Developing highly conserved microsatellite markers: a case study in the filamentous fungal genus *Aspergillus*

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Abstract

Microsatellite markers are highly variable and very commonly used in population genetics studies. However, microsatellite loci are typically poorly conserved over large evolutionary distances and cannot be used across distantly related species. Thus, the development of highly conserved microsatellite markers that amplify homologous loci in distantly related species would increase efficiency and allow investigation of the same questions in multiple lineages using the same marker set. Here we describe a protocol for the development of such microsatellite markers from species with complete genome / transcriptome sequences. Application of this protocol to the filamentous fungal genus *Aspergillus* resulted in the generation of 9 and 11 variable microsatellite markers in two phylogenetically distinct clades.

Main Text

Microsatellites are short units of DNA (1-6bp) repeated in tandem (Goldstein & Schlötterer 1999). Partly due to their abundance in eukaryotic genomes (Katti *et al.* 2001) and partly due their high levels of polymorphism (Ellegren 2004), microsatellite loci are very popular genetic markers for molecular ecology studies. Because microsatellites are usually poorly conserved between distantly related species (Barbara *et al.* 2007; Gibbons & Rokas 2009), they are typically developed and applied within a single species or between very close relatives (Barbara *et al.* 2007; Ellis & Burke 2007). However, there is a growing need for the development of highly conserved microsatellites for two reasons: (1) they allow more efficient experimental designs, and (2) they can be used to address

the same question in multiple independently-evolving lineages using the same set of markers.

We used the filamentous fungal genus *Aspergillus* as a model for the development of highly conserved microsatellite markers for several reasons. First, ten genomes from eight species have been fully sequenced (Fedorova *et al.* 2008; Galagan *et al.* 2005; Machida *et al.* 2005; Nierman *et al.* 2005; Payne *et al.* 2006; Pel *et al.* 2007; Rokas & Galagan 2008; Rokas *et al.* 2007; Yu *et al.* 2005). Second, the genus has an evolutionary history extending back well over 200 million years and exhibits varying levels of sequence divergence (Fedorova *et al.* 2008; Galagan *et al.* 2005). Specifically, pair-wise amino-acid divergence within the clades examined in this study is 21% and 16% for the *Flavi-Terrei* and *Fumigati-Clavati* clades, respectively (Figure 1) (Rokas & Galagan 2008). Lastly, *Aspergillus* species exhibit diverse ecologies and reproductive strategies (Balajee *et al.* 2008; Geiser 2009; Geiser *et al.* 1998; Nierman *et al.* 2005; Pel *et al.* 2007; Pringle *et al.* 2005; Rokas 2009), making the genus an ideal candidate for comparative population genetic studies between pairs of related species that differ with respect to life history characteristics (e.g., pathogenicity, mode of reproduction).

We first attempted to construct genus-wide microsatellites. However, very few microsatellites were conserved across all 8 sequenced *Aspergillus* transcriptomes and were typically flanked by highly divergent sequences, hindering primer design. Thus, we restricted our search for highly conserved primers to the *Flavi-Terrei* and *Fumigati-Clavati* clades (Peterson 2008; Rokas *et al.* 2007) (Figure 1).

Microsatellite primers were designed from the fully sequenced transcriptomes of *Aspergillus* species belonging to the *Flavi-Terrei* and *Fumigati-Clavati* clades separately (Peterson 2008; Rokas *et al.* 2007). Section *Flavi* includes *A. flavus* and *A. oryzae* and section *Terrei* includes *A. terreus.* Section *Fumigati* includes *A. fumigatus* and *Neosartorya fischeri* (the sexual state of *A. fisherianus*) and section *Clavati* includes *A. clavatus* and *A. giganteus*. We began by predicting orthologs between all species pairs (within clades) using the reciprocal best BLAST hit algorithm, with an e-value cutoff of $1e^{-06}$ (Koonin 2005). Microsatellites were then detected *in silico* in each transcriptome using the EMBOSS ETANDEM software (Rice *et al.* 2000). Next, conserved microsatellite-containing transcripts were aligned using ClustalW (Chenna *et al.* 2003) and spatial conservation of the microsatellite sequence was manually verified. Finally, the ClustalW consensus sequence was used to design microsatellite primer pairs (two forward and two reverse primers per locus) using the Primo Degenerate program of Change Bioscience's ® BioToolKit 320 package (www.changbioscience.com/biotoolkit2.html).

