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Variation of plastid and mitochondrial DNAs in the genus *Hedysarum*

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Summary. Plastid and mitochondrial DNAs from *Hedysarum* species of the western Mediterranean basin, *H. spinosissimum* ssp *eu-spinosissimum, H. spinosissimum* ssp *capitatum, H. carnosum, H. coronarium* and *H. flexuosum,* were compared by restriction endonuclease fragment analysis. ctDNA fragment patterns for ssp *eu-spinosissimum* and ssp *capitatum* were indistinguishable in different enzyme digests. An identical ctDNA variation was found in Hpa II digests with two Sardinian populations of ssp *capitatum.* Each of the two subspecies was characterized by specific mt DNA patterns with Pst I, Bam HI, Sma I and EcoRI. No variation was detected in populations of different geographical origins for a given subspecies. *H. carnosum, H. coronarium* and *H. flexuosum* generated specific ct and mt DNA patterns. Comparison of mitochondrial fragments indicated: – a strong homology between the two subspecies, – a closer homology among the three other diploids, each being closer to the other two than to *H. spinosissimum* subspecies – as was also the case for the plastid genomes.

Key words: *Hedysarum* – Chloroplast DNA – Mitochondrial DNA – Restriction patterns

Introduction

The genus *Hedysarum (leguminosae)* contains various species distinguishable by different morphologies, mating systems, biological cycles and geographical origins. These plants all possess flowers of the papilionacea type and articulated cloves. Among the species of the Mediterranean group, the five diploids (2n = 16) *H. carnosum* L., *H. coronarium* L., *H. flexuosum* L., *H. spinosissimum* L. subspecies *eu-spinosissimum Briq,* and *capitatum Desf* are the most widely spread in the western basin, where they grow wild. These diploids are nutritious and highly palatable to sheep. In addition, the Spanish sainfoin, *H. coronarium,* is grown for fodder in a number of countries: Spain, Italy and North Africa.

Several ecological studies followed by genetic evaluation studies were started recently to improve these species by breeding: Tunisia 1975; southern France 1976, 1977; Morocco 1979; Tunisia and Algeria 1981; Tunisia, Sardinia and Malta 1983. An important genetic variability was found within each diploid species (Baatout et al. 1976; Figier et al. 1978; Baatout 1980; Boussaid 1980; Figier 1982; Chriki et al. 1982; Chriki and Harborne 1983; Trifi-Farah et al. 1983). Distinctive morphological and mating characteristics are summarized in Table 1. It is noticeable that the subspecies *H. capitatum* appeared mostly allogamous and the subspecies *H.-eu-spinosissimum* mostly autogamous (Baatout 1980). Although both these subspecies are found in all western Mediterranean countries, such is not the case for the three other species. *H. coronarium* seems to be present in all sampled countries except France; *H. carnosum* appears to be distributed in North Africa only; *H. flexuosum* has been found only in Morocco. Further sampling programmes may indicate a wider area for these species. In Tunisia, the ecological domain of *Hedysarum* species has been well described by Baatout et al. (1976). The Tunisian Dorsal has been shown to separate the semi-temperate ecological domain of *H. capitatum* and *H. coronarium* to the north and the arid ecological domain of *H. eu-spinosissimum* and *H. carnosum* to the south. A zone with mixed populations of the two subspecies has been found slightly south of the Dorsal. The biochemical characteristics of *Hedysarum* species are still unknown. Chriki et al. (1982) have described two types of acyanic mutants in *H. coronarium.* Variability in carboxylic esterases has been shown to occur in natural populations of *H. coronarium* and *H. capitatum* (Trifi-Farah et al. 1983). Recent results from studies on *Hedysarum* species populations suggest controlled gene exchanges among

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Table 1. *Hedysarum* populations used in experiments

