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Developing an appropriate strategy to assess genetic variability in plant germplasm collections

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Abstract The value of molecular biology for monitoring the genetic status of germplasm collections is subject to practical limitations. The large number and variability of accessions held usually dictates the approach that can be employed. A quick, simple but reliable molecular protocol must be combined with an appropriate strategy for handling large sample sizes. In this study, ISSR-PCR was used to reveal genetic variability within and between accessions held in a collection of lupin germplasm. Pooling of DNA from individuals within accessions was found to be the most appropriate strategy for assessing large quantities of plant material. Band profiles generated from pools containing five individuals were fully representative of all constituent individuals used in the mix. Pools comprising 10 or 20 individuals, however, sometimes failed to contain minor bands that had been present only in the profile of one individual. Variation was observed between pools containing five different genotypes from the same accession. Routine large-scale screens are required to assess the genetic diversity and homogeneity of the lupin germplasm collection held in Reading. It is concluded that 2–3 pools of five genotypes may be sufficient to represent the genetic variability within and between accessions in the lupin and similar collections.

Key words Lupin · *Lupinus albus* · Germplasm collection · ISSR-PCR · Microsatellite · Genebank

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Introduction

Ex situ conservation of land races and wild relatives provides vital insurance against excessive erosion of a crop's genetic base. For this reason, genebank collections have been established for all major and most minor crops. These repositories typically contain hundreds or even thousands of accessions originating from several geographic regions and representing a range of genetic backgrounds. Their utility for breeding purposes rests largely on the accuracy of evaluation and passport data, and also on the genetic fidelity of the material held. In time, there is considerable scope for the accumulation of documentation errors that lead to wasteful duplication of stocks and also for genetic erosion to occur within accessions. Such events can be extremely difficult to detect but dramatically reduce the practical value of collections. There is a need, therefore, for a simple system to test the genetic identity and diversity of individuals within accessions and also to compare all accessions held within a collection.

A comparison of plant morphology is the simplest approach for the detection of mislabelled accessions and the assessment of genetic diversity. This strategy is sensitive to environmental influences and cannot always distinguish between closely related samples. Molecular methods of identification have the distinct advantage of being independent of climatic variables but can be limited by other considerations. For example, it is important that the technique used must be able to distinguish most or all genotypes held in a collection and also be able to provide evidence of genetic erosion. At the same time, the large number of accessions held in most collections dictates that the protocol used should be quick, uncomplicated and cheap. Inter Simple Sequence Repeat PCR (ISSR-PCR) is a highly informative technique that has been used to DNA-fingerprint a wide range of crops (e.g. Kantety et al. 1995; Charters et al. 1996; Nagaoka and Ogihara

1997). ISSR-PCR is also a quick and simple technique with low running costs and requiring only small quantities of template DNA. This approach therefore has potential for the routine testing of the genetic identity and purity of accessions held in germplasm collections. White lupin (*Lupinus albus*) is an emergent crop with a seed protein content comparable to that found in soyabean (Garcia et al. 1997; Huyghe 1997). There is increasing interest in the potential of white lupin as an alternative to soyabean in areas unsuitable for soyabean cultivation. Efforts to improve the crop have centred on the introduction of determinate phenotypes and traits allowing the range of the species to be expanded into North and Western Europe. Concurrent with this selection process will be a narrowing of the genetic base of the crop. The University of Reading, UK, holds a substantial collection of land races and current cultivars of white lupin. This represents a valuable genetic resource that will have utility for future breeding programmes of the crop. It is important that the collection is periodically screened for the genetic erosion of stocks, to detect documentation errors, to identify areas of genetic duplication and to characterise the genetic diversity represented. In this study we examine the potential of ISSR-PCR to provide a routine screening protocol for assessing the genetic status of this collection.

Materials and methods

Plant material

Seeds of 28 *L. albus* accessions (Table 1) were taken from the Lupin germplasm collection housed in the Department of Agricultural Botany, The University of Reading, UK, and Dr. Christian Huyghe, INRA, Lusignan, France, provided a further 12 accessions. Batches of 20 seeds were germinated on moist filter paper at room temperature in the dark. DNA was extracted from 2-cm radicles of 5–10 seedlings from each accession according to the method described by Tai and Tanksley (1991). DNA was re-suspended into a final volume of 50 µl TE buffer and quantified using a DyNA Quant fluorimeter (Amersham Pharmacia Biotech).

