ASSESSMENT OF POPULATION GENETIC VARIATION AND STRUCTURE OF *ACACIA WOODMANIORUM*, AND ITS PHYLOGENETIC RELATIONSHIP TO OTHER *ACACIA* SPECIES

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Our environment, our future

Summary

This report describes the progress made in the second six months of the project 'Assessment of population genetic variation and structure of *Acacia woodmaniorum*, and its phylogenetic relationship to other *Acacia* species' undertaken by the Department of Environment and Conservation for Karara Mining Ltd. The project commenced in September 2008 and in the first six months initial germplasm collections commenced, DNA extraction protocols were optimised, microsatellite libraries produced and PCR primers optimised. During the second six months of the project comprehensive germplasm collections have been conducted across the species range, primer optimisation has been finalised and reported in the publication 'Characterisation of microsatellite DNA markers for the rare *Acacia woodmaniorum* (Leguminosae: Mimosaceae)' and genotyping of the main germplasm collection is currently being conducted. Germplasm collections and DNA extraction of *Acacia alata* populations has also commenced. The maturation of seed pods from the 2009 flowering season is currently being monitored.

Progress to Date

Field Work

A number of field trips to the Karara site have been conducted and comprehensive germplasm collections conducted across the species range. 566 individual plants have been sampled using a sampling strategy that incorporated discontinuous sampling at evenly distributed points throughout the main populations as well as exhaustive sampling of all individuals in the smallest most isolated populations located off the main ridges as well as in the Blue Hills and Terapod populations (Figure 1). In total, sampling included 279 individuals from Mungada/Windaning Ridge, 137 individuals from Jasper Hills and all individuals at the Blue Hills (140) and Terapod (10) populations (Table 1). Twenty individuals were sampled at regular distance intervals in the large continuous populations along Mungada Ridge (MA1-MA6) and Windaning Ridge (WA1-WA3), and from the larger populations on the more disjunct ironstone outcrops at Jasper Hill (JHA, JHB, JHD and JHE). Five individuals were sampled from smaller populations located just off the main ridges (MB-MD, WC-WD and JHC and JHF-JHG) and all individuals were sampled in small populations (WD-WE and various outliers). The smaller populations where exhaustive sampling has been possible will be suitable for gene flow studies as well as for the assessment of genetic structure across the species range. All sampled individuals were tagged and recorded by differential Global Positioning System (dGPS).