Species or taxon	Geographical origin	Mating system	Important characters
H. spinosissimum ssp *capitatum*	Tunisia Sardinia Malta France	mostly allogamous	narrow leaves, violet corolla 6–12 flowers per inflorescence, rigid cloves
H. spinosissimum ssp *eu-spinosissimum*	Tunisia Sardinia Algeria France	mostly autogamous	narrow leaves, pale pink corolla, 3–6 flowers per inflorescence, rigid cloves
H. carnosum	Tunisia	mostly allogamous	oval leaves, pale violet corolla, 15–20 flowers per inflorescence, rigid cloves
H. flexuosum	Morocco	allogamous	oval leaves, red violet corolla, 15–20 flowers per inflorescence, flexible cloves
H. coronarium	Tunisia Sardinia Malta	allogamous	oval leaves, red violet corolla, 15–20 flowers per inflorescence, rigid cloves

the various compartments of known species complexes (Baatout 1980).

Restriction enzyme patterns of chloroplast (ct) and mitochondrial (mt) DNAs have already been used to probe the taxonomic relationships in different genera (Vedel et al. 1978, 1980; Timothy et al. 1979; Rhodes et al. 1981; Kung et al. 1982; Gordon et al. 1983; Metzlaff et al. 1981; Lebacq and Vedel 1981; Berthou et al. 1983; Palmer et al. 1983; Bowman et al. 1983; Tsunewaki and Ogihara 1983; Kemble et al. 1983; Clegg et al. 1984; De Bonte et al. 1984). These studies have demonstrated a diversity among cytoplasmic genomes which grew more pronounced as the taxonomic plant groups diverged. In this paper we present a restriction enzyme analysis of cytoplasmic DNAs from the five *Hedysarum* diploids which was done in order to estimate their intra and interspecific genetic variability and to explore phylogenetic relationships in the genus *Hedysarum*.

Material and methods

Plant material

Populations used in the study of cytoplasmic DNA polymorphism are listed in Table 1. Seeds harvested during the different selection programmes (dates indicated above) were grown either on vermiculite in a greenhouse of the Phytotron of the CNRS in Gif-sur-Yvette for one month at 22 °C and 16 h day-length, or in Petri dishes for four days at 25 °C in the dark.

Organelle and DNA isolation

Chloroplasts were prepared from one month-old green plants. The leaves were homogenized at 4 °C in a Waring blender 3×5 s at maximum speed in a buffer containing 0.5 M mannitol, 0.05 M Tris, 3×10^{-3} M EDTA, 10^{-3} M mercaptoethanol, 0.1% BSA, pH 8.0. The homogenate was filtered through a 20 µm nylon net and the filtrate centrifuged at 1,000 g, 12 min. The crude chloroplast pellets were gently dispersed in the homogenization buffer and fractionated by centrifugation at 4 °C in discontinuous sucrose gradients 30%, 60%, steps in the same buffer at 2,000 g, 20 min. Material banding at the interface was carefully removed, diluted with three volumes of the homogenization buffer and pelleted at 1,200 g, 12 min. DNase I treatment was omitted because these fractions are composed of broken organelles and DNase treatment destroys all the ct DNA.

Mitochondria were prepared from 4 day-old seedlings grown in the dark, using the same homogenization buffer described above. The mitochondrial fractions were pelleted and then purified successively by DNase treatment and centrifugation in discontinuous sucrose gradients as previously described (Berthou et al. 1983). Ct and mt pellets were lysed with sodium lauroyl sarcosine and autodigested pronase as described previously (Herrmann et al. 1975). Lysates were made, 0.2 M with respect to sodium acetate, and extracted once by chloroform and phenol (1 vol. of each). The DNA was precipitated from the aqueous phase by cold ethanol. The DNA pellets were washed with cold ethanol prior to resuspension in 0.05 M Tris, 0.02 M EDTA pH 8.0. Centrifugation in CsCl-ethidium bromide gradients was conducted as described previously (Herrmann et al. 1975).

