

## USING AFLP MARKERS FOR SPECIES DIFFERENTIATION AND ASSESSMENT OF GENETIC VARIABILITY OF *IN VITRO*-CULTURED *PAPAVER BRACTEATUM* (SECTION *OXYTONA*)

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### SUMMARY

Amplified fragment length polymorphism (AFLP) markers were employed to detect genetic variation among species of *Papaver* (section *Oxytona*) and assess genetic fidelity between *in vitro* cell lines of *Papaver bracteatum* and mature plants derived from the propagation of their callus cultures. Regenerated plants exhibited morphological and phytochemical characteristics dissimilar to those of their source material. Thebaine, the dominant alkaloid produced by *Papaver bracteatum*, was not detected in capsules from mature regenerated accessions, indicating that there may have been a loss of genetic uniformity. Instead, the dominant alkaloid produced by the regenerated plant was shown to be isothebaine (by TLC and GC/MS), a metabolic characteristic of *P. pseudo-orientale*. A Neighbor-Joining tree constructed from AFLP fingerprints distinctly separates the three species of *Oxytona* while firmly grouping the *in vitro*-cultured plants with *P. pseudo-orientale*. Additionally, phytochemical data and chromosome counts indicate that the seed used to initiate cultures was of hybrid origin and that the loss in genetic uniformity was not due to somaclonal variation occurring during the *in vitro* culture process. AFLP fingerprinting was therefore able to differentiate *Oxytona* species and investigate allopolyploidy in closely related *Papaver* species.

**Key words:** AFLP fingerprinting; isothebaine; *Oxytona*; regeneration; *Papaver bracteatum*; somaclonal variation; thebaine.

### INTRODUCTION

The genus *Papaver* L. is of continuing commercial interest because of its medicinally important alkaloids. *Papaver somniferum* L. is grown for its morphine and codeine content, while *Papaver bracteatum* Lindl. can be used as a source of the morphinan alkaloid thebaine, which can be converted to a number of opiate analgesics, including codeine, oxymorphone, and oxycodone (McNicholas and Martin, 1984). During the 1970s, the United Nations called for extensive studies into the utilization of *P. bracteatum* as a source of thebaine and the possible replacement of *P. somniferum* as the major source of morphinan alkaloids (United Nations Report, 1976). However, the perennial nature of *P. bracteatum* renders it uneconomical as a replacement to the annual *P. somniferum* because it cannot be harvested until the second growing season (Ilahi and Ghauri, 1994). This has stimulated research into the potential of *in vitro* cell culture technologies for morphinane alkaloid production (Kamo and Mahlberg, 1988), which would obviate the need for field cultivation of poppies.

*Papaver bracteatum* is a member of the taxonomic section *Oxytona* which comprises a polyploid series including the diploid *P. bracteatum* ( $2n = 14$ ), the tetraploid *P. orientale* Fedde ( $2n = 28$ ), and the allohexaploid *P. pseudo-orientale* Fedde ( $2n = 42$ ). The three can be differentiated using morphological, cytological, and

phytochemical characters (Table 1) but the distinction is not always clear (Goldblatt, 1974; Nyman and Bruhn, 1979). Goldblatt (1974) reported that the morphological ranges of variation of *P. bracteatum*, *P. orientale*, and *P. pseudo-orientale*, when nearing their extremes, can often overlap, especially with *P. orientale* and *P. pseudo-orientale*. Interspecific hybridization does occur (Goldblatt, 1974; Milo et al., 1986; Ojala et al., 1990; Levy and Milo, 1991) and due to phenotypic similarities, the misidentification of plant material used to initiate cell cultures is common. The identification of species within this section is therefore problematic and is exacerbated by the abundance of ornamental cultivars and garden varieties of 'oriental poppies' that have been developed.

Molecular techniques offer the potential to accurately differentiate species before initiating cultures and bypass the reliance on diagnostic morphological and phytochemical characters that take time to collect, since flowering and maturity do not occur until the second growing season for members of section *Oxytona*. Such techniques involve the generation of characteristic genetic fingerprints (or the detection of species-specific molecular markers), which are then used to differentiate between closely related species, varieties, and cultivars. Characteristic DNA fingerprints can be generated using amplified fragment length polymorphism (AFLP; Vos et al., 1995). AFLP fingerprinting is based on the selective amplification of restriction fragments from a digest of genomic DNA. The resulting complex fingerprints are reproducible and provide a wide number of informative markers derived from the many loci dispersed throughout the genome (Ridout and Donini, 1999;

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TABLE 1

DIAGNOSTIC MORPHOLOGICAL CHARACTERS USED TO DEFINE THE SPECIES OF *PAPAVER* SECTION *OXYTONA* (MANY OF THE CHARACTER STATES CAN OVERLAP AND CAUSE CONFUSION WHEN IDENTIFYING PLANTS TO THE LEVEL OF SPECIES)

