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TECHNICAL NOTE

Isolation and characterization of thirteen polymorphic tetranucleotide microsatellite markers in the Tokay gecko (*Gekko Gecko*)

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Abstract Thirteen tetranucleotide microsatellite loci were isolated from tetranucleotide microsatellite enriched libraries for the Tokay gecko (*gekko gecko*). The species is endemic to Southeast Asia, known to be a habitat specialist, and exposed to severe hunting pressures in the mountainous areas of Southeast Asia. These highly polymorphic markers (6–25 alleles) will facilitate the population genetic analyses of this species. In particular, they will enable estimates of gene flow between the two types of the Tokay gecko: the black-spotted Tokay gecko and the red-spotted Tokay gecko-a critical element in assessing classification between the two.

Keywords *Gekko gecko* · Microsatellite markers · Enriched library · Polymorphism

Introduction

Tokay geckos (*Gekko gecko*) are mainly distributed in the tropical mountain areas of southern China, Thailand, Vietnam, Malaysia, Indonesia, and other Southeast Asian countries. They have suffered severe habitat destruction and excessive hunting during the past few decades and as such they are becoming endangered (Li et al. 1996). Therefore, the Tokay is now listed as a second class key species in China (Yuan and Li 2008). Tokay geckos from Guangdong, Guangxi, southern Yunnan and northern Vietnam are called black-spotted tokays and are smaller, darker, and possess sundry spots while those in southeast Asia and much of southern Asia, are called red-spotted tokays and are larger, lighter in color and possess more eye-catching red spots (Liu et al. 2000). Although remarkable morphological and habitat differences exist in the two geckos, they are still considered one species taxonomically, *Gekko gecko* (Zhang et al. 1997).

Some microsatellite loci have been developed for investigating the population genetics of tokay geckos (Peng et al. 2010). These markers were mostly based on dinucleotide AC repeats. However, tetranucleotide repeat markers typically give fewer stutter bands in polymerase chain reaction (PCRs), and so are easier to be genotyped (Ellegren 2004). To distinctly quantify the genetic variation between the black-spotted and red-spotted tokays, tetranucleotide microsatellites enrichment libraries were constructed.

Twenty-five black-spotted tokays and 25 red-spotted tokays were collected from two different locations: Daxin county, China and Lang son county, Vietnam. Genomic libraries were constructed based on the protocol described by Zou et al. with minor modifications (Zou et al. 2005). DNA samples from tail tissue were isolated by the standard phenol–chloroform extraction procedure with Proteinase K (Sambrook and Russell 1989). Approximately 6 μg of genomic DNA was digested with the restriction enzyme *Mbo*I at 37°C overnight and fragments of 300–900 bp were selected on an agarose gel and recovered with a DEAE-cellulose membrane in a method described by Sambrook

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Table 1 Primer sequences and characteristics of thirteen microsatellite loci isolated in *Gekko Gecko*

Locus	Primer sequence (5′–3′) F	Repeat motif	Ta (°C)	Size range (bp)	N	The black-spotted tokay gecko				The red-spotted tokay gecko				GenBank accession no.
						A	H_E	H_O	PIC	A	H_E	H_O	PIC	
GGT01	F-CATTGTTGTTAGTCATAAGGGAGAG R-GGATCCATTATGGTCGATTCTT	$(GATA)_{12}$	63	174–214	25	8	0.79	0.756	0.772	8	0.817	0.763	0.79	HQ605711
GGT02	F-GTCTGAAAAGAAAGGAGGACAAT R-GAGGAAAAACCAGCAGCG	$(GATA)_{16}(GACA)_{21}$	64	263–347	25	23	0.917	0.934	0.922	25	0.927*	0.978	0.954	HQ605712
GGT03	F-GACAGAGTGCCTGAAGAACTATGG R-GCCTCCTTATCTCTCCTTGCTGT	$(AAAG)_{18}(AAGG)_{14}$	60	304–364	25	14	0.88	0.813	0.849	16	0.878	0.859	0.862	HQ605713
GGT04	F-GGTGGATGGATGGTATGGATG R-TGATGCCAAAGAGTCTGCTGTAT	$(GATA)_{15}GAT(GATA)_{11}$	64	205–281	25	20	0.917	0.9	0.903	23	0.938	0.911	0.927	HQ605715
GGT05	F-GAGGAGGAGAAGATTGATAGGTGAC R-CTTGGAAGTTGGACTCTATGGCA	$(GATA)_{18}(GATG)_4$	66	244–296	25	15	0.836	0.887	0.856	16	0.871	0.89	0.88	HQ605716
GGT06	F-TCCCAACACTCTGGAAGTAAGC R-GGCAATGGCAAAGACACCT	$(AAAG)_{19}$	58	287–327	25	11	0.799	0.851	0.833	13	0.824	0.869	0.837	HQ605717
GGT07	F-GATGTGTTAATAACCACATGGATGT R-CCTAAGATAAGTGAGTGCGTAAGAG	$(GA)_9AA(GATA)_{20}$	65	281–345	25	16	0.854	0.893	0.86	19	0.883*	0.906	0.899	HQ605718
GGT08	F-TCTACTGTAAAATAAGACATCGAGACA R-TTGTGACCTAACTCTACCTCATCAT	$(GATA)_{33}$	64	343–371	25	6	0.723*	0.788	0.763	8	0.744	0.798	0.763	HQ605719
GGT09	F-CAGGTATTTCCCAACCCAGAG R-TGCTATTGTTCGGTGCTTCA	$(AAAG)_{26}$	58	271–319	25	9	0.821	0.776	0.799	10	0.806	0.786	0.791	HQ605720
GGT10	F-TGGAGGTTCCAACCACAGAG R-CGCAGCCATACATTACTACAGG	$(TATC)_{12}(ATCT)_9$	64	230–294	25	13	0.866	0.793	0.834	15	0.877	0.836	0.859	HQ605721
GGT11	F-CAATCTTTTGGGTATGTTGTAGGTAT R-TGTGTGTATGTCTGATGTTTGTCTTTA	$(GATA)_{16}(GACA)_{12}$	63	311–351	25	9	0.768*	0.831	0.804	10	0.827	0.796	0.807	HQ605722
GGT12	F-AGCTGCTTTACGTATATCCACTG R-GGTGAAAGGTGGGAGATTGA	$(TATC)_{14}ATCC(ATCT)_{14}$	64	153–229	25	19	0.879	0.9	0.896	22	0.900*	0.916	0.909	HQ605723
GGT13	F-CTGCACCAGGAAGGAAGATTT R-CCATGGCAGATGTTTGAAGAA	$(ATCT)_{15}…(ATCC)_{15}$ …$(TACC)_8$	63	334–374	25	10	0.846	0.788	0.812	11	0.856	0.811	0.827	HQ605724
Mean					25	13.3	0.838	0.84	0.839	15.1	0.858	0.855	0.854	