DNA restriction and agarose gel electrophoresis

Cytoplasmic DNA's were digested with restriction endonucleases Hpa II, Sal I, Bam HI, Pst I, EcoRI and Bgl I (Boehrin-

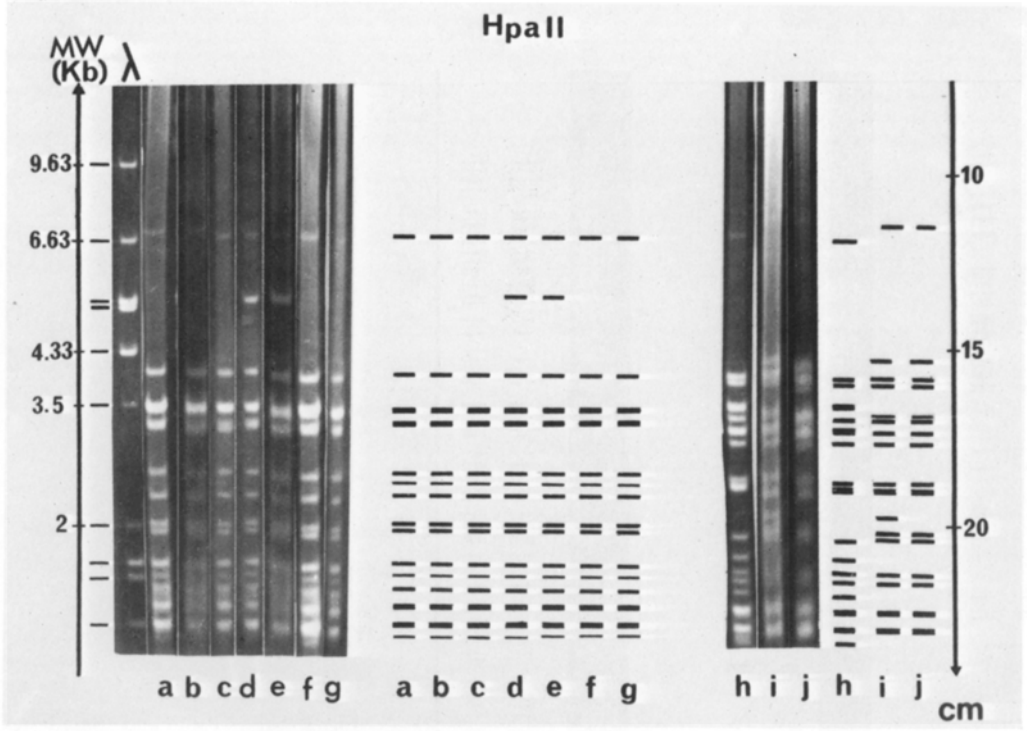

Fig. 1. Hpa II restriction patterns of ct DNAs from: *a H. eu-spinosissimum* from Tunisia; *b H. eu-spinosissimum* from France; *c H. eu-spinosissimum* from Sardinia (Sa 008); *d H. eu-spinosissimum* from Sardinia (Sa 002); *e H. eu-spinosissimum* from Sardinia (Sa 021); *f H. capitatum* from Tunisia; *g H. capitatum* from Sardinia; *h H. carnosum* from Tunisia; *i H. coronarium* from Sardinia; *j H. flexuosum* from Morocco; λ, molecular weight standard (Marker II + Marker III, Boehringer Mannheim). On the *right* are the corresponding schematic representations

ger Mannheim, FRG) using conditions specified by the suppliers. The restriction fragments were separated by electrophoresis on 35 cm-long vertical slab gels containing 0.7% agarose. A mixture of DNA fragments generated from λ DNA by Hind III and from λ DNA by Hind III + EcoRI (Marker II and Marker III, respectively, from Boehringer Mannheim) was used as a molecular weight standard.

