High levels of genetic divergence between Tasmanian and Victorian platypuses, *Ornithorhynchus anatinus*, as revealed by microsatellite loci

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ABSTRACT

The platypus, *Ornithorhynchus anatinus* is a unique, iconic mammal endemic to Australia. Despite being listed as 'common' throughout its range, platypus abundance is poorly understood. Dependence on aquatic habitats in Australia renders this species potentially vulnerable to a variety of processes including drought, climate change and habitat loss. To assist with understanding population processes, 180 individuals from Tasmania and Victoria were characterised across thirteen microsatellite loci. Large genetic differences were evident between Tasmanian and Victorian *O. anatinus*. Within Tasmania, high levels of allelic diversity were detected with genetic differentiation identified among some populations. Similarly, allelic diversity was high within Victorian platypuses, along with significant genetic differentiation among populations. The large genetic differences found between Tasmanian and mainland platypuses indicate long-term isolation and it is likely that the lack of past/present catchment connectedness contributes to differentiation found between populations within these regions. Understanding patterns of genetic differentiation within and between catchments will help guide future conservation management decisions for platypus.

Keywords: platypus, *Ornithorhynchus anatinus*, microsatellite, population structure

The uniquely Australian platypus, *Ornithorhynchus anatinus*, (Monotremata: Ornithorhynchidae), is one of only three commonly recognised species of extant egg-laying mammals (Grant 2007). This semi-aquatic monotreme is confined to inland waterways and its distribution is largely restricted to the east coast of Australia (Figure 1) from Cooktown in the north to Tasmania in the south (Grant & Temple-Smith 1998). Despite being listed as common throughout their range, platypuses are still considered potentially vulnerable (Carrick 1983). This is largely due to a dependence on freshwater aquatic habitats with continuing degradation of suitable water bodies caused by damming, drainage, pollution and drought likely to lead to an increase in the isolation and fragmentation of populations (Grant & Temple-Smith 2003). Understanding demographic and population processes such as genetic diversity, population structure, gene flow, population size and distribution are therefore critical for the longer-term management of this species.

Previous research has identified significant differentiation among *O. anatinus* populations in morphological characteristics, life history traits and, potentially, in disease susceptibility (Carrick 1983; Munks et al. 2000; Otley et al. 2000a; Stewart 1996; Strahan 1995). Morphological studies have found body size varies from north to south along the *O. anatinus* distribution as well as in rivers flowing either side of the Great Dividing Range (Grant & Temple-Smith 1983). This raises the issue of whether these population differences have arisen in response to environmental conditions or whether they reflect underlying genetic variation.

Mitochondrial DNA (mtDNA) analysis has detected some geographic partitioning between populations (Gemmell 1994), while genetic structure analysis revealed four separate genetic clusters from samples around Australia (Warren et al. 2008). High levels of genetic divergence between Tasmanian and mainland *O. anatinus* individuals has been identified through microsatellite analysis (Akiyama 1998), however limited sample sizes restricts the conclusions that can be drawn. Increasing the knowledge of population processes in this species is likely to become increasingly important given that, under a climate change model, drought conditions in south-eastern Australia are anticipated to occur with increased frequency and intensity (Howe et al. 2005). To assist with interpreting contemporary patterns of genetic divergence in *O. anatinus*, we have collected a large number of samples from catchments in Victoria and Tasmania and individuals have been genotyped at thirteen microsatellite markers (see Supplementary Table 1).

MATERIALS AND METHODS

Sample collection

Hair or web-tissue samples were collected from 70 Victorian and 110 Tasmanian platypuses between October 2007 and December 2008 (Figure 1). Hair follicles were removed with forceps or alternatively, a section of skin webbing approximately 2 mm^2 was cut from the distal margin of webbing on the rear foot

using sharp, sterile scissors. All samples were immediately placed in 2 mL Eppendorf tubes containing 100% ethanol.

