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TECHNICAL NOTE

Isolation of simple sequence repeats from groundnut

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Abbreviations: AFLP: amplified fragment length polymorphism
MW: molecular weight
PCR: polymerase chain reaction
SDS: sodium dodecyl sulfate
SSR: simple sequence repeat

SSRs have proved to be the most powerful tool for variety identification in groundnut of similar origin, and have much potential in genetic and breeding studies. To facilitate SSR discovery in groundnut, we proposed a highly simplified SSR isolation protocol based on multiple enzyme digestion/ligation, mixed biotin-labeled probes and streptavidin coated magnetic beads hybridization capture strategy. Of the 272 colonies randomly picked for sequencing, 119 were found to have unique SSR inserts.

Groundnut or peanut (*Arachis hypogaea* L.), is an important crop worldwide, distributed across the vast area in tropical, subtropical and temperate zones. It is a valuable source of edible oil and protein for human beings, and of fodder for livestock. In contrast to its apparent diversified variations in traits, its genetic variations at molecular level as detected by RAPD, RFLP, and SSR analysis, proved to be unexpectedly low (Halward et al. 1993; Krishna et al. 2004). In that case, the genetic linkage maps published were constructed using wild *Arachis* species (Halward et al. 1993; Burow et al. 2001; Moretzsohn et al. 2005).

Several workers (Hopkins et al. 1999; Gao et al. 2003; Ferguson et al. 2004; Moretzsohn et al. 2004) have reported groundnut SSR primers developed either based on traditional library construction and screening or by exploiting an AFLP pre-amplification protocol, with variable rate of success. Yang et al. (2005) identified 24 new groundnut SSR-containing sequences by means of GenBank inquiry. To facilitate SSR marker development in groundnut, we presented a highly simplified SSR DNA isolation protocol with good results.

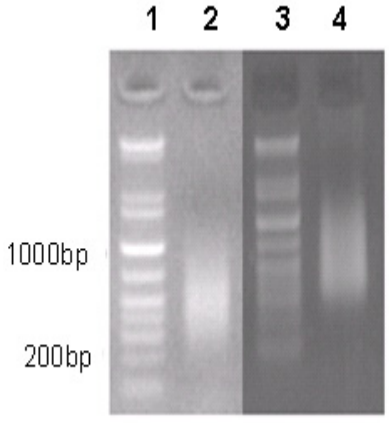

Figure 1. Preamplification product (Lane 2) and PCR product of captured DNAs (Lane 4). Lanes 1 and 3: 1 Kb plus DNA ladder (Tiangen).

MATERIALS AND METHODS

DNA was extracted from leaves of field-grown groundnut plants of 24-3, a hybrid derivative of *Arachis hypogaea* L. x *A. glabrata* Benth PI262801, following a modified CTAB method as described earlier (Wang et al. 2004). DNA digestion and ligation mixture (60 µl) containing 10 x NEBuffer4 6 µl, BSA (100x) 0.6 µl, groundnut genomic DNA 0.6 µg, AP11/AP12 adaptor 15 pmol (AP11: 5'→3' CTCTTGCTTAGATCTGGACTA, AP12:5'→3' pTAGTCCAGATCTAAGCAAGAGCACA), 10 mM ATP 6 µl, *Dra I* (NEB) 0.5 µl, *Hae III* (NEB) 1 µl, *Rsa I* (NEB) 0.5 µl, *PshA I* (NEB) 0.5 µl and T4 DNA Ligase (NEB cat # M0202T) 2 µl, was incubated at 37ºC overnight, and at 80ºC for 20 min to de-activate the enzymes. Ten microliters of digestion and ligation product were pre-amplified using 3 µl of AP11 primer (10 µM) in a volume of 50 µl, and the PCR profile was 72ºC for 2 min, 94ºC for 2 min, and 10 cycles of 1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and a final extension step of 72ºC for 10 min.

The hybridization mixture (30 µl), made up of 100 ng of the pre-amplification product, 6XSSC, 0.1% SDS, and 200 ng each of 5' biotinylated $(TA)_{30}$, $(CA)_{20}$, $(GA)_{20}$, $(AGA)_{15}$, $(TGA)_{15}$, $(ACA)_{15}$ (Sangon Ltd), was subjected to 5 min of denaturation at 95ºC and 1 hr of re-naturation at 60ºC. Two hundred micrograms of streptavidin-coated paramagnetic beads (Promega), previously equilibrated with 6xSSC for 3 times and 6xSSC, 0.1% SDS for 1 time, were then added to the mixture. The mixture was incubated at 60ºC for 15 min with gentle agitation at 5 min intervals. Liquid was removed using a magnetic separation stand (Promega). Beads were washed with gentle agitation with 300 µl of 6xSSC, 0.1% SDS at room temperature for 15 min for 2 times, with pre-warmed 6xSSC, 0.1% SDS (60ºC) at 60ºC for 15 min for 2 times, and then with 300 µl of 6xSSC at room temperature for 15 min for 2 times to remove SDS. After removal of final wash, captured DNAs were eluted from the beads with addition of 200 µl of T.E preheated to 95ºC, gentle flicking of the Eppendorf tube, and incubation at 95ºC for 10 min. With the aid of the magnetic stand, eluted DNAs in T.E buffer were quickly transferred to an aseptic tube in ice bath, and then desalted at 4ºC using a Millipore Microcon YM-100 column according to producer's recommendation. The probes in the captured DNAs were also removed during this process, so were the ssDNAs with MW lower than 300 nt.

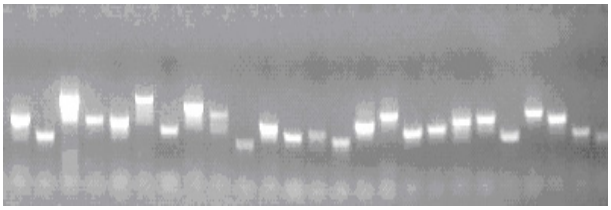

Figure 2. PCR screening of the white colonies for transformants harbouring plasmids with inserts. Rightmost lane: 1 Kb plus DNA ladder. The rest lanes: PCR product from individual colonies with inserts of varied length.

The resultant DNAs were amplified using primer AP11, purified and ligated into a pCF-T vector (Tiangen Biotech). Chemically competent cells of TOPO 10 were utilized in heat-shock transformation. Length of inserts was determined using a colony PCR procedure involving heat treatment of white colonies with TTE buffer. DNA sequence was analyzed on an ABI 3730XL sequencer using the M13 forward/reverse primer. After removal of the sequence of vector and adaptor and exclusion of redundant sequences, SSRs in the inserts were identified by exploiting the SSR Hunter and Tandem Repeat Finder search tools.

RESULTS AND DISCUSSION

Agarose electrophoresis of pre-amplification product showed that multiple enzyme digestion/ligation procedure produced DNA fragments of expected size (200-around 1000 bp) (Figure 1). PCR product of captured DNAs was in the similar MW range (Figure 1). Sixty colonies were randomly picked for colony PCR using AP11 primer. All of them harbouring plasmids with inserts of expected size (Figure 1 and Figure 2).

Plasmids were extracted from the colonies and inserts sequenced using M13 forward/reverse primer. Of the 272 colonies for sequencing, 259 were non-redundancy sequences, and 119 were found to have unique SSR inserts (Table 1). All of the six probes used could be directly related to these sequences; the (cgc) 4 SSR was an only exceptional case. The ratio of non-redundant SSR inserts was 43.7%. Although it may not be the highest in groundnut SSR isolation, due to the judicious choice of restriction enzymes, and a probe removal step for uprooting probe-primed PCR, most of these SSRs identified were found to possess flanking sequences needed for primer design; we were able to design 123 “good” primer pairs for further evaluation. In Hopkins's report, 66 (55.0%) out of the 120 sequenced “positive” clones had SSRs, but only 26 (21.7%) primer pairs could be designed, where both the occurrence of short tandem repeats (<6 core unit) and the close proximity of the SSR to the end of insert DNA limited the ability to design primers for the majority of the SSRs identified (Hopkins et al. 1999). Gao et al. (2003) identified 14 (5.5%) unique SSR-containing sequences in 256 clones. He et al. (2003) sequenced 401 randomly picked clones resulting from AFLP pre-amplification based protocol, 83 (20.7%) of which were unique SSRs, and 56 (14.0%) primer pairs were designed. Moretzsohn et al. (2004) pre-screened the clones before sequencing using SSR-anchored PCR strategy and found 162 of the 750 clones had SSRs. There were 91 unique sequences, but only 67 were suitable for primer design (41.4% of positive clones). Ferguson et al. (2004) identified 348 (21.3%) SSRs by sequencing 1,627 clones, merely 226 (13.9%) primers could be designed.