Results

Hpa II restriction analysis of Hedysarum ct DNAs

The green material in *Hedysarum* species is represented by narrow, ribbed leaflets. Because of their peculiar structure, the leaflets even after mild homogenization, give pellets of broken chloroplasts. Consequently, the DNase treatment of the chloroplast pellets previously used to remove the nuclear DNA selectively cannot be applied. Among the six restriction enzymes (Sal I, Bgl I, BamH I, Xho I, Hpa II, EcoRI) used in the ct DNA analysis, Hpa II gives the most useful restriction patterns for comparing *Hedysarum* ct DNAs contaminated with nuclear DNA. The enzyme produces the largest number of ct DNA fragments and therefore has a high resolving power for uncovering differences. Furthermore, the low molecular weight of the Hpa II fragments prevents interference with the nuclear DNA contamination (which appears as a smear in the upper part of the patterns).

Figure 1 shows the Hpa II restriction patterns obtained with the ct DNAs isolated from different populations of the two subspecies *H. capitatum* and *H. eu-spinosissimum* (lanes a–g), and from *H. carnosum, H. coronarium* and *H. flexuosum* (lanes h–j). With the exception of the two Sardinian populations of *H. eu-spinosissimum* Sa 002 and Sa 021, the different populations of *H. capitatum* and *H. eu-spinosissimum* (described in Table 1) have identical ct DNAs. Sa 002 and Sa 021 possess an additional Hpa II band of about 5 kb in size. Other enzymes mentioned above do not allow us to distinguish the two subspecies (patterns not shown). Each of the three species *H. carnosum, H. coronarium,* and *H. flexuosum* appear to be characterized by a specific ct DNA pattern (lanes h–j) quite distinct from the other two as well as from the subspecies *H. capitatum* and *H. eu-spinosissimum.* Taking the *H. capitatum* ct DNA as a reference, ct DNAs from *H. carnosum, H. coronarium* and *H. flexuosum* contain respectively six, nine, and eight specific bands as

