Molecular Ecology Resources (2009) **9**, 402–404 doi: 10.1111/j.1755-0998.2008.02250.x

PERMANENT GENETIC RESOURCES

Anonymous nuclear markers for Malagasy plated lizards (*Zonosaurus*)

BRICE P. NOONAN*,† and ANNE D. YODER*
Department of Biology, Duke University, Box 90338, Durham, NC 27708,USA, †Department of Biology, University of Mississippi, PO Box 1848, University, MS 38677, USA

Abstract

We report the development of 18, single-copy, anonymous nuclear loci from the Malagasy plated lizard *Zonosaurus madagascariensis*. More than 140 clones from a genomic library were examined and 38 potential loci tested across both closely and distantly related lizards. Of the 18 loci reported here, more than half (10) work in closely related zonosaurines although only one successfully amplified a homologous fragment in the distantly related iguanid (*Oplurus*). Sequences of these loci revealed a high frequency of single nucleotide polymorphisms, supporting previous reports of high levels of intraspecific variation in lizards.

Keywords: genomic library, single nucleotide polymorphism, *Zonosaurus*

Received 16 November 2007; revision accepted 16 January 2008

The remarkable diversification of the largely endemic biota of Madagascar has intrigued biologists for decades. This biodiversity hotspot is one of the most imperilled environments on the planet with less than 10% of the original land cover remaining. Intensive bio-inventory and systematic studies have long sought to quantify the island's diversity. Investigators have recently turned to phylogeographical studies, employing molecular data, in order to identify factors effecting this diversification (Craul *et al*. 2007; Heckman *et al*. 2007; Olivieri *et al*. 2007). Herein we report the development of 18 anonymous nuclear markers for *Zonosaurus madagascariensis*, a member of an endemic subfamily of lizards (Zonosaurinae), to be used in analyses of population structure and historical demography. Given that we hope to employ these markers in comparative studies of multiple species of *Zonosaurus*, we have tested these markers to assess the breadth of their utility by attempting to amplify other zonosaurines.

We assembled a genomic library from the pooled DNA of two individuals of *Z. madagascariensis* (field nos APR 7123 and HER 2193). Total genomic DNA was extracted using the high-salt precipitation method of Crandall *et al*. (1999). These low-yield extractions were subjected to whole genome amplification using the QIAGEN Repli-G kit. Products of the amplification were then combined and concentrated to ~550 ng/μL (assayed using a NanoDrop ND-1000). Restriction digest (using Rsa1) of ~12.0 μg of genomic DNA (50 μL total volume) was employed to fragment the DNA. Digested DNA was visualized on a 1% agarose gel and fragments of 1–3 kb in size were excised and purified using the QIAGEN Gel Extraction kit eluting with water. The concentration of purified, size-selected DNA was assayed and volume adjusted via vacufuge concentration to obtain concentrations of 25 ng/μL or greater to provide the proper molar ratio (10:1) of insert to vector for blunt-ended ligation. Approximately 100 ng of size-selected DNA was ligated into 25 ng of pCR Blunt vector, transformed into competent *Escherichia coli* One Shot TOP10 cells (Invitrogen), and plated on agar plates containing kanamycin (50 μg/mL).

Colonies were individually picked with a pipette tip, grown overnight in 2.0 mL LB-kan liquid medium, and purified using the QIAGEN plasmid mini prep kit. Plasmid inserts were amplified using the M13F (–20) and M13R primers. Alternatively, single colonies were replated to a grid and grown to a large size (1–2 mm). These large colonies were scraped and added directly to a 25 μL polymerase chain reaction (PCR). A total of 141 colonies were amplified and run on a 1% agarose gel to determine product size. Eighty-eight of these 141 (63%) ranged in size from 500 to 1600 bp (despite our initial size selection lower limit of 1 kb, smaller fragments were carried through) and were selected for sequencing and primer development. Once

