**Dispersal patterns and population structuring among platypuses, *Ornithorhynchus anatinus*,
throughout south-eastern Australia**

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Abstract

Dispersal patterns can have a major impact on the dynamics and viability of populations, and understanding these patterns is crucial to the conservation and management of a species. In this study, patterns of sex-biased dispersal and waterway/overland dispersal are investigated in the endemic Australian platypus, *Ornithorhynchus anatinus*, a semi-aquatic monotreme. Analyses of over 750 individuals of south-eastern Australia at thirteen microsatellite loci and two mitochondrial genes, *cytochrome b* and *cytochrome oxidase* subunit II, provide genetic insight into dispersal patterns. For the first time, western Victorian individuals are shown to be genetically distinct from other populations in Victoria. Despite distinct morphological differentiation either side of the Great Dividing Range, populations remain genetically similar between coastal and inland areas suggesting gene flow is likely to occur across these ranges. Landscape genetic analyses indicate variability in dispersal patterns between Victorian and Tasmanian platypuses with a greater avoidance of overland travel indicated in Victoria compared to Tasmania. Females appear to remain within their natal area or return to breed, maintaining greater genetic structure in maternally inherited mitochondrial DNA in comparison to nuclear DNA and sharing genetic similarity within a short river distance (i.e. ≤ 1.4 km). The results of this study provide a valuable spatial framework for the management of wild platypus populations within south-eastern Australia and a baseline for future monitoring of populations that are likely to be impacted by environmental and anthropogenic change.

Keywords: Sex-biased dispersal, semi-aquatic, microsatellites, mtDNA, genetic diversity, phylogeography, landscape genetics

1 Knowledge of the population structure and dispersal patterns of a species can direct conservation
2 and management plans. Uncovering patterns of dispersal can provide important insight into
3 population dynamics including the extent of relatedness among individuals or between regions. In
4 this study, population structure and patterns of genetic dispersal are investigated in a semi-aquatic
5 monotreme; the platypus, *Ornithorhynchus anatinus*. The platypus is endemic to Australia with a
6 distribution extending along much of eastern mainland Australia and throughout Tasmania
7 (Figure 1). It is the only extant member of its family and, along with several species of echidna, is
8 one of the few monotremes that exist worldwide.

9
10 The platypus is listed as a species of least concern by the International Union for Conservation of
11 Nature (Lunney et al. 2008) but, acknowledging its dependence on waterways, must be
12 considered potentially vulnerable (Carrick et al. 2008). A number of factors could combine to
13 threaten populations including invasive predators (Connolly et al. 1997), disease (Gust and
14 Griffiths 2009), human-mediated environmental change (Grant and Temple-Smith 2003) and
15 drought (Grant 1992b). Localised declines or extinctions have been recorded, particularly in
16 urban and agricultural areas (Grant 1992b). Climate change is predicted to further impact the
17 distribution of the platypus with increasing temperatures forecast to reduce suitable habitat by >
18 30% by 2070 (Klamt et al. 2011). Despite the species' iconic status, fundamental gaps persist in
19 the contemporary understanding of platypus biology, ecology, and behaviour (Grant 2007). A
20 paucity of knowledge on abundance, demographics and platypus behaviour in the wild currently
21 limits our understanding of the precise impacts of many potentially threatening processes.

22
23 Currently, the platypus is listed as a single species, despite differences in the morphology and
24 behavior of individuals over a variety of spatial scales. For instance body size increases with
25 increasing latitude and larger individuals are found inland of the Great Dividing Range (Furlan et
26 al. 2011). Breeding seasons also occur earlier in the north of Australia (Carrick et al. 2008).
27 Variation in morphology or life history traits can occur in response to environmental conditions
28 as a result of phenotypic plasticity (Nylin and Gotthard 1998; Riek and Geiser 2012; Stillwell and
29 Fox 2009). Alternatively, genetic components can contribute to body size variation (Aulstad et al.
30 1972; Boyko et al. 2010; Flint and Mackay 2009; Refstie 1980; Weedon et al. 2008) and
31 variability in breeding behaviour (Pierre et al. 2011; Solovyeva and Pearce 2011). Among
32 platypus populations, genetic differences may contribute to observed differences in morphology
33 and behaviour, although this has not been investigated previously.

1 Broad levels of population structure have been detected within the species. Genetic differentiation
2 between Tasmanian and mainland platypuses has been well established at both the mitochondrial
3 and nuclear level (Akiyama 1998; Furlan et al. 2010; Warren et al. 2008). Evidence of further
4 geographic partitioning exists on the mainland with some nuclear differentiation observed
5 between populations of Queensland and New South Wales (Kolomyjec 2010) and distinct
6 mtDNA haplotypes revealed among individuals of Queensland, New South Wales and Victoria
7 (Gemmell 1994; Gongora et al. 2011). Genetic population structure at a finer spatial scale has not
8 yet been explored.

9
10 Many factors can potentially influence genetic differentiation by impacting gene flow. Dispersal
11 distances or the role of various barriers will alter according to the physiology and/or habitat
12 preferences of a species. For example, dispersal patterns differ extensively between terrestrial and
13 aquatic-restricted species. For aquatic-restricted species, movement is confined to waterways and
14 therefore, waterway connectivity is fundamental to dispersal and ultimately, gene flow. Large
15 genetic divergence has been observed between disconnected river systems for molluscs,
16 crustaceans and fish (Bernatchez et al. 1992; Coleman et al. 2010; Hughes 2007; Musyl and
17 Keenan 1992; Rourke 2008; Rourke et al. 2011; Sepulveda-Villet et al. 2009). Frequently, genetic
18 relatedness among aquatic populations also reflects historic changes in stream connections
19 (Hughes et al. 2009; Kotlik and Berrebi 2001; McGlashan and Hughes 2000; Sepulveda-Villet et
20 al. 2009). The platypus appears to be well adapted to an aquatic environment; it possesses webbed
21 feet for efficient swimming, a streamlined body, well-insulated fur (Grant and Dawson 1978) and
22 depends upon freshwater to provide food (Faragher et al. 1979; McLachlan-Troup et al. 2010).
23 Consequently, waterways are likely to facilitate dispersal in the platypus. Within south-eastern
24 Australia, distinct separation between river basins occurs between inland and coastal regions of
25 New South Wales and Victoria, where the Great Dividing Range imparts a significant
26 biogeographical barrier to numerous fish species (Musyl and Keenan 1992). Considerable levels
27 of genetic differentiation have been detected either side of the Great Divide in other aquatic and
28 semi-aquatic species (Georges et al. 1998; Jerry 2008; Musyl and Keenan 1992; Symula et al.
29 2008) suggesting a lack of migration occurring across the ranges. It seems likely that significant
30 morphological differentiation in platypus body size, identified in individuals either side of the
31 divide (Carrick et al. 2008; Furlan et al. 2011; Grant 2007), may also have a genetic basis. Steep
32 terrain and an absence of interconnected waterways either side of the divide are likely to restrict
33 dispersal and lead to genetic differentiation among platypuses.

