Theor Appl Genet (2002) 104:1150–1156
DOI 10.1007/s00122-001-0863-3

**K.G. Gilbert (nee Stoker) · S. Garton · M.A. Karam
G.M. Arnold · A. Karp · K.J. Edwards · D.T. Cooke
J.H.A. Barker**

A high degree of genetic diversity is revealed in *Isatis* spp. (dyer's woad) by amplified fragment length polymorphism (AFLP)

Received: 8 August 2001 / Accepted: 26 November 2001 / Published online: 9 March 2002

Abstract Genetic diversity in 38 genotypes, representing 28 individual genotypes from five landraces of *Isatis tinctoria* (three German: Tubingen, Potsdam and Erfurt, one Swiss and one English), five genotypes of *Isatis indigotica* (Chinese woad) and five genotypes of *Isatis glauca*, were investigated using AFLP analysis. Five primer combinations detected a total of 502 fragments of which 436 (86.9%) were polymorphic. The level of polymorphism recorded within each species was 29.8, 86.9 and 35.8% for *I. indigotica*, *I. tinctoria* and *I. glauca*, respectively. Clearly, genetic diversity within *I. tinctoria* was greater than that observed in *I. indigotica* or *I. glauca*. Cluster analyses of the AFLP data using UPGMA and PCO revealed the complete separation of the genotypes of each species into distinct groups. *I. indigotica* separated as an entirely independent group, whereas *I. glauca* formed a separate cluster within the *I. tinctoria* group. Indeed, *I. tinctoria* and *I. glauca* are more closely related to each other than either is to *I. indigotica*. In addition, the genotypes of each landrace, apart from one from the English group, were clearly discriminated. However, the anomalous genotype did associate with the rest of its group when it was linked with the Erfurt group. These results provide new and useful information about the make-up of the *Isatis* genome, which has not previously been evaluated. They will be useful in the selection of plant material for variety development and conservation of the gene-pool.

Keywords Indigo · Marker-assisted breeding · Indican · Isatan B

Communicated by P. Langridge

K.G. Gilbert (nee Stoker) (✉) · K.J. Edwards · D.T. Cooke
Department of Biological Sciences, University of Bristol,
c/o Long Ashton Research Station, Bristol, BS41 9AF, UK
e-mail: kerry.gilbert@bbsrc.ac.uk
Tel.: +44-(0)-1275-549408, Fax: +44 (0)1275 394007

S. Garton · G.M. Arnold · A. Karp · J.H.A. Barker
IACR-Long Ashton Research Station,
Department of Agricultural Sciences,
University of Bristol, Long Ashton, Bristol, BS41 9AF, UK

M.A. Karam
Botany Department, Faculty of Science, Fayoum Branch,
Cairo University, Fayoum, Egypt

Introduction

The earliest known source of indigo in northern Europe was *Isatis tinctoria* (woad) (Epstein et al. 1967). It was grown as a dye crop until the 1930s, when it was superseded by synthetic indigo. *I. tinctoria* is one species from a genus of around 30 (Clapham et al. 1958), most of which produce indigo. *I. tinctoria* is a biennial or perennial herb, indigenous, but not native, to northern Europe. It is a native of southeast Russia and was recorded as growing on exposed hillsides and in breezy places (Dunn 1905). A genus of the *Cruciferae*, *Isatis* spp. belong to the same family as the brassicas and have many similarities with them. Woad is a diploid species with a chromosome number of 14 (2n = 28) (Clapham et al. 1958). It is widely believed that it is a self-incompatible, obligate outbreeder, although recent work has suggested there may be some self-compatibility (Gilbert, K.G., Hill, D.J. and Cooke, D.T., unpublished results).

Isatis spp. produce two indoxyl-forming substances in their leaves, indican (Schunk 1855) and isatan B (Beijerinck 1900), which, when exposed to the air form the blue compound, indigo (Epstein et al. 1967). *Isatis indigotica* (Chinese woad or tein-cheing), which has not been widely used as a dye-plant, was first described by Fortune (1846). It is very closely related to its European counterpart, *I. tinctoria*, containing the same indigo precursors, although it has a greater indigo-production capacity, and several morphological differences (Stoker 1997). However, it is more susceptible to diseases found in temperate climates, such as cabbage root fly and cabbage flea beetle (Gilbert and Cooke, unpublished observations). *Isatis glauca* can also be used to produce indigo, although it has not been used previously in large-

scale production. It was included in this study, as it may be commercially useful in breeding.

