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Conserv Genet Resour. Author manuscript; available in PMC 2015 June 01.

Published in final edited form as:
Conserv Genet Resour. 2014 June 1; 6(2): 429–431. doi:10.1007/s12686-013-0115-2.

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Development of 14 microsatellite markers in the Queensland koala (*Phascolarctos cinereus adustus*) using next generation sequencing technology

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Abstract

We report the development of 14 new microsatellite markers in the Queensland koala (*Phascolarctos cinereus adustus*). Ten unrelated Queensland koala individuals from the San Diego Zoo, USA, were genotyped. The number of alleles per locus ranged from 2 to 7, with an average of 5.14 alleles per locus. Across all loci, the average observed and expected heterozygosity values were both 0.69. These polymorphic microsatellite loci will be useful for genetic studies relevant to the conservation of the koala, a species listed as vulnerable.

Keywords

454 GS-FLX Technology; conservation genetics

The koala (*Phascolarctos cinereus*) is listed as vulnerable by the Australian government, and as threatened under the U.S. Endangered Species Act. Persistent threats to the long-term survival of koalas include habitat loss and fragmentation, and virus-related diseases, with motor vehicle accidents and dog attacks greatly impacting some populations (Department of the Environment 2013). Evaluating genetic variation and structure among koala populations in Australia continues to be the focus of conservation genetic studies (Lee et al. 2010). Koala microsatellite markers have been developed using DNA only from southern Australian koalas, while mitochondrial DNA has been widely used in genetic studies on Queensland koalas (Lee et al. 2010) (see supplementary material). In this study, we developed novel microsatellite markers using next-generation sequences from a Queensland koala, and tested them for effectiveness on ten unrelated Queensland koalas, all housed at the San Diego Zoo, USA.

Genomic DNA from one koala (Pci–SN404) was sequenced using 454 GS-FLX Technology (Roche Applied Science). We obtained 35,942 fragment reads from the 454 sequencing with

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an average read length of 549 base pairs. MSATCOMMANDER 1.0.8 (Faircloth 2008) was used to identify microsatellites by screening the sequence data for di-, tri-, tetra- and penta-nucleotide motifs with a minimum of 10 repeats each. MSATCOMMANDER interfaces with PRIMER3 software allowing for design of primers while minimizing potential structural or functional defects. The MSATCOMMANDER program was modified to ensure that the flanking region between the microsatellite and primer sequence would generate an amplicon size range of 100–250 base pairs, inclusive of the lengths of both primers (Brandt et al. 2013).

After excluding primers that appeared to match non-target loci or species, 34 primer pairs were subject to further testing, including loci that had di-, tri-, tetra- and penta-nucleotide repeats. An M13 forward tail (5′-TGTAAAACGACGGCCAGT-3′) was added at the 5′ end of all forward primers to facilitate fluorescent-dye labeling (Ishida et al. 2012). Primers were then tested using DNA from ten unrelated Queensland koalas from San Diego Zoo. Amplification of all loci was performed using the same thermocycling conditions previously described by Ishida et al. (2012); see the supplementary materials included with this report for precise details. PCR amplicons were diluted and run on an ABI-3730xl DNA Analyzer using the GenScan-500LIZ (Applied Biosystems) as a size standard. Alleles were visualized and scored using GeneMapper Version 3.7 (Applied Biosystems).

A total of 14 polymorphic loci were successfully genotyped in all ten individuals except for the three loci *Phci5*, *Phci12*, and *Phci16*, which amplified in 9 individuals each (Table 1). Sequences obtained from the Roche 454 sequencer for the 14 loci are listed in supplementary Table S1. Allelic diversity ranged from 2 to 7 alleles per locus with an average of 5.14 alleles. Observed and expected heterozygosity averaged across all loci were both 0.69. Between pairs of loci, no significant linkage disequilibrium was detected after Bonferroni correction ($P < 0.0005$). No evidence of deviation from Hardy-Weinberg equilibrium was detected with the exception of marker *Phci12*. Locus *Phci12* had 5 alleles and showed high *He* (0.89), while *Ho* was low (0.11) (8 out of 9 individuals were homozygous for this locus). Given these results, we would not recommend the use of *Phci12*. Additionally, we would recommend that the other markers be used before *Phci16* and *Phci19*, as these two had relatively lower allelic diversity.

In summary, we developed novel microsatellite markers in Queensland koalas after using 454 GS-FLX technology to obtain whole genome shotgun sequences. We searched for short tandem repeats in the shotgun sequences and designed suitable microsatellite primers to test for polymorphisms in ten unrelated koalas from the San Diego Zoo, USA. The new microsatellite markers developed should be useful for examining population structure and genetic diversity among Australian koalas, and may be used in studies to aid in the genetic management of captive populations or to examine wild koala genetic diversity and structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

For generously providing koala samples, we thank the San Diego Zoo, Columbus Zoo, San Francisco Zoo, and Dallas Zoo. We thank K. Zhao for modifying the MSATCOMMANDER program and J. R. Brandt for creating a PERL script to search primer sequences against the 454 sequence database. We thank the UIUC Core Sequencing Facility for fragment analysis. The project described was supported by grant number R01GM092706 from the National Institute of General Medical Sciences (NIGMS).

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Table 1

Characteristics of 14 novel microsatellite markers developed in the Queensland koala

Locus	Primer sequence (5′-3′)	*N*	Alleles	H_o	H_e	Allele range	Motif
Phci2	F: ACCTCCTACCACATTGAGCC R: GGGAGTGACAAATTCCACAAC	10	7	0.90	0.83	142–166	$(AC)_{12}$
Phci5	F: GGGATGAAGTTGAGGTGGTG R: TGGTACAGGGCTCTCACATAG	9**	5	0.89	0.81	155–175	$(AATG)_{10}$
Phci9	F: AAGGACACTGTACTGGGCTC R: CTGCTACCATGCTTCTGTTCC	10	4	0.80	0.65	174–183	$(AGC)_{18}$
Phci10	F: ATTGGAGAGTGTGGGTGAGG R: TCCAGGTTCAGCACTCTAGAG	10	6	0.90	0.83	198–224	$(AGAT)_{13}$
*Phci12**	F: GAGGGCAGGAAGGATAGGC R: TCCAAACTCCCTCGGTTCAG	9**	5	0.11	0.79	221–254	$(ACG)_{13}$
Phci15	F: TAATTCTTTGAGCGTCGCCC R: GAACTGGTGTGGCATAGTGG	10	5	0.60	0.63	219–223	$(AGA)_{15}$
Phci16	F: CATCTTGGACCTGGAGTAGC R: ACCCTGACTCTTGAGACCAC	9**	2	0.22	0.21	164–172	$(AC)_{10}$
Phci17	F: TACCTCTCCAGTTCACCCAC R: CCCTAGACTCCAGGCATGAG	10	6	1.00	0.84	155–167	$(AC)_{17}$
Phci18	F: ATATTGTCATGTGCCCTGCC R: GCCCTTACTGTGTTTCCAGC	10	6	0.90	0.74	157–181	$(AC)_{19}$
Phci19	F: GCCTTTCTCTCAGTGCATTCC R: TAAGGGCAGTTGGAAGGAGC	10	3	0.20	0.28	169–175	$(AC)_{18}$
Phci22	F: CGTCTTCCCATGAACTGCTC R: GGAGAGGGAAAGTGGAGCC	10	6	0.80	0.76	184–208	$(AC)_{17}$
Phci27	F: CACAGTCAGTATGTTGGCCG R: AACACCTTAACTCACCCAGC	10	5	0.90	0.81	193–208	$(AC)_{15}$
Phci28	F: ATTCTGGGCTACGTCTTGGG R: GCAAGGGAACAGCTGATCTC	10	6	0.90	0.84	187–203	$(AC)_{22}$
Phci31	F: CTTGAATTCCCAGGGCCAAC R: TGGTAGGCAGAAGTGAAACG	10	4	0.50	0.64	195–201	$(AC)_{16}$

*Significant deviation of locus *Phci12* from Hardy-Weinberg expectations after Bonferroni correction ($P < 0.0005$)

**The same koala DNA sample (Pci-SN192) failed to amplify for these three loci

N is the number of individuals successfully amplified; H_O is observed heterozygosity; H_e is expected heterozygosity

An M13-tail (5′-TGTAAAACGACGGCCAGT) was attached to the 5′ end of all forward primers

For each motif, the copy number listed is the one that was present among the next generation sequencing results