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In silico identification of expressed sequence tags based simple sequence repeats (EST-SSRs) markers in *Trifolium* species

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ABSTRACT: The EST-SSRs (genic SSRs) are the microsatellites in expressed sequence tags (ESTs). Advancement in functional genomics has contributed mainly for the identification of ESTs in different species and made them available in databases. They are of transcribe regions, so are more conserved and considered with more cross transferability across the taxonomic borders. In *Trifolium alexandrinum* (Berseem), no expressed sequence tags (ESTs) are available in NCBI database yet. Therefore, in this study by considering the cross transferability potential of EST-SSRs, expressed sequence tags of *Trifolium pratense* and *Trifolium repens* were retrieved from NCBI database and their EST-SSRs were *in silico* identified. We retrieved 1014 ESTs for SSR identification from both *Trifolium* species and identified 198 EST-SSRs from them. In identified EST-SSRs, trinucleotide SSRs repeats were found to be more frequent. Of these 198 *in silico* identified EST-SSRs, 44 showed cross species amplification in *T. alexandrinum* and was proved for their transferability potential. The identified EST-SSRs could be a great source for genetic diversity and population genetics studies of *Trifolium* species. These EST-SSRs could be further screened to investigate their linkage to disease resistance in *Trifolium* species.

KEYWORDS: expressed sequence tags, simple sequence repeats, microsatellite, berseem, *Trifolium* species

INTRODUCTION

Leguminous crops have global importance towards the sustainable agriculture. These crops are rich in protein and oil contents that make them good nutritional source for the members of kingdom Animalia [1]. Nitrogen fixation is one of the remarkable properties of their candidate species that is being used to enhance soil fertility. However, the important constituents of these species are their natural composite or secondary metabolites that involve in symbiosis as well as in plant stress responses [2, 3]. Apart from all, their recognition is also existed as major intercropping species. In this respect, clover (*Trifolium* spp. L) is distinguishable group of legume crops that belongs to family Fabaceae and subfamily Faboideae. Whereas this genus; *Trifolium* consisted of mainly 250 annual or perennial species, native to Middle East, Europe, America and Africa [4]. Of them, twenty species (10%) are important to feed animals and are well known forage crops of several regions [5]. Normally these species are grown with companion grasses like rangeland but their silages also have many uses.

The most important species of *Trifolium* genus are *T. repens* (white clover), *T. pratense* (red clover) and *T. alexandrinum* (Egyptian clover). Among them, *T. alexandrinum* is of great importance in terms of animal feed [6]. It has been widely cultivated as multi-cut annual grazing crop in Africa and Asia, particularly in Indo-Pak [7]. The good quality, high yielding fodder crops with high digestibility in livestock are valuable features of berseem crop [8]. Moreover, the genetic analysis and characterization of this crop for diversity and for marker-assisted breeding is an important and preliminary step toward crop improvement [9].

Simple-sequence repeats (SSRs) markers are gaining importance due to their high polymorphic rate, high reproducibility, multi-allelic and codominant nature [10]. These features impart distinctness to SSR markers for exploring genomic variation and thereby make them valued for diversity analysis and marker assisted selection [11]. SSRs are abun-

dant, frequently occur in eukaryotic genome, and widely used in variation study of several legume crops including, white clover, peanut, pigeon pea, mung bean and berseem clover [12]. However, *de novo* development of genomic SSR is problematic in terms of cost, time and species specific property thus makes them not readily transferable to the other species. Therefore, the possible solution is to identify the genic SSRs or EST-SSRs to cross-taxonomic boundaries [13].

Expressed sequence tags (ESTs) are a short sequence of cDNA (complementary DNA) that give more feasibility and specificity of results towards high quality nuclear marker development. The cross transferability of EST-SSRs has been identified and evaluated in several legume crops including pea, fababean and chickpea, mungbean and dolichos bean [14]. Hence, in this research study, we have tried to identify the SRRs from EST sequences of *T. repens* and *T. pratense* through bioinformatic tools, which may have cross transferability into *T. alexandrinum* (Berseem) and thereby could be putative EST-SSR markers for molecular characterization.

METHODS

Mining of ESTs sequences

A total of 1014 ESTs sequences of *T. pratense* and *T. repens* were retrieved from the NCBI (National Center for Biotechnology Information) database. The non-redundancy of these ESTs were analyzed using SeqMan Pro v. 7.1.0.

EST-SSRs detection

The FASTA formatted file were uploaded in WEB-SAT program (microsatellite identification software tool). The criteria set for the detection of SSRs from ESTs sequences were 6 repeat units for di-, 4 for tri-, and 3 for tetra-, penta- and hexanucleotides.

Primer designing for SSR markers

For SSR markers, primers were designed from the flanking region of identified SSRs. Primer designing and analyzing tools provided by Integrated DNA Technology (IDT) services (eu.idtdna.com/pages) were used to design EST-SSR based primer pairs.

DNA extraction and PCR analysis

Three cultivars of *T. alexandrinum* (berseem), Agaite, Pachate and Anmol, were used to assess the cross transferability of identified EST-SSR. Total genomic DNA of these cultivars was extracted using modified CTAB method [15], and maintained in TE buffer. The quantification was performed by UV visible NANODROP (8000 Spectrophotometer, Thermo Scientific). PCR were carried out in 96 well thermal cycler (peqSTAR) with 50 µl of reaction mixture comprising of High-Fidelity PCR Master Mix (Thermo Scientific). The amplified PCR products were resolved on 2% high resolution Agarose gel and were visualized under UV light using Gel Documentation system (GDS) of BioRad, USA.

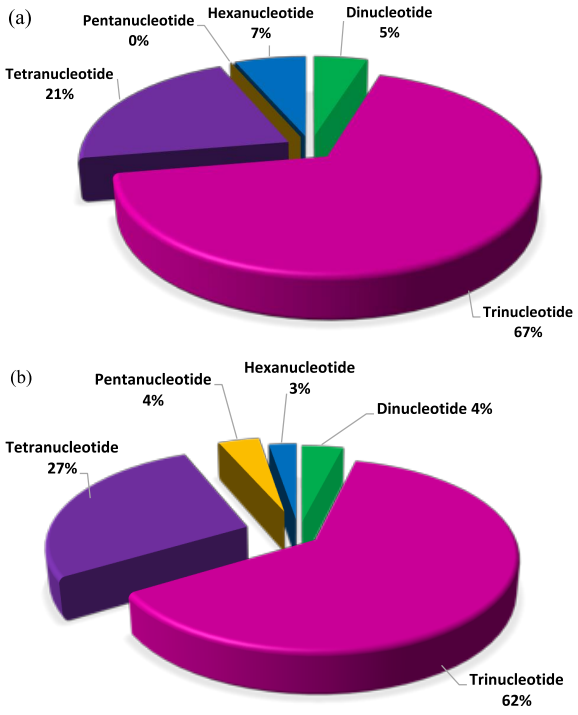

Fig. 1 Distribution frequency of dinucleotide, trinucleotide, tetranucleotide, pentanucleotide and hexanucleotide EST-SSRs in ESTs of (a) *T. repens* and (b) *T. pratense*.

