High-throughput microsatellite markers discovery for the Sichuan Hill Partridge (*Arborophila rufipectus*) and assessment of genetic diversity in the Laojunshan population

Abstract

Sichuan Hill Partridge (*Arborophila rufipectus*) is a globally endangered species endemic to China. However, the genetic diversity of this species was poorly studied due to lack of molecular markers. We used 454 pyrosequencing to discover microsatellites from the *A. rufipectus* genome in this study. A total of 6,280 di-nucleotides, 8,139 tri-nucleotides, and 11,987 tetra-nucleotides with sufficient flanking region for primer design were screened with *in silico* mining. Ultimately, 18 tetra-nucleotide polymorphic loci were first identified and used to examine the genetic diversity in the Laojunshan population. The number of alleles per locus ranged from 5 to 13, observed and expected heterozygosity varied from 0.292 to 0.917 and 0.722 to 0.909, respectively. These results revealed that this endangered species' genetic diversity as not as low as expected. However, a heterozygote deficit was found in the Laojunshan population. We proposed that the management should focus on increasing the connectivity of remnant patches and fragments to increase the opportunity of greater gene flow among fragmented populations of *A. rufipectus*. This is the first time that polymorphic microsatellite markers were reported for *A. rufipectus*. These markers provide a valuable resource for future population genetics studies and for the management and conservation of this species.

Key words: *Arborophila rufipectus*; 454 pyrosequencing; microsatellites; genetic variation

1. Introduction

The Sichuan Hill Partridge (*Arborophila rufipectus*) is an endangered species endemic to montane southwestern China, where it inhabits subtropical broadleaf forest (Liao et al., 2008). Human activities, deforestation, poaching, and agricultural expansion have resulted in the disappearance of large areas of the subtropical broadleaf forest where this species lives. The present forest habitat of this species has been highly fragmented by the high mountains and encroachment of farming in the area. Currently it is reported to be found only in evergreen and deciduous broadleaf forests in the Pingshan, Mabian, Ganluo, and adjacent mountains in the south-central part of Sichuan Province, China (Johnsgard, 1988; Xu et al., 1994). This species is categorized as ‘endangered’ under the Red List criteria (IUCN, 2014) because of their restricted range, small populations, and severely fragmented habitat (Dai et al., 1998; He et al., 2009).

Genetic variation, an important component for both short- and long-term persistence of populations, is essential to ensure the conservation of the evolutionary potential for this species to adapt to changing environments (England et al., 2003; Guan et al., 2009; Huang et al., 2013). Furthermore, information about effective population size, population differentiation, and genetic connectivity of this endemic species would shed light on critical evolutionary processes as well as on conservation management (Gu et al., 2013). Maintaining genetic diversity is key for keeping a population stable and healthy. Previous studies of *A. rufipectus* were all focused on their distribution, habitat utilization, breeding biology, ecology, calls, and behavior (Liao et al., 2007, 2008; Dai et al., 2009). The population genetics of the Sichuan Hill Partridge has still been poorly studied due to lack

of molecular markers. Given these concerns, we determined that obtaining enough molecular markers was a prerequisite for improving the population genetic studies of this endangered species.

Microsatellites, also known as SSRs, have been proven in recent years to be powerful tools for investigating intra-species genetic variation and ecological characteristics such as gene flow, genetic diversity, population structure, mating systems, and cooperative breeding (Guan et al., 2009; Huang et al., 2013; Page et al., 2014). Next generation sequencing (NGS) technologies have become popular for their success