DNA extraction and microsatellite analysis

DNA was extracted from either web-tissue or hair samples using a CTAB- phenol/chloroform extraction (Endersby et al. 2005) and genotyped at thirteen microsatellite loci (see Supplementary Table 1). Each microsatellite locus was amplified in a 10 μL Polymerase Chain Reaction (PCR) containing: 1.0 μL of DNA, 1 X PCR buffer (ThermoPol) 2mM $MgCl_2$, 2mM dNTPs, 0.25 μg purified bovine serum albumin (New England Biolabs), 0.5 units *Taq* polymerase (NEB), 0.3 μM forward primer end-labelled with [γ33P]-ATP, 0.1 μM unlabelled forward primer, and 0.4 μM reverse primer.

PCRs were carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). PCR cycling conditions consisted of an initial denaturation step at 94 °C (3 min) followed by 35 cycles of 94 °C (30 s), primer annealing (see Supplementary Table 1) (30 s) and extension at 72 °C (30 s), with a final extension at 72 °C (5 min). PCR products were separated through a 5% polyacrylamide denaturing gel at 65 W for 2–3.5 hours and exposed for 24–72 hours to autoradiographic film (OGX, CEA, Strängnäs, Sweden). Allele sizes were determined by comparison with a λgt11 ladder (*f*mol DNA Cycle Sequencing System, Promega, Madison, WI, USA).

To ensure accurate scoring of genotypes and determine PCR/scoring repeatability, 48 individuals were selected at random, re-amplified for six microsatellite loci and scored as above. The genotype scores were highly repeatable, with an error rate of less than 0.61%

Microsatellite data analysis

FSTAT version 2.9.3 (Goudet 2001) was used to calculate allelic richness averaged over loci and Weir & Cockerham's (1984) measure of F_{IS}. Observed (H_O) and expected heterozygosities (H_E) were estimated and deviations from Hardy–Weinberg equilibrium (HWE) were determined by exact tests and permutation in ARLEQUIN version 3.11 (Excoffier et al. 2005).

Ornithorhynchus anatinus were grouped into potentially interbreeding populations on the basis of water catchment borders in Tasmania (TASMAP©, State of Tasmania) and river connectivity in Victoria (Olinda Creek, despite falling within the borders of the Yarra catchment, remains isolated from the main Yarra system). The Yarra catchment was initially split into two populations because of the large distance between the upper and lower sections of the catchment. However, analyses indicated no difference between these two areas (data not shown) and therefore the samples were combined into one population for subsequent analyses. Minimum population size was set at 8 individuals which, although a small number for constructing definitive conclusions, constitutes a reasonable size to provide the preliminary genetic

population analyses sought within this study. A global estimate and population pairwise estimates of F_{ST} were calculated in FSTAT (2800 permutations). Isolation by distance (Wright 1943) was tested using a regression analysis of Slatkin's (1995) linearized F_{ST} transformation ($F_{ST}/1 - F_{ST}$) onto the natural log of geographic distance (Rousset 1997). A Mantel test (Mantel 1967) (10000 permutations) was performed in POPTOOLS (HOOD 2002) to determine the significance of this relationship.

Genetic population structure was investigated with a hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992), partitioning the genetic diversity among regions (Tasmania and Victoria), among populations within regions and within populations. The AMOVA was performed in ARLEQUIN version 3.11 (Excoffier et al. 2005) with pairwise F_{ST} as the distance measure. Patterns of genetic differentiation were summarised between populations sampled using a multivariate factorial correspondence analysis implemented in GENETIX version 4.03 (Belkhir et al. 2004). The two factors that explain the majority of the variation in multilocus genotypes were plotted.

All tests involving multiple comparisons were corrected at the table-wide $\alpha' = 0.05$ level (Sokal & Rohlf 1995).