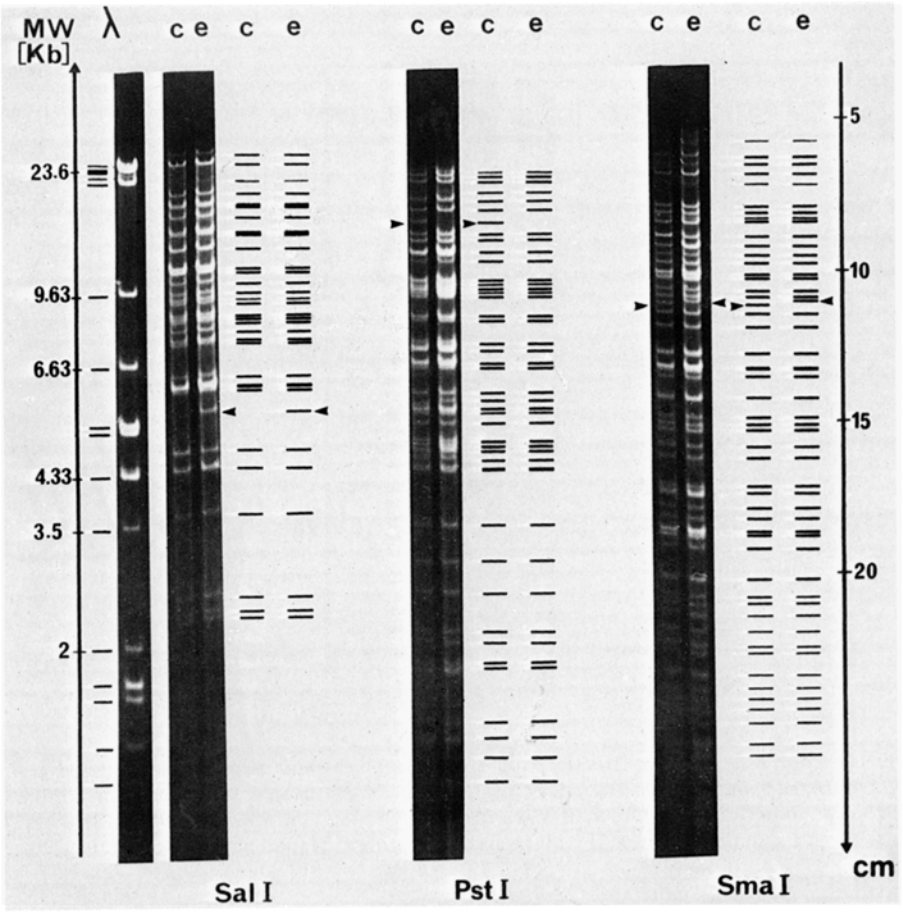

Fig. 2. Sal I, Pst I and Sma I restriction patterns of mt DNAs from: *c H. capitatum; e H. eu-spinosissimum;* the two subspecies originate from Tunisia. On the *right* are the corresponding schematic representations with *arrows* locating the specific fragments

Table 2. Distribution of restriction fragments (larger than 1 kb) obtained by Hpa II hydrolysis of ct DNA from different *Hedysarum* populations

	H. capitatum and *H. eu-spinosissimum* (Sa 002 and Sa 021 excepted)	*H. eu-spinosissimum* Sa 002 Sa 021	*H. carnosum*	*H. coronarium*	*H. flexuosum*
T	15	16	17	17	16
C	–	15	11	8	8
S	–	1	6	9	8

T = Total no. of fragments; C = no. of common fragments with *H. capitatum;* S = no. of specific fragments (by comparison to *H. capitatum*)

opposed to eleven, eight, and eight common bands (Table 2).

Restriction analysis of mt DNAs isolated from Hedysarum species

The situation encountered with *Hedysarum* mt DNAs is quite different from that described above with ct DNAs because the nuclear DNA contamination can be removed easily by DNase treatment of the mitochondrial pellets without loss of mt DNA. Such a difference between the two cytoplasmic compartments may be explained by the different plant material used in organelle isolation (see "Materials and methods").

Comparison of the Pst I, Sma I, EcoRI and Bam H I patterns of the mt DNAs isolated from *H. capitatum*

