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Comparison of Molecular Cytogenetic and Genetic Analyses in Accessions of the Two Biotypes of *Vicia benghalensis* L.

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In *Vicia benghalensis* two different biotypes, named A and B, had been observed which differed for several cytological, genetical and biochemical characters. In the present study 27 accessions of this species belonging to the two biotypes were investigated using different molecular analyses, supported by an attempt to produce hybrids between selected accessions. RAPD fingerprinting of the 27 accessions demonstrated that accessions belonging to biotype A show a high degree of genetic similarity, while the opposite is true for the accessions of biotype B. The total genomic DNA from one reference accession was used as a probe to Southern blots of the DNA extracted from all the accessions in use. This analysis demonstrated that cross hybridization among the DNA of the two biotypes occurs only to a limited extent. The chromosomal localization of 18S-5·8S-25S and 5S rRNA gene clusters was determined by *in situ* hybridization. The results indicate that the two biotypes differ in the position of one of the 5S rRNA gene clusters. This indicates a major chromosomal rearrangement. Cell synchronization experiments on two reference accessions suggested that the two biotypes might differ in the duration of the single phases of the cell cycle. Finally, in crossing experiments three pods were obtained which developed for only a few days and proved to bear no viable seeds. These results confirm the existence of two divergent gene-pools and demonstrate the reproductive barrier between the two biotypes, thus suggesting the possibility of a specific ranking.

Key words: *Vicia benghalensis* L., genetic differentiation, *in situ* hybridization, Southern hybridization, crosses, RAPDs, cell synchronization.

INTRODUCTION

Differences in a small number of morphological characters, mainly of the reproductive organs of plants, often provide the most conspicuous means of distinguishing closely related species, or are used in studying the differentiation level among conspecific populations. In *Vicia benghalensis* L. ($2n = 14$) belonging to section *Cracca* (Leguminoseae), and considered a synonym of *V. atropurpurea* Desf. (Mettin and Hanelt, 1967), morphological characters described in the botanical keys to the species (Ball, 1968) fail to reveal any large scale differentiation. In turn, this result is supported by cytological and biochemical studies (Chooi, 1971; Galasso *et al*., 1994; Piergiovanni *et al*., 1995).

Previous studies demonstrated that within a collection of *V. benghalensis* held at the Germplasm Institute in Bari, two separate groups could be identified, which differed for heterochromatin content and distribution, and for seed storage protein profiles (Galasso *et al*., 1994; Piergiovanni *et al*., 1995). The latter results suggested, additionally, a difference in the mating behaviour of the two groups. Subsequent analyses using allozyme markers suggested the existence of a reproductive barrier and, hence, the presence of two genetically isolated gene-pools (Sonnante, Piergiovanni and Pignone, 1997). As a result the two groups were identified as 'biotype A' and 'biotype B': the former showed a less heterochromatic karyotype and lower genetic variability than the latter.

Southern-blot hybridization of total genomic DNA as a probe to membrane-bound DNA fragments, digested with restriction endonucleases, from a related species has proved to be an effective tool to examine, at the qualitative level, the phyletic relationships of the species involved (Anamthawat-Jónsson *et al*., 1990; Heslop-Harrison and Schwarzacher, 1996). Moreover, RAPD (Random Amplified Polymorphic DNA) fingerprinting has been used to provide quantitative estimation of the degree of genetic divergence or taxonomic relationships among conspecific or heterospecific populations (Maass and Klaas, 1995; Prince *et al*., 1995).

The aim of the present study is to clarify the extent of genetical and functional differentiation using different approaches to help understand the genetic relationships among the accessions. Moreover, to better elucidate the observed genetic isolation, hybridization experiments were made to ascertain the level of fertility in hybrids between the two biotypes.

