CSIRO PUBLISHING

Australian Journal of Zoology, 2016, **64**, 390–394
http://dx.doi.org/10.1071/ZO16072

Short communication

Characterisation of microsatellites for *Litoria nannotis* (Amphibia : Hylidae), an endangered waterfall frog endemic to the Australian Wet Tropics

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Abstract. *Litoria nannotis* is an endangered waterfall frog from the wet tropics region in north Queensland that has suffered significant population declines due to the emerging fungal disease known as chytridiomycosis. The species has two deeply divergent lineages, and we used 454 shotgun sequencing of DNA extracted from one individual of the northern lineage to identify and design PCR primers for 576 microsatellite loci. Thirty markers were tested for amplification success and variability in a population sample from each lineage. Of these, 17 were found to be polymorphic in the northern lineage and 10 loci were polymorphic in the southern lineage. Numbers of alleles per locus ranged from 2 to 14 (mean = 6.47, s.d. = 4.02) for the northern lineage (17 polymorphic loci), and from 2 to 8 (mean = 5.40, s.d. = 2.55) in the southern lineage (10 polymorphic loci). Levels of heterozygosity were high in both lineages (northern mean $H_E = 0.63$, s.d. = 0.21, range = 0.27–0.89; southern mean $H_E = 0.57$, s.d. = 0.25, range = 0.18–0.81). These loci will be useful in understanding the genetic variation and connectivity amongst populations of this species recovering from mass population declines due to disease.

Additional keywords: 454 GSFLX, population declines, shotgun sequencing.

Received 18 October 2016, accepted 2 March 2017, published online 23 March 2017

The waterfall frog (*Litoria nannotis*) is an endangered species from the Australian Wet Tropics. High-elevation populations declined significantly in the early 1990s due to the emergence of the fungal disease known as chytridiomycosis caused by the fungus *Batrachochytrium dendrobatidis* (Berger *et al*. 1998), but lowland populations persisted (Richards *et al*. 1994). *L. nannotis* is part of the torrent frog group, which comprises four species, two of which were feared extinct during the declines (Richards *et al*. 1994). All species in this group seem to have a similar biology (Cunningham 2002), and understanding population dynamics and potential gene flow between high and low elevations as well as between dry and wet forest sites is crucial when designing conservation strategies for these amphibians in this system. This species comprises at least two distinct lineages, a product of historical climatic shifts and expansions and contractions in their habitat (Schneider *et al*. 1998; Cunningham 2002; Bell *et al*. 2012). Knowledge of current and recent historical population structure, gene flow and levels of genetic diversity is especially pertinent for *L. nannotis*, as some higher-elevation populations are showing some signs of recovery (Puschendorf *et al*. 2011).

We isolated genomic DNA (1 µg) from the liver of one individual *L. nannotis* from the northern lineage (16.466291°S, 145.152538°E, WGS84, 668 m elevation) using a DNeasy spin column tissue extraction kit (Qiagen) and following manufacturer's instructions. DNA was then sent to the Australian Genomic Research Facility in Brisbane, Australia, for shotgun sequencing on a Titanium GS-FLX (454 Life Sciences/Roche FLX) following Gardner *et al*. (2011). The sample occupied 12.5% of a plate and produced 110 205 individual sequences, with an average fragment size of 314.2 (s.d. = 132.2). Raw sequences are available on DRYAD (doi: 10.5061/dryad.jd183; Meglécz *et al*. 2012). We used the program QDD 1.3 (Meglécz *et al*. 2010) to screen the raw sequences for more than eight di-, tetra- or penta-base repeats, and to remove redundant sequences and design primers for PCR amplification of products of 80–480 base pairs (automated in QDD using Primer3: Rozen and Skaletsky 2000). We identified 576 *in silico* microsatellite loci and ordered primer pairs for 30 of these. Initially, the loci were trialed for amplification success in eight individuals, four from each lineage using the Type-it microsatellite PCR kit (Qiagen). We performed

Table 1. Details for 19 *Litoria nannotis* microsatellite loci developed from 454 shotgun sequence data