RESULTS AND DISCUSSION

Molecular markers are the promising tools to measure genetic divergence in several plant species including isozyme, AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphic DNA), ISSR (inter simple sequence repeats) and SSR (simple sequence repeats) [16–18]. Hence, in this study, 198 SSRs were identified from 1014 ESTs sequences of *T. pratense* and *T. repens* (Table S1). Of these total identified SSRs, 121 EST-SSRs were identified from ESTs sequences of *T. pratense*, whereas 77 EST-SSRs were identified from ESTs sequences of *T. repens*. Trinucleotide

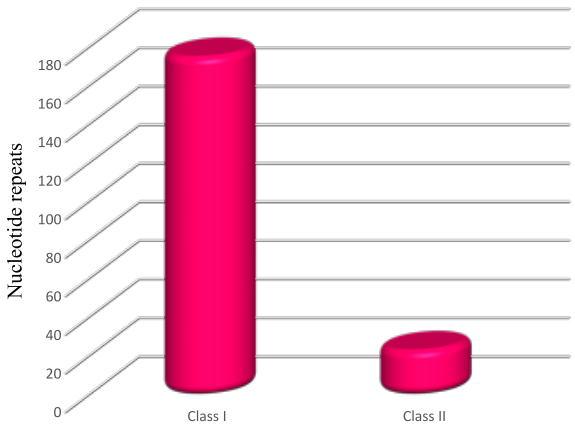

Fig. 2 Classes of EST-SSRs based on nucleotide repeats.

Table 1 EST-SSRs primers that showed (✓) and did not show (✕) cross species amplification in *T. alexandrinum* in PCR analysis.

Primer	Agaite	Pachate	Anmol	Primer	Agaite	Pachate	Anmol
TP5	✓	✓	✓	TR11	✓	✕	✓
TP8	✓	✓	✓	TR17	✓	✕	✕
TP10	✓	✓	✕	TR19	✓	✓	✓
TP11	✓	✕	✕	TR28	✓	✓	✓
TP14	✓	✓	✓	TR29	✕	✓	✓
TP21	✕	✕	✓	TR31	✓	✕	✕
TP22	✕	✓	✕	TR35	✓	✓	✓
TP23	✓	✓	✓	TR37	✓	✓	✓
TP29	✓	✓	✓	TR45	✕	✓	✓
TP37	✕	✕	✓	TR48	✕	✕	✓
TP45	✕	✓	✓	TR54	✓	✕	✕
TP48	✕	✓	✕	TR55	✕	✓	✕
TP50	✓	✓	✓	TR57	✓	✓	✕
TP51	✓	✓	✓	TR71	✕	✓	✓
TP55	✓	✓	✓	TR75	✓	✓	✓
TP59	✓	✓	✓	TR84	✓	✓	✓
TP63	✓	✕	✕	TR86	✓	✓	✓
TP65	✕	✓	✓	TR94	✕	✓	✕
TP72	✕	✓	✕	TR109	✕	✓	✓
TR6	✓	✓	✓	TR114	✓	✕	✕
TR9	✕	✕	✓	TR115	✓	✓	✓
TR10	✓	✓	✓	TR120	✓	✓	✓

was observed to be more frequent type of repeat in both *Trifolium* species. In case of *T. repens*, 82 trinucleotide (67%), 25 tetranucleotide (21%), 8 hexanucleotide (7%), 6 dinucleotide (5%) and zero pentanucleotide SSRs (0%) were observed (Fig. 1a). However, in *T. pratense*, 48 trinucleotide (62%), 21 tetranucleotide (27%), 3 dinucleotide (4%), 3 pentanucleotide (4%) and 2 hexanucleotide SSRs (3%) were observed (Fig. 1b). The nucleotide repeats of identified EST-SSR were grouped into two classes; Class I (12–20 nt) and Class II ($\geqslant$ 20 nt). The Class I repeats were found to be more frequent (Fig. 2).

A total of 104 different types of EST-SSRs motifs were identified which belong to 5 different types of nucleotide repeats, 8 different types of dinucleotide repeats, 44 different types of trinucleotide repeats, 35 different types of tetranucleotide repeat, 3 different types of pentanucleotide repeats and 9 different types of hexanucleotide repeats. The repeat type CT/AG (4) was more abundant in dinucleotide EST-SSRs, followed by TC/GA (2), AT/TA (1), GA/TC (1) and CA/TG (1) (Fig. 3a). However, repeat type GAA/TTC (10) was abundant in trinucleotide repeats followed by CAA/TTG (7), TGG/CCA (7), CAC/GTG (6), GAT/ATC (6), ACC/GGT (6), ATC/GAT (6), TTC/GAA (6), TCA/TGA (5), TCT/AGA (5), ATT/AAT (4), AAG/CTT (4), ACA/TGT (3), AGG/CCT (3), CTT/AAG (3), GGT/ACC (3), CCG/CGG (3), TGT/ACA (3), TAA/TTA (2), AAT/ATT (2), ATA/TAT (2), AAC/GTT (2), CCT/AGG (2), CTC/GAG (2), CCA/TGG (2), TGC/GCA (2), GTA/TAC (2), CGC/GCG (2), CAT/ATG (2), CAG/CTG (2), TAC/GTA (2), GCT/AGC (2), GCC/GGC (1), CTG/CAG (1), AGA/TCT (1), TCG/CGA (1), GTT/AAC (1), TTA/TAA (1), GAG/CTC (1), GGA/GCC (1), TGA/TCA (1), GCG/CGC (1), GTA/CAT (1), and ACT/AGT (1) (Fig. 3b). In tetranucleotide repeats, repeat type TTCC/GGAA (3) was frequently present followed TTAT/ATAA (2), ATTT/AAAT (2), TTTC/GAAA (2), TGTT/AACA (2), TCTT/AAGA (2), CCAA/TTGG (2), CATA/TATG (2), AATA/TATT (2), CTTC/GAAG (2), AAAC/GTTT (1), GAAA/TTTC (1), ACCT/AGGT (1), TAGA/TCTA (1), TAGT/ACTA (1), CTAG/CTAG (1), TTAA/TTAA (1), TGAT/ATCA (1), CCAT/ATGG (1), TCAC/GTGA (1), CTTT/AAAG (1), TAAA/TTTA (1), TAAT/ATTA (1), TCGC/GCGA (1), TTTG/CAAA (1), ATTC/GAAT (1), TTCA/TGAA (1), TCTA/TAGA (1), CAAT/ATTG (1), ATTA/TAAT (1), AATC/GATT (1), GCAT/CGTA (1), ATCA/TGAT (1), ACTA/TAGT (1), and AGTG/CACT (1) (Fig. 3c). In case of pentanucleotide repeats, no type was found frequent and all pentanucleotide repeat types were appeared singly as GAAAA/TTTTC (1), CAAAC/GTTTG (1), and GAATC/GATTC (1) (Fig. 3d). In hexanucleotide repeats, the repeat type CCAAAC/GTTTGG (2) was frequent followed by GATTTT/AAAATC (1), CCATCA/TGATGG (1), TTCTCT/AGAGAA (1), TTTGAT/ATCAAA (1), ACCTCC/GGAGGT (1), TGCACC/GGTGCA (1), TGATGG/CCATCA (1), and GTTGGT/ACCAAC (1) (Fig. 3e).

We identified 198 SSRs from 1014 sequences (121 from *T. pratense*, 77 from *T. repens*) that added

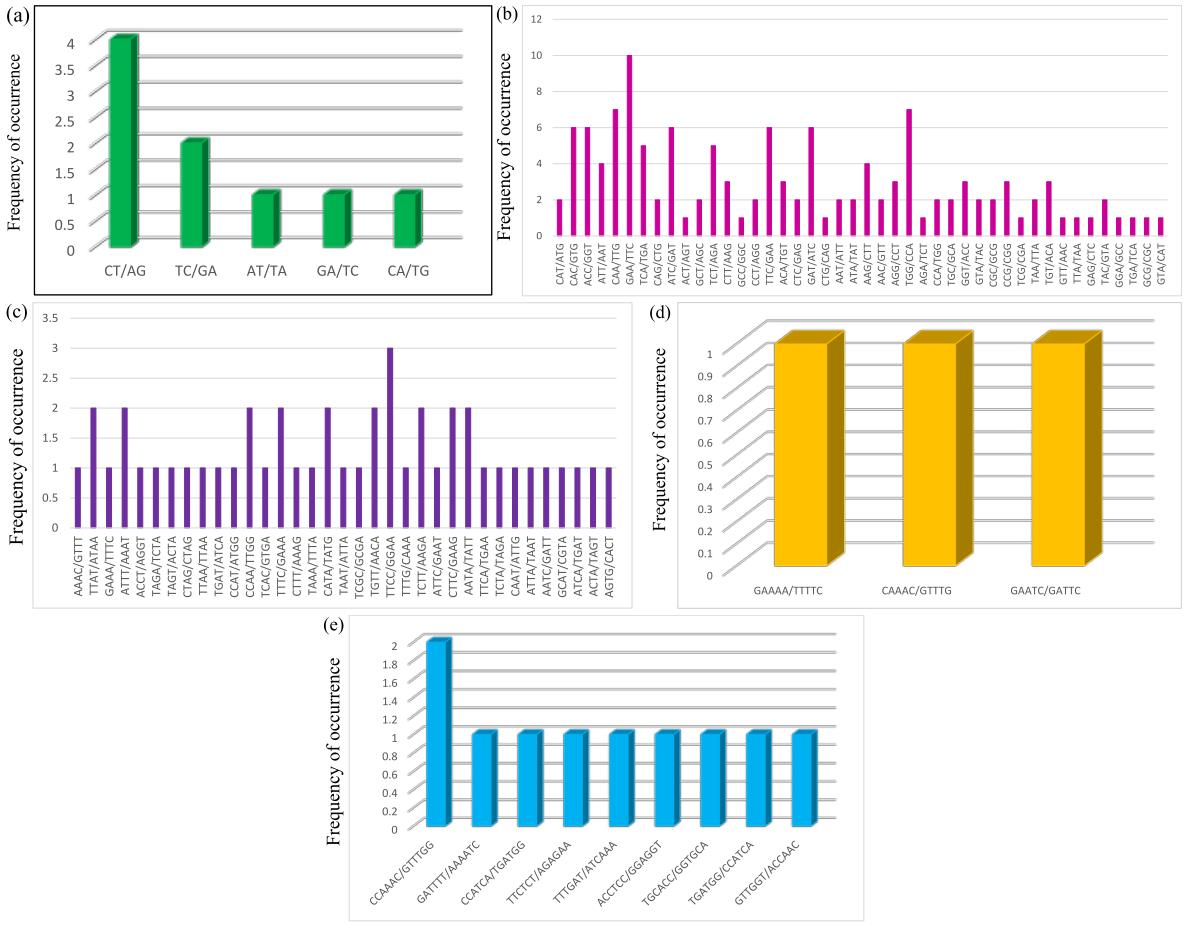

Fig. 3 Frequency of occurrence of different types of EST-SSRs motifs comprising of (a) dinucleotide, (b) trinucleotide, (c) tetranucleotide, (d) pentanucleotide, and (e) hexanucleotide in ESTs sequences.

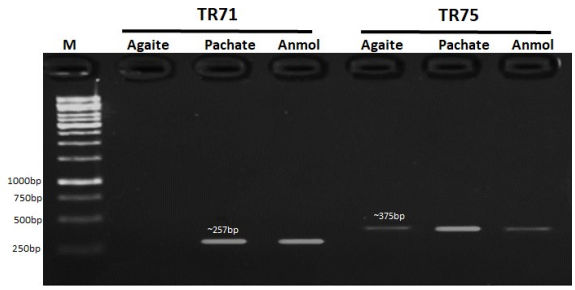

Fig. 4 EST-SSRs primers based PCR amplification in *T. alexandrinum*. M = 1 Kb DNA ladder; TR71 and TR75 = EST-SSRs specific primers; Agaite, Pachate, Anmol = cultivars of *T. alexandrinum*.

confirmation towards presence of SSR in EST sequence as identified in mungbean. The SSR development through EST database has become an efficient choice with time saving and low cost option for germplasm characterization, comparative genome mapping and linkage analysis [19]. Moreover, the identified genic SSRs from *Trifolium* spp. were able to be applied for their cross transferability with berseem (*T. alexandrinum*). This would be helpful to study different aspects of its genomics in a more precise way, which was still missing due to the unavailability of ESTs in *T. alexandrinum*. From the PCR analysis of these 198 EST-SSRs based primers, 44 primers showed cross-species amplification in *T. alexandrinum*. PCR pattern of some representative markers is given in Fig. 4. Of these 44 primers, 25 primers were from EST-SSRs of *T. repens* and 19 were from *T. pratense* (Table 1).

CONCLUSION

Simple sequence repeats are the molecular markers of choice for genetic diversity studies due to their highly specific nature and reproducibility. As the unavailability of genetic information record, functional

genomic studies are very limited in *T. alexandrinum*. The inaccessibility of genic SSRs for diversity analysis and population genetics of *T. alexandrinum* in more meaningful way has been becoming the major hurdle. Because in this species, expressed sequence tags are not available yet. Hence, this study would be an imperative contribution for population genetics studies in *T. alexandrinum*. As the ESTs of its closely related species, *T. pratense* and *T. repens* are available. Therefore, in this study, their ESTs were retrieved, subjected to *in silico* characterization to find EST-SSRs and were then analyzed for their cross species transferability in *T. alexandrinum*. In 198 *in silico* identified EST-SSRs based primers, 44 primers gave cross species amplification and showed their transferability in *T. alexandrinum*. These identified EST-SSRs may be used in diversity analysis and population genetics studies of germplasm of *T. alexandrinum* as well as for further breeding programs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2020.001.

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Appendix A. Supplementary data

Table S1 Identified EST-SSRs and their primers sequences with melting temperatures (TM), length of primers (L) and product size for Trifolium pratense (TP) and Trifolium repens (TR).

Serial	SSRs	Forward primer	TM	L	Reverse primer	TM	L	Size
TP1	(AAG)5	CCAACAACAACAAAACAGAGAGAG	60.231	24	CAACTCACCAAATCAGAAGCAG	59.914	22	184
TP2	(TCG)4	TAAACTCCACCCAAAACCACTC	60.254	22	CAACAACTCATCCACCGACTTA	60.030	22	367
TP3	(CCG)4	GTTGTGCTGCGCTCTCTCTT	60.886	20	AACAGCTCTCTGTCCATAAGGC	59.913	22	317
TP4	(CTC)4	TCTCCATTCTTCCACCATCTTT	59.940	22	CTTTCAATGCTTTCATCGTGTC	59.752	22	273
TP5	(CAA)5	TCTCCATTCTTCCACCATCTTT	59.940	22	CTTTCAATGCTTTCATCGTGTC	59.752	22	273
TP6	(TAA)7	CTGCTTCCACTTTCCTTCAGTT	59.922	22	GGTGTCTCCTTTGAATACCTGC	60.004	22	392
TP7	(TTC)4	CTGCTTCCACTTTCCTTCAGTT	59.922	22	GGTGTCTCCTTTGAATACCTGC	60.004	22	392
TP8	(TGG)4	GAATCGCTTAATTGCTCGTTCT	59.890	22	TGCGTTGAAGAATACCATCTTG	60.131	22	221
TP9	(AAAC)3	GTCACTTTAGAGGAGCCGGA	59.430	20	CTTGGAAATGCTTCACCTTTCT	59.762	22	386
TP10	(TGG)4	GATACCGGATTTCCATGTCAAC	60.439	22	CACTTCCAAACTCTTCTTTCGG	60.267	22	395
TP11	(TTAT)4	ATTACCGGAACCAGAGGGTTA	59.712	21	TTAAACTCATTCCTCCACCACC	60.221	22	304
TP12	(TGG)4	ACAACTAAAGATCAACGGCCAC	60.413	22	GCAGCTTCCAACCACTGACTAC	61.237	22	192
TP13	(GAAA)3	AAAGAGTGAGTTGGCTTCCATT	59.282	22	TCTAGCAAATGATCCAGACCAA	59.708	22	330
TP14	(ATTT)3	GTCCCAGTTTTGTTGAGAGAGG	60.146	22	CGTCGGAGAGTGATGATTATTG	59.592	22	298
TP15	(AAT)5	GTCCCAGTTTTGTTGAGAGAGG	60.146	22	CGTCGGAGAGTGATGATTATTG	59.592	22	298
TP16	(TGT)4	GTCCCAGTTTTGTTGAGAGAGG	60.146	22	CGTCGGAGAGTGATGATTATTG	59.592	22	298
TP17	(TCA)5	TTCCAATAATCATCACTCTCCG	59.043	22	TTGGTCAGCTATGCCTGTAGTC	59.406	22	223
TP18	(TGATGG)3	ACCTTCTCCACAAATCTGAAGC	59.757	22	TTCCAATTCCTTCTTCTTCCCT	60.408	22	320
TP19	(TTC)7	CACAACCACACCCACACTACTC	60.379	22	CAGCATCAGCCATCTTTTACTG	59.904	22	159
TP20	(ACCT)3	CTGCTTTCAACCCTTTTGAGAT	59.762	22	TAGTGCCATCGTTGTTGTTTGT	60.461	22	352
TP21	(CT)7	CCTTTAGTGAGGGTTAATTGCG	60.017	22	GCCGAGCAGATCAAAGAGTAAG	60.522	22	331
TP22	(ACC)11	GTATGTTCCTCCGCACTTGAG	59.749	21	GACCTGATGGTGCTAATGTTCA	60.000	22	180
TP23	(GA)6	ATTCCCGATACAGATGGTTCAG	60.208	22	ATTCGGACCTTTCCATATACCA	59.572	22	286
TP24	(ATTT)3	CAATTTCTCCTCCTTCTCCTCC	60.547	22	AAGAGAGTTGTGTTTGGGTGCT	60.209	22	107
TP25	(ACC)8	ACAACAATCTCACCCCTCAAAC	60.268	22	AGGCGATTAAGTTGGGTAACG	60.369	21	372