MATERIAL AND METHODS

Plant materials, DNA extraction and Southern hybridization

Twenty-seven accessions of *V. benghalensis* stored at the Germplasm Institute (CNR) of Bari were used (Table 1). Total genomic DNA was extracted from young leaves as

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TABLE 1. Vicia benghalensis *accessions used in this study*

Identification number*	Accession number†	Biotype‡	Origin§
1	105608	B	Italy
2	105647	A	France
3	101765	B	n.r.
4	103298	B	Italy
5	104347	A	n.r.
6	104632	B	n.r.
7	104637	B	n.r.
8	104746	B	Latvia
9	104749	B	Krasnodar
10	104762	B	Ukraina
11	105154	A	Israel
12	105158	A	Italy
13	105821	A	n.r.
14	105302	A	Italy
15	105563	A	Italy
16	105596	A	n.r.
17	105597	A	Italy
18	105598	A	n.r.
19	105599	A	n.r.
20	105600	A	France
21	105281	B	Italy
22	105826	A	n.r.
23	105832	A	n.r.
24	105912	B	n.r.
25	106199	B	n.r.
26	106200	B	Greece
27	108257	A	n.r.

* Used to identify lanes in Figs 2 and 3.
† MG numbers used at the Istituto del Germoplasma.
‡ Following Piergiovanni *et al*. (1995).
§ n.r., Not reported by the donor.

described by Dellaporta, Wood and Hicks (1983). DNA was digested with BamHI, EcoRI, EcoRV and HpaII restriction endonucleases, fractionated by electrophoresis on a 1·5% agarose gel (about 5 μg/lane) and transferred onto a positively charged nylon membrane by Southern blotting, using 0·4 M NaOH solution overnight. Membranes were hybridized, without blocking DNA, using as a probe the total genomic DNA from *V. benghalensis* biotype A (accession MG 105647) mechanically sheared to about 500 bp. The genomic probe was labelled using ECL™ (Amersham) direct labelling and detection system according to Anamthawat-Jonsson *et al*. (1990). Hybridization and washings were performed in 6 M urea for high stringency (0·5 × SSC), allowing probe with over 86% homology to remain hybridized.

RAPD fingerprinting

Forty 10-mer oligonucleotides (OPP 01–20 and OPQ 01–20, Operon Technologies Inc., USA) were tested as single primers for the amplification of RAPD sequences. Nine primers (OPP-03, OPP-04, OPP-05, OPP-06, OPP-09, OPP-10, OPQ-02, OPQ-09, OPQ-10), which showed a relatively simple, clear and reproducible banding pattern were chosen for further analysis.

Amplification reactions had a final volume of 25 μl containing 10 mM Tris-HCl pH 9, 50 mM KCl, 1·5 mM $MgCl_2$, 100 μM each of dATP, dTTP, dCTP, and dGTP, 0·2 μM of 10-mer random primer, 1 unit of Taq DNA polymerase, and 25 ng of genomic DNA. RAPD reactions were performed in a Perkin-Elmer thermal cycler. PCR was initiated by a denaturation step at 94 °C for 3 min, followed by 45 cycles of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min; a final elongation at 72 °C for 5 min was allowed. Amplification products were resolved by electrophoresis on a 1·5% agarose gel stained with ethidium bromide and visualized under UV illumination.

PCR products were scored as presence (1) or absence (0) of bands for each of the 27 accessions analysed. Only reproducible bands were scored. Data were used to calculate a Jaccard (1908) similarity index from which a UPGMA dendrogram was constructed.

Cell synchronization

For cell synchronization experiments, seeds of accession MG 105647 (biotype A) and MG 105608 (biotype B) were treated with 5% HClO in distilled water for 15 min and soaked in running tap water overnight, prior to treatment with 0·02% gibberellic acid for 4 h at room temperature; germination took place in Petri dishes at 25 °C in the dark.