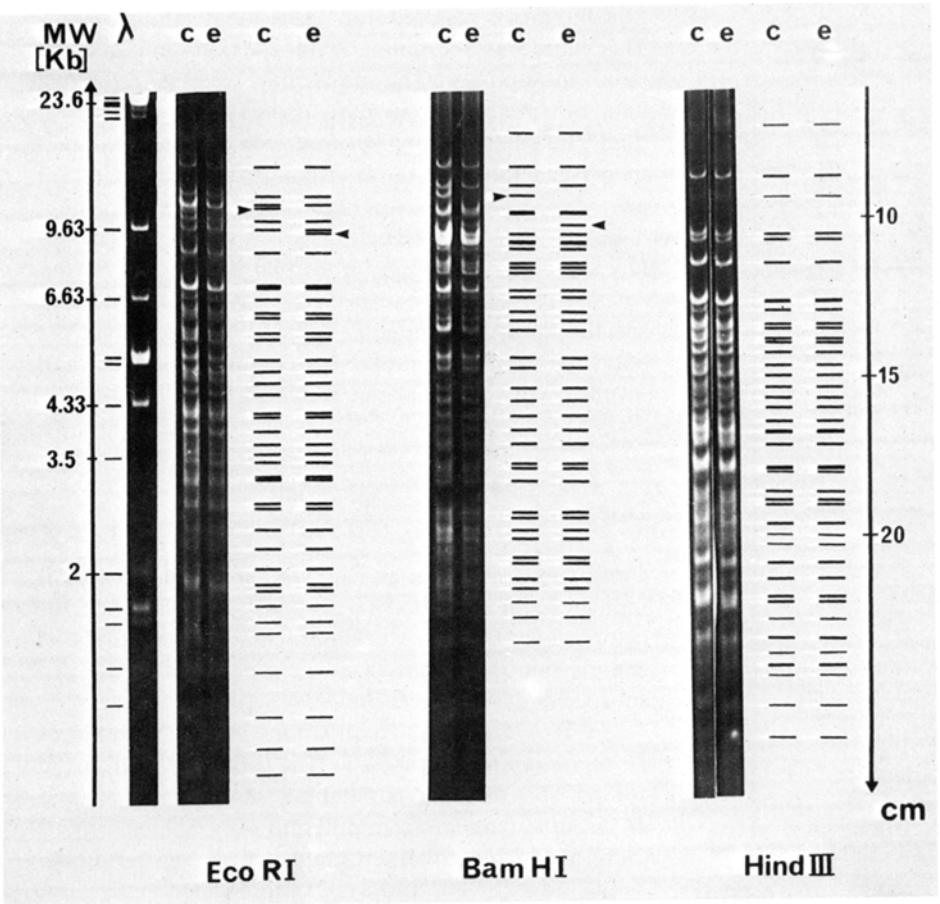

Fig. 3. EcoRI, Bam HI and Hind III restriction fragments of mt DNAs from: *c H. capitatum; e H. eu-spinosissimum;* the two subspecies originate from Tunisia. On the *right* are the corresponding schematic representations with *arrows* locating the specific fragments

Table 3. Distribution of restriction fragments (larger than 1 kb) obtained by Bam HI and Sal I hydrolysis of mt DNA from different *Hedysarum* species

Enzyme		*H. capitatum*	*H. eu-spinosissimum*	*H. carnosum*	*H. coronarium*	*H. flexuosum*
	T	37	37	37	37	35
Bam HI	C	–	36	32	31	31
	S	–	1	5	6	4
	T	30	30	30	29	30
Sal I	C	–	30	22	19	23
	S	–	1[a]	8	10	7

T = Total no. of fragments; C = no. of common fragments with *H. capitatum;* S = no. of specific fragments (by comparison to *H. capitatum*)

[a] This fragment is either absent, or present as a very faint band in *H. capitatum* Sal I patterns

and *H. eu-spinosissimum* revealed that each subspecies contains a specific mt DNA (Figs. 2 and 3). Differences between *H. capitatum* and *H. eu-spinosissimum* mt DNAs do not exceed two DNA bands with each of the four enzymes. In the case of the Pst I analysis, the two subspecies differ only by the presence of an additional band of about 14 kbp in the *H. capitatum* diagram. Electrophoregrams of Sma I mt DNA fragments contain 50 fragments each and may be distinguished by their eighteenth fragment: 9.1 kbp in the allogamous subspecies as opposed to 9.3 kbp in the autogamous one. Each subspecies has EcoRI patterns containing 39 fragments of which only two are specific (the fourth, 11 kbp in size in *H. capitatum* and the sixth, 9.5 kbp in size in *H. eu-spinosissimum*). Bam H I restriction patterns are composed of 37 fragments of which the fourth (11.5 kbp) and the fifth (10 kbp) are specific for mt DNA s from *H. capitatum* and *H. eu-spinosissimum,*

