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Phylogenetic and genetic studies in *Papaver* section *Oxytona*: cytogenetics, isozyme analysis and chloroplast DNA variation*

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Summary. Chromosome behaviour at meiosis, isozyme studies and analysis of the chloroplast DNA restriction fragments were used to assess the phylogenetic relations among the three *Papaver* species of the section *Oxytona*. The multivalents observed in diplotene – diakinesis stages of meiosis of the hexaploid *P. pseudo-orientale* and its tetraploid hybrid with *P. bracteatum* indicate the autopolyploid nature of this section. Further evidence supporting this conclusion was obtained from isozyme analysis. The same number of isozymes was expressed in all the species regardless of their ploidy level. Inheritance studies conducted with Pgi, Dia and Acp allozymes demonstrated, for the first time, the transfer and expression of genetic material among these species. The differences found in the chloroplast DNA restriction fragments of the *Oxytona* species and of *P. somniferum* indicate intensive evolution of the chloroplast DNA in the genus *Papaver*. The similarity of the chloroplast DNA restriction patterns and of the isozymes in *P. orientale* and *P. pseudo-orientale* suggested that *P. orientale* was the female parent in the cross generating *P. pseudo-orientale* and that the latter species is of recent origin.

Key words: *Papaver oxytona* – Interspecific hybrids – Polyploidy – Isozymes – Chloroplast DNA

Introduction

The *Papaver* species of section *Oxytona* are currently receiving increased attention for the therapeutic properties of their alkaloids; *P. bracteatum* is particularly important since it might represent a safer substitution for the opium poppy *P. somniferum*. The species of the section *Oxytona* were studied, mainly from the chemical point of view, and a comprehensive review of the alkaloids of this section was recently published (Theuns et al. 1987). Genetic aspects were barely investigated, and in several studies great confusion prevailed in the correct identification of the species as emphasized by Theuns et al. (1987).

The three species of the *Oxytona* section constitute a polyploid series: *P. bracteatum*, diploid ($2n = 14$); *P. orientale*, tetraploid ($2n = 28$); and *P. pseudo-orientale*, hexaploid ($2n = 42$). Goldblatt (1974), in his review on the morphology, chemistry and ecology of this section, suggested that *P. orientale* is allotetraploid, originating from an interspecific cross between *P. bracteatum* and another alpine diploid species; *P. pseudo-orientale* is considered to result from a backcross of *P. orientale* to *P. bracteatum*.

In order to assess the phylogenetic relationships among the species of this series, we have employed various experimental approaches: cytogenetics, isozyme and chloroplast DNA variation. These approaches are powerful tools for determining the structure, origin and evolutionary trends of polyploids (Cauderon 1985; Crawford 1983; Gottlieb 1981a; Palmer et al. 1983; Vedel et al. 1981). This work presents combined evidence on the phylogeny of this section, and the value of the findings for future breeding is briefly discussed.

Materials and methods

Plant material

One accession from each species: *P. bracteatum* = PB (P.I. 381442), *P. orientale* = PO (P.I. 376815), and *P. pseudo-*

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orientale = PPO (P.I. 375952), were used in this study. Plants of the parental species, F_1 hybrids, F_2 and backcross (BCF_1) families were examined. For a detailed description of the crosses and growing conditions of the plant material, see Milo et al. (1986). Outcrossing is predominant in the polyploid species and *P. bracteatum* is self-incompatible. In outcrossing species, one population provides most of the isozyme variation encountered in the species (Gottlieb 1981a). For chloroplast DNA analysis, *P. somniferum* = PS (P.I. 253162) was included.

Cytogenetics

Flowering buds were examined to study the chromosome pairing in early stages of meiosis in *P. pseudo-orientale,* and the hybrid between *P. pseudo-orientale* and *P. bracteatum.* PMC's at diplotene – diakinesis stages were analysed as described previously (Milo et al. 1986).

