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TECHNICAL NOTE

Primers for novel microsatellite markers in "fire-specialist" lizards (*Amphibolurus norrisi*, *Ctenotus atlas* and *Nephrurus stellatus*) and their performance across multiple populations

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Abstract We developed 45 microsatellite markers for three lizard species with fire-related distributions: *Amphibolurus norrisi*, *Ctenotus atlas* and *Nephrurus stellatus* (17, 12 and 16 markers respectively). To isolate microsatellites we used an enrichment technique for *N. stellatus* and next-generation sequencing for *A. norrisi* and *C. atlas*. Fluorescent tags were attached to primers during PCR for flexible genotyping. All loci were polymorphic with 2–24 alleles and expected heterozygosities of 0.043–0.927. These markers will facilitate studies of post-fire dispersal and recolonisation.

Keywords Dispersal · Fire ecology · Gene flow · Population genetics · Reptiles

Fire related distributions in many species are well documented but the processes behind these patterns are not, hindering predictions of the impact of fire regimes on biodiversity (Whelan et al. 2002). Fire-specialists (i.e. species which specialise on a specific post-fire stage) may become threatened if fire is not managed at appropriate scales. Dispersal affects post-fire recolonisation (Clarke 2008), and may critically influence how species respond to different fire regimes. Driscoll and Henderson (2008) identified a number fire-specialist Australian woodland reptiles including: *Nephrurus stellatus*, a burrowing gecko with an early successional response; *Ctentous atlas*, a spinifex specialist skink with a mid to late successional response; and the semi-arboreal agamid *Amphibolurus norrisi* whose fire response varied with location. We developed 45 novel microsatellite markers to study post-fire dispersal through analysis of gene flow in these three species.

A microsatellite library was constructed for *N. stellatus* using an enrichment technique on genomic DNA from liver tissue of two individuals (Australian Biological Tissue Collection, South Australian Museum (ABTC): 40819, 80029). We followed the protocol of Gardner et al. (2008) but hybridised the DNA with 3′ Biotinylated oligos for AC and AAC microsatellite motifs (Murphy et al. 2009). We screened 128 insert positive clones (64 per motif) for microsatellites using the three primer PCR method of Gardner et al. (1999). We sequenced 64 clones (32 per motif) potentially containing microsatellites. MICRO-FAMILY 1.2 (Meglécz 2007) was used to remove redundant sequences and primers were designed for 32 microsatellites with suitable flanking regions.

Microsatellite libraries were compiled for *A. norrisi* and *C. atlas* using next-generation sequencing. Genomic DNA was extracted from liver tissue of two individuals of each species (ABTC: 80032, 53146 (*A. norrisi*) and 88779, 53770 (*C. atlas*)) and sequenced on a Roche 454 GS-FLX system at AGRF (www.agrf.org.au) with each sample occupying 12% of a plate. The run produced 98,407 and 90,498 individual sequences of which 4.2 and 3.6% contained microsatellites for *A. norrisi* and *C. atlas*, respectively. We used the program iQDD 0.9 (Meglécz et al. 2010) to identify sequences with a minimum of eight di-, tetra- or penta-base repeats, remove redundant sequences,

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Table 1 Microsatellite primer sequences and locus information for the lizards *Amphibolurus norrisi*, *Ctenotus atlas*, and *Nephrurus stellatus*