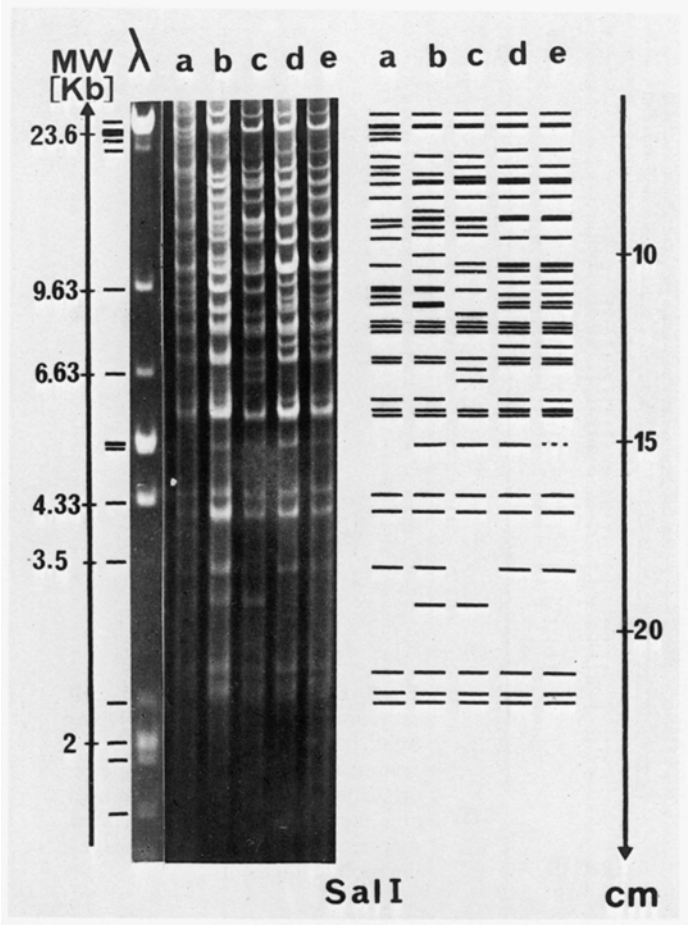

Fig. 4. Agarose slab gel electrophoresis of Sal I digests of mt DNAs from: *a H. flexuosum* from Morocco; *b H. carnosum* from Tunisia; *c H. coronarium* from Sardinia; *d H. eu-spinosissimum* from Tunisia; *e H. capitatum* from Tunisia. On the *right* are the corresponding schematic representations

respectively. In the case of the Sal I cleavage, the *H. capitatum* pattern is either missing a fragment of 5.3 kbp (always present with *H. eu-spinosissimum*) or contains it as a faint band (compare Fig. 2 and Fig. 4, lane e). Hind III did not allow us to distinguish between the two subspecies (Fig. 3). It has been verified that *H. capitatum* populations of different geographical origins have identical mt DNAs. Identical patterns have also been found among mt DNAs of different *H. eu-spinosissimum* populations, including Sa 002 and Sa 021.

The enzymes mentioned above gave specific mt DNA patterns with *H. carnosum, H. coronarium* and *H. flexuosum.* Sal I and Bam HI restriction patterns obtained with the five diploids have been presented as examples (Figs. 4 and 5) because of their reduced complexity – about 30 and 37 fragments, respectively. The molecular weight of the five mt DNAs estimated by adding the molecular weight of all the Sal I restriction fragments resulted in a value greater than 300 kb. This value was determined without taking into account band multiplicity. The molecular weights estimated with other enzymes used in experiments were lower than 300 kb, probably because small restriction fragments generated by these enzymes migrate out of the gel.

Using the *H. capitatum* mt DNA as a reference, mt DNAs from *H. carnosum, H. coronarium* and *H. flexuosum* are characterized respectively by five, six, and four specific Bam HI fragments (Table 3). In the case of the Sal I analysis, these mt DNA's contain eight, ten, and seven specific bands, respectively.

Discussion

Comparison of the ct and mt DNA restriction patterns indicates that each *Hedysarum* species contains specific cytoplasmic DNAs. More particularly, two kinds of cytoplasmic DNA variations were detected in the two subspecies of *H. spinosissimum:*

1) Two Sardinian populations of the subspecies *H. capitatum* possess specific Hpa II ct DNA patterns in comparison to other populations of *H. capitatum* and *H. eu-spinosissimum* from different geographical origins (Sardinia included). Interestingly, these two Sardinian populations (Sa 002 and Sa 021) are located in the same peninsula, which suggests that their ct DNA has recently evolved from the ct DNA commonly found in *H. capitatum.* Further knowledge about this variation requires the construction and the comparison of the ct DNA physical maps corresponding to the two types of populations.