Isozyme analysis

Crude extracts were obtained by macerating fresh leaves of 2-month-old plants in Tris 0.1 M pH 7.5 buffer, with glutation 0.06 M and 0.06 mg/ml PVP40. Three gels and electrode buffer systems were used to screen the parental plants for isozymic variation: 1) tris citrate/boric acid mixture pH 7.6; 2) tris citrate/boric acid mixture, pH 8.1 (Tanksley 1979), and 3) histidine/tris citrate mixture, pH 7.0 (Zamir and Ladizinsky 1984). The gels were run for 6 h at 200 V for systems 1) and 2), and at 100 V for system 3). Staining for enzyme activity was done as described by Vallejos (1983), except the assay solution for Diaphorase, which contained 1 mg of 2.6 dichlorophenol indophenol, 1 mg NADH and 10 mg MTT in 50 ml Tris 0.1 M, pH 8.5.

Chloroplast DNA analysis

Chloroplast DNA was purified from leaves of one-month-old plants of the three *Oxytona* species and of *P. somniferum* belonging to the *Mecones* section, according to the procedure of Saltz and Beckman (1981). Restriction endonucleases Bgl I, Xho I, Kpn I, EcoR I, Hind III, Sma I, Sal I and BamH I were purchased from New England Biolabs and digestion was performed according to the supplier's instructions. Restriction fragments were separated by electrophoresis in 0.5%–1.0% agarose slab gels in buffer containing: 40 m*M* tris-base, 20 m*M* sodium acetate and 1 m*M* EDTA (pH 7.5).

Results and discussion

Cytogenetics

In a previous study (Milo et al. 1986) only bivalents were observed at Metaphase I of the three species: *P. bracteatum* (PB) $2n = 14$, *P. orientale* (PO) $2n = 28$ and *P. pseudo-orientale* (PPO) $2n = 42$. The formation of bivalents in the polyploid species suggested their alloploid origin. However, the interspecific hybrid between PB and PPO showed the formation of 14 bivalents. This result indicates that in addition to the homology between the chromosomes of the two species, autosyndesis prevails among the PPO chromosomes. These contradictory results, and the decrease of chiasma per chromosome in the polyploid species compared with the diploid, led us to examine early stages of the meiosis. The hexaploid PPO and its tetraploid hybrid with PB were examined at diplotene – diakinesis, the earliest stage that individual chromosomes could be clearly distinguished. Multivalents were observed in both cases (Fig. 1). The average and range of chromosome configurations per cell in diplotene and in MI stages are presented in Table 1. The multivalent configurations found in PMC's of PPO and in its hybrid with PB during early stages of meiosis are further evidence for the homology among the three genomes of PPO, as well as between them and the PB genome. These results indicate that PPO is an autopolyploid originating from PB. The bivalents and – at a very low frequency – univalents that are observed at metaphase I in PPO apparently result from early terminalization of chiasmata.

Isozyme analysis

Out of 19 enzymes tested, only 4 – phosphoglucomutase (Pgm), phosphoglucose isomerase (Pgi), diaphorase (Dia) and acid phosphatase (Acp) – showed variation within and/or between species. All remaining enzymes

Table 1. Mean and range (in *parentheses*) of chromosome configurations per cell in metaphase I and diplotene of *Papaver pseudo-orientale* and its F_1 hybrid with *P. bracteatum*

Species or hybrid	Meiosis stage	No. of cells analyzed	No. per cell[a]					
			I	II	III	IV	V	VI
PPO	diakinesis	21	0.9	9.8	0.8	0.8	0.09	0.2
$2n = 42$			(0–3)	(6–12)	(0–2)	(0–2)	(0–1)	(0–1)
	metaphase I	31	0.4	20.8	–	–	–	–
			(0–4)	(19–21)				
PB × PPO	diakinesis	30	0.8	10.2	0.7	1.2	–	–
$2n = 28$			(0–6)	(8–14)	(0–2)	(0–2)		
	metaphase I	34	2.2	12.9	–	–	–	–
			(0–6)	(11–14)				

[a] I = Univalents, II = bivalents, III = trivalents, IV = quadrivalents, V = pentavalents, VI = hexavalents

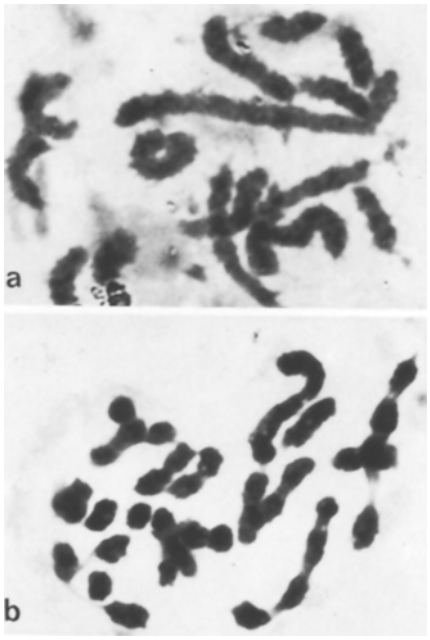

Fig. 1 a, b. Chromosome associations at diakinesis showing multivalents in: **a** F_1 hybrid *Papaver bracteatum* × *P. pseudo-orientale* ($2n = 28$); **b** *P. pseudo-orientale* ($2n = 42$)

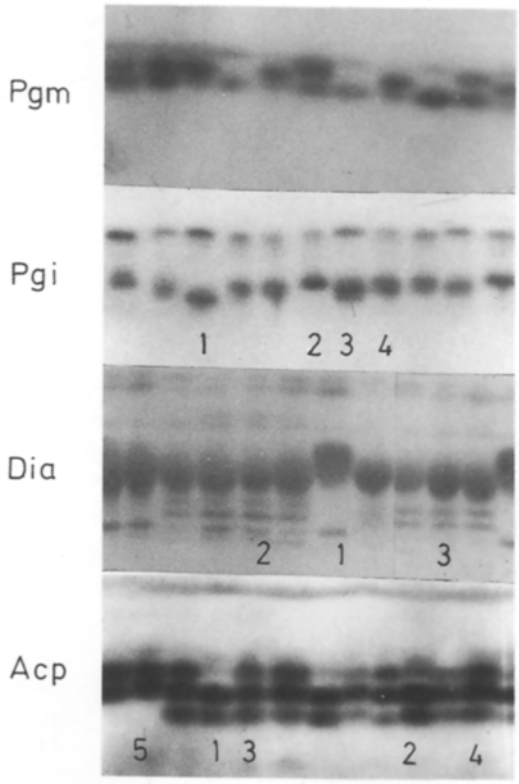

Fig. 2. Zymograms showing electrophoretic pattern of *Pgm, Pgi, Dia* and *Acp* of *Papaver* species and hybrids of section *Oxytona; Pgi 1* slow band in *P. bracteatum; Pgi 2* fast band in *P. orientale; Pgi 3* three-band heterozygote phenotype in the diploid *P. bracteatum; Pgi 4* two-band heterozygote phenotype in the polyploids; *Dia 1* slow band in *P. bracteatum; Dia 2* three-band phenotype in hybrid of *P. bracteatum* × *P. orientale; Dia 3* two-band phenotype in the polyploids; *Acp1-5,* see legend to Fig. 3

displayed a monomorphic band pattern in the tested individuals of the three species.

Phosphoglucomutase

Up to four bands of this enzyme were expressed in the examined material. These bands were organized in two groups. For each group a slow and/or a fast band were present (Fig. 2), and were presumed to be specified by two genes with two alleles (fast and slow) for each. Similar findings were reported in lentil (Zamir and Ladizinsky 1984) and in tomato (Tanksley 1979). Homozygote and heterozygote plants for the two genes were present in the three species. It was observed that the polyploid species had no additional bands, as compared with the diploid species.

Phosphoglucose isomerase

One cytosol Pgi locus, with two different alleles, was expressed in all species. The slow allele was present mainly in the diploid PB and relatively rare in the polyploids PO and PPO. Heterozygote plants were found in all ploidy levels, but differences in the relative band intensity between diploid and polyploid plants were observed. In the diploid PB, the heterozygote phenotype showed three bands as expected for a dimeric enzyme, the middle band of the heterodimer (F/S) being more intense. However, in the polyploid plants, the heterozygote displayed a particular pattern: the F/F and F/S bands were of the same intensity, while the S/S band was very weak and in some cases difficult to detect, as shown in Fig. 2.

This kind of variation in the polyploids suggests that more than two alleles for the same Pgi enzyme are expressed. If, for example, in a tetraploid plant three copies of the fast allele and only one copy of the slow one are present, then the expected proportion of F/F, F/S, S/S will be 9 : 6 : 1, respectively. In a triploid case of two fast alleles to one slow, the expected ratio is 4 : 4 : 1 for F/F, F/S and S/S. Similar differences in band intensities were used by Tanksley (1979) to detect trisomic tomato plants, and by Chyi and Weeden (1984) to identify the $2n$ gamete parent of triploid apples. Hence, only one Pgi gene is expressed in the polyploid species as in the diploid one and the differences in band intensity result from dosage effect of the alleles in the polyploids. These findings constitute additional evidence for the autopolyploid nature of the series.

Table 2. Segregation of parental, F_1 hybrids, BCF_1 and F_2 progenies for Pgi and Dia

Parents and hybrids	Progeny distribution									
	Pgi[a]				Dia[b]					
	Phenotype	S	SF	F	Phenotype	Null	S	SM	SMF	MF
PB		24	12	1		1	32	–	3	–
PO		–	5	14		–	–	–	–	25
PPO		–	6	15		–	–	–	–	34
F_1										
PB × PO	S × F	–	8	2	S × MF	–	–	–	13	–
PB × PPO	SF × SF	–	13	5	S × MF	–	–	–	8	10
PO × PPO	F × SF	–	10	11	MF × MF	–	–	–	–	21
BCF_1										
(PB × PO) × PB	SF × S	3	14	–	SMF × S	–	2	–	15	–
	F × S	–	20	5	SMF × S	–	2	–	23	21
BCF_1										
(PB × PPO) × PB	SF × S	–	18	2	SMF × S	–	14	5	75	31
	F × S	–	10	12						
	F × S	–	–	53						
F_2										
(PB × PPO)	SF ×	–	19	3	SMF ×	–	1	–	12	21
	SF ×	–	5	10						
BCF_1										
(PB × PPO) × PPO	SF × F	–	13	10	SMF × MF	–	–	–	5	58
	F × F	–	–	44						

[a] S = slow band, SF = heterodimer band, F = fast band
[b] S = slowest band, SM = slowest and middle bands, SMF = slowest, middle and fastest bands, MF = middle and fastest bands (see Fig. 2)

The proportions of the various phenotypes in the parental lines and in their hybrids are given in Table 2. All S/F plants were scored together as a heterozygote phenotype. All F1 hybrids, regardless of their genetic constitution, segregated for S/F and F phenotypes only. In most of the hybrids of PB, some plants did not express the expected slow band, although the PB plants used as male parents had this band only. Moreover, in the backcross (PB × PPO) × PB, several families did not segregate, and had the fast band only. (The possibility of contamination must be discarded, since segregation for the other enzymes and morphological characters of the two parents were observed.) These results may be explained by assuming PB plants heterozygous for slow and null alleles, or by partial dominance of the fast allele of PPO. The same feature of dominance of the female parent was reported for malate dehydrogenase isozymes in interspecific hybrids in *Nicotiana* (Sheen 1972).

Diaphorase

Several regions of activity were recovered for this enzyme. A clear-cut variation between species was found for the migration of the slowest bands. The slowest band was observed in all but one plant of PB which was null for this locus; in two plants of this species two additional fast bands were expressed. In the polyploid species all the plants had these two bands only (Fig. 2). Distribution of the progenies resulting from crosses between different genotypes is shown in Table 2. The zymograms of the F1 hybrids between PB × PO and PPO × PO were uniform and expressed the bands of the two parents. The plants of the PB × PPO hybrid segregated in a 1 : 1 ratio for the slow band of the PB parent, indicating that the PB parent plant was heterozygous for null allele. In the BCF_1 and F_2generations, all the families segregated for the three bands. However, the segregation ratios were not mendelian, probably due to the high level of sterility of the interspecific hybrids. The behaviour of the Dia enzyme suggests that three loci are involved in this system for the following reasons: 1) in the diploid species all three bands were present in a few plants, 2) segregation for the fast and middle band indicates that they are controlled by different genes, and 3) the polyploids do not possess additional genes for this particular enzyme.

