and by the presence/absence of a *Pst*I restriction site (Fig. 5). The chloroplast genome is generally a very conservative genetic system (Sears 1983). Factors such as the absence of direct selective pressures on chloroplasts, the slow mutation rate, the difficulty of establishing mutations within the chloro-

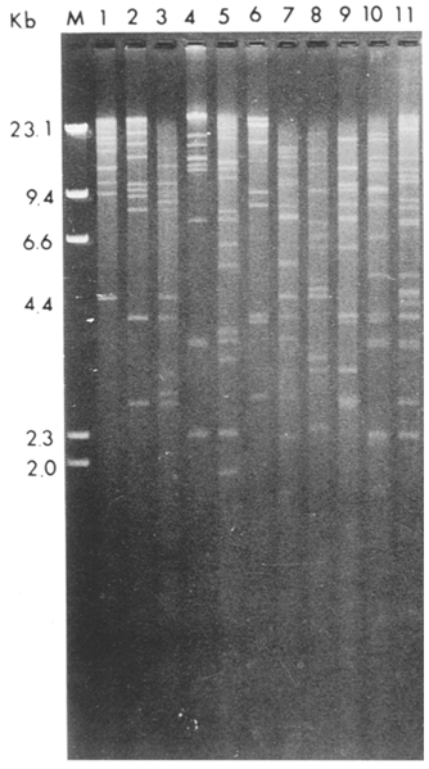


Fig. 3 Single and double restriction digest profiles of cpDNA of *H. lupulus*. cpDNA was digested with *Pst*I (*lane 1*) *Pvu*II (2), *Pst*I and *Pvu*II (3), *Sal*I (*4*), *Pst*I and *Sal*I (*5*), *Kpn*I (*6*), *Pst*I and *Kpn*I (*7*), *Pst*I and *Xho*I (*8*), *Kpn*I and *Xho*I (*9*), *Kpn*I and *Xho*I (*10*), *Pvu*II and *Sal*I (*11*). Fragment sizes of lambda DNA-*Hin*dIII (*M*) digest are shown in the *left margin*

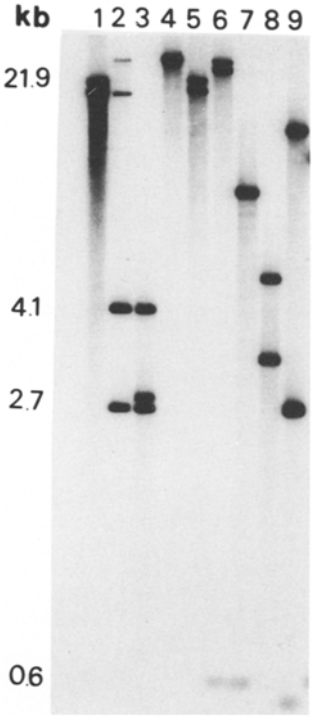


Fig. 4 Autoradiograph of molecular hybridization of P8 barley clone to a nylon membrane containing hop cpDNA. The lanes represent cpDNA digests of *Pst*I (*1*), *Pvu*II (*2*), *Pst*I and *Pvu*II (*3*), *Sal*I (*4*), *Pst*I and *Sal*I (*5*), *Kpn*I (*6*), *Pst*I and *Kpn*I (*7*), *Pst*I and *Xho*I (*8*), *Kpn*I and *Xho*I (*9*). Approximate fragment sizes (kbp) are shown in *left margin*

plast and the absence of cpDNA recombination are thought to be responsible for the evolutionary conservatism of the chloroplast genome (reviewed by Sears 1983).

In contrast to the gross structural similarity of their chloroplast genomes, the two hop species have strikingly different chromosome numbers, probably the result of aneuploidy. Therefore, it is conceivable that the aneuploid reduction/addition of chromosomes in the two hop species occurred after species divergence from a common maternal ancestor. It has been reported that both hop species are able to tolerate gross imbalances in their chromosome complement (Parker and Clark 1991).

The two hop species are morphologically distinguishable. The main differences between *H. lupulus* and *H. japonicus* include their perennial versus annual habit, large and small female inflorescences and the presence and absence of an underground stem, respectively. This study showed that the chloroplast genomes of the two hop species can also be distinguished by restriction site and minor structural differences. It is generally argued that the loss of a restriction site is more likely than the net gain of a site (Templeton 1983; DeBry and Slade 1985). If this is true, then this study suggests that *H. japonicus* has lost a *Pst*I site. In addition to the single *Pst*I restriction site difference, the chloroplast genomes of the two species are characterized by three small insertion/deletion events located in three different regions of the chloroplast genome (Fig. 5). The insertion/deletion present in the small single-copy region was easily noticeable in the *Bcl*I, *Eco*RV and *Hin*dIII digests. This insertion/deletion was almost imperceptible in the *Sal*I and *Pvu*II digests, probably because of the large fragment sizes that these enzymes produced in this region. The insertion/deletion was also not visible in the *Xho*I digest, possibly because it occurred near the junction of the 14.5- and 9.4-kbp fragments (Fig. 5). One of the two insertion/deletions found in the large single-copy region is within the V5 *Pvu*II fragment. This insertion/deletion was also detected by hybridization to the 0.8-kbp P12N clone, suggesting that this may be the exact site of the insertion/deletion. The third insertion/deletion appeared in the region of the 26.6-kbp *Pst*I fragment of *H. japonicus*. This insertion/deletion was not clearly evident in the *Sal*I digest. Further hybridization experiments, perhaps with very small cpDNA clones, are required to find the exact location of these insertions/deletions. Small insertions/deletions, although difficult to observe, are common in the structural evolution of chloroplast genomes (Palmer et al. 1988). Some authors, such as Timothy et al. (1979) and Kung et al. (1982), have suggested the existence of coevolutionary changes between the chloroplast and nuclear genomes. Whether this is true in *H. japonicus* is a matter of conjecture.