RESULTS AND DISCUSSION

Population genetic structure

One hundred and eighty *O. anatinus* individuals from two regions (110 from Tasmania and 70 from Victoria) were genotyped at thirteen microsatellite loci. All loci were polymorphic in both the Tasmanian and Victorian populations (Supplementary Table 1). In total, 135 alleles were detected across the thirteen loci. Despite many (86) unique alleles being recorded in Tasmania and Victoria, average allele numbers per locus within populations remained comparable (mean of 3.95 for Tasmanian populations and 4.925 for Victorian populations). Similarly, observed heterozygosities did not differ between regions when averaged over loci (0.532 and 0.519 respectively). Both Victorian and Tasmanian samples deviated significantly from HWE across the majority of loci (data not shown). This excess of observed homozygotes is potentially an artifact of combining several populations into one, resulting in a Wahlund effect (Wahlund 1928). Consequently, samples were divided into populations according to waterway connectivity or water catchment location. This resulted in loci approaching HWE (Table 1). However, some caution is warranted when interpreting results as sample sizes were small for some populations and familial relationships in samples could also lead to an excess of homozygotes (i.e. Olinda Creek $n = 8$).

The global estimate of F_{ST} over all populations and loci was significantly different from zero ($F_{ST} = 0.226$, 99% CI 0.132-0.344) indicating population differentiation and limited gene flow. The pairwise F_{ST} estimates indicated significant differentiation between Tasmanian and Victorian *O. anatinus* populations

after correction for multiple comparisons, with F_{ST} values ranging from 0.301 to 0.407. Within Tasmania, populations exhibited low levels of genetic differentiation with F_{ST} values ranging from 0 to 0.096. Populations of the Great Forester-Brid catchment and of the Lower Derwent catchment, however, showed significant differentiation from all other populations (F_{ST} =0.049 to 0.096, $P < 0.001$). Within Victoria, significant genetic differentiation was observed between Olinda Creek and the Yarra River population (F_{ST} = 0.048, $P < 0.001$).

The AMOVA results provide further evidence for highly significant genetic divergence between Tasmanian and Victorian *O. anatinus*. The majority of variation in the microsatellites was explained by differences within populations (67.89%, $F_{ST} = 0.3211$, $P < 0.001$). However, a significant amount of variation was also explained by differences among regions (30.64%, $F_{CT} = 0.3064$, $P < 0.001$), with a small but significant percentage of variation accounted for among populations within regions (1.47%, $F_{SC} = 0.0211$, $P < 0.001$). This suggests large genetic differences between *O. anatinus* from Tasmania and Victoria and some differences occurring between populations within both states.

The relationship between individuals from different populations and different regions is best represented by the two-dimensional factorial correspondence analysis of the microsatellite variation (Figure 2). The factor that explained the majority of the variance (factor 1, 64.51%) differentiated the Tasmanian from the Victorian populations. Samples from Victoria were spread most widely across the second axis (factor 2, 11.16%), while Tasmanian populations remained clustered indicating a lower level of genetic variation found within this region.

The large genetic differentiation detected between Tasmanian and Victorian populations of *O. anatinus* is not surprising given the long-term isolation of these two regions. During the last glacial maximum Tasmania was connected to the mainland permitting the migration of *O. anatinus* between the two states via a series of freshwater networks and land bridges (Lambeck & Chappell 2001). Historic patterns of river flows, however, show limited potential for migration between populations from the regions analysed (Harris et al. 2005) suggesting that the isolation of these populations dates back much earlier. The significant genetic differentiation between Tasmanian and Victorian *O. anatinus* detected here is likely to reflect independent evolution occurring in these regions and may underlie divergence recorded in some morphological and life history traits (Carrick 1983; Munks et al. 2000; Otley et al. 2000a; Strahan 1995).