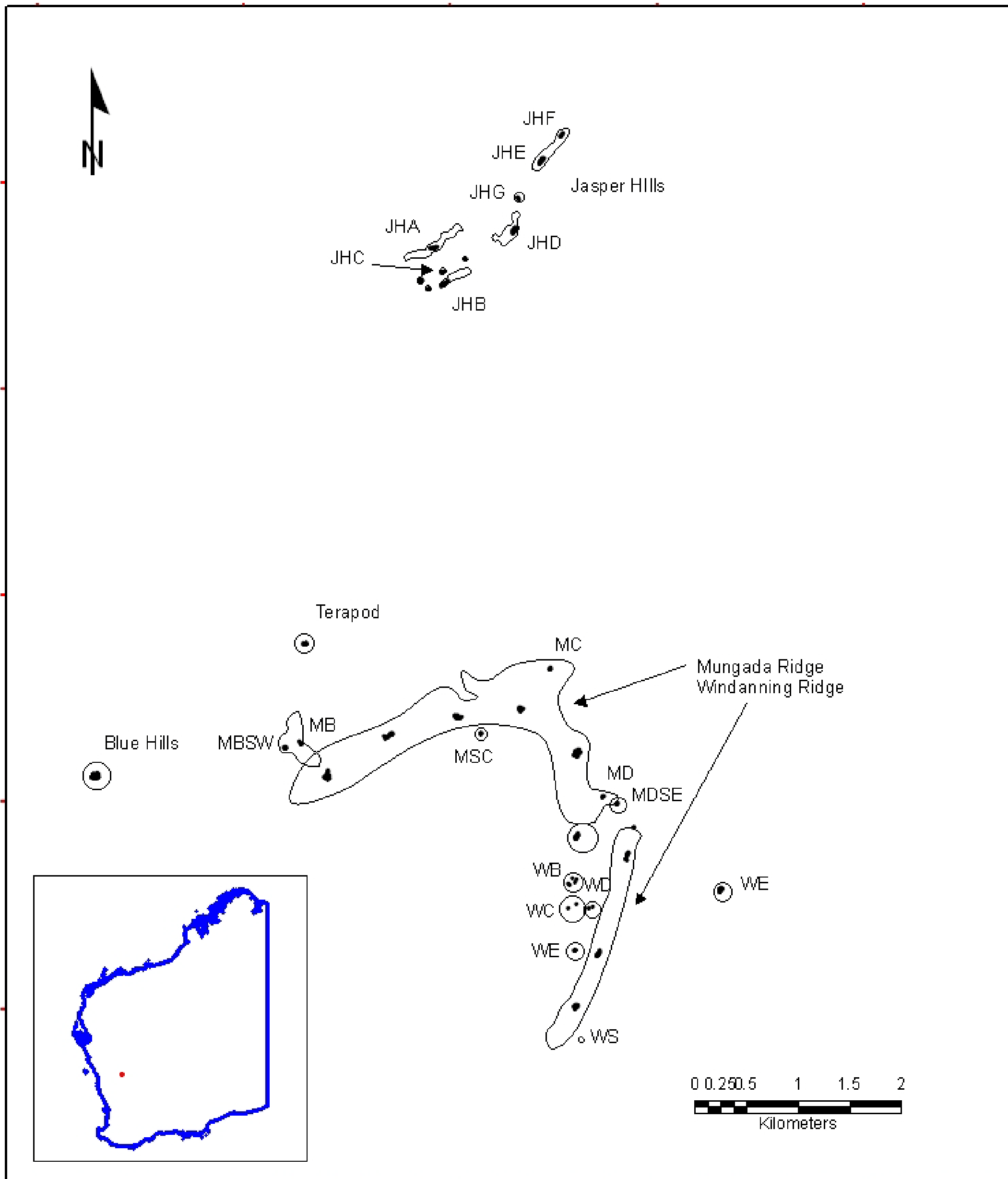

Figure 1 Distribution of *Acacia woodmaniourm*, location of subpopulations and sampled individuals (dark circles).

Table 1 Population names, subpopulation names, the number of individual sampled and the total number of individuals recorded for *Acacia woodmaniorum*.

Population	**Subpopulation** **Number of individuals sampled** **(Total number of individuals)**								**Total**
Mungada Ridge	**MA1-6**	**MAB**	**MAC**	**MAD**				**Outliers**	
	120 (15 850)	5 (1 164)	5 (880)	5 (270)				24	159 (18 186)
Windaning Ridge	**WA1-3**	**WAB**	**WAC**	**WAD**	**WAE**			**Outliers**	
	60 (6 169)	5 (105)	5 (375)	29 (29) All	16 (16) All			5	120 (6 699)
Jasper Hill	**JHA**	**JHB**	**JHC**	**JHD**	**JHE**	**JHF**	**JHG**	**Outliers**	
	20 (729)	20 (5 70)	5 (120)	20 (1 047)	20 (444)	5 (125)	5 (32)	42	137 (3108)
Blue Hills				All					140 (140)
Terapod				All					10 (10)
Total									566 (28 143)

Currently the maturation of seed pods from the 2009 flowering season is being monitored so that seed can be collected from smaller populations suitable for gene flow and for assessment of correlated paternity within pods.

Germplasm collections have also commenced for the *Acacia alata* species complex which occurs from north of Geraldton south to Albany. This species complex, specifically *A. alata* var *biglandulosa*, is thought to be the closest relative to *Acacia woodmaniorum* and will be used in the phylogenetic/phylogeographic component of the project (Phase Two). We are collaborating with Dr Gillian Brown of the University of Melbourne and the Royal Botanic Gardens Melbourne and others who are conducting a phylogenetic review of the Pulchelloidea clade of *Acacias*. This clade includes members of the section Alatae to which *A. woodmaniourm* and *A. alata* belong. So far germplasm has been collected from four populations of *A. alata* var *biglandulosa*, six populations of var *platyptera* and two populations of var *tetrantha*.

