European Arachnology 2002 (F. Samu & Cs. Szinetár eds.), pp. 49-56.
 ISBN 963 214 791 X
(Proceedings of the 20th European Colloquium of Arachnology, Szombathely 22-26 July 2002)

RAMS technique fails in developing microsatellite primers for *Coelotes terrestris*

SHIRLEY GURDEBEKE[1] & JEAN-PIERRE MAELFAIT[1,2]
[1] *Ghent University, Department of Biology, Unit of Animal Ecology, Zoogeography and Nature Conservation, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium* (Shirley.Gurdebecke@chello.be)
[2] *Institute of Nature Conservation, Kliniekstraat 25, B-1070 Brussel, Belgium*

Abstract
Microsatellites are considered to be neutral, highly polymorphic and codominant markers and have therefore become very useful for genome mapping, paternity and kinship analysis and for studying the genetic structure of natural populations. One drawback of microsatellites, however, is that they need to be isolated for every organism that is investigated for the first time. Traditionally, for every species, genomic libraries were constructed and screened with repeat-containing probes. RAMS (Randomly Amplified MicroSatellites), an alternative method, is based on the RAPD technique (Randomly Amplified Polymorphic DNA) and avoids time-consuming library construction and screening. We applied this technique to isolate microsatellite loci from the spider Coelotes terrestris (Wider, 1834). Although the method has been successfully used for microsatellite marker development on e.g. the genome of Cladocera, Odonata and two species of flatfish, we were not able to characterize polymorphic microsatellite sequences of the spider species Coelotes terrestris. It is possible that the genome of this species contains only very few dinucleotide microsatellites, which would correspond with statements by other investigators that microsatellites are not as ubiquitous as previously expected. To avoid possible problems caused by scarcity of microsatellite loci, and with the advent of cheaper and faster enrichment protocols, we would recommend the use of enriched genomic libraries when trying to isolate microsatellites from spider (or arachnid) DNA.

Key words: Araneae, primer characterization, microsatellites

INTRODUCTION

The spider *Coelotes terrestris* (Wider, 1834) (Amaurobiidae) is one of many organisms that are stenotopically bound to mesophilous forests in Flanders and that have to survive in a highly disturbed environment (Gurdebeke 2002). It is expected that organisms bound to these patchy forest habitats and with limited dispersal capacities, will experience strong effects of genetic drift. In a first attempt to study population genetics of *C. terrestris,* allozyme polymorphisms were studied. However, the number of polymorphic loci and alleles were too low to make a thorough analysis of the population genetic structure (Gurdebeke et al. 2000). To test whether the low level of polymorphism in *C. terrestris* was an artefact of the allozyme markers, a follow-up study by means of the RAPD technique was performed. By using these dominant markers, we were able to discover more genetic variation in the spider (Gurdebeke et al. 2003). Being able to develop microsatellites for this organism would make it possible to confirm our RAPD findings with co-dominant microsatellite markers.

Microsatellites, as a new class of polymorphic DNA markers, became available only after the discovery of multilocus fingerprinting with minisatellites by Jeffreys et al. (1985) and

the invention of the polymerase chain reaction (PCR) by Saiki et al. (1988) (Schlötterer & Pemberton 1998). These markers, also called simple sequence repeats (SSR) or short tandem repeats (STR), typically contain 10-50 copies of a short repeat motif (1-10 base pairs, usually 1-5 bp) (Tautz 1989; Rosenbaum & Deinard 1998; Scribner & Pearce 2000). They are considered to be ubiquitous in both eukaryotic and prokaryotic genomes and are present in coding, as well as in non-coding regions (Hamada et al. 1982; Jarne & Lagoda 1996; Schlötterer & Pemberton 1998; Zane et al. 2002). Microsatellite alleles are the result of mutations, causing changes in repeat number during DNA slippage, an intramolecular mutation mechanism. Normally, these mutations are recognized and repaired by the DNA mismatch repair system (Strand et al. 1993) and observed differences in allele length have thus probably escaped the repair system (Schlötterer & Pemberton 1998). Repeat number can vary between individuals of the same species and the markers are considered to be neutral, highly polymorphic and codominant (Schlötterer 1998; Fagerberg et al. 2001). Microsatellites have therefore become a very useful marker system for genome mapping, paternity and kinship analysis and for studying the genetic structure of natural populations (Schlötterer & Pemberton 1998; Scribner & Pearce 2000; Fagerberg et al. 2001; but see Rosenbaum & Deinard 1998).