As a consequence of its decline as a dye crop, there has been no attempt to breed woad to improve indigo precursor content or crop architecture (Gilbert and Cooke 2001). Recent research has shown that the method by which indigo was obtained from woad and also the large variation between plants in terms of their indigo-production capacity, were mainly responsible for the low yields (Stoker 1997). Changes in legislation and a growing demand from consumers and manufacturers for naturally derived products from renewable resources have led to a revived interest in woad as a source of indigo for industry, leading to the need for new systematic research on this crop.

In northern Europe, *I. tinctoria* was grown as the sole source of indigo until the 17th century and continued to be grown as a fermentation aid in the woad dye vat until the first part of the 20th century (Plowright 1901; Burkhill 1921). Large numbers of workers were employed solely for the purpose of woad cultivation (Hurry 1930). Consequently, individual groups of woad growers selected their own seed for subsequent crops, which led to the development of characteristic populations (landraces) unique to each group of growers. Analysis of plants derived from different species and landraces has shown that they each have distinct phenotypic traits. It has also been shown that different landraces produce varying amounts of indigo precursors (Hill 1992). Therefore, the choice of landrace is important when contemplating using woad to produce indigo commercially. Furthermore, the amount of indigo per unit weight can vary enormously within each landrace and with environmental conditions (Stoker et al. 1998). Therefore, the ability to distinguish between landraces and, ultimately, between individual genotypes within each landrace would provide a diagnostic tool for the selection of useful plants for use in a marker-assisted breeding programme.

DNA fingerprinting techniques offer the opportunity to characterise diversity at the genetic level. A variety of DNA fingerprinting techniques have been developed during the last decade, most of which use PCR for the amplification of specific DNA fragments. These include randomly amplified polymorphic DNA (RAPD; Williams et al. 1990), arbitrary primed PCR (AP-PCR; Welsh and McClelland 1990), DNA amplification fingerprinting (DAF; Caetano-Anolles et al. 1991) and amplified fragment length polymorphism (AFLP) analysis (Zabeau and Vos 1993; Vos et al. 1995). The latter has the capacity to detect polymorphism at a greater number of loci (Clerc et al. 1998; Barker et al. 1999; Garcia-Mas et al. 2000). Similar to RAPD analysis, the AFLP assay requires no prior sequence knowledge. However, it detects up to a 10-fold greater number of loci than those detected by RAPD (Karp et al. 1996; Maughan et al. 1996). The AFLP method is a robust and reproducible technique (Erschadi et al. 2000), which will generate fingerprints of any DNA, regardless of the origin or complexity (Vos et al. 1995). In this case, AFLP analysis was selected because it detects a large number of loci and provides an effective means of covering a large proportion of the genome in a single assay (Karp and Edwards 1997).

In the present work, the genetic diversity in landraces of three species, namely *I. tinctoria*, *I. indigotica* and *I. glauca*, was examined in an attempt to determine their genetic relatedness and to elucidate and identify genetic markers specific for each species and landrace. As *I. tinctoria* has been widely cultivated for the production of woad indigo, there are several landraces originating from areas where the crop was grown. However, *I. indigotica* and *I. glauca* have not been subject to the same selection, so there are not the variety of landraces available for these species.

Materials and methods

Plant material

Seeds were provided from three different sources: *I. tinctoria* (English) and *I. indigotica* were obtained from the private collection of Dr. David Hill (University of Bristol, UK), *I. tinctoria* (Swiss and Erfurt) came from the collection of Thüringer Landesanstalt für Landwirtschaft, Jena, Germany, and *I. tinctoria* (Potsdam and Tübingen) and *I. glauca* were obtained via the Botanic Gardens of the University of Bristol, UK.

A total of 38 individual plants (Table 1) were grown in pots containing compost, under glasshouse conditions, with a 16-h day length and a minimum temperature of 16 °C, for 6 weeks.

DNA extraction

Young leaf material (approximately 125 mg) was collected from each plant and frozen in liquid nitrogen. Genomic DNA was extracted using the Nucleon Phytopure DNA Extraction Kit (Amersham Pharmacia Biotech) with the addition of 10 mM of 2-mercaptoethanol in Reagent One.