Table 1 Accessions of *L. albus* used for ISSR-PCR

Code	Collection area	Cultivar name/collection site
RLC1	USA	ARK 10
RLC3	South Africa	BUTTERCUP
RLC5	Egypt	EGYPTSKA
RLC7	Germany	PFLUGS GELA
RLC9	Germany	HANSA
RLC10	USA	HOPE
RLC19	Italy	LUPINI BEAN
RLC21	Germany	NAHRQUELL
RLC26	Hungary	SHARKIA 13
RLC28	USA	SSK-79
RLC32	Germany	PFLUGS ULTRA
RLC70	France	LUDET
RLC73	France	LUCYANNE
RLC89	Sudan	TURMUS
RLC171	Greece	DELPHI
RLC174	Greece	MESSINA
RLC177	Greece	PIRGOS
RLC180	Greece	ATHENS
RLC443	Spain	MONTORO, CORDOBA
RLC444	Spain	AZUEL, CORDOBA
RLC456	Spain	CONSTANTINA, SEVILLA
RLC505	Portugal	SETUBAL
RLC806	Former Yugoslavia	MULINE ISLAND, UGLJANE
RLC807	Former Yugoslavia	LUKORAN ISLAND, UGLJANE
RLC1001	Turkey	ADANA
RLC1009	Turkey	KAYSERI
RLC1020	Italy	SOUTH OF VITERBO
RLC1068	Poland	HETMAN
cv adam	France	ADAM
cv alban	France	ALBAN
cv alex	France	ALEX
cv athos	France	ATHOS
cv lucrop	France	LUCROP
cv kalina	Poland	KALINA
cv kievsky	Russia	KIEVSKY
cv lunoble	France	LUNOBLE
cv lublanc	France	LUBLANC
cv lucky	France	LUCKY
cv lumunieux	France	LUMUNIEUX
cv lutop	France	LUTOP

PCR amplification and detection

The PCR conditions, amplicon separation by PAGE, and staining were all as described by Charters et al. (1996). Amplifications were performed using a Hybaid Omnigene Thermocycler using the following programme: 30 cycles of 1 min at 94°C, 2 min at 3°C less than the Tm of the primer, 30 s at 72°C, followed by 5-min extension at 72°C. PCR products were fractionated on a pre-cast polyacrylamide gel (48S, Amersham Pharmacia Biotech) and stained with silver nitrate.

Gel scoring and cluster analysis

Band profiles generated by PCR were compiled onto a data matrix on the basis of the presence (1) or absence (0) of selected bands. Dendrograms were constructed by UPGMA cluster analysis from a Jaccard dissimilarity matrix using the NTSYS software package (Numerical Taxonomy and Multivariate Analysis system, Exeter Software, New York).

Experimental design

Detection of genetic variability

DNA was extracted from ten representatives of accessions RLC3, RLC19 and RLC174 (Table 1), and subjected to ISSR-PCR using primers 836, 840, 842, 888 and 891 (Table 2).

The effect of template dilution by pooling

It is possible that the pooling of template DNA may lead to the poor amplification of certain rare markers such that they become absent from the collective band profile. Several factors could contribute to

Table 2 ISSR primers used to screen *L. albus* accessions

Primer code	DNA sequence
836	AGAGAGAGAGAGAGAGYA
840	GAGAGAGAGAGAGAGAYT
842	GAGAGAGAGAGAGAGAYG
888	BDBCACACACACACACA
891	HVHTGTGTGTGTGTGTG
818	CACACACACACACACAG
812	GAGAGAGAGAGAGAGAA
809	AGAGAGAGAGAGAGAGG
825	ACACACACACACACACT
890	VHVGTGTGTGTGTGTGT

the production of undetected markers of this kind, including template DNA concentration and purity, primer selection, amplicon size, PCR conditions, and the sensitivity of product detection. Thus, dilution of template DNA could have variable effects on the appearance of different markers in pooled band profiles. Two genotypes (denoted A and B) of accession RLC19 are known to differ in the band profiles generated by several ISSR primers (unpublished data). These individuals were selected to study the disappearance of rare markers from pooled samples attributable to template dilution. Pooled DNA (20 ng) comprising the following mixtures of the two genotypes were used as a template for PCR: 20% A, 80% B; 10% A, 90% B; and 5% A, 95% B. ISSR-PCR was conducted using primers 840, 842, 888 and 891. The mixing of pooled samples and the ISSR-PCR was duplicated and the products fractionated on the same gel for comparative purposes.