TP26	(GTT)5	CACTACAAAATCCAGAAGCACG	59.807	22	CCTTTCACTCTCTCAATCCGTC	60.247	22	141
TP27	(AAG)6	ACGAGGAAGAAGAGATTTGTGC	59.892	22	AGGAGAGTTGGTGAGAGAGTGG	59.910	22	393
TP28	(CCA)4	GTGCTTTACCCCTAGATGTCCA	60.373	22	TCATGTGGAAGAAGGAATGTTG	59.971	22	372
TP29	(AAG)4	CACTAAAACAACCCCACTCTCC	59.901	22	CGTATCCTTACCCACCTTTGAC	59.762	22	265
TP30	(GTTGGT)3	GGTGGGAAACAAATGAGGTTTA	60.089	22	AATCTCCGTCAGCTCTATGAGG	59.870	22	190
TP31	(TTA)6	GTAGACAAGGCCATATTGGGAG	59.856	22	GGTAGGCACCACCTGAGTTTAG	60.054	22	341
TP32	(TAGA)5	CTTACACGTTCTAGGAGCAGCA	59.715	22	TTGGAACAAATTCCTCAGTCCT	59.976	22	229
TP33	(GAT)5	GCAATTAGCTTCAAACACTGACAAG	61.476	25	ATGGGTTTCACAAAGCAAGAAG	60.514	22	199
TP34	(CAT)4	ACCAAGACTCCTCCTCCTTTTC	60.109	22	TGGATGGATGGTAACAAACAGA	60.227	22	283
TP35	(TAGT)4	TGAAACCCATTCATTCTCCTCT	59.940	22	CGTCAACGGGAACATTCTCTAT	60.373	22	364
TP36	(GAT)6	TGAAACCCATTCATTCTCCTCT	59.940	22	AGAGTCGTCAACGGGAACATT	60.927	21	369
TP37	(GAG)10	CCGCAGTATTAGCAAAGGTTCT	59.813	22	AGCTTTCTGCAACTCCTCAAAC	60.061	22	292
TP38	(CTAG)3	TTAATCCATTTGCTCCACACAC	59.867	22	CTTCCAGAAACAAGAACAAGGC	60.275	22	194
TP39	(TAC)4	TTTCCTTTCCTTTCCTTTCCTC	60.050	22	GTGTGTGTGTGTGTGTGTGTGA	60.052	22	147
TP40	(TTAA)5	TTTCCTTTCCTTTCCTTTCCTC	60.050	22	GTGTGTGTGTGTGTGTGTGTGA	60.052	22	147
TP41	(TGAT)3	AAAGAGTGAGTTGGCTTCCATT	59.282	22	TCTAGCAAATGATCCAGACCAA	59.708	22	330
TP42	(CAA)4	CCATCTTCTCTTCAAAACCCAC	59.976	22	ACAACAACAACAACGCGAGTAT	59.607	22	362
TP43	(ATC)4	CCACTAATTTTGACATTCCCGT	60.109	22	GGTGATTGATGGGATCGAGTAT	60.046	22	288
TP44	(CCAT)3	GCCACTTATCTCTGCCAATTTC	60.102	22	CAAGTTTGCTGCTGACGCTA	60.338	20	220
TP45	(CCAA)3	GTTCTCCAATCCCTATGAGCAC	59.968	22	CTAACAAGCAGCACCATGAAGA	60.440	22	196
TP46	(TCAC)3	TTTTGTAAGTGAGGTTTGGGGT	59.778	22	TGGAGAGAGAAGAAAGAGGTGG	59.988	22	184
TP47	(TAC)6	ATCCCTTCTCCAAAGTCAAAGG	60.811	22	TTGCTTCATTGCTGTAGTGCTT	60.082	22	331
TP48	(CA)6	GGATTCAACTCGCAATGTAAAC	58.631	22	CATGTTCCAGCACAAGAAGATT	59.242	22	343
TP49	(CAA)4	GCTTGATGAGGGACCAGTAGAC	60.139	22	ATCAGTGGATTTATTGGGTTGG	59.945	22	236
TP50	(TTTC)4	CCTTCACTCACTCTCTTCAGCA	59.786	22	ATCCAAATCCAGCACCAGAATA	60.692	22	269
TP51	(ACA)4	GCTTCTTTTCTTTCCTTATCTCTTCCC	63.157	27	CCTCACATCCATCATCTTTCCA	62.168	22	259
TP52	(GGC)4	AGACCCTTCACAGTTGATTGCT	60.175	22	GAAAGAGCCTTGATTAGCAGGA	59.987	22	289
TP53	(CTT)4	CAACCTCTGCTCTCTCAACTCA	59.786	22	AATGAACCCGTAGTTATGTGGG	60.001	22	370
TP54	(CTTT)3	CGTGTGTTTGTTCCATAGCGTA	60.962	22	TCATTCGATCCATAGGGGATAA	60.478	22	218
TP55	(TGT)4	TGAATCTTCTTCCTCCGCTG	60.474	20	GAATACCTTCCAAGCCAACAAG	60.001	22	193
TP56	(CCT)6	ATTTGAATGTGAGGGAGAAGGA	59.940	22	CAGGACCATTGTTAGGAGGGTA	60.234	22	324
TP57	(GAT)4	ATTGTTAGCACCAAACCAAACC	60.149	22	TGAAACCTTAGAACCAACAGCA	59.785	22	141
TP58	(TAAA)3	CCTGGTGAAATAATACAAGTCCTCT	59.050	25	TATGGAAAACAACACAGTTCCG	59.898	22	259
TP59	(TAA)4	ACTACGCCAACGCACTCAC	59.920	19	ACTGGAACTGGTATCAACCCTG	60.281	22	375
TP60	(TGA)7	CAACGCACTCACCACCTAATAA	60.054	22	ACTGGAACTGGTATCAACCCTG	60.281	22	368
TP61	(GAATC)3	CAAAACGAGAGAACAAGGGA	57.924	20	TTTCCGAGACCGTGATATTTAG	58.274	22	346
TP62	(GAT)4	TATTACATCACCAACTCGTCGC	60.025	22	ATAACACCATCAGCACCAACAC	59.786	22	339
TP63	(CATA)4	AACTGCTCCAATTCACGTTAGG	60.533	22	CTATGGTTGCATTCCTTGGTTT	60.237	22	264
TP64	(TGG)4	TGTTGTGACCCTCCTGATGTAG	60.028	22	GGGTGCTCTTATTCCATCTCAA	60.451	22	357
TP65	(GAAAA)3	AGCCAGGGTGAAGACAATTTA	58.707	21	AATGTTGCGTGGTGTAGTGAGT	59.590	22	219
TP66	(CATA)4	AACTGCTCCAATTCACGTTAGG	60.533	22	TACCCAGGTGGAAAATTCTTTG	60.210	22	361
TP67	(GCG)4	TCCTCTCATCATTCTCCTCCTC	59.778	22	GCCTCTGTATCTTGGCTCTTTG	60.393	22	360
TP68	(CCAA)3	GTTCTCCAATCCCTATGAGCAC	59.968	22	CTAACAAGCAGCACCATGAAGA	60.440	22	196
TP69	(TAAT)3	GCAGGACCCATTTAGTTCAATC	59.841	22	TCCTCTTCCTCTTTCTCTGATCC	60.323	23	378
TP70	(ATA)5	GCAGGACCCATTTAGTTCAATC	59.841	22	TCCTCTTCCTCTTTCTCTGATCC	60.323	23	378

