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# Cross-species amplification of *Medicago truncatula* microsatellites across three major pulse crops

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**Abstract** Model plants are facilitating the genetic characterization and comparative mapping of a number of traditional crops. *Medicago truncatula* has been widely accepted as a model plant to this end as it provides the essential tools for multiple aspects of legume genetics and genomics. A large set of markers from highly conserved *M. truncatula* gene regions is being created and used to establish a worldwide framework for comparative genomic studies in legumes. We have investigated the potential for cross-species amplification of 209 expressed sequence tag (EST)-based and 33 bacterial artificial chromosome (BAC)-based microsatellites from *M. truncatula* in the three most important European legume pulses—pea, faba bean and chickpea—that might facilitate future comparative mapping. Our results revealed significant transferability of *M. truncatula* microsatellites to the three pulses (40% in faba bean, 36.3% in chickpea and 37.6% in pea). The percentage of *M. truncatula* EST-SSRs (simple sequence repeats) amplified in the three crops (39–43%) was twofold higher than that of the genomic SSRs (21–24%). Sequence analysis determined that the level of conservation in the microsatellite motif was very low, while the flanking regions were generally well conserved. The variations in the sequences were mainly due to changes in the number of repeat motifs in the microsatellite region combined with indel and base substitutions. None of the functional microsatellites showed direct polymorphism among the parental genotypes tested, consequently preventing their immediate use for mapping purposes.

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## Introduction

The legume family (Leguminosae or Fabaceae) is the second most important food and forage source after grasses. Most cultivated legumes are found within the Papilionoideae, which include the so-called tropical or phaseloid legumes and the temperate or galegoid legumes, with the genera *Melilotus, Trifolium, Medicago, Pisum, Vicia, Lotus, Cicer* and *Lens* (Young et al. 2003). The large genome size of some of these species (up to 12,000 Mbp in *Vicia faba*) has impeded their mapping and genetic characterization, thereby hampering gene isolation and map-based cloning.

Within the legume family, there are four well-studied model systems—*Medicago truncatula*,*Lotus japonicus*, *Glycine max* and *Pisum sativum*. While these cover only a tiny portion of the legume diversity, they are providing valuable tools for the genetic characterization and comparative mapping of some of the more traditional crops. Among them, *M. truncatula*, with a small genome of approximately 500 Mbp is gaining wide acceptance as a model plant for multiple aspects of legume genetics and genomics (Cook 1999). Several large-scale projects on *M. truncatula* genomics have been initiated by the international community, and essential tools are being

developed for structural and functional genomics along with the development of bioinformatics resources (Frugoli and Harris 2001).

With respect to synteny, the ultimate goal is to develop a composite legume genetic map that includes the most agronomically important legumes. Through the exploration of highly conserved gene regions, primer pairs can be designed and used for linkage mapping in multiple taxa. At present a very large set of such markers is being created and used to establish a worldwide framework for comparative genomic studies in legumes. If genome structures are highly conserved, it will be possible to quickly identify candidate genes in the model species corresponding to loci mapped in the agronomically important species. Initial results indicate a high degree of macrosynteny between the genomes of pea, alfalfa and *Medicago* (Frugoli et al. 1999) or alfalfa and *Medicago* (Choi et al. 2004), as well as significant levels of microsynteny among legumes and even with *Arabidopsis thaliana* (Grant et al. 2000; Lee et al. 2001; Marek et al. 2001; Foster-Hartnett 2002; Yan et al. 2003).

Among the possible markers available for use, microsatellites, also referred to as simple sequence repeats (SSRs), are widely employed for a multitude of genetic projects due to the highly reproducible and reliable identification of alleles when these markers are applied. These arrays of short (1–6 pb) tandem DNA repeats which are amplified by PCR using primers that complement the flanking regions (usually conserved), and they are co-dominantly inherited and highly polymorphic. The major constraint of using SSRs as molecular markers is the cost and effort required for their development, and this has restricted their use to only a few of the agriculturally important crops. A more widespread use of SSRs in plants would be facilitated if such loci were transferable across species.