Laboratory Work

DNA has been extracted from all 566 sampled individuals of *A. woodmaniourm* for the analysis of species genetic variation and population genetic structure. 60 primer pairs were trialled for amplification of microsatellites in *A. woodmaniourm* and 28 primer pairs have been optimised for use. This work is described in further detail in the article 'Characterisation of microsatellite DNA markers for the rare *Acacia woodmaniorum* (Leguminosae: Mimosaceae)' which has been accepted for publication in the journal Conservation Genetics Resources (Appendix 1). 16 primer pairs have been selected for genotyping of the main germplasm collection and this work is currently being conducted.

DNA extractions protocols have been successfully optimised for *Acacia alata* samples and extractions are currently being conducted. DNA from two *A. woodmaniorum*, two *A. alata* var *biglandulosa*, one var *platyptera* and one var *tetrantha* accession have been sent to Dr Gillian Brown for inclusion in the phylogenetic analysis of the Pulchelloidea clade of Acacia.

Challenges

There is currently some difficulty monitoring the maturation of seed pods in the field. Seed pods from the 2009 flowering season must be collected this year as they mature in order to genotype progeny for an assessment of correlated paternity within pods and gene flow among populations in *A. woodmaniorum*. The distance and long travel times to site as well as the dependence of seed pod maturation on weather conditions (i.e. rainfall and temperatures at the site) make it difficult to predetermine the appropriate time for collections. We are seeking the assistance of John Tomich the Senior Environmental Officer with the monitoring of pod and seed maturation.

Appendix 1

Technical Note for Conservation Genetic Resources

Characterisation of microsatellite DNA markers for the rare *Acacia woodmaniorum* (Leguminosae: Mimosaceae)

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Keywords: microsatellites, M13 tagged primer, *Acacia woodmaniorum,* banded ironstone, Western Australia,

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Running Title: Microsatellite DNA markers for *Acacia woodmaniorum*

Abstract
Acacia woodmaniorum is a rare endemic shrub restricted to the banded ironstone ranges of Western Australia. In order to conduct studies of genetic structure and gene flow in *A. woodmaniorum*, a genomic library was constructed and novel polymorphic microsatellite markers developed. Markers were assessed in individuals from across the species range and polymorphism further investigated for 28 selected markers in 30 individuals from the Blue Hills population. Ten loci showed significant departure from Hardy-Weinberg equilibrium ($p < 0.01$), and two locus combinations showed evidence of Linkage Disequilibrium ($p < 0.001$). Ten loci showed evidence of null alleles. Levels of polymorphism were moderate in the Blue Hills population; the number of alleles per locus ranged from one to 11 and averaged 4.429, and both expected heterozygosities and observed heterozygosities ranged from 0.000 to 1.00 averaging 0.539 and 0.511 respectively.

Acacia woodmaniorum is a declared rare flora with a restricted range on the banded ironstone formations of the Yilgarn craton in the Midwest region of Western Australia. This land system is located on the border of the species-rich Transitional Rainfall Zone and the Arid zone and consists of a series of highly weathered low ranges rich in hematite and magnetite. *A. woodmaniorum* is a sprawling, prickly shrub, with high substrate specificity for the skeletal soils and rock crevices of hematite/magnetite rich outcrops. Plants typically occur high in the landscape on western and southern facing slopes. The species geographic distribution covers an area of <40 km^2 (Maslin & Buscomb 2007) and is comprised of two discrete banded ironstone ranges. Both ranges are composed of mineral deposits of significant economic value and covered by mining exploration leases. Knowledge of population genetic variation and structure in *A. woodmaniourm* is required to meet environmental management commitments that aim to minimise potential impacts to environments and species biodiversity by mining activities.