Table S1 Continue.

Serial	SSRs	Forward primer	TM	L	Reverse primer	TM	L	Size
TP71	(TCA)9	CCAATAAGAATGGGGAAAGAGA	59.437	22	AGGATGGAATATGGAACATGGA	60.399	22	284
TP72	(TCT)4	GTTTCCGCTACCACATCTTTTC	60.007	22	GTTTCCAACTCTCTTCACACCC	60.016	22	162
TP73	(TGT)4	GTTTCCGCTACCACATCTTTTC	60.007	22	TCTTCTGTTCTAACCAGCCCTC	59.886	22	314
TP74	(TCA)4	AAACGATAGTGTGGATTTTGCC	60.244	22	ATGAAGATTTCCGTGTCATTCC	60.194	22	129
TP75	(GTA)4	AACACTTCACCGGACACTCTTT	60.074	22	TCTTTCCTATCCACAACTGGCT	60.130	22	297
TP76	(CAAAC)4	CCTCTTCTCATCTCTCCTTCCA	59.950	22	CAATGATGGGTTGGTGCTAAA	60.737	21	184
TP77	(ATG)4	CTTAACTCAAATGACTCGCCTT	57.715	22	GAAACATCTTCCCTTTCAACAC	57.695	22	309
TR1	(CAT)4	TGATGGAATGGATGAAGATGAG	59.894	22	AGTGATTCGGCAACCTTCTCTA	60.262	22	333
TR2	(CAC)4	GATCCATCTCCCACACTTAAACA	60.235	23	TTGGTGTTGTGCTTCTGGACTA	60.721	22	199
TR3	(ACC)5	CCCAAAACCTAAACCTCAAAAC	58.948	22	TGCTCTGTACCTTTCTTGACCA	59.914	22	386
TR4	(ATT)6	TTAACCACCACCACCACCTTAT	60.385	22	TGCTCTGTACCTTTCTTGACCA	59.914	22	333
TR5	(CAA)4	TGCTCAAATTCAAGTATGCCAC	60.138	22	CAAAGTCACCTCACATCTACGC	59.803	22	281
TR6	(GAA)4	GCGTAGATGTGAGGTGACTTTG	59.803	22	TAAGGGAGGCATGTCGAAGTAT	59.987	22	313
TR7	(TCGC)3	CACTAACATTCACTTGCATACACAC	58.694	25	GATTCTTCGTCCAGGCTATTGT	59.620	22	232
TR8	(GATTTT)3	TCCGATTTCACAACCTTCCTTA	60.821	22	ATTCAAAGACGGGAATCTGTGT	59.870	22	164
TR9	(TGTT)3	TTCTGATTTCACACCGTTTCAG	60.147	22	ATAACGACCCTTTCGGGTTAAT	59.975	22	368
TR10	(TCA)5	ACTACTACTGATGGCGTGTCTCC	59.718	23	GCGATGTTGTTGTTGATAGGAA	60.003	22	109
TR11	(TTCC)3	ATTTCCCTCTAACCTGCATCTG	59.615	22	GATACTTGCTACCGGAATCGAC	59.993	22	309
TR12	(TTTC)3	ATTTCCCTCTAACCTGCATCTG	59.615	22	GATACTTGCTACCGGAATCGAC	59.993	22	309
TR13	(CAG)4	ACACGCCATCCTTATTCACTTT	59.898	22	CATTTGGTCTTCGGTCTACTCC	59.998	22	388
TR14	(CAC)4	GAAGCTATGGAATCAAGGAAGC	59.359	22	TCAACAAACAAACCAAGAGTGG	60.045	22	361
TR15	(CAC)4	GAAGCTATGGAATCAAGGAAGC	59.359	22	TCAACAAACAAACCAAGAGTGG	60.045	22	361
TR16	(TTTG)3	TATTTCCACCGAAGAATCAAGG	60.305	22	TCAAGCCAAACTTCCTACAACA	59.785	22	107
TR17	(GAA)4	GCGTAGATGTGAGGTGACTTTG	59.803	22	TAAGGGAGGCATGTCGAAGTAT	59.987	22	313
TR18	(ATC)5	ACAAAACTCAGACCCATCACCT	59.901	22	GTATTTCCTGCTGACCTGCTTC	60.270	22	324
TR19	(CAC)7	GAAATGTGTTCTTGGCGTTGTA	60.039	22	CCGCTTCTTTTAACAATCCATC	59.970	22	194
TR20	(ACT)4	TTGAAGATGGCTATGAAACGTG	60.131	22	AGGCATGAAGATTTCTGGTGAT	59.968	22	115
TR21	(GCT)4	GTATGCTTCATCGAATCCCAAT	60.178	22	GTAACCAGGCACAAAATTCCTC	59.877	22	385
TR22	(CCATCA)3	CTGCCTAACTCCATCGCTCT	59.598	20	GCTGCAATTCCCTGTCTGTTA	60.264	21	376
TR23	(CT)14	CTCTCATCCCTCTTCCACTCTC	59.439	22	GGGAGTTCGAGTTCAGTTATGG	59.998	22	109
TR24	(TCT)6	TCCTTCATTCTTTCTTTCGG	57.007	20	TCGTCTTCATCATCATCTTCAG	57.893	22	283
TR25	(TTCC)3	TTGAAGATGGCTATGAAACGTG	60.131	22	AGGCATGAAGATTTCTGGTGAT	59.968	22	115
TR26	(GCT)4	GTATGCTTCATCGAATCCCAAT	60.178	22	GTAACCAGGCACAAAATTCCTC	59.877	22	385
TR27	(ACC)4	TCTCTCTCTCTCTCTGCTCGGT	60.036	22	TGAGTCTTGAAATCGGAGGAGT	60.247	22	198
TR28	(CTT)4	CTCAAACCAAACAAACCCTTTC	59.886	22	CAACGGCAACAACACAGTAAGT	60.131	22	364
TR29	(TCTT)3	TCCTTTCTCTCTCTACAACCGC	60.019	22	CAACGGCAACAACACAGTAAGT	60.131	22	296
TR30	(CAA)4	TTTCCTTACAATTTCCACACCC	60.089	22	CGCCTTTAGTCCAGTGTCTTTT	59.820	22	389