Correspondence: Brice P. Noonan, Fax: 662-915-5144; E-mail: bnoonan@olemiss.edu

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd

Table 1 PCR primers for 18 anonymous nuclear loci developed from a genomic library of *Zonosaurus madagascariensis*. GenBank Accession numbers of original, cloned fragment, forward and reverse primer sequences (5′–3′), size of amplicon, number of *Z. madagascariensis* samples sequenced (SS), number of indels, number of variable sites (S) and haplotypes (H), G + C content (G–C), per-site nucleotide diversity (π), Watterson's theta (θ_W), and Tajima's *D* (D) (statistics from DnaSP, Rozas & Rozas 1999). Superscripts of SS number indicate that the primer pair worked for other *Zonosaurus* species (1), *Trachyloptychus* (2), and the iguanian *Oplurus* (3)

Locus	Accession	Primers	Size	SS	Indels	S (H)	G–C	§	¶$_W$	D
29	EU268548	F: TTTTCATGGCAAGACAGCAG R: CTGTTCCCTCAATTCCCAGA	509	22	2	10 (8)	0.439	0.0066	0.0067	–0.0736
30	EU268549	F: CCAAGCTCAAATTCCTGCTC R: GAAGCCCTGCTTGTATGCTC	437	69[1]		6 (4)	0.354	0.0033	0.0064	–1. 1798
31	EU268550	F: CATAAAGATCAACGCCAGCA R: TTCAGGCTCCTTGCCTAAAA	565	20	1	9 (11)	0.377	0.007	0.0063	0.3853
32	EU268551	F: CAGCCAGAACCTCTCTGCTT R: CCTGGATATTTCCACCCAGA	559	36[1,2]	1	10 (7)	0.454	0.012	0.0191	–1.1414
35	EU268552	F: CCCAGATCAAGATGGCAGAT R: AAGCCCTAGCATGTGGTCTG	499	70[1,2]	1	24 (8)	0.413	0.0047	0.0138	–2.0416
36	EU268553	F: AGCTGGAAAGTCCCCAGTTT R: CCCTTTGACTACACCCCTGA	467	84[1]	1	19 (10)	0.449	0.0043	0.0119	–1.8713
37	EU268554	F: TCTTGAAGCAGGGCTTTTGT R: GGGAAACTGTGCCATGATCT	418	72[1,2]	3	31 (9)	0.351	0.0145	0.0213	–1.0239
38	EU268555	F: CCCACATGACATTTGCACTC R: CTTCTGTCCCTGTGCCTCTC	436	24[1,2]		5 (5)	0.561	0.0039	0.0036	0.2858
41	EU268556	F: AGTTCGCATCTCCTCTGGAA R: TTCCTATGTCTCCCCCTCCT	411	30	2	18 (13)	0.343	0.0102	0.0121	–0.5166
42	EU268557	F: GATGCCAGGAAGGGAACTT R: TCTGTTGGGATCTGCAAGTG	429	21	2	13 (12)	0.398	0.0109	0.0109	0.0038
43	EU268558	F: CAACCCCCTTGTTGCTCTTA R: CACCACGGCTAATAGGAGA	459	26	1	17 (18)	0.569	0.0121	0.0121	–0.0143
45	EU268559	F: CTACACGGCCTGTTCCATTT R: AGCAGTGGCACTTCAAGGTT	406	24		17 (13)	0.422	0.0151	0.0164	–0.2838
46	EU268560	F: TATTGGCTAGGCCAAAATGC R: TTATGGGTTTCCTGGGTGAG	450	24[1,2,3]		9 (8)	0.392	0.0058	0.0062	–0.2177
49	EU268562	F: AGCCCCTGATTAAGCCAGTT R: TGCTGAAAATTCCTGGGTTC	430	24[1,2]	1	21 (11)	0.423	0.0093	0.0134	–1.1237
91	EU268563	F: CTGATGCCCAGGATGAAACT R: ACACCGACTTGACGGAAAT	577	24		20 (10)	0.449	0.0102	0.0111	–0.2764
93	EU268564	F: GCCCCACTTTTGCATTACTC R: CATGTTCCTGCTGCTGAAAA	438	27[1]		17 (12)	0.248	0.0109	0.011	–0.0583
102	EU268565	F: GAAGCCATTTCTTGCAGAGG R: GATGAGGGTGTGAACAGGA	587	30		25 (12)	0.455	0.0085	0.012	–1.0596
103	EU268566	F: ACTGAGGGCTCCTTCACTCA R: GGCACTTAGCAAGAGCTGA	525	20[1,2]	3	8 (9)	0.478	0.0063	0.0054	0.6023