1 Being a semi-aquatic mammal, the platypus also has the capacity for terrestrial dispersal, which
2 means that gene flow patterns may differ from aquatic-restricted species. For example, terrestrial
3 dispersal in the freshwater shrimp, *Caridina zebra*, resulted in gene flow across river basins
4 creating one widespread clade (Hurwood and Hughes 2001). Unfortunately, little is known of the
5 dispersal patterns of semi-aquatic mammals; a limited number of radio-tracking or live-trapping
6 studies have provided some information on dispersal patterns in other semi-aquatic species (e.g.
7 Caley 1987; Reid et al. 1994; Stone and Gorman 1985), although the time and cost involved in
8 such studies often restrict data to a localised area and/or a limited number of individuals. The
9 platypus appears to be capable of migrating between adjacent river basins, having been reported
10 covering distances up to 7 to 8 km overland (Burrell 1927) and occasionally negotiating steep
11 terrain (Burrell 1927; Furlan pers. obs.). A higher frequency of overland movement has been
12 reported to occur in Tasmanian platypuses in comparison to mainland individuals (Connolly et al.
13 1997; Otley et al. 2000) but it is currently unclear how often, or over what distance platypuses are
14 able to make successful terrestrial movements between water bodies.

15
16 Genetic connectivity has been found between platypuses of two adjacent, disconnected river
17 systems in NSW suggesting overland dispersal (Kolomyjec et al. 2009). Such dispersal is costly
18 however, since terrestrial movement in this species requires proportionally more energy than
19 swimming (Fish et al. 2001), increases the risk of predation (especially in more recent times of
20 human habitation and invasive predators) and may expose individuals to lethal heat stress (Grant
21 and Dawson 1978). Several observations of terrestrial dispersal recorded during times of drought
22 (Ellis 2000) may have occurred out of necessity and have not necessarily contributed to a
23 successful genetic or effective dispersal event (i.e. movement of an individual to a location where
24 it successfully reproduces). Accordingly, terrestrial dispersal may remain sporadic, and probably
25 contributes less to gene flow than movements along streams and rivers.

26
27 Many species display a sex-bias in their dispersal patterns. Studies in mammals indicate a
28 propensity for males to disperse while females remain within their natal area (Greenwood 1980;
29 Lawson Handley and Perrin 2007) although, some notable exceptions exist (Clutton-Brock 1989;
30 Sweitzer and Berger 1998; Walker et al. 2008; Zhan et al. 2007). Sex-biased dispersal can
31 influence the social behaviour and dynamics of a population as well as its genetic structure
32 (Bohonak 1999; Surridge et al. 1999). The majority of studies into mammalian sex-biased
33 dispersal have been conducted in eutherian mammals and, to a lesser extent, marsupial mammals.
34 Thorough investigations into sex-biased dispersal in monotremes have not yet been conducted.

1 Previous radio-tracking and mark-recapture studies have provided some limited information on
2 movement patterns of platypuses. These studies indicate that, although home ranges are typically
3 confined to a couple of kilometers (Ellis 2000; Grant 2007; Gust and Handasyde 1995;
4 McLachlan-Troup 2007), significant dispersal events of greater than 20 km have been recorded in
5 platypuses, most frequently among immature (< 2 years of age) or adult males (APC 2001, J.
6 Griffiths personal observation). Although migration events can be detected through methods such
7 as radio-tracking or mark-recapture, these do not differentiate between ecological dispersal and
8 effective dispersal (i.e. movement of an individual to a location where it successfully reproduces)
9 (see Box 2 in Lawson Handley and Perrin 2007). These research methods are often costly and
10 time-consuming and consequently, provide information on a limited number of individuals. Using
11 genetic analyses to identify dispersal patterns necessarily focuses on effective dispersal and relies
12 on DNA samples collected from a single capture event allowing dispersal patterns to be detected
13 across numerous individuals.

14
15 In this study we utilise extensive sampling throughout south-eastern Australia to conduct the
16 largest genetic study on the platypus. We investigate population structuring and dispersal patterns
17 of the platypus throughout its south-eastern geographic range incorporating individuals of three
18 New South Wales river basins and the majority of Victorian and Tasmanian river basins in which
19 they are known to occur (19/24 and 11/15 respectively). Collections of the majority of samples
20 occurred within a relatively short time span (2007-2010), providing the distinct advantage of
21 detecting any genetic structuring and gene flow without concern for temporal changes in allele
22 frequencies. We addressed two main hypotheses in relation to platypus dispersal. Firstly, that
23 overland dispersal will incur a greater cost than aquatic dispersal for platypuses and, secondly,
24 that platypus dispersal is male-biased. To address overland/waterway dispersal, 752 individuals
25 were genotyped at nuclear loci to investigate friction models and levels of genetic correlation
26 between individuals. We anticipate that friction models with high dispersal costs for land in
27 comparison to waterways will best predict the rate and pattern of gene flow within and among
28 river basins. Due to occasional overland dispersal events, we also expect to observe low genetic
29 structure among neighbouring river basins at the regional scale. Given the higher frequency of
30 overland dispersal purported to occur in Tasmania (Connolly et al. 1997; Otley et al. 2000), we
31 expect that dispersal costs for land in comparison to waterways will be reduced in Tasmania
32 compared with the mainland and we therefore expect to see lower genetic structuring in
33 Tasmanian platypuses. To investigate sex-biased dispersal patterns, a subset of individuals were
34 sequenced at the mitochondrial genes *cytochrome oxidase* subunit II (COII) and *cytochrome b*

(*cytb*) to provide a comparison to nuclear genetic structure. We anticipate that the maternally inherited mitochondrial DNA will be more highly structured than bi-modally inherited nuclear DNA due to male-biased dispersal. In spite of increased overland movement in Tasmania, mitochondrial genetic structuring is predicted to remain similar among platypuses of the mainland and Tasmania, provided the majority of overland movement on the island is attributed to males. In addition, we anticipate that females will maintain greater nuclear genetic relatedness within their natal area as a result of female philopatry.

Methods

Sample collection and DNA extraction

Hair or skin samples were collected from a total of 752 unique *O. anatinus* individuals. In total, 152 samples originated from New South Wales, 417 from Victoria and 183 from Tasmania. The majority of samples ($n = 541$) were collected from Victoria or Tasmania between October 2007 and December 2010 and build on the studies of Furlan *et al.* (2010) ($n = 180$). Additional samples were collected from New South Wales, Victoria and Tasmania between 1990-2002 [partially published in Akiyama (1998), Handasyde *et al.* (1992) and Handasyde *et al.* (2003)]. Platypuses were aged and sexed according to their spur morphology following the techniques of Temple-Smith (1973). Hair follicles were removed with forceps or alternatively, a section of skin webbing approximately 2 mm² was cut from the distal margin of webbing on the front or rear foot using sharp, sterile iris scissors. DNA was extracted from either web-tissue or hair samples using a CTAB-phenol/chloroform extraction method (Endersby *et al.* 2005) and genotyped at thirteen microsatellite loci as described in Furlan *et al.* (2010).