In contrast to previous reported SSR isolation protocols, our simplified protocol utilized 4 enzymes to cut groundnut DNA into ideally sized fragments which were ligated to adaptors in a single tube. The present SSR enrichment protocol adopted a multiple enzyme digestion/ligation procedure apparently similar to AFLP pre-amplification based protocol, but the product in our protocol was in the range of 200-1000 bp, whereas in groundnut EcoR I/Mse I AFLP protocol, the pre-amplification product was generally between 70 and 500 bp. Too short DNA sequences in the latter case may increase the possibility of lack of adequate flanking sequences. With the advance in sequencing facility and technology, the number of base pairs of DNA readable in a single sequencing reaction tends to be longer and longer, and DNA inserts of ~1000 bp do not necessarily mean more cost.

It can be seen from the Figure 3 that ct/ag repeat motif had the highest frequencies, followed by ga/tc, ttc/gaa and ca/tg. Indeed, ct/ag repeat was reported to be rich in other plant species, and was the most frequently dispersed SSRs of groundnut in He's report (He et al. 2003). The results to some extent may reveal the relative abundance of different repeat motifs as well as the ease of capture.

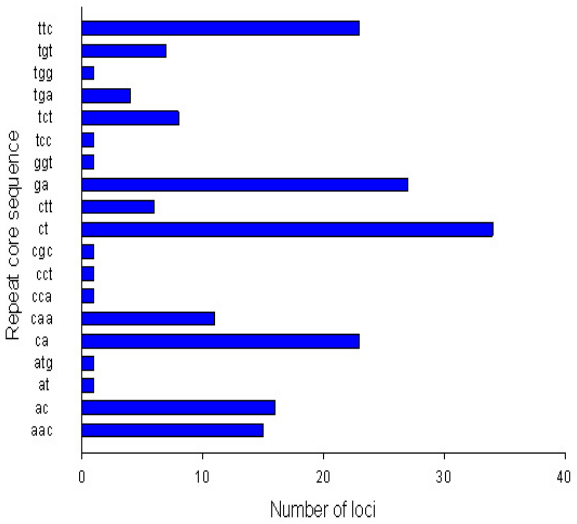

Figure 3. Frequencies of SSRs with different core sequences.

The copy number of the SSR core sequences was also highly variable. SSRs, even for 3-nucleotide core sequences, with copy number higher than 40 were not strange. The number of repeats may exceed 80.

In the present study, of the 123 newly designed primer pairs tested in 12 peanut varieties/lines mainly bred in Shandong province, China, only 44 (35.8%) produced polymorphic bands (Huang et al. 2006). Despite the fact that several hundreds of SSRs have been isolated from groundnut, only a small portion of them showed polymorphic in the cultivated groundnut, far from the need for map construction let alone QTL mapping. Strengthening groundnut SSR development is absolutely necessary. Compared to previous protocols reported in groundnut, the present protocol was efficient, time-saving and easy to follow. In all previous reports without exceptions, the cultivated groundnut was the only plant material used to isolate groundnut SSRs; in this study, a hybrid derivative was exploited instead. Considering the polyploidy nature of the groundnut crop and frequent occurrence of multiple banding patterns in groundnut SSR analysis, use of the inter specific hybrid derived material makes it possible to isolate SSRs originated from both cultivated and wild groundnut.

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APPENDIX

Table 1. Property of the newly identified groundnut SSRs.