In metaphase synchronization experiments, 2 cm long root tips were incubated for 18 h in Murashige and Skoog (1962) medium containing 2·5 mM hydroxyurea (HU) and then transferred to HU-free MS medium following Dolezel, Cihalikova and Lucretti (1992). Root tips were sampled at 1 h intervals, up to 8 h, to monitor mitotic activity. The mitotic activity was calculated as the mitotic index (MI), i.e. the proportion of dividing cells over the total number of cells observed. Each time at least 1000 cells from at least three different root tips were scored.

Chromosome preparation and in situ *hybridization*

For metaphase preparations the same protocol and accessions as for synchronization were used, except that root tips were excised around the time assuring the highest MI and treated in saturated *p*-dichloro-benzene solution for 2 h at 13 °C or in 0·05% colchicine at room temperature for 2·5 h, before fixation in ethanol: acetic acid (3:1, v/v), and storage at 4 °C until use.

Chromosome preparations for *in situ* hybridization followed the protocol described by Schwarzacher, Leitch and Heslop-Harrison (1994). Mitotic metaphases were hybridized *in situ* with the probes pTa71 and pTa794. The clone pTa71 (Gerlach and Bedbrook, 1979), containing a 9Kb EcoRI fragment of *Triticum aestivum* L. consisting of the 18S-5·8S-25S rDNA and the transcribed and non-transcribed intergenic spacer regions, was labelled with rhodamine-4-dUTP by nick-translation. The clone pTa794 (Gerlach and Dyer, 1980), including a 410 bp 5S rDNA and the intergenic spacer isolated from *Triticum aestivum* L., was labelled with digoxigenin-11-dUTP using the polymerase chain reaction.

The hybridization mixture and the chromosome preparations were denaturated together at 70 °C for 5 min; the temperature was then gradually decreased to 37 °C using a

Hybaid Omnislide temperature cycler (Heslop-Harrison *et al*., 1991).

Crossing experiments

An accession of biotype A (MG 105647) was reciprocally crossed with one of biotype B (MG 105608). Generally one or two flowers per inflorescence were used, while the remaining flowers were removed. Emasculation and pollination were performed manually. After pollination the flowers were bagged using clear bags and pod development monitored.

RESULTS

Southern hybridization

In ethidium bromide-stained gels of size-fractionated DNA digested with BamHI, EcoRI, EcoRV and HpaII, many repetitive DNA families gave restriction fragment bands. Some bands were present in both biotypes, but many were only present in one biotype and absent in the other (Fig. 1A). After Southern hybridization with labelled genomic DNA of biotype A, strong hybridization was observed only on the lanes relative to biotype A (Fig. 1B)

The luminographs in Fig. 2 show the hybridization of labelled genomic DNA of *V. benghalensis* biotype A as a probe to size-fractionated EcoRI DNA digests of the 27 accessions of *V. benghalensis* examined. Strong hybridization signals to the DNA lanes of biotype A are evident, while very weak or no hybridization signals occurred on lanes representing biotype B.

RAPD fingerprinting

A total of 88 RAPD bands were obtained with nine primers, with a mean of 9·8 and a range of 2–16 bands per primer. An example of a typical RAPD pattern is shown in Fig. 3. Only one band of primer OPP-05 was monomorphic across all accessions; all the other 87 bands showed polymorphism and were used in cluster analysis. Sixty-two bands were only found in accessions of biotype B, while those remaining were detected either in accessions of both biotypes or of biotype A only. Accessions belonging to biotype A revealed a lower level of polymorphism and 12 bands were shared by all accessions of this group. In contrast, all accessions of biotype B revealed only one common band and a higher level of variation was observed.