The emphasis on SSRs has lately shifted towards the use of EST (expressed sequence tag)-SSRs, which are relatively inexpensive compared with the development of genomic SSRs (Gupta et al. 2003). Such markers are expected to be more transferable to closely related genera since they are anchored within more conserved transcribed regions (Cordeiro et al. 2001; Hempel and Peakall, 2003; Decroocq et al. 2003). However, the level of polymorphism detected with EST-SSRs has been lower than that detected with the genomic SSRs (Cho et al. 2000; Scott et al. 2000; Eujayl et al. 2002). EST-SSRs also have a higher probability of being functionally associated with differences in gene expression than the genomic SSRs. Sequence alignment of mapped EST-SSR loci will enable a tentative assignment of function to the other related species; for example, a homology search in rice was used to place genes with a putative function onto a wheat map, thus facilitating functional assignment of wheat ESTs (Gao et al. 2004).

A growing number of plant studies have reported different levels of SSR primer sequence conservation in *Eucalyptus* ssp. (Byrne et al. 1996; Brondani et al. 1998), *Quercus* ssp. (Steinkellner et al. 1997), *Prunus* ssp. (Dirlewanger et al. 2002), within Melilaceae (White and Powell, 1997) and Myrtaceae (Rosetto et al. 2000), within the genus *Brassica* (Lagercrantz et al. 1993), between the model species *A. thaliana* and several *Brassica* species (Westman and Kresovich 1998) among cereals (Sourdille et al. 2001; Kuleung et al. 2004; Saha et al. 2004; Yu et al. 2004) and among *Vitaceae* and *Rosaceae* (Decroocq et al. 2003).

Several investigators have used SSRs derived from total genomic DNA to obtain cross-species amplification between legume genera or species. Dayanandan et al. (1997) reported primer sequence conservation among Leguminosae tropical trees, and Peakall et al. (1998) described up to 65% cross-amplification within the genus *Glycine* and lower rates (3–13%) outside the genus. Conservation of chickpea microsatellites between accessions and species of *Cicer* has recently been reported (Choumane et al. 2000). Finally, the transferability of pea and chickpea SSRs across several mayor pulse crops was examined, revealing a high level of sequence conservation (Pandian et al. 2000). EST-SSRs from *M. truncatula* have been recently evaluated for transferability and polymorphism within the genus (Julier et al. 2003; Eujayl et al. 2004), and in both of these studies, high levels of both cross-amplification and polymorphism were detected in several *Medicago* ssp.

Among legumes, the availability of SSRs is limited to species for which sequencing databases already exists and, with the exception of soybean and *Medicago*, the number remains very low for many of the major pulse crops. In this study we investigated the potential for cross-species amplification of 209 EST-based and 33 bacterial artificial chromosome (BAC)-based microsatellites from *M. truncatula* within the three most important European legume pulses—pea, faba bean and chickpea. *M. truncatula* is also part of the Galegoid phylum, and this genetic proximity offers the potential to easily transfer information on the genome structure of the model legume to the other legume crops. Extensive conservation might facilitate future comparative mapping and the identification of candidate genes among these species.

## Materials and methods

### Plant material and DNA isolation

Analyses were carried out using plant tissue from the three temperate pulses, pea (*Pisum sativum*), faba bean (*Vicia faba*) and chickpea (*Cicer arietinum* and *C. reticulatum*) as well as *Medicago truncatula* (cv. Jemalong) as a positive control. The following crop accessions were assayed: in pea, P665 (belonging to *P. sativum* L. ssp. *syriacum* Berg), and *P. sativum* L. cv. *Messire*; in faba bean, Vf 6, Vf 136, Vf 27 and 29H; in chickpea, ICCL81001, Wr315, ILC72, JG62 (*C. arietinum*

accessions) and Cr5-10 (*C. reticulatum*). All materials are parental lines that have been used in previous mapping studies by our group. The progenies derived from these lines have been used to detect genes and QTLs (quantitative trait loci) for important agronomic traits such as seed weight or resistance to broomrape, *Fusarium, Uromyces* and *Ascochyta* (Roman et al. 2002, 2003; Avila et al. 2003, 2004; Millán et al. 2003; Rubio et al. 2003; Valderrama et al. 2004).

DNA extractions were carried out from young leaf tissue using the method reported by Lassner et al. (1989) and modified by Torres et al. (1993). Co-precipitated RNA was eliminated by adding 0.7 U RNase. DNA was dissolved in TE, and the final concentration was estimated by agarose gel electrophoresis and ethidium bromide staining using known concentrations of uncut $\lambda$-DNA as a standard. The working concentration of DNA was adjusted to 30 ng/µl.