The chloroplast genome of hop (Fig. 5) has the same physical and structural organization as those found in most angiosperms. The hop cpDNA molecule is organized into three regions with the typical large and small single-copy regions of approximately 81 kbp and 20 kbp, respectively, and two inverted repeat regions of

Table 1 *Humulus* cpDNA fragment sizes, copy number and barley and tobacco probes to which they hybridized. With the exception of *Pst*I (shown separately) the probe-fragment hybridization results were identical in both hop species. Fragment sizes in parentheses are those for *H. japonicus* where a distinct difference was noticed

Enzyme	Fragment designation	Size (kbp)	Copy number	Probe-fragment hybridization
*Kpn*I	K1	39.8 (39.0)	1	P2B, P3B, P4B, P8B, P2bN, P3bN, P5N
	K2a[a]	30.4 (29.6)	1	P5B, P7B, P2aN, P3aN
	K2b[a]	29.6	1	P2B, P3B, P8B, P3aN, P3bN, P5N
	K3	20.0 (19.2)	1	P6B, P9B, P10B, P3bN
	K4	9.1	1	P4B, P2aN, P2bN
	K5	8.4	1	P6B, P9B, P3bN
	K6	4.1	1	P1B, P3aN
	K7	4.0	1	P1B, P3aN
	K8	2.5	1	P1B, P5B, P3aN
	K9	0.6	2	P6B, P9B, P3bN
*Sal*I	S1	39.9 (39.1)	1	P6B, P8B, P9B, P10B, P3bN
	S2	22.4 (21.6)	1	P5B, P7B, P2aN
	S3	18.2	1	P1B, P3aN
	S4a	14.1	1	P2B, P3B, P2bN, P5N
	S4b	14.1	1	P4B
	S5	12.9	1	P2B, P3B, P5N
	S6	11.5	1	P2B, P2bN
	S7	7.5	1	P1B, P3aN
	S8	3.8	1	P4B, P2bN
	S9	2.3	2	P2B, P3B
*Pvu*II	V1	40.2 (39.4)	1	P2B, P3B, P4B, P2bN, P3bN, P5N
	V2	22.8 (22.0)	1	P6B, P9B, P10B, P3bN
	V3	19.0	1	P2B, P3B, P8B, P3bN, P5N
	V4	17.0 (16.2)	1	P5B, P7B
	V5	10.5	1	P1B, P3aN
	V6	9.8	1	P1B, P3aN
	V7	8.9	1	P7B, P2aN
	V8	8.1	1	P5B, P7B, P2aN
	V9	4.1	2	P6B, P8B, P9B, P3bN
	V10	2.7	2	P8B, P3bN
*Xho*I	X1	38.4 (37.6)	1	P1B, P3aN
	X2	25.9 (25.1)	1	P4B, P2aN
	X3	14.5	1	P6B, P9B, P3bN
	X4	12.9	2	P2B, P3B, P8B, P3bN, P5N
	X5	10.4	1	P2B, P3B, P3aN, P5N
	X6	9.4	1	P6B, P9B, P10B, P3bN
	X7	6.3	1	P7B, P2aN
	X8	4.2	1	P2aN
	X9	3.5	2	P8B, P3bN
	X10	3.3	1	P5N
	X11	0.6	2	P6B, P3bN
*Pst*I				
H. lupulus	P1	22.7	1	P6B, P9B, P3bN
	P2	21.9	1	P6B, P8B, P9B, P3bN
	P3	20.9	1	P1B, P3aN
	P4	19.2	1	P6B, P8B, P9B, P10B, P3bN
	P5	14.5	1	P2B, P5N, P8N, P9N
	P6a	12.9	1	P2B, P3B, P5N
	P6b	12.9	1	P5B, P7B, P3aN
	P7	10.4	1	P2B, P3B, P5N
	P8	9.1	1	P4B
	P9	4.6	1	P2B, P3B
	P10	1.0	1	P3B
H. japonicus	P1	26.6	1	P2B, P5N, P8N, P9N
	P2	21.9	1	P6B, P8B, P9B, P3bN
	P3a	20.9	1	P5B, P7B, P2aN
	P3b	20.9	1	P1B, P3aN
	P4	19.2	1	P6B, P8B, P9B, P10B, P3bN
	P5	12.9	1	P5B, P7B, P3aN
	P6	10.4	1	P2B, P3B, P5N
	P7	9.1	1	P4B
	P8	4.6	1	P2B, P3B
	P9	1.0	1	P3B

