DEVELOPMENT AND APPLICATION OF SEQUENCE-TAGGED MICROSATELLITE SITE (STMS) MARKERS IN CHICKPEA (*CICER ARIETINUM*), BANANA (*MUSA* SPP.) AND THEIR MAJOR PATHOGENS, *ASCOCHYTA RABIEI* AND *MYCOSPHAERELLA FIJIENSIS*

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Abstract

DNA markers of various kinds have found widespread application in many facets of plant breeding and plant pathogen control. Yet another marker type, sequence-tagged microsatellite (STMS) markers, provides the markers of choice for nearly every crop because of their co-dominant nature, reliability, ease of application and high polymorphic information content. We report here on the development of a whole set of STMS markers and the respective, selected primer sequences for two important crops, chickpea (*Cicer arietinum* L.) and banana (*Musa acuminata*), and for their most devastating fungal pathogens, *Ascochyta rabiei* and *Mycosphaerella fijiensis*, respectively. These markers were generated either by direct screening of size-selected genomic libraries with microsatellite-complementary oligonucleotides, or by enrichment of DNA fragments containing microsatellite sequences. A total of 69 markers for chickpea, 15 markers for *M. acuminata*, 19 markers for *A rabiei* and 11 markers for *M. fijiensis*, selected on the basis of their high information content and ease of use are presented here. These can be applied for mapping of the respective genomes, for various population studies, and cultivar and isolate identification. We further demonstrate that several of these markers can potentially be applied across species boundaries and thus could increase the marker repertoire also for other species of the genus *Cicer*, *Musa* and for *Ascochyta*-type pathogens of bean, and potentially also of lentil and pea.

1. INTRODUCTION

In recent years, plant breeding has profited enormously from the advent of molecular marker technologies. Molecular markers, and especially those based on selectively neutral DNA polymorphisms, facilitate the reliable identification of breeding lines, cultivars, clones and hybrids, allow the monitoring of introgression of alien DNA into cultivated germplasm, and the estimation of genetic diversity. Moreover, advanced high-density DNA marker maps are now available for most important crops, providing a basis for marker-assisted selection of useful traits, pyramiding of resistance genes, and the isolation of these and other agronomically important genes via map-based cloning (reviews in [1, 2]).

Set their importance for genome analysis of plants aside, molecular markers also catalysed the characterisation of plant pathogens. The unequivocal identification of pathogenic fungi, for example, which in former times required a skilled pathologist, can now be performed routinely. Molecular markers allow to rapidly and reliably identify fungal races and pathotypes, and to monitor their population structure [3, 4].

The requirement for more and more easily applicable markers has led to the development of a plethora of different marker techniques (review in [2]). A particular type of markers, i.e. those based on microsatellite variability has gained popularity in the past years, thanks to the many advantages it provides for research and applications. A special type of microsatellite markers are the subject of this article.

The term "microsatellite" [5], also *s*imple *s*equence *r*epeat (SSR), or *s*hort *t*andem *r*epeat (STR) characterises short, reiterated sequence motifs of about 1 to 6 nucleotides that, like classical satellite DNA, are organised in more or less perfect tandem arrays of a few up to even thousands of repeat units. The key feature of SSR repeats that makes them the preferred target for marker development is a strong tendency to change their overall length by slipped-strand mispairing and other less well understood processes (reviews in [6, 7]) leading to *v*ariable *n*umbers of *t*andem *r*epeats (VNTR) [8] and resulting in *s*imple *s*equence *l*ength *p*olymorphisms (SSLPs) [9].

Mutation rates of SSRs are generally high. Consequently, up to 30 alleles were reported for particular loci in plants [10, 11]. Also in fungi, hypervariable SSRs have been observed [12]. However, microsatellite mutation rates vary considerably among loci and organisms. STRs are abundant and usually more or less evenly dispersed throughout eucaryotic genomes but appear to be less frequent in plants as compared to vertebrates [13]. Reported estimates of microsatellite frequencies vary considerably, with average inter-SSR distances ranging from 10 kb to more than 1 Mb, depending on the motif and the organism. For example, in the chickpea genome $[TAA]_n$, $[GA]_n$ and $[CA]_n$ arrays are present at more than 12 000 loci with an average spacing of around 60 kb [14].

In contrast to plants, studies of microsatellite sequences in fungi are scarce [12, 15–18], as are reports on the systematic development and application of markers from microsatellite sequences of these lower eucaryotes [19–22].

1.1. Amplification of single microsatellite loci: STMS markers

Currently the most popular method to exploit SSR variability for the generation of genetic markers uses primers targeted to SSR-flanking sequences to amplify the enclosed SSR. The results are locus-specific amplification products that often exhibit considerable length differences among different individuals or populations of the same species, mostly due to the variable number of tandem repeats within the SSR. These sequence-tagged microsatellite site (STMS) [23] markers are the markers of choice for nearly every organism. They represent single-locus, co-dominant, easy-to-use and reliable markers with high polymorphic information content possessing the potential for automated, non-radioactive detection. Moreover, besides the availability of standard instrumentation (thermocycler and gel apparatus) the knowledge of STMS primer sequences is the only prerequisite for their application. Since these can easily be exchanged between laboratories, STMS markers can be applied by any interested lab without time-consuming preparation and mailing of probes [24, 25].

In spite of the many advantages of STMS, they also have some limitations, which have precluded their widespread application for a while. Main obstacles for an extensive use are the high costs for cloning, sequencing and primer synthesis. Further, standard protocols still use radioisotopes and sequencing gels to detect the amplified SSRs. Finally, the efficiency of primer generation suffers from a number of problems including redundancy of clones and the occurrence of artificial chimeras (recent reviews in [24, 25]).

However, in recent years, the advent of microsatellite enrichment techniques (reviews in [24, 25]) and dropping costs for DNA sequencing and primer synthesis reduced the necessary investment for the generation of large numbers of STMS markers opening an avenue also for application in more orphan species, such as the tropical tree *Simarouba* [26].