## Microsatellite markers (SSRs)

A total of 242 oligonucleotide primer pairs designed to amplify SSR loci in *M. truncatula* were assayed (Julier et al. 2003; Huguet, unpublished data). Of these 209 were identified in the public EST databases, and some have been mapped in *M. truncatula* recombinant inbred lines (RILs) (Huguet et al. 2001). The remaining 33 are BAC-based microsatellites and do not necessarily correspond to expressed genomic DNA regions. The sequences of these SSRs primer pairs along with the SSR marker name, linkage group in *M. truncatula*, GenBank EST identification number (ID), repetitive motif and cross-amplification with the three legume crops assayed are available as electronic supplementary material.

## PCR analysis

Reaction mixtures of 20 µl contained 10 m *M* Tris-HCl (pH 8), 50 m *M* KCl, EDTA 1 m *M*, 0.1% Triton X-100, 50% (v/v) glycerol, 30 ng of template DNA, 0.4 $\mu M$ of each forward and reverse primer, 1.5 m *M* of each dNTP, 1.5 m *M* $MgCl_2$, and 1 U *Taq* polymerase (Biotools). Amplifications were carried out in a gradient thermocycler (T-Gradient PCR; Biometra) with a 4-min initial denaturation at 94°C, followed by 40 cycles of 94°C for 30 s, with the annealing temperature ranging from 50°C to 57°C (the optimum annealing temperature was determined for each legume crop and primer pair analyzed) for 30 s and an extension at 72°C for 30 s, and a final extension step at 72°C for 6 min before cooling to 4°C. The standard annealing temperature for the *M. truncatula* SSRs was determined to be 55°C (Huguet, unpublished data). The optimum annealing temperature for the three pulse crops was determined to be the highest one that amplified a fragment of the approximate size of the corresponding locus in *M. truncatula*.

Amplified products were separated on 3.5% agarose gels. Electrophoresis was performed at 90 V and maximum amperage for 3 h. Gels were stained with ethidium bromide and photographed under UV light using the software KODAK DIGITAL SCIENCE 1D VER. 2.0 AND 3.5. Results were confirmed in replicated assays. Samples that produced complex banding patterns under the above conditions were reamplified by a touchdown protocol for greater stringency. The touchdown protocol was carried out on a GeneAmp PCR System 9700 (Applied Biosystem, Foster City, Calif.). The thermal profile varied from the previous PCR program in that the annealing temperature used in the first cycle was 59°C. This temperature was then dropped by 0.5°C every cycle until a temperature 3°C below the one used for *M. truncatula* PCR amplification was reached (i.e. from 59°C to 52°C). The remaining 26 cycles were performed at the latter temperature.

## Analysis of amplification products

All *M. truncatula* SSRs were 300 bp or smaller, and most had length variants between 100 bp and 200 bp (Huguet, unpublished data). Amplification products from legume crops were classified into several groups according to their banding pattern and staining intensity. Amplified bands with a size similar to that of the corresponding *M. truncatula* SSR displaying strong, moderate or light staining intensity were classified into groups I, II or III, respectively. SSRs displaying a weak signal and complex patterns even under the touchdown protocol were placed into group IV.

## DNA sequencing

To confirm the amplification of the expected *M. truncatula* microsatellites, we sequenced several amplified PCR products with the corresponding forward and reverse microsatellite primers in one individual per species. Selection was based on consistent amplification and staining intensity of the bands within the expected size range in each of the three legume crops analyzed. PCR reactions were performed as described above, and amplification products were assessed by electrophoresis on 2% agarose gels in TBE for 2 h at 90 V and staining with ethidium bromide. Specific DNA bands were excised from the gel and purified by using the QIAquick Gel Extraction kit (Qiagen, Valencia, Calif.). Sequencing was performed using a BigDye terminator cycle sequencing v 3.1 kit (PE Biosystems, Foster City, Calif.) on an ABI Prism 3100 Genetic Analyzer apparatus (Applied Biosystems) at the Servicio de Secuenciación Automática de DNA, SCAI (University of Córdoba, Spain).

## Results and discussion

### Microsatellite cross-species amplification

This study was carried out to determine the transferability of *M. truncatula* microsatellites to the three most important European legume pulses, pea, faba bean and chickpea. In the initial assays, we identified the primer pairs giving amplification in these three species using *M. truncatula* cv. Jemalong as a control.