Cluster analysis of the similarity values was performed to generate a UPGMA dendrogram showing similarity between accessions (Fig. 4). In the graph, two main clusters can be detected: the upper group comprises all accessions belonging to biotype A, whereas the lower cluster includes all accessions corresponding to biotype B. The two groups separate at a value of 0·153. The similarity index ranges

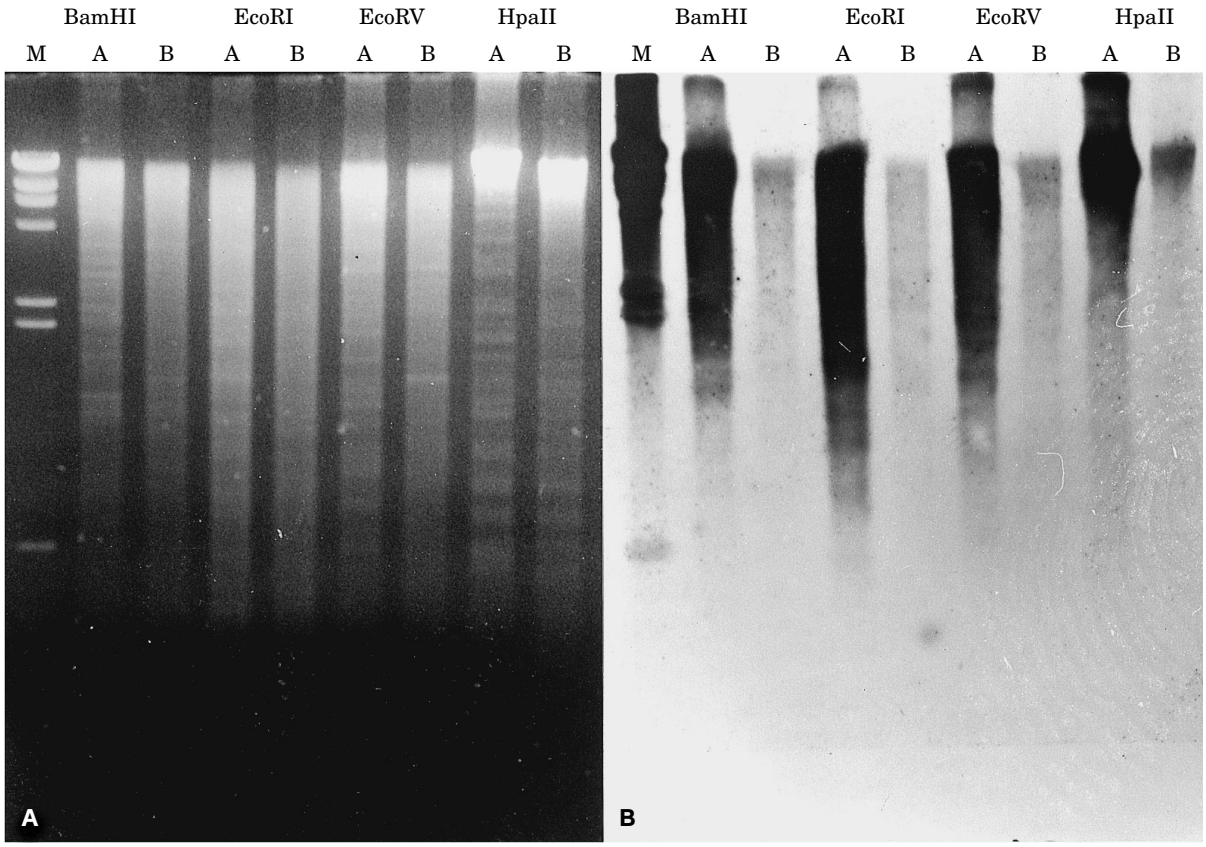

FIG. 1. Southern hybridization using total genomic DNA from *V. benghalensis* MG 105647 (biotype A), as a probe to digests of *V. benghalensis* biotype A (MG 105647) and B (MG 105608). **A**, Ethidium bromide stained gel. **B**, Luminograph of (**A**) after transfer and probing with *V. benghalensis* biotype A. M, Lambda HindIII marker bands (from top to bottom) 23·1, 9·4, 6·6, 4·4, 2·3, 2·1, 0·5 Kb.

