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Isolation of microsatellites from an enriched genomic library of the plant-parasitic nematode *Meloidogyne incognita* and their detection in other root-knot nematode species

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Abstract The root-knot nematode *Meloidogyne incognita* is a polyphagous pest distributed from temperate to tropical regions. However, the lack of suitable markers leads to a poor knowledge of its population genetic structure and colonization process. Here we describe the first characterization of 15 microsatellite loci from this nematode, that were developed from an enriched genomic library. Although the variability of these microsatellites was generally low, three of them exhibited a significant level of intrapopulation polymorphism, with three to seven alleles detected. The observed and expected heterozygosities ranged from 0.025 to 0.385 and from 0.024 to 0.779, respectively. Thus, these new microsatellite markers have potential value for the implementation of genotyping experiments in this nematode. Furthermore, successful cross-amplification of the variable microsatellite loci in seven other *Meloidogyne* species provides the opportunity of using these markers for population genetic studies in these damaging plant-parasitic nematodes.

Keywords Genetic diversity · Molecular marker · Phytoparasitic nematode · Simple sequence repeats

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The Southern root-knot nematode, *Meloidogyne incognita* (Heteroderidae) is a serious pest characterized by both its world-wide distribution and its very large host range (Trudgill and Blok 2001), which raise questions about the origin, the processes of dispersal and the resulting genetic structure of the populations. In recent years, studies of genetic diversity have been carried out in this mitotic parthenogenetic organism using molecular markers such as RAPD or AFLP, and revealed rather unexpected levels of clonal diversity among populations (reviewed in Castagnone-Sereno 2006). But surprisingly, microsatellites, which are usually regarded as among the most appropriate tools to study variation at the individual level (Selkoe and Toonen 2006), have been very poorly investigated in this taxon. Indeed, only one microsatellite locus had been characterized so far in root-knot nematodes, i.e. in *M. artiellia*, a species assumed to reproduce by both amphimix and facultative meiotic parthenogenesis (De Luca et al. 2002). Recently, simple sequence repeats were identified by a bioinformatic data-mining analysis in ESTs from phytoparasitic nematodes, including some root-knot nematode

species, but no information was provided about regions flanking these loci (Huang et al. 2010). Here, we evaluate the feasability of microsatellite isolation by screening an enriched genomic library from *M. incognita*, and report the subsequent characterization of 15 microsatellite loci in this nematode. Furthermore, these loci were also tested for their presence in 11 additional root-knot nematode species.

Genomic DNA was isolated from a pool of *M. incognita* eggs from a population from Kursk, Russia according to standard procedures (Sambrook et al. 1989) and used to construct an enriched partial genomic library. Total genomic DNA (4–5 μg) was digested to completion with *Rsa*I enzyme. After migration on agarose gel, the 400–1200-bp DNA fraction was purified and ligated to *Rsa*I linkers. The enrichment procedure followed the protocol of Kijas et al. (1994) based on streptavidin-coated magnetic particles (Magnesphere, Promega). The 5′-biotinylated $(CT)_{10}$ and $(GT)_{10}$ oligonucleotides were used as probes. The enriched single stranded DNA was amplified using one of the *Rsa*I linkers as primer to recover double stranded DNA. The PCR products were purified and ligated into pGEM-T Easy vector (Promega), and the plasmid transformed into *Escherichia coli* super-competent cells (XL1 blue, Stratagene). Recombinant clones were transferred to positively charged Hybond-N+ membranes (Amersham). DNA was fixed on the membrane by baking at 80°C for an hour. Positive colonies (i.e. containing a microsatellite motif) were identified by hybridization at 48°C with $(CT)_{10}$ and $(GT)_{10}$ probes labelled with digoxigenine using the DIG Oligonucleotide Tailing kit (Boehringer Mannheim). Of the 1400 recombinant colonies analyzed, 280 positive clones were visually identified, picked and sequenced by Genome Express (France). Among them, 231 contained a repeat region. However, after removing redundant sequences and clones with the microsatellite motif at the end of the cloned insert, 183 clones proved suitable for primer design. Forward and reverse primers were designed for 25 of these sequences using the online version of the Primer 3 program (http://frodo.wi.mit.edu/primer3/input.htm). Primers were screened in a two-step process using the plasmid followed by the original DNA sample as template. Primers that amplified a single band in the control samples were further screened for polymorphism on genomic DNA extracts prepared from single *M. incognita* second-stage juveniles as previously described (Plantard and Porte 2003). PCR reactions were performed using a T3 Thermocycler (Biometra), in a final volume of 25 μl containing 1 μl of the DNA template solution, 0.4 mM of each dNTP, 0.48 μM of each designed primer, 1x Incubation Mix buffer containing 1.5 mM $MgCl_2$ (Qbiogene) and 0.25 U *Taq* DNA polymerase (Qbiogene). Three different PCR procedures were used depending on the locus (Table 1). PCR products were separated by electrophoresis in nondenaturing 8% acrylamide gels, stained with ethidium bromide and visualized under UV light. Molecular sizes of the amplified fragments were estimated using a 10-bp ladder (Invitrogen). For each of the 25 loci, polymorphism was assessed on 12 to 78 individuals from two populations from Sevilla, Spain and Kurk, Russia. Of the 25 amplified microsatellite loci, 10 did not yield clearly defined amplified products and were discarded. Except for the locus 1 H02, the other microsatellite loci revealed variable banding patterns, the total number of bands detected on agarose gels ranging from two to 15 per locus (Table 1). Quite unexpectedly, for eight of these 15 loci, a significant difference in size (> 100 bp) was detected between the original clone from the enriched library and the amplified products, suggesting that some of these bands at least may not be true alleles, but rather primer-sequence related DNA fragments. Since the definitive identification of true alleles *versus* such false positives would have been a laborious and time-consuming task (i.e., cloning and sequencing each amplified band), we thus decided to focus our analyses on the seven remaining loci, i.e., 1A03, 1A06, 1 C12, 1 F11, 1 H02, 3A01 and 3 C05.