Character	<i>Papaver bracteatum</i>	<i>Papaver orientale</i>	<i>Papaver pseudo-orientale</i>
Dominant alkaloid	Thebaine	Oripavine	Isothebaine
Petal characteristics	Deep red, base black streak	Orange no markings	Orange/red black center
Ploidy	Diploid $2n = 2x = 14$	Tetraploid $2n = 4x = 28$	Hexaploid $2n = 6x = 42$
Flowers	Bractate	Ebractate	Bractate/ebractate
Petals	6 rarely 4	4 rarely 6	4 or 6
Petal color	Dark red, dark markings running to base	Pale orange	Orange, black rectangular markings near base
Leaves	Dentate-bidentate	Serrate	Deeply pinnatisect-subcompound
Leaf margins	Dentate-bidentate	Dentate	Irregularly dentate
Floral bracts	3–8	Absent	5–6
Bud (during development)	Erect, oval, oblong before opening	Pendulous	Absent or present 1–4 Erect/oval
Calyx	3-valved	2-valved	2–3-valved
Bristles	Thick/adpressed	Slender/subpatent	Slender/subpatent
Anthers	Linear/dark purple	Oblong/yellow or pale violet	Linear/pale violet
Mean pollen diameter ( $\mu\text{m}$ )	25.5	27.4	28.8
Ovary	Ovoid	Ovoid	Ovoid
Ovary disc	Flat/concave or conical	Slightly convex	Slightly convex
Stigmatic rays	12–24	8–15	9–19
Capsule	$\approx 3.0$ cm wide	$\approx 2.0$ cm wide	$\approx 2.5$ cm wide

TABLE 2

*PAPAVER* MATERIAL USED IN STUDY

Accession	Accession code	Source/information	Chromosome numbers	Dominant alkaloid
<i>P. bracteatum</i>	22	ARYA II P.I 381605	$2n = 2x = 14$	Thebaine
<i>P. bracteatum</i>	30	ARYA II P.I 381605	$2n = 2x = 14$	Thebaine
<i>P. bracteatum</i>	31	ARYA IIS P.I 381605	$2n = 2x = 14$	Thebaine
<i>P. pseudo-orientale</i>	36	SoP	$2n = 6x = 42$	Isothebaine
<i>P. pseudo-orientale</i>	40	SoP (cv. Salmon Queen)	$2n = 6x = 42$	Isothebaine
<i>P. pseudo-orientale</i>	37	SoP	–	Isothebaine
<i>P. pseudo-orientale</i>	18	NBG 000341	–	Isothebaine
<i>P. pseudo-orientale</i>	02	TCD BG	–	–
<i>P. orientale</i>	35	SoP	$2n = 4x = 28$	Oripavine
<i>In vitro</i> hybrid	43	Culture initiated from seed of second-generation ARYA II	$2n = 4x = 28$	Isothebaine
Regenerated hybrid	34	Micropropagated through tissue culture of accession 43	$2n = 4x = 28$	Isothebaine

NBG: National Botanic Gardens, Glasnevin, Ireland; TCD BG: Trinity College Dublin, Botanic Gardens; SoP: School of Pharmacognosy, Department of Pharmacy, Trinity College Dublin.

Dominant alkaloids (identified by TLC and GC/MS) and chromosome numbers were counted for a selection of accessions.

Hodkinson et al., 2000). AFLP has been used to detect genetic variation in highly inbred lines of cereal crops and for the study of clonal variation in plants (Mackill et al., 1996; Paul et al., 1997; Waugh et al., 1997; Hodkinson et al., 2002). It has also been used for species differentiation in a range of higher plant species such as *Phyllostachys* (Hodkinson et al., 2000), but not in *Papaver* section *Oxytona*.

Several cell cultures were developed by Hook et al. (1988) from seeds of *P. bracteatum* originally supplied as ARYA II. Plants developed from these seeds exhibited morphological and phytochemical characteristics typical of *P. bracteatum* as described by Goldblatt (1974). On repeated subculture one cell line exhibited organogenesis, and subsequently differentiated into *in vitro*

plantlets, some of which were grown on to maturity under garden conditions. The resulting plants did not resemble the parent, exhibiting morphological characters similar but not identical to those of *P. pseudo-orientale*. This therefore brought into question the identity of the original seed used to initiate the cultures or indicated that the cell culture process was not stable and had induced morphological change possibly via somaclonal variation.