AFLP analysis

The primer and adapter sequences were synthesised and de-salted by Sigma-Genosys Limited, UK. Enzymes and radioactive nucleotides were obtained from Amersham Pharmacia Biotech, UK Limited, unless otherwise stated. AFLP analyses were essentially done according to the method described by Zabeau and Vos (1993). Briefly: genomic DNA (about 0.5 µg) was digested with *Pst*I and *Mse*I (New England Biolabs) restriction endonucleases for 1 h at 37 °C in 1 × One-Phor-All buffer (OPA) (10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, pH 7.5). The digested DNA was ligated to biotinylated *Pst*I (5 pmol) and

Table 1 Origin of *Isatis* spp. used for AFLP analyses

Sample no.	Species	Region	Country of origin
1–5	*I. glauca*	–	Romania
6–11	*I. tinctoria*	Tubingen	Germany
12–16	*I. tinctoria*	Potsdam	Germany
17–22	*I. tinctoria*	Erfurt	Germany
23–28	*I. tinctoria*	–	Switzerland
29–33	*I. tinctoria*	Tewkesbury	England
34–38	*I. indigotica*	–	China

non-biotinylated *Mse*I (50 pmol) adapters (see below) with T4 DNA ligase in the presence of 1 mM ATP and 1 × OPA buffer at 37 °C overnight.

*Pst*I adapter:

5′Biotin-CTCGTAGACTGCGTACATGCA-3′,
3′-CATCTGACGCATGT-5′

*Mse*I adapter:

5′-GACGATGAGTCCTGAG-3′
3′-TACTCAGGACTCAT-5′.

Biotinylated fragments were selected by binding to Dynabeads M-280 streptavidin (Dynal). Primers for AFLP amplification were based on the *Mse*I and *Pst*I adapters and restriction sites, as follows:

*Mse*I: 5′-GATGAGTCCTGAGTAA-3′

*Pst*I: 5′-GACTGCGTACATGCAG-3′

For selective PCR, five primer combinations were used: *Mse*I + GAA and *Pst*I + AC or CC, *Mse*I + ACA and *Pst*I + AC, *Mse*I + ACC and *Pst*I + CA or CC. In each case, the *Mse*I primer was end-labelled with [γ^{33}P]ATP and T4 polynucleotide kinase, according to Vos et al. (1995). Each PCR reaction contained a 1-µl beaded DNA template, 2.5 ng of ^{33}P-labelled and 12.5 ng of unlabelled *Mse*I primer, 15 ng of *Pst*I primer, 0.2 mM of each dNTP, 20 mM Tris-HCl (pH 8.5), 1.5 mM $MgCl_2$, 50 mM KCl and 0.25 U of *Taq* DNA Polymerase (Gibco BRL), in a total volume of 10 µl. The reactions were transferred to a GeneAmp PCR system 9700 (Applied Biosystems) pre-heated to 94 °C and DNA was amplified using ten cycles comprising: 40 s at 94 °C (denaturation), 60 s at 62 °C (annealing) and 60 s at 72 °C (extension), followed by a further 25 cycles with a lower annealing temperature of 56 °C. The final 72 °C extension step was extended for 10 min. Following PCR, the amplification products were mixed with an equal volume of 2 × AFLP loading buffer (98% formamide, 10 mM EDTA, 0.01% xylene cyanol and 0.01% bromophenol blue) and denatured at 95 °C for 3 min. Samples (4 µl) were loaded onto 4.5% denaturing polyacrylamide gels and electrophoresed at a constant 65 W for 2.5 h. Gels were dried and exposed overnight to Kodak Biomax MR-2 film.

Data analysis

AFLP fragments were scored manually as present (1) or absent (0). Only the polymorphic bands were used in subsequent analyses, as the inclusion of monomorphic bands made no difference to the overall relationship between individuals (data not shown). The polymorphic data were used to determine similarities between the genotypes and groups using the statistical package Genstat 5 Release 4.1 (Payne et al. 1997). A Jaccard similarity matrix (Sneath and Sokel 1973) was calculated and used in an unweighted pair-group method arithmetic average (UPGMA) cluster analysis and a principal co-ordinates analysis (PCO). A generalised Procrustes analysis was used to determine whether there were any major differences between primer combinations in terms of relationships between samples (Barker et al. 1999). The binary data set was subject to bootstrapping, to evaluate the robustness of the groupings formed, using the program WINBOOT (Nelson et al. 1994; available from http://www.irri.org/winboot.htm).

Results and discussion

Since *Isatis* spp. were last used for indigo production, breeding methods have evolved and new techniques to accelerate the breeding process are in place. Furthermore, the ability to determine the genetic relationships between different species and landraces is now deemed highly important in the selection of plants in any breeding programme, because it allows the organisation of plant material and facilitates the selection of parental genotypes (Barker et al. 1999). Moreover, the assessment of germplasm diversity and the management of genetic resources are pivotal for plant breeding in introgressing exotic genes and characteristics into established cultivars (Tanksley and McCouch 1997). Previously, there has been no attempt to characterise the genetic diversity in *Isatis* spp. This is unusual in that most useful species have undergone at least some studies, such as those using isozyme markers or RAPD analysis (e.g., *Morus* spp., Sharma et al. 2000; *Brassica juncea*, Srivastava et al. 2001).

With the 38 genotypes of *Isatis* spp. used here, five AFLP primer combinations produced a total of 502 fragments, of which 436 (86.9%) were polymorphic between two or more genotypes. An example of the variation for a single AFLP primer combination is shown in Fig. 1. The number of fragments per primer combination ranged from 82 (*Mse*I + ACA/*Pst*I + AC) to 122 (*Mse*I + ACC/*Pst*I + CA). The number of polymorphic fragments detected per combination ranged from 72 (*Mse*I + ACA/*Pst*I + AC) to 101 (*Mse*I + ACC/*Pst*I + CA and *Mse*I + ACC/*Pst*I + CC), with an average of 87.2 polymorphic fragments per primer combination. Based on the percentage of polymorphic fragments, primer combinations also detected different levels of polymorphism ranging from 82.8% (*Mse*I + ACC/*Pst*I + CA) to 92.7% (*Mse*I + ACC/*Pst*I + CC). This compares favourably with studies on 45 genotypes of *Morus* spp. (Sharma et al. 2000), where five AFLP primer combinations also revealed a high level of polymorphism (81.2%) from a similar number of polymorphic fragments (92–123, compared with 72–101 in this study).

Of the 436 polymorphic fragments present, 41 were present only in the genotypes of *I. indigotica* and 71 were present only in the genotypes of *I. tinctoria*. The percentage of polymorphic fragments recorded in one or more genotypes within *I. indigotica* ranged from 19.4% (*Mse*I + GAA/*Pst*I + AC) to 47.4% (*Mse*I + ACA/*Pst*I + AC) with an average level of polymorphism of 31.2%. In *I. glauca* there was slightly more diversity, ranging from 28.3% (*Mse*I + ACC/*Pst*I + CA) to 46.9% (*Mse*I + GAA/*Pst*I + AC) with an average of 36.7%. In contrast, the diversity within *I. tinctoria* was greater, ranging from 80.3% (*Mse*I + GAA/*Pst*I + CC) to 92.6% (*Mse*I + ACC/*Pst* + CA) with an average of 86.8%, although, in part, this may be attributed to the fact that this species was represented by five different landraces, comprising 28 different genotypes.

Only a single fragment was found exclusively in the genotypes of *I. glauca* (<1%) and a further single fragment was shared exclusively between *I. glauca* and *I. indigotica*, whereas 175 (40.1%) fragments were shared between *I. tinctoria* and *I. glauca*.

I. tinctoria was represented by either five or six genotypes from each of five landraces: Tubingen, Potsdam, Erfurt, Swiss and English. Of 393 fragments recorded as present within the species, 341 (86.8%) were polymorphic between genotypes of the five landraces. Of these,

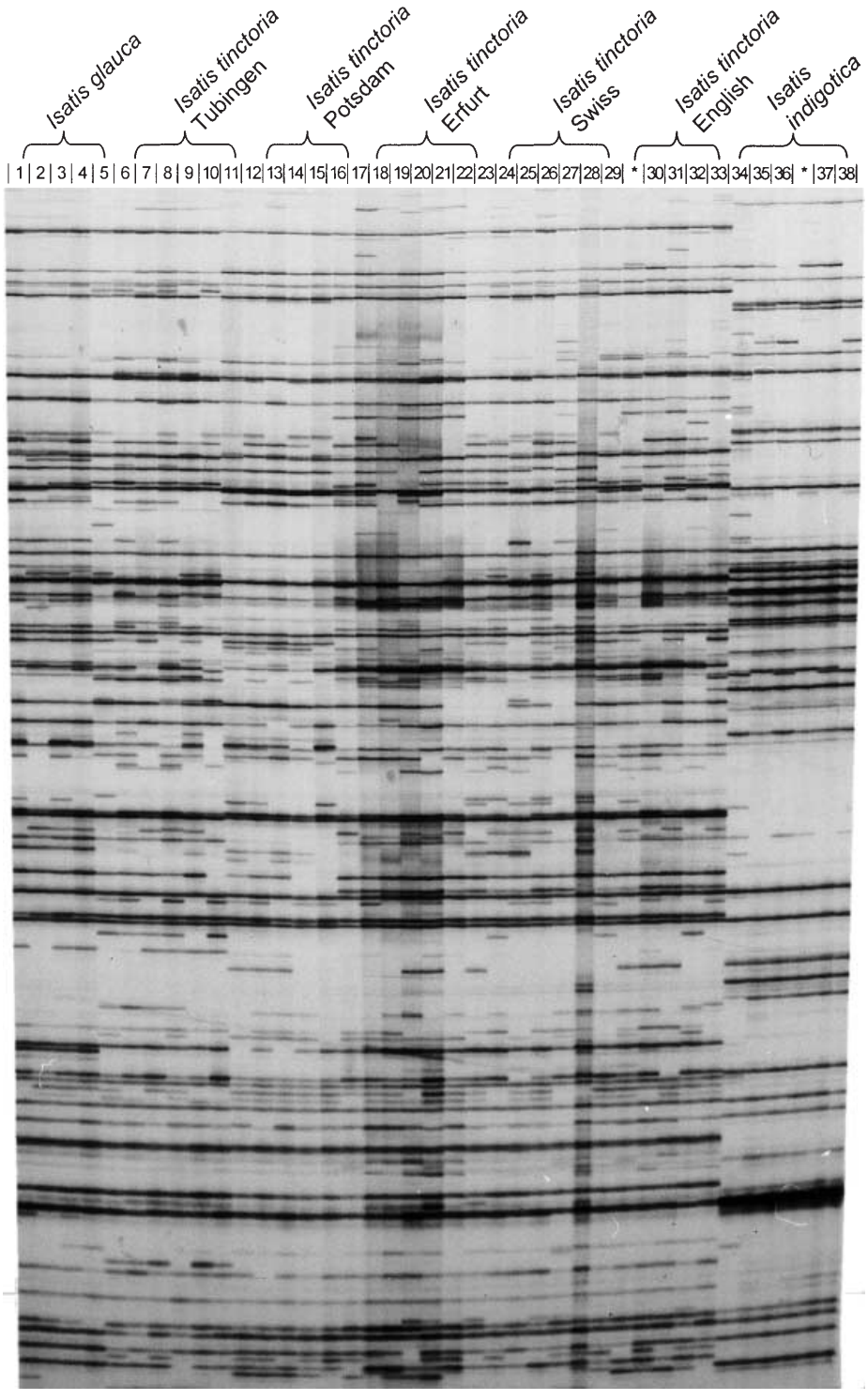

Fig. 1 AFLP fingerprints of 38 genotypes of *Isatis* spp. using primer combination *Mse*I + ACA/*Pst*I + AC. *Lane numbers* correspond to the numerical codes used to identify each genotype as described in Table 1. *Asterisks* indicate samples that were not included in this study

polymorphism within individual landraces ranged from 41.0% (Potsdam) to 65.6% (English) with between two (English) and 17 (Tubingen) fragments being exclusive to individual groups (Table 2).

The generalised Procrustes analysis, comparing the sets of polymorphic fragments from the five primer combinations, showed no evidence of any combination giving large differences in relationships between the genotypes. Therefore, it was deemed appropriate to combine

Table 2 Polymorphism within landraces of *I. tinctoria*

Landrace	Total no. of polymorphic fragments (%)	Total no. of exclusive fragments (%)
Potsdam	96 (41.0)	5 (2.7)
Tubingen	158 (52.1)	17 (6.8)
Erfurt	174 (56.9)	5 (2.0)
Swiss	210 (64.4)	6 (2.2)
English	193 (65.6)	2 (0.7)

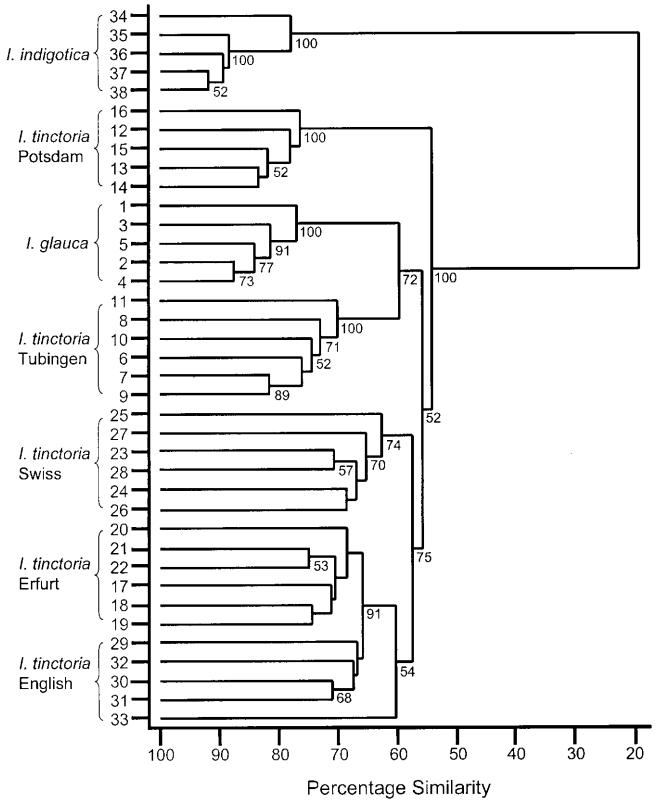

Fig. 2 Genetic similarity among 38 genotypes of *Isatis* spp. revealed by UPGMA cluster analysis based on 436 AFLP polymorphic fragments. Bootstrap confidence intervals are indicated for clusters present in the 50% majority rule consensus of 2,000 UPGMA searches. The numerical codes used to identify each genotype are described in Table 1

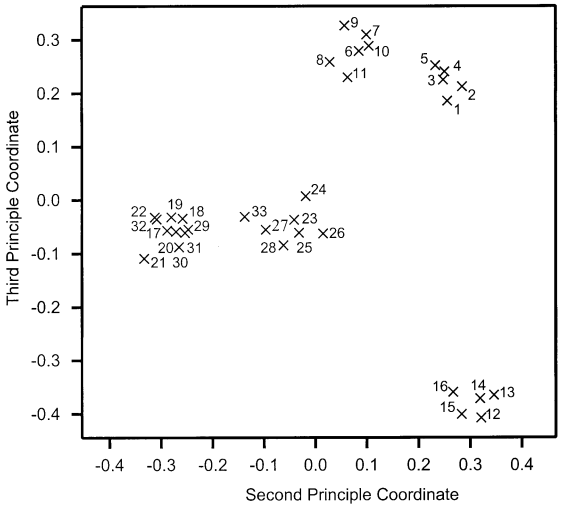

Fig. 3 Principal coordinate plot (3rd vs 2nd dimension) for 33 genotypes of *Isatis* spp., estimated with 436 AFLP polymorphic fragments using the Jaccard similarity matrix. The numerical codes used to identify each genotype are described in Table 1. The *I. indigotica* genotypes have been excluded, as in a PCO plot of the 2nd vs 1st dimension (data not shown) these grouped separately

all 436 polymorphic fragments, derived from AFLP analyses, for the calculation of the Jaccard similarity matrix and subsequent UPGMA and PCO cluster analyses.

The dendrogram resulting from the UPGMA cluster analysis revealed two main groups, where the five genotypes of *I. indigotica* formed one distinct group and genotypes of *I. tinctoria* and *I. glauca* formed the other (Fig. 2). Within this second group, *I. tinctoria* (Potsdam), *I. tinctoria* (Tubingen) and *I. glauca* formed separate groupings, although the latter two groups appeared to be more closely related. Of the remaining landraces, the genotypes from the Swiss group were more similar to each other, but this grouping was not particularly well separated. The Erfurt and English were also separated, apart from sample 33 (English). These results corresponded well to those from the PCO analysis. The first dimension accounted for 25.5% of the total variation, virtually all of which (99.6%) separated the *I. indigotica* genotypes from the rest. The second and third dimensions accounted for 9.0% and 6.2% of the total variation, respectively, and separated all other groups, except for the two *I. tinctoria* landraces, Erfurt and English (Fig. 3). Thus, the first three dimensions accounted for 42.7% of the total variation and the next three accounted for a further 14.4%; these dimensions all showed significant differentiation between different groups, whereas later dimensions did not.

The mean percentage similarities between and within groups are shown in Table 3. This again indicated the distinctness of the *I. indigotica* group (84.7% similarity within group, 18.6–21.9% similarity between it and other groups). Also, the *I. glauca* group (80.7% within, 55.3–59.6% between it and *I. tinctoria* groups), the *I. tinctoria* groups from Potsdam (78.6% within, 52.7–55.1% between it and other *I. tinctoria* groups) and Tubingen (73.2% within, 53.2–56.3% between it and other *I. tinctoria* groups).

Table 3 Mean % similarities for between and within groups

			(a)	(b)	(c)	(d)	(e)	(f)	(g)
(a)	*I. glauca*		80.7						
(b)	*I. tinctoria*	Tubingen	59.6	73.2					
(c)	*I. tinctoria*	Potsdam	56.4	53.2	78.6				
(d)	*I. tinctoria*	Erfurt	55.3	56.3	53.4	70.5			
(e)	*I. tinctoria*	Switzerland	56.9	55.5	55.1	56.6	65.4		
(f)	*I. tinctoria*	England	55.4	54.9	52.7	64.6	58.3	65.1	
(g)	*I. indigotica*		19.0	21.9	19.0	18.7	18.6	18.6	84.7

Taken together, the results successfully discriminated each species and even landraces into distinct groups, with the exception of the one English genotype (sample 33). It was also evident that the English landrace was more similar to Erfurt than to any of the other landraces. Furthermore, the analyses showed that *I. indigotica* separated as an entirely independent group, which clearly differed from all other groups. Interestingly, this species also has a greater indigo-production capacity (Stoker 1997). The genotypes of *I. glauca* clustered within the *I. tinctoria* group and were more similar to the *I. tinctoria* landraces than to *I. indigotica*. It appeared to be most closely related to the *I. tinctoria* landrace, Tubingen.

At present, indigo production from woad is approaching commercial viability, in terms of the amount of indigo produced per unit weight against cost of production and extraction. However, in order to guarantee an economic return it is imperative that indigo production is improved. This can be increased up to 10-fold by careful selection of phenotype and environmental conditions (Stoker et al. 1998). By selecting plants with a greater indigo-production capacity under the prevailing conditions and subjecting them to classical breeding methods, it is envisaged that a population with a greater production capacity can be generated. *I. indigotica* provides an interesting source of genetic material as it has an increased capacity for indigo production (Stoker 1997). However, its susceptibility to disease reduces its usefulness as a crop plant. If *I. tinctoria* genotypes can be crossed with those of *I. indigotica* it should be possible to produce a higher indigo yielder with less disease susceptibility, whilst maintaining genetic diversity.

The results illustrate the usefulness of the AFLP technique in discriminating not only between *I. indigotica* and *I. tinctoria* but also between the five landraces of *I. tinctoria*. AFLP fragments present exclusively in only one species or landrace were identified. If these fragments were isolated, re-amplified and sequenced to design specific primers for use in a simple diagnostic PCR assay, larger sample sizes could be screened routinely for use in the identification of these species/landraces. At present, it is necessary to grow plants for 6 weeks prior to screening the indigo-production capacity. If fragments exclusive to higher yielding genotypes can be found, then marker-assisted selection of useful plants could be utilised, at an earlier stage. This would save time and resources in maintaining plants prior to screening and, therefore, significantly improve plant selection for breeding of the *Isatis* genus.

Acknowledgements K. G. Gilbert and D. T. Cooke received funding for the first part of this work from a MAFF-LINK grant (CSA4308/LK0801) and the work was completed using funding from an EU Framework V grant (QLK5-CT-2000-3096). The authors thank Mr. Ken Williams for photographic expertise. M.A. Karam wishes to thank Prof. P.R. Shewry, Head of IACR, Long Ashton Research Station, for support and encouragement. IACR receives grand-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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