Locus name	GenBank accession no.	Forward primer sequence 5′–3′	Reverse primer sequence 5′–3′	Repeat motif	Size range (bp)	Primer conc (Nm)	Fluorescent tag	Scoring error rate %
Amphibolurus norrisi								
AmNo02	HQ283281	CAATGGTTTTCAGGAACTGGA	TTTGTCTCCTCCCTCCTTCC	$(AG)_{13}$	114–162	40	PET	6.3
AmNo04	HQ283282	TATTGTTCCCTCCCCTTCCT	GGGTTGCAGGGATTTGTTAG	$(AT)_{9}$	137–157	10	VIC	0.0
AmNo05	HQ283283	TAACCGACTGGATAGGCGAG	TGGTTTTGAATCATTGCTGC	$(AAAC)_{12}$	106 –146	40	NED	7.1
AmNo11	HQ283285	GCCATTAACTGTGCTGGCTT	TTTCTAACATAACTACTGCACAGCAA	$(AG)_{16}$	167–231	40	PET	0.0
AmNo12	HQ283286	TCCTGATGAGGATGAGGAGG	CTCCAGTTGCACAGCAACAC	$(AC)_{10}$	162–208	20	NED	0.0
AmNo18	HQ283287	AAAACAGCACTGTATCTTTCAATTT	AAATGAGTTTGGGGCATGAG	$(AC)_{14}$	238–278	20	VIC	6.3
AmNo20	HQ283288	GCCCACAACAGAAGTTTTGC	GGCTGGACTCCTGGTTATCA	$(AG)_{12}$	233–253	60	NED	0.0
AmNo24	HQ283290	CAGACCAGATAGGCGGGATA	CCATAAGTTCCACCGATTCAA	$(AAAT)_{16}$	222–342	20	FAM	0.0
AmNo25	HQ283291	AAGAAGTGCCAGGCCACC	GGTGTGTTTTCCATTTGCTG	$(AG)_{8}$	311–321	10	NED	0.0
AmNo26	HQ283292	TGGTTCCAGAGTGCCTCATT	TGTTCCTCTTTGGACCAACC	$(AC)_{11}$	316–332	40	NED	0.0
AmNo29	HQ283293	GGGCCTACTTTGTGACTTGC	TTGACTAGATAGGACGGGTAACAA	$(AAAT)_{18}$	264–378	20	FAM	0.0
AmNo30	HQ283294	GTTTCCTTTCCCTTTCCCAA	AAGGCACAATGGCTGAAATC	$(AAAT)_{13}$	288–380	40	PET	0.0
AmNo31	HQ283295	TGAAACCAGATCTTCTCAAAAGG	CCAATCCATTTCTAGGACCA	$(AAAT)_{13}$	294–402	10	PET	16.7
AmNo33	HQ283296	CAACAAAACTAAATCTTAGTGGCA	CGTTGCCTTGAGGGTGTATAA	$(AC)_{11}$	418–430	20	NED	0.0
AmNo36	HQ283297	CAGACATTTTCCAACTTTTAAGGA	GCAGACAAAAGACTCGTCTGAA	$(AATG)_{10}$	459–571	20	VIC	0.0
AmNo37	HQ283298	CAGTCATTTAGGATACTGGGAATG	TCAAATACAATCTAAAGTCTATTGCAG	$(AAAT)_{10}$	420–504	10	FAM	0.0
AmNo39	HQ283299	TGCAGTTGAATGTTTAAGGGTT	GCTTAGTCAATCCTATCCATTTCC	$(AAAT)_{16}$	392–480	60	VIC	0.0
Ctenotus atlas								
CtAt01	HQ283300	AAACCCGAAAGCATGATGAG	GTCAGCACCTTGAATGCAAAT	$(AG)_{13}$	93–135	10	FAM	5.6
CtAt02	HQ283301	ATGAAATGCTTACGCAGACG	TTGACAAAAGGGCAATGTAGG	$(AC)_{14}$	117–159	20	FAM	0.0
CtAt03	HQ283302	TCAAACAAGGAATTATTGTTCATTACA	GCAAACCAGTCTTGTCTGGTAAA	$(AG)_{10}$	116–168	10	NED	7.9
CtAt04	HQ283303	TCAATCCTCAGTTGCCTCCT	TGCCTGTATTATTTCATGCCAA	$(AAGT)_{13}$	101–173	20	NED	0.0
CtAt08	HQ283304	TATCAGTAACGCAGTCGCGG	ACTTCGGACCAAACCTCCTT	$(AG)_{8}$	177–199	20	VIC	1.9
CtAt09	HQ283305	GTTGGCTGTAAACCCAGCAT	CCTCTTCAAAGCCAAGCATC	$(AG)_{8}$	162–194	10	PET	0.0
CtAt12	HQ283306	TGTTAGAGACGGAACTTTGATGA	CTCTAAGGGTGTGGTGCGTT	$(AATG)_{14}$	141–205	20	PET	0.0
CtAt15	HQ283307	CCCTTGTGCTGGTGAAACTT	TGCGCTCAGCAAATGTAATC	$(AATG)_{8}$	254–311	10	NED	1.7
CtAt18	HQ283308	GATGAAGCTCAGGAAGCCAG	TACATGGCCACTTTGCTGAA	$(AC)_{11}$	346–396	20	PET	0.0
CtAt20	HQ283309	CTCCACGACTTCCTCACCAT	ATGATCCAGATTACCGGTCG	$(AC)_{8}$	308–368	20	VIC	4.5
CtAt24	HQ283311	GCTACCTGCATCGCTGTTG	TTCTGGAAGACTGTGGCTCC	$(ACAG)_{11}$	325–389	10	NED	0.0
CtAt30	HQ283312	AGCCATTGCTACATGCTGTG	CAGCCAACGTTGTCCCTA	$(AAGT)_{16}$	377–445	20	VIC	10.0
Nephrurus stellatus								
NeSt05[1]	HQ283314	TGCATTATCTAGTTGTGGACTG	CACTGCTCATGGTAACACAC	$(TG)_{20}$	142–172	20	VIC	3.2
NeSt06	HQ283315	CATGTGTTCACACACTTACACAC	GTCTGTGGTCTCTTGCTGG	$(AC)_{19}$	97–137	20	NED	0.8