The objectives of this work were to generate characteristic genomic fingerprints using AFLP, which could be used to differentiate between the three species of section *Oxytona* and detect their hybrids. We wished to develop a marker system capable of identifying the genetic constitution of *in vitro*-generated plants and assessing the stability and quality of repeatedly subcultured cell

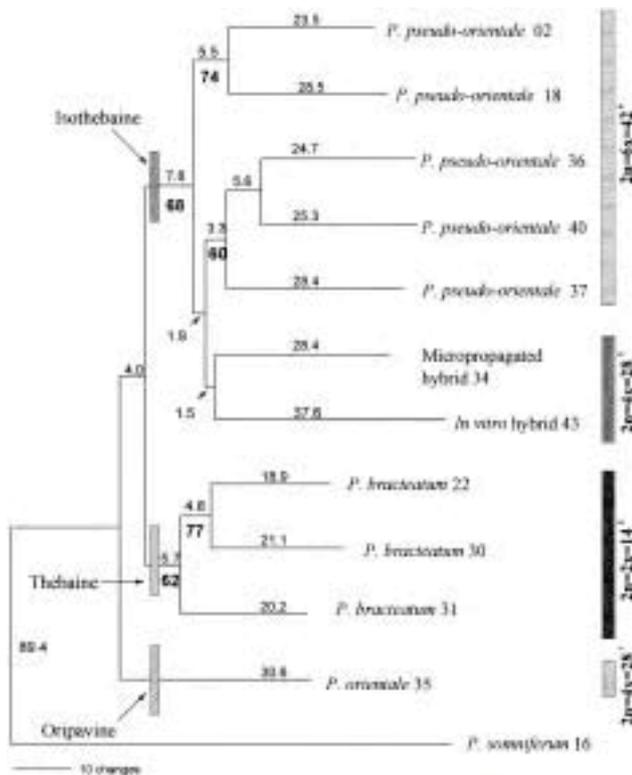


FIG. 1. Neighbor-Joining (NJ) tree based on AFLP data. The NJ tree has branches proportional to genetic distance (see scale bar). Three groups representing the three species of *Oxytona* are apparent. The *in vitro* plant and the regenerated plant resolve within the *P. pseudo-orientale* group.<sup>1</sup>Based on chromosome count for accession 36. <sup>2</sup>Based on chromosome count for accessions 34 and 43. <sup>3</sup>Based on chromosome count for accessions 22 and 31. <sup>4</sup>Based on chromosome count for accession 35.

lines. The collection of basic phytochemical (dominant alkaloid) and cytological data (chromosome counts) were to aid the interpretation of our results.

## MATERIALS AND METHODS

**Plant material.** Plant material was obtained from a number of sources listed in Table 2, and included four species: *Papaver orientale*, *P. bracteatum*, *P. pseudo-orientale*, and *P. somniferum*. Capsules were collected from mature plants from May to July 2000, 4 wk after flowering, and dried in a fan-assisted oven at  $<35^{\circ}\text{C}$ . Not all accessions were available for alkaloid analysis and chromosome counts.

**In vitro culture of *Papaver bracteatum*.** Callus cultures were originally established in 1984 (Hook et al., 1988) from surface-sterilized seeds plated onto an agar-solidified (0.9%) MS salt medium (Murashige and Skoog, 1962) containing 2,4-dichlorophenoxyacetic acid (2,4-D;  $1\ \mu\text{M}$ ),  $\alpha$ -naphthaleneacetic acid (NAA;  $1\ \mu\text{M}$ ), glycine ( $26.64\ \mu\text{M}$ ), nicotinic acid ( $4.06\ \text{mM}$ ), pyridoxine HCl ( $2.43\ \mu\text{M}$ ), mesoinositol ( $1.10\ \text{mM}$ ), thiamine HCl ( $1.48\ \mu\text{M}$ ), and sucrose (3%), using Technical Agar number 3 (Oxoid Ltd., UK). Cultures grew as beige-colored aggregates and were subcultured every 4–6 wk onto fresh medium. Subsequently, aggregates showing organogenesis were transferred to hormone-free MS agar where shoot and root regeneration occurred. Regenerated *in vitro* plantlets were also subcultured onto fresh medium every 4–6 wk, and maintained to the present day. For alkaloid analysis, *in vitro*-grown plantlets were harvested after 6 wk growth and dried as above. Roots formed and these were used as the material for cytological analysis.

**Cytological investigations.** Seeds from identified accessions were germinated under constant light at  $25^{\circ}\text{C}$  on wet filter paper in plastic Petri dishes. Root tips were taken when growth reached 1.5 cm. Chromosome counts were also made on young rapidly growing roots of *in vitro*-cultured plantlets. Root tips were pretreated in ice-cold water for 24 h, fixed in 3:1 absolute ethanol:acetic acid and stored at  $4^{\circ}\text{C}$  until use. Root tips were hydrolyzed in 1 N HCl at  $60^{\circ}\text{C}$  for 8 min, transferred into 5 ml of Feulgen solution (Feulgen solution stains exposed aldehyde groups following hydrolysis), placed in the dark for 30 min, and viewed with a light microscope at a magnification of  $\times 1000$ .

**DNA extraction.** DNA was extracted from 0.5 g of silica gel (Sigma) dried leaf material using a modification of the 2XCTAB procedure of Doyle and Doyle (1987) and precipitated using isopropanol for 24 h at  $-20^{\circ}\text{C}$ . The DNA was then pelleted by centrifugation at 2000 rpm, washed with 70% ethanol, and stored in TE buffer (10 mM Tris-HCl, pH 7.6 and 1 mM EDTA) at  $-80^{\circ}\text{C}$ . DNA was further purified using Concert PCR purification columns (Gibco BRL) and ranged in quantity from 70 to  $250\ \text{ng}\ \mu\text{L}^{-1}$ , as estimated by fluorescence of ethidium bromide-stained DNA compared to known standards.

**AFLP analysis.** AFLP reactions used the AFLP Plant Mapping Kit of Applied Biosystems Inc. (ABI), following their standard protocols. Firstly, the restriction enzymes *EcoRI* and *MseI* were used to digest total genomic DNA. Oligonucleotide primers specific to these restriction sites were ligated to the fragments. Secondly, a preselective amplification was used according to the manufacturer's instructions. Thirdly, two primer combinations *MseCTC/EcoACG* and *MseCAG/EcoAGC* were used for the selective PCR step (each including a fluorescently labeled primer), which amplified a subset of the fragments, created in the initial PCR. Finally, detection of DNA fragments was carried out on an ABI 377 Automated Sequencer with ABI Genescan 2.0.2.

**Data analysis.** AFLP profiles were scored using the Genotyper 1.1 software of ABI. DNA fragments ranging between 50 and 500 base pairs in size were scored as presence/absence characters and analyzed with Neighbor-Joining (NJ) using Nei and Li distances (Nei and Li, 1985) with the phylogenetic analysis program PAUP 4.0 (Swofford, 1998). Internal support for groupings was assessed using the bootstrap procedure with 1000 replicates (Felsenstein, 1985). Principal coordinates analysis (PCO) was performed with Le Prodigier R v4.0d (Casgrain, 1999) using Dice distances (Dice, 1945).

**Alkaloid analysis.** Mature, but unripe, poppy capsules were dried in a fan-assisted oven at  $<35^{\circ}\text{C}$ . The stigmatic discs, seeds, and placentae were removed and samples were powdered, and 0.5 g of powdered capsule was extracted twice with 5% aqueous acetic acid (50 ml) by shaking on an orbital shaker for 1 h. The combined acid extracts were made alkaline to pH 9 with 10% ammonium hydroxide and then extracted ( $\times 3$ ) with chloroform (50 ml). The chloroform extracts were dried over anhydrous sodium sulfate, combined and evaporated *in vacuo*. The resultant residue was dissolved in 0.5 ml methanol and examined by thin-layer chromatography (TLC) using silica gel plates (Silica gel 60 F<sub>254</sub>; Merck) developed in acetone:toluene:ethanol:conc. ammonia (40:40:12:2.5). Visualization of alkaloids was with UV light and Dragendorff's reagent. Reference compounds were thebaine ( $2.0\ \text{mg}\ \text{mL}^{-1}$ ), isothebaine ( $1.4\ \text{mg}\ \text{mL}^{-1}$ ), oripavine ( $0.3\ \text{mg}\ \text{mL}^{-1}$ ), and salutaridine ( $2.5\ \text{mg}\ \text{mL}^{-1}$ ).

For gas chromatographic mass spectrum (GC/MS) analysis, samples were injected in splitless mode onto a GC capillary column (30 m  $\times$  0.25 mm i.d., Va 35MS) coupled to a Saturn 2000 MSD (EI 70 eV). The injector temperature and the GC-MS transfer line was held at  $260^{\circ}\text{C}$  and the column programmed from  $100^{\circ}\text{C}$ , held for 3 min, rising to  $300^{\circ}\text{C}$  at  $15^{\circ}\text{C}\ \text{min}^{-1}$ . Helium was used as carrier gas at a flow rate of  $1\ \text{ml}\ \text{min}^{-1}$ .