Loci shown in bold are in Hardy–Weinberg equilibrium. N_a, no. of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information content; P_{HWE}, probability value from a test for deviation from Hardy-Weinberg Equilibrium

Locus	Primer sequence 5′–3′	Repeat motif	Primer conc. (μM)	T_a (°C)	N	Allele size range	N_a	H_O	H_E	PIC	P_{HWE}	Multiplex group	GenBank accession no.
Northern													
Lnan03	**F: GCCATGCACATGAGCTTTTA** **R: CCAATACGCGCCAATTTTAC**	**(AT)8**	**0.2**	**58**	**44**	**140–142**	**2**	**0.568**	**0.500**	**0.375**	**0.364**[A]	**4**	**KX518722**
Lnan04	F: GGTGGACATCATGTGGATCA R: CCAATACGCGCCAATTTTAC	(AT)8	0.2	58	44	190–192	2	0.068	0.107	0.101	0.016[A]	5	KX518723
Lnan06	**F: GAGTTTCCTTCCCAAAAGCA** **R: GCATCAATCCCTGTCTCCAA**	**(TG)9**	**0.2**	**58**	**44**	**100–106**	**3**	**0.250**	**0.271**	**0.24**	**0.118**[A]	**5**	**KX518724**
Lnan08	**F: GTATAACAGGGCGGAACTGC** **R: GTGTAACTCGCCTTCCTTGC**	**(GT)9**	**0.2**	**58**	**44**	**131–139**	**4**	**0.727**	**0.667**	**0.611**	**0.644**[A]	**2**	**KX518725**
Lnan10	**F: TGTGTAAATTGCTCCAGGCA** **R: TGAATGATGCCAGACCAAGA**	**(AT)11**	**0.2**	**58**	**44**	**140–184**	**10**	**0.750**	**0.761**	**0.734**	**0.654**[A]	**4**	**KX518726**
Lnan14	**F: GCAACCAATATGGGTGACATT** **R: GCACTTATGTTGCGATGCAC**	**(AT)12**	**0.2**	**58**	**44**	**210–216**	**4**	**0.591**	**0.582**	**0.504**	**0.285**[A]	**5**	**KX518728**
Lnan15	**F: TGCAGATCCATGCAATACTGA** **R: TCAACGTTCAATGGTCAAGG**	**(AAT)8**	**0.2**	**58**	**44**	**149–167**	**7**	**0.636**	**0.774**	**0.74**	**0.021**[A]	**1**	**KX518729**
Lnan16	**F: ACTTTGTTAGGTGCTGCGGA** **R: GCACCCTTAATGTGTTCCTGA**	**(AAT)8**	**0.2**	**58**	**43**	**103–109**	**2**	**0.419**	**0.381**	**0.308**	**0.514**[A]	**3**	**KX518730**
Lnan17	**F: GCGGTTACAGGGTACAGCAT** **R: TGTACTTTGTTAGGCGCTGC**	**(TTA)8**	**0.2**	**58**	**44**	**207–219**	**4**	**0.432**	**0.440**	**0.377**	**0.960**[A]	**1**	**KX518731**
Lnan18	**F: CCAAAACCGCTTTTCTGTTG** **R: TGGGTTAATAACATGAGGAAGAGTT**	**(CTA)8**	**0.2**	**58**	**44**	**136–142**	**2**	**0.386**	**0.363**	**0.297**	**0.675**[A]	**2**	**KX518721**
Lnan20	**F: AAGTGCTCCGGATACCAATG** **R: TTGTTGATGAATCTGGTGCC**	**(TAT)11**	**0.2**	**58**	**43**	**285–294**	**4**	**0.721**	**0.653**	**0.589**	**0.466**[A]	**3**	**KX518720**
Lnan21	**F: TACTTTGTTAGTCGCTGCGG** **R: CTCTTGTTGGCCTCCCATAA**	**(ATT)12**	**0.2**	**58**	**44**	**124–136**	**4**	**0.386**	**0.326**	**0.296**	**0.866**[A]	**4**	**KX857664**
Lnan22	**F: CAAGGTTGACACCAAGCAGA** **R: TGTAACTTTGTTAGGCGCTGC**	**(TTA)12**	**0.2**	**58**	**44**	**107–134**	**7**	**0.864**	**0.808**	**0.781**	**0.519**[A]	**1**	**KX518732**
Lnan24	**F: GCCATTTAAGACACCTGGGA** **R: CCATTGTGTGCTGCAGTGAT**	**(ATCT)12**	**0.2**	**58**	**43**	**136–170**	**9**	**0.884**	**0.858**	**0.841**	**0.771**[A]	**3**	**KX518733**
Lnan25	**F: TAAGGGGATTGGTATGCTGG** **R: GAAGTGCCACTACCATTCTTTTG**	**(CTAT)13**	**0.2**	**58**	**44**	**155–187**	**9**	**0.818**	**0.793**	**0.771**	**0.441**[A]	**5**	**KX857663**
Lnan26	**F: CTTTCACGTCATAGGAACCCA** **R: CAACAGGGCTTTCAACCATT**	**(GATA)13**	**0.2**	**58**	**43**	**133–171**	**12**	**0.837**	**0.839**	**0.822**	**0.997**[A]	**3**	**KX518734**
Lnan27	F: CCACTCCTGTTGGGGAGATA R: AAATGTGGGAAAAGTGAAGCA	(GATA)14	0.2	58	44	81–159	9	0.886	0.839	0.821	0.081[A]	1	KX518719
Lnan29	**F: CTATGCGGCCATCTTCTCTC** **R: GTGACTTGCAGCCTGTTGAG**	**(ATCT)17**	**0.2**	**58**	**44**	**178–249**	**13**	**0.909**	**0.894**	**0.885**	**0.499**[A]	**4**	**KX518735**
Lnan30	**F: GTGAAAAGCAATGCCACCTT** **R: TCAGTAGACCACAAAGAGCGTT**	**(ATCT)17**	**0.2**	**58**	**43**	**127–210**	**14**	**0.791**	**0.860**	**0.847**	**0.266**[A]	**2**	**KX518736**