TR31	(GCC)4	AATGTCTCGACCCTAACTAACCTG	59.954	24	ATGCACAGGGAAGAAGATGAAT	59.968	22	115
TR32	(ATTC)4	TCAATAAGAGAGGGTGTGAGGAA	60.116	23	TTGGAGAAGTTTTGAGGTTGGT	60.012	22	398
TR33	(CCT)4	AGGGGATGTTTGTAGTGCTTGT	59.928	22	GCAGTGGACTTTGAAGGAGC	59.997	20	267
TR34	(CAA)4	TGCTCAAATTCAAGTATGCCAC	60.138	22	CAAAGTCACCTCACATCTACGC	59.803	22	281
TR35	(GAA)4	GCGTAGATGTGAGGTGACTTTG	59.803	22	TAAGGGAGGCATGTCGAAGTAT	59.987	22	313
TR36	(ATC)8	CTCACACACCTTTTAACACCCA	59.929	22	CCATGTCCATTAACTCCTCCTC	59.827	22	395
TR37	(TTC)8	GGGTGGACCTATTTACCTCTCC	60.075	22	GGTTGAAAAGTGGTGGATTGTT	60.130	22	233
TR38	(ACA)6	GTCGAACACCAAAACCTTTCTC	60.019	22	TCCATTCCTACTCCTCCTCCTT	60.438	22	272
TR39	(CAG)4	GCTGTCTCCTTAATCTTCGTGC	60.401	22	CTGAGAATGTTGTTGTTGGAGG	59.633	22	229
TR40	(CAC)8	TCCAATTATCCGAATCAGCACC	63.228	22	TGCCCAGTGAGGGACGAA	63.928	18	105
TR41	(TTC)5	TACCCAACTCAACTGTCCCTCT	60.031	22	CGACAACACTATCACCTTTGGA	60.030	22	329
TR42	(TTCTCT)3	TACCCAACTCAACTGTCCCTCT	60.031	22	CGACAACACTATCACCTTTGGA	60.030	22	329
TR43	(CTC)4	GCTCAAGCCAAAGCTAAATCTG	60.508	22	GTGGAGGAGGAGAAGACTGAGA	59.991	22	115
TR44	(TCT)4	TTCAACAACCCTAAAACCAACC	60.122	22	GCATCGACCTAGTGCTTTAACAT	59.706	23	188
TR45	(GAA)4	GCGTAGATGTGAGGTGACTTTG	59.803	22	TAAGGGAGGCATGTCGAAGTAT	59.987	22	313
TR46	(ATT)5	CTCTCTCATCACCATCTCCTCC	60.216	22	ATCGTTTTGGTATTCATGGCTC	60.211	22	296
TR47	(TCTT)3	TTCGTCATTCTTCTTTCTCTCTCTC	59.685	25	GCCTTTCACTGTCTCCGTAATC	60.137	22	390
TR48	(GAT)4	GACTTCAAAGGCCGTGATTG	60.636	20	CCGCATGACAGTTACCAAGAT	60.008	21	243
TR49	(CTG)5	ATCACCTTTCATCTCAACTCCG	60.496	22	ACACCACATCACATCCAAACAT	60.020	22	358
TR50	(TCA)6	CAAGACAGCCAATTACTTTCCC	60.001	22	AAGGTTCTCGCTACCTGCTTTA	59.581	22	379
TR51	(ATT)4	TCACACACTCTCATCTTTCTCCA	59.899	23	GTCTTAGGATTGCCCATACGAG	59.987	22	234
TR52	(AAT)5	AGAAAGATTCCATTCATCACCG	60.320	22	GAGGATCAGGTTCACTCCACAC	60.953	22	400
TR53	(TTTGAT)4	CACTTCCATCTCCAATTTCACA	59.971	22	AATTTTCCCCAAGGTCTTCTTC	59.826	22	376
TR54	(CTTC)3	CTTCACAATCACAACACACACC	58.959	22	CCATCAGTTTATGCTTCACTGC	59.775	22	209
TR55	(AATA)3	TCCTCTTCCTTCCTTCCTTTCT	59.833	22	GCTCCCATAAATCTCTTGTTGC	60.102	22	350
TR56	(ATA)7	GTTGGGGACTTGCTCTTGATAC	60.004	22	CTTCACTGGTTATTTTCCGGTC	59.871	22	198
TR57	(CT)12	ACTATCCCATTGTCTCCATTCC	59.175	22	AGCCACTTGTTCAGTTGGTTTT	60.075	22	170
TR58	(AAG)4	GCACTTGATGAACTCAAACAGC	59.927	22	ACAACCTCCACAACAGACTCCT	60.074	22	290
TR59	(GAA)4	GCACTTGATGAACTCAAACAGC	59.927	22	ACAACCTCCACAACAGACTCCT	60.074	22	290
TR60	(AAC)4	GACTTGGCAGCTCTGATTCTTT	60.026	22	CACCCTTCTTCATCTAATCCTTCT	59.196	24	136
TR61	(CT)8	GCGCTCATTTCCTTATCTTCTT	59.041	22	GTCCTAAAAGCAACCTTTGGTG	60.035	22	306
TR62	(TCT)4	GCGCTCATTTCCTTATCTTCTT	59.041	22	GTCCTAAAAGCAACCTTTGGTG	60.035	22	306
TR63	(ACC)4	ATAATGGCTACCACCACCGTA	59.238	21	CATGGTTCTTCTTATCTCCTCTGAA	60.134	25	196
TR64	(TTC)4	AATAACGCTGAAAACGCTAACC	59.705	22	AATTTGGTCTTGAGAGGGTGAA	59.976	22	137
TR65	(AGG)4	AGTGGTGGAAGTGGAGTAAAGC	59.682	22	GTAATTCCTAACGGCTCGATGA	60.459	22	372
TR66	(TTC)4	GCAGCAGCAACTACCTAACAGA	59.721	22	ACAAAGCAACAACCCCAATTAC	60.149	22	378
TR67	(GAA)4	CACAAGGTGTTGAAGATAGCGA	60.305	22	GAGGAGAAAGAGGAGATGCTGA	60.096	22	183
TR68	(GAA)6	TGTTGAAGATAGCGACGAAGAA	60.018	22	TCACACTCACCATGACAAATCA	60.008	22	387