2) Each subspecies is characterized by distinct mt DNA patterns with the enzymes Pst I, Bam HI, Sma I, EcoRI and to a much lesser extent Sal I. However, Pst I, Bam HI, Sma I and EcoRI restriction patterns from the two subspecies present differences limited to one restriction site. At the present time, we have a very restricted understanding of both the extent and the molecular basis of any variations (or mutations) in higher plant mitochondrial genomes which affect mitochondrial function. However, previous results indicate that the mitochondrial genome, rather than the chloroplast genome, is the cytoplasmic determinant of male sterility in a range of plants (Leaver and Gray 1982; Belliard et al. 1979). In the case of the two subspecies of *H. spinosissimum,* we can further speculate that the mt DNA polymorphism is in some way related to the different mating systems.

The degree of divergence in organelle DNAs from the five diploids conforms to a great extent with variations in important characteristics (detailed in Ta-

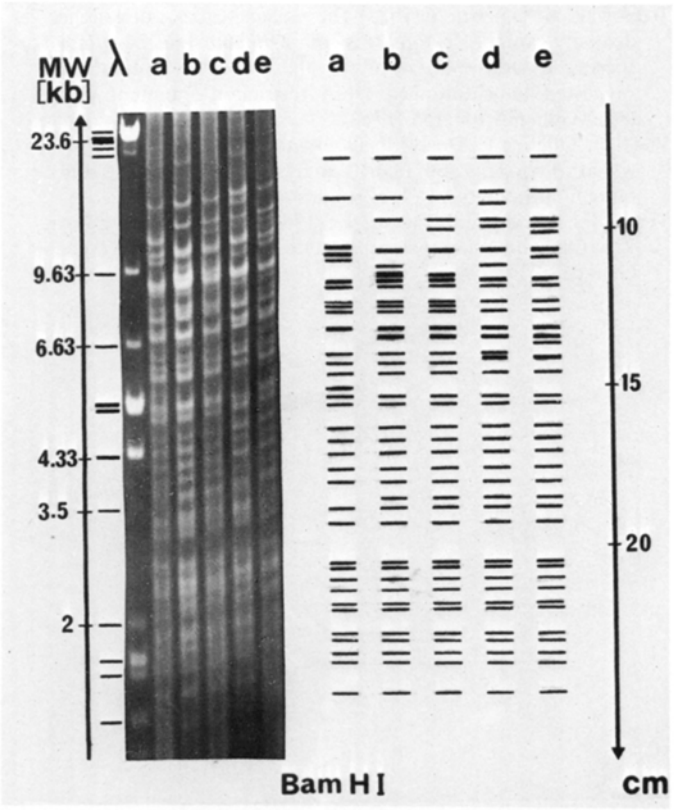

Fig. 5. Agarose slab gel electrophoresis of Bam HI digests of mt DNAs from: *a H. flexuosum; b H. eu-spinosissimum; c H. capitatum; d H. carnosum; e H. coronarium.* Geographical origins are as indicated in Fig. 4

ble 1). Two subsets are distinguished: subset I with the two subspecies of *H. spinosissimum,* and subset II grouping *H. carnosum, H. coronarium* and *H. flexuosum.* According to theories of evolution, one can hypothesize that *H. capitatum* (mostly allogamous with variant ct DNA populations) has evolved from *H. eu-spinosissimum* (mostly autogamous). On the other hand, the mt DNA analysis suggests the sequence *H. flexuosum, H. carnosum, H. coronarium.* We are unable to choose between the two possibilities: either the diploids of the western Mediterranean basin have evolved from *H. eu-spinosissimum,* or from an ancestor common to subset I and subset II. At the present time, relationships in the genus *Hedysarum* are hampered by the lack of natural hybrids and the complete deficiency of intra and interspecific hybridization (Baatout 1980).

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