Here, we describe the development and sequences of STMS primer pairs for chickpea and banana as well as their major fungal pathogens *Ascochyta rabiei* and *Mycospaerella fijiensis*, respectively.

2. DEVELOPMENT OF STMS MARKERS FOR CHICKPEA, BANANA, *A. rabiei* AND *M. fijiensis*

2.1. STMS markers for chickpea (*Cicer arietinum* L.)

2.1.1. Isolation of microsatellite-containing clones

The STMS primer pairs described here were derived from a size-selected genomic library of 280 000 colonies that represented ~18% of the chickpea genome and was screened for $(GA)_n$, $(GAA)_n$ and $(TAA)_n$ micosatellite-containing clones. The plasmids of 389 positive colonies were sequenced. The majority (~75%) contained perfect repeats. Interrupted, interrupted-compound and compound repeats were only present from 6 to 9%. Microsatellites of the (TAA)-type contained the longest repeats with unit numbers ranging from 9 to 131. For 218 loci, primers could be designed and used for the detection of microsatellite length polymorphisms in 6 chickpea cultivars, *C. echinospermum and C. reticulatum* (chickpea's closest relatives). Altogether, 174 primer pairs gave interpretable banding patterns, 137 (79%) of which revealed at least 2 alleles on native polyacrylamide gels. Genetic mapping of 120 of these STMS in a population of recombinant inbred lines from an inter-species cross between *C. reticulatum* and the cultivated chickpea line ICC 4958 is described in [27]. A more comprehensive map, spanning around 2000 cM is reported in a forthcoming paper [28]. The table given below contains only those STMS markers that revealed at least 4 alleles. More STMS primer pairs for chickpea can be found in [14, 27].

2.1.2. Detection of microsatellite polymorphisms in chickpea: PCR conditions and gel electrophoresis

Primers were 22 to 32 nucleotides long allowing annealing temperatures of 55°C. Thirty-five cycles of PCR were performed on 50 ng DNA in 20 µl "Silverstar" reaction buffer containing 2 µM primers, 1.5 mM $MgCl_2$, 250 mM nucleotides and 0.4 U "Silverstar" polymerase (Eurogentec, Belgium). The DNA was first denatured for 2 min at 94°C, annealing was at 55°C for 50 sec, and elongation at 60 °C (TAA-repeats) or 72°C (GA and GAA-repeats) for 50 sec. The reduced elongation temperature for TAA-repeats (as compared to the optimal 72°C) was necessary to stabilise the long (TAA)-microsatellites. Between 4 to 12 µl of the reaction mix were separated either on 2% agarose gels for a first analysis of amplification success, or on 8% native polyacrylamide gels for determination of allele length and numbers.

For those familiar with Spanish language, detailed protocols for PCR with STMS primer pairs and gel electrophoresis of amplification products can be found in [29].

TABLE 1. HIGHLY POLYMORPHIC STMS MARKERS FOR CHICKPEA

Locus	Repeat Type	Primer Sequence (5`-3`)	Expected Product (bp)
TA1	P	L TGAAATATGGAATGATTACTGAGTGAC	243
		R TATTGAAATAGGTCAGGCTTATAAAAA	
TA2	I	L AAATGGAAGAAGAATAAAAACGAAAC	175
		R TTCCATTCTTTATTATCCATATCACTACA	
TA5	P	L ATCATTTCAATTTCCTCAACTATGAAT	205
		R TCGTTAACACGTAATTTCAAGTAAAGAT	
TA8	P	L AAAATTTGCACCCACAAAATATG	246
		R CTGAAAATTATGGCAGGGAAAC	
TA11	P	L CATGCCATAAACTCAATACAATACAAC	230
		R TTCATTGAGGACAATGTGTAATTTAAG	
TA13	P	L TAAGTTAAGGGACCAACGAA	243
		R CAAGTTGGAGTCAAACCAAT	
TA14	I	L TGACTTGCTATTTAGGGAACA	250
		RTGGCTAAAGACAATTAAAGTT	
TA18	P	L AAAATAATCTCCACTTCACAAATTTTC	147
		R ATAAGTGCGTTATTAGTTTGGTCTTGT	
TA20	I	L ATTTTCTTTATCCGCTGCAAAT	299
		R TTAAATACTGCCTTCGATCCGT	
TA21	P	L GTACCTCGAAGATGTAGCCGATA	347
		R TTTTCCATTTAGAGTAGGATCTTCTTG	
TA25	P	L AGTTTAATTGGCTGGTTCTAAGATAAC	247
		R AGGATGATCTTTAATAAATCAGAATGA	
TA27	P	L GATAAAATCATTATTGGGTGTCCTTT	241
		R TTCAAATAATCTTTCATCAGTCAAATG	
TA28	I	L TAATTGATCATACTCTCACTATCTGCC	300
		R TGGGAATGAATATATTTTTGAAGTAAA	
TA34	P	L AAGAGTTGTTCCCTTTCTTTT	230
		R CCATTATCATTCTTGTTTTCAA	
TA37	P	L ACTTACATGAATTATCTTTCTTGGTCC	282
		R CGTATTCAAATAATCTTTCATCAGTCA	
TA39	P	L TTAGCGTGGCTAACTTTATTTGC	249
		R ATAAATATCCAATTCTGGTAGTTGACG	
TA42	C	L ATATCGAAATAAATAACAACAGGATGG	209
		R TAGTTGATACTTGGATGATAACCAAAA	
TA43	P	L GGTTGTGTTCTCCAGATTTT	183
		R AAGAGTTGTTGGAGAGCAA	
TA44	P	L ACCGAAATGGAAACAAATAA	193
		R ACAAAACTGGGGGACTAAAT	
TA45	C	L ATGCGTATAAAACCCAGAGA	190
		R TGTTTTTATTGGATTTTCAGTTTCA	
TA53	P	L GGAGAAAATGGTAGTTTAAAGAGTACTAA	249
		R AAAAATATGAAGACTAACTTTGCATTTA	
TA59	P	L ATCTAAAGAGAAATCAAAATTGTCGAA	258
		R GCAAATGTGAAGCATGTATAGATAAAG	
TA64	P	L ATATATCGTAACTCATTAATCATCCGC	239
		R AAATTGTTGTCATCAAATGGAAAATA	
TA71	P	L CGATTTAACACAAAACACAAA	225
		R CCTATCCATTGTCATCTCGT	

TABLE 1. (cont.)