The BCF_1 and F_2 segregation pattern displayed the transfer and expression of Dia loci between the species. The gametes of the two hybrids of PB and the polyploid

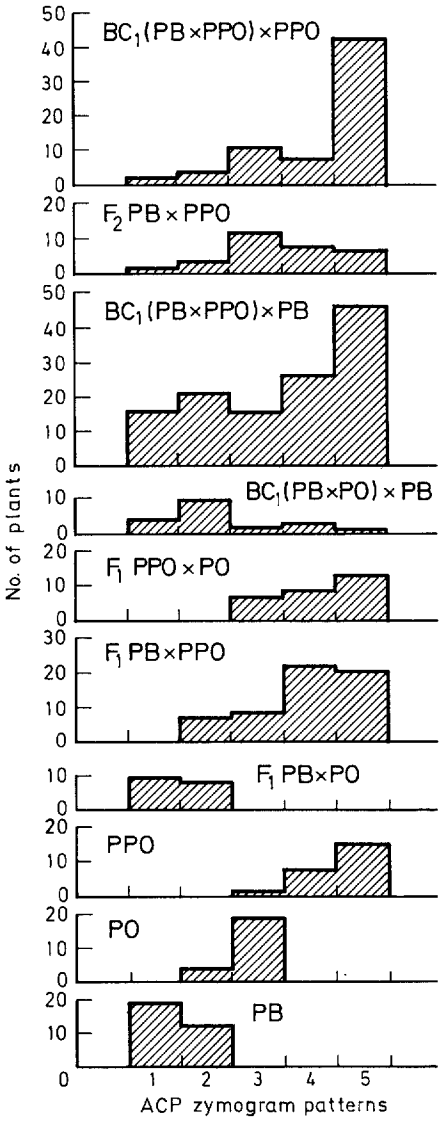

Fig. 3. Distribution of parental, *F1* hybrids, BCF_1 and F_2 progenies for acid phosphatase, classified into five phenotypes: *1* the upper band was absent; *2* the upper band was lighter than the lower band; *3* the upper and the lower bands had the same intensity; *4* the upper band was darker than the lower band; *5* the lower band was absent

species had 14 chromosomes (Milo et al. 1986). These genomes could either be exclusively from the polyploid species, or a combination of chromosomes from both parents. BCF_1 and F_2 plants with the *P. bracteatum* phenotype (slow band only) show that genetic material from this parent is transmitted in viable gametes of the F_1 plants.

Acid phosphatase

Several bands of Acp activity occurred on the zymogram (Fig. 2). The variation between the species was found in the faster three bands that were most intensively coloured. The plants were classified into five phenotypes: 1) The upper band was absent, 2) The upper band was lighter than the lower band, 3) The upper and the lower bands had the same intensity, 4) The upper band was darker than the lower band, and 5) The lower band was absent.

Phenotypes 1 and 2 were observed in PB, 3 and 4 in PO and 3, 4 and 5 in PPO. The distribution of the different phenotypes in the parents and crosses is summarized in Fig. 3. The distribution of the phenotypes in segregating populations showed a wide variation, almost typical of a quantitative trait. The segregation data fit a two-gene model: Acp 1 (+/–) gives the slow and middle bands, Acp 2 (+/–) gives the fast and middle bands. Dosage effects of Acp loci in the polyploid species could account for the "quantitative" nature of the progeny distribution. No additional isozymes were found in the polyploid species when compared with the diploid. The tetraploid PO had an intermediate phenotype between the diploid PB and the hexaploid PPO.

Isozyme analysis is a powerful tool to distinguish between auto and alloploid mode of origin (Crawford 1983). In autopolyploidy the same genes and alleles are usually present both in the diploid and the polyploid species (Gottlieb 1981b; Desborough 1983). On the other hand, in allopolyploidy the mumber of genes in the polyploid species is greater than in any of the diploid progenitors (Roose and Gottlieb 1976; Hart 1983; Weeden 1983). In ancient allopolyploids, silencing can reduce the number of duplicated loci (Ferris and Whitt 1977; Li 1980). In the section *Oxytona* the same loci were observed in the three species for 19 enzymes examined. These results indicate, therefore, that the polyploid species are actually autopolyploids. The possibility that the similarity of loci in the diploid and the polyploid species is a result of a silencing mechanism seems less probable.