Despite their application in many fields, a major drawback of microsatellites is the fact that they need to be isolated for every organism that is investigated for the first time. Because microsatellite sequences are mostly situated in the noncoding regions of the DNA, where higher nucleotide substitution rates are observed than in coding regions, it is not possible to develop universal primers that work for several (groups of) organisms. Some cross-species amplification has been observed, but only, for species belonging to the same genus or to recently separated genera (Zane et al. 2002). For spiders, as far as we know, only one article has been published related to microsatellite isolations and characterizations (Rütten et al. 2001). Therefore, this work can be considered among the first testing out a new method for arachnids.

Traditionally, for microsatellite detection and isolation, partial genomic libraries were screened with repeat-containing probes (Rassmann et al. 1991). This method however was very labour intensive and time consuming and often yielded few microsatellite loci. Therefore, it is mostly useful when investigating organisms with a high frequency of microsatellite regions in their genome, or when few loci are needed (e.g. parentage assignment or population allocation studies) (Zane et al. 2002). When more loci are needed, or when organisms with low frequencies of microsatellites need to be investigated, this traditional method may not be sufficient and therefore, alternative strategies were developed. An overview of the methods available for microsatellite detection and isolation is given in the review by Zane et al. (2002). RAMS (Randomly amplified microsatellites) (Ender et al. 1996), is an alternative method which avoids library construction and screening, and is based on the randomly amplified polymorphic DNA (RAPD: Williams et al. 1990; Welsh & McClelland 1990) technique. This technique was chosen because of our previous experience with RAPDs (Gurdebeke et al. 2000, 2003) and because we were given the opportunity to conduct our research in the laboratory of and with help from the investigators who described this technique. In this contribution, we report the results of the application of this technique in our investigation to isolate microsatellite markers from the spider *Coelotes terrestris* and comment on the varying success in microsatellite isolation from arachnid DNA.

MATERIAL AND METHODS

Genomic DNA of *C. terrestris* was extracted with the PureGene DNA isolation kit (Type D-

5000A, Gentra Systems, Inc., Biozym, Landgraaf, The Netherlands). RAPD profiles were generated with primers of Kit A, B, C, G, M, P and AB (Operon Technologies Inc., Alameda, CA). RAPD-PCR amplifications were carried out in 25 µl volumes containing 10 ng DNA template, 1x *Taq* polymerase buffer (incl. 1.5 mM $MgCl_2$, Biotherm™), 0.5 mM $MgCl_2$, 0.1 mM dNTPs each, 3 pM RAPD primer and 0.7 U *Taq* polymerase (Biotherm™). Initial denaturation was 2 min at 93°C, followed by 40 cycles (93°C for 20 s, 38°C for 15s (ramp 3 s/1°C), 72°C for 30s). RAPD profiles were size separated on a 1.4% agarose gel and were blotted onto positively charged nylon membranes (Boehringer) using alkaline transfer. These were checked for microsatellite motifs by means of Southern hybridisation with biotin and digoxigenin labelled $(GA)_{10}$ and $(GT)_{10}$ probes. Hybridization was done overnight at 42°C containing 20-40 ng/ml of the probe. Colorimetric detection was performed with NBT and X-phosphate. Where hybridisation had taken place, several signals were observed and those regions of the blot were cut out from the membrane. After denaturation these were heated in 10 µl bidistilled water for 10 min at 95°C to separate the probes from the RAPD fragment, the DNA of the cut out pieces was used for re-amplification (25 µl volumes containing 1x *Taq* polymerase buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs each, 3 pM of the corresponding RAPD primer and 0.75 U *Taq* polymerase). The re-amplified bands were purified with the MinElute Gel Extraction Kit (Qiagen), following the manufacturers instructions and ligated into the pGEM®-T Easy Vector System (Promega). The plasmids were transformed into JM109 High Efficiency Competent Cells (Promega). The ligated fragment of the positive colonies was boiled in 40 µl sterile water for 5 min at 99°C and 10 µl was PCR amplified by means of BacPCR with primers T7 and SP6. The BacPCR amplifications were carried out in 25 µl of a mixture containing 1 x *Taq* polymerase buffer (incl. 1.5 mM $MgCl_2$), 0.5 mM $MgCl_2$, 0.1 mM of each dNTP, 2.5 pM of each primer and 0.7 U of *Taq* polymerase. Initial denaturation was 2.30 min at 92°C, followed by 35 cycles (92°C for 30 s, 51°C for 30 s, 72°C for 60 s) and 3 min at 72°C. The resulting PCR product was precipitated with 5 M NH_4Ac and isopropanol and resuspended in sterile water. Cycle sequencing was carried out with ABI 310 BigDye terminator by using 7 µl of PCR product and the primers T7 and SP6, at 5 pM concentrations. The PCR protocol was 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. These PCR products were purified with 3 M NaAc and 98% ethanol and resolved in 20 µl HPLC water and 4 µl was directly transferred for sequencing with an automated ABI 310 DNA Sequencer (Applied Biosystems). After interpretation of the sequences, specific primers were developed containing a GT repeat or GT-rich regions with the online program Primer 3 (Rozen & Skaletsky 2000) and PCR conditions were optimised for amplification of the loci from different individuals, belonging to different populations. Fragments were labelled with [F] dNTP reagents (PE Applied Biosystems), used to incorporate fluorescent dNTPs during PCR, for subsequent size determination with an automated ABI 310 DNA Sequencer.