In order to assess genetic diversity in *A. woodmaniorum*, primer pairs were produced for 60 microsatellite loci isolated from di-nucleotide and tri-nucleotide microsatellite enriched genomic libraries, prepared by Genetic Identification Service (GIS, Chatsworth, CA, USA) (Table 1). DNA was extracted from 10g of fresh material of one individual, located on Mungada Ridge, essentially by the methods of (Wagner et al. 1987) with the following changes; phyllode material was ground to a fine powder using an electric coffee grinder and 40ml of half strength extraction buffer (with 5% (wt/vol) polyethylene glycol 8000, 0.05% bovine serum albumin, and 0.1M sodium sulfite) added. Pelletisation was by centrifugation for 7 min with resuspension in 4ml wash buffer (minus spermine and spermidine), addition of 1ml 5M NaCl, 1.6ml 8.6%CTAB/0.7M NaCl, and 2ml 10% sarcosyl, and samples incubated at 65°C for 15 min prior to chloroform extraction. DNA was tested for restriction with RSA1 and BSTU1 and four libraries constructed, each enriched for one of the following repeat sequences: CA, GA, AAC and ATG. DNA was partially restricted and the resulting fragments ligated into the *Hin*dIII site of pUC19 plasmid. Ligation products were introduced into *Eschericia coli* DHα (ElectroMax^{TM}, Invitrogen) by electroporation. Positive colonies were amplified with universal M13 primers, and clones with inserts between 350 and 700 bp were sequenced.

Phyllodes were sampled from further individuals located across the species range and DNA extraction conducted on 40mg of homogenised, freeze dried material, following the method of Doyle and Doyle (1987), with the addition of PVP-40T polyvinyl pyrrolidine to the extraction buffer and two chloroform extraction steps. Primer pairs for the initial 60 loci were screened for polymorphism in ten individuals from across four populations using an M13 tailed three primer PCR system. The forward primer of each primer pair incorporated a 5´ universal M13 tail and a third forward primer was modified with a 5´ fluorophore in addition to the complement of the universal M13 tail. Microsatellite loci were amplified in a total volume of 15 μl per reaction containing 20 ng template DNA, 50 mM KCl, 20mM Tris HCl pH 8.4, 0.2 mM each dNTP, 0.08 μM M13 tailed forward primer and reverse primer, 0.016 μM M13 tailed fluorescently labelled forward primer and 0.75 units of *Taq* DNA polymerase and $MgCl_2$ at 2.0mM for PCR program one and at 2.5mM for PCR program two. Program one consisted of 95°C for 4 min, 15 cycles of 30 s at 94°C, 30 s of step

down from 65°C to 50°C, 45 s at 72°C followed by 30 cycles of 15 s at 94°C, 15 s at 50°C, 45 s at 72°C followed by 8 min at 72°C. Program two consisted of 95°C for 4 min, 25 cycles of 30 s at 94°C, 30 s of step down from 65°C to 50°C, 80 s at 72°C followed by 30 cycles of 15 s at 94°C, 15 s at 50°C, and 45 s at 72°C followed by 8 min at 72°C. PCR conditions are presented for all loci in Table1. Reactions were completed in a Touchdown Thermal Cycler (Hybaid Ltd) and PCR products visualised on 8% non-denaturing polyacrylamide gels stained with ethidium bromide.

Of the 60 primer pairs tested, 43 could initially be interpreted as amplifying single loci in tested individuals. Nine primers failed to amplify and eight appeared to amplify multiple loci producing numerous bands that could not be resolved even under high stringency conditions. Amplification of the 43 loci was further tested in 24 individuals from across five populations of *A. woodmaniorum*. Fragments were visualised via automated fluorescent scanning detection using an Applied Biosystems 3730 DNA Analyser (Applied Biosystems) and genotypes scored using Genemapper™ v3.7 analysis software (Applied Biosystems). Of these loci, a further 15 appeared to amplify multiple loci producing numerous peaks in at least some populations, although this is possibly as the result of slippage during the PCR.

Marker utility and genetic diversity was investigated for 30 individuals from the Blue Hills North population with the remaining 28 loci (Table 1). One µl of diluted PCR product was added to 12 µl LIZ500(-250)/formamide, fragment analysis was carried out by automated fluorescent scanning detection and multilocus genotypes scored. Tests for Hardy-Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) were conducted using exact tests in GENEPOP version 3.4 (Raymond & Rousset 1995) with Bonferroni correction for multiple comparisons. Evidence for large allele drop-out, scoring error due to stutter and the frequency of null alleles were assessed with MICRO-CHECKER (Oosterhout et al. 2004) using a Bonferroni adjusted 95% confidence interval and 10 000 repetitions and genetic diversity parameters estimated using GenAlEx 6.2 (Peakall & Smouse 2006) (Table 1).