sequenced, anonymity was verified via BLAST search. The majority of these inserts returned no significant BLAST hits, although a few (six) were similar to known lizard SINE elements and one was nearly identical to 28S sequence of *Xenopus* (GenBank Accession no. X59733). These seven loci were excluded from development. Of those that remained anonymous (*n* = 81), 43 were excluded based on suboptimal base composition, small size, or the inability of Primer 3 (Rozen & Skaletsky 2000) to recognize suitable priming sites within the fragment. Primers were developed for the 38 remaining anonymous loci and these were tested on a number of *Z. madagascariensis* samples as well as two other zonosaurines (*Z. laticaudatus*, *Trachyloptychus madagascariensis*) and an iguanian (*Oplurus madagascariensis*).

As we had a large number of loci to select from, we chose to develop only those that amplified without significant optimization of reaction conditions. Amplification employed a standard touchdown cycle: 95°C–1.5min; 10× [95°C–35s, 63°C–35s (–0.5°C/cycle), 72°C–1min]; 10× (95°C–35s, 58°C–35s, 72°C–1min); 15× (95°C–35s, 52°C–35s, 72°C–1min); 72–10min, and standard reaction conditions [2.0 mM $MgCl_2$, 0.3 mM each dNTP, 0.4 U *Taq* polymerase (Sigma); 14.5 µL total volume] for all loci. Of the 38 loci tested, 18 reliably produced a single product in *Z. madagascariensis* under these conditions (Table 1). Many of the loci (10/18) successfully amplified in *Z. laticaudatus* and *Trachyloptychus*, although only one produced a likely homologous sequence in *Oplurus* (AL46). When direct sequencing revealed an

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd

individual to be heterozygous for a particular locus, PCR products were cloned using one-fifth recommended volume reactions (Invitrogen TOPO TA Cloning kit) and four colonies were sequenced. Levels of pairwise sequence divergence within *Z. madagascariensis* ranged from 0.9% (AL-31) to 5.0% (AL-37). The observed frequency of single nucleotide polymorphisms (SNPs) averaged 33 SNPs per kilobase, a number consistent with the unusually high number reported in a previous study of the iguanid lizard *Sceloporus* (38 SNPs/kb; Rosenblum *et al*. 2007).

Acknowledgements

We thank Dean Choi and Samantha Pearlman for their assistance in the laboratory. This project was funded in part by a grant from the National Science Foundation to A.D.Y. (DEB-0621279).

References

Crandall KA, Fetzner JW Jr, Lawler SH, Kinnersley M, Austin CM (1999) Phylogenetic relationships among the Australian and New Zealand genera of freshwater crayfishes (Decapoda: Parastacidae). *Australian Journal of Zoology*, **47**, 199–214.

Craul M, Zimmermann E, Rasoloharijaona S, Randrianambinina B, Radespiel U (2007) Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species. *BMC Evolutionary Biology*, **7**, 83.

Heckman KL, Hanley CL, Rasoloarison R, Yoder AD (2007) Multiple nuclear loci reveal patterns of incomplete lineage sorting and complex species history within western mouse lemurs (*Microcebus*). *Molecular Phylogenetics and Evolution*, **43**, 353–367.

Olivieri G, Zimmermann E, Randrianambinina B *et al*. (2007) The ever-increasing diversity in mouse lemurs: three new species in north and northwestern Madagascar. *Molecular Phylogenetics and Evolution*, **43**, 309–327.

Rosenblum EB, Belfiore NM, Moritz C (2007) Anonymous nuclear markers for the eastern fence lizard, *Sceloporus undulatus*. *Molecular Ecology Notes*, **7**, 113–116.

Rozas J, Rozas R (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey. http://www.genome.wi.mit.edu/genome_software/other/primer3.html.

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd