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Genetic variability of the tokay gecko based on microsatellite analysis

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ABSTRACT

The black-spotted tokay gecko and red-spotted tokay gecko have different distribution areas and are significantly different in appearance but are classified into the same species *Gekko gecko*. Twelve microsatellite loci were isolated, characterized and evaluated from wild black-spotted tokay geckos for the first time. Of them, nine loci were successfully amplified in red-spotted tokay geckos using multiplex polymerase chain reactions (PCRs). A total of 208 different alleles were observed in the 70 wild black-spotted and red-spotted tokays, and the average number of alleles per locus was 17.3. The average values for observed heterozygosity, expected heterozygosity and polymorphism information content were 0.762, 0.891 and 0.871, respectively, which showed that the wild *G. gecko* population had a high level of genetic variability. Both black-spotted tokays and red-spotted tokays showed a significant ($P < 0.001$) deficit of heterozygotes. The red-spotted tokay ($H_E = 0.881$, $A = 16.4$) had a higher level of genetic variability than black-spotted tokay ($H_E = 0.804$, $A = 10.7$). The pairwise F_{ST} ($P < 0.001$) estimates of the two types of tokay were 0.143, which indicated that there was a significant level of genetic differentiation between the two.

1. Introduction

Tokay geckos (*Gekko gecko*) are mainly distributed in southern China, Thailand, Vietnam, Malaysia, Indonesia, and other southeast Asian countries. As a traditional Chinese drug, the tokay population has been declining dramatically due to habitat destruction and excessive hunting and as such they are becoming endangered (Li et al., 1996). Therefore, the tokay is listed as a second class key species in China and a first class key species in Guangxi Zhuang Autonomous Region, China.

Tokay geckos from Guangdong, Guangxi, southern Yunnan and northern Vietnam are called black-spotted tokays and are smaller, darker, and possess sundry spots compared against those in southeast Asia and much of southern Asia, which are called red-spotted tokays and are larger, lighter in color and possess more eye-catching red spots. In addition to their significantly different appearance and distribution areas, they have different pharmacodynamic actions as an ingredient in traditional Chinese medicine (Yuan and Li, 2008). However, they are considered one species taxonomically, *G. gecko*, and this has generated much controversy. Nowadays, more and more work has been done on the genetic differentiation of these two types of tokay geckos (Liu et al., 2000; Zhang et al., 2006; Qin et al., 2007).

Genetic studies on *G. gecko* using DNA markers are very rare. In order to have a better understanding of the genetic diversity and the degree of differentiation across and within black-spotted and red-spotted tokays, in the current study, 12 novel microsatellite loci in *G. gecko* were isolated from an $(AC)_n$ enrichment library for the first time and used to explore the genetic variation of *G. gecko*.

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2. Materials and methods

2.1. Sample collection and DNA isolation

A total of 70 wild tokays (*G. gecko*) were obtained from four different locations. Thirty-five black-spotted tokays were sampled from Daxin, Nanning and Chongzuo in Guangxi Zhuang Autonomous Region, China and 35 red-spotted tokays were collected from Lang Son, Vietnam (Fig. 1). Sampling localities are in the karst mountain areas at the edge of southeast YunNan–KweiChow Plateau. DNA samples from tail tissue were isolated by the standard phenol–chloroform extraction procedure with Proteinase K (Sambrook and Russell, 1989).

2.2. Construction of the $(AC)_n$-enriched library

The $(AC)_n$-enriched library was constructed with DNA from one black-spotted tokay based on the protocol described by Zou et al. (2005) with minor modifications.

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 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. PCR was carried out to test the success of the ligation of adaptors to the digested DNA with oligoA as primers. The ligated DNA was hybridized to a biotin-labeled dinucleotide repeats $(AC)_{12}$ probe at 50 °C for 3 h and captured on streptavidin-coated magnetic beads (M-280 Dynabeads, USA). To increase the quantity, the enriched fragments released from the probe were amplified with 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* JM109 competent cells which were then plated on LB-ampicillin media supplemented with IPTG/X-Gal to construct an $(AC)_n$-enriched library.