Microsatellite amplification and data analysis

Microsatellites were amplified using either fluorescence or radio-labeling protocols according to Furlan *et al.* (2010). Briefly, each radio-labeled microsatellite locus was amplified in a 10 µL Polymerase Chain Reaction (PCR) containing; 1.0 µL of DNA, 1X PCR buffer (ThermoPol) 2 mM MgCl₂, 2 mM dNTPs, 0.25 µg purified bovine serum albumin (New England Biolabs), 0.5 units Taq polymerase (NEB), 0.3 µM forward primer end-labeled with [³²P]-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 (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 h and exposed for 24–

72 h to autoradiographic film (OGX, CEA, Strängnäs, Sweden). Allele sizes were determined by comparison with a λ gt11 ladder (fmol DNA Cycle Sequencing System, Promega, Madison, WI, USA). Loci analysed via fluorescence had the forward primer labeled with a unique fluorophore (FAM, NED, VIC, PET) and were pooled into three groups for co-amplification using multiplex PCR following the protocols outlined in Blacket et al. (2012). Products were genotyped using an Applied Biosystems 3730 capillary analyser (AGRF, Melbourne, Australia) and product lengths were scored manually in GeneMapper version 4.0 (Applied Biosystems). At each locus, at least 20 individuals were analysed using both methods across all thirteen loci to ensure consistency across the two amplification techniques. To ensure accurate scoring of genotypes and determine PCR repeatability, 152 individuals were selected at random, re-amplified for all thirteen microsatellite loci and scored as above. Genotypes across all loci were highly repeatable, with an error rate of less than 0.68%.

River basin boundaries and waterway connectivity were defined according to *Australian River Basins* (Geoscience Australia, 1997) and *Drainage Australia* (Geoscience Australia, 2000). Geographic distances between individuals were calculated in GenALEX (Peakall and Smouse 2006) and river distance was calculated in the geographic information system program ArcGIS 9.3.1 (ESRI, Redlands, CA) using the network analyst add-in.

An estimate of the null allele frequency for each locus was performed in GENELAND (Guillot et al. 2005; Guillot et al. 2008; Guillot et al. 2009). Null alleles were found to be at low frequency across all loci (< 0.07) with a very low frequency of homozygous null individuals (< 0.012) and therefore they were not considered for any further analyses.

Given the high levels of genetic differentiation known to exist between Tasmania and the mainland (Akiyama 1998; Furlan et al. 2010; Warren et al. 2008), investigations of broad scale population structure were carried out using two independent analyses within these locations. To ensure the effects of isolation by distance are not mistaken for genetic discontinuity [see Schwartz and McKelvey (2009)], mainland cluster analyses excluded individuals of the Shoalhaven, Gwydir and Border River Basins that are separated from other sampling locations by > 230 km.

Genetic structure was analysed separately within Tasmania and the mainland using STRUCTURE version 2.3 (Pritchard et al. 2000). This was determined using only genetic data without any *a priori* allocation of individuals to locations. Five independent simulations were run for $K = 1-20$

1 with 100,000 burn-in iterations and 500,000 data iterations using an uncorrelated allele frequency
2 model. The true K was determined according to methods developed by Evanno *et al.* (2005) and
3 implemented on the STRUCTURE HARVESTER website
4 (http://taylor0.biology.ucla.edu/struct_harvest). Because STRUCTURE often only detects the upper
5 hierarchical level of structure (Evanno *et al.* 2005), the STRUCTURE analyses were rerun (as
6 described above) to investigate the presence of sub-structure.

7
8 Further genetic population structure was investigated with a hierarchical analysis of molecular
9 variance (AMOVA) (Excoffier *et al.* 1992), partitioning the genetic diversity among regions,
10 among populations within regions and within populations. The AMOVA was performed in
11 ARLEQUIN version 3.11 (Excoffier *et al.* 2005) with pairwise F_{ST} as the distance measure.
12 Individuals sampled within a river basin were grouped into putative populations. Two AMOVA's
13 were performed to investigate genetic diversity among Tasmanian and mainland regions and
14 either side of the Great Dividing Range in Victoria. Genetic diversity was also investigated
15 among populations within regions and within populations (i.e. within a river basin). Patterns of
16 genetic differentiation were summarised between individuals of Tasmania and the mainland
17 separately using a discriminant analysis of principle components (DAPC) (Jombart *et al.* 2010)
18 using the R package ADEGENET 1.3-5 (Jombart 2008). This analysis extracts information from
19 genetic data by transforming the genotypes into uncorrelated components using principal
20 components analysis then applying a discriminant analysis to a number of principal components
21 in order to maximize the among-population variation and minimize the variation within
22 predefined groups. We used river basins as *a priori* groups with 60 principle components used for
23 the mainland and 40 for Tasmania representing 91.2% and 94.9% of the total genetic variance
24 respectively.

25
26 Isolation by distance (Wright 1943) was assessed by Mantel tests (Mantel 1967) in POPTOOLS
27 (Hood 2002) (10,000 permutations) using genetic distance against the natural log of Euclidian
28 distance. Genetic distance was calculated using the GenAlEx (Peakall and Smouse 2006)
29 calculation for co-dominant genetic distance. Tests were carried out for individuals of Tasmania
30 and the mainland separately and relationships were also explored by linear regression.

31
32 To further explore patterns of dispersal, the cost-distance between individuals within Tasmania
33 and Victoria was generated using the ArcGIS 9.3.1 (ESRI, Redlands, CA) landscape genetics
34 toolbox (Etherington 2010). Water and land were weighted to create least cost path distances

1 among individuals. Friction values of water versus land were assigned as 1:1 (no difference
2 between water and land hence equivalent to using Euclidean distance), 1:2, 1:10, 1:100, 1:1000
3 on a cell size of 20 x 20 m. To study the effect of landscape structure across a level of reasonable
4 dispersal distance, individuals within river basins were analysed to test patterns of overland and
5 waterway dispersal with analyses restricted to individuals with reasonably contiguous sampling.
6 This meant individuals of the Bunyip River Basin were split into two groups due to a large
7 sampling gap between individuals (> 30 km) and four remote individuals of the Upper Murray
8 River Basin (separated by > 65 km) were excluded from analyses of this basin. Moreover,
9 analyses were restricted to river basins where > 90 comparisons were possible (i.e. $n \geq 14$
10 individuals). Partial mantel tests based on Pearson correlation statistic [adapted from Manly
11 (1991)] were performed for each cost matrix to compute the correlation between cost distance and
12 co-dominant genetic distance (Peakall and Smouse 2006; Smouse and Peakall 1999) corrected for
13 Euclidean distance using packages raster and vegan implemented in R (R Development Core
14 Team 2012). Significant results were determined after correction for multiple comparisons
15 according to the false discovery rate (Benjamini and Hochberg 1995).