Ta Annealing temperature of primer pair, *N* number of individuals genotyped, A number of alleles, *Ho* observed heterozygosity, H_E Expected heterozygosity, *PIC* polymorphism information content

* Statistically significant deviation from Hardy–Weinberg equilibrium

and Russell (2001). Adaptors (oligoA: 5′-pGGC CAG AGA CCC CAA GCT TCG-3′; oligoB: 5′-pGAT CCG AAG CTT GGG GTC TCT GGC C-3′) were ligated to size-selected DNA to serve as primer binding sites for amplification by PCR. The ligated DNA was hybridized to a biotin-labeled tetranucleotide repeats $(GATA)_6$ and $(AAAG)_6$ probe at 55 and 67°C for 3 h, respectively and captured on streptavidin-coated magnetic beads (M-280 Dynabeads, USA). To increase the quantity, enriched fragments released from the probe were amplified by PCR. The 400–900 bp PCR products were purified using the Gel Extraction Kit (Omega, USA) and cloned into pMD19-T vectors (TaKaRa, China). The ligation mixture was transformed into *Escherichia coli JM*109 competent cells which were then plated on LB ampicillin media supplemented with IPTG/X-Gal to construct microsatellite-enriched libraries.

To screen the positive clones containing tetranucleotide repeats, PCR amplifications were performed using oligoA, $(GATA)_6$ and $(AAAG)_6$ as primers. The annealing temperatures were 50 and 57°C, respectively. About 480 clones were screened, and 135 positive were obtained and sequenced with an ABI Prism 3700 sequencer (Perkin-Elmer). The primers amplifying the microsatellites were designed based on the flanking sequences of the repeat in each clone using the software PRIMER3 (Rozen and Skaletsky 2000). Of the 135 positive clones sequenced, 28 loci had suitable flanking sequences for primer design. Finally, 13 primer pairs were characterized using PCRs (Table 1). PCRs in a reaction volume of 25 μL contained approximately 20 ng of genomic DNA, 10buffer 2.5 μL, 10 pmol reverse primer, 10 pmol forward primer, 1.5 mM $MgCl_2$, 150 mM of each dNTPs, and 0.5 U of Taq DNA polymerase (TaKaRa, China) on a program of 94°C for 5 min, 35 cycles of 94°C for 30 s, at the primer-specific annealing temperature (Table 1) for 30 s, 72°C for 30 s, followed by a 5 min final extension at 72°C. Then 3–5 μL of PCR products were separated on an 8% urea–polyacrylamide gel along with pUC19 DNA/MspI (HapII) (Fermentas, Lithuania) and visualized with the silver staining protocol as described by Bassam et al. (1991). The allele size of each microsatellite was determined by the GeneTool software (BioTools Incorporated, Canada).

The average of number of alleles per locus (A), polymorphism information content (PIC), and observed and expected heterozygosities (H_O and H_E) in the black-spotted tokays were 13.3, 0.839, 0.840, and 0.838, respectively, and in the red-spotted tokays were 15.1, 0.854, 0.855, and 0.858, respectively. Two loci (GGT08 and GGT11) in 25 black-spotted tokays and three loci (GGT02, GGT07 and GGT12) in 25 red-spotted tokays showed significant deviations from Hardy–Weinberg equilibrium ($P < 0.05$). High levels of HWE deviation may be due to reasons such as a small population size, genetic drift, Wahlund effects (Hartl and Clark 1997), non-random sampling, and/or inbreeding. No significant linkage association was found among all loci. The average polymorphisms of the 13 microsatellite loci in the black-spotted tokay (A = 13.3, PIC = 0.839) and red-spotted tokay (A = 15.1, PIC = 0.854) were comparable with those (black-spotted tokay: A = 10.7, PIC = 0.760; red-spotted tokay: A = 16.4, PIC = 0.852) from the $(AC)_n$ loci isolated by Peng et al. (2010).

In conclusion, these 13 tetranucleotide microsatellite loci were highly polymorphic, suggesting that these polymorphic markers will be a valuable tool for studies on genetic structure of gecko populations.

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