Variation between accessions

DNA extracted from five individuals belonging to the same accession was quantified and adjusted to a final concentration of 20 ng/μl before being mixed. Pools were made in this way for all 40 accessions studied (Table 1). These were compared using the ten ISSR primers described in Table 2 obtained from the University of British Columbia, Vancouver, Canada (set #9). PCRs and gels were replicated at least twice for each ISSR primer used. The ability of the five most-informative primers to distinguish between accessions was assessed by calculating their Resolving Power, Rp (Prevost and Wilkinson 1998). This function has been found to correlate strongly with the ability to distinguish between taxa and is given by the formula: $Rp = \varepsilon I_b$, where band informativeness, $I_b = 1 - (2 \times |0.5 - p|)$ and p is the proportion of accessions containing band I.

Detection of rare markers in pooled samples

The effect of pooling template DNA may be more complex than suggested by dilution experiments, particularly when a range of germplasm is used. For instance, there is the possibility that the genetic composition of the pool may influence the likelihood of rare band amplification. On a practical basis, problems in the accuracy and reliability of DNA quantification and variation in the purity of extracted DNA may also affect the likelihood of rare band amplification. The effect of pool composition on the amplification of rare bands was therefore examined using pools compiled from three accessions (RLC3, RLC19 and RLC174).

DNA was extracted from ten individuals of each accession and adjusted to a concentration of 20 ng/μl. Pools were then compiled by randomly selecting five of the ten samples using the random number generator in Excel (Microsoft Ltd). There were 15 pooled samples made in this way for each of the three accessions (all pools contained a different mix of individuals). ISSR-PCR was conducted on all 45 pools using primer 888.

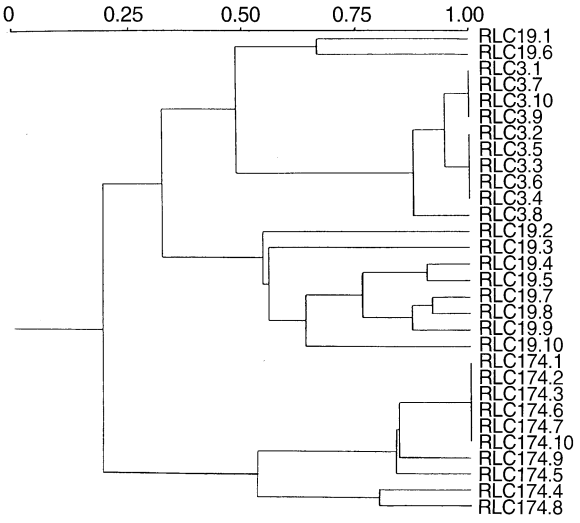


Fig. 1 UPGMA phenogram using Jaccard similarity of ten representatives each of accessions RLC3, RLC19 and RLC173

Results

Detection of genetic variability

The first requirement of any molecular approach for the assessment of germplasm held in a collection is the ability to distinguish between genotypes and accessions. The band profiles of ten representative genotypes from the three selected accessions were compared by Jaccard similarity and clustered with UPGMA (Fig. 1). Each of the accessions formed fairly distinct clusters, although two individuals of accession RLC19 showed a greater affinity to members of accession RCL3 than to other representatives of the same sample. There was also variation between individuals belonging to the same accession. Representatives of accession RLC3 showed the least variability, with two groups of individuals proving impossible to differentiate (plants 3.1, 3.7, 3.9 and 3.10, and plants 3.2, 3.3, 3.4, 3.5, 3.6). In contrast, accession RLC19 showed far more diversity and all ten individuals could be distinguished on the basis of their band profiles. Individuals of accession RLC174 showed a mixed pattern of variability, with six representatives proving indistinguishable (plants 174.1, 174.2, 174.3, 174.6, 174.7 and 174.10) and the remaining four generating unique profiles.