Sequence ID	Repeat motifs	Type	Primer ID	Primer sequence (Foward)	Primer sequence (Reverse)
CTW-06	(ct)27	perfect	S-01	TGGACTAGACAAGGAACAACCA	GAGCCATGAGCACACAACAC
CTW-06	(ct)4	perfect			
CTW-07	(gaa)5	perfect	S-02	TTGTTGCTATTTAGGGTGATTGA	GTGGGACAAGGCTTTGTTGT
CTW-07	(gaa)5	perfect			
CTW-07	(aac)5	perfect			
CTW-10	(tct)5	perfect	S-03	GCACCAATTTTGTCCCTGAT	AAGGGGTTTGCACGTAAATG
CTW-11	(ca)10(ct)8	compound	S-04	GAACGCCAGTTTACGTCGTC	TTGGGACACTTACCGAAGAGTT
CTW-11	(ct)4	perfect			
CTW-13	(ac)53	perfect	S-05	CCGGCTAGAGAATACACACACA	CCGGCTAGAGAATACACACACA
CTW-13	(ca)4	perfect			
CTW-13	(ca)4	perfect			
CTW-14	(ac)84	perfect	S-06	CCGGCTAGAGAATACACACACA	TCCTCCTTCCTCCTTGAACA
CTW-15	(tct)5	perfect	S-07	GCACCAATTTTGTCCCTGAT	CAGAAGGGGTTTGCACCTAA
CTW-16	(ac)12	perfect	S-08	AAGTCCAAAATGCATGCTCA	GGCTCTGTGTGGTAGGGTGT
CTW-18	(ag)5	perfect	S-09	CGCTGTCCTTATCGAACCAT	CTCTCACTCGCGCTTTCTCT
CTW-18	(ca)4	perfect			
CTW-18	(ga)4	perfect			
CTW-18	(ga)4	perfect			
CTW-19	(gt)32	perfect	S-10	CAAGCCAAAAGTGGAAAACC	TCCTTTTGCTAATGCGGTCT
CTW-19	(gt)4	perfect			
CTW-19	(ct)5	perfect			
CTW-20	(ct)4tt(ct)9	imperfect	S-11	ATGACGGCAGTAGCAGAAGC	TTGAGGAGAAGACGCTGTTG
CTW-20	(tc)4	perfect			
CTW-20	(ct)4	perfect			
CTW-21	(caa)3cca(caa)2	imperfect	S-12	TCATTGACCTAGCCGAATCC	GAGGGACCAATTGTTGGTTG
CTW-23	(aac)5	perfect	S-13	TTGTTGCTATTTAGGGTGATTGA	CGTCGTTTGATTCATGTAGCC
CTW-23	(gaa)5	perfect			
CTW-23	(gaa)5	perfect			
CTW-25	(ca)12	perfect	S-14	AGGCAAACCACTGCAAGAGT	CGCTTCCCTGGGATACTTAG
CTW-26	(ctt)5	perfect	S-15	TGAACGAAAAATGCTAATGTGG	CGCAGAGACGTGTTGAAGAA
CTW-26	(ctt)5	perfect			
CTW-26	(ctt)4	perfect			
CTW-28	(tc)9	perfect	S-16	TGGTAGTGGAGTCAGAGTGTGTG	GTTGCATTGCCCAACTCTTT
CTW-28	(gt)4	perfect			
CTW-29	(ct)16	perfect	S-17	CATTGGAAAGATCCGACGAT	GTTGCAACAACGACGATGG
CTW-29	(ct)4	perfect			
CTW-31	(ct)5	perfect	S-18	CAATAAATTCGTCGTAT	GAGAGAAGAGAAGGTTAGAGA
CTW-33	(ct)24	perfect	S-19	GCTCCACTAGTGCCGAAATC	CAGACACCCGGAGGCTTA