[a] For *Kpn*I fragment K2, designation a and b is relevant only to *H. japonicus*

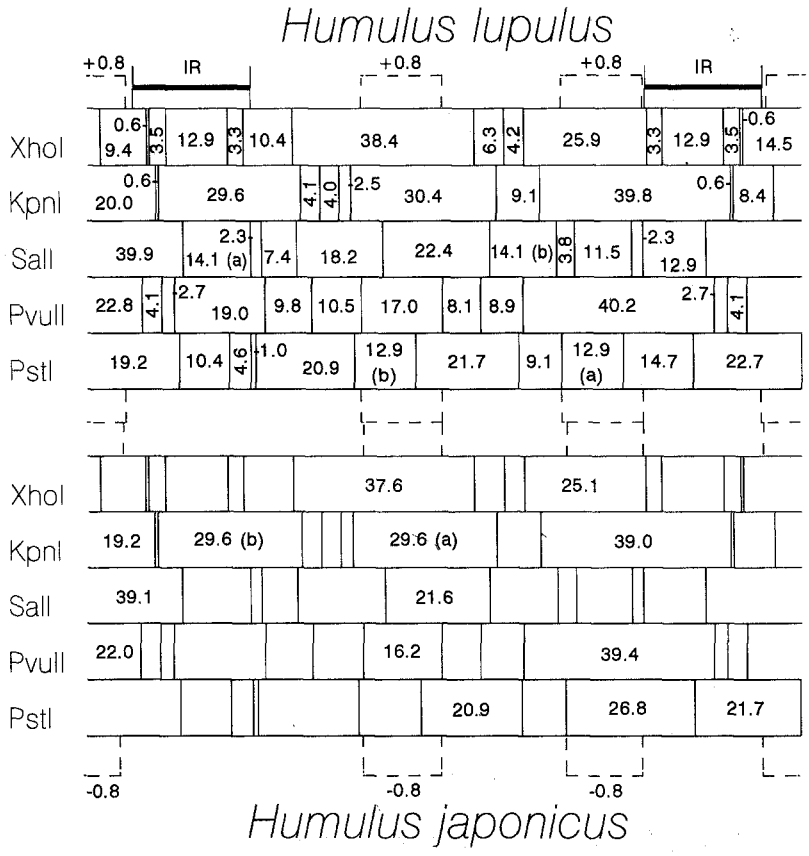


Fig. 5 Comparative cpDNA restriction site maps of *H. lupulus* and *H. japonicus*. The cleavage sites for *Pst*I, *Pvu*II, *Sal*I, *Kpn*I and *Xho*I are shown. All the fragment sizes (kbp) are shown for *H. lupulus*. In *H. japonicus*, only fragments differing in size are indicated. The approximate location of the three insertions/deletions are shown. The positions of the inverted repeat regions are designated *IR*. The maps are presented in linearized form starting at the center of the small single-copy region

23 kbp each. The size of the hop cpDNA molecule is approximately 148 kbp. This is similar to the chloroplast genome size of other dicotyledonous plants such spinach (150 kbp; Herrmann et al. 1980), soybean (150 kbp; Spielmann et al. 1983), *Cucumis* (150 kbp; Perl-Treves and Galun 1985), *Brassica* (151 kbp; Palmer et al. 1983) and safflower (151 kbp; Smith and Ma 1985). The hop cpDNA is smaller than those found in tobacco (160 kbp; Fluhr and Edelman 1981), kiwifruit (160 kbp; Hudson and Gardner 1988), and Citrus (166 kbp; Green et al. 1986). The physical maps indicate that the chloroplast genome of hops (*Cannabaceae*) is colinear with that of barley (*Poaceae*). This is not surprising since DNA-DNA hybridization studies indicate that there is at least 30–50% sequence homology between the cpDNAs of even unrelated plant species (Bisaro and Siegel 1980; Lamppa and Bendich 1981).

Chloroplast DNA has been widely used in phylogenetic and evolutionary studies (Palmer et al. 1988). Considerable morphological differences are found between hop plants from different parts of the world (Neve 1991). On the basis of morphological differences attempts have been made to classify the different hop variants into sub-species or even different species (Small 1978, 1980; Rybacek 1991). We envisage that knowledge of the structural organization and the restriction site map of hop cpDNA will be useful in providing information on the systematics of hop. Chloroplast DNA has found widespread application in biosystematic and phylogenetic studies. We have examined cpDNA restriction site patterns in hop cultivars from our worldwide collection of plants. A manuscript on this work is in preparation.

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