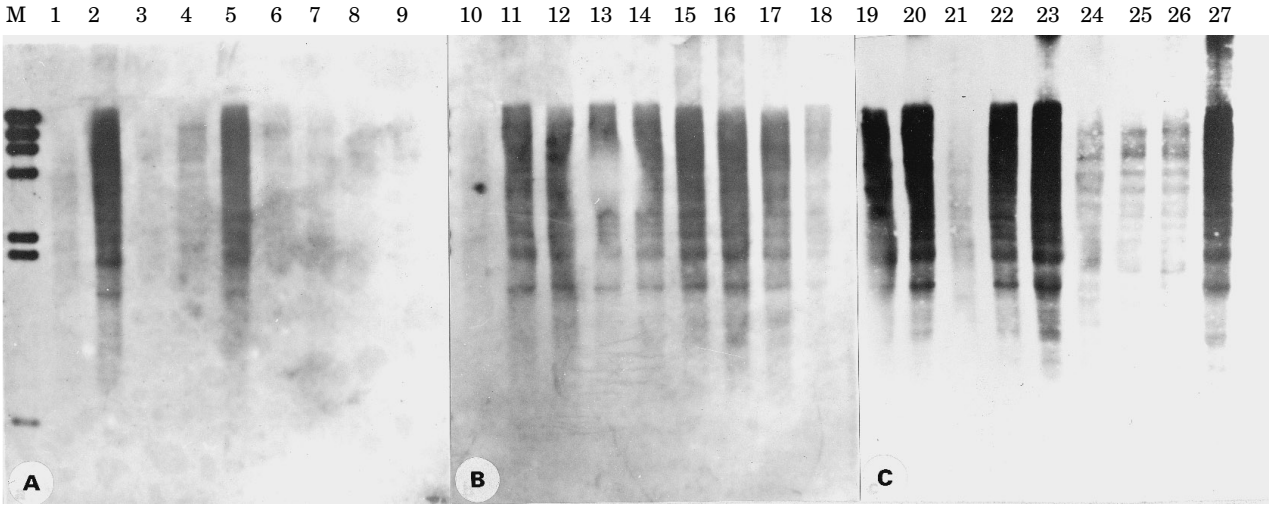

FIG. 2. Southern hybridization of *V. benghalensis* MG 105607 (biotype A) total genomic DNA probe to a target blot containing EcoRI restricted and fractionated total genomic DNA digest from 27 *V. benghalensis* accessions listed in Table 1. M, Lambda HindIII marker.

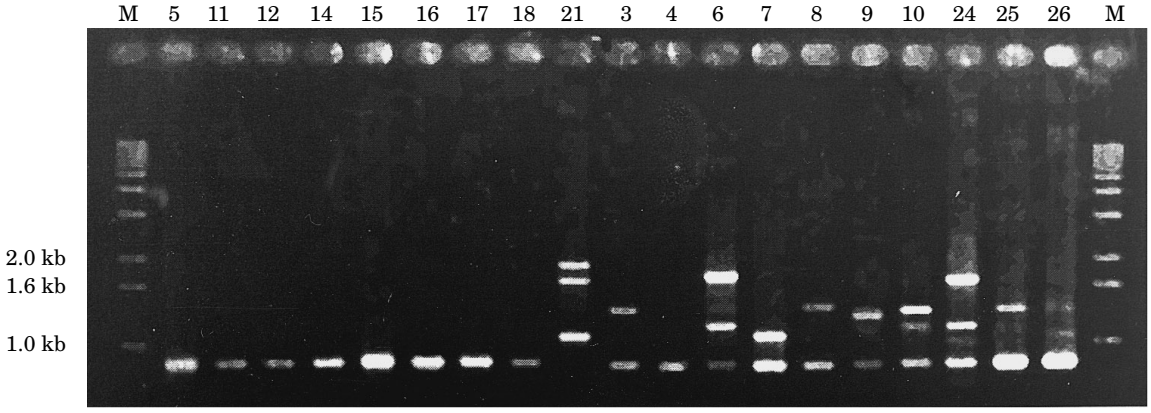

FIG. 3. RAPD profiles of 19 representative *V. benghalensis* accessions using the primer OPQ 10. Numbers refer to accessions listed in Table 1. M, 1 Kb DNA ladder.