Locus	Repeat Type	Primer Sequence (5`-3`)	Expected Product (bp)
TA72	P	L GAAAGATTTAAAAGATTTTCCACGTTA	256
		R TTAGAAGCATATTGTTGGGATAAGAGT	
TA76s.	I	L TCCTCTTCTTCGATATCATCA	206
		R CCATTCTATCTTTGGTGCTT	
TA78	P	L CGGTAAATAAGTTTCCCTCC	205
		R CATCGTGAATATTGAAGGGT	
TA80	P	L CGAATTTTTACATCCGTAATG	211
		R AATCAATCCATTTTGCATTC	
TA89	I	L ATCCTTCACGCTTATTTAGTTTTTACA	233
		R CAAGTAAAAGAGTCACTAGACCTCACA	
TA93	P	L TTTCTCACACAAATAACAAATTAACTGA	192
		R TCAACATTAATTAACTACTATGATCTGTCA	
TA96	C	L TGTTTTGGAGAAGAGTGATTC	275
		R TGTGCATGCAAATTCTTACT	
TA103	P	L TGAAATATCTAATGTTGCAATTAGGAC	184
		R TATGGATCACATCAAAGAAATAAAAT	
TA106	P	L CGGATGGACTCAACTTTATC	248
		R TGTCTGCATGTTGATCTGTT	
TA110	P	L ACACTATAGGTATAGGCATTTAGGCAA	220
		R TTCTTTATAAATATCAGACCGGAAAGA	
TA113	P	L TCTGCAAAAACTATTACGTTAATACCA	203
		R TTGTGTGTAATGGATTGAGTATCTCTT	
TA114	P	L TCCATNTAGAGTAGGATNTTNTTGGA	298
		R TGATACATGAGTTATTCAAGACCCTAA	
TA116	I	L AATTCAATGACGAATTTTTATAAGGG	182
		R AAAAAGAAAAGGGAAAAGTAGGTTTTA	
TA117	P	L GAAAATCCCAAATTTTTCTTCTTCT	248
		R AACCTTATTTAAGAATATGAGAAACACA	
TA125	P	L TTGAAATTGAACTGTAACAGAACATAAA	235
		R TAGATAGGTGATCACAAGAAGAGAATG	
TA130	P	L TCTTTCTTTGCTTCCAATGT	219
		R GTAAATCCCACGAGAAATCAA	
TA135	P	L TGGTTGGAAATTGATGTTTT	192
		R GTGGTGTGAGCATAATTCAA	
TA140	IC	L TTTTGGCATGTTGTAGTAATCATATTT	180
		R TGAAATGAAAAAGAAAAGGAAAAAGTA	
TA142	P	L TGTTAACATTCCCTAATATCAATAACTT	135
		R TTCCACAATGTTGTATGTTTTGTAAG	
TA144	P	L TATTTTAATCCGGTGAATATTACCTTT	241
		R GTGGAGTCACTATCAACAATCATACAT	
TA146	P	L CTAAGTTTAATATGTTAGTCCTTAAATTAT	161
		R ACGAACGCAACATTAATTTTATATT	
TA180	P	L CATCGTGAATATTGAAGGGT	205
		R CGGTAAATAAGTTTCCCTCC	
TA194	P	L TTTTTGGCTTATTAGACTGACTT	132
		R TTGCCATAAAATACAAAATCC	

TABLE 1. (cont.)

Locus	Repeat Type	Primer Sequence (5`-3`)	Expected Product (bp)
TR1	P	L CGTATGATTTTGCCGTCTAT	224
		R ACCTCAAGTTCTCCGAAAGT	
TR3	P	L GAAGTATCAGTATCACGTGTAATTCGT	244
		R CTTACGGAGAACATGAACATCAA	
TR7	P	L GCATTATTCACCATTTGGAT	204
		R TGTGATAATTTTCTAAGTGTTTT	
TR19	P	L TCAGTATCACGTGTAATTCGT	227
		R CATGAACATCAAGTTCTCCA	
TR20	P	L ACCTGCTTGTTTAGCACAAT	172
		R CCGCATAGCAATTTATCTTC	
TR26	P	L TCATCGCAGATGATGTAGAA	215
		R TTGAACCTCAAGTTCTCTGG	
TR29	I	L GCCCACTGAAAAATAAAAAG	220
		R ATTTGAACCTCAAGTTCTCG	
TR44	P	L TTAATATTCAAAAACTCTCTTGTGCAAT	289
		R TTTACAACAGCGCTTGTATTTAGTAAG	
TR56	P	L TTGATTCTCTCACGTGTAATTC	248
		R ATTTTGATTACCGTTGTGGT	
TR59	C	L AAAAGGAACCTCAAGTGACA	174
		R GAAAATGAGGGAGTGAGATG	
TR60	P	L TGAGTCAAAACAAAGAACTTG	250
		R CTACCGGAAATTTCATTGAC	
TS29	P	L AACATTCATGAACCTACCTCAACTTA	342
		R CCATATATGAGTACACTACCTCTCGG	
TS43	P	L AAGTTTGGTCATAACACACATTCAATA	212
		R TAAATTCACAAACTCAATTTATTGGC	
TS53	P	L GATCNTTCCAAAAGTTCATTTNTATAAT	267
		R TTAAAGAACTGATACATTCCGATTATTT	
TS57	IC	L TCAATTTATAATCATAGAGAATCNGAGA	321
		R CCTAAAACAAATAAAATCTTAAATAATA	
TS104	P	L TCAAGATTGATATTGATTAGATAAAAGC	214
		R CTTTATTTACCACTTGCACAACACTAA	
GA 6	P	L ATTTTTCTCCGGTGTTGCAC	221
		R AAACGACAGAGAGTGGCGAT	
GA 16	P	L CACCTCGTACCATGGTTTCTG	247
		R TAAATTTCATCCTCTCCGGC	
GA 26	P	L GATGCTCAAGACATCTGCCA	234
		R TCATACTCAACAAATTCATTTCCC	
GA 34	P	L CCTTTGCATGTATGTGGCAT	133
		R CCGTTTATAAAGGATGTAZGAGAC	