Table 1 continued

Locus name	GenBank accession no.	Forward primer sequence 5′–3′	Reverse primer sequence 5′–3′	Repeat motif	Size range (bp)	Primer conc (Nm)	Fluorescent tag	Scoring error rate %
NeSt09[1]	HQ283316	TAAGATCACAGCACCTGAGC	TTCCATTGCCTATTTCCG	$(AAC)_6A(AAC)_4$	225–267	20	VIC	0.0
NeSt11[2]	HQ283317	CATCAGTGAATCCCTGCTG	CGATTCTCAGCAAACACAC	$(TG)_{24}$	294–330	20	NED	0.0
NeSt16	HQ283318	ACCCTTCTCTTGATGAGGTG	TTAAGGAAGACAGCTTGCC	$(AC)_{24}$	215–259	40	FAM	2.3
NeSt18[3]	HQ283319	CCCGTGTTGCCATATTAAG	CAAAACACCTCAATCATTGC	$(GT)_{25}$	108–146	20	FAM	1.4
NeSt23[3]	HQ283320	AGGGTCAGGTGACACAGTATC	CATTTAATAGTGGCATGACATC	$(TG)_{22}$	208–246	20	FAM	0.7
NeSt28	HQ283321	ACCAATTCAATCATAGGATCAC	ACAGCCTAACATACATCACAAG	$(GTT)_{27}$	230–287	20	VIC	1.4
NeSt31	HQ283322	AAGCTGCCTTGAGATATTATG	GAGAGTAGCATGGGACGAAC	$(TTC)_{10}TCC(TTC)_5(N)_{18}(TCC)_4$ $(TTG)_{17}(TTC)_{22}$	293–383	20	FAM	2.6
NeSt32	HQ283323	GAGTTCACAATTACCCAGACAG	TAATTCCAATAGAACACAGCG	$(TTG)_{15}$	278–311	20	PET	5.5
NeSt33	HQ283324	GCCATCTGTTTGAGACTATTG	AGAATCCAGCTTGGAGTCTAG	$(ATT)_9(GTT)_{15}$	214–262	20	PET	0.0
NeSt35	HQ283325	ACTGAATGAAGTGAGACATAAGTC	AACGTGCCTCCTCCTCAC	$(AAC)_{11}$	197–241	20	NED	0.0
NeSt38	HQ283326	CACCAACAAGGCAAATAGC	TCCTTTCTGGATTGTGTGG	$(GTT)_{17}$	289–349	20	NED	2.0
NeSt43	HQ283327	GTGATGGCATCATCCTCAG	AGCAGCAGCCTGACTCTG	$(GTT)_{14}$	154–220	20	PET	0.0
NeSt46	HQ283328	CTGTCCTCAACAGCTAGTGC	AAGCCTAACAGTGCTATTCTAAG	$(GTT)_{13}$	270–303	20	PET	2.0
NeSt47[2]	HQ283329	GATCTTGAATGACATCGTGC	CTCTTCTGCATTAGTCTGAGTTC	$(CAA)_{17}$	193–253	20	NED	2.1