CTW-36	(caa)5	perfect	S-20	CACGAACAGCCACTCAAAGA	CTCTGGGGGACTAGCTGTTG
CTW-39	(ct)15	perfect	S-21	AGTCCTACTTGTGGGGGTTG	TCCCTTTTGCAGTGAAATCC
CTW-41	(ac)8(at)5	compound	S-22	CGTGACAAACATGTGCTGCT	TTTTGGAATCTGTTTATGGGAAA
CTW-51	(tgt)4	perfect	S-23	CTGGAAGTGGTCCTGTTGGT	GCTGCTCCTGTCTCTGGAAT
CTW-52	(ga)22	perfect	S-24	GGCAATGCACACGCTACTCT	CGTGAGGCGTGAGAGTTCAT
CTW-54	(aga)7	perfect	S-25	GCTATGCTTTTACCACACCAAA	CCATTCATGGTCATCCCTTC
CTW-54	(ag)4	perfect			
CTW-56	(aac)4	perfect	S-26	ACATGAGTGCCCAACTAGCC	TGCAGAGCTTCAACAACCAC
CTW-66	(ca)4	perfect	S-27	ATCCGGCTCACAGTTCAATC	GCCAAGGCTGAAAAGAGTTG
CTW-68	(ttg)4	perfect	S-28	TTGCAAGATGTGCATCAAAA	TGACAAACCAACAAACGACA
CTW-68	(gt)4	perfect			
CTW NEW_67	(ttc)15	Perfect	S-29	CACCGCCGCCCGTTTCTTCTCCT	GGGCAACGGCTCGACGGTGGTATC
CTW NEW_67	(cct)5	perfect	S-30	CTTCTTCTTCTTCCCGCCACC	GGCGGGCGACGGGCAAC
CTW NEW_124	(tc)3tt(tc)10tt(tc)4	imperfect	S-31	GGCGGCGATGTAGAACCCTCCAGTAG	ACCGCCATCGCCATCGTTGTTGT
CTW NEW_174	(ct)2cc(ct)19	imperfect	S-32	GCATTCGCGCAGCAACC	CCAGAGTAGAGCGGCAGTCC
CTW NEW_270	(caa)4	perfect	S-33	AGATCGCCGCCCTTACCAAAACCT	AGAAAGCCCCAAAATCGTGAGTAACAT
CTW NEW_263	(tgt)4	perfect	S-34	ACCTTCTTCCGCGCTTGTTTCAG	TCCCAGCTCCGATCCTCATACTTCA
CTW NEW_74	(ga)5	perfect	S-35	GCGGTTGCCTGGGTCGTC	CCGCAATGGAAGTGGGAAAGTAT
CTW NEW_227	(tct)6	perfect	S-36	GGCAACGCGTGGTAGCAGTG	GAGTGAGTGAACCAGAAGGAAGGA
CTW NEW_33	(gt)8	perfect	S-37	GACCGCGGCTCCACTTCTTTCTCT	ACATTCCCCTTTCACCCCTCACAAC
CTW NEW_51	(ga)17	perfect	S-38	GGCAGCGAAGCACCCATTGTTA	GTAGGGTTGCGTTTCGTTTTCTTATCG
CTW NEW_72	(aca)4	perfect	S-39	TCCAAAATCAACCAGAAAGCAGAAGCAGATG	AGGAAGAGAAGCGGAGAGGGAGAGAAG
CTW NEW_204	(ca)7	perfect	S-40	ACCCAACACTAGCCGCCACTGA	GCAACGCCTCCTCCTCTTCCTCTA
CTW NEW_16	(aac)4	perfect	S-41	AGAGTATGCGGAATTTGTGCTGAT	CCCGTTGTTGGTTGTGATGG
CTW NEW_209	(caa)4	perfect	S-42	GAGGGGGCGAACGTTGGACTTG	GCCGGAGCACTTGAGCATTTTT
CTW NEW_7	(aga)6	perfect	S-43	ATTCTTTGGACTCGGGTTCATACTTTG	ACACCATCCCTCACTCTCCTCCATA
CTW NEW_197	(tg)17(ag)17	compound	S-44	GGTGTTGAGGGATGGTTGTTCTAA	CTTTCCCGCCTCTCCCTCTC
CTW NEW_62	(tga)5	perfect	S-45	AGGTGTTGTGGCATTGTTCTTCAT	CGGCGGTAGCGGTAGCGGTTAT
CTW NEW_77	(gaa)8	perfect	S-46	ATGGCGAATCGGAGGGTAGGTT	TCCAATCGTGCGTTTCAATCATCT
CTW NEW_19	(gtt)5	perfect	S-47	ATTCTGAGGCTGCTTCCCAAACT	CTGCCATGTAAGCCGTGAATAAG
CTW NEW_86	(gtt)5	perfect	S-48	ATTCTGAGGCTGCTTCCCAAACT	CTGCCATGTAAGCCGTGAATAAG
CTW NEW_36	(gt)11(ga) 7ggaggaa(ga)6	compound	S-49	GGCAGCGAAGCACCCATTGT	CGTTTCGTTTTCTTATCGCACTTC
CTW NEW_241	(aac)4	perfect	S-50	ATGCACGCAACTACAGGAAGATAAC	TGCGCAAGAGAACGGAACAT
CTW NEW_71	(tc)7	perfect	S-51	CCCAATTCGCATAAAAACAGAGAC	CGAGCCGCAATCCAACACT
CTW NEW_74	(ga)5	perfect	S-52	CCCTGAGAATGAAAGAAAGAAACA	CAACCGCAGCGACGATAGATG
CTW NEW_92	(tct)6	perfect	S-53	CACACCCATCCATCTCCTCCATA	TGTCTTTGTTGCTCCTCCCTCATT
CTW NEW_234	(ca)5(ga)35	compound	S-54	GTGTGCCATGTAGGTGTGACTG	GTTTGCCCTCTTGTTTTCTCC
CTW NEW_37	(ag)5	perfect	S-55	ACCCCCAACTGCACTACTATTCATTTT	CGACGCGGCGAGGCTTCC
CTW NEW_202	(tc)16	perfect	S-56	CATAGGCGTCCCATTGCTTACAG	GATTACGCGCTCTTTCATTTG
CTW NEW_252	(ttg)9	perfect	S-57	AGGGCGAAAGGCAGAGGAAGA	AAAGGGGTGAGACAGCCAATAACAT
CTW NEW_231	(tc)5	perfect	S-58	GAGCGAAAGAGAACGAGACAACAA	TCGGGGAGGATCAACCAAATAG
CTW NEW_62	(gtt)4	perfect	S-59	TTGGTGGAAGCCCTAGAGTGAGTGAA	ATGGAAATGAAGCCGATAAGAGA
CTW NEW_92	(tc)5	perfect	S-60	TTGGTGCAGGGATGTAAATG	ATATGGAGGAGATGGATGGGTGTG
CTW NEW_137	(aac)4	perfect	S-61	GAGGAGGCAGAGATAATCAGG	GAGAGGTCTGCTGTTGGGTAT
CTW NEW_166	(tg)6	perfect	S-62	CAAGTGGGGGGTTTATGGTG	CCCCCTCCATCACCCCT