Listed in the Table are only those markers that reveal at least 4 alleles in 6 chickpea accessions and *C. reticulatum* and *C. echinospermum* on native polyacrylamide gels. Additional primer sequences can be found in [14,27]. The locus name and the sequences of the left (L) and right (R) primers are given. It is further indicated whether the microsatellite is of the perfect (P), interrupted (I), compound (C) or interrupted compound (IC) type. The expected sizes of amplification products in chickpea accession ILC 3279, from which the clones were derived, are given in bp. A more detailed description of the microsatellites and position of the respective genomic localisation for many of them can be found in [27].

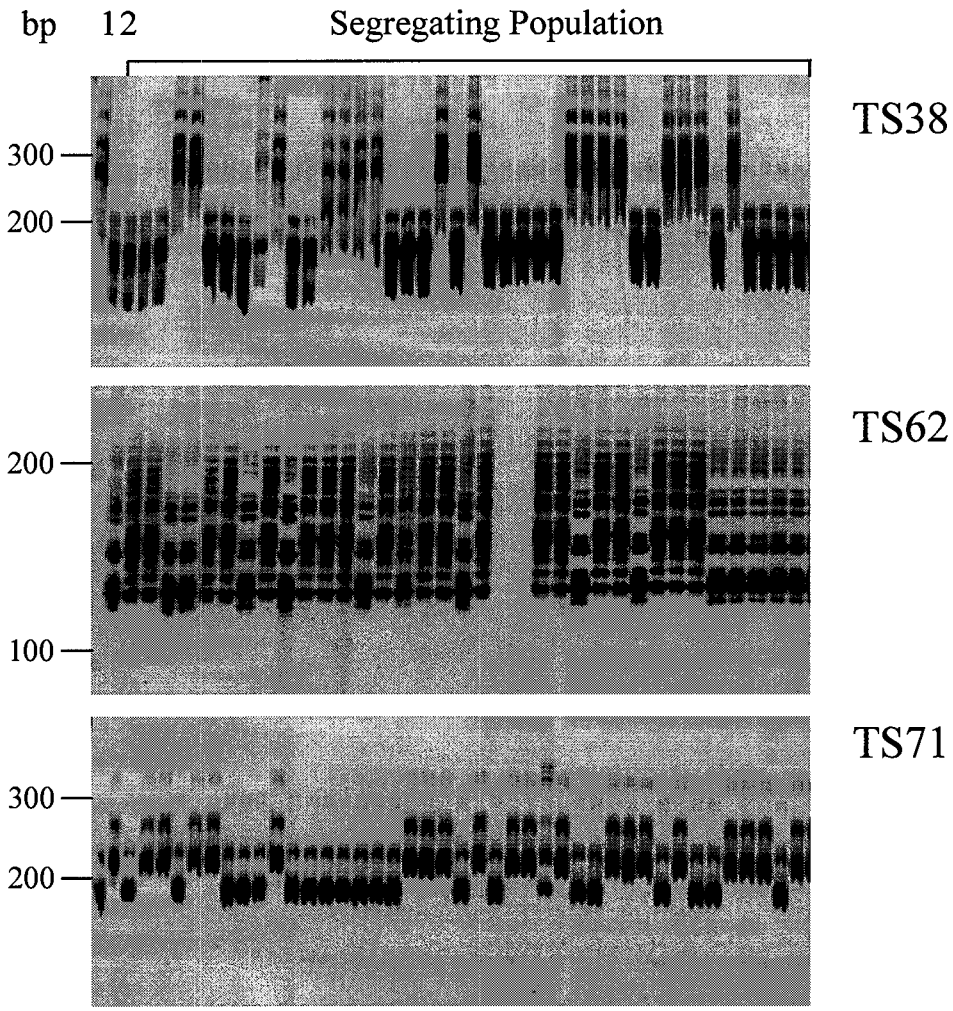

Fig. 1. Mapping of STMS markers in chickpea. Mapping of STMS marker loci TS38, TS62 and TS71 in recombinant inbred lines from a wide cross between a cultivated chickpea accession (ICC4959) and an accession of C. reticulatum (P.I. 498777, slot 2), chickpeas wild, intercrossable relative. Nondenaturing 8% polyacrylamide gel after staining with ethidium bromide (courtesy of Ruth Jungmann, Frankfurt). Numbers on the left indicate molecular weights in base pares. Parental lines ICC 4958 and P.I. 498777 are loaded in slots 1 and 2, respectively. Note, that in all cases several bands are visible that segregate the same way and thus are derived from the same locus

2.2. STMS markers for banana and plantain

2.2.1. Isolation of microsatellite-containing clones

CsCl-gradient purified DNA was isolated from leaf tissue of *M. acuminata* ssp. *malaccensis* [30]. The DNA was restricted with *TaqI*, separated in low melting point agarose gels and fragments in the size range of 300 to 600 bp were eluted from the gel. These were then cloned into the *ClaI* site of pGEM-7Zf (Promega), approximately 8000 clones were plated on Petri dishes, lifted onto nitrocellulose membranes and their DNA fixed on the membrane using standard procedures [31]. The membranes were probed with radiolabled synthetic oligonucleotides $(GT)_{11}$, $(AT)_{11}$ and $(CT)_{15}$ for the presence of microsatellite-containing sequences [30]. After several rounds of screening, the inserts of 30 clones were sequenced. Perfect, compound and imperfect repeats were identified. Primers could successfully be designed for the loci shown in Table 2.