The effect of template dilution by pooling

The use of individuals as a basis for comparing the genetic variability within and between accessions is

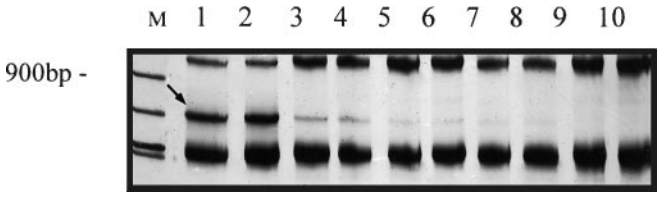


Fig. 2 Effect of template DNA dilution on ISSR band intensity. The intensity of a band diagnostic of genotype A (*arrowed*) in ISSR-PCR profiles from DNA mixtures of genotypes A and B. *Lanes* are in the following order: *M* 100-bp ladder; *1*, *2* genotype A; *3*, *4* 80% genotype B, 20% genotype A; *5*, *6* 90% genotype B, 10% genotype A; *7*, *8* 95% genotype B, 5% genotype A; *9*, *10* genotype B

subject to practical constraints. Small numbers of genotypes may not be fully representative of the genetic diversity contained in an accession. On the other hand, the use of ten or more genotypes significantly reduces the number of accessions that can be handled. Pooling template DNA from several genotypes therefore offers an attractive alternative strategy. This approach is useful only if all bands in the profiles of all genotypes contained within the pool are also represented in the composite profile. The appearance of diagnostic bands in the profiles of mixed samples of genotypes A and B varied with the composition of the mixture and also with the primer used. All pools containing genotype mixtures of 20% A, 80% B generated profiles possessing all of the bands that had been present in the constituent profiles. This was also true in the 10% A, 90% B and 5% A, 95% B mixtures for the majority of markers, although bands diagnostic of the rarest genotype were frequently faint and difficult to score (Fig. 2). Minor bands that were diagnostic of the rare genotype could not always be scored with certainty in pools comprising 10% A, 90% B or 5% A, 95% B mixtures. For this reason, all subsequent pools were comprised of DNA mixtures from five genotypes.

Fig. 3 Variation in band profile in pooled DNA samples from 40 lupin accessions. *Lane* order: *M* 100-bp ladder (700 bp is *arrowed*); *1*, RLC1; *2*, RLC3; *3*, RLC5; *4*, RLC7; *5*, RLC9; *6*, RLC10; *7*, RLC19; *8*, RLC21; *9*, RLC26; *10*, RLC28; *11*, RLC32; *12*, RLC70; *13*, RLC73; *14*, RLC89; *15*, RLC171; *16*, RLC174; *17*, RLC177; *18*, RLC180; *19*, RLC443; *20*, RLC444; *21*, RLC456; *22*, RLC505; *23*, RLC806; *24*, RLC807; *25*, RLC1001; *26*, RLC1009; *27*, RLC1020; *28*, RLC1068; *29*, cv adam; *30*, cv alban; *31*, cv alex; *32*, cv athos; *33*, cv lucrop; *34*, cv kalina; *35*, cv kievsky; *36*, cv lunoble; *37*, cv lublanc; *38*, cv lucky; *39*, cv luminieux; *40*, cv lutop

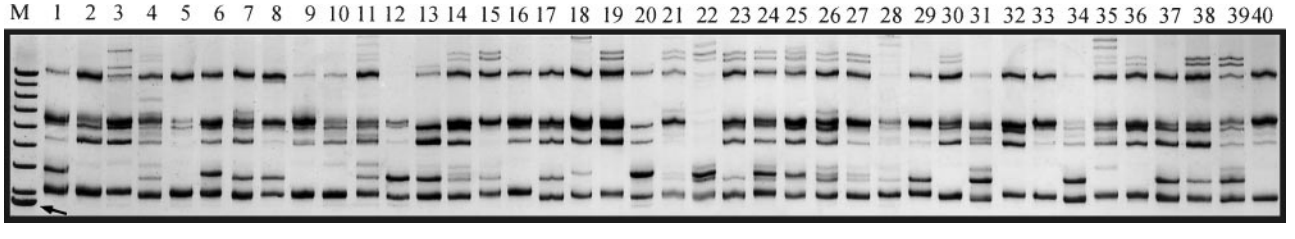
Table 3 Characteristics of the band profiles produced by five selected ISSR primers