We estimated the level of polymorphism of these seven loci by genotyping 26 to 66 second-stage juveniles from the *M. incognita* Seville population. DNA amplifications were performed essentially as described previously, except that the forward primer of each microsatellite locus was labelled with a fluorescent dye (either FAM, NED or VIC). One microlitre of each PCR was mixed with 9.5 μl of Hi-Di formamide (Applied Biosystems) for denaturing and 0.15 μl of GeneScan-500LIZ Size Standard (Applied Biosystems). Separation and detection of PCR products were carried out by a capillary electrophoresis with an automatic sequencer (ABI

Table 1 Characterization of 15 microsatellite loci isolated from an enriched partial genomic library of the nematode *Meloidogyne incognita*

Locus	Repeat motif	Primer sequence (5′ to 3′)	T_a (°C)	P	Clone size (bp)	Size range (bp)	n	N_a	H_o	H_e[a]	Accession number
1A01	$(GATA)_3(GA)(GATA)_5(GA)_5(GATA)_3(GA)_{11}$	CGGTTAGGTGTTACATGCCG CTGTTCCAATATCCTATAGC	48	1	197	197–300	22	3	—[b]	—	AM490042
1A03	$(GA)_3\ldots(GA)_{10}\ldots(GA)_9$	CCTGCACTAAGAGAACGAGAGAA CCCCCGTTACAGGTCTTAAT	55	1	170	166–172	65	4	0.385	0.462	AM490043
1A06	$(AG)_{11}$	CCGTTCTCACCTCCACAAAT GGAGCTGCAGAGATGAACAA	62	1	151	135–151	74	7	0.288**	0.779	AM490044
1B12	$(GA)_{20}$	CGGTTGTGTTATGCACTCGA CTCACCGTTTCGTCCTCAAC	50	2	191	115–300	78	6	—	—	AM490045
1C12	$(GA)_9(GT)_2$	GGCAGTGCGGATAAAATAGG TCACTCGTCGGCACACTATC	58	1	116	114–118	39	3	0.025	0.024	AM490046
1D08	$(TC)_4\ldots(TC)_3\ldots(CT)_{15}$	AGTGACTTTGGGGTTGGATG GCCCAACAAACAACGTGTAG	48	3	137	100–400	23	8	—	—	AM490047
1D09	$(TC)_{17}\ldots(TC)_5\ldots(GC)_4\ldots(TC)_3\ldots(AT)_6$	CGCGCATTAGGTCGTATATG GTTTTTATTACAAGGGGACG	48	1	287	60–300	40	10	—	—	AM490048
1F08	$(GA)_{17}\ldots(GA)_3\ldots(GA)_5\ldots(GA)_3\ldots(GA)_4\ldots(GA)_5$	GCTAGTCTAATGCTTTCCG CTCTTTTCCCTCTCCCTG	48	1	253	83–300	63	15	—	—	AM490049
1F11	$(GA)_{12}$	AAGAGATGCCGTCTCCTGAA TCAACTTCGCGCCATACTTT	59	2	128	128–130	70	2	0	—	AM490050
1G12	$(TC)_{10}\ldots(CA)_7\ldots(GC)_3\ldots(TAA)_4(TA)_2\ldots(TA)_3$	GACCGTCCTGCTGTCAAG ATTACTGGGGGTGATACGAC	50	1	498	300–500	38	5	—	—	AM490051
1H02	$(GA)_{10}\ldots(AG)_3$	TCGTGTATTCCACGGGGTCA CCACGACCTATAGCGTGAATG	50	1	135	135	57	1	—	—	AM490052
3A01	$(TC)_{10}$	CTCTGCTGGATGCGAAACAC CGATGTGACACCAATACGGC	54	1	190	160–200	12	3	0	—	AM490053
3B02	$(GA)_{16}\ldots(GA)_3\ldots(GA)_3$	GAAGAAATCGGCGGGTAGGC ACCGGACGAGATCGCCTTTC	55	1	117	100–400	13	5	—	—	AM490054
3C05	$(AG)_9\ldots(AG)_4\ldots(AG)_6$	GTCCTTAGGTGTCACTTGCC AATCTTGGCTTCTGTCTTCG	54	1	126	150–190	59	3	0	—	AM490055
3C11	$(TC)_3\ldots(CT)_8$	ATGCCCGACTCTGATCTTTC CCACATTTTGTTGTATCAGC	50	1	363	210–370	56	3	—	—	AM490056