Ten loci showed deviation from HWE (Table 1, $p < 0.01$) and LD was detected for two locus combinations (AwB002 x AwB108 and AwA117 x AwB109, $p < 0.001$) in the Blue Hills population. There was no evidence of large allele drop-out. Homozygotic excess with an uneven distribution across alleles, may be evidence of null alleles and was detected for ten loci, at estimated frequencies ranging from 0.1031 to 0.3652 (Table 1, Oosterhout et al. 2004). An excess of homozygotes for alleles differing in size by a single repeat can be interpreted as evidence of scoring error due to stutter peaks and was detected for locus AwB106.

Overall, marker polymorphism was moderate in the Blue Hills population of *A. woodmaniorum* (Table 1). 96.43% of loci were polymorphic with one monomorphic locus. The number of alleles per locus ranged from 1 to 11 averaging 4.429, and the effective number of alleles ranged from 1 to 8.108 averaging 2.750. Both expected heterozygosities and observed heterozygosities ranged from 0.000 to 1.00, averaging 0.539 and 0.511 respectively. Values of the fixation index varied from -1.00 to 0.863 averaging 0.019 over all loci. The microsatellite loci identified in this

study will be further assessed for suitability in studies of population genetic structure and gene flow among populations of *Acacia woodmaniorum*.

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Table 1. Characterisation of 28 microsatellite loci in the Blue Hills population of *Acacia woodmaniorum*. Shown are locus names, primer sequence, GenBank accession number, repeat motif, program used for PCR amplification, the size range of observed alleles in base pairs (including the M13 tail), number of alleles (N), observed (H_o) and expected (H_e) heterozygosities, estimate of the fixation index (F) and the frequency of null alleles, where suspected, for 30 individuals. * denotes loci not in Hardy-Weinberg Equilibrium for this population.

Locus	Primer sequence (5′-3′)	GenBank accession no.	Repeat motif	PCR program	Allele size range	N	N_e	H_o	H_e	F	Null allele
AwA012	F:AAATCATAAACCCCGAACAG R:GGGAATGGGACTAACAAAAG	GQ452302	(GT)$_{11}$	2	232-240	3	1.729	0.367	0.422	0.130	-
AwA116	F:TTCCTCTCTTTCCTGTCTTGT R:GCCATTTGCCAGAACTTAT	GQ452303	(CA)$_{113}$	1	230-246	3	2.219	0.400	0.549	0.272	0.1437
AwA117*	F:TCACCCTTGTCTAAGCAAAG R:CTGAGCCACATTTTCTTGAG	GQ452304	(TC)$_{16}$(CA)$_{13}$	1	216-231	6	3.198	0.448	0.687	0.348	0.1687
AwA122*	F:GATGGGGTGATAAGGATTG R:TGTGCTTGTAAGGTTTCAAGT	GQ452305	(CA)$_{44}$	1	190-202	3	2.156	0.962	0.536	-0.793	-
AwA124	F:TTTCTTTCTCAGTTCTGACCAC R:TAGCGTGAGTTATTTCCTGATC	GQ452306	(CA)$_9$	1	192-197	5	1.622	0.379	0.383	0.011	-
AwA125*	F:ATGGGCGAGGTAGAACTC R:TCTTGTATGGTTTGTCTCAGTG	GQ452307	(CA)$_{12}$	1	161-164	4	3.986	0.586	0.749	0.217	0.1057
AwA129	F:TTTGTCAGTGCCATTTCATTAG R: GGGATAGTGGAACCGTGTC	GQ452308	(CA)$_{13}$	1	294-298	2	1.071	0.069	0.067	-0.036	-
AwB001	F:TAAGCCTAACCACACAGGGTC R:GGTGATCCAATGGCATTTC	GQ452309	(GA)$_{22}$	2	166-204	5	2.314	0.633	0.568	-0.115	-
AwB002*	F: AGGTGATAATCGGCGTCATC R:AGGCACGAACCCTGTAGC	GQ452310	(CT)$_{24}$(GA)$_{11}$ imperfect	1	292-330	7	3.719	0.433	0.731	0.407	0.1996
AwB003	F:GCCAGTTCATATCTGAATGTTC R:AATGGATCGTAGGAGTATTGTG	GQ452311	(CT)$_7$	2	200-220	7	3.409	0.533	0.707	0.245	0.1031
AwB008	F:CCTGTGCAGTTGTAACTACTCC R:GGCTTGTTTAACATTTCTCAAG	GQ452312	(GA)$_{12}$	1	287-289	2	1.147	0.138	0.128	-0.074	-
AwB009	F:ATGCTCGGAGTCTATCTATGG R:TCATCTTCCTTGTTCAGTGTC	GQ452313	(CT)$_{17}$	1	294-313	8	4.358	0.733	0.771	0.048	-
AwB106	F:TCATCTTCCTTGTTCAGTGTC R:AAAGCTAGGCAAGCTAGAAATG	GQ452314	(CT)$_{10}$	2	229-247	3	1.439	0.167	0.305	0.454	0.1750