ISSR primer	ISSR-PCR bands		No. of distinguishable groups/individuals	Resolving power
	Scored	Polymorphic		
836	11	10	21/37	3.8
840	15	14	35/37	7.4
842	16	16	33/37	6.3
888	18	16	29/37	6.2
891	18	17	30/37	5.7

Variation between accessions

Of the 40 accessions initially investigated, 37 gave reliable banding patterns with all the primers and so were used in further analysis. The number of markers generated by the ten ISSR primers varied between 7 (primer 818) and 18 (primers 888, 890 and 891). No variation was observed between replicates in the presence or absence of any of the bands selected for analysis. All, or the vast majority, of bands produced by each primer were polymorphic between accessions. Overall, the 137 bands scored for the ten primers studied included 122 (89%) that were polymorphic between accessions. The number of accessions or groups of accessions that could be distinguished by each primer varied from 9/37 (primer 818) to 35/37 (primer 840, Fig. 3). Diagnosis of all 37 accessions was possible using any two of the ten primers screened. The ability of the five most-informative primers to diagnose lupin accessions was also assessed on the basis of Resolving power (Rp). The Rp values varied between 3.8 for primer 836 and 7.4 for primer 840. Primers with higher Rp values were generally able to distinguish more genotypes (Table 3). The collective Rp value for all five selected primers was 29.4.

The UPGMA tree of a combined data set using the five most-informative primers provided some evidence of clustering (Fig. 4). The poor documentation associated with these lines, coupled with widespread transportation of stocks between countries, meant that it was not possible to relate clustering to geographical origin.



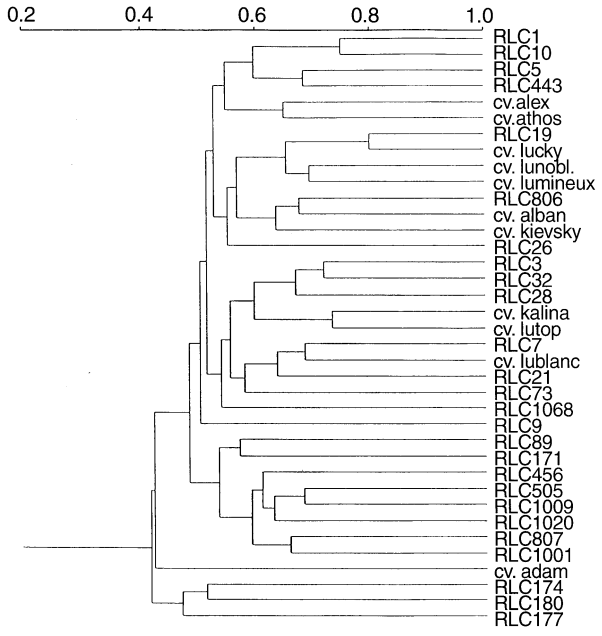


Fig. 4 UPGMA phenogram using the Jaccard similarity of ISSR-PCR band profiles from pooled samples of 37 lupin accessions

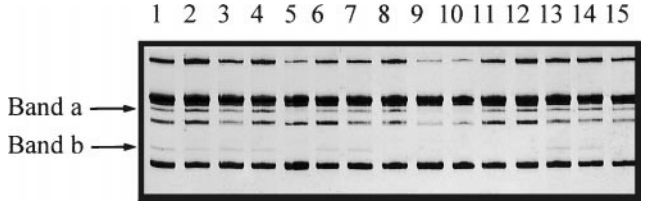


Fig. 5 Effect of pool composition on the appearance of two bands (*a and b*) in composite profiles using primer 888. Band *a* is unique to one genotype that is present in the pools in *lanes 1–4*, *6–8* and *11–15*. *Band b* is unique to a second genotype present in the pools in *lanes 1–4*, *6–7* and *13–14*

Variation within accessions

Pools were made containing combinations of five genotypes from ten characterised representatives of three accessions and used to generate ISSR profiles. Bands diagnostic to one or more genotypes were detected in all pools containing an individual with the marker, regardless of the size of the marker or the genetic composition of the remaining genotypes used in the pool (Fig. 5) as predicted by template-dilution experiments. Thus, the profile of the pool accurately represented a composite of all components of the pool, irrespective of the accession being studied. The pattern of genetic diversity found using pooled samples within accessions mirrored that found using individual genotypes. The profiles of accession RLC3 did not vary between pools whereas those of accession RLC174 showed variability in two band positions (Fig. 5). An intermediate level of variation was observed between pools of accession RLC19, where polymorphisms were noted in the presence/absence of one band position.

Discussion

There is a wide range of molecular techniques available to assess genetic variability of populations and individuals. Speed, reproducibility and the ability to detect genetic variation within and between accessions determine the utility of these for genebank management. The method for ISSR-PCR used here can be completed within 9 h and produced consistent band profiles between replicates. This is in accordance with previous work demonstrating reproducibility of the protocol between PCRs, DNA extractions, gels (Charters et al. 1996), and also between laboratories (Charters et al. 1999). The results presented here also show that ISSR-PCR primers are able to reveal variability both between and within lupin accessions. These polymorphisms have three main functions for germplasm management: (1) as a means of identification, (2) to detect genetic erosion (measure genetic diversity), and (3) to reveal genetic relationships.

The practical utility of any molecular approach for germplasm management is partly determined by the ability to differentiate between large numbers of accessions. The ISSR primers used here varied in their ability to diagnose lupin accessions, and primers with the highest Rp values were generally the most effective in distinguishing between accessions. This is in agreement with the findings of Prevost and Wilkinson (1999) on potato cultivars. Resolving power provides a basis for comparing the diagnostic effectiveness of primers used in one study with those of another in which different numbers of accessions are included. The combined Rp value of two primers also provides a measure of their collective performance for identification purposes. In this work, the combination of any two primers allowed the diagnosis of all 37 accessions. The two least-informative of the five selected primers possessed a joint Rp value of 9.5. Interestingly, the primer with the highest individual Rp (primer 840) fell short of this value (7.4) and was only able to distinguish 35/37 accessions. The collective Rp value for all five selected primers was 29.4. Should the relationship between Rp and diagnosis hold over larger sample sizes, this value suggests that the combined use of all five primers would allow complete diagnosis of over 120 accessions. This extrapolation is subject to a number of assumptions, including that the expanded set of accessions has a broadly similar genetic make-up to those used here.

It is important that any technique used for germplasm management should provide information on the genetic relationships of the material held. This allows

valuable atypical accessions to be identified and conserved, whereas duplicates (or near duplicates) can be removed. The clustering by UPGMA of individuals into accessions and of accessions into larger groups demonstrates that ISSR profiles could also have value for revealing the genetic relationships of lupin stocks. In the present case there was a tendency for accessions obtained from a particular geographic region or breeding programme to cluster, but there were also many exceptions. It is clear that more detailed information on pedigrees and exchange of germplasm would be needed in order to clarify the genetic relationships within these data.

Collectively, these results show that ISSR-PCR is able to provide information on the identity (diagnostic ability), genetic diversity and genetic relationships of material held in a lupin germplasm collection.

The number of individuals required for the replenishment of seed stocks of an accession will depend partly on the variability of the accession being managed. In general, fewer parental plants would be required to maintain the diversity of genetically uniform accessions than for more variable stocks. A measure of the genetic variability contained by the accessions therefore allows curators to optimise the use of resources (e.g. glasshouse space, labour) required for seed multiplication. At the same time, knowledge of genetic variation between accessions held in a genebank has direct importance for the management of the collection as a whole. The size of most collections is close to the operating capacity of the institution where it is being maintained. Size is generally limited by manpower and/or space constraints. Under these circumstances, the introduction of fresh accessions is made possible only by the removal of others. Even where a collection is in an expansive phase, choices are often required over which accessions obtained from a collecting expedition should be entered into the collection. Curators aim to target accessions containing many new alleles and avoid those that represent duplications of stocks already held. This need is particularly acute where phytosanitary regulations require the passage of material through quarantine, usually at a cost to the importer. It is of vital importance, therefore, that there is a reliable means of ranking the importance of accessions held and of potential new material to be added to a collection. This can be achieved simply by reference to the geographical source of the material or, in collections containing several related species, by taxonomic identity. Neither approach has value for single-species collections and those where the relationship between geography and genetic diversity is poorly characterised. In the case of the lupin collection, documentation relating to the geographic origins of accessions is often vague. Furthermore, this collection is comprised largely of landrace and primitive cultivated material that may have been transported away from the point of origin. A quick measure of the genetic diversity between accessions therefore provides the most appropriate method of identifying valuable genetically distinct stocks from those that represent genetic duplications of other accessions. The ability of all ISSR primers screened to reveal variability both within and between accessions therefore has potential value for the management of the lupin collection.

The ability to measure genetic diversity within accessions improves as the number of individuals compared increases. At the same time, however, larger sample sizes also limit the number of accessions that can be assessed. The use of pools of individuals offers a practical alternative that would enable rapid comparisons to be made between many accessions (Virk et al. 1995 a, b). This approach is subject to certain constraints. Band profiles amplified from large pools (ten or more genotypes) would tend to contain more of the alleles present in the whole accession than would profiles generated from small pools. Large pools therefore provide a more representative measure of the genetic architecture of the accession. On the other hand, the results presented here suggest that faint bands present in only one representative of a 10 or 20-plant pool may not always amplify in the composite profile. This throws into question the reliability of large pools as representatives of the accession as a whole. A more appropriate strategy may therefore be to use two or more pools containing fewer individuals, where all bands are represented in the composite profile.

These results also have implications for the bulk-screening of accessions to associate traits of interest with molecular markers. This approach requires markers that are present in very few genotypes of a large pool to be absent (rather than present) from the composite band profile. Variation in the appearance of bands in 10 and 20-plant pools suggests that the sensitivity of the approach may fluctuate between markers produced by the same primer.

The most accurate measure of genetic variability within accessions would be to compare the band profiles of large numbers of individuals. However, the large numbers of accessions held in most germplasm collections renders this approach impractical. A practical alternative might also be to use pools. Large pools provide little information on the genetic variability within the accessions being studied. For instance, consider the variation between pools comprised of 10-plant mixes in which a particular allele (x) appears in 20% of the genotypes. The probability of this allele being absent from any one pool is given by the equation:

$$\text{Prob. allele x being absent from pool} = (\text{prob. of allele x is absent in a random individual})^{\text{number of individuals in the pool}}$$

or,

$$\text{Prob. allele x being absent from pool} = 0.8^{10} \text{ or } 0.13.$$

It follows that the vast majority (i.e. 87%) of 10-plant pools would contain allele x and so these pooled profiles would be largely invariant. In pools comprising five plants, the same allele would be absent from only 0.8^5 of pools ($P = 0.33$). Thus, approximately 1/3 of pools would lack the allele and 2/3 would contain it. The use of two or three small pools would therefore identify infrequent alleles and also provide a measure of genetic diversity within an accession. This approach is not a test specifically for rare alleles but nevertheless would help prevent genotypes that contain infrequent alleles from becoming rarer or even lost from an accession. This is particularly significant since it is questionable whether alleles can be maintained in collections over prolonged periods once they have been allowed to become rare within an accession (Brown et al. 1997).

It can be inferred that 2–3 pools of five genotypes could be used to provide a measure of genetic diversity within accessions and, at the same time, also enable the genetic comparison of large numbers of accessions. The combination of this type of pooling strategy and ISSR-PCR analysis would reduce the effort and resources required for fingerprinting but not for the DNA extraction, unless plant material was pooled prior to extraction. Thus the approach would allow routine periodic screening of relatively large numbers of accessions to monitor and manage the genetic status of a germplasm collection such as the lupin collection.

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