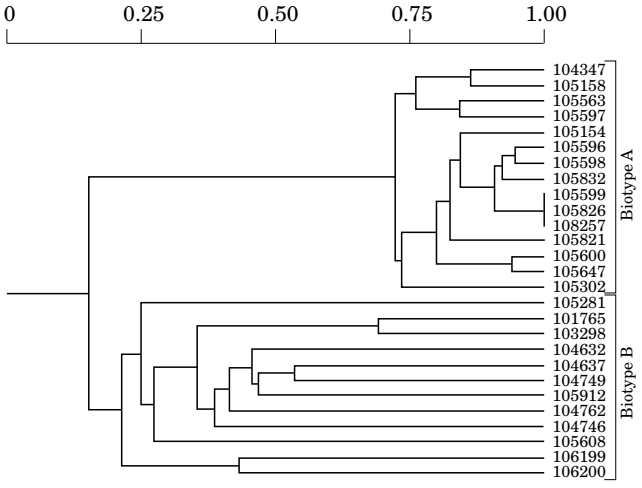

FIG. 4. UPGMA dendrogram based on Jaccard (1908) similarity index.

from 0·054, between accessions 105600 (biotype A) and 104762 (biotype B), to 1, between accessions 105599, 105826, and 108257, all belonging to biotype A and possessing the same banding pattern for all the primers analysed. However,

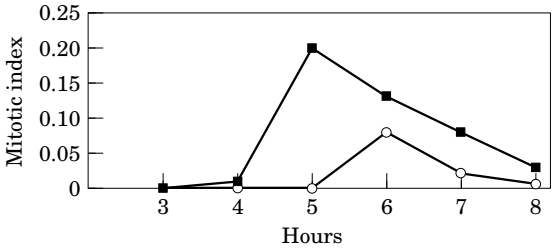

FIG. 5. Variation of the proportion of dividing cells after hydroxyurea treatment. ■, Biotype A; ○, Biotype B.

such a high value might be the consequence of a low number of polymorphic bands in biotype A, i.e. 13. Within biotype A, similarity ranges from 0·522 (between accessions 104347 and 105600) to 1, whereas within group B values range from 0·143 (between 105608 and 104762) to 0·692 (between 101765 and 103298).

Cell synchronization

The results of the synchronization experiments are shown in Fig. 5. Hydroxyurea proved to be a very good