Of the 242 microsatellites assayed, 97 (40%) gave consistent cross-amplification in faba bean, 88 (36.3%) in chickpea and 91 (37.6%) in pea (Table 1). Amplicons for each of these primer pairs were classified into three groups based on their reproducibility and staining intensity. Consequently, of the respective SSRs showing cross-amplification, 38 in faba bean, 44 in chickpea and 36 in pea displayed strong amplification products similar to those found in *M. truncatula* (group I) . The number of products with a moderate signal compared with the source species (group II) were 28 (faba bean), 22 (chickpea) and 24 (pea), while the number of SSRs with a light staining intensity (groups III) were 31, 22 and 31, respectively. In addition, several primer pairs displayed complex banding patterns with several minor bands showing a weak signal (group IV). Modifications in the annealing temperature and $MgCl_2$ concentration did not improve the amplification outcome in this latter group, which included 47 SSRs in faba bean, 44 in chickpea, and 22 in pea. These primer pairs were considered to be negative with respect to SSR amplification and were omitted from further analysis. Finally, group V included those primers that did not amplify in any of the crops tested (Table 1).

In most positive primer pair amplifications (groups I, II and III), a single PCR fragment similar in size to *M. truncatula* SSRs was predominant. However, multiple products were sometimes observed, mostly for EST-SSR primer pairs (Table 1). Given that SSRs are considered to be locus-specific markers, this outcome might seem unexpected since a single band or co-migrating twin bands are generally predicted. Nevertheless, the appearance of multiple bands among EST-SSRs has been previously reported in different cereal species as the result of amplification of more than one homoeolocus by each EST-SSR (Holton et al. 2002; Gupta et al. 2003; Saha et al. 2004). Contrary to this, when SSRs derived from *M. truncatula* genomic DNA were assayed in our study, no multiple loci were detected, a result which is in agreement with those of previous investigations (Gupta et al. 2003). With the aim of assessing the identity of these secondary products, we sequenced several of the additional bands displaying intense amplification, but none of these shared homology with known *M. truncatula* sequences (data not shown). This result underscores the importance of verifying the sequence homology of fragments amplified with heterologous primers before drawing inferences concerning mapping or evolutionary relationships.

Fifty-eight of the functional SSRs (46%), yielded fragments of a similar size in the three legume species tested, 34 (27%) showed transferability in two of the crops, and 34 (27%) were successfully amplified in only one of the tested species (see S1 in the electronic supplementary material). The amplified fragments of the target species were usually weaker than those of the donor species.

The use of SSRs based on ESTs greatly increased the success rate of cross-amplification among crops (Table 2). The percentage of *M. truncatula* EST-SSRs amplified in faba bean, chickpea and pea was twofold higher (39–43%) than that of genomic SSRs (21–24%). These results are in agreement with those obtained in previous studies that reported higher transfer rates and lower levels of polymorphisms with EST-SSRs than with genomic SSRs (Cho et al. 2000; Eujayl et al. 2002; Holton et al. 2002; Liewlaksaneeyanawin et al. 2004), the latter being frequently more polymorphic but not as easily transferable to related species (Röder et al. 1995; Sourdille et al. 2001). The 55 *M. truncatula* ESTs-SSRs that cross-amplified in the three legume species had no specific characteristics. They belonged to different functional classes, including signal transduction, post-translational regulation, primary metabolism, gene expression or RNA metabolism, and approximately one-half of them have an unknown function. Twenty-one different SSR motifs were found in the 55 ESTs, and these were equally distributed among the 5′-untranslated regions (UTRs), the coding sequences and the 3′-UTRs. Forty-eight of these ESTs have been mapped in *M. truncatula* and are distributed on all eight of the

**Table 1** Amplification of *Medicago truncatula* microsatellites in faba bean, chickpea and pea

| Group | Amplification[a] | *Vicia faba* | *Cicer sp.* | *Pisum sativum* |
|---|---|---|---|---|
| I | + + + | 38 | 44 | 36 |
| II | + + | 28 | 22 | 24 |
| III | + | 31 | 22 | 31 |
| IV | I | 47 | 44 | 22 |
| V | - | 98 | 110 | 129 |
| Total | | 242 | 242 | 242 |

[a]Strong, moderate and weak amplification patterns are indicated as + + +, + + and +, respectively. I, Complex and unspecific pattern; -, no amplification

**Table 2** Number and percentage (%) of *M. truncatula* SSRs amplified in faba bean, chickpea and pea on the basis of their origin (EST or BAC)

| | 209 *M. truncatula* EST-SSRs | 33 *M. truncatula* BAC-SSRs |
|---|---|---|
| *Vicia faba* | 89 (43%) | 8 (24%) |
| *Cicer sp.* | 81 (39%) | 7 (21%) |
| *Pisum sativum* | 84 (40%) | 7(21%) |