T_a = annealing temperature; P = PCR procedure used: (1) 94°C for 5 min; 40 cycles of 94°C for 1 min, T_a for 1 min, 72°C for 30 s; 72°C for 5 min; (2) 94°C for 5 min; 40 cycles of 94°C for 1 min, T_a for 1 min, 72°C for 90 s; 72°C for 5 min; (3) 94°C for 2 min; 40 cycles of 94°C for 20 s, T_a for 30 s, 72°C for 30 s; 72°C for 5 min; n = number of individuals tested; N_a = total number of bands observed in agarose gels; H_o = observed heterozygosity; H_e = expected heterozygosity

[a] According to Nei (1978)

[b] Only the loci for which the amplified products showed a similar size range than the original clone were analyzed for polymorphism (see text)

** indicates significant deviation to Hardy-Weinberg equilibrium ($P<0.01$)

PRISM 3100 Genetic Analyser, Applied Biosystems). Genotypes were scored using Gene Marker version 1.4 (SoftGenetics). After analysis, three loci (1A03, 1A06 and 1 C12) were found polymorphic, with 3, 7 and 2 alleles detected in the nematodes tested, respectively. The observed and expected heterozygosities calculated using GENEPOP version 4.0.10 (Raymond and Rousset 1995) ranged from 0.025 to 0.385, and from 0.024 to 0.779, respectively (Table 1). The 1A06 locus exhibited a significant heterozygote deficiency, maybe due to the presence of null alleles and/or genetic structuring within the population. The four other loci (1 F11, 1 H02, 3A01 and 3 C05) were found to be monomorphic in the analyzed population, although previous data have shown that three of them (1 F11, 3A01 and 3 C05) display several bands in agarose gels (Table 1). Further experiments are needed for determining whether polymorphism may be detected for these loci in other *M. incognita* populations.

Cross-species amplification of the seven previously selected microsatellites was further investigated in 11 additional root-knot nematode species (Table 2). Using the PCR procedure developed for *M. incognita*, cross-species amplification was observed in *M. arenaria*, *M. enterolobii*, *M. exigua*, *M. hapla*, *M. javanica*, *M. maritima* and *M. paranaensis*, suggesting that some of the isolated loci are largely distributed in the whole genus. Indeed, the observed distribution appeared quite independent from the phylogenetic relationships of these species, since cross-amplification was noticed for representatives of the three main clades of the genus *Meloidogyne*, as inferred from rDNA and/or mtDNA sequence analyses (Tigano et al. 2005; Holterman et al. 2009). Overall, part of the primers defined in the present study might be useful to diversity studies within other root-knot nematodes, even though further studies are required, in order to analyze the polymorphism of the microsatellite motifs for such species.

Table 2 Cross-amplification patterns of seven microsatellites isolated in *Meloidogyne incognita* in 11 other root-knot nematode species

	1A03	1A06	1C12	1F11	1H02	3A01	3C05
Meloidogyne arenaria	+	+	+	+	0	+	0
M. chitwoodi	0	0	0	0	0	0	0
M. enterolobii	0	+	0	+	0	+	+
M. exigua	0	0	0	0	+	0	0
M. fallax	0	0	0	0	0	0	0
M. floridensis	0	0	0	0	0	0	0
M. hapla	+	0	+	+	0	0	0
M. hispanica	0	0	0	0	0	0	0
M. javanica	+	+	+	+	0	+	0
M. maritima	0	+	+	+	0	+	+
M. paranaensis	0	0	0	0	+	0	0

+ = amplification product(s) in the expected size range

0 = no or not reliable amplification products (under the PCR procedures developed for *M. incognita*)

Even if successful, the procedure used here remains time-consuming and costly. Moreover, although we used an enrichment strategy, our approach revealed a relative paucity of polymorphic loci in the nematode. Indeed, enrichment strategies in general have been shown to introduce potential bias in genome representativeness of the selected loci (Castoe et al. 2010). Conversely, *in silico* mining of whole-genome sequence data has recently been proven as a successful approach for high-throughput identification of microsatellite loci (Sharma et al. 2007). In this respect, the recent completion of the *M. incognita* and *M. hapla* genomes (Abad et al. 2008; Opperman et al. 2008) should open a new area for microsatellite development in these pests. Indeed, more than 2,000 di- to hexanucleotide perfect repeat loci were very recently identified in the *M. incognita* genome through data mining (Castagnone-Sereno et al. 2010), that could be used as a strong basis for the further development of molecular markers in this species. However, to our knowledge, the present study reports the first characterization of polymorphic microsatellite loci in the plant-parasitic nematode *M. incognita* using a conventional bench approach. Although their overall diversity was generally low, some of the loci identified here appeared promising for *M. incognita* genotyping, since reliable multilocus polymorphisms were detected between individual nematodes. From this point of view, they demonstrate the feasibility of microsatellite isolation in *M. incognita*, and constitute a first step into the development of population genetic studies in this nematode.

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