The genetic homogeneity of four of the Tasmanian populations may reflect current and widespread gene flow throughout these catchments. However, geographic distance could also be influencing levels of genetic divergence: the four genetically homogeneous populations lie within the north-central part of the state (Figure 1) while the Lower Derwent population, yielding the highest levels of pairwise genetic differentiation, is located approximately 130km to the south. Preliminary Mantel tests detected a weak

correlation between geographic and genetic distance within Tasmanian *O. anatinus* ($r = 0.071$, $P < 0.05$). Significant levels of genetic differentiation apparent between the Great Forester-Brid population and the adjacent North Esk population, however, suggests additional factors are likely to contribute to genetic partitioning. Terrestrial dispersal is thought to be less common on the mainland (Otley et al. 2000b), possibly due to increased predator pressure, land clearing and urbanization, which may contribute to genetic differentiation between *O. anatinus* populations of different river systems. Further sampling within Victoria will help to determine whether populations on the mainland are more divergent than their Tasmanian counterparts.

In conclusion, clear genetic differentiation was identified between populations of *O. anatinus* from Tasmania and those in Victoria. This differentiation is characterized by the many unique alleles found within these states. Despite similar levels of allelic diversity occurring within Tasmania and Victoria, the microsatellite results indicate that there is low genetic differentiation among populations of *O. anatinus* within Tasmania. Further investigation of population genetic structure through additional sampling will provide important information for management bodies to assist in the conservation of this unique Australian monotreme.

ACKNOWLEDGEMENTS

We thank the Holsworth Wildlife Research Fund, Melbourne Water, and the Australian Government via both NRM North, and the 'Caring for Our Country' Program for funding. We thank Michael Driessen and Annie Philips from DPIW, and the many volunteers and Melbourne Water staff for help collecting samples. This work was carried out with ethics approval (DPI 09.07, DPI fisheries RP907, DSE 10004130, DPIW 16/2007-08, and Tasmanian Inland Fisheries 2007/47).

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Table 1. *Ornithorhynchus anatinus* population statistics for Tasmanian and Victorian samples across thirteen microsatellite loci. *O. anatinus* individuals were allocated to populations based on water catchments (in the case of Tasmania) or river connectivity (in the case of Victoria) where a sample size of ≥ 8 had been collected.

