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Development of 22 Microsatellite Markers for Assessing Hybridization in the Genus *Gekko* (Squamata: Gekkonidae)

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Abstract: *Gekko yakuensis* and *G. tawaensis*, both endemic to western Japan, are threatened by genetic introgression from *G. hokouensis* and *G. japonicus*, respectively. To know detailed situation of their hybridizations for planning relevant conservation measures, development of sensitive genetic markers is desired. We here developed microsatellite markers based on the sequences obtained from *G. hokouensis* using 454 GS Junior sequencer, and tested stability of PCR amplification and species-specificity of alleles at each locus using *G. hokouensis*, *G. yakuensis*, *G. japonicus*, and *G. tawaensis*. The results showed that 22 loci were almost constantly amplified in more than one species. We further confirmed that there were fixed or nearly fixed allelic displacement between *G. hokouensis* and *G. yakuensis*, and between *G. japonicus* and *G. tawaensis* at 14 loci. Thus, these 22 loci are considered to be useful for evaluation of hybridizations between these pairs of species.

Key words: *Gekko hokouensis*; Hybridization; Microsatellite; Diagnostic markers; Population genetics

Introduction

Eight species of the genus *Gekko*, including two putative undescribed species, are known from Japan (Tokunaga, 1988; Ota, 1989;

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Toda et al., 2001a, 2008). *Gekko yakuensis* and *G. tawaensis* are endemic to the southern part of Kyushu and the Osumi Island Group, and the coastal areas facing to the Seto Inland Sea and the remaining coastal area of Shikoku, respectively. By use of allozyme method, Toda et al. (2001b, 2006) detected natural hybridizations between *G. hokouensis* and *G. yakuensis*, and between *G. japonicus* and *G. tawaensis*, each in several areas where the two species involved occur sympatrically. Habitat alterations, such as coastal revetment, have been facilitating the spread of *G. hokouensis* and *G. japonicus*, both prefer open habitat, and might have induced introgressive hybridizations with *G. yakuensis* and *G. tawaensis*, respectively. For this reason, *G. yakuensis* and *G. tawaensis* are listed in the Red List of Japan as Vulnerable and Near Threatened, respectively (Ministry of the Environment, Japan, 2014). For effective conservation managements of the two species, it is necessary to know detailed situation of their hybridizations with those recently spreading species. Although the effectiveness of allozyme genotyping for detection of hybridization was elucidated in the previous studies, the method also has undesirable features in view of conservation purposes, for instance, necessities in collecting relatively large and fresh tissue samples by much damaging or even killing animals and in keeping tissues frozen with high cost. Therefore, it is desired to develop genetic markers that can detect hybrid genotypes less invasively with less cost.

Microsatellite is a popular molecular marker in population genetics and is disseminated by recent technical advances in their development and genotyping (Guichoux et al., 2011). Microsatellite markers in *Gekko* have ever been reported for *G. swinhonis* (Li and Zhou, 2007) and *G. japonicus* (Wei et al., 2015). We tested several of the markers for samples of *G. hokouensis* and *G. yakuensis*, but most of them were not amplified nor variable between these two species. Hence, we have developed another series of microsatellite markers that is suitable in assessing states of hybridizations for each of the two species pairs.

Materials and Methods

Total genomic DNA was extracted from liver tissue of *G. hokouensis* from Yonaguni-jima Island (Zoological Collection of the Kyoto University Museum: KUZ R71161) using standard phenol–chloroform method, and was used for isolation of microsatellite loci. Microsatellite enrichment was conducted using 3' biotin-labeled oligonucleotide probes–magnetic bead complex following Glenn and Schable (2005) and Kurita et al. (2013). After recovering DNA concentration by PCR, the PCR products with length of 300–800 bp were obtained from the gel and purified by QIAquick Gel Extraction Kit (Qiagen). The amplicon was prepared with the GS Rapid Library Preparation Kit (Roche), and pyrosequencing was performed with the 454 GS Junior sequencer (Roche) to determine a series of sequences with microsatellite motifs. Procedures for sequencing with 454 GS Junior and screening of candidates of microsatellite loci were same as Kurita et al. (2014).

To develop microsatellite markers that are applicable to many of the *Gekko* species, we checked presence/absence of homologous microsatellite loci in *G. japonicus* draft genome sequences provided by Liu et al. (2015). Although both *G. hokouensis* and *G. japonicus* are assigned to the *japonicus* species group by Rösler et al. (2011), they are phylogenetically distant from each other in this species group, and most of the *Gekko* species distributed in Japan are located between the two species on phylogenetic tree (Toda, 2000). Therefore, microsatellite loci conserved in both *G. hokouensis* and *G. japonicus* are expected to be applicable to other *Gekko* species in Japan. We downloaded the draft genomic sequences of *G. japonicus* (GenBank accession no. LNDG01000000), and conducted homology search of the sequences obtained from *G.*

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hokouensis against the *G. japonicus* genome using the NCBI BLAST+ program (Camacho et al., 2009). According to the result, we selected 47 loci that had homologs in the *G. japonicus* genome and satisfied other a few conditions (e.g., suitable repeat number of motif, composition by a single motif, enough length of flanking regions, etc.), and designed primers using Primer3Plus (Untergasser et al., 2012). We further tested the primers' specificity with the target region of the *G. japonicus* genome by using the default setting of Primer-BLAST (Ye et al., 2012). In this setting, primers with six or more mismatch bases with the target region and/or those with high sequence similarity (less than two base mismatches) with non-target region/species were judged as "less-specific primers".

The 47 potential loci were assigned to several sets of multiplex PCR system according to expected sizes of PCR products and the multiplex PCR was conducted for several representative samples using Type-it Microsatellite PCR Kit (Qiagen) and fluorescent-tagged universal primers (Blacket et al., 2012). PCR was carried out in 5 µl reactions containing 10–100 ng of extracted DNA, 2.5 µl of Type-it Multiplex PCR Master Mix, 0.01 µM of each tailed forward primer, 0.2 µM of each reverse primer, and 0.01 µM of each fluorescent-tagged universal primer (Tail A, Tail B, Tail C, and Tail D labelled with 6-FAM, VIC, NED, and PET, respectively). The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 30 s; final extension at 60°C for 30 min. The PCR products were subjected to fragment analysis by ABI3130xl Genetic Analyzer (Applied Biosystems) and sizes of alleles at each locus were determined by Geneious software (version 10.1.3; Biomatters Ltd.) with GeneScan 600LIZ size standard (Applied Biosystems).

Applicability and allele composition at each locus were evaluated using wild samples of *G. hokouensis* (n=20; Kawanabe, Kagoshima Pref.), *G. yakuensis* (n=20; Tanegashima Island, Kagoshima Pref.), *G. japonicus* (n=10; Usa, Saeki, and Oita, Oita Pref.), and *G. tawaensis* (n=10; Saeki, Oita Pref.), which were considered to be pure populations without any hybridizations. The samples of *G. hokouensis* and *G. yakuensis* were collected from mutually allopatric populations. Those of *G. japonicus* and *G. tawaensis* were collected from partially sympatric regions, but all of the geckos were considered to be pure individuals on the basis of morphological characters. Based on the genotypes of the above samples, we compared allele frequencies among the four species. We also calculated number of alleles per locus (N_A), observed and expected heterozygosities (H_O and H_E, respectively) with GenAlEx 6.5 (Peakall and Smouse, 2012) for each sample. Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using GENEPOP on the web (version 4.2; Raymond and Rousset, 1995; Rousset, 2008). The significance level of the deviation from HWE and LD were adjusted using the sequential Bonferroni procedure (Rice, 1989). Presence of null alleles was tested using Micro-Checker (version 2.2.3; van Oosterhout et al., 2004).

Results and Discussion

We obtained 3,740 reads from the enriched library of *G. hokouensis* by a 454 GS Junior run. We detected 161 sequences containing single microsatellite motif and having 50 bp or more flanking regions on both sides of the microsatellite region, but many of these were still unsuitable for designing primers because of inappropriate sequences in the flanking regions. Among the designed 47 pairs of primers whose loci were expected to have homologs in the *G. japonicus* genome, the sequences of 22 primer pairs were judged as "highly specific" to the target regions of the genomic sequences of *G. japonicus*. As a result of the amplification trials using the 47 primer pairs, another set of 22 loci (two tetra-, three tri-, and 17 di-nucleotide loci)

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were successfully amplified in more than one of the four species examined (Tables 1 and 2). Fifteen out of the 22 amplified loci were the ones whose primer sequences showed high specificity to the target region of the *G. japonicus* genome. BLAST searches of flanking regions (30 bps) of the 22 isolated loci against DDBJ database confirmed that none of these loci was the same as previously developed microsatellite loci, although only 5' flanking region of Gh147 showed certain similarity with those of Gs217 of *G. swinhonis* (Li and Zhou, 2007) and Hm122 of *Hemidactylus mabouia* (Short and Petren, 2008). We allocated these 22 loci to three multiplex reactions for efficient genotyping (Table 1).

In *G. hokouensis* and *G. yakuensis*, 20 and 16 (out of the 22) loci were mostly well amplified respectively. In *G. hokouensis*, N_A, H_O, and H_E ranged from 1–8, 0–0.78, and 0–0.80, respectively (Table 2). Significant deviations from HWE were detected in Gh35, Gh71, and Gh147 loci ($P<0.05$). In *G. yakuensis*, N_A, H_O, and H_E ranged from 1–6, 0–0.53, and 0–0.72, respectively (Table 2), and significant deviation from HWE was not detected in any locus. No significant LD was detected in any pair of loci in both species. Null alleles were suggested at Gh35, Gh71, and Gh147 in *G. hokouensis* and at Gh147 in *G. yakuensis*. Among the 16 loci that were commonly amplified in both species, allelic displacements were observed between the two species at four loci (Gh27, Gh91, Gh105, Gh143), and remarkable allele frequency differences were observed at other 10 loci (Gh35, Gh64, Gh71, Gh76, Gh82, Gh84, Gh95, Gh144, Gh147, Gh150) (Table 3).

In each of *G. japonicus* and *G. tawaensis*, 17 loci were mostly well amplified, and 16 loci were amplified in common between the two species, though a few individuals of *G. japonicus* could not be genotyped in several loci. In this species, N_A, H_O, and H_E ranged from 1–4, 0–0.50, and 0–0.59, respectively (Table 2). A significant deviation from HWE was detected in Gh144 locus ($P<0.05$). In *G. tawaensis*, N_A, H_O, and H_E ranged from 1–2, 0–0.20, and 0–0.18, respectively (Table 2), and no significant deviation from HWE was detected in any of the 17 loci. No significant LD was detected in any pair of loci in both species. Presence of null alleles was suggested at Gh144 in *G. japonicus*. Among the 16 commonly amplified loci, allelic displacements were observed at 12 loci (Gh27, Gh35, Gh42, Gh50, Gh53, Gh64, Gh70, Gh78, Gh95, Gh143, Gh146, Gh147), and the predominant alleles were different between the two species at other two loci (Gh28, Gh144) (Table 3).

The microsatellite markers developed here are not highly variable in *G. yakuensis* and *G. tawaensis* (Table 2), being consistent with the results of previous allozyme studies (average heterozygosity of *G. hokouensis*, *G. yakuensis*, *G. japonicus*, and *G. tawaensis* were 0.054, 0.017, 0.110, and 0.005, respectively) (Toda et al., 2001b, 2003). Nonetheless, these markers will be still useful for studies of hybridizations because the species-specificity of alleles is sufficiently high (Table 3).

In this study, 11 out of the 22 loci were mostly well amplified in the four species examined. Because all of the 11 loci had primers that were judged as highly specific to the target region of the *G. japonicus* genome, it is considered that the homolog search and specificity evaluation for the candidate primer sequences against the *G. japonicus* genome were effective to develop the loci that have high universality within the genus. Cross-amplifiable microsatellite markers were successfully developed for a variety of taxa by comparison of genome-derived sequences with multiple closely- or distantly-related species (e.g., Gotoh et al., 2013; Wang et al., 2015). Whole genome information becomes available for many non-model species in recent years, and thus it is recommended to add these procedures in developing effective genetic markers when the relevant information is available.

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Table 1. Twenty-two microsatellite markers developed in *Gekko hokouensis*.

Locus	Repeat motif	Primer sequences (5'–3')	Multiplex	Dye	Accession No.
Gh27	(GT)12	F: Tail A-CTGGGAGAGAATCACTGCAAC	III	6-FAM	LC490625
		R: GAGGGCATCCATGTGTACCT			
Gh28	(GT)10	F: Tail C-TGATAAGGTGACTGCCATCG	III	NED	LC490626
		R: GGAGCTAATCTGCACAAGCA			
Gh34	(AC)6	F: Tail B-TACCCTAAGTCCACCGCAAC	II	VIC	LC490627
		R: TCTCCAAAATTAAGCAGATATTCAGA			
Gh35	(GT)11	F: Tail D-TTTCAGCTGCCAATGTCTTG	III	PET	LC490628
		R: GATTAAGCATTTCGCCTCCA			
Gh42	(AC)7	F: Tail D-TGGGGTGTATGAAGCTGTGA	I	PET	LC490629
		R: AGCCCAACCTGAAGAAAACT			
Gh50	(AC)6	F: Tail C-GTCCACTTGGCACAAGTCCT	II	NED	LC490630
		R: TGGGGTCTTGCTAACAGTGA			
Gh53	(AC)10	F: Tail A-GAAATGCCCAAGCTCCAATA	III	6-FAM	LC490631
		R: AATTACTGAGAGCCGCATCA			
Gh64	(AC)9	F: Tail D-ACACAGCACAACGAGGAATG	II	PET	LC490632
		R: ATGTGGCTGCAATGATGTGT			
Gh70	(AC)9	F: Tail C-TGCTTGATTTTGGGTTGAGA	I	NED	LC490633
		R: GGGACTTTGCGGAGACAGTA			
Gh71	(AC)16	F: Tail C-GGCACTGCTTCTTTGGGTAA	III	NED	LC490634
		R: GAGAAACGGACTCGAACCAG			
Gh76	(AC)11	F: Tail D-TAGAGGAGCACCGGAAATGT	III	PET	LC490635
		R: TTCAGGGACCTGCTTGAGAT			
Gh78	(GT)7	F: Tail A-AGGATGGGCGACATGATAAC	II	6-FAM	LC490636
		R: TGTACAACTCAAGTGCGAAGC			
Gh82	(GT)7	F: Tail A-CTCCGGGAGCTCACTTTATG	III	6-FAM	LC490637
		R: TGCTTTTTACACAGCACTCTCTG			
Gh84	(AC)10	F: Tail A-CACCACTACTCAGCCCAGAA	I	6-FAM	LC490638
		R: GGGAATCACAGCCTCTTAGG			
Gh91	(GT)8	F: Tail D-GCATCCTTTGGGTTACGTGT	I	PET	LC490639
		R: CGTGTGCTGCTTACACCAAT			
Gh95	(AC)11	F: Tail C-ACAGAGGCTGCCACTTATCC	II	NED	LC490640
		R: TGCGATTGTGCTATCCCATA			
Gh105	(AC)14	F: Tail C-TGCTTAATCTACGTAGGGCAGA	II	NED	LC490641
		R: CCCACCCAGAGCCTCTTCTA			
Gh143	(GTTT)12	F: Tail B-TGTGACCAGTTCTGCCACTG	I	VIC	LC490642
		R: AAGAGCACCGCACTTTGTTT			
Gh144	(ACGC)6	F: Tail A-GCAGGTCCCAAGTCAATCAT	I	FAM	LC490643
		R: GGGGCAAGTTTCTGTGTTGT			
Gh146	(GTT)7	F: Tail C-GGAGCACATTGAAGGTACGG	III	NED	LC490644
		R: GTCCCACACAGCTCCAAAGT			
Gh147	(CCT)7	F: Tail A-CCCGACACTTGTGCCATTAC	II	6-FAM	LC490645
		R: CTGTGGCAAGGTGCTAGAAGA			
Gh150	(ATC)8	F: Tail A-TCCTGGCCACAATCAAGGAC	I	6-FAM	LC490646
		R: AAACCTTTAGCAGTTTTTCTAAGTCT			

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TABLE 2. Characteristics of 22 microsatellite loci in four *Gekko* species.

Locus	*G. hokouensis* (N=20)				*G. yakuensis* (N=20)				*G. japonicus* (N=10)				*G. tawaensis* (N=10)				N_A (all)
	N_{Amp}	N_A	H_O	H_E	N_{Amp}	N_A	H_O	H_E	N_{Amp}	N_A	H_O	H_E	N_{Amp}	N_A	H_O	H_E	
Gh27	19	4	0.21	0.20	20	1	0	0	8	1	0	0	10	1	0	0	7
Gh28	20	1	0	0	—	—	—	—	10	2	0.30	0.26	10	1	0	0	3
Gh34	20	2	0.35	0.40	—	—	—	—	10	1	0	0	—	—	—	—	3
Gh35	19	7	0.26*	0.78	20	1	0	0	10	4	0.40	0.35	10	1	0	0	9
Gh42	—	—	—	—	—	—	—	—	9	2	0.11	0.28	10	1	0	0	3
Gh50	20	1	0	0	16	1	0	0	10	1	0	0	10	1	0	0	3
Gh53	20	3	0.25	0.23	—	—	—	—	6	1	0	0	10	1	0	0	5
Gh64	20	3	0.15	0.22	20	1	0	0	10	1	0	0	10	1	0	0	4
Gh70	20	1	0	0	—	—	—	—	10	1	0	0	10	1	0	0	3
Gh71	18	5	0.22*	0.66	19	2	0.05	0.15	6	1	0	0	10	1	0	0	6
Gh76	20	8	0.75	0.72	20	2	0.25	0.29	—	—	—	—	10	2	0.20	0.18	11
Gh78	—	—	—	—	—	—	—	—	10	1	0	0	10	1	0	0	2
Gh82	18	6	0.78	0.71	20	1	0	0	—	—	—	—	—	—	—	—	6
Gh84	20	7	0.75	0.80	20	1	0	0	10	1	0	0	10	1	0	0	8
Gh91	14	1	0	0	20	1	0	0	—	—	—	—	—	—	—	—	2
Gh95	20	2	0.10	0.10	20	1	0	0	10	1	0	0	10	1	0	0	4
Gh105	20	1	0	0	18	1	0	0	—	—	—	—	—	—	—	—	2
Gh143	20	1	0	0	20	3	0.15	0.14	10	1	0	0	10	1	0	0	6
Gh144	20	2	0.05	0.05	14	1	0	0	8	3	0*	0.59	10	1	0	0	5
Gh146	20	2	0.35	0.29	20	1	0	0	10	2	0.50	0.50	10	1	0	0	4
Gh147	11	6	0*	0.78	20	2	0.20	0.50	10	2	0.40	0.32	10	1	0	0	8
Gh150	20	2	0.05	0.05	19	6	0.53	0.72	—	—	—	—	—	—	—	—	7
Average		3.25	0.21	0.30		1.63	0.07	0.11		1.53	0.10	0.13		1.06	0.01	0.01	5.05

N: number of individuals assayed, N_{Amp}: number of individuals successfully genotyped, N_A: number of alleles per locus, H_O and H_E: observed and expected heterozygosity: *Significant deviation from HWE after the sequential Bonferroni correction.