2.3. Screening the positive clones and sequencing

To screen the positive clones containing $(AC)_n$ repeats, PCR amplifications were performed using oligoA and $(AC)_{12}$ as primers. The annealing temperature was 57 °C. Analysis of the amplification products was achieved by electrophoresis on a 1.5% agarose gel and those clones that showed a double or multiple band (Fig. 2) were cultured overnight in LB medium containing ampicillin and sequenced with an ABI Prism 3700 sequencer (Perkin-Elmer).

2.4. Analysis of microsatellite polymorphism and genetic diversity in wild G. gecko

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). PCR was performed to confirm the optimized annealing temperature of each pair of primers. PCRs in a reaction volume of 25 μL contained approximately 20 ng of genomic DNA, 10×buffer 2.5 μL, 10 pmol reverse primer, 10 pmol forward primer, 1.5 mM $MgCl_2$, 150 μM of each dNTPs, and 0.5 U of Taq DNA polymerase (Tiangen, 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.

Fig. 1. Localities for *Gekko gecko* samples. Filled circles represent the localities of black-spotted tokay samples and the open circle represents the locality of red-spotted tokay samples.

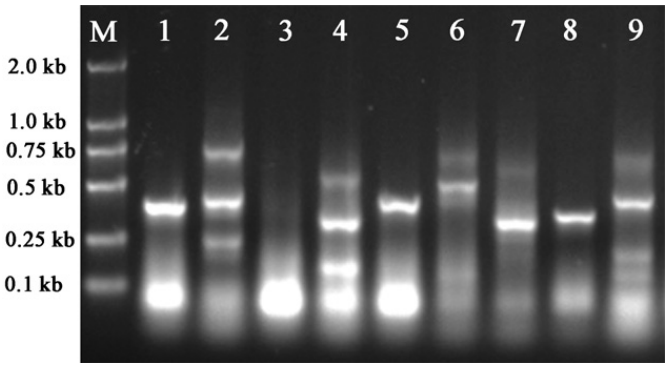

Fig. 2. PCR screening of the positive clones; M: DL2000 DNA marker; lanes 1–9: clones for PCR screening; lanes 2, 4, and 9: positive clones.

Polymorphism analysis was conducted using 35 black-spotted tokays collected from the Guangxi Zhuang Autonomous Region, China. The primers that were characterized successfully using PCR were labeled with fluorescence (Invitrogen, USA) and used to assess the degree of polymorphism. Three multiplex PCRs were optimized for genotyping all 12 markers. These multiplex PCRs could amplify 3–5markers per reaction (Zhu et al., 2009). All multiplex PCRs work under the PCR conditions described for single locus PCR and the procedure is the same as given above. PCR products were analyzed using an ABI PRISM 377 sequencer and GeneMapper version 3.2 (USA).

Cross-amplification was performed using template DNA from red-spotted tokay geckos because the microsatellite $(AC)_n$-enriched library of *G. gecko* was constructed using DNA from one black-spotted tokay. Also the fluorescent PCR products were detected using the same sequencer mentioned above.

To guarantee data efficiency, null allele frequencies of the data sets were estimated by Micro-Checker software (van Oosterhout et al., 2004). Allele frequency and private alleles were analyzed through the software CONVERT 1.31 (Glaubitz, 2004). General estimates of genetic polymorphism including the number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and the polymorphism information content (PIC) were calculated using CERVUS 3.0.3 (Marshall et al., 1998). The Wilcoxon signed-rank test was used to determine significance. For the same reason, a more powerful Markov chain test of Hardy–Weinberg equilibrium (HWE) was conducted with GenePop 3.4 (Raymond and Rousset, 1995) instead of in CERVUS. All probability tests were based on the Markov chain method using 1000 dememorization steps, 100 batches and 1000 iterations per batch. To examine the independence of the microsatellite loci, linkage disequilibrium between all pairs of loci was tested. Significant levels for multiple comparisons of loci across samples were adjusted using a standard Bonferroni correction (Rice, 1989). Wright's F-statistics (F_{ST}, F_{IS}, F_{IT}) were calculated using the program FSTAT, version 2.9.3.2 (Goudet, 2001).

Table 1
Primer sequences generated for microsatellite amplification for the study of *Gekko gecko*.

Locus	Primer (5′–3′) (F, forward; R, reverse)	Repeat motif	Labeled dye	Annealing temperature (°C)	Size range (bp)	GenBank Accession No
GGD01	F:TGTGGTGCCATCTTGTGGTGTT[a] R:CACATCAGGCTTCTGCCCAAT	$(GT)_{27}(AG)_{27}$	FAM	67	242–314	GQ246224
GGD02	F:CCTCAAAATCTGCCACCCTCA[a] R:GACTCCCCATGCCAATCACAA	$(GT)_{20}$	HEX	66	284–312	GQ246222
GGD03	F:GTGTAGCAGCAGAATATGTAGGG[a] R:CCACAAACCACCATGAGACTA	$(AC)_{24}$	TAMAR	57	198–248	GQ245672
GGD04	F:ACACCTGCTTGCCTTATTCTGG[a] R:GAGTTACGCTCTTCAAAGCCCAT	$(TC)17(AC)_{10}$ $(ACGC)3(AC)_7$	TAMAR	67	202–262	GQ246220
GGD05	F:GAGAGTTGGCTTGGCTTTAAG[a] R:TGTCCCTCACTTGCCTGC	$(AC)_{23}$	FAM	58	118–136	GQ245671
GGD06	F:ATCTGGGAGGACCGAAATGTG[a] R:TGAGAGTTCAGTCAGCAGATGGAG	$(TG)_{17}(AG)_{23}$	FAM	66	256–326	GQ246225
GGD07	F:ACAGCAGGGTCCAAAGTCAATG[a] R:CACTTGGTGGGCGTTCATGTT	$(TG)_{22}(AG)_{29}$	HEX	66	224–274	GQ246223
GGD08	F:GGCCTATGGAATCTAAGAGCACTG[a] R:ATCCCATCCTCATCGCCAAT	$(GT)_{21}$	TAMAR	67	211–237	GQ246221
GGD09	F:TCCTTCCTTCCCTGGTTCTAA[a] R:TGTGCCGTGTTGAGCGA	$(AC)_{27}$	TET	58	150–190	GQ245673
GGD10	F:GCAGAATCAAGGGACTCAGAG[a] R:TAGAGAAGAGCCTGGCATATTAG	$(AC)_{18}$	TAMAR	59	206–226	GQ245674
GGD11	F:GAAGCTCATTCCACCAGTGTG[a] R:CCAGCCATTGACTTTCCTAACT	$(AC)_{28}$	TET	64	182–208	GQ245675
GGD12	F:CATTTTGACTGCTGCCCTTATTC[a] R:CTTTCTATACCTCTTGGGAGTTGTGA	$(TC)_{24}(AC)_{21}$	FAM	62	142–170	GQ245676

[a] Labeled primers.

3. Results

3.1. Screening of $(AC)_n$-containing clones

About 192 clones were screened and 87 positive clones with two or more amplification bands were obtained. Forty-five clones were sequenced and had $(AC)_n$ motifs. These 45 insertion sequences were all different from each other. Of the 45 microsatellite loci, 10 loci were excluded from further analyses because they had no suitable flanking sequences for primer design. Finally, 12 primer pairs were characterized using PCRs (Table 1).

3.2. Analysis of microsatellite polymorphism and genetic diversity in G. gecko

All 12 microsatellite loci were found to be highly polymorphic and showed considerable variation in the black-spotted tokays. Micro-Checker software analysis showed that no loci in the black-spotted tokays had null alleles but two loci (GGD05, GGD06) in the red-spotted tokay did. However, there was no evidence for large allelic dropout in the data set of these two loci. Based on these results, we know the data were sufficient for further analysis. The number of alleles ranged from 5 to 18, with an average of 10.6. H_O (observed heterozygosity) and H_E (expected heterozygosity) varied from 0.621 to 0.833 and 0.626 to 0.909, with an average of 0.755 and 0.804, respectively. Two loci (GGD07, GGD08) showed significant deviation from Hardy–Weinberg Equilibrium ($P < 0.01$) using the exact test for conformance to HWE frequencies. No significant linkage association was found among all loci. All 12 loci were successfully amplified in the red-spotted tokay except for three loci (GGD02, GGD08, GGD11). The number of individuals successfully genotyped (N), the number of alleles per locus (A), the polymorphism information content (PIC), expected heterozygosity (H_E), observed heterozygosity (H_O), and F_{IS}, probability of deviation from Hardy–Weinberg equilibrium per locus are given in Table 2.