(*continued next page*)

Table 1. (*continued*)

Locus	Primer sequence 5′–3′	Repeat motif	Primer conc. (μM)	T_a (°C)	*N*	Allele size range	N_a	H_O	H_E	PIC	P_{HWE}	Multiplex group	GenBank accession no.
Southern													
Lnan03	**F: GCCATGCACATGAGCTTTTA**	**(AT)8**	**0.2**	**58**	**40**	**140–142**	**2.000**	**0.200**	**0.180**	**0.164**	**0.482**	**4**	**KX518722**
	R: CCAATACGCGCCAATTTTAC												
Lnan04	F: GGTGGACATCATGTGGATCA	(AT)8	0.2	58	39	192	1.000	n.a.	n.a.	n.a.	n.a.	5	KX518723
	R: CCAATACGCGCCAATTTTAC												
Lnan08	F: GTATAACAGGGCGGAACTGC	(GT)9	0.2	58	40	131	1.000	n.a.	n.a.	n.a.	n.a.	2	KX518725
	R: GTGTAACTCGCCTTCCTTGC												
Lnan10	**F: TGTGTAAATTGCTCCAGGCA**	**(AT)11**	**0.2**	**58**	**40**	**139–162**	**4.000**	**0.575**	**0.641**	**0.574**	**0.115**	**4**	**KX518726**
	R: TGAATGATGCCAGACCAAGA												
Lnan12	**F: TCAAATCCATTGTGGTGGTG**	**(TA)11**	**0.2**	**58**	**40**	**191–221**	**8.000**	**0.700**	**0.681**	**0.631**	**0.997**	**2**	**KX518727**
	R: CCACATGTTGCCTACTCCCT												
Lnan14	**F: GCAACCAATATGGGTGACATT**	**(AT)12**	**0.2**	**58**	**39**	**206–232**	**6.000**	**0.718**	**0.673**	**0.624**	**0.198**	**5**	**KX518728**
	R: GCACTTATGTTGCGATGCAC												
Lnan15	F: TGCAGATCCATGCAATACTGA	(AAT)8	0.2	58	39	148	1.000	n.a.	n.a.	n.a.	n.a.	1	KX518729
	R: TCAACGTTCAATGGTCAAGG												
Lnan16	**F: ACTTTGTTAGGTGCTGCGGA**	**(AAT)8**	**0.2**	**58**	**39**	**112–127**	**5.000**	**0.538**	**0.617**	**0.583**	**0.228**	**3**	**KX518730**
	R: GCACCCTTAATGTGTTCCTGA												
Lnan17	F: GCGGTTACAGGGTACAGCAT	(TTA)8	0.2	58	40	210–213	3.000	0.100	0.184	0.174	<0.001[B]	1	KX518731
	R: TGTACTTTGTTAGGCGCTGC												
Lnan18	**F: CCAAAACCGCTTTTCTGTTG**	**(CTA)8**	**0.2**	**58**	**40**	**133–136**	**2.000**	**0.200**	**0.180**	**0.164**	**0.482**	**2**	**KX518721**
	R: TGGGTTAATAACATGAGGAAGAGTT												
Lnan20	**F: AAGTGCTCCGGATACCAATG**	**(TAT)11**	**0.2**	**58**	**39**	**273–283**	**3.000**	**0.359**	**0.325**	**0.296**	**0.710**	**3**	**KX518720**
	R: TTGTTGATGAATCTGGTGCC												
Lnan21	F: TACTTTGTTAGTCGCTGCGG	(ATT)12	0.2	58	40	121	1.000	n.a.	n.a.	n.a.	n.a.	4	KX857664
	R: CTCTTGTTGGCCTCCCATAA												
Lnan24	F: GCCATTTAAGACACCTGGGA	(ATCT)12	0.2	58	39	123–145	6.000	0.718	0.739	0.705	0.023	3	KX518733
	R: CCATTGTGTGCTGCAGTGAT												
Lnan25	F: TAAGGGGATTGGTATGCTGG	(ATCT)12	0.2	58	37	142–224	13.000	0.676	0.874	0.861	0.005[B]	5	KX857663
	R: GAAGTGCCACTACCATTCTTTTG												
Lnan26	**F: CTTTCACGTCATAGGAACCCA**	**(GATA)13**	**0.2**	**58**	**39**	**121–151**	**8.000**	**0.744**	**0.811**	**0.787**	**0.508**	**3**	**KX518734**
	R: CAACAGGGCTTTCAACCATT												
Lnan27	**F: CCACTCCTGTTGGGGAGATA**	**(GATA)14**	**0.2**	**58**	**39**	**106–138**	**8.000**	**0.769**	**0.812**	**0.786**	**0.862**	**1**	**KX518719**
	R: AAATGTGGGAAAAGTGAAGCA												
Lnan30	**F: GTGAAAAGCAATGCCACCTT**	**(ATCT)17**	**0.2**	**58**	**40**	**123–153**	**8.000**	**0.775**	**0.814**	**0.789**	**0.414**	**2**	**KX518736**
	R: TCAGTAGACCACAAAGAGCGTT												

[A]Not significant after FDR correction, FDR value = 0.00263.
[B]Significant after FDR correction, FDR value = 0.012.

amplifications in 10-μL reactions, containing 20–50-ng template, 1x Type-it Multiplex PCR Master Mix (Qiagen) and 0.2 μM each primer (forward and reverse). Indirectly labelled reactions contained a tailed forward primer and a reporter primer (5′ labelled with fluorescent dye modification HEX, TET or FAM) at a 1 : 4 ratio (total = 0.2 μM). PCR cycling conditions were as follows: initial 5 min denaturation at 95°C, followed by 28 cycles of 95°C for 30 s (denaturation), 58°C for 90 s (annealing), 72°C for 30 s (extension), with a final extension 30 min at 60°C. Following visualisation by electrophoresis through a 1.5% agarose gel, loci exhibiting reliable amplification of a single product of expected size were assessed for polymorphism. We separated DNA fragments on a MegaBACE 1000 capillary sequencer and sized with GeneMarker 2.2 software (SoftGenetics) using a 400 base pair DNA ladder as an internal size standard.