Only one RAPD primer pair was used per reaction. Although possible to generate RAPD bands when combining more than one primer pair, it proved to be very difficult to retrieve bands with two different primers in the later stages of the protocol (e.g. reamplification of cut out pieces of membrane).

RESULTS

Southern hybridisation with biotin and digoxigenin labelled $(GA)_{10}$ and $(GT)_{10}$ probes of RAPD profiles rendered 19 potential microsatellite motifs (Table 1). A small piece of the blot, at the height of the signal, was cut out from the membrane and used for re-amplification with the corresponding RAPD protocol. Fourteen of these reactions were suc-

Table 1. Overview of the positive hybridisation signals and the results of the subsequent analyses. The first part of the fragment name refers to the RAPD primer that generated the band, the second part describes the estimated length of the fragment in base pairs. [1] Fragment too long for ligation; [2] Non successful ligation; ł Sequence contained no repeat.

	Used for reamplification	Used for ligation	Used for sequencing	Repeat region	Length variation
B1-600	+	+	+	GT-rich	No length variation
M2-250	+	+	+	TAAA and CAAA-	No length variation
M2-350	+	+	+	TAAA(A)-rich	Amplification not in
B7-450	+	+	+	$(GT)_{25}$-repeat	Amplification
M2-450	+	+	+	GTTT-rich	Amplification
C8-350	+	+	+	- ł	-
C8-450	+	+	+	- ł	-
M3- 850	+	+	- [2]	-	-
M3-800	+	+	- [2]	-	-
C8-1000	+	- [1]	-	-	-
A2-1700	+	- [1]	-	-	-
B7-850	+	-	-	-	-
C2-550	+	-	-	-	-
C2-500	+	-	-	-	-
B6-1150	-	-	-	-	-
B20-1500	-	-	-	-	-
B20-1450	-	-	-	-	-
B17-650	-	-	-	-	-
B17-400	-	-	-	-	-

cessful, but many reactions gave result to several non-specific products instead of one clear PCR product. Nine of these resulting PCR fragments were selected for subsequent ligation into a pGEM®-T Easy Vector and transformation into JM109 High Efficiency Competent Cells. The selection was mainly based on the length of the PCR product, which had to be smaller than 1 kB for successful ligation. Despite the selection, however, of the 9 ligation reactions, the two with the longest DNA fragment failed (M3-850 and M3-800), which resulted in seven potential microsatellite containing plasmids. Sequencing of these fragments brought to light that one of the plasmids indeed contained a $(GT)_{25}$ repeat (B7-450) and 4 others were GT(TT)-rich regions or harboured imperfect or compound TAAA(A) or CAAA repeats (see Appendix). In two of the seven sequenced fragments (C8-350 and C8-450), no (im)perfect repeats could be detected. The primers that were developed for amplification of the five former fragments from the *Coelotes terrestris* genome, were only able to amplify 3 of the 5 wanted regions. Two of these were amplified in all tested individuals, but no length variations were observed in these sequences (B1-600 and M2-250). The primers for generating the third fragment, M2-350, were not able to generate a PCR product in all tested individuals and not one amplification took place when using the primers developed for M2-450 and B7-450. The failure of amplifying the region containing the perfect

GT repeat lead us to believe that a recombination had taken place in the flanking region, making suitable primer design impossible or even that the microsatellite sequence might not have originated from *C. terrestris* DNA.

DISCUSSION

Despite successful microsatellite marker development by means of the RAMS protocol on the genome of, for example, Cladocera (*Daphnia*) (Ender et al. 1996), Dover sole (*Solea solea*) (Iyengar et al. 2000a), turbot (*Scophthalmus maximus*) (Iyengar et al. 2000b) and the Odonate *Megaloprepus coerulatus* (Fincke & Hadrys 2001), we were not able to characterise polymorphic microsatellite sequences of the spider species *Coelotes terrestris*. Although this research was carried out in the laboratory of and with help from the authors who originally described the RAMS technique, only one bright hybridisation signal, indicating a perfect microsatellite repeat (fragment B7-450), was found. On the other hand, this single signal proved that the technique and chemicals used were working well. One possibility to explain these findings is that this species does not possess a high frequency of microsatellites, and that the used technique was therefore unable to locate regions that were complementary to the probes $(GA)_{10}$ and $(GT)_{10}$. Zane et al. (2002) noted that repeat preference varies between species and that the probes that are used may be underrepresented in the considered genome. Moreover, several authors have stated that the frequency of microsatellites (absolute numbers of microsatellite loci), varies across taxa and that some species contain only very few microsatellites (Jarne & Lagoda 1996; Schlötterer & Pemberton 1998; Hancock 1999). That microsatellites are not as ubiquitous in all taxa as previously expected, was already mentioned by Nčve & Meglécz (2000). Also in the Acari, an arachnid order closely related to spiders, polymorphic di- and trinucleotide microsatellites are not always present with a high frequency and were reported to be extremely rare in the spider mite *Tetranychus urticae*, the predatory mite *Amblyseius fallacis* and the tick *Ixodes scapularis* (Navajas et al. 1998; Navajas & Fenton 2000; Fagerberg et al. 2001). For the latter, only a method that was based on a number of protocols and that involved the enrichment of the genomic library, lead to successful microsatellite isolation (Fagerberg et al. 2001).

Moreover, it has only been recently, that the first microsatellite isolations and characterizations were published for a spider species (Rütten et al. 2001). By means of a library enriched for CA repeats, these investigators were able to develop fourteen polymorphic microsatellite markers for the spider *Pholcus phalangioides*. According to the authors, their method uses standard techniques and commercially available kits, and when combined, are efficient even for investigators with little expertise in molecular biology. In view of our results and these recent findings and with the advent of cheaper and faster enrichment protocols, we recommend the use of enriched genomic libraries to isolate microsatellites from spider DNA in future. This can indeed avoid possible waste of time and money by using alternative microsatellite development methods, until it has been undoubtedly ascertained that there is no scarcity of microsatellite loci in the spider (or other arachnid) genome, and that the use of enriched libraries is not required.

ACKNOWLEDGEMENTS

Our thanks goes to Prof. Dr. G. Haegeman and Dr. K. Van Craenenbroeck for assistance during a preliminary investigation. Most of the analyses, however, were carried out in the lab of Prof. Dr. B. Schierwater (Ecology and Evolution Lab, Tieräztliche Hochschule Hannover, Germany), who we are very much indebted to. We thank Dr. A. Ender for assistance during the practical implementation of this research and two anonymous referees for the provision of helpful comments on an earlier version of this manuscript. The first author acknow-

ledges a grant from the Flemish Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT).

REFERENCES

Ender, A., Schwenk, K. , Städler, T. , Streit, B. & Schierwater, B. 1996. RAPD identification of microsatellites in *Daphnia*. *Molecular Ecology* 5, 437-441.

Fagerberg, A.J., Fulton, R.E. & Black IV, W.C. 2001. Microsatellite loci are not abundant in all arthropod genomes: analysis in the hard tick, *Ixodes scapularis* and the yellow fever mosquito, *Aedes aegypti*. *Insect Molecular Biology* 10, 225-236.

Fincke, O.M. & Hadrys, H. 2001. Unpredictable offspring survivorship in the damselfly, *Megaloprepus coerulatus*, shapes parental behavior, constrains sexual selection, and challenges traditional fitness estimates. *Evolution* 55, 762-772.

Gurdebeke, S. 2002. *Interdemic variation in genetic structure and life history traits of the forest-dwelling spider Coelotes terrestris (Wider, 1834).* Unpublished PhD thesis, Ghent University.

Gurdebeke, S., Neirynck, B., & Maelfait, J.-P. 2000. Population genetic effects of forest fragmentation in Flanders (Belgium) on *Coelotes terrestris* (Wider) (Araneae: Agelenidae) as revealed by allozymes and RAPD. *Ekologia* 19, supp. 3, 87-96.