A total of 208 different alleles were observed in the 70 individuals analyzed. Both black-spotted and red-spotted tokays were found to contain private alleles (18 in the black-spotted tokay and 26 in the red-spotted tokay). One hundred sixty-four alleles were shared between them. The mean expected and observed heterozygosities and PIC per locus across the two types of tokay was 0.891, 0.762 and 0.871, respectively. Both positive and negative inbreeding coefficient (F_{IS}) values were found in all loci and both types of gecko. Furthermore, we found that the mean values of F_{IS} in the two were 0.062 and 0.126. F-statistics (F_{IS}, F_{IT}, F_{ST}) for each of the nine markers across black-spotted and red-spotted tokays are presented in Table 3.

4. Discussion

In this study, all 12 microsatellite loci showed considerable variation in the two types of *G. gecko* examined. The polymorphism (100%) in all 12 microsatellite loci found in *G. gecko* was similar to the results of other gekkonid species such as

Table 2
Genetic variability at 12 microsatellite loci in *Gekko gecko*.

Gekko gecko	Locus												
	GGD01	GGD02	GGD03	GGD04	GGD05	GGD06	GGD07	GGD08	GGD09	GGD10	GGD11	GGD12	Mean
Black-spotted tokay													
N	35	35	35	35	35	34	34	34	35	35	34	35	
A	18	15	8	9	7	14	12	11	8	5	13	8	10.7
H_O	0.833	0.833	0.800	0.667	0.767	0.759	0.759	0.621	0.767	0.633	0.828	0.800	0.755
H_E	0.854	0.884	0.809	0.784	0.809	0.909	0.809	0.756	0.739	0.626	0.867	0.811	0.804
PIC	0.826	0.858	0.773	0.740	0.765	0.884	0.775	0.707	0.691	0.539	0.836	0.767	0.760
F_{IS}	0.025	0.058	0.011	0.151	0.053	0.168	0.064	0.181	−0.038	−0.012	0.046	0.013	0.062
P	0.045	0.469	0.772	0.020	0.121	0.000	0.118	0.006	0.993	0.496	0.211	0.076	
Red-spotted tokay													
N	35		35	34	33	33	33		34	34		34	
A	21	–	16	22	13	25	21	–	6	10	–	14	16.4
H_O	0.933	–	0.733	0.931	0.567	0.679	1.000	–	0.586	0.828	–	0.690	0.771
H_E	0.953	–	0.840	0.943	0.859	0.954	0.952	–	0.702	0.857	–	0.869	0.881
PIC	0.933	–	0.814	0.922	0.832	0.933	0.931	–	0.638	0.824	–	0.840	0.852
F_{IS}	0.021	–	0.128	0.012	0.344	0.292	−0.051	–	0.168	0.035	–	0.209	0.126
P	0.376	–	0.056	0.510	0.012	0.000	0.204	–	0.070	0.284	–	0.110	
Total													
A	24	15	18	21	15	29	21	11	12	13	13	16	17.3
H_O	0.883	0.833	0.767	0.797	0.667	0.719	0.877	0.621	0.678	0.729	0.828	0.746	0.762
H_E	0.928	0.884	0.903	0.931	0.906	0.965	0.930	0.756	0.862	0.869	0.867	0.895	0.891
PIC	0.916	0.858	0.887	0.918	0.890	0.955	0.917	0.707	0.839	0.848	0.836	0.877	0.871

Number of individuals successfully genotyped (N), Number of alleles per locus (A), observed heterozygosity (H_O), expected heterozygosity (H_E), polymorphism information content (PIC), inbreeding coefficient (F_{IS}), and probability of significant deviation from Hardy–Weinberg equilibrium (P) are given for each type of tokay and each loci.

Table 3
F-statistics for each of the nine markers across black-spotted tokays and red-spotted tokays.

Locus	F_{IT}	F_{ST}	F_{IS}	Relatc
GGD01	0.037	0.048	−0.012	0.023
GGD03	0.151	0.145	0.007	−0.014
GGD04	0.115	0.124	−0.007	0.014
GGD05	0.214	0.139	0.086	−0.189
GGD06	0.157	0.006	0.098	−0.216
GGD07	0.016	0.101	−0.095	0.173
GGD09	0.322	0.278	0.061	−0.129
GGD10	0.082	0.262	−0.244	0.392
GGD12	0.110	0.117	−0.007	0.014
Mean[a]	0.135	0.143	−0.009	0.014
S.E.[a]	±0.031	±0.027	±0.033	

Relatc estimates the inbreeding corrected relatedness (Pamilo, 1985).
[a] Mean and standard errors (S.E.) – estimate from jackknife over loci and significance from *t*-test using these estimates.