For all polymorphic loci, forward primers were synthesised with a 5′ flourescent tag: FAM (GeneWorks), NED, PET or VIC (Applied Biosystems). Loci were then screened for variation in 44 individuals from a single locality within the northern *L. nannotis* lineage (16.236250°S, 144.935690°E, WGS84, 959 m above sea level) and 40 individuals collected from a single locality representing the southern lineage (18.992422°S, 146.191184°E, WGS84, 742 m above sea level) (Table 1). We used the same PCR conditions and allele-scoring software described above, with allele binning to ensure consistent scoring across genotyping runs. Due to consistent differences in allele profiles among lineages, independent scoring panels were used for each lineage. Multiplex PCR combinations (Table 1) were later designed *in silico* with the aid of MULTIPLEX MANAGER 1.0 software (Holleley and Geerts 2009), and tested using PCR conditions described above. Characteristics of each locus in each lineage are summarised in Table 1. Data are presented for 19 loci that amplified consistently in the northern lineage, and similarly for 17 loci in the southern lineage. Basic summary statistics (number of alleles, observed and expected heterozygosities) were calculated in GENALEX 6.5 (Peakall and Smouse 2012), which was also used to test for deviations from Hardy–Weinberg Equilibrium (HWE). Polymorphic Information Content values were calculated for each locus in CERVUS (Kalinowski *et al*. 2007). Potential linkage disequilibrium (LD) between pairs of loci was investigated using GENEPOP 4.2 online, with 10 000 iterations (http://genepop.curtin.edu.au/) (Raymond and Rousset 1995; Rousset 2008) (Table 1). *P* values from HWE and LD tests were adjusted for multiple tests of significance using the false discovery rate (FDR) correction and included in Table 1. (Benjamini and Hochberg 1995). 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, using a 95% confidence level and 10 000 iterations.

In the northern lineage, 17 of 19 polymorphic loci conformed to HWE expectations and are considered suitable for population genetic studies (shown in bold in Table 1). In the southern lineage, 10 of 17 polymorphic loci met HWE expectations. Of those loci not in HWE, there was evidence for null alleles at locus Lnan15 in the northern lineage, and Lnan17 and Lnan25 in the southern lineage. There was no evidence of large allele drop-out at any locus. Following FDR correction, all loci were found to be inherited independently (north, $P > 0.002$, FDR = 0.0003; south, $P > 0.02$, FDR = 0.0006). Overall, the markers exhibit high levels of polymorphism in northern and southern *L. nannotis* lineages suitable for studies of relatedness, population genetic structure and connectivity. For polymorphic loci also in HWE, numbers of alleles per locus ranged from 2 to 14 (mean = 6.47, s.d. = 4.02) for the northern lineage (17 polymorphic loci), and from 2 to 8 (mean = 5.40, s.d. = 2.55) in the southern lineage (10 polymorphic loci). Levels of heterozygosity were high in both lineages (northern mean $H_E = 0.63$, s.d. = 0.21, range = 0.27–0.89; southern mean $H_E = 0.57$, s.d. = 0.25, range = 0.18–0.81). Overall, the markers exhibit high levels of polymorphism in northern and southern *L. nannotis* lineages suitable for studies of relatedness, population genetic structure and connectivity.

These markers will be used to document patterns of gene flow, population structure and genetic diversity in *L. nannotis* and to investigate their recovery from the amphibian population declines linked to chytridiomycosis documented since the early 1990s (Berger *et al*. 1998). More recently, high-elevation populations seem to be recovering, and larger, seemingly healthy populations have been described in the western slopes of the wet tropics region, including one sister species, *Litoria lorica*, which was previously thought to be extinct (Puschendorf *et al*. 2011). How these populations are interconnected, and the source of the recovering populations, are key aspects of frog conservation in this region.

Acknowledgements

David Blair, Martin van der Meer, Blanche Danastas and Giana Gomez provided help in the laboratory as well as in the design of these primers. We acknowledge the assistance and support of Alison Fitch from Flinders University. This project was funded by the Australian Research Council (DP1094540).

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Handling Editor: Steven Cooper