TABLE 3. Allele frequencies in four *Gekko* species at 22 loci. Values for diagnostic alleles (i.e., alleles found only in one of the four species) are given in bold. The predominant alleles are indicated with asterisks.

Locus	Allele	*G. hokouensis*	*G. yakuensis*	*G. japonicus*	*G. tawaensis*	Locus	Allele	*G. hokouensis*	*G. yakuensis*	*G. japonicus*	*G. tawaensis*
Gh27	198				**1.000***	Gh78	275	—	—		**1.000***
	204	**0.026**					277	—	—	**1.000***	
	206	**0.053**				Gh82	273	0.083	1.000*	—	—
	207		**1.000***				277	**0.028**		—	—
	209	**0.895***					279	**0.194**		—	—
	211	**0.026**					281	**0.028**		—	—
	214			**1.000***			287	**0.444***		—	—
Gh28	164	**1.000***	—				291	**0.222**		—	—
	166		—	**0.850***		Gh84	241			1.000*	1.000*
	168		—	0.150	1.000*		243	0.125	1.000*		
Gh34	129		—	**1.000***	—		253	**0.075**			
	136	**0.725***	—		—		258	**0.100**			
	140	**0.275**	—		—		262	**0.100**			
Gh35	188				**1.000***		271	**0.325***			
	189	**0.211**					278	**0.050**			
	191	0.289*		0.100			289	**0.225**			
	193	0.263	1.000*			Gh91	183	**1.000***		—	—
	199	0.079		0.050			187		**1.000***	—	—
	201	0.105		0.050		Gh95	152			**1.000***	
	203	**0.026**					154	**0.950***			
	205			**0.800***			156	0.050	1.000*		
	209	**0.026**					162				**1.000***
Gh42	324	—	—		**1.000***	Gh105	225	**1.000***		—	—
	327	—	—	**0.167**			243		**1.000***	—	—
	332	—	—	**0.833***		Gh143	193				**1.000***
Gh50	313	1.000*	1.000*				196		**0.025**		
	318			**1.000***			197			**1.000***	
	323				**1.000***		200		**0.050**		
Gh53	125	**0.050**	—				202	**1.000***			
	128		—		**1.000***		204		**0.925***		
	133	**0.875***	—			Gh144	175	0.025	1.000*		
	149	**0.075**	—				179	**0.975***			
	158		—	**1.000***			184			**0.500***	
Gh64	328				**1.000***		188			0.125	1.000*
	341	0.875*		1.000*			192			**0.375**	
	343	**0.025**				Gh146	220				**1.000***
	346	0.100	1.000*				222	**0.175**			
Gh70	143		—	**1.000***			229			**0.550***	
	151		—		**1.000***		244	0.825*	1.000*	0.450	
	153	**1.000***	—			Gh147	468			**0.800***	
Gh71	286			1.000*	1.000*		477	0.091		0.200	
	304	**0.083**					480	**0.364***			
	310	0.111	0.921*				483	**0.091**			
	312	0.194	0.079				484				**1.000***
	314	**0.528***					486	**0.091**			
	323	**0.083**					489	0.182	0.450		
Gh76	390	0.025	0.175	—			492	0.182	0.550*		
	394		**0.825***	—		Gh150	400	**0.975***		—	—
	396	**0.225**		—			410	0.025	0.132	—	—
	398	**0.025**		—			413		**0.053**	—	—
	400	**0.100**		—			416		**0.395***	—	—
	402	**0.050**		—			419		**0.053**	—	—
	404	**0.450***		—			422		**0.316**	—	—
	406	**0.025**		—			425		**0.053**	—	—
	409	**0.100**		—							
	410			—	**0.900***						
	414			—	**0.100**						

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