TABLE 2. SELECTED STMS PRIMER PAIRS FROM *Musa acuminata* SSP. *malaccensis*

Locus	Repeat Type	Primer Sequence (5′-3′)	Expected Product (bp)
MaSSR 1	P	L TGAGGCGGGGAATCGGTA	126
		R GGCGGGAGACAGATGGAGTT	
MaSSR 5	P	L AGATGGCGGAGGGAAGAG	120
		R CCGGATCCAAGCTTATCGA	
MaSSR 7	P	L AAGAAGGCACGAGGGTAG	212
		R CGAACCAAGTGAAATAGCG	
MaSSR 8	P	L GGAAAACGCGAATGTGTG	250
		R AGCCATATACCGAGCACTTG	
MaSSR 9	IC	L ATGTCGCTTCGGACCAGA	162
		L R GCAGGACGAAGAACTTACC	
MaSSR 10	P	L ATGATCATGAGAGGAATATCT	127
		R TCGCTCTAATCGGATTATCTC	
MaSSR 11	I	L GGTTGGAACGAAGGTATACTAA	270
		R TCCAAGCTTATCGATCTACG	
MaSSR 12	C	L TGTCGAAGCATCCTACATC	262
		R CTTGGAAACATGAGAAACATAC	
MaSSR 14	P	L TTGAAGTGAATCCCAAGTTTG	131
		R AAAACACATGTCCCCATCTC	
MaSSR 15	P	L TGCTCTTCCACATCTCAAGAAC	247
		R GATTGCACGGAGATTCAACA	
MaSSR 16	I	L ATGGTTAGCTCCGCTTGAAT	294
		R GAGGTGGAAACCCAATCATT	
MaSSR 18	P	L ATGGTTAGCTCCGCTTGAAT	179
		GAGGTGGAAACCCAATCATT	
MaSSR 19	I	L CGTCACAGAAGAAAGCACTTG	144
		R AACCCGGATATTTCATTGTA	
MaSSR 20	P	L GAAATGGAGTTGGAGAAACA	222
		R CACATATCCTTGTCGGAAGT	
MaSSR 24	P	L GAGCCCATTAAGCTGAACA	172
		R CCGACAGTCAACATACAATACA	

The locus name and the sequences of the left (L) and right (R) primers are given. It is further indicated whether the microsatellite is of the perfect (P), interrupted (I), compound (C) or interrupted compound (IC) type. The expected sizes of amplification products in *Musa acuminata* spp. *malaccensis*, from which the clones were derived, are given in bp. A more detailed description of the microsatellites and position of the respective genomic localisation for many of them can be found in [30].

2.2.2. Detection of SSLPs in Musa species

PCR started at 94°C for 4 minutes for initial denaturation followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at T_a (T_a depends on the primer pair, usually 55 °C), 30 seconds extension at 72°C and a final extension at 72°C for 10 minutes.

Reaction volume ranged from 10 to 25 µl. Concentration of the genomic DNA template in the PCR reaction mixture was 2 ng/µl. Final concentrations in the reaction solution were 1.5 mM $MgCl_2$, 150 µM dNTP, 0.2 µM of each primer and 0.2 U/10µl of thermostable polymerase. Before analysis, 1:1 volume 'stop mix' (95% formamide, 0.05% xylene cyanole,

0.05% bromophenol blue, 12.5% sucrose, 10mM NaOH) was added to the aliquots. Denaturing was for 15 minutes at 75°C.

In order to promote the exportation of microsatellite markers to developing countries, we adapted a previously described non-radioactive procedure [32], based on silver stained, denaturing polyacrylamide gels (urea-PAGE), to allele length detection. The procedure starts with the separation of PCR products were separated on denaturing 5% polyacrylamide gels (8 M urea, 1 × TBE, 40 to 60 cm long). After electrophoresis, the gels were soaked in 10% acetic acid for 20 minutes, then washed 3 times for 2 minutes in deionized water and stained for 30 minutes in 0.1% $AgNO_3$ containing 0.06% formaldehyde. After a brief (5–15 seconds) rinse in double-distilled water, signals are developed in 500 ml of 3% Na_2CO_3 with 200 µL of a 10% solution of $Na(S_2O_3)_2$ and 1.5 ml of 38% formaldehyde prepared with double distilled water. After bands become visible, gels are soaked in 10% acetic acid to stop development. The gels are then rinsed with deionized water, and might either be kept in 10% glycerol and dried for 2 hours at 80°C on a gel drier, or dried in a fume hood at room temperature for 24 hours.

That way, up to 20 alleles could be detected in a collection of 69 *Musa* accessions including diploid and various polyploid accessions, plantains and cooking bananas, covering most of the *Musaceae* family [30].

2.3. STMS markers for *Ascochyta rabiei*

Ascochyta rabiei (Pass.) Labrousse is a major fungal pathogen of chickpea. The ascomycete causes severe yield losses in Africa, Asia, Australia, Northamerica, and the Mediterranean basin. The sexual stage of its life cycle, the teleomorph *Didymella rabiei* (Kovachevski) v. Arx has been identified and two compatible *Ascochyta rabiei* mating type isolates were deposited at ATCC (*A*merican *T*ype *C*ulture *C*ollection, Rockville, Md., USA) accession numbers 76501 and 76502. Based on virulence to different chickpea cultivars the pathogen was classified into distinct pathotypes. The pathotype of an isolate may be related to fingerprint patterns obtained from in-gel hybridization of short synthetic oligonucleotides containing microsatellite sequences to restricted genomic DNA as has been shown in an extended survey of *A. rabiei* isolates from Pakistan [4].

2.3.1. Development of STMS markers for Ascochyta rabiei

Our research aimed at developing STMS markers for the characterisation and monitoring of *A. rabiei* populations, and for the construction of a genetic linkage map. For this purpose, DNA was isolated from lyophilized mycelia of ATCC76501 as described [3]. Genomic libraries for the isolation of microsatellite-loci were established from DNA digested separately with either *Alu*I, *Rsa*I, *Hae*I or *Sau*IIIA, size selected on agarose gels (fragment sizes between 300 and 750 bp), ligated into the *Eco*RV- and *Bam*HI-site, respectively, of the pBluescript II SK phagemid (Stratagene) and electroporated into *E. coli* SURE cells. Approximately 5.000 clones were plated on selective 2YT medium, and transferred onto nylon filters for colony hybridization. The filters were hybridized to cocktails of radioactively endlabeled, synthetic single-stranded oligonucleotide probes [$(CA)_{10}$ and $(GA)_{10}$ or $(CAA)_{10}$ and $(GAA)_{10}$] complementary to microsatellite sequences. The dinucleotide mix was hybridized at 43°C and the trinucleotide mix at 48°C, using standard procedures [31]. Microsatellite-containing clones were picked and rescreened by hybridizing the same

cocktails. Plasmids of the 50 positive clones were isolated and inserts sequenced. Primers were designed from microsatellite flanking regions as 20- to 23–mers with a GC-content of at least 45% and a T_m optimum of 56°C. Primer design was possible with 38 of 50 sequences, three clones did not carry a microsatellite at all, but other CA- or GA-rich sequences and nine inserts contained microsatellites too close to the cloning site to allow primer design [22].

2.3.2 Detection of SSLPs in four A. rabiei isolates

PCR was performed in 25 µL volumes containing 1.6 mM $MgCl_2$, 0.2mM dNTPs, 5 pmol of each primer and 0.5 units *Taq* DNA polymerase (Eurogentec, Belgium) as well as the provided 10 × buffer and 10 ng of template DNA. After an initial denaturation (95°C, 20 s) PCR was run for 35 cycles (94°C for 20 s, 53°C for 25 s, 65°C for 23 s) followed by a 20 s final extension step at 65°C in a Perkin-Elmer 2400 thermocycler. Samples were separated on 2% agarose gels and stained with ethidium bromide. Under these conditions 31 of 38 primer pairs worked immediately. Decreasing the annealing temperature to 48°C allowed to amplify a product of the expected molecular weight from six of the remaining seven primer pairs, but three primer pairs displayed more than one band. One primer pair yielded no amplicon at all and was excluded without further testing. More than one band was produced by five primer pairs at an annealing temperature of 53°C. An increase to 57°C produced less bands but still at least two loci were amplified. Major products in the expected molecular weight ranges were observed [22].

Here we present several STMS markers from *Ascochyta rabiei* isolate ATCC76501, and the sequences of the flanking primers. We further investigated, whether these primer pairs would also amplify loci from the related bean pathogen *A. fabae*, and thus could be transferred between *Ascochyta* species [22].

2.4. STMS markers for *Mycosphaerella fijiensis*

M. fijiensis DNA was digested with either the 4 bp blunt end cutter *AluI* or *RsaI* followed by ligation of an adapter allowing PCR amplification of the restriction fragments after enrichment. Microsatellite enrichment was performed with biotinylated oligonucleotides complementary to either $(GA)_{10}$ or a mixture of $(CAA)_8/(GAA)_8/(CA)_{10}$. Biotinylated duplex molecules were fished out of the hybridization solution using streptavidine-coated magnetic beads (Dynal®). After PCR amplification a second enrichment round was performed. PCR products of the second amplification were cloned into a pGEMT vector (Promega®). Single white colonies were selected for colony-PCR which uses a vector-derived primer pair to amplify the cloned insert. All amplified inserts were separated in an agarose gel and thereafter blotted onto a nylon membrane. This Southern blot was hybridized with radioactively labelled $(GA)_{10}$ or a mixture of $(CAA)_8/(GAA)_8/(CA)_{10}$. Positive inserts were sequenced by an automatic fluorescence sequencer (Applied Biosystems ABI 3700®) using a vector-derived nested primer. Primer pairs were designed from the microsatellite-flanking unique sequences using the Primer 3 program (www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) [20].

TABLE 3. STMS MARKERS FOR *A. rabiei*

Locus	Primer sequence (5'- 3')	Expected size (bp)	Annealing (°C)	Amplicons in *A. fabae*
*ArA*03T	L TAGGTGGCTAAATCTGTAGG R CAGCAATGGCAACGAGCACG	379	53	2
*ArA*05T	L CGAGGATGAAGAGATTCTCAAG R CACTTCTGATGCTACGCTTAC	162	53	2
*ArA*06T	L CTCGAAACACATTCCTGTGC R GGTAGAAACGACGAATAGGG	162	53	1
*ArA*08T	L CAGAGGGGAATTGTTGTTC R ACGACGAGGATGAGGACTTC	249	53	0
*ArA*12T	L AAAGTCAAGTAGACCTGAATACG R GAGAAATTTGACCAAGTGAGAA	175	48	1
*ArR*01T	L AAGAGTCCAGCGTATCGTTT R GCTGTGTCTGTCTCCATCTC	206	57	1
*ArR*04T	L ACGCGTGGAAGAGTCCAGCG R ATGCTCGACAACCTCTCTGGC	262	57	2
*ArH*01T	L CCAAGCTTGGGGACATGGACA R TCAGTTGGCAGACCGTGGTGGC	274	57	1
*ArH*02T	L CTGTATAGCGTTACTGTGTG R TCCATCCGTCTTGACATCCG	365	53	3
*ArH*05T	L CATTGTGGCATCTGACATCAC R TGGATGGGAGGTTTTTGGTA	197	53	1
*ArH*06T	L CTGTCACAGTAACGACAACG R ATTCCAGAGAGCCTTGATTG	167	53	1
*ArH*07T	L CGACAATAACAACCAGTACGAT R CTATGCTGTCTGCCCTCAGT	248	48	0
*ArS*01T	L GAGAAAGAGAAGCGCTATTAAA R GAAGGTATTTCCCTAGCAGAAGA	166	48	2
*ArS*03T	L ATGGAGAAGTCGAGGTCCAT R CTCTTGCGTGGCCTAGAAGG	152	53	2
*ArR*05D	L GTCTAGTTTGCGGAGAGAGTG R CTAGGATGGACACGTTAAAGC	259	53	1
*ArA*02D	L CTATCACCATGCCTCCATCA R TGTTCCCTTTGAGTTGAAGAG	150	53	0
*ArH*02D	L AGAAAGGGGAGATTCGAGAC R AGGTCAGCACGAGATAGCAC	141	53	1

All STMS markers for *A. rabiei* listed here are of the perfect type. The expected size of amplification products in ATCC76501, from which the DNA was derived is given in base pares. The annealing temperature best suited for the respective locus and the number of alleles in two accessions of *A. fabae* are also indicated. More detailes can be found in [22].

The results of three enriched libraries are shown in Tables 4 and 5. GA repeats seem to be more abundant (27%) as compared to CA repeats (15–20%). Trinucleotide repeats are present in the enriched libraries between 4 and 9 percent. Thirty percent (106) of the white colonies (348) contained a microsatellite. However, the sequences were not always good enough to design primer pairs, because the microsatellite was either too close to the cloning site, or it was too short. Also, 10 duplicates were detected in the libraries. From the 26 designed primer pairs, those described in Table 6 have been selected with respect to the number of polymorphisms they detect in the populations.

TABLE 4. EFFICIENCY OF MICROSATELLITE ENRICHMENT IN 3 INDEPENDENT PARTIAL GENOMIC LIBRARIES OF *M. fijiensis* [33]

Genomic Library	Colonies Selected	Positive Signals	Enrichment Factor
1. library	140	CA : 28	CA : 20%
[Pool, Rsa I]		CAA: 10	CAA: 7%
		GAA: 12	GAA: 9%
Total of Positive Clones		49	35%
2. library	112	CA : 17	CA : 15%
[Pool, Alu I]		CAA: 9	CAA: 8%
		GAA: 5	GAA: 4%
Total of Positive Clones		31	28%
3. library	96	26	27%
[GA, Alu I]			
Total of Positive Clones		26	27%
Total of Three Libraries	348	106	30%

TABLE 5. NUMBERS OF SEQUENCES, DUPLICATES AND PRIMER PAIRS FROM 3 INDEPENDENT GENOMIC LIBRARIES OF *M. fijiensis* [33]

Genomic Library	Positive Colonies	Sequences	Duplicates	Primer Pairs
1. Library [pool, *Rsa I*]	49	26	8	12
2. Library [pool, *Alu I*]	31	19	2	8
3. Library [GA, *Alu I*]	26	12	-	6
Total	106	57	10	26

TABLE 6. STMS MARKERS FOR *M. fijiensis*

Locus	Repeat Type	Primer Sequences (5′-3′)	Expected size (bp)	Nig/Mex/ Col
MfSSR-005	P	TCCAAATTCCATCGTTGTCA CGATGATTTGGGTGGTCAAGCTA	158	Mex
MfSSR-025	P	CATGACTGACGTCCTCTTCTCA ATATGGGAAGGGGAAAGGTG	176	Mex/Nig
MfSSR-061	P	TGCAAACTCTGATGCTGGAC TTCAGAGGCTCGTCTTTGGT	124	-
MfSSR-137	P	GGCTCGAAGTGGACTAGCAC CTGGTCGAGGGTCGGG	243	Mex
MfSSR-175	C	AACCTCACATAGGCTGCCAC TATACCTTTCGTTCGGCCTG	286	-
MfSSR-203	P	CTCTGTGGCGTAAGTGGGTG TGATTGCACAGCAGGAAGAG	227	-
MfSSR-230	P	ACAAACTCCCAAGCATCACC GATCGATTCTATTGGCGGAA	265	Nig
MfSSR-244	P	GGCCATTTCATTTGCAAGAC ATGCCACAAAATCTCCATCC	215	Mex/Nig
MfSSR-304	P	TACATACCAGGCCGTCAACA TGACGCATGCATGATACAGA	232	Mex
MfSSR-308	P	TGCAGACTTTCCGATTCCTT TTACGTGGAAAACGCTACCC	152	Mex
MfSSR-316	P	TCCCAGCCAAATCAAAACTC AAGAAATTCGGCATTGAACG	110	Mex/Nig

The outermost left column indicates polymorphisms within or between isolates from Nigeria (Nig), Mexico (Mex) and Colombia (Col) that are visible in agarose gels [33, 36].

3. PERSPECTIVES: A BROAD SPECTRUM OF APPLICATIONS FOR STMS MARKERS

We present here a set of STMS markers for two important crops, chickpea and banana, and for two of their most important fungal pathogens. These can now be used for genome mapping, population and genetic diversity studies in the species from which they have been derived, thereby providing a readily applicable and reliable common basis for data exchange between populations and laboratories. However, first experiments indicate that the range of species for which they can be used is broader than anticipated. For example, chickpea STMS primer pairs amplify loci in the genomes of its wild relatives, markers from *Musa acuminata* can be used in other *Musaceae*, and primers from *A. rabiei* amplify loci from *A. fabae*. These results are portrayed below to encourage the application of these markers also in other species of the respective genus and across genus boundaries. First studies suggest that STMS primers from chickpea, for example, also amplify loci in genomic DNA of both, pea and lentil. However, our results also show, that amplification products from a distinct locus in chickpea are not necessarily allelic to a locus amplified from another species, and thus are not syntenic. Yet, if results are interpreted cautiously, the presented STMS markers may be valuable tools not only for the original species but across species and genus boundaries.

3.1. Transferability of STMS markers from chickpea between species of the genus *Cicer*

It has frequently been observed that SSR-flanking sequences are conserved in closely related species. For example, human STMS amplified corresponding loci from chimpanzees, and bovine STMS alleles from goats and sheep. Transferability of markers was observed in *Canidae*, *Cetaceae*, different species of pines, and *Medicago* (see [34] for references).