16
17 Tests for sex-biased dispersal were carried out on individuals of the Shoalhaven River Basin,
18 NSW where reasonable sample sizes were obtained along a short stretch of river in the upper
19 reaches ($n = 122$ along 13.5 km of stream). Tests were conducted on adults (likely to represent
20 the post-dispersal population) in FSTAT version 2.9.3 (Goudet 2001; Goudet et al. 2002). Ten
21 thousand permutations were performed to generate statistical descriptors including Weir and
22 Cockerham's (1984) F_{ST} , relatedness and mean assignment index (mAI/c) (Favre et al. 1997).
23 Differences in these statistics between males and females were used to identify biases in
24 dispersal. Spatial autocorrelation tests were conducted on adult females at six distance classes
25 with > 100 comparisons per distance class to determine patterns of relatedness between
26 individuals of this sex. Analyses were carried out in GenAlEx (Peakall and Smouse 2006) with
27 genetic distance between individuals calculated using the GenAlEx (Peakall and Smouse 2006)
28 calculation for co-dominant genetic distance and compared to distance along the river. Significant
29 results were determined after correction for multiple comparisons according to the false discovery
30 rate (Benjamini and Hochberg 1995). Small sample size prohibited conducting similar spatial
31 autocorrelation tests among adult males.

32
33 *Mitochondrial DNA amplification and data analysis*

1 Mitochondrial primers were designed either side of the COII and *cytb* genes (see Supplementary
2 Material Table 1) according to published platypus mitochondrial sequence data (Janke et al.
3 1996).

4
5 Five hundred and forty-nine individuals were sequenced for 603 bp of the COII gene. For each
6 COII haplotype, a subset of individuals ($n = 265$ in total) was also sequenced for 835 bp of the
7 *cytb* gene. Polymerase Chain Reactions (PCRs) were performed in a total volume of 30 μ L
8 containing: 3.0 μ L of DNA, 1X PCR buffer (ThermoPol), 2 mM $MgCl_2$, 0.24 mM dNTPs, 0.5 μ g
9 purified bovine serum albumin (New England Biolabs), 0.3 units Taq polymerase (NEB), 0.4 μ M
10 forward primer and 0.4 μ M reverse primer.

11
12 PCRs were carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). PCR
13 cycling conditions consisted of an initial denaturation step at 94°C (3 min) followed by 35 cycles
14 of 94°C (30 s), primer annealing at 55°C (30 s) and extension at 72°C (30 s), with a final
15 extension at 72°C (5 min). PCR products were sent to AGRF laboratories (Melbourne, Australia)
16 for forward sequencing on an AB 3730xl DNA Analyzer. All unique sequence results were
17 sequenced in the reverse direction to ensure accuracy. PCR products were sequenced using the
18 above primers and a modified version of OAm₁cytb R:
19 OAm₁cytb_2 R-5'TAAGGATTGAGGCGACAAGG3'.

20
21 Sequences were aligned in BioEdit (Hall 1999) using the CLUSTAL W algorithm and edited
22 manually where required. Sequences were then imported into MEGA version 4 (Tamura et al.
23 2007). A partition-homogeneity test was run in PAUP version 4.0b10 (Swofford 1998) and found
24 COII and *cytb* to have the same evolutionary history ($p = 1.00$). Consequently, analyses were also
25 conducted for concatenated sequences of COII and *cytb* for 265 individuals. Phylogenetic trees
26 were constructed using both Bayesian inference and maximum parsimony methods (see below).

27
28 Given the evolutionary distinctiveness of the platypus and the rate of change in the mitochondrial
29 genes under analysis, no suitable outgroup species exists. The closest relative of the platypus, the
30 short-beaked echidna (*Tachyglossus aculeatus*), is separated by over 20 million years (Warren et
31 al. 2008) and is highly evolutionarily divergent. Incorrect or inappropriate outgroup selection has
32 been shown to significantly affect tree topology (see Miller and Austin 2006) and consequently,
33 relationships for the platypus are represented as networks within the species.

Pairwise distances between haplotypes and groups were calculated using the Kimura-2-parameter model in MEGA (Tamura et al. 2007). The optimum nucleotide substitution model was determined using JMODELTEST (Posada 2008) and results implemented in MRBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Bayesian inference phylogenetic trees were constructed using the Metropolis-coupled Monte Carlo Markov Chain (MCMC) method. Four chains were run for 5,000,000 generations, and trees were sampled every 500 generations. The first 40 trees were discarded as burn-in prior to calculating a 50% majority rules consensus tree.

Maximum parsimony phylogenetic trees were constructed in PAUP version 4.0b10 (Swofford 1998) to represent relationships between haplotypes. Bootstrapping analysis with 1,000 replicates was conducted to estimate support for nodes within the tree. Fu and Li's *D* (Fu and Li 1993) and Fu's *F* (Fu 1997) were calculated in DNASP V5.10 (Librado and Rozas 2009) to test whether observed mutation patterns in the mtDNA sequences were consistent with a neutral model of molecular evolution.

Results

Nuclear Microsatellite Variation

Implementing methods developed by Evanno *et al.* (2005) to determine the greatest change in *K*, STRUCTURE (Pritchard et al. 2010) analysis identified two population clusters (*K* = 2) within Victoria. Although many individuals show signs of admixture (mean *Q* values of cluster 1 = 0.78 and cluster 2 = 0.82), individuals in western Victoria (i.e. from the Glenelg, Wimmera, Hopkins, Otways, Barwon and Loddon River Basins) associated most strongly with cluster one while individuals of central Victoria (i.e. Maribyrnong, Goulburn, Yarra and Bunyip River Basins) associated most strongly with cluster two (Figure 2a). Interestingly, some individuals of eastern Victoria (i.e. Snowy, Ovens and La Trobe River Basins) also appeared to show an association with individuals of cluster one (i.e. western Victoria). Further analysis within the two clusters revealed the presence of substructure. Cluster one separated into a further three clusters clearly identifying individuals of the Glenelg River Basin and two individuals of the Wimmera River Basin as a unique cluster (Figure 2b) (mean *Q* values of 0.90). The remaining two clusters revealed no discernable association between individuals and geographic location (mean *Q* values of cluster 2 = 0.89 and cluster 3 = 0.87). Individuals assigned to cluster two in the original STRUCTURE analysis were assigned to a further three clusters, although *Q* values were low (< 0.7) and no discernable association was evident between individuals and geographic location (data not

shown). No genetic differentiation was identified among individuals of Tasmania (data not shown).