Table S1 Continue.

Serial	SSRs	Forward primer	TM	L	Reverse primer	TM	L	Size
TR69	(TGG)4	CACCAAAGAGAGGACGTGGTAG	61.083	22	GACCATCAACATCATCAGCATC	60.357	22	255
TR70	(TTCA)3	TGTTTCACCACAACAACAACAC	59.410	22	CCTTGTCCTGTTCGTCTTCTCT	59.916	22	257
TR71	(TC)8	TGTTTCACCACAACAACAACAC	59.410	22	CCTTGTCCTGTTCGTCTTCTCT	59.916	22	257
TR72	(TGTT)3	TTCTGATTTCACACCGTTTCAG	60.147	22	ATAACGACCCTTTCGGGTTAAT	59.975	22	368
TR73	(ACCTCC)3	AATCTGCTGGAATGATGGTTG	59.947	21	GAGGTGATTGTGATGACTGAGC	59.734	22	141
TR74	(AGG)4	AACACCAGAAACTGAACGACCT	60.074	22	GGCATTTGACCATAAACAACAG	59.384	22	264
TR75	(AT)8	AAGATGGAGCATTTCCGAGTAG	59.747	22	CTAATTCAAGGGGTTTTGCTTG	59.997	22	375
TR76	(TCT)4	CACGAGGTTCTCTTCTTCCATT	59.751	22	CCTTTAGCAGCAGCAGATTGA	60.663	21	373
TR77	(TTAT)6	TCTCTTCTTCCATTCCCTCTTC	58.920	22	GTGATAAGCATGAACCCAGTTG	59.499	22	252
TR78	(TCTA)3	TCTCTTCTCTTCTCTTCTCTATCAAGG	59.416	27	CCACCACCACCATTCATATTC	59.925	21	210
TR79	(TGG)4	TCTATATGCAGCTTGGTGATGC	60.259	22	TCCTTCTGATTTTCCACTTCTACC	60.005	24	389
TR80	(CAAT)3	CTTTCAGAGACACTTCGCTTCA	59.797	22	GAAGAACTCATCTCGCTCCAGT	60.023	22	149
TR81	(ATT)4	TAGCTCAGAAATGCAAAAGTGG	59.536	22	GTTGATACTCGTCCACAACGAC	59.525	22	362
TR82	(AGA)5	TATTGCTCTGCGAAGGGTTATT	60.115	22	ATGATCGGAAGACCTGATAGGA	59.925	22	195
TR83	(ACC)4	GGTTGTGCATCTTCTAAACGTG	59.677	22	TGTGGTTGTGATTGTTGTGATG	60.324	22	320
TR84	(CCAAAC)4	CGTCTCACTTGTTCTTCCCTCT	59.916	22	GAATCTCCTTGCTGCCTTAGAA	59.987	22	345
TR85	(CCAAAC)4	TTCAAGGACCACTACCAATGC	59.985	21	AGCGAAGAACCTGAAGAAATTG	59.892	22	341
TR86	(ATC)5	CTTTTCCAACACCACCATCATA	59.724	22	AGACCGAAAGGGAGATAGAACC	59.968	22	218
TR87	(TTCC)3	AGAGGCACCGTCAAAAGAGAT	60.261	21	GCACGAACCAATTACATAACGA	59.896	22	233
TR88	(CTT)10	CACTCAAACCAAATCCAGTTCA	60.008	22	TTCGGTAGCGGTCTCTTACTTC	59.913	22	345
TR89	(CCA)5	CAAGCACTCAACAACCATGAAT	60.037	22	CCATCATCATCCACAGAAGCTA	60.096	22	200
TR90	(CTTC)3	TCACAATCACAACACACACCC	60.326	21	CCATCAGTTTATGCTTCACTGC	59.775	22	220
TR91	(AATA)4	TCCTCTTCCTTCCTTCCTTTCT	59.833	22	GCTCCCATAAATCTCTTGTTGC	60.102	22	363
TR92	(TGC)4	CATCAACAAGAACAAGAACCCA	60.008	22	TAGCACCATAAATCCCAATTCC	60.042	22	400
TR93	(ATTA)3	TGAGAGAAGAAACCGTGATGTG	60.291	22	GCACAAACCACCCAAAATCTTA	61.092	22	386
TR94	(GGT)4	CGGCACGAGGATTTAAGTTT	59.225	20	GTAATGGCTACCACCACCATAA	58.766	22	292
TR95	(AATC)3	TGAACCCTAAACCTAGACCAGC	59.653	22	GTCATACTCAGCCCTCGTCATT	60.525	22	170
TR96	(CAC)4	AACCTTGTTTCTCACGCACAC	60.208	21	TTCAGCTCCACCTTGTGATTTA	59.747	22	366
TR97	(TGCACC)3	TAAATCACAAGGTGGAGCTGAA	59.747	22	GGCGATACTTCTGTAAATGGCT	59.656	22	266
TR98	(ACA)4	TGTACTTCGATCCCTTTCACCT	59.998	22	GGTTGGCTACTGCTCTGTTTCT	59.950	22	276
TR99	(GTA)4	GTCAGAATACGGAAACCTGGG	60.728	21	GGGATAGAACCGGGATTAAGAC	60.043	22	198
TR100	(GGT)4	CATGGTTCTTCTTATCTCCTCTGAA	60.134	25	ATAATGGCTACCACCACCGTA	59.238	21	196
TR101	(GGT)4	CACTTCCATCTCCAATTTCACA	59.971	22	AATTTTCCCCAAGGTCTTCTTC	59.826	22	373
TR102	(ATC)4	CATGTCGCCTTAGAAAACCATT	60.364	22	CTGATGGACCGTGTGAGACTT	60.166	21	249
TR103	(GAA)4	GCGTAGATGTGAGGTGACTTTG	59.803	22	TAAGGGAGGCATGTCGAAGTAT	59.987	22	314
TR104	(GAA)5	CAACAAACACAAACCGAAGAAC	59.557	22	AGCTACCCATTTACCCCACTTT	60.127	22	175
TR105	(GCAT)3	AAAGAGAAACGGTGGTGTTGTT	59.943	22	GCGGCGATAGGTGTAGAATAAG	60.135	22	168
TR106	(AAC)8	TGGACCTTCTGCTCGTCTTAAT	60.262	22	CCCTAGCACCAACTTCAGTAGC	60.315	22	137
TR107	(CGC)4	GGGGTAAATGGGTAGCTGAAAT	60.424	22	CAAAATCACCAGAACCACCAC	60.262	21	379
TR108	(ATCA)4	TTAGGGCTACTTTCCCCTTTTC	59.969	22	CGATTGACGAGCTTATCTTCCT	59.877	22	383
TR109	(GAA)4	TTGTTCCCATAACACTTGACCA	60.262	22	ATCAAATCATCCCATCCTGAAC	60.024	22	349
TR110	(GAT)4	GTGGCTCTTTTCCTAATGTTGC	60.136	22	TTTCCTCTGCTGCCTTCTTATC	59.987	22	219
TR111	(TC)18	GCCGATTCATACTGCTGCTTA	60.378	21	GGAATGTCCAATACAACTGCAA	59.867	22	356
TR112	(ACTA)3	CTTGCCCGTTATGGTTTAGTTG	60.739	22	AATCCCAGCATGTTTGTTATCC	60.082	22	358
TR113	(CCG)4	CTCACTCCAATCAGAAAATCCC	59.940	22	AGAAGCATCAAGAACACGAACA	59.920	22	301
TR114	(AGG)4	GTGCTGGTGGAAAAGGAAATAA	60.344	22	TTGGCGAGTGTCGTAGTTATCA	60.689	22	230
TR115	(CCG)4	GAACCTTTCTACGACTCCGCT	59.899	21	GCAGTGATTGTTCTGGAAGTTG	59.780	22	264
TR116	(ATC)4	GAAGTTTGTCCCCTGTCTCATC	59.979	22	ACGAGGTGATTCTTCTGTTGCT	60.310	22	218
TR117	(CGC)4	TCAGTCCACTTTCCTTAAACCC	59.499	22	GGAGTTCCGTTACATCGTCTTC	60.004	22	174
TR118	(TGC)5	AATAGGTGGTGGATTGGATCTG	60.074	22	CCCCTTGATTACTCCTACCTGA	59.477	22	269
TR119	(AGTG)4	CGGAACACCACCATCACTC	59.945	19	GGTACTCCTCCTGACTCGTTGT	59.668	22	162
TR120	(CAA)4	CATTGAGTGCCTTTTCTCCAG	59.861	21	ACTCCTACCCAAAACAAAACCA	59.778	22	287
TR121	(TGG)5	ACTGAGCTTTGTCTTGGTTTGC	60.831	22	CTACTTGAGCCTTAGCTGGTGG	60.436	22	226