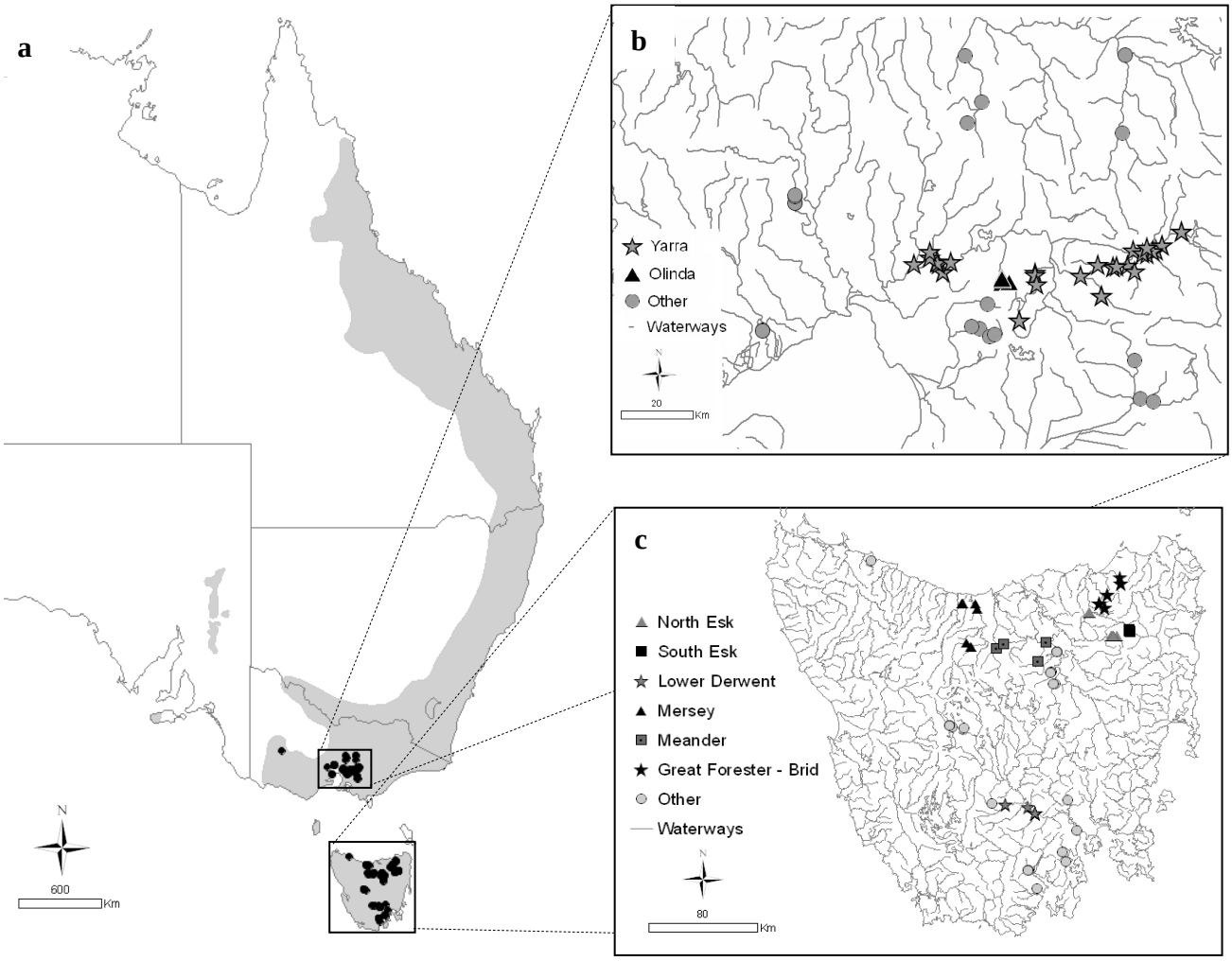
Region	Population	*n*	*a*	*r*	H_O	H_E	F_{IS}	HWE (*P* value)
Tasmania	All	110	6.54	5.92	0.532	0.584	0.088	0.204
	Great Forester-Brid	13	3.77	3.31	0.473	0.553	-0.014	0.820
	Lower Derwent	14	3.77	3.33	0.610	0.604	0.064	0.549
	Meander	12	4.08	3.51	0.568	0.568	0.050	0.631
	Mersey	12	3.69	3.27	0.577	0.530	0.123	0.497
	North Esk	15	4.08	3.36	0.534	0.560	0.017	0.292
	South Esk	16	4.31	3.56	0.445	0.536	0.181	0.064
Victoria	All	70	7.92	7.80	0.519	0.601	0.137	<0.001*
	Yarra	37	6.62	4.20	0.522	0.582	0.105	0.051
	Olinda Creek	8	3.23	3.14	0.570	0.492	-0.172	0.008

(*n*), the number of individuals genotyped for each population; (*a*), mean number of alleles; (*r*), allelic richness; (H_O), observed heterozygosity; (H_E), expected heterozygosity; F_{IS}, multilocus estimates; HWE, Hardy–Weinberg equilibrium *P* values;

* indicates significance after correction for multiple comparison

Fig. 1 a Distribution map of the platypus, *Ornithorhynchus anatinus*, modified from Grant and Temple-Smith (1998). Shaded region represents the current distribution and closed circles indicate collection sites within Victoria and Tasmania. Insets show the population groupings according to water catchments or river connectivity within **(b)** Victoria and **(c)** Tasmania

Fig. 2 Two-dimensional plot showing the relationships among populations of *Ornithorhynchus anatinus* based on a factorial correspondence analysis of thirteen microsatellite genotypes. Only the first two factors are shown, with the percentage of variance given in parenthesis