Chloroplast DNA variation

The restriction fragments detected after digesting the chloroplast DNA's of the four species (three of the section *Oxytona,* and *P. somniferum*) with eight restriction enzymes are summarized in Table 3 and presented in Fig. 4. The chloroplast DNA of all the species contained 136 kb. All the enzymes, except Sma I, are able to distinguish between PS and the species belonging to section *Oxytona.* Differences between the species of the section were found in the restriction patterns of EcoR I, BamH I and Hind III that produced relatively more fragments (25, 24 and 27, respectively). The main source of variation is a result of site mutations, creating new restriction sites. In the EcoR I pattern, the 14.0 kb and 4.9 kb fragments of PB were absent in the polyploids,

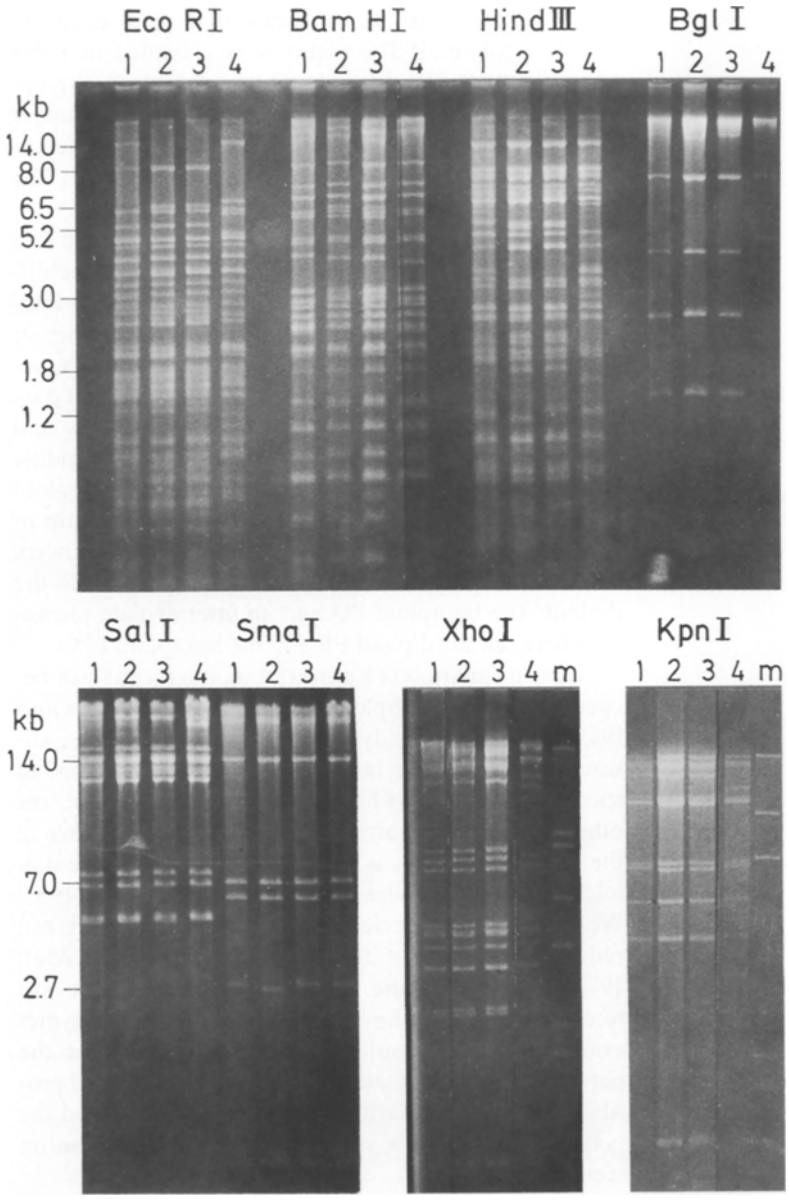

Fig. 4. Restriction endonuclease fragment patterns of chloroplast DNA from: *1 Papaver bracteatum; 2 P. orientale; 3 P. pseudo-orientale; 4 P. somniferum; m* HindIII digests of phage λ DNA