Gurdebeke, S., Maelfait, J.-P., & Backeljau, T. 2003. Contrasting allozyme and RAPD variation in spider populations from patchy forest habitats. *Genetica* 119, 27-34.

Hamada, H., Petrino, M.G. & Kakunaga, T. 1982. A novel repeated element with Z-DNA-forming potential is widely found in evolutionarily diverse eukaryotic genomes. *Proceedings of the National Academy of Sciences, USA* 79, 6465-6469.

Hancock, J.M. 1999. Microsatellites and other simple sequences: genomic context and mutational mechanisms. In: *Microsatellites. Evolution and Applications* (D.B. Goldstein & C. Schlötterer eds.), pp. 1-9. Oxford University Press, New York.

Iyengar, A., Piyapattanakorn, S., Stone, D.M., Heipel, D.A., Howell, B.R., Baynes, S.M. & Maclean, N. 2000a. Identification of microsatellite repeats in turbot (*Scophthalmus maximus*) and Dover sole (*Solea solea*) using a RAPD-based technique: Characterization of microsatellite markers in Dover sole. *Marine Biotechnology* 2, 49-56.

Iyengar, A., Piyapattanakorn, S., Heipel, D.A., Stone, D.M., Howell, B.R., Child A.R. & Maclean, N. 2000b. A suite of highly polymorphic microsatellite markers in turbot (*Scophthalmus maximus* L.) with potential for use across several flatfish species. *Molecular Ecology* 9, 368-371.

Jarne, P. & Lagoda, P.J.L. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* 11, 424-429.

Jeffreys, A.J., Wilson, V. & Thein, S.L. 1985. Hypervariable ' minisatellite' regions in human DNA. *Nature* 314, 67-73.

Navajas, M. & Fenton, B. 2000. The application of molecular markers in the study of diversity in acarology: a review. *Experimental and Applied Acarology* 24, 751-774.

Navajas, M.J., Thistlewood, H.M.A., Lagnel, J. & Hughes, C. 1998. Microsatellite sequences are under-represented in two mite genomes. *Insect Molecular Biology* 7, 249-256.

Nčve G. & Meglécz, E. 2000. Microsatellite frequencies in different taxa. *Trends in Ecology and Evolution* 15, 376-377.

Rassmann, K., Schlötterer, C. & Tautz, D. 1991. Isolation of simple-sequence loci for use in polymerase chain reaction-based DNA fingerprinting. *Electrophoresis* 12, 113-118.

Rosenbaum, H.C. & Deinard, A.S. 1998. Caution before claim: an overview of microsatellite analysis in ecology and evolutionary biology. In: *Molecular Approaches to Ecology and Evolution* (R.

DeSalle & B. Schierwater eds.), pp. 87-106. Birkhäuser Verlag, Basel.

Rozen, S. & Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (S. Krawetz & S. Misener eds.), pp. 365-386. Humana Press, Totowa, NJ.

Rütten, K.B., Schulz, I., Olek, K. & Uhl, G. 2001. Polymorphic microsatellite markers in the spider *Pholcus phalangioides* isolated from a library enriched for CA repeats. *Molecular Ecology Notes* 1, 255.

Saiki, R.K., Gelfand, D.H. , Stoffel, S., Scharf, S.J. , Higuchi, R. , Horn, G.T. , Mullis, K.B. & Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-491.

Schlötterer, C. & Pemberton, J. 1998. The use of microsatellites for genetic analysis of natural populations - a critical review. In: *Molecular Approaches to Ecology and Evolution* (R. DeSalle & B. Schierwater eds.), pp. 71-86. Birkhäuser Verlag, Basel.

Schlötterer, C. 1998. Microsatellites. In: *Molecular Genetic Analysis of Populations. A Practical Approach* (A.R. Hoelzel ed.), pp. 237-261. Oxford University Press, Oxford.

Scribner, K.T. & Pearce, J.M. 2000. Microsatellites: evolutionary and methodological background and empirical applications at individual, population and phylogentic levels. In: *Molecular Methods in Ecology* (A.J. Baker, ed.), pp. 235-273. Blackwell Sciences Ltd., Oxford.

Strand, M., Prolla, T.A., Liskay, R.M. & Petes, T.D. 1993. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 365, 274-276.

Tautz, D. 1989. Hypervariability of simple sequence as a general source for polymorphic DNA markers. *Nucleic Acids Research* 17, 6463-6471.

Welsh, J. & McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research* 18, 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J. A. & Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18, 6531-6535.