One of the aims of our work is the marker-assisted utilisation of the primary and secondary gene pool of chickpea for the improvement of the crop. Therefore, we explored whether and to which extent STMS primers designed for the cultigen could also be applied to genome analysis of wild *Cicer* species. We explored if conservation of microsatellite-flanking sequences reflects the known evolutionary relationship between these species. Further, we exemplarily investigated what underlies the differences in number and size of amplification products derived from the same or different species observed in preliminary experiments [34].

The conservation of 90 microsatellite-flanking sequences from chickpea was investigated in 39 accessions of 8 annual, and one accession of a perennial species of the genus *Cicer*. All primer sequences successfully amplified microsatellites in related species, indicating conservation of microsatellite-flanking sequences in chickpea's relatives. However, conservation of primer sites varied between species depending on their known phylogenetic relationship to chickpea. It ranged from 92.2% in *C. reticulatum*, chickpea's closest relative and potential ancestor, down to 50% for *C. cuneatum*. A phylogenetic tree generated using PAUP revealed a closer relationship between chickpea and the other members of its crossability group to the perennial *C. anatolicum* than to other annual species of the genus. In many cases variation in size and number of amplification products between and within species was observed. Sequence analysis of highly divergent amplification products revealed that differences in their size are either due to large variation in the number of microsatellite repeats of the same allele (in the case of chickpeas closest relatives), or to the amplification of another locus unrelated to the one amplified from chickpea (in the case of more distantly related species). Both, sequence information and bootstrapping suggested that STMS derived from chickpea may efficiently and reliably be used for synteny studies in chickpea's

crossability group including *C. anatolicum*. However, care should be taken when applying these markers to more distantly related species of the genus [34].

3.2. Transferability of STMS markers from *M. acuminata* between species of the genus *Musa*

It is not surprising that STMS primers developed from *M. acuminata* ssp. *malaccensis* (AA) also amplify loci in other subspecies and in the related species *M. balbisiana* (BB). Almost all of the banana and plantain cultivars are either intraspecific (AAA) or interspecific (AAB/ABB) hybrids which can be characterised by the developed STMS markers [30, 35]. Moreover, some of the primer pairs also amplify polymorphic fragments in other *Musa* species and even related taxa, e.g. other members of the family of *Musaceae* [30, 36]. Levels of expected and observed heterozygosity were highest among diploid *M. acuminata* accessions and wild types. Variation among triploid banana cultivars, plantains, and cooking bananas was considerably lower, although the "frozen" alleles in these sterile hybrids allow to trace back potential ancestors [30, 36].

3.3. *Ascochyta rabiei* microsatellite markers for population studies of *Ascochyta* pathogens of chickpea, pea, bean and lentil

Applications of STMS markers to natural field populations detect polymorphic allele sizes that are observed more frequently at loci with comparatively high repeat numbers. The analysed *A. rabiei* microsatellites remain stable during vegetative propagation and mutations have only very rarely been observed after sexual reproduction and are therefore well suited for this purpose [3, 22]. The use of only few of the presented primer pairs flanking microsatellites with highest repetition grades and single-locus performance should result in lineage- or strain-specific allele patterns with high discriminatory potential. Reliable monitoring of *A. rabiei* populations in chickpea-growing regions of the world can now be performed with a fast PCR approach.

It is striking, that the STMS markers suggested for widespread use here, were developed from genome screening with trinucleotide repeats. Although the experiments were performed in parallel and under identical conditions, $[CAA]_n/[GAA]_n$ screening was more efficient [25 markers] than the use of $[CA]_n/[GA]_n$ [only 12 markers]. In addition, trinucleotide-based markers revealed more polymorphisms [about two thirds of the bands were polymorphic on agarose gels] as compared to dinucleotide repeats [only 25% polymorphisms]. The same holds for the transferability. Only one third of the dinucleotide markers were transferable to *A. fabae* as compared to 80% of the primer pairs from sequences flanking trinucleotide repeats. One advantage of the primers flanking dinucleotide repeats is single-locus amplification in *A. rabiei* and *A. fabae* [22]. Furthermore, transferability to other species of the genus *Ascochyta* can be expected as has been exemplified for *A. fabae*. These plant pathogens are strictly host specific, and are highly virulent to leguminous crops such as pea, bean or lentil. The conservation of primer binding sites, especially those flanking trinucleotide repeats, is demonstrated by unique amplification products in most of the cases [22]. It remains to be examined whether these fragments are also polymorphic and contain microsatellites in heterologous species.

3.4. *Mycosphaerella fijiensis* microsatellites for population studies

STMS primers derived from a Nigerian isolate (*Mf*-Nig-862) can successfully be applied to isolates from Latin-American *M. fijiensis* populations. Although the distribution of alleles varied considerably between both continents due to geographic isolation, the markers detected unique haplotypes as well as internal structures of the populations [20]. In some countries (e.g. Colombia) banana are grown in lowlands and highlands. In the highlands a second *Musa* pathogen, *M. musicola*, the causative agent of Yellow Sigatoka is observed. This fungus has a lower temperature optimum than the Black Sigatoka pathogen *M. fijiensis*. On the other hand, *M. fijiensis* is more aggressive because of its shorter life cycle, and could eventually replace *M. musicola* in the lowlands. We are now applying STMS markers from *M. fijiensis* for monitoring both species in Latin America asking if adaptive processes occur in the *M. fijiensis* populations of the lower highlands. If *M. fijiensis* would be adapting to lower temperatures it could replace *M. musicola* even in the highlands.

Another problem caused by these mainly sexually propagating ascomycetes is their resistance to fungicides. First benomyl-resistant isolates have been observed, and the appearance in regions of extensive spraying (e.g. banana plantations in Central America) of strains carrying more than a single resistance factor is only a matter of time. A molecular pathogen forecast system allowing to monitor the population before spraying would help to reduce the risks for more and more resistant strains. Such strains can be characterised by STMS markers and be identified by their unique multi-locus haplotypes to improve plant protection.

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