**Table 3** Number of *M. truncatula* SSRs motifs[a] in faba bean, chickpea and pea and BLAST scores (E-values)[a] against the corresponding nucleotide sequences of the donor species

| SSR primer | Motif | *Vicia faba* | | *Cicer sp.* | | *Pisum sativum* | |
|---|---|---|---|---|---|---|---|
| | | Number of motifs | E-value | Number of motifs | E value | Number of motifs | E-value |
| MTIC62 | $(AT)_{10}$ | $(AT)_2$ | 0.83 | n.a. | n.a. | n.a. | n.a. |
| MTIC82 | $(TC)_{11}$ | $(TC)_2$ | 2.8 | n.a. | n.a. | n.a. | n.a. |
| MTIC221 | $(ATT)_5$ | $(ATT)_3/(ATT)_2$ | 0.018 | n.a. | n.a. | $(ATT)_4$ | 1.1 |
| MTIC235 | $(TAA)_5$ | - | 6.00E-04 | $(TAA)_8$ | 1.00E-11 | $(TAA)_3$ | 2.00E-04 |
| MTIC236 | $(ATT)_8$ | $(ATT)_2$ | 0.02 | $(ATT)_3$ | 3.00E-05 | n.a. | n.a. |
| MTIC258 | $(CAT)_5$ | - | 1.00E-08 | - | 0.55 | - | 0.53 |
| MTIC268 | $(CAT)_9$ | - | 1.0 | - | 0.038 | n.a. | n.a. |
| MTIC279 | $(TGC)_5$ | $(TGC)_2$ | 1.00E-07 | $(TGC)_2$ | 8.00E-17 | $(TGC)_3$-ATAGA-$(TGC)_2$ | 0.67 |
| MTIC327 | $(CTT)_5$ | (CTT)-C-(CTT) | 0.21 | n.a. | n.a. | n.a. | n.a. |
| MTIC333 | $(CTT)_6$ | n.a. | n.a. | n.a. | n.a. | - | 0.52 |
| MTIC339 | $(GAA)_5$ | $(GAA)_2$ | 3.00E-27 | $(GAA)_1$ | 2.00E-18 | (GAA)-AGT-$(GAA)_2$ | 4.00E-28 |

[a] –, No conservation; n.a., Not analysed

chromosomes, with no evidence of clustering or preferential location with respect to centromeres (Huguet, unpublished results).

Rossetto (2001) collected and summarized information from the major published studies on cross-transferability of plant microsatellites and reported a success rate of 35%. In the present study, the mean percentage of successful transferability was comparable in the three crops tested and ranged from 36% to 40%. The transferability of legume SSRs has been examined across several major pulse crops and has revealed different levels of sequence conservation. Pandian et al. (2000) reported that approximately 53% of the field pea SSR markers tested amplified across lentil, vetch and chickpea accessions, whereas for chickpea SSR primers the mean rate of successful transferability to lentil, vetch and pea was remarkably lower (9%). Similarly, Peakall et al. (1998) described up to 65% cross-amplification within the genus *Glycine* and significantly lower rates (3–13%) outside the genus, a result that is in agreement with the general trend of decreasing amplification with increasing evolutionary distances from the source species (Butcher et al. 2000). Eujayl et al. (2004) reported a higher level of transferability (89%) of EST-SSR markers across six species within *Medicago*.

Our results did not reveal significant differences in the transferability of *M. truncatula* microsatellites among the three pulses. This is likely due to the high conservation of the primer binding sites in the coding regions assayed in the present study. Analysis of microsynteny in *Arabidopsis* showed that the structure and spacing of genes are largely conserved between genomes, even though the sequences of non-coding regions (i.e. introns and intergenic sequences) are hardly conserved at all (Barnes, 2002). Although this colinearity can be seen most clearly in short sequences, it can also be found using dense genetic maps that are based on ESTs. Initial studies with legumes have also documented high degrees of macrosynteny between the genomes of pea, alfalfa and *Medicago* (Frugoli et al. 1999) or between those of alfalfa and *Medicago* (Choi et al. 2004) as well as significant levels of macro- and microsynteny among legume genomes and model species such as *M. truncatula* and *A. thaliana*, (Grant et al. 2000; Lee et al. 2001; Marek et al. 2001; Foster-Hartnett 2002; Yan et al. 2003). One major application of defined colinearity is the

