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TECHNICAL NOTE

Isolation and characterisation of novel microsatellite and mitochondrial DNA markers for the Eastern Water Dragon (*Physignathus lesueurii*)

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Abstract Habitat fragmentation as a result of urbanisation is a growing problem for native lizard species. The Eastern Water Dragon (*Physignathus lesueurii*) is a social arboreal agamid lizard, native to Australia. This species represents an ideal model species to investigate the effect of urbanisation because of their prominent abundance in the urban landscape. Here we describe the isolation and characterisation of a novel set of 74 di-, tri-, and tetra-microsatellites from which 18 were selected and optimised into two multiplexes. The 18 microsatellites generated a total 148 alleles across the two populations. The number of alleles per locus varied from 2 to 18 alleles and measures of Ho and He varied from 0.395 to 0.877 and from 0.441 to 0.880, respectively. We also present primers for four novel mitochondrial DNA (mtDNA) markers. The combined length of the four mtDNA marker pairs was 2,528 bp which included 15 nucleotides changes. In comparison to threatened species, which are generally characterised by small population sizes, the Eastern Water Dragon represents an ideal model species to investigate the effect of urbanisation on their behavioural ecology and connectivity patterns among populations.

Keywords Paternity · Agamid lizards · Agamidae · Gene flow · Kinship · 454 Pryosequencing

The Eastern Water Dragon (*Physignathus lesueurii*) is a social, semi-aquatic and arboreal agamid lizard (Agamidae), native to Australia. This species is distributed throughout riparian zones of rivers and wetlands, natural and artificial, from Northern Queensland, eastern New South Wales, to northeastern Victoria. The species is extralimital in southern New Guinea (Cogger 2000). Eastern Water Dragons exhibit elaborate social behaviours and a harem like mating system (Greer 1999). They are generally found in large social groups comprising several females, juveniles of various ages, subordinate males and a dominant male (Greer 1999). Sexual maturity is reached between 2 and 3 years of age and show strong sexual dimorphism. Males have larger body size than females and a dull red flush on the chest (Greer 1999). Males are territorial and engage in ritualised combats during the mating season (spring to late summer). While recognised as a 'common' species (Thompson 1993), Eastern Water Dragons are nonetheless a protected species under the Environment Protection and Biodiversity Conservation Act (1999). Despite their intriguing social and mating system as well as their prominent abundance in the urban landscape, little is known about the behavioural ecology and population genetics of this species. In comparison to threatened species, which are generally characterised by small population sizes, the Eastern Water Dragon represents an ideal model species to investigate the effect of urbanisation on their behavioural ecology and connectivity patterns among

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populations. Although microsatellite marker panels exist for three other agamid lizards [n = 8 for the painted dragon, *Ctenophorus pictus* (Austin et al. 2006) and n = 15 for the Australian agamid lizards *Amphibolurus muricatus* and *Ctenophorus pictus* (Schwartz et al. 2007)], high-resolution markers are lacking for The Eastern Water Dragon (*P. lesueurii*).

As a first step toward understanding the patterns of gene flow among urban populations of the Eastern water dragon we sequenced genomic DNA using 454 pyrosequencing technology. This study identified a novel set of 74 di-, tri-, and tetra- microsatellites (Table S1) from which 18 were selected and optimised into 2 multiplexes (Table 1). We also present 4 novel mitochondrial DNA marker pairs (Table 3). DNA was extracted using the QIAGEN DNeasy extraction kit using the manufacturers protocol. Individual DNA extractions of seven animals collected from geographically isolated populations within the Brisbane CBD, Australia, were pooled. In total 5 μg of DNA was sent to the Australian Genome Research Facility for 454 pyrosequencing. DNA was checked for quality on an Agilent bioanalyzer and used to construct libraries for 454 GS-FLX titanium libraries. Sequencing of this library generated 74,317reads with an average length of 361 bp.

Msatcommander ver. 0.8.1 (Faircloth 2007) was used to identify pure di-, tri-, and tetra-nucleotide microsatellite loci that were a minimum of six tandem repeats in length. In total we identified 2,297 microsatellites in the *P. lesueurii* sequences that met the above search criteria. Primers were designed for 600 of these microsatellite loci with Batch Primer 3 with same settings as Sexton et al. (2010). Of these 600 loci, 74 primer pairs were screened for PCR amplification success and polymorphism using 8 samples from the initial 7 animals collected from geographically isolated populations within the Brisbane CBD (Table S1). PCR amplification was performed according to the protocol of Sexton et al. (2010). Eighteen of the primer pairs tested showed evidence of polymorphisms and were thus chosen for further optimisation and polymorphism testing using two populations: Roma Street Parklands (n = 115, 27° 27′ 46″ S, 153° 1′ 11″ E) and Brisbane Botanic Gardens, Mt Coot-tha (n = 52, 152°58′39″E, 27°28′26″S). Forward primers of the 18 loci were 5′ labelled with 6FAM, NED, VIC or PET fluorescent dyes (Table 1) and amplified in two multiplex PCRs using Qiagen's Multiplex kit. PCR thermocycling conditions for both multiplexes are as follow: denaturing at 94°C for 10 min; 35 cycles at 94°C for 1 min, 58.5°C for 30 s, and 72°C for 1 min; with a final extension of 72°C for 20 min. PCR products were resolved on an ABI 3730 capillary sequencer using the LIZ 500 (−250) internal size standard and scored using GeneMapper ver. 4.0 software. PCR thermocycling conditions for the mtDNA are as follow: denaturing at 94°C for 10 min; 35 cycles at 94°C for

Table 1 Details of the 18 microsatellites loci developed from 454 shotgun sequence data in Eastern Water Dragon *Physignathus lesueurii*. Genbank submission number 1464831

Locus name	5′	3′	T_A (°C)	Repeat motif	Multiplex #	Size range (bp)	GenBank accession #
EWD1	CGGGCAATTTAAAGCTGCAG	TTACTTCTGAAGAGTCGTTCCC	58.5	$(AC)_8$	M2	89–95	JN208353
EWD3	CTGCCCAGAGTAGTAGCCTG	ACAAGTTATGATCATGCAACCG	58.5	$(AAAC)_6$	M1	94–102	JN208354
EWD5	TGCCAGCCTGTGTTTGAAAG	GAAGTCACTCAGAGCAACTTTG	58.5	$(AG)_9$	M1	100–116	JN208355
EWD6	TGGGAGGAAGTGAAACAGGG	TTGTCAAACACCATCCAATGC	58.5	$(AG)_{10}$	M1	102–124	JN208356
EWD9	ACAGCGCTTTAATAAATGTGGG	GATTGTTGTGCGGCTGTTAC	58.5	$(AG)_{10}$	M1	88–100	JN208357
EWD15	AGGCCATGCTATCTCAGAGG	TGCCCAGAGTAGTGCTTCAG	58.5	$(AATT)_8$	M2	96–116	JN208358
EWD16	CAAGTCTGTGAAGCCTCGTG	TCAAGCAGCACTGATGAAGG	58.5	$(AT)_8$	M2	111–121	JN208359
EWD21	CTCCTCCAACACATAGACTCTC	GCCTTCTCTGCTTGTCTTTCG	58.5	$(AC)_9$	M1	145–171	JN208360
EWD24	CCTTTCCCACTCTGCAGTTC	CTTGACGCTGCTGTCTCATG	58.5	$(AAGT)_{16}$	M2	129–175	JN208361
EWD26	ACCTCCCGCTTTAAACCAATC	TTTCAACTGCAGTGTACCGC	58.5	$(AATC)_6$	M1	162–236	JN208362
EWD30	TTGTTCAGAGGGTGGGTGTC	CCAAGCAAGAATTAACAGGTGC	58.5	$(AG)_8$	M2	157–159	JN208363
EWD34	ATCTACCTCCCGCTTTAAACC	TTTCAACTGCAGTGTACCGC	58.5	$(AATC)_8$	M1	167–241	JN208364
EWD44	TTGACATCAGCCTCGGAGAC	AGGTATCATTAGGTTGCTTGCC	58.5	$(AAAT)_7$	M1	202–242	JN208365
EWD46	AGCCAACCATTACCTAGGGC	ACACACACACACTGTATGCTTG	58.5	$(AC)_{11}$	M2	218–228	JN208366
EWD51	TCAGAAAGGTAAACATGGGCTC	GGTCCCTTCCAGCTCTGTAG	58.5	$(AAAT)_9$	M2	182–248	JN208367
EWD59	CCCTCCTGCAGTAAGACTCG	TGGCTCTTTGACCTACACCC	58.5	$(AAAT)_6$	M2	223–271	JN208368
EWD62	TGGGTGGCTGTACTCTACTG	GTGTCATTTCAGGGCGCATG	58.5	$(ACCT)_{19}$	M2	255–237	JN208369
EWD69	GCAGTTCCAACCACCAAGTC	ACCAGAGCTTAGCGGTAACTAG	58.5	$(ACAT)_{16}$	M2	285–361	JN208370

Table 2 Basic statistics of the 18 microsatellites loci developed from 454 shotgun sequence data in Eastern Water Dragon *Physignathus lesueurii*. Hardy–Weinberg equilibrium (HWE) was measured across 6 subpopulations

Locus name	Na	Ho	He	HWE
EWD1	2	0.874	0.495	NS**
EWD15	5	0.413	0.509	NS
EWD16	7	0.689	0.712	NS
EWD21	8	0.416	0.508	NS*
EWD24	11	0.820	0.803	NS
EWD26	18	0.784	0.877	NS
EWD3	5	0.395	0.441	NS*
EWD30	2	0.311	0.478	NS
EWD34	14	0.780	0.874	NS
EWD44	6	0.390	0.455	NS*
EWD46	3	0.479	0.517	NS
EWD5	7	0.593	0.731	NS
EWD51	12	0.749	0.776	NS*
EWD59	8	0.412	0.494	NS
EWD6	8	0.635	0.691	NS
EWD62	11	0.784	0.816	NS*
EWD69	17	0.777	0.869	NS
EWD9	4	0.339	0.470	NS**

Overall no loci were consistently found to deviate from HWE

* A locus was found to be significantly out of HWE in one subpopulation

** A locus was found to be significantly out of HWE in two subpopulations

1 min, 53°C for 1 min, and 72°C for 1 min; with a final extension of 72°C for 20 min. PCR products were purified using ExoSAP-IT (Affymetrix), sequenced with the BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and separated on an ABI 3730 DNA Sequencer (Applied Biosystems). The sequences were visualised and edited using Geneious Pro version 5.0.

Measures of genetic diversity, tests for linkage disequilibrium (LD) and departures from Hardy–Weinberg equilibrium (HWE) of the 18 loci were performed in GenAlex ver. 6.4 (Peakall and Smouse 2006) and Genepop ver. 4.1 as implemented on the web (Rousset 2008). Micro-Checker ver. 2.2.3 was used to examine if any loci exhibited patterns consistent with null alleles (Van Oosterhout et al. 2004). The characteristics of the 18 loci are summarised in Table 2. In total 148 alleles were observed at the 18 loci across the two populations. The number of alleles per locus varied from 2 to 18 alleles and the expected (He) and observed (Ho) heterozygosities varied from 0.395 to 0.877 and from 0.441 to 0.880, respectively. No significant LD was detected after correction for false discovery rate (Strimmer 2008) and no microsatellite markers showed consistent departure from HWE after taking into account

Table 3 Details for the 4 mtDNA markers developed using 454 shotgun sequence data in Eastern Water Dragon *Physignathus lesueurii* GenBank submission number 1464831

Locus name	5′	3′	T_A (°C)	Sequence length bp	Sample size	Haplotype number	Nucleotide changes	GenBank accessions #
mtDNA_EWD1	TTATGGTGCGGGGGCATTGGT	ACATCATTTGGCCGCTCTGGCA	53	620	7	2	6	JN381508–JN381509
mtDNA_EWD2	GGCGCGCGCTTTGTCTCTTG	CTCCGCACAAGCCGGACTCC	53	718	7	2	4	JN381510–JN381511
mtDNA_EWD3	GAGTCCGGCTTGTGCGGAGG	GGCAACCCACGCCACGTTCA	53	420	7	2	2	JN381512–JN381513
mtDNA_EWD4	GGCGTGGGGCCTTCTATGGC	TCTTCCCGGCCGCTGGTTA	53	770	7	2	3	JN381514–JN381515

population genetic structure and applying Bonferroni correction. The presence of null alleles was identified at loci 1, 5, 8, 13 due to excess homozygosity. We also developed 4 new mtDNA marker pairs which were amplified across 7 animals from the Roma Street Parklands. The four mitochondrial DNA marker pairs resulted in a total of 2,528 bp mtDNA sequence data characterised with a total of 15 nucleotides changes (13 point mutations and two indels). Each mtDNA fragment showed a total of 2 haplotypes, each showing identical clustering of the 7 animals (5 and 2). The number of nucleotide changes, however, varied from 2 to 6 (Table 3).

In combination, these microsatellite and mitochondrial markers will be very useful to address significant evolutionary questions about social (Frère et al. 2011) and sexual (Pryke et al. 2010) evolution as well as landscape genetics in an urban setting (Noël and Lapointe 2010).

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