Genomic DNA was extracted from cultures of sixty (*A. flavus* (9), *A. oryzae* (31) and *A. terreus* (20)) and 41 (*A. fumigatus* (28), *N. fischeri* (4), *A. clavatus* (6) and *A. giganteus* (3)) strains from the *Flavi-Terrei* and *Fumigati-Clavati* clades, respectively, using a modified CTAB protocol (Stewart & Via 1993). Fungal strains were grown in potato dextrose broth in a tissue culture rotator for 3-4 days at room temperature. Mycelium was ground in liquid nitrogen and incubated in CTAB buffer. Following two organic

extractions with chloroform, DNA was precipitated with isopropanol, washed twice with 70% ethanol and resuspended in 1X TE buffer. To verify that the strains were not contaminated, we sequenced approximately 580bp of the internal transcribed spaced (ITS) region from all samples and performed BLAST (Altschul *et al.* 1990) searches using the sequence of each strain against the NCBI non-redundant sequence database.

Primer pairs were first screened to verify amplifications in 25 µl reactions consisting of 20 ng of template DNA, 1 X Promega GoTaq™ reaction buffer, 0.8 mM dNTPs, 2.5 µM primers and 0.05 U Promega Flexi™ Taq polymerase. A touchdown PCR protocol (Don *et al.* 1991) was implemented to limit nonspecific amplification and consisted of the following cycling profile: 95ºC for 3 min, 11 cycles of 94ºC for 30 s, 65ºC for 30 s (with annealing temperature dropping 1ºC per cycle) and 72ºC for 45s, followed by 29 cycles of 94ºC for 30 s, 53ºC for 30 s, 72ºC for 45 s, followed by a final extension of 72ºC for 20 min. Amplicons of two strains of each species were sequenced at Genewiz (Northbrunswick, NJ) to confirm target sequence. Forward primers of successful pairs (there were 9 successful primer pairs for the *Flavi-Terrei* clade and 11 for the *Fumigati-Clavati* clade) were fluorescently labeled for use with the ABI DS-33 (G5) dye set (Table 1). PCR products were resolved on an ABO 3730xl Genetic Analyzer at Genewiz, using GENESCAN 500 LIZ size standard. Genotypes were determined using Peak Scanner Software v1.0 (ABI). Haploid diversity was calculated for each locus using GenAlEx version 6.2 (Peakall & Smouse 2006). Haploid linkage disequilibrium was independently calculated between all polymorphic loci using Multilocus 1.3b (Agapow & Bert 2001) assessing significance via analysis of 999 randomized datasets.

All loci displayed variation in at least one species (Table 1). All loci exhibited multiple alleles in all species from the *Flavi-Terrei* clade with one exception (locus 07647 in *A. flavus* and *A. oryzae*; Table 1). In the *Fumigati-Clavati* clade, all loci exhibited multiple alleles in *A. fumigatus*, whereas 7, 9 and 8 of the 11 loci displayed variation in *N. fischeri*, *A. clavatus* and *A. giganteus*, respectively (Table 1). Haploid diversity ranged from 0.00-0.79 in *A. flavus/A. oryzae* and 0.07-0.67 in *A. fumigatus*. Importantly, the average haploid diversity for our "coding-region" microsatellite loci was comparable to the average haploid diversity reported for "non-coding region" microsatellite loci developed in *A. flavus* and *A. oryzae* (Grubisha & Cotty 2009; Tomimura *et al.* 2009). The same three locus pairs in *A. flavus/A. oryzae* and *A.* terreus independently displayed evidence of linkage disequilibrium (06964 with 09130, 09292 and 09308) after a multiple-test corrected *p* value of 0.006 (0.05 / # polymorphic loci). However, locus 06964 resides on a different contig from loci 09130, 09292 and 09308 and is separated by at least 1 Mb of sequence. No statistical evidence of linkage was detected between any locus pairs in *A. fumigatus*.

We have reported a novel and efficient method for developing microsatellite markers from two diverse clades of the filamentous fungal genus *Aspergillus.* Our work focused on microsatellite loci nested within coding regions because the lower conservation of flanking regions in non-coding sequence constrains the development of highly conserved markers. Nevertheless, a recently study identified ~5,800 conserved non-coding sequences in genome comparisons between *A. oryzae*, *A. fumigatus* and *A. nidulans*

(Galagan et al. 2005), a number very similar to the number of orthologous genes shared by the three species (~5,900 genes), suggesting that this approach might also be useful in the identification of highly conserved microsatellites from non-coding regions. Although only a few eukaryotic clades of the tree of life are as densely sequenced as the genus *Aspergillus*, the advent of next-generation DNA sequencing technologies and their use to address ecological and evolutionary questions in non-model organisms (Gibbons *et al.* 2009; Hudson 2008; Rokas & Abbot 2009), suggest that our approach for generating highly conserved microsatellites will soon be widely applicable.

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Figure Legend

Figure 1. Phylogeny of *Aspergillus* species used in this study (Peterson 2008; Rokas & Galagan 2008). Full genome data are available for all species in boldface. The strains analyzed are as follows: *A. flavus*: 1-22[†], 7-4[†], A111[†], A120[†], A150[†], F35[†], F60[†], F67[†], NRRL 3357[†]; *A. oryzae*: RIB 127 081031[‡], RIB 211 081031[‡], RIB 215 081031[‡], RIB 330 081125[‡], RIB 331 081125[‡], RIB 40[†], RIB 430 081031[‡], RIB 505 081031[‡], RIB 537 081031[‡], RIB 621[‡], RIB 624 081031[‡], RIB 629 081031[‡], RIB 630 081031[‡], RIB 632 081031[‡], RIB 633 081031[‡], RIB 638 081031[‡], RIB 642 081031[‡], RIB 646 081031[‡], RIB 910 081125[‡], RIB 919 081125[‡], RIB 934 081125[‡], RIB 935 081125[‡], RIB 936 081031[‡], RIB 940 081031[‡], RIB 941 081031[‡], RIB 944 081031[‡], RIB 949 081031[‡], RIB 1031 081031[‡], RIB 1032 081031[‡], RIB 3005 081125[‡], NRRL 00469[†]; *A. terreus*: NIH2624[†], UAB2[§], UAB3[§], UAB4[§], UAB6[§], UAB8[§], UAB10[§], UAB11[§], UAB12[§], UAB14[§], UAB15[§], UAB17[§], UAB19[§], UAB22[§], UAB26[§], UAB29[§], UAB30[§], UAB31[§], UAB34[§], UAB36[§]; *A. clavatus*: 18[¥], 19[¥], 315[¥], 1423[¥], 2373[¥], NRRL 1[†]; *A. giganteus*: 52[¥], 355[¥], 373[¥]; *A. fumigatus*: 2006[¥], 2569[¥], 5860[§], B5355[§], B5856[§], B5357[§], B5857[§], B5359[§], B5361[§], B5602[§], B5852[§], B5854[§], B5861[§], B5865[§], B6069[§], B6070[§], B6072[§], B6073[§], B6074[§], B6075[§], B6076[§], B6077[§], B6078[§], B6081[§], B6083[§], B6269[§], CEA10[†], NRRL5109[§], *N. fischeri*: 2192[¥], 2388[¥], 2389[¥], NRRL 181[†] ([†]Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; [¥]USDA ARS, Southern Regional Research Center, New Orleans, LA, USA; [‡]National Research Institute of Brewing in Higashi-Hiroshima, Japan; [§]Center for Disease Control and Prevention. Atlanta, USA).

References

Agapow PM, Burt A (2001) Indices of multilocus linkage disequilibrium. *Molecular Ecology Notes* **1**, 101-102.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Balajee SA, de Valk HA, Lasker BA, Meis JFGM, Klaassen CHW (2008) Utility of a microsatellite assay for identifying clonally related outbreak isolates of Aspergillus fumigatus. *Journal of Microbiological Methods* **73**, 252-256.

Barbara T, Palma-Silva C, Paggi GM*, et al.* (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. *Molecular Ecology* **16**, 3759-3767.

Chenna R, Sugawara H, Koike T*, et al.* (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**, 3497-3500.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* **19**, 4008.

Ellegren H (2004) Microsatellites: Simple sequences with complex evolution. *Nature Reviews Genetics* **5**, 435-445.

Ellis JR, Burke JM (2007) EST-SSRs as a resource for population genetic analyses. *Heredity* **99**, 125-132.

Fedorova ND, Khaldi N, Joardar VS*, et al.* (2008) Genomic islands in the pathogenic filamentous fungus *Aspergillus fumigatus*. *PLoS Genet* **4**, e1000046.

Galagan JE, Calvo SE, Cuomo C*, et al.* (2005) Sequencing of *Aspergillus nidulans* and comparative analysis with *A. fumigatus* and *A. oryzae*. *Nature* **438**, 1105-1115.