Zane, L., Bargelloni, L. & Patarnello, T. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology* 11, 1-16.

Appendix. Sequences of promising clones. Priming sites indicated in bold. $(GT)_{25}$ repeat underlined.

B1-600 (565 bp)

5′—GTTTCGCTCCTGGATGACATCCCGAATGTCTGCAGGTAATTTTTGGTGTTTGTGGGGA
ATGTAGCTGGATGTAGAGTCCGAAATTGCAGTCTTTATGTGTTGTGTGACGTCAAGGACG
GCATTGTCCAATTCGTCAGTGTTGTGTATGTCAGTGAGGGGAAGGGGGGTTCTATCAAGA
ATTTTAGTAAAGCTAGCCCAGTCCACACGAGAGGGGAGAGGGGGGCCGGGAAGAGGA
GAATCAAGATGTAAAGTGAGTAGGACTGGGCGGCGATCGGAAGTGAAAAGGTTGAGTA
CGTTAACTGTGTATGTGTAGGGAATATTTTTAAAAAGGGCCAGATCAATAGTAGTGGGA
TTATTGTGTCTGGAAAAACGTGTAGGTCCGGGAGGAACTGCAACATCAAATCGATTATG
TAAAAGAAACTCGTGGAGGTCTCTGCCTACACGGGAAGTAGTATTGGAGCCCTACCGGG
GGTGATGAGCATTAAAGTCCCCGGCAAGGATGACATTAGGTGCCAAATTGAAAAGATT
ACGGAGGTCACAAGTTACTGAGTGGGGGGAGCGAAAC—3′

M2-250 (249 bp)

5′–ACAACGCCTCGCACAAAGAAACAAACACATAAATACATGAAAAAACAAACACACA
AAGAAATAAGTAAACAAATAAACACACAAAACAAACAAACATTCACACATACAAAGA
CACACTACGCCACTCCTCCTAATTACGATGAGGCGATATCATCTTAAATAATGGACCGA
GAGTTACATAAATGGTACCGGGGGACTGAGAAGTTACGTGCGAACTACTACCACTCTCT
ACCTACGATGAGGCGTTGT–3′

M2-350 (336 bp)

5′–ACAACGCCTCGAAAGGATGGAGTAAGTCTGGCAATCGACACTGACGCTGTGCGCAG
ACGATACGTACGTGTTGCCACAATGAATGAAAGAAAGAAAATATTAAAAACTTTGAGC
CAAAATAAAAGAAGCAACGTAAGGGAAATAAAACAATCAACAAACGGTGGAGAAGA
ATATGCGTAAAAAATGGGAAAAACAAACAAAATAAAACAAAAGTAAATAAATAAATA
AAAATAAAATGATATAAAATAAAATAAAAATTAATAAAAATAAAATCTAAATCTAAAA
ATCAACAAATGCCAAGTAGAACTAAGAGAAAAAGTGGGGGGGAGGCGTTGT–3′

B7-450 (252 bp)

5′–GGTGACGCAGCAGGTATAAAGCACGAAGAACTAAGAATACGTATGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTGTGTTTATTTCTTCGTATATTTA
TTTGTGCGTGTGTTTGTTTGTTTGTGTATTTAATCACTAGTGAATTCGCAGCCGCCTGCAG
GTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGATTCTATAGTGT
CACCTGCGTCACC–3′

M2-450 (465 bp)

5′–ACAACGCCTCATCGTAGGTGGAGCATGGAAGTGGTTCGCACGAAATTTCTCAGTTCA
CTGGTACCACTTATATAACTATCAGTTCATTATTTAATATGATATCGCCCCATTGTAGGTG
GGAGGAGCAGCGTGGTGTGTGTTTGTTTGCGTATTTGTATGCATTTCTTTGTTCGTGTCTTT
GTTTGCTTGTGTACGTTTGTTTGTTTGTGTATTTGTATGCATTTGGTTGGTTGTACCTTTGTT
TGTTTCTGTGTGTGTTTGTTTGTTTGTTTATTTATGCTAGTTTATGCTTGTTTGTATTTTCGTA
TATTTACTTGTTTGTTTGTGTTTTTATATTTCAATGGGCGTGTATTTTGTTATTCTATATTCT
ATTTAGCCCCTGAAGCGTGTGTCCTTCTATATCGTTTTTATTTCAATTGAGCGTTTTATAAT
TCCATATTCTAATATACCCCTGGAGGCGTTGT–3′