CTW NEW_200	(ag)13a(ag)2	imperfect	S-63	CACCGTGGTATGATCGTTTCTTTT	GTTCGCGTGGGATTGTTTGTGT
CTW NEW_51	(gt)11(ga) 7gtgagga(ag)6	compound	S-64	GGCAGCGAAGCACCCATTGT	TCCTTCGACCCTATCTATCAGTATCAC
CTW NEW_67	(ctt)9	perfect	S-65	CGATACCACCGTCGAGC	CAAGAACCCAGAATCAGGAAG
CTW NEW_130	(tg)15	perfect	S-66	ACCCCCATTGAGCGATTTG	AGTCCCATTGCCTTTCTTCTGTAT
CTW NEW_157	(aac)6	perfect	S-67	TCTCCTTCCCGAACAACCCTATTA	ATTGTTGACTTGGCTTCGTTCCTA
CTW NEW_137	(aac)6	perfect	S-68	AATCAAGGTGGCAACTACAGC	AGACACTATACTTGCAACGAGGAT
CTW NEW_17	(ttc)4	perfect	S-69	GGGGAGTCGTGTCAAGCCATTA	ACCCCAAACCCAACCCTCAC
CTW NEW_43	(ttg)5	perfect	S-70	CCTTTCCCATTCCATTAGC	GTCCGAGTTGAGGAACAACAA
CTW NEW_88	(tct)7	perfect	S-71	ACCTCTTTCCCTCTCCTCCATA	TTCCTTGCCTCTGTTGTTTGAT
CTW NEW_139	(ag)6	perfect	S-72	TACAGCCCAAATGGAATGAGAA	GAGTTGGGAAGAAAGGATGAAGAT
CTW NEW_68	(aag)8	perfect	S-73	AGTCCACTGAACCGAACACCAATC	TCCCTACCACCGAACGAAACAAT
CTW NEW_20	(aac)9	perfect	S-74	GCACGCGCTCAGGACAAAT	AGGGCGAAAGGCAGAGGAA
CTW NEW_249	(ttc)4	perfect	S-75	ACACCCTCCTCAACATCAAAT	ATACCCAAGCGAAACAAGAATC
CTW NEW_36	(ga)18	perfect	S-76	ATACTGATAGATAGGGTCGAAGGAGAG	CAACGAAAGAAAAATAAGGACATAGTG
CTW NEW_194	(ct)9	perfect	S-77	CACCCCTCACTACAAGAAAAATAC	ATGGCGGAGAAGAGGGAGGAG
CTW NEW_199	(caa)6taa(caa)4	Imperfect	S-78	TCCAATTCAATCTCACTAAAAACT	CAAAGGGGAGCACGAACATAAG
CTW NEW_193	(ttc)5	perfect	S-79	AAACCACGCAGTCCGAATACA	CTTGATGGGCTTTGGAGATAA
CTW NEW_202	(tct)6	perfect	S-80	GGCGTCCCATTGCTTAC	AGAATGCGTTGATGTTATGAA
CTW NEW_119	(tc)26	perfect	S-81	GCTTCAGTGGTGGGCTCAT	TATCATAGTAAAAAGGTGGGAACAAT
CTW NEW_219	(tgt)4	perfect	S-82	TTGCAAAGTAGCGTTCAGAC	CATGGATGGCAGGACAAT
CTW NEW_271	(ca)15ta(ca)11	Imperfect	S-83	CTTGAACTTATTTTTGGTGGGTGAAC	CAAGGGAGAATGAAGAATGCTAAG
CTW NEW_274	(ga)9	perfect	S-84	CAGCCAATATGTCACAACCCTAAT	CTCCCACTACAAATCTCCAATCAAT
CTW NEW_178	(ct)12cc(ct)14	Imperfect	S-85	AAACTATCACCGACAAAAA	AGAGACATAAGCCGAGAGG
CTW NEW_32	(ttc)14	perfect	S-86	TCCATGAGGGGTTATAGGTGTTT	GGGTGTATTTCTGAAGTTCCATTATC
CTW NEW_67	(ggt)8	perfect	S-87	TCTGAGTTCTGGCTTTTGAT	CACCACCACCATCATCATCAT
CTW NEW_128	(ag)43	perfect	S-88	TCAAAGAAGCAATAAAAATC	CTCCACCGGCAAGCACCTC
CTW NEW_82	(ct)19	perfect	S-89	ATCTATGGCCGGGTTGGTT	AGGTGGTGGGTAGTGCTTCTG
CTW NEW_231	(ttc)11	perfect	S-90	GAGAGCGAAAGAGAACGAGAC	GAATTGGAATCCATAGCCAT
CTW NEW_162	(ttc)8(tcc)2(ttc)4	compound	S-91	TGAGGGCAGGGGAAGAT	CGTCGGTGGTTGAAGCAGAG
CTW NEW_219	(tgt)4	perfect	S-92	ATTGGCAGATGAAGAAGGA	GGGAAATCAGAGGTGGAATAA
CTW NEW_38	(tg)10(ag)14	compound	S-93	TTGGGGAAATACAGAATAACG	CTCCCACATCCCCACCAT
CTW NEW_185	(ag)25	perfect	S-94	TTCCCAAAAATAGTCAACCA	TCTTCCTCTGCCTTTCATCCA
CTW NEW_40	(ca)5	perfect	S-95	AACCCCAACCATCAAACAAACA	ATGGTATCACTGGGAAATG
CTW NEW_193	(tct)4	perfect	S-96	ATACACATTCCTCTCCATCTCCT	TTTTTCTTCCCTTTCTTCTTTCTA
CTW NEW_206	(ttg)13	perfect	S-97	GAATCGCGTCTCAGGTG	TATTGCTTACGATTATTTTGT
CTW NEW_225	(Ac)7	perfect	S-98	TTAATGAACCCAAATACACA	AGCCAAAACCCTAAAAACTA
CTW NEW_162	(ttc)7	perfect	S-99	GGGCAGGGGAAGATCAATA	ATGAGGGTGAATTGGAATTGG
CTW NEW_74	(ac)5	perfect	S-100	AAGCGCCATATGTGTTTGA	CCCGTCTTGGCTTTCTTCT
CTW NEW_220	(tca)4	perfect	S-101	AGTGCGTTTGGCTCATCA	ATTTCGTTCATTTAGTCCATAGA
CTW NEW_67	(tga)5	perfect	S-102	TCCTGATTCTGGGTTCTTGA	CCATCCACTGCCACTCCAT
CTW NEW_152	(gt)14	perfect	S-103	ATGTGGGAATTATGGGTAGC	ATGGCGTGACAAAAGAATC
CTW NEW_253	(ttg)8	perfect	S-104	GAATCGCGTCTCAGGTGGTTT	TTAGATGAGTATGAAGAGATTAT
CTW NEW_211	(ag)9	perfect	S-105	AAGCTCATTTCATCACAA	CCACAAACGGCTCATCAATC
CTW NEW_78	(gaa)11	perfect	S-106	GCCAGCATAGAAGCATAATAACA	GAGTAATAGTGAATCAATGAGAAGAGG
CTW NEW_277	(gaa)6	perfect	S-107	TTCAATAATCCAAACCTCATCA	CTGTTTGCGTTTTTCTACTCTG

CTW NEW_177	(tc)14(ac)15	compound	S-108	GCTTACATTACACGTCATCTC	CCGAACTTACAGTTAGGAG
CTW NEW_27	(ag)21	perfect	S-109	AAGGGAGCACAATCATA	GAGCACGAGTTCATACAC
CTW NEW_9	(aac)37	perfect	S-110	TTCTAGTAGTAAAAATAAAAACAC	GTCAAAGGGAGGCACGAACATAAGT
CTW NEW_136	(gt)20	perfect	S-111	TGAAAATTAAAACTACCAACTACA	TGCCCCAAGATAACACAAT
CTW NEW_254	(atg)4	perfect	S-112	ACTGCTAGCGTTGTTTTCTTCC	CATTACACCTTCACCAACACCA
CTW NEW_78	(tc)9	perfect	S-113	TTGCATGTAGGAAAGAAAGATT	TTGGATGTGGTGGTGATGT
CTW NEW_133	(ct)12	perfect	S-114	AAGAGACGAAAGTGAGTTAGC	GGGAGCATGTTTAGGGAGAC
CTW NEW_182	(cca)5	perfect	S-115	GGTAATATGCCTTGGTGAC	TTCTTGATAATTCTGTGGAT
CTW NEW_217	(ttc)4	perfect	S-116	GATTTGTTTTCTTCTTCGTTTTT	CATAATCCACTTCGCCCTAAT
CTW NEW_266	(ttg)8	perfect	S-117	GGATAAAATAAGGAATGA	TTGCAAGTAAGTAATACAA
CTW NEW_78	(aat)6	perfect	S-118	TATATGATGCTTGATTGAGACT	CATGTAGAAGGCTTGGAGGGTAT
CTW NEW_217	(ttc)4	perfect	S-119	CTTCTTCGTTCTTCTTCC	ACGCGTTAGTCTCACAGTCA
CTW NEW_35	(tc)25	perfect	S-120	TTCAAACTACATCTCAAACTAT	TGTGCCAGGACCCAAAAT
CTW NEW_8	(aca)4	perfect	S-121	TTCTCAAAGTCTGTCTGG	TTTAGCAATTGGTTCTTA
CTW NEW_32	(gaa)4	perfect	S-122	TTTTTCGATTTTCATGGTTTCTG	TTTCTCTTTCTCCTCATCTTCTGC
CTW NEW_12	(ct)11	perfect	S-123	GTATGGTGACTGTAGTTCTC	AGTGACCAAAATAGAAGC
CTW NEW_103	(tg)12	perfect			
CTW NEW_104	(tgt)15	perfect			
CTW NEW_12	(cgc)4	perfect			
CTW NEW_171	(aac)6	perfect			
CTW NEW_184	(tc)24	perfect			
CTW NEW_190	(ttg)7	perfect			
CTW NEW_194	(tc)8	perfect			
CTW NEW_215	(gga)4	perfect			
CTW NEW_222	(ct)24	perfect			
CTW NEW_223	(tg)35	perfect			
CTW NEW_227	(ct)21	perfect			
CTW NEW_23	(ca)28	perfect			
CTW NEW_257	(tg)26	perfect			
CTW NEW_259	(tg)11(ag) 13tg(ag)10	compound			
CTW NEW_277	(gaa)4gga (gaa)gag (gaa)6cgc (gaa)taga (gaa)40	imperfect			
CTW NEW_52	(tg)51	perfect			
CTW NEW_58	(ct)31	perfect			
CTW NEW_67	(tga)4(tgg)7	compound			
CTW NEW_68	(agg)4	perfect			
CTW NEW_85	(tg)7	perfect			
CTW NEW_97	(tc)13	perfect			
CTW NEW_98	(tg)12	perfect			