Geiser DM (2009) Sexual structures in *Aspergillus*: morphology, importance and genomics. *Med Mycol* **47 Suppl 1**, S21-26.

Geiser DM, Taylor JW, Ritchie KB, Smith GW (1998) Cause of sea fan death in the West Indies. *Nature* **394**, 137-138.

Gibbons JG, Janson EM, Hittinger CT*, et al.* (2009) Benchmarking next-generation transcriptome sequencing for functional and evolutionary genomics. *Mol Biol Evol*, in press.

Gibbons JG, Rokas A (2009) Comparative and functional characterization of intragenic tandem repeats in 10 *Aspergillus* genomes. *Mol Biol Evol* **26**, 591-602.

Goldstein DB, Schlötterer C (1999) *Microsatellites : evolution and applications* Oxford University Press, Oxford ; New York.

Grubisha LC, Cotty PJ (2009) Twenty-four microsatellite markers for the aflatoxin-producing fungus Aspergillus flavus. *Molecular Ecology Resources* **9**, 264-267.

Hudson ME (2008) Sequencing breakthroughs for genomic ecology and evolutionary biology. *Molecular Ecology Resources* **8**, 3-17.

Katti MV, Ranjekar PK, Gupta VS (2001) Differential distribution of simple sequence repeats in eukaryotic genome sequences. *Mol Biol Evol* **18**, 1161-1167.

Koonin EV (2005) Orthologs, paralogs, and evolutionary genomics. *Annu Rev Genet* **39**, 309-338.

Machida M, Asai K, Sano M*, et al.* (2005) Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **438**, 1157-1161.

Nierman WC, Pain A, Anderson MJ, *et al.* (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438**, 1151-1156.
Payne GA, Nierman WC, Wortman JR, *et al.* (2006) Whole genome comparison of *Aspergillus flavus* and *A. oryzae*. *Med Mycol* **44 Suppl**, 9-11.
Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295.
Pel HJ, de Winde JH, Archer DB, *et al.* (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. *Nat Biotechnol*.
Peterson SW (2008) Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. *Mycologia* **100**, 205-226.
Pringle A, Baker DM, Platt JL, *et al.* (2005) Cryptic speciation in the cosmopolitan and clonal human pathogenic fungus *Aspergillus fumigatus*. *Evolution* **59**, 1886-1899.
Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* **16**, 276-277.
Rokas A (2009) The effect of domestication on the fungal proteome. *Trends Genet* **25**, 60-63.
Rokas A, Abbot P (2009) Harnessing genomics for evolutionary insights. *Trends in Ecology & Evolution* **24**, 192-200.
Rokas A, Galagan JE (2008) The *Aspergillus nidulans* genome and a comparative analysis of genome evolution in *Aspergillus*. In: *The Aspergilli: Genomics, Medical Applications, Biotechnology, and Research Methods* (eds. Goldman GH, Osmani SA), pp. 43-55. CRC Press.
Rokas A, Payne G, Fedorova ND, *et al.* (2007) What can comparative genomics tell us about species concepts in the genus *Aspergillus*? *Stud Mycol* **59**, 11-17.
Stewart CN, Via LE (1993) A Rapid Ctab DNA Isolation Technique Useful for Rapid Fingerprinting and Other Pcr Applications. *Biotechniques* **14**, 748-&.
Tomimura K, Iwashita K, Yamada O, Kobayashi K (2009) Development of VNTR markers for three Aspergillus species used in brewing. *Molecular Ecology Resources* **9**, 613-615.
Yu J, Cleveland TE, Nierman WC, Bennett JW (2005) Aspergillus flavus genomics: gateway to human and animal health, food safety, and crop resistance to diseases. *Rev Iberoam Micol* **22**, 194-202.