## RESULTS

**AFLP analysis.** The two primer combinations from AFLP generated 254 polymorphic markers, of which 146 were shared between individuals. A NJ tree from the AFLP analysis, that groups accessions according to genetic similarity, is given in Fig. 1. There are three major divisions, representing the three species of section *Oxytona*, respectively (*P. orientale*, *P. bracteatum*, and *P. pseudo-orientale*). The *in vitro*-grown plant and the regenerated plant

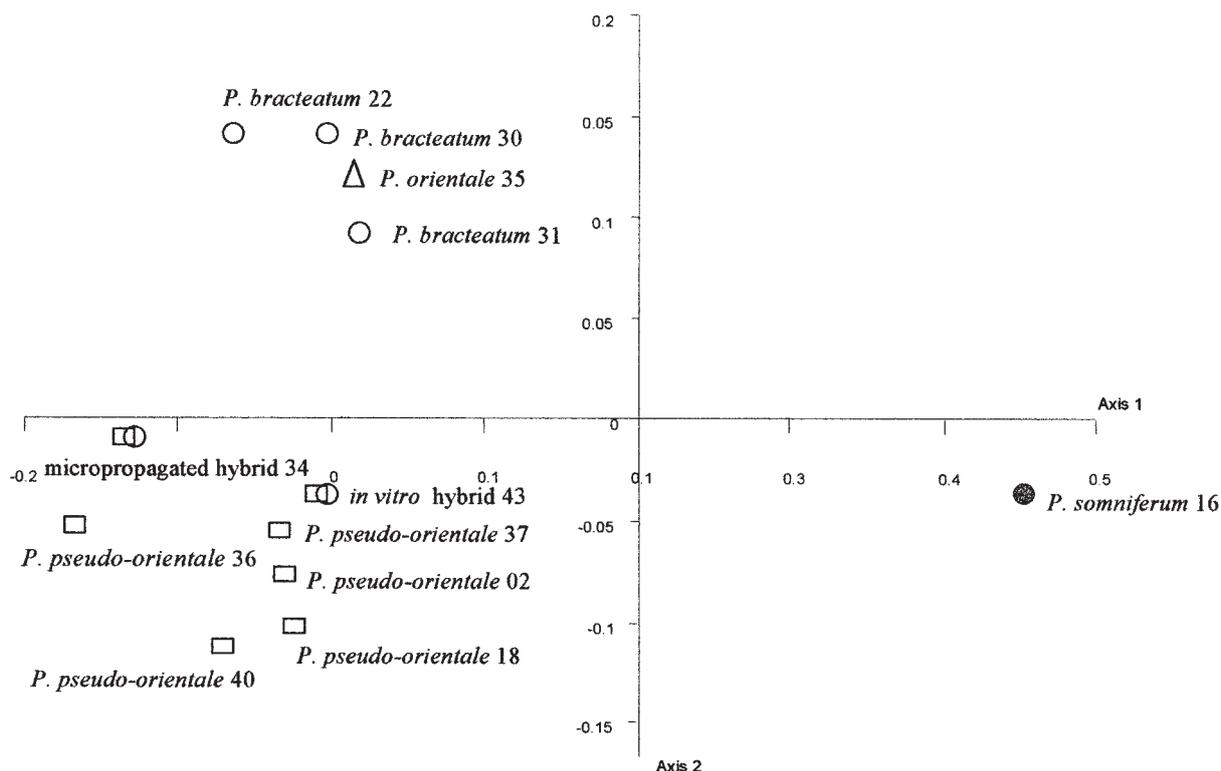


FIG. 2. Principal coordinates analysis (PCO) of AFLP data. The first two axes cumulatively accounted for 60.4% of the data variance (38.3 and 22.1, respectively). The *in vitro* plant and the regenerated plant group separately from the *P. bracteatum* accessions.

TABLE 3

SELECTED IONS, RETENTION TIMES, AND ION RATIOS OF *PAPAVER* ALKALOIDS: THEBAINE, ISOHEBAINE, AND FOUR SELECTED ACCESSIONS

Compound/accession	Retention time (min)	Monitored ions	Ion ratio percentage (abundance)
Thebaine	15.2	311,296	100.75
Isothebaine	16.0	311,294	100.50
Accession 30	15.2	311,296	100.25
Accession 31	15.2	311,296	100.25
Accession 40	16.0	311,294	100.60
Accession 43	16.0	311,294	100.60

grouped with the *P. pseudo-orientale* accessions. Dendrograms produced for each set of primers separately were in congruence with the composite dendrogram for both primers.

The results of the PCO analysis are illustrated in Fig. 2. The first two axes cumulatively accounted for 60.4% of the data variance (representing 38.3 and 22.1, respectively). The PCO analysis is congruent with the NJ tree and shows that the *in vitro*-grown plant and the regenerated plant were distinct from *Papaver bracteatum* but shared the majority of markers with *P. pseudo-orientale*.

**Alkaloid analysis.** Alkaloid profiles for accessions where capsules were available are given in Table 2. Reference standards of thebaine, isothebaine, oripavine, and salutaridine had  $R_f$  values of 0.54, 0.57, 0.42, and 0.46, respectively. The *in vitro*-grown plant and

the regenerated plant produced isothebaine ( $R_f$  0.57) as the dominant alkaloid with several unidentified alkaloids present as minor constituents. For reference, capsules from *P. bracteatum* (accessions 22, 30, and 31) and *P. pseudo-orientale* (accessions 36 and 40) produced thebaine ( $R_f$  value of 0.54) and isothebaine ( $R_f$  value of 0.57) as dominant alkaloids, respectively, with *P. orientale* (accession 35) producing oripavine ( $R_f$  value of 0.42) as its dominant alkaloid.

To validate the TLC data, alkaloid extracts for selected accessions and standards were subjected to GC/MS. Their retention times and mass fragmentation spectra are given in Table 3. *Papaver bracteatum* (accessions 30 and 31) and *P. pseudo-orientale* (accession 40) produced thebaine (RT 15.2 min,  $m/z$  311) and isothebaine (RT 16 min,  $m/z$  311), respectively, as their dominant alkaloids. The extract from the *P. bracteatum*  $\times$  *P. pseudo-orientale* hybrid (accession 43) contained isothebaine (RT 16 min,  $m/z$  311) as the dominant alkaloid (Table 3).

**Cytological analysis.** Cells from root tips of *in vitro*-cultured *P. bracteatum* contained 28 chromosomes as detected in metaphase of mitosis. Chromosome counts for available accessions are given in Table 2. Members of the *P. bracteatum* AFLP group were diploid with  $2n = 14$ , while members of the *P. pseudo-orientale* group were hexaploid with  $2n = 42$  (excluding the hybrid). *Papaver orientale* had 28 chromosomes and is tetraploid ( $2n = 28$ ).

## DISCUSSION

*Identification of a hybrid in vitro cell line using AFLP fingerprinting.* The NJ tree generated from the AFLP markers

showed three major groups, which correspond to the three species, *P. bracteatum*, *P. orientale*, and *P. pseudo-orientale*. *Papaver bracteatum* accessions resolved independently from a group which contains the *P. pseudo-orientale* accessions. *Papaver orientale* resolved as a sister group to the other two species but bootstrap support for this position is weak (<50). The results of the PCO analysis are congruent with these conclusions. Accessions labeled as *P. orientale* shared morphological characters with *P. pseudo-orientale*, having orange petals with large dark spots on each. These two accessions (02 and 18) grouped with other *P. pseudo-orientale* plants. AFLP-generated markers are therefore highly efficient when used in conjunction with morphological, phytochemical, and cytological characters to identify plant material.

Regenerated plantlets grown on from the *P. bracteatum* cultures should group with *P. bracteatum* if they have remained genetically stable before and during tissue culture. However, this *in vitro*-derived plantlet did not associate with *P. bracteatum* and instead grouped more closely with *P. pseudo-orientale*. These AFLP groupings show that genomic material from *P. pseudo-orientale* is present within the *in vitro*-grown plant and the micropropagated accession, indicating that a hybridization event may have occurred between *P. pseudo-orientale* and the maternal *P. bracteatum*, from which seed was obtained to initiate the *in vitro* plants. The *in vitro* plant and the regenerated accession group together but are not identical, which suggests that somaclonal variation may have occurred during their culture. AFLP-generated fingerprints would therefore have the potential to detect loss of genetic uniformity due to somaclonal variation in poppies. We are currently investigating them further in this regard.

The results from the alkaloid analysis (Tables 2 and 3) support the conclusion that the *in vitro*-grown plant is a hybrid. If the *in vitro*-grown plant bred true from *P. bracteatum*, thebaine would be the expected dominant alkaloid (Hook et al., 1988). Environmental influences and the genotype  $\times$  environment interaction have considerable effects on the quantity of alkaloids produced by particular accessions (Bernath and Nemeth, 1998). However, for species of section *Oxytona*, phytochemical spectrum and to some extent alkaloid quantity are influenced primarily by parent genotype and ploidy (Milo et al., 1990; Levy and Milo, 1991). For example, hybrids between *Oxytona* species produce characteristic phytochemical spectra dependent on the inherited genetic information (Milo et al., 1990; Levy and Milo, 1991). The wide spectrum of alkaloids found in the differentiated *in vitro* plant (including isothebaine) demonstrates the presence of *P. pseudo-orientale* genomic material and its resulting biosynthetic pathways. Hybrids between *P. bracteatum* and *P. pseudo-orientale* have been reported to produce both isothebaine and thebaine, inherited from the two parental genotypes, respectively (Levy and Milo, 1991). Our results contrast with Levy and Milo (1991), as no traces of thebaine were recorded for the hybrid accession. The alkaloid spectrum found within each species is congruent with the groupings of the AFLP analysis with three groups all producing diagnostic dominant alkaloids.

To confirm that the material used to initiate the *in vitro* plant arose from hybrid seed, chromosomes were counted and compared to counts for other studied accessions. *Papaver bracteatum*, *P. orientale*, and *P. pseudo-orientale* had 14, 28, and 42 chromosomes, respectively. The *in vitro* plant had 28 chromosomes and is therefore a tetraploid. We have provided evidence to show that the cultured

plant is likely to be an allopolyploid with genomes from both *P. bracteatum* and another species (probably *P. pseudo-orientale*). The hybrid clusters closer to *P. pseudo-orientale* than *P. bracteatum* (in both NJ and PCO analyses), which indicates that it might have inherited more chromosomes from *P. pseudo-orientale* than *P. bracteatum*. In this case, one explanation is that it inherited seven chromosomes from *P. bracteatum* and 21 from *P. pseudo-orientale*.

Goldblatt (1974) cites examples of *Papaver* (section *Oxytona*) hybrids with somewhat intermediate characteristics, and stresses that some of the diagnostic characters when used alone could lead to misidentifications. Hybrids are not always morphologically intermediate between their parents (Riesberg, 1995) and great care needs to be taken when evaluating their characters. They are often just as likely to exhibit the characteristics of one or other of the parental species. The regenerated plant resembled *P. pseudo-orientale* more closely in its gross morphology than *P. bracteatum*, having six orange petals with faded central purple spots. Single bracts were present but not found on all flowering stems. The two-valved calyx was borne on a pendulous flowering stem becoming erect when mature. Uncharacteristic fine setae were subpatent on the lower portion of the flowering stem, becoming adpressed further up. The capsule bore a concave stigmatic disc comprising 14 stigmatic rays. There was, therefore, little indication from morphological data that *P. bracteatum* was a parent to this particular accession. The original *P. bracteatum* plant had, in fact, been garden grown in the presence of *P. pseudo-orientale* and a hybridization event was possible. Molecular AFLP data in combination with chromosome counts confirm that such an event did occur. This is therefore an example of a situation where a hybrid is not morphologically intermediate between its parents as it inherited more characters from one of them.

*In vitro technology and molecular markers.* Genetic variation among micropropagated plants is well documented (Rani and Raini, 2000). Somaclonal variation, whether it is at the molecular, morphological, or the phytochemical level, must be detected and minimized if true-to-type plants are to be produced. The use of molecular techniques has allowed rapid detection of such variation and helped elucidate the mechanisms that cause the loss of genetic uniformity.

AFLP fingerprints are highly reproducible and generally produce a large number of polymorphic markers (Becker et al., 1995; Ridout and Donini, 1999; Hodkinson et al., 2000). We have demonstrated that they can be applied to the study of genetic variation in poppies. They have the potential to identify ambiguities between parent material and regenerated plantlets and to detect somaclonal variation in *Papaver in vitro* clones and propagated plants. In our research, the rapid identification of the hybrid cell line allowed us to review the various sources of plant material used to initiate callus and suspension cultures.

The mass propagation of high alkaloid-producing cell lines demonstrates one of the beneficial uses of *in vitro* technologies to the successful utilization of *Papaver* species. *Papaver bracteatum* plants are almost always self-incompatible, which makes the creation of phytochemically pure lines through selfing very difficult to achieve. Day et al. (1986) reported successful regeneration of micropropagated plants from embryogenic callus culture with little phytochemical variation occurring between micropropagated offspring and parent plant material. The effects of somaclonal variation, however, have the potential to diminish effectiveness of tissue culture as a means of producing significant quantities of genetically and

TABLE 4  
 MEDIA AND CULTURE CONDITIONS REPORTED FOR THE GROWTH OF *PAPAVER BRACTEATUM* CELL CULTURES (AFTER HOOK ET AL., 1988)