The variation in microsatellite data within the mainland and Tasmania (Figure 3a and b respectively) was plotted after implementing a DAPC (Jombart et al. 2010) using the R package ADEGENET 1.3-5 (Jombart 2008). The DAPC grouped mainland individuals into eight clusters along two axes with eigenvalues of 355.7 and 243.7 respectively. Some individuals of far western Victoria (the Glenelg River Basin, cluster 1) were separated by the first axis. The second axis produced slight separation of clusters 2 and 4. Cluster 2 was a mix of individuals from 16 mainland basins while cluster 4 consisted mainly of individuals from the Shoalhaven River Basin in NSW (Supplementary Material Table 2a). The remainder of mainland clusters did not produce any distinct separation along these two axes. The DAPC of Tasmanian platypuses grouped individuals into five clusters along two axes with reasonably low eigenvalues of 132.7 and 94.51 respectively. None of the five clusters correspond clearly to river basins or geographic location of individuals (Supplementary Material Table 2b). Minimal separation was evident among the five clusters.

AMOVA analysis of Tasmania and the mainland detected significant differentiation among regions (27.57%, $p < 0.001$) (Table 1). An additional AMOVA analysis detected no significant genetic differentiation either side of the Great Dividing Range in Victoria (0%, $p = 0.817$) (Table 1).

Further population sub-division was identified within an otherwise connected system, possibly due to instream barriers. This occurred within the Yarra River Basin where Olinda Creek is divided from the main waterway by a 440 m dam wall. Genetic differentiation was found between individuals of Olinda Creek ($n = 16$) and the rest of the Yarra system ($n = 104$) ($F_{ST} = 0.046$, 95% CI 0.020 – 0.079, $G'_{ST_est} = 0.140$, 95% CI 0.072 – 0.305, $D_{est} = 0.111$ 95% CI 0.058 – 0.246).

Mantel tests revealed a significant positive linear correlation between genetic and Euclidean distance among individuals within Tasmania and individuals within the mainland (Tasmania; $r = 0.222$, $p < 0.001$, Mainland; $r = 0.287$, $p < 0.001$). Partial correlations between cost-distance and genetic distance (corrected for Euclidean distance) were generally stronger in Victoria compared to Tasmanian (Figure 4). Partial correlations were significantly positive at a cost-distance of 1:10, 1:100 or 1:1000 for individuals of the Glenelg, Goulburn, Upper Murray and Yarra River Basins of Victoria indicating a reduced frequency of overland dispersal in these localities. No

significant partial correlations were detected within the four Tasmanian river basins analysed. Further cost-distance analyses were performed grouping individuals of Victoria and Tasmania with adjacent river basins. Similar patterns were found with individuals of the mainland showing a preference for waterway dispersal while no significant partial correlations were detected among Tasmanian individuals.

Consistent with patterns of male-biased dispersal, adult males from the upper Shoalhaven River had lower F_{ST} , lower relatedness and lower $mAIC$ than adult females, although differences between the sexes were not statistically significant (Table 2). Spatial genetic structure autocorrelograms within the upper Shoalhaven River revealed adult female pairs within 100 m or 1.4 km of river were significantly positively genetically correlated while female pairs separated by 2.8 or 4 km of river were negatively genetically correlated (Figure 5).

Mitochondrial DNA Variation

Individuals were sequenced at two partial mitochondrial genes; 518bp of COII and 738bp of *cytb*. Nineteen COII haplotypes were detected among 549 individuals and 30 *cytb* haplotypes were detected among 265 individuals. Sequences fully translated in all individuals. Mitochondrial DNA sequence variation was shown to be selectively neutral for both COII (Fu and Li's D test statistic = -1.015, $p > 0.10$, Fu's F statistic = -12.322) and *cytb* (Fu and Li's D test statistic = -0.314, $p > 0.10$, Fu's F statistic = -21.907). For both genes, phylogenetic reconstruction of sequences according to maximum parsimony produced networks with a clear separation between haplotypes found in Tasmania and the mainland and identified two distinct clades within Tasmania (Supplementary Material Figures 1 and 2). Bayesian phylogenetic reconstructions also revealed common relationships at both partial mitochondrial gene sequences. In addition to the above relationships, further geographic partitioning was evident on the mainland; regional differences among haplotypes were identified within the mainland with distinct lineages assigned to northern New South Wales and western Victoria (i.e. Glenelg River Basin and Wimmera/Hopkins River Basins) (see Supplementary Material Figures 1 and 2).

Concatenated sequences across 265 individuals showed a total of 82 nucleotide changes resulting in 37 unique haplotypes. Genetic distances between haplotypes throughout Australia were small (0.001-0.031). Clear separation between Tasmania and the mainland was evident with 26 fixed nucleotide changes (corresponding to 24 transitions and two transversions) and a genetic distance of 0.027. Within Tasmania, 14 haplotypes were identified, corresponding to two distinct clades

1 separated by nine fixed nucleotide changes (corresponding to eight transitions and one
2 transversion) and a genetic distance of 0.009. Further structure was found on the Australian
3 mainland with clades again corresponding to northern New South Wales (haplotypes U and V),
4 and western Victoria (haplotypes Q, R, S and T) (Figure 6). Within the rest of the mainland, two
5 distinct clades were present, one of which predominantly resided within eastern
6 Victoria/Shoalhaven (haplotypes K, L, M, N, O and W) and the other found throughout the
7 remainder of Victoria. In total, 23 haplotypes were detected among mainland individuals.

9 Of the 23 haplotypes detected on the Australian mainland, 20 were confined to a river basin
10 (Figure 7 and Supplementary Material Table 3 and Figure 3). Some haplotypes were also
11 confined to a location within a river basin. For instance, three haplotypes identified in the
12 Goulburn River Basin were restricted to a tributary; haplotype H was found only in Seven Creeks,
13 haplotype G in Gaffneys Creek and haplotype I in King Parrot Creek. Within the Yarra River
14 Basin, two unique haplotypes (E and J) were found only among individuals of Olinda Creek,
15 distinct from the common haplotype (A) identified in all other individuals sequenced from within
16 this basin ($n = 23$).

18 In Tasmania, haplotypes did not appear to produce any discernable patterns relating to geographic
19 location (Supplementary Material Table 3 and Figure 3). Haplotypes were rarely unique to a river
20 basin (Figure 7). Rather, haplotypes were widely distributed across the island and the majority of
21 haplotypes were found in multiple river basins. Consequently, several Tasmanian samples of
22 unknown origin (Supplementary Material Table 3) were unable to be allocated to a geographic
23 location based on mtDNA sequence data.

25 All nuclear and mitochondrial analyses were reduced to investigate Tasmanian and Victorian
26 samples collected over a contemporary time span (2007 - 2010, $n = 542$) (data not shown).
27 Results within these regions remained consistent with data presented above.