a
N
600
Km
b
Yarra
Olinda
Other
Waterways
N
20
Km
c
North Esk
South Esk
Lower Derwent
Mersey
Meander
Great Forester - Brid
Other
Waterways
N
80
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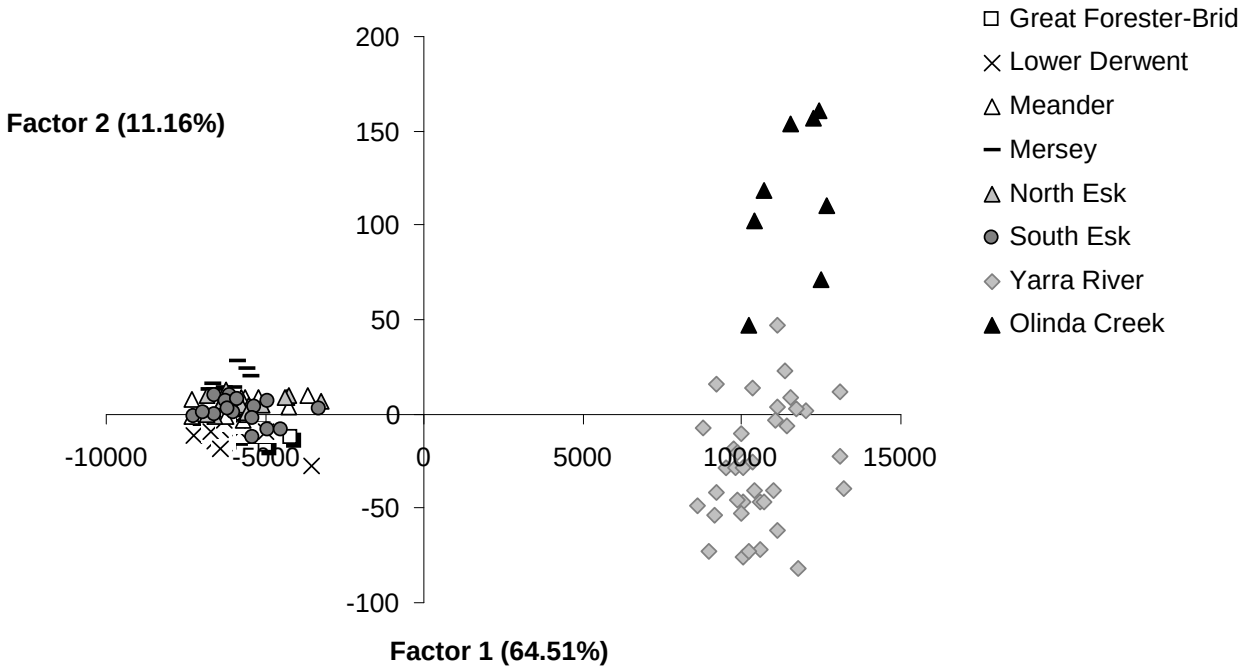
Factor 2 (11.16%)
200
150
100
50
0
-50
-100
-10000
-5000
0
5000
10000
15000
Factor 1 (64.51%)
Great Forester-Brid
Lower Derwent
Meander
Mersey
North Esk
South Esk
Yarra River
Olinda Creek

Supplementary Material – Isolation and characterization of microsatellite loci in *Ornithorhynchus anatinus*

The recent sequencing of the platypus genome (WARREN *et al.* 2008) has allowed us to better investigate population processes through genetic research by providing a reference for sequence information. Previous microsatellite studies in the platypus have relied upon markers isolated from humans with the chromosome location or microsatellite motif of the platypus unknown (AKIYAMA 1998). Microsatellite repeat regions are now more readily identifiable throughout the platypus genome and are able to be placed to their exact chromosome location.

Thirteen microsatellite loci have been isolated and optimised for amplification in *Ornithorhynchus anatinus* (see supplementary Table 1). Characterisation of 180 individuals from Tasmania and Victoria revealed all loci to be polymorphic (3-24 alleles per locus) with observed heterozygosities ranging from 0.445 – 0.610. The microsatellite loci show sufficient variation to permit analyses of the population genetics of *O. anatinus* throughout their geographical range. These markers, together with those developed by Kolomyjec *et al.* (2008) provide an important tool for assessing population processes affecting platypuses and will assist conservation of this unique Australian monotreme.