and another five fragments of 8.0 kb (a single band in PB and double band in PO and PPO), 6.5 kb, 2.5 kb, 1.8 kb and 1.3 kb were gained. In the Hind III pattern, two PB fragments of 3.9 kb and 1.0 kb were lost in the polyploids and one 5.0 kb band was added. In the BamH I pattern, two fragments of 2.5 kb and 2.9 kb were absent and one 6.5 kb fragment was gained in the polyploids. Another source of variation, similar to the chloroplast DNA Sma I patterns, is the increased mobility of 2.7 kb fragment in PO. This kind of variation is probably the result of a deletion in this fragment and not the creation of a new restriction site near the end of the fragment (Palmer and Zamir 1982). The same pattern of increased mobility in PO was observed in the 1.2 kb fragment in Hind III patterns. In the BamH I patterns two small alterations in electrophoretic mobility were found: one between PB and the two polyploid species, and the other between them and PS. A total of 17 restriction site mutuations was observed at 159 sites sampled. PS differed by 12 mutations from PB, indicating the remoteness of PS from section *Oxytona.* PB differed from PO and PPO by five mutations, and the two polyploids (PO and PPO) differed only by a small deletion in the PO chloroplast DNA.

The variation found between the species of the section shows that PO and PPO are closely related to each other and more distant from the diploid PB. Therefore, it can be concluded that the tetraploid species PO gave rise to the hexaploid PPO by contributing both the chromosomes and the cytoplasm. On the other hand,

Table 3. Number of bands scored in chloroplast DNA restriction patterns of the *Oxytona* species: *Papaver bracteatum, P. orientale, P. pseudo-orientale,* and *P. somniferum* from the *Mecones* section. Numbers in parentheses are fragments different from those of *P. bracteatum*

Enzyme	No. of bands scored			
	PB	PO	PPO	PS
Sal I	12	12	12	13 (1)
Sma I	5	5 (1)[a]	5	5
Xho I	15	15	15	11
Kpn I	11	11	11	11 (3)
EcoR I	25	28 (6)	28 (6)	27 (9)
BamH I	24	24 (3)	24 (3)	24 (5)
Hind III	27	26 (3) + (1)[a]	26 (3)	23 (9)
Bgl I	8	8	8	6 (4)
Total	127	129 (14)	129 (12)	120 (31)

[a] The difference is referred to as a small deletion in this fragment

the role of the diploid in the evolution of the polyploid species is not clear-cut. Indeed, the cytogenetic evidence, and to some extent the isozyme study, are indicative of the autopolyploid nature of PO and PPO which have evolved from PB. However, the differences in the chloroplast DNA restriction patterns between the diploid and the polyploids do not support this conclusion, as reported for wheat (Vedel et al. 1981) and potato (Hosoka 1986). The discrepancy between the two sets of evidence may be explained by assuming that the diploid species, being older than the polyploids, is also more variable in its plastid genome; likewise, only one of the cytotypes of PB gave rise to the polyploid species. This hypothesis can be tested by examining additional accessions of PB. Another possibility is that considerable evolution of the chloroplast DNA had occurred in the genus *Papaver,* and the difference between the species reflects independent evolution at the diploid and polyploid levels. This implies, however, that the hexaploid species is of relatively recent origin.

Concluding remarks

Several pieces of evidence emerging from the cytogenetic, isozyme and chloroplast DNA studies establish the autopolyploidy nature of the species of section *Oxytona.* The findings underline the importance of using various approaches in phylogenetic studies. The final evidence for the polyploidy nature of PO and PPO should come from intraspecific genetic studies, using genetic markers such as the Pgi and Pgm isozymes investigated in this study, which may enable us to distinguish between disomic and polysomic inheritance.

We demonstrated the transfer and expression of genetic material between the species and have shown, for the first time, that genetic information from PB is transferred and expressed in the segregations of the interspecific hybrids, although most of the chromosomes are contributed by the polyploid parent.

The three species can be used as one gene pool in breeding programs aimed at the development of ideotypes combining desirable agronomic and chemical characters. For example, the high thebaine content and the predominance of this alkaloid in PB could be associated with the profuse flowering during the first growing season and the lodging resistance of PPO. The development of such breeding lines could have a great impact on the introduction of alternative plant sources for the production of morphinan alkaloids.

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