Gekko swinhonis (Li and Zhou, 2007), and higher than the polymorphism (84.4%) among five *G. gecko* populations detected by RAPD technology (Qin et al., 2005). The black-spotted tokay and red-spotted tokay had two (GGD06, GGD08) and one (GGD06) microsatellite loci departing from HWE, respectively. Three loci in the red-spotted tokay (GGD02, GGD08 and GGD11) had not been tested with HWE because their PCR products showed multiple bands and was undetected by the DNA sequencer. 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. However, in the present study, we inferred that the deviation from HWE was related to Wahlund effects (presence of population substructure).

Genetic diversity ($H_E = 0.891$, $A = 17.3$) observed in wild *G. gecko* was higher than that observed in the mourning gecko ($H_E = 0.654$, $A = 5.1$; Wilmhoff et al., 2003), *Oedura reticulata* ($H_E = 0.712$, $A = 13.7$; Hoehn and Sarre, 2005), the tropical house gecko ($H_E = 0.568$, $A = 5.0$; Short and Petren, 2008), and *G. swinhonis* ($H_E = 0.790$, $A = 15.4$; Li and Zhou, 2007) and comparable with that in the Australian geckos ($H_E = 0.874$, $A = 17.5$; Hoehn and Sarre, 2006). There was no significant difference for the number of alleles (A), expected heterozygosity (H_E), observed heterozygosity (H_O), and polymorphism information content (PIC) between the two types of tokay. However, the red-spotted tokay showed higher levels for all parameters ($A = 16.4$, $H_O = 0.771$, $H_E = 0.881$, PIC = 0.852) than the black-spotted tokay ($A = 10.7$, $H_O = 0.755$, $H_E = 0.804$, PIC = 0.760). The current findings confirmed previous results (Qin et al., 2005) that red-spotted tokay had a higher level of genetic variation than the black-spotted tokay. Inbreeding coefficient (F_{IS}) values ranged from −0.012 to 0.181 in the black-spotted tokay, and varied from −0.051 to 0.344 in the red-spotted tokay, suggesting a heterozygote deficiency in some loci and a heterozygote excess in others. The significant heterozygote deficiency found in the tokay gecko population could be due to one or more of the following reasons: segregation of non-amplifying (null) alleles, Wahlund effects (presence of population substructure), scoring biases (heterozygotes scored incorrectly as homozygotes), or inbreeding. However, the presence of null alleles was excluded, and scoring bias may be possible for a few but not for all loci. All tokay gecko samples were collected from the wild randomly. Similarly, the possibility of inbreeding was excluded. Since the level of the global deficit of heterozygotes ($F_{IT} = 0.135$) was significantly different with F_{IS} (−0.009), this indicates that Wahlund effects may be the main reason for it.

F_{ST} values ranged from 0.006 (GGD06) to 0.278 (GGD09) in the 12 microsatellite loci and the overall F_{ST} was 0.143 ($P < 0.01$). In general, F_{ST} values below 0.05 are expected with current gene flow, values between 0.05 and 0.1 indicate that populations are semi-isolated, and values above 0.1 suggest that populations are isolated from each other (Wilson et al., 2003). Thus, in this study the two types of tokay gecko seem to be isolated. The results were comparable with those reported by Qin et al.'s (2005) RAPD analysis and comparative study of allozyme polymorphism (Qin et al., 2006) and mitochondrial Cyt *b* (Qin et al., 2007) but the genetic distance between black-spotted and red-spotted tokays estimated by pairwise F_{ST} (0.143) in the present study was significantly higher than those reported previously.

In conclusion, 12 identifiable, highly polymorphic microsatellite markers in the genome of *G. gecko* have been developed. High genetic diversity in terms of allelic diversity and mean heterozygosity at microsatellite loci was found in *G. gecko*. The evidence that there was significant genetic differentiation between black-spotted tokays and red-spotted tokays indicated there was a tendency to differentiate into two subspecies. However, more knowledge of the genetic structure and molecular characterization of *G. gecko* are required to confirm this speculation.

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