Microsatellite development

Microsatellite repeat regions were identified from the assembled platypus genome (WARREN *et al.* 2008) using the web-based computer program Tandem Repeat Occurrence Locator (TROLL) (http://wsmartins.net/webtroll/troll.html). Potential microsatellite regions were assessed based on repeat motif, repeat length and characteristics of the flanking regions. The chromosome locations of repeat regions were identified from the platypus genome assembly. To ensure a broad coverage of the genome and to minimise any risk of linkage disequilibrium, microsatellite loci selected for analysis were located on different chromosomes or chromosome arms. Primers were then designed to amplify repeats using OLIGO Primer Analysis 4.04 (National Biosciences, Plymouth, MN, USA).

Table 1 Thirteen polymorphic microsatellite loci isolated from *Ornithorhynchus anatinus* and tested on 180 samples collected from Tasmania and Victoria

Locus	Repeat Motif	Primer sequence 5'-3'	Motif Start Position	T_a	Size range (bp)	*k*
OA 1.3	$(GA)_5$ N $(AG)_5$ N_{20} $(AG)_{14}$	F: GACCTCTTTGCCACTTTGCTA R: GGATTAGAACCCACGATCTGTT	524364	59.4	187-213	10 (4)
OA 3.2	$(GA)_5$ $(AT)_5$ N_{10} $(TG)_{12}$	F: GCCCTATGTACCTTGAATATAA R: ACAGTTGGTGGACTTGATTC	1742787	48.7	115-127	6 (3)
OA 4.5	$(AC)_{14}$	F: ACGCCCCACCCGTTCCCTTTC R: ATCCATTCGCCGATCTCCTGTGC	10343093	61.4	184-207	11 (6)
OA 5.1	$(GT)_{14}$	F: CTTGGAAAGCATACACAGATG R: GAAATTGTTGGACTATGGGTAT	1302697	52.0	218-228	4 (2)
OA 6.2	$(TC)_{22}$	F: TAGGGTGGTTTGAAAGGTTTTG R: AGACAGCCGTAGGAGCACTAAA	592875	56.1	191-239	24 (19)
OA 7.3	$(TG)_{12}$	F: AATCTGAAAAGGCAACAATCT R: GGGCTTATCATTTGTCCTCTA	196957	48.4	135-163	13 (9)
OA 10.5	$(TC)_9$ N $(CA)_{10}$	F: GCTCTGATGGCTAATACTGCTA R: ATCCCTTCCCTCTCCATTATTA	2805189	50.3	244-250	4 (2)
OA 11.9	$(GA)_5(GT)_{10}$	F: GGTCAAAGAGTCCCAGAATGAC R: GAGACAGGAAACTTGGCATAGG	2799505	60.1	130-134	3 (0)
OA 12.6	$(CA)_{14}$	F: GATCTCCCACTACCGACAGTTT R: CAGGGTGGAATGATTACAGAAA	3263043	56.1	190-210	10 (8)
OA 14.3	$(CA)_{14}$ $(CACAC)_2$ $(AC)_5$ $(CA)_{13}$	F: GAAGGAGGAGGAGAGGTTGACA R: TTCAGCGACTTTTCTGTTCCATAG	623741	54.0	164-188	15 (11)
OA 17.6	$(GA)_{10}$	F: GTAACTTCTCACGGGGCAACTT R: GGCATTTTATTTTCTCGCCTCTA	1343141	53.5	220-228	5 (3)
OA 18.5	$(TG)_{11}$ N_3 $(GC)_6$	F: TTGTCTATATTCTTGGAAGGGCTC R: ATTGCAGGTAAAGTGAAGGGAA	1215676	60.0	138-148	11 (6)
OA 20.12	$(TC)_{15}(CT)_{17}$	F: GTTCCCTTGAGGACGGAGA R: CAGTGGGCTTTTCCATTCATA	892777	60.1	204-244	19 (9)

*T*a, annealing temperature; *k*, number of alleles observed (number of unique alleles observed between Tasmania and Victoria are given in parenthesis)