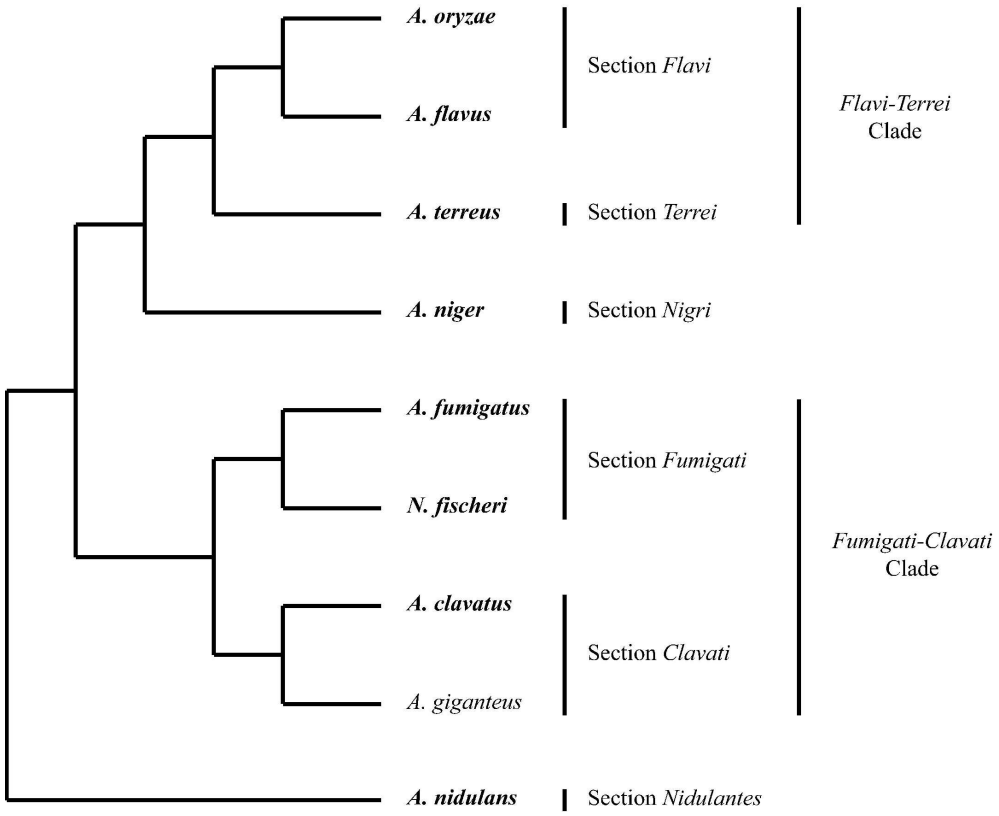

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Table 1. Characteristics of microsatellite loci from the *Flavi-Terrei* (first panel) and *Fumigati-Clavati* (second panel) clades, including forward and reverse primer sequences, reference transcript IDs from which primers were designed, fluorescently labeled dye, conensus repeat motif, allele size range, number of alleles (N_A) and haploid diversity (h). Because *A. oryzae* is a domesticated ecotype of *A. flavus* (Geiser et al. 1998a; Rokas 2009; Rokas et al. 2007), data from the two organisms were combined. For the *Fumigati-Clavati* clade, haploid diversity is only reported for *A. fumigatus*. All highly conserved microsatellite markers reported in this study are distinct from previously developed *A. fumigatus*, *A. flavus* and *A. oryzae* species-specific markers (Balajee et al. 2008; Grubisha & Cotty 2009; Pringle et al. 2005; Tomimura et al. 2009).

Species	Locus Name	Primer Sequence (5'-3')	Reference Transcript ID	Label	Consensus Repeat Motif	Allele Size Range (bp)	N_A	h
A. flavus/A.oryzae	03100	F: RGCGGAAARCARGCC	AFL2G_03100.2, AO090012000172	NED	AAG	323 - 341	4	0.19
A. terreus		R: TSGAGCTGAGACGRTC	ATEG_04806.1			320 - 338	3	0.27
	03661	F: GGAARGACAAACGRCGC	AFL2G_03661.2, AO090012000799	NED	AAG	413 - 416	2	0.05
		R: CGTCCRATWCKTGCMGC	ATEG_04066.1			398 - 413	2	0.10
	04631	F: CGTCGCARTTCACSTC	AFL2G_04631.2, AO090023000819	NED	GCA	336 - 356	6	0.71
		R: GTCTCMCGCTTCTTGG	ATEG_05447.1			330 - 357	2	0.10
	06964	F: AACAGGCCCGTGARG	AFL2G_06964.2, AO090026000289	NED	CAG	480 - 492	3	0.30
		R: GRGCAATSGASGTGG	ATEG_01306.1			480 - 492	2	0.10
	07647	F: GCGGTCAGCAGYTGAACC	AFL2G_07647.2, AO090001000487	NED	CAG	235	1	0.00
		R: CGSAGAATACCGGCSACK	ATEG_02933.1			231 - 234	2	0.10
	09130	F: GCTGCAAAAGCTGCGCG	AFL2G_09130.2, AO090001000653	VIC	GAA	438 - 441	2	0.05
		R: CGGGGTCTTKGGRAACG	ATEG_06732.1			414 - 441	2	0.11
	09292	F: GCMGAGAAACAAGCCC	AFL2G_09292.2, AO090001000672	VIC	AGA	340 - 361	2	0.05