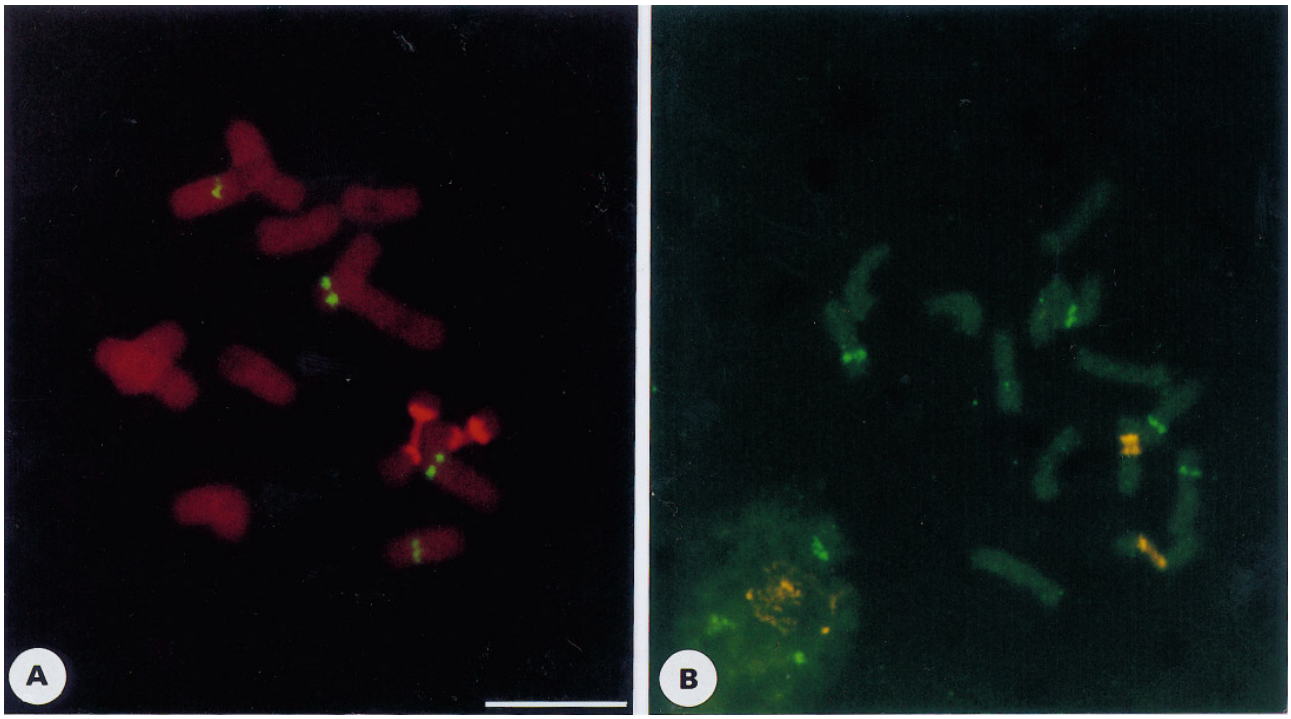

FIG. 6. **A**, *V. benghalensis* biotype A and **B**, *V. benghalensis* biotype B metaphases after target *in situ* hybridization with rhodamine-4-dUTP labelled 18S-5·8S-25S rDNA (red-yellow) and digoxigenin-11-dUTP labelled 5S rDNA (green); arrows indicate the 5S rRNA gene cluster located on the long arm in biotype A. Bar = 10 μm.

synchronizing agent at the concentration used in the experiments (2·5 mM). Its efficiency is demonstrated by the absence of any dividing cells within the first 3 h (biotype A) and 5 h (biotype B) after the end of HU treatment, which blocks cells in the S phase. Comparison of MI showed differences between biotype A with a peak of MI (0·1998) at 5 h after HU treatment, while biotype B reached a peak of MI (0·0782) after 6 h (Fig. 5).

Eight hours after HU treatment the difference in the MI of both biotypes was significantly reduced. Comparing the areas below the two curves, it becomes evident that the number of dividing cells is much higher for biotype A than for biotype B.

In situ *hybridization*

After simultaneous *in situ* hybridization with rhodamine-4-dUTP labelled pTa71 and digoxigenin-11-dUTP labelled pTa794 on metaphase spreads, it was possible to observe that the sites of annealing of the two probes are located on different chromosomes. In both biotypes, one pair of pTa71 hybridization sites was detected in association with the secondary constriction of the satellited chromosomes (Fig. 6A and B). The sites of annealing of the pTa794 probe were located on two chromosome pairs, but with different locations in the two biotypes. In biotype B the probe hybridized to regions belonging to the short arms of both chromosome pairs (Fig. 6B); while in biotype A one site was observed on the short arm, and the other on the long arm of another chromosome pair (Fig. 6A). Due to the similarity of the non-satellited chromosomes it is impossible to determine exactly which chromosomes bear the 5S rRNA genes clusters.

Crossing experiments

Flowers of both biotypes were used as female parents in crossing experiments. A few days after pollination all pollinated flowers produced a pod. In most cases, young pods started to turn brown when about 1 cm in length and dried out within a couple of days. In only three cases, out of about 80 crosses, did a complete pod develop in which very small seeds displaying no differentiated features were formed.