29 Discussion

30 Genetic analysis has been used to investigate the population structure and dispersal patterns of the
31 platypus within south-eastern Australia. Consistent with previous studies (Akiyama 1998; Furlan
32 et al. 2010; Warren et al. 2008), very high levels of genetic differentiation are evident between
33 Tasmanian and mainland platypuses at both the mitochondrial (minimum 2.9% sequence
34 divergence) and nuclear level (27.57% variation among regions, $p < 0.001$). Further population

1 structure is evident on the mainland with separation between individuals of western Victoria
2 (particularly individuals of the far-west Glenelg River Basin) and central/eastern Victoria.
3 Individuals sampled within locations along the upper Shoalhaven River in NSW and the Gwydir
4 and Border rivers in northern NSW also display genetic differentiation from the remainder of
5 mainland individuals, although, this is not surprising given the large distance between sampling
6 points (> 230 km). Individuals from the island state of Tasmania exhibited no additional genetic
7 structure. Investigation of dispersal patterns in the platypuses indicate an association between
8 genetic distance and geographic distance in both Tasmania and the mainland. As hypothesised,
9 overland movement of the platypus occurs at a higher cost than waterway movement in the
10 majority of mainland river basins. While we anticipated that an increased frequency of overland
11 movement in Tasmania would likely reduce cost of overland movement, we were somewhat
12 surprised to find no significant preference for waterway dispersal within Tasmania. While the
13 hypothesis surrounding male-biased dispersal was unable to be fully evaluated, greater
14 mitochondrial structure compared to nuclear genetic structure and patterns of average pairwise
15 genetic relatedness among males and females along a short stretch of the Shoalhaven River
16 provided some evidence for male-biased dispersal in the platypus.

17
18 Mitochondrial DNA haplotypes show a high level of structuring on the mainland with the
19 majority of haplotypes restricted to an individual river basin. Further population subdivision can
20 be detected within river basins where particular haplotypes are restricted to localised segments of
21 waterways, such as those detected within the Goulburn and Yarra River Basins. In Tasmania
22 however, mtDNA haplotypes show a lack of population structure and do not appear to exhibit any
23 geographic partitioning. Microsatellite data revealed evidence for isolation by distance in
24 Tasmania but failed to reveal any additional population structuring throughout the island.
25 Tasmanian platypuses appear to show less genetic structuring than is found on the mainland. This
26 low level of population structure evident in Tasmania appears to indicate greater gene flow across
27 river basins, suggesting migration may occur overland between disconnected river systems. This
28 is in accordance with indications of a higher frequency of overland movement in Tasmanian
29 platypuses in comparison to mainland individuals (Connolly et al. 1997; Otley et al. 2000).
30 Within Tasmanian river basins, the cost of overland dispersal for platypuses was not found to be
31 significantly greater than the cost of waterway dispersal. The greater densities of rivers in
32 Tasmania compared to Victoria may facilitate overland dispersal; despite representing only 0.9%
33 of Australia's landmass, Tasmania contains 12% of the nation's freshwater resources (Australian
34 Government 2011) and has far fewer ephemeral streams than the mainland (Robson 2008). This is

likely to decrease the distances platypuses would be required to traverse between disconnected waterways and could potentially explain the low levels of population structuring and low cost of overland movement. Overland movement demands higher energy expenditure (Fish et al. 2001) and exposes the individual to heat stress from ambient and metabolic heat sources (Grant and Dawson 1978). The closer proximity of water refuges and generally cooler conditions prevailing in Tasmania, may facilitate overland movement for platypuses. In addition, variations in land use, reduced human population density and an absence of predatory foxes from the island, until recent times (Parkes and Anderson 2009; Saunders et al. 2006), may permit overland dispersal to successfully occur more frequently than it does on the mainland.

Despite both nuclear and mitochondrial DNA showing little evidence of contemporary population structure within Tasmanian platypuses, evidence for historic population structure does exist. Two distinct mtDNA lineages are present on the island. It is likely that populations of platypuses on Tasmania were historically separated and evolved independently. This may date back to the Pleistocene when glaciers covered regions of high elevation, dominated the Central Highlands (Kiernan 1990) and potentially prevented gene flow between populations on either side. Subsequent glacier melting may have allowed populations to reconnect, leading to the mtDNA haplotypes being redistributed across most of the state, as identified in this study.

Isolation by distance appears to occur within both Tasmanian and mainland individuals, with a decrease in genetic similarity apparent with an increase in geographic distance. Consequently, the genetic differentiation evident in individuals of the Gwydir and Border River Basins of northern New South Wales from the rest of the mainland Australian platypuses sampled in this study is not surprising given the large geographic separation. Individuals of northern NSW display unique mitochondrial haplotypes (V and U; Figures 5 and 6). Of greater interest, however, is evidence of genetic similarity between platypuses of these distant locations. A haplotype common throughout much of Victoria (haplotype A) is also present within this New South Wales region and microsatellite variation does not clearly distinguish northern NSW individuals from the remainder of sampled mainland individuals (Figure 3a). The Murray-Darling drainage system connects the Gwydir and Border River Basins to northern Victoria. Given the propensity for aquatic dispersal on the mainland, this river connectivity may facilitate migration of individuals north and south resulting in populations of mixed ancestry despite the vast distance. Further sampling throughout New South Wales would allow for a better spatial understanding of population structure occurring within this state.

1
2 Individuals from the upper Shoalhaven River in New South Wales also appear genetically distinct
3 at both the nuclear and mitochondrial level (Figure 3a and 5). This basin is likely to have been
4 historically disconnected from inland regions of southern Victoria given that the river system
5 drains north then east towards the coast. Large geographic distance may also contribute to the
6 genetic isolation of this population. Consistent with this, mitochondrial haplotypes specific to the
7 upper Shoalhaven River showed more similarity to haplotypes present in eastern Victoria than
8 those of the more distant central Victorian areas. Populations of eastern Victoria, however, also
9 possessed a haplotype common to central Victoria (haplotype A), providing evidence for
10 migration between populations of eastern and central Victoria. This is supported at the nuclear
11 level with microsatellite analysis revealing an absence of genetic differentiation among
12 populations of eastern and central Victoria also suggesting admixture among these regions.