AwB107	F:CCAATATCTACGTCACCAACTC R:TAGCTTCTTCATCGTCGAATAC	GQ542315	(TC)$_{16}$	1	197-218	11	8.108	0.933	0.877	-0.065	-
AwB108	F:AATGTTCCCTTCATCGTTCTC R:AGCACTTCATCTTGTTCACCA	GQ452316	(GA)$_{15}$	1	213-235	5	2.217	0.600	0.549	-0.093	-
AwB109	F:GAGAATTGAAGTCGCTTAGATG R:AAGTAAGGAATGGACGTGTATG	GQ452317	(CT)$_{22}$	2	156-178	4	3.651	0.667	0.726	0.082	-
AwC001	F:TCCTGAACCTTGACTCTGTG R:GATGGATGCTATGATGAAAATC	GQ452318	(TTG)$_{12}$ imperfect	1	159	1	1.000	0.000	0.000	-	-
AwC008	F:CATCAGCCAGTCCCATCTC R:TGGAGGCGAAGCATAGTG	GQ452319	(AAC)$_{28}$	1	277-317	9	5.813	0.652	0.828	0.212	-
AwD003*	F:ACCGCTTGAATCTTCTAACC R: CAGCCAACACAAAGGAATC	GQ452320	(TCA)$_{6}$	1	140-150	4	2.017	0.069	0.504	0.863	0.3652
AwD007*	F:AGATTGATGCAATGCTTGTC R:GTTCTTGCGTGCTCTCTG	GQ452321	(ATG)$_{10}$ imperfect	2	217-230	5	2.628	0.367	0.619	0.408	0.2148
AwD008	F:GGGAGAAAGCAACGAAAT R:GCATCAGTAGGCGTAGGAC	GQ452322	(TCA)$_{8}$ imperfect	1	193-209	3	2.597	0.633	0.615	-0.030	-
AwD009*	F:ACCTCCACCTTTCATTCTCC R:TTCCACCCATTGTTCCTTC	GQ458232 3	(TCA)$_{7}$	1	221-230	5	3.114	0.467	0.679	0.313	0.1515
AwD010	F:GCCTTGAGAGGAGCTTCTG R:CCTGAGGATGGAGGACAAG	GQ452324	(TCA)$_{7}$	1	213-216	3	1.479	0.379	0.324	-0.171	-
AwD012	F:TTTACCTTCAACATCCACCTC R:CCACCGTCGGAGACTCTA	GQ452325	(TCA)$_{9}$ imperfect	1	233-239	3	2.039	0.767	0.509	-0.505	-
AwD113*	F:GGAAGAACAACACACAGAACTG R:CTATCCCTTCATGCGTAAGAAC	GQ452326	(TCA)$_{10}$	1	198-221	7	3.984	0.370	0.749	0.505	0.2412
AwD116	F:ATCCTACGGGTAGTTTCAGTTC R:GAACAACGATCTTCAAGACTTG	GQ452327	(ATG)$_{6}$	1	151-154	2	1.998	0.567	0.499	-0.135	-
AwD117*	F:CTAACCATCATTGCGACTTCTT R:ATTGGCAAGCTAAAGTTACAGC	QQ452328	(ATC)$_{7}$	1	246-252	2	2.000	1.000	0.500	-1.000	-
AwD120*	F:TGGGGACATTCAGACTTG R:CAAAGGGATTGCTCTAAAGAC	GQ452329	(ATC)$_{7}$ imperfect	1	230-242	2	2.000	1.000	0.500	-1.000	-
Mean (standard error)						4.429 (0.456)	2.750 (0.291)	0.511 (0.053)	0.529 (0.043)	0.019 (0.083)	