DISCUSSION

The results of the molecular analyses confirm the previous assumption that in *V. benghalensis* the two biotypes are markedly different from each other. The results of Southern hybridization using genomic DNA as a probe are unexpected for conspecific populations. When this analysis is used to study similar species it is generally necessary to block with related unlabelled DNA to reduce the cross hybridization of homologous sequences (Anamthawat-Jònsson *et al.*, 1990). In our case this procedure was not necessary since at the stringency of 86%, cross hybridization between the two biotypes was very weak. Genomic DNA contains both

repetitive and single-copy sequences, but in plant genomes the proportion of the latter is very low: *in situ* hybridization experiments have proved that genomic probes are enriched with repetitive dispersed sequences (Heslop-Harrison and Schwarzacher, 1996). Repetitive sequences may evolve very rapidly as soon as a mating barrier is formed, and are, therefore, particularly useful in phylogenetic studies (Anamthawat-Jònsson and Heslop-Harrison, 1993). Thus the strong divergence in repetitive DNA sequences between the two biotypes indicates that the reproductive barrier did not arise recently and permitted independent evolution of the repetitive fractions of the two biotypes.

RAPD analysis confirmed the genetic distance between the previously detected biotypes A and B. In fact the clusters of biotypes A and B revealed only 15% similarity. This represents a very low value for accessions belonging to the same species; a similar or higher Jaccard index value separated different species of *Hordeum* (Gonzàlez and Ferrer, 1993). Results from many other species are not directly comparable, due to the utilization of different similarity or distance indices. RAPD banding pattern revealed a great uniformity for biotype A, whereas biotype B showed a high level of polymorphism. A similar behaviour has been observed using isozyme markers (Sonnante *et al*., 1996), and the calculation of Nei's diversity parameters has led to the conclusion that biotype A should have an autogamous mating system, while accessions belonging to biotype B are highly outcrossing.

The cell synchronization experiments indicate that a difference in the length of mitotic cycle exists between the two biotypes. Since HU blocks cells in the S phase, the interval between the release of meristems and the appearance of the first divisions actually gives a measure of the length of the G_2 phase. Moreover the observation of a higher level of dividing cells in biotype A gives an indication that the overall length of the mitotic cycle in biotype A is shorter than in biotype B. The two biotypes differ for heterochromatin content (Galasso *et al*., 1994) and it has been reported that heterochromatin content can affect the duration of the mitotic cycle (Nagl, 1974; Ahmad and Narayan, 1994). In our samples it appears that higher heterochromatin content increases the duration of the cell cycle as observed by Ahmad and Narayan (1994) in the closely related genus *Lathyrus*.

It is also worthy to note that one of the two 5S rRNA gene clusters has a different location in the two biotypes (Fig. 6A and B). Galasso *et al*. (1994) found evidence that, in addition to the difference in heterochromatin content, a chromosomal restructuring involving the non-satellited arm of the satellite-chromosome differentiated the two biotypes. The results of pTa71 hybridization confirm that the satellited arm is unchanged in the two biotypes and retains active 18S-5·8S-25S rRNA genes, as demonstrated by the positive reaction to silver staining of the same regions (Galasso *et al*., 1994). No silent loci such as described for the genome of the legume genera *Cicer* or *Vigna* (Galasso *et al*., 1995, 1996) were detectable.

Crossing experiments have demonstrated that after artificial pollination the two biotypes fail to produce hybrids. Moreover, the barrier appears to be active at a very early stage after pollination; the fact that pods are formed indicates that fertilization occurs, but embryo development is disturbed. The fact that populations belonging to different biotypes do not mate raises the question whether the two biotypes are actually two different species. Biochemical, cytological and molecular data provided evidence that *V. benghalensis* does not form a single, genetically cohesive, species. Further studies are in progress to define the systematics and nomenclature of the two biotypes better.

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