13
14 Significant genetic differentiation is evident between western Victoria and the rest of the state.
15 Both nuclear microsatellites and mitochondrial sequences show distinct variation between these
16 two regions although the location of the division between east and west varies. There appears to
17 be a cline in the genetic composition of individuals with genetic differentiation decreasing west to
18 east. For instance, individuals from the Glenelg River Basin (the most westerly point of the
19 platypus' natural distribution) are invariably differentiated from the remainder of central/eastern
20 Victorian individuals (Figure 3a). STRUCTURE output reveals two individuals of the Wimmera
21 River Basin share genetic similarity with those of the Glenelg River Basin (Figure 2b). At the
22 mitochondrial level, individuals of the Glenelg and Wimmera River Basins and some individuals
23 of the Hopkins River Basins display haplotypes distinct from the rest of Victoria (Figure 6).
24 Genetic divisions between populations of eastern and western Victoria have been found in
25 numerous aquatic species (Coleman et al. 2010; Hammer 2001; Unmack 2001), although the
26 exact locality of the split varies. For the platypus, this differentiation possibly reflects historic
27 drainage patterns where the coastal-flowing rivers of the Glenelg and Hopkins River Basins
28 drained towards the southwest, isolated from the river basins of central and eastern Victoria
29 reaching the coast in the south or south-east (Harris et al. 2005). Alternatively, or in addition,
30 more recent phenomena may be contributing to functional genetic variation. Western Victoria
31 represents the most westerly range of the platypus' natural geographic distribution and
32 experiences hot, dry conditions. Individuals found along species borders such as this may exhibit
33 adaptive genetic differentiation as a result of local selection pressures leading to certain traits
34 being genetically selected for (Hoffmann and Blows 1994) with most studies of peripheral

1 populations showing increased differentiation at neutral genetic marker loci (Eckert et al. 2008).
2 More recent gene flow across this western Victorian region is likely to be responsible for mixing
3 genotypes. The propensity for ephemeral streams in these western Victorian river basins,
4 however, may continue to restrict contemporary dispersal somewhat (Robson 2008). Both
5 contemporary and historic dispersal patterns are likely to have contributed to the pattern of
6 genetic differentiation observed today, indicating overland dispersal between multiple river
7 basins.

8
9 Despite evidence for morphological variation either side of the Great Dividing Range (Carrick et
10 al. 2008; Furlan et al. 2011; Grant 2007) and an absence of interconnected waterways,
11 differentiation in neutral genetic markers does not exist among populations either side of the
12 divide in the nuclear or mitochondrial DNA regions investigated here. The Great Dividing Range
13 imposes a significant barrier to dispersal for many aquatic or semi-aquatic species (Georges et al.
14 1998; Jerry 2008; Musyl and Keenan 1992; Symula et al. 2008), although there are numerous
15 examples of genetic similarity across the divide for semi-aquatic and even aquatic-restricted
16 species (Cook et al. 2006; McGlashan and Hughes 2001; Watson and Littlejohn 1985). Despite
17 significant uplift of this region dating back over 5 million years (Dickinson et al. 2002), historic
18 geomorphological events, such as river capture, may have occurred to alter flows of headwater
19 streams (Bishop 1995), and consequently alter species' migration patterns. A lack of
20 mitochondrial and neutral genetic variation in the platypus, however, suggests a recent connection
21 across the Great Dividing Range. Overland migration across the Great Dividing Range could
22 potentially maintain genetic connectivity among populations of the platypus either side of the
23 divide in Victoria. Variation in genes contributing to morphological differences, however, may
24 still show differentiation either side of the divide due to the influence of selection. Consequently,
25 even in the absence of neutral genetic variation, adaptive variation may mean individuals either
26 side of the Great Divide still constitute separate populations for the purpose of conservation.
27 Alternatively, external factors may be contributing to this size variation (e.g. environmental
28 conditions).

29
30 The cost of overland dispersal among individuals of some Victorian river basins was found to be
31 10, 100 or 1000 times greater than waterway dispersal, suggesting movement via waterways
32 occurs with a much greater frequency than overland movement in these localities. The genetic
33 structuring observed within Victoria (detailed above), however, suggests that a low frequency of
34 overland movement is likely to occur to maintain genetic connectivity between adjacent river

basins. Two Victorian river basins did not produce significant cost-distance correlations with geographic distance once corrected for Euclidean distance. It is possible that the close correlation with Euclidean distance within the Maribyrnong River Basin (where individuals were sampled along a linear stretch of river) masks any associations with cost-distance. The two analyses of Bunyip River individuals approached significance at a cost-distance ratio of 1:10 ($p = 0.029$ for Bunyip 1 and $p = 0.069$ for Bunyip 2) but these were not significant after correction for multiple comparisons. Increased sampling within these locations will provide a better indication of whether the cost of overland dispersal is greater than waterway dispersal.

Within the Yarra River Basin, the Olinda Creek platypus population exhibits genetic divergence from other individuals of the basin. Significant nuclear variation ($F_{ST} = 0.046$, $G'_{ST_est} = 0.140$, $D_{est} = 0.111$) and two unique mitochondrial haplotypes exist among individuals of this waterway. A number of recent modifications have occurred along Olinda Creek which may have contributed to the genetic separation of platypuses; the 2 km downstream section of creek has undergone extensive modification and straightening to maximise agricultural productivity in the surrounding farmland, a sewage treatment plant operated downstream from 1968, and, more recently, a 440m long dam wall was constructed partway up the creek in 1990 to form a small lake, Lillydale Lake (Young 2005). A study on the genetic relationship among platypus populations above and below the Nepean dam in New South Wales (completed in 1935) also detected significant genetic differentiation either side of the dam ($F_{ST} = 0.077$) (Kolomyjec 2010). In other aquatic species, recent (~30–100ya) anthropogenic modifications occurring along waterways such as dams and weirs have been associated with genetic differentiation among upstream and downstream populations (Heggenes and Røed 2006). While it is unknown whether the genetic differentiation detected in this study pre-dates the human modification of the stream, it is evident that the Olinda Creek population is currently genetically isolated from individuals throughout the remainder of the Yarra River Basin.

Along a short stretch of the upper Shoalhaven River, females were found to maintain genetic similarity within 1.4 km of stream while genetically dissimilar females were separated by > 2.8 km of stream. The genetic relatedness patterns occurring among males could not be determined due to low sample size, prohibiting comparisons between the sexes. Patterns of genetic relatedness, genetic differentiation and population assignment among males and females however, suggested a higher frequency of male dispersal. Unfortunately, small adult male sample size inhibits our ability to confidently detect dispersal patterns with significance. Several

behavioural observations support the possibility of male-biased dispersal; aggressive male-male interactions involving the spurs on their rear ankles are known to occur and these have been recorded to increase in frequency during the breeding season (Grant 2007). The crural gland which supplies venom to the spurs also increases its secretion during the breeding season (Temple-Smith 1973). Together, this suggests that dispersing males are likely to compete for females or for mating territories. Mark-recapture studies in the upper Shoalhaven River population also provide evidence for the occurrence of female philopatry and male-biased dispersal in this population, with a number of juvenile females breeding in their natal area (Grant et al. 2004). In addition, these studies showed a low male recapture rate with only 14% (n=94) of male juveniles and 36% (n=165) of adult males recaptured, compared to 32% (n=135) and 51% (n=278) of juvenile and adult females in the same area (Grant 1992a; Grant 2004). Inbreeding avoidance is thought to be the primary cause of sex-biased dispersal in mammals (Greenwood 1980; Perrin and Mazalov 2000) as males disperse in search of unrelated females. Across Victoria, variation in population structure detected using mitochondrial or microsatellite DNA may also provide evidence for a male-biased dispersal pattern. While many maternally inherited mtDNA haplotypes remain unique to a river basin (Figure 7), bi-modally inherited microsatellite loci reveal low levels of differentiation. Given the trends indicated here, further research with increased statistical power should be carried out to determine whether significant differences in male and female platypus dispersal patterns exist.