		R: CYGCTTCACYTTGKCCACC	ATEG_06748.1			330 - 339	3	0.19
	09308	F: ACKAGTTGGGCTACSG	AFL2G_09308.2, AO090102000614	VIC	CAG	353 - 365	4	0.31
		R: GCTCTCRTACTCRAGG	ATEG_08001.1			361 - 373	5	0.55
	09988	F: GCYGGMTGTATCATGG	AFL2G_09988.2, AO090038000283	VIC	GCA	368 - 386	7	0.79
		R: WACCATCCCYCCRTAC	ATEG_00332.1			373 - 382	2	0.10
A. fumigatus	1g10200	F: CAACTACGCGCGGTTCGAG	Afu1g10200	6FAM	GAG	314 - 317	2	0.07
N. fischeri		R: CTTGCGYCGCTTCTTGACCC	NFIA_015450			312 - 315	2	
A. clavatus			ACLA_025340			332 - 335	2	
A. giganteus			N/A			369 - 372	2	
	1g11490	F: GTRTCACCSAGYSTRGTTCC	Afu1g11490	6FAM	AGA	313 - 328	5	0.67
		R: CCRAGCCATGTCAATGGC	NFIA_014080			324	1	
			ACLA_024160			316	1	
			N/A			307 - 310	2	
	1g12120	F: GARGCTCGYCGAAARGCC	Afu1g12120	PET	CAGGGA	349 - 376	5	0.60
		R: GGCTCCTTYGGWGTARCGG	NFIA_013390			376	1	
			ACLA_023350			327 - 381	3	
			N/A			313 - 391	2	
	1g14430	F: YCARTGGTACTGGTTCGCC	Afu1g14430	PET	CGGCTC	295 - 313	2	0.14
		R: CTKCTCYTCAGCRSTGCC	NFIA_010990			312 - 345	4	

		ACLA_020990			362 - 368	2	
		N/A			300 - 306	3	
2g13290	F: MRGCGASGARGCCCCKCTCAC	Afu2g13290	6FAM	CAG	194 - 203	3	0.20
	R: GCTGWGCGGCAGGRGCR	NFIA_088470			194 - 197	2	
		ACLA_072090			234 - 240	3	
		N/A			242 - 254	3	
3g09600	F: CSGATTACGATGGCGARGAAGARCC	Afu3g09600	PET	AGA	530 - 542	4	0.45
	R: ACRTACAYKCCTTCCCTCTGGCGR	NFIA_067690			504 - 540	2	
		ACLA_037370			542 - 596	3	
		N/A			591 - 600	2	
4g02990	F: AYGCCGARTGGCARCARAC	Afu4g02990	PET	AAG	311 - 317	3	0.59
	R: CTTTTGCTCRAGBTCGGYC	NFIA_030470			310	1	
		ACLA_055890			316 - 322	2	
		N/A			307 - 322	2	
4g09070	F: TYGCCTTGRTMTCAGGCGG	Afu4g09070	6FAM	GAA	289 - 355	6	0.66
	R: CGGCTTCGTAGAGCGG	NFIA_107100			235 - 238	2	
		ACLA_048430			272 - 287	2	
		N/A			258	1	
5g01780	F: GATTGCYCGGGAGAGCATC	Afu5g01780	PET	CAG	511 - 520	3	0.14

	R: CTCCAGSGGWCTTTTSTCC	NFIA_040320			513 - 516	2	
		ACLA_003540			609 - 615	3	
		N/A			531 - 594	3	
6g02510	F: TSGTGGTTCCKGAGTGGG	Afu6g02510	6FAM	TCTCAG	313 - 328	3	0.14
	R: TCATCCGCKCGMGGYTGG	NFIA_048780			322 - 334	2	
		ACLA_097970			323 - 368	4	
		N/A			357	1	
7g04870	F: CTACGCCGGYCAYCAAGY	Afu7g04870	6FAM	AAG	233 - 251	5	0.51
	R: ASGARGCGGARAAGTTGCC	NFIA_025830			238	1	
		ACLA_006570			254	1	
		N/A			245	1	