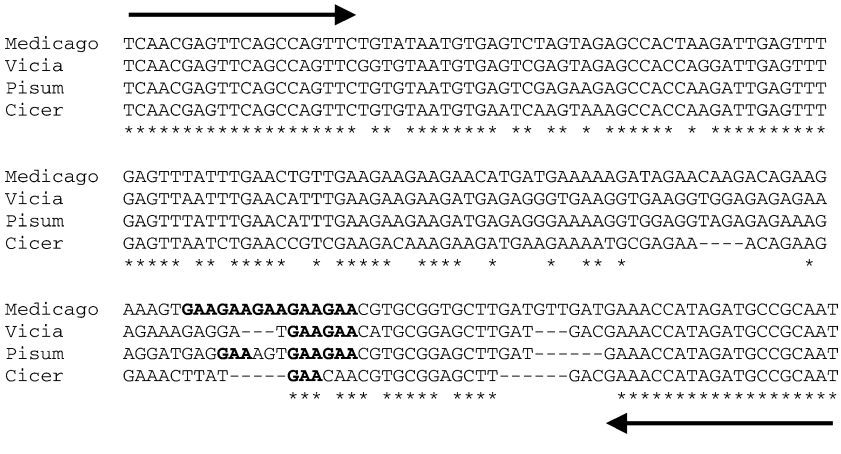


**Fig. 1** CLUSTALX alignment of sequences obtained from PCR bands amplified by the *Medicago truncatula* primer pair MTIC 339 in *Vicia faba*, *Pisum sativum* and *Cicer arietinum*. Alignments include primer sequences (*arrows*). Repetitive sequences are indicated in *bold*. Conserved nucleotide positions are marked by *asterisks*

possibility of predicting the positions of QTLs across species boundaries or of identifying candidates genes responsible for a specific trait.

None of the functional microsatellites tested showed a direct size polymorphism among the parental genotypes tested, thereby preventing their immediate use for mapping purposes. In case of EST-SSRs, this outcome may be due to selection against mutations in these conserved sequences (Scott et al. 2000) or to the reduced number of nucleotide motif repeats. Additional reasons might be the limited number of parental lines assayed for each of the legume crops or the need for using higher resolution systems, such as polyacrylamide gel electrophoresis (PAGE). To verify this hypothesis, we re-examined some of the SSRs using polyacrylamide denaturing gels. In all of the cases tested, the lack of size polymorphism was confirmed by this technique, thereby supporting the premise that the EST-SSR markers are of limited use for mapping purposes.

## DNA sequencing

In general, all of the primers amplified genomic regions of the expected size in the majority of crops tested. This can be explained by the conservation of the sequence repeats and flanking regions in the legume species assayed. To confirm the identity of the amplified fragments and to explore the reasons of this lack of polymorphism, we sequenced the amplification products of 11 SSRs (Table 3) and then compared these with the nucleotide sequences in the EST databases using the BLASTN program to locate regions of similarity with the donor species *M. truncatula*.

All 11 sequences displayed significant homology with the corresponding *Medicago* EST from which the primers were originally derived (Table 3). Detailed sequence analysis determined that the level of conservation in the microsatellite motif was low, while the flanking regions were generally well conserved in all three crop species (Fig. 1). The variations in the sequences were mainly due to changes in the number of repeat motifs in the microsatellite region combined with indel and base substitutions. The success of heterologous amplification was therefore confirmed, although none of the sequenced products contained the original number of tandem repeats. Thus, as observed in previous studies (Peakall et al. 1998; Rosetto et al. 2000; Saha et al. 2004), size similarity with the *M. truncatula* SSRs was not a good predictor of whether the product actually contained the original repetitive region.

The lack of polymorphism of *M. truncatula* EST-SSRs observed in the present study indicates that they may have a limited utility for mapping purposes in other legume species. One possible strategy to overcome this limitation would be to search for SNPs (single nucleotide polymorphisms) within the EST sequences from which the SSRs were derived. This approach should significantly increase the probability of detecting polymorphisms, especially if the entire EST sequence was taken into account as opposed to only the SSR region.

Mapping a large number of EST may be highly useful because genes of known function could be related to phenotypic variations and QTLs. Progenies derived from the parental lines assayed in this study have been used to detect genes and QTLs for important agronomic traits such as seed weight and broomrape, *Fusarium*, *Uromyces* and *Ascochyta* resistance (Roman et al. 2002; 2003; Avila et al. 2003, 2004; Millán et al. 2003; Rubio et al. 2003; Valderrama et al. 2004). The increasing number of gene/QTL studies among legumes raises the need for standard markers for producing consensus maps from different progenies and/or different species.

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