Six *N. stellatus* loci were amplified in three duplex reactions, indicated by 1, 2 and 3

Table 2 Population statistics for microsatellite markers in the lizards *Amphibolurus norrisi*, *Ctenotus atlas*, and *Nephrurus stellatus*

Locus name	Target sample site					Comparison sample sites	
	No. alleles	H_O	H_E	P	Freq. null alleles	% sites with HW disequilibrium	% sites with null allele
Amphibolurus norrisi (target site $N = 23$, no. comparison sites = 6)							
AmNo02	10	0.476	0.771	< 0.001*	0.194†	17	33
AmNo04	6	0.826	0.808	0.945	−0.009	0	0
AmNo05	8	0.826	0.821	0.501	−0.009	0	0
AmNo11	12	0.682	0.781	0.096	0.073	0	0
AmNo12	6	0.600	0.666	0.092	0.039	0	33
AmNo18	11	0.652	0.836	0.036	0.113†	0	50
AmNo20	7	0.609	0.664	0.209	0.048	0	0
AmNo24	18	0.739	0.913	0.043	0.096†	83	83
AmNo25	5	0.545	0.655	0.062	0.096	17	0
AmNo26	6	0.818	0.693	0.634	−0.123	0	0
AmNo29	17	0.850	0.915	0.006	0.033	17	0
AmNo30	18	0.783	0.925	<0.001*	0.079†	0	0
AmNo31	14	0.643	0.885	0.001*	0.140†	50	67
AmNo33	2	0.043	0.043	0.915	−0.022	17	17
AmNo36	17	0.783	0.926	0.004	0.077†	0	17
AmNo37	15	0.864	0.916	0.566	0.030	17	33
AmNo39	15	0.909	0.895	0.591	−0.008	0	0
Ctenotus atlas (target site $N = 54$, no. comparison sites = 6)							
CtAt01	12	0.510	0.751	<0.001*	0.158†	33	83
CtAt02	6	0.519	0.557	0.036	0.041	0	0
CtAt03	21	0.902	0.905	0.543	0.003	0	0
CtAt04	12	0.522	0.838	<0.001*	0.187†	33	100
CtAt08	9	0.756	0.852	0.035	0.054	0	0
CtAt09	8	0.740	0.734	0.954	−0.004	0	0
CtAt12	14	0.827	0.885	0.666	0.032	0	0
CtAt15	24	0.884	0.925	0.052	0.025	0	17
CtAt18	8	0.653	0.742	0.559	0.065	0	0
CtAt20	15	0.647	0.884	<0.001*	0.137†	50	50
CtAt24	12	0.875	0.902	0.716	0.015	0	0
CtAt30	14	0.744	0.883	0.114	0.079†	33	33
Nephrurus stellatus (target site $N = 63$, no. comparison sites = 7)							
NeSt05	11	0.831	0.878	0.440	0.028	29	57
NeSt06	14	0.949	0.878	0.946	−0.043	0	0
NeSt09	14	0.836	0.852	0.214	0.009	0	0
NeSt11	22	0.857	0.880	0.107	0.016	0	0
NeSt16	13	0.839	0.845	0.631	0.006	0	0
NeSt18	15	0.852	0.900	0.407	0.029	14	0
NeSt23	15	0.885	0.875	0.511	−0.007	0	0
NeSt28	13	0.787	0.730	0.923	−0.084	0	14
NeSt31	20	1.000	0.927	0.766	−0.040	0	0
NeSt32	10	0.695	0.773	0.371	0.047	14	43
NeSt33	14	0.885	0.898	0.514	0.007	0	0
NeSt35	15	0.755	0.766	0.738	0.001	0	0
NeSt38	15	0.857	0.887	0.552	0.017	0	0
NeSt43	11	0.870	0.855	0.780	−0.011	0	0