Medium	Auxin ( $\mu M$ )	Cytokinin ( $\mu M$ )	Other supplement	Culture type	Other conditions	Alkaloids present	Reference
MS (no glycine)	2,4-D (4.53)	Kinetin (0, 0.47)	Coconut milk (7%)	Callus	26-h dark	Sanguinarine Nor-sanguinarine Dihydrosanguinarine Oxysanguinarine Chelirubine Not investigated	Ikutsu et al. (1974)
MS	2,4-D (4.53)	Kinetin (0, 0.47)	Polypeptone Coconut milk	Suspension	26-h dark, 3% sucrose		Kamimura and Akutsu (1976)
MS	NAA (5.33)	Kinetin (0, 0.47)	Coconut milk (10%)	Callus suspension	3% sucrose	Thebaine Protopine	Kamimura and Nishikawa (1976)
MS	2,4-D (4.53)	Kinetin (0, 0.47)	Coconut milk (10%)	Callus suspension	26-h dark, 3% sucrose	Stylophine	Kamimura et al. (1976)
MS	2,4-D (0.45)			Callus	24°C, 24-h light	Thebaine	Shafiee et al. (1976)
MS	2,4-D (4.53)	Kinetin (0, 0.47)	Various	Callus		Sanguinarine	Lockwood (1981)
MS	IAA (5.7)			Root suspension	15-h light	Orientalidine	Zito and Staba (1982)
MS	2,4-D (45.24)	Kinetin (0, 0.47)	Vitamin C (0.001%)	Suspension	12-h light	Sanguinarine	Lockwood (1981)
MS	2,4-D (1.0)			Suspension	Continuous dim light	Orientalidine	Hook et al. (1988)
MS	NAA (1.0)			Suspension	18-h light	Dihydrosanguinarine	
MS	2,4-D (1.0)		Copper	Suspension		Sanguinarine	Lecky (1992)
MS	NAA (1.0)			Suspension		Dihydrosanguinarine Sanguinarine	

IAA, indoleacetic acid.

phytochemically uniform accessions. Additionally, seeds taken from mature plants grown under garden conditions have the potential of being of hybrid origin. If we had not established that the seeds used to initiate cultures were of hybrid origin and proceeded with our work on a larger scale, subculturing and mass propagation of this particular accession would have resulted in a crop of hybrids, none of which would have possessed thebaine, the desired phytochemical constituent.

*In vitro production of alkaloids in Papaver bracteatum.* There has been little substantial success in producing good yields of thebaine from callus or suspension cultures derived from poppy cells. The production of morphinan alkaloids is known to be associated with cell differentiation and morphogenetic processes (Constabel, 1985; Kutchan et al., 1985; Kamo and Mahlberg, 1988; Szoke, 1998). Morphinan alkaloid synthesis is dependent on the presence of specialized cells known as laticifers (Roberts et al., 1983; Kutchan et al., 1985; Rush et al., 1985), which are absent in undifferentiated poppy cultures. The detection of such alkaloids often coincides with the development of laticifers in differentiated plant material that develop in a similar way to young seedlings. The majority of reported alkaloids from callus and suspension *Papaver bracteatum* cultures are the benzophenanthridine type including sanguinarine, dihydrosanguinarine, and oxysanguinarine (Table 4), which are produced in minor quantities in intact plants of the genus *Papaver* but are found as the dominant constituents of undifferentiated callus and suspension cultures (Cline and Coscia, 1988). The biosynthesis of these alkaloids occurs at loci different to the laticifers associated with morphinan alkaloids (Kutchan et al., 1985). The characteristic phytochemical characters often used to aid species differentiation with species of section *Oxytona* are not produced by undifferentiated *in vitro* tissues. The use of genetic fingerprinting offers the potential to differentiate between closely related species, identify hybrid material, and assess the amount of genetic stability during the tissue culture processes.

The variability of alkaloids reportedly produced by the cell culture (Table 4) has often been explained by differences in growth conditions and by the degree of tissue differentiation. It is possible, however, that such variability could also be due to misidentification of the plant material used as the seed source (Phillipson et al., 1981). We witnessed a departure from the expected alkaloid profile of a *P. bracteatum* plant but were able to attribute this to misidentification. Unfortunately, other studies that report high variability in alkaloid profiles often fail to provide evidence for correct identification of their material.

#### CONCLUSIONS

The most fundamental requirement for the use of any plant species in the pharmaceutical industry is its correct identification. Poppy species belonging to section *Oxytona* are not sufficiently distinct to base identification solely on morphological characters. Regenerated *Papaver* plantlets from tissue culture could be grown on to maturity and identified using morphological characters and chromosome numbers but a growing period of 2 yr is required to produce flowers. AFLPs combined with cytogenetic analysis offer a powerful way to rapidly differentiate species and identify potential hybrids. AFLP clearly has great potential as a way of monitoring the genetic stability of *in vitro* plants, particularly those that possess superior phytochemical traits and that are multiplied by tissue

culture methods. We are also investigating the potential of flow cytometry (Rabinovitch, 1994; Wilkinson et al., 2000) for rapidly screening the ploidy level and genetic constitution of *Papaver*.

The DNA fingerprinting technique, AFLP, combined with standard cytogenetic techniques offers the possibility to improve the utilization of *Papaver bracteatum* as a pharmaceutical resource. The markers can be used for marker-aided selection and for the study of quantitative trait loci (Mackill et al., 1996; Paul et al., 1997; Waugh et al., 1997). We have also demonstrated that the markers have wider utility in the field of plant systematics for the study of hybridization and introgression in poppies.

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