Conclusion

Significant population structure has been detected among platypuses of south-eastern Australia. Further evidence of a significant genetic separation between Tasmania and mainland platypuses has been found, while genetic divergence between individuals of western Victoria and the remainder of the state has been identified for the first time. Platypuses of three New South Wales river basins were found to be genetically distinct from Victorian individuals. Identification of these genetically diverse populations can assist with the management and conservation of the species, in particular, for captive breeding or translocation events. Further sampling within the river basins in the north of the species' distribution (i.e. throughout New South Wales and Queensland) will expand our knowledge of the population structure of platypuses. In a separate study, small numbers of platypuses from two additional New South Wales river basins were investigated at microsatellite loci and found to cluster with the three New South Wales river basins analysed in this study (Kolomyjec 2010). In addition, five Queensland river basins were assigned to a distinct 'northern' population cluster (Kolomyjec 2010). Many river basins still

1 remain to be sampled to generate an Australia-wide perspective of the population structure of the
2 species.

3
4 The genetic differentiation identified between platypus populations in this study provides
5 evidence for both aquatic and terrestrial dispersal. Dispersal patterns in the platypus suggest a
6 greater cost of overland compared to waterway movement in Victoria than within Tasmania. The
7 genetic differentiation observed among individuals of disconnected waterways also indicates that
8 the platypus shows some dependence on waterways for migration. An absence of genetic
9 differentiation either side of the Great Dividing Range in Victoria, however, suggests the
10 occurrence of overland dispersal. The platypus is likely to be exposed to a range of habitat
11 modifications in the future as a result of a warming climate (IPCC 2007) and continued damming
12 and draining of freshwater environments. These are likely to increase fragmentation and isolation
13 along waterways, potentially restricting waterway migration for platypuses. The suggestion of
14 sex-biased dispersal indicated in this study will have obvious implications for isolated
15 populations. These populations are likely to experience a breakdown of the natural dynamics of
16 breeding group structuring. Restricted dispersal can lead to an increased rate of inbreeding and
17 decreased genetic diversity (Greenwood 1980). Maintaining river connectivity is likely to remain
18 the most important conservation priority in the future to facilitate or maintain gene flow within
19 the species.

20
21 Detecting future changes in the population structure among platypuses will benefit from the
22 baseline data provided in this study. Future habitat modification may lead to isolation and genetic
23 differentiation among platypus populations. An absence of previous data makes it difficult to
24 ascertain the impacts of various threatening processes on platypus populations and has precluded
25 the identification of genetically distinct populations (such as Olinda Creek) as historic or recent
26 phenomena. Data presented in this study will benefit future research by providing a temporal
27 comparison to detect any future changes in migration patterns and population structure.

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Table 1. AMOVA comparing genetic variation in microsatellite data among populations of *Ornithorhynchus anatinus* among Tasmania and the mainland and either side of the Great Dividing Range in Victoria.

	Source of Variation	df	Sum of squares	Variance components	% Variation	Stat	Fixation indices	P value
Tasmania vs. Mainland	Among Regions	1	732.957	1.34033	27.57	F _{CT}	0.276	< 0.001
	Among Pops	42	480.183	0.27482	5.65	F _{SC}	0.078	< 0.001
	Within Pops	1432	4648.94	3.24647	66.78	F _{ST}	0.332	< 0.001
	Total	1475	5862.08	4.86163				
Great Dividing Range – Victoria	Among Regions	1	9.886	-0.04959	0.00	F _{CT}	-0.014	0.817
	Among Pops	17	236.401	0.28165	7.94	F _{SC}	0.078	< 0.001
	Within Pops	815	2702.039	3.31538	93.46	F _{ST}	0.065	< 0.001
	Total	833	2948.326	3.54745				

1 **Table 2.** Average pairwise relatedness values within and between six locations along the Shoalhaven River, NSW, for male and female adult
 2 platypuses.
 3

	<i>N</i>	<i>F_{ST}</i>	Relatedness	mAIC
Females	47	0.0169	0.0339	0.20075
Males	13	-0.0133	-0.0279	-0.64859
<i>P</i> value		0.0998	0.1006	0.1215

Figure 1. The current distribution of the platypus throughout Australia is indicated by light grey shading. Dark grey shading indicates areas > 700 m in elevation along the Great Dividing Range. Insert shows sampled river basins in dark grey shading.

Figure 2. Summary plot of the estimated membership coefficient for each *O. anatinus* individual within a) Victoria across two population clusters ($K = 2$) and b) sub-structuring of cluster 1 into three further clusters ($K = 3$). Each individual is represented by a single vertical line divided into shaded segments representing the proportional membership to each of the population clusters. Individuals are grouped according to river basin from west to east across Victoria with broad locations indicated above the figure.

Figure 3. Scatterplots of the Discriminant Analysis of Principal Components of platypuses across 13 microsatellite loci. The first two principal components of the DAPC are shown for a) mainland individuals and b) Tasmanian individuals. Clusters are indicated by different colours and inertia ellipses, while dots represent individuals. (Further details of individuals contained in each cluster are presented in Supplementary Material Table 2).

Figure 4. Partial correlation between cost distance and genetic distance corrected for Euclidean distance. Solid lines indicate Victorian river basins and dotted lines indicate Tasmanian river basins. The number of individuals within each group is shown in the legend in parenthesis. Asterisks indicate partial correlations that are significant after correction for multiple comparisons.

Figure 5. Spatial genetic structure autocorrelograms for adult female platypuses of the upper Shoalhaven River, NSW. The genetic correlation coefficient (r) is shown as a function of geographical distance across six distance classes with 95% CI showing a random spatial genetic structure. The number of comparisons within each distance class is shown in parentheses and asterisks indicate where genetic correlations within distance classes significantly differ from random after correction for multiple comparisons.

Figure 6. *Ornithorhynchus anatinus* Bayesian inference phylogenetic tree generated from a concatenated segment of two partial gene sequences, *cytochrome oxidase* subunit II (518 bp) and *cytochrome b* (738 bp). Haplotypes are indicated at the termination of branches. Bootstrap support for parsimony (above node in bold) and maximum likelihood (below node) are indicated

where support is greater than 0.5.

Figure 7. The proportion of platypus haplotypes unique to river basins of the mainland or Tasmania are indicated by dark grey shading while light grey shading represents haplotypes that are shared across two or more river basins. Total numbers of unique or common haplotypes are indicated inside the columns. Haplotypes have been generated from concatenated segments of two partial mitochondrial gene sequences, *cytochrome oxidase* subunit II (518 bp) and *cytochrome b* (738 bp). (Further details of haplotypes identified in river basins of south-eastern Australia are presented in Supplementary Material Table 3 and Supplementary Material Figure 3.

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