Table 2 continued

Locus name	Target sample site					Comparison sample sites	
	No. alleles	H_O	H_E	P	Freq. null alleles	% sites with HW disequilibrium	% sites with null allele
NeSt46	10	0.847	0.782	0.608	−0.042	0	0
NeSt47	15	0.803	0.872	0.083	0.042	0	0

Observed (H_O) and expected (H_E) heterozygosity, probability value from Hardy–Weinberg equilibrium test (P). * significant Hardy–Weinberg disequilibrium after sequential Bonferroni adjustment, † presence of a null allele

and design primers for 39 *A. norrisi* and 32 *C. atlas* loci with PCR product lengths of 80–480 base pairs.

Multiplex-ready PCR tags were attached to the forward (5′–3′: ACGACGTTGTAAAA) and reverse (5′–3′: CATTAAGTTCCCATTA) primers (Hayden et al. 2008). After optimising primer concentrations (Table 1) we selected 21 (54% of total screened) *A. norrisi*, 16 (50%) *C. atlas*, and 18 (56%) *N. stellatus* loci for genotyping using the method of Hayden et al. (2008). We amplified six loci in three duplex reactions (Table 1) and all other loci individually. PCR products were combined into two pools per species and genotyped on an ABI 3730 instrument (Applied Biosystems) with the size standard GS500 (-250) LIZ. We processed a total of 180 *A. norrisi*, 379 *C. atlas*, and 840 *N. stellatus* samples. We genotyped 8% of samples on each plate twice to calculate scoring error rates per locus (Table 1) as the number of errors per number of alleles tested (DeWoody et al. 2006). Mean error rates were 2.1, 2.6 and 1.5% for *A. norrisi*, *C. atlas* and *N. stellatus* respectively, which should not substantially bias estimates of population differentiation (Bonin et al. 2004). Two loci from each species failed to amplify consistently and were removed from the data set.

To assess the suitability of markers for analysis we used adult lizard genotype data from a target site within Hincks Conservation Park, South Australia. *N. stellatus* samples ($N = 63$) were collected from an area that last burnt in 1999 and *A. norrisi* ($N = 23$) and *C. atlas* ($N = 54$) samples were collected from an area that last burnt in 1966. For each locus we calculated the number of alleles, observed and expected heterozygosity using GENALEX 6.4 (Peakall and Smouse 2006), and deviation from Hardy–Weinberg Equilibrium (HWE) using GENEPOP 3.4 (Raymond and Rousset 1995) (Table 2). P values from HWE tests were adjusted for multiple tests of significance using the sequential Bonferroni method (Hochberg 1988) (Table 2). We used MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004) to check each locus for evidence of null alleles, scoring error due to stuttering, and large allele drop out. Six *A. norrisi* and four *C. atlas* loci showed significant null allele frequencies at the target site (Table 2). None of the loci showed evidence for large allele drop out, but one *A. norrisi* locus (AmNo02) showed evidence of scoring error due to stuttering consistent with our calculations (Table 1). We checked all pairs of loci in each species for linkage disequilibrium in GENEPOP and none were significant after sequential Bonferroni adjustment.

Because some loci showed deviation from HWE or null alleles, we analysed data from a number of additional sample sites to determine if these patterns were either locus or population specific. We used data from seven additional sample sites for *N. stellatus* ($N = 23$–48) and six additional sites for *A. norrisi* ($N = 10$–24) and *C. atlas* ($N = 14$–35). For each locus we calculated the percentage of comparison sites with evidence for a null allele and deviation from HWE (Table 2). Two loci each from *C. atlas* and *A. norrisi* failed both HWE and null allele tests at all sites and were removed from the data set leaving a final panel of 45 loci suitable for further research (Tables 1, 2).

These markers will enhance our ability to understand dispersal and implement fire management at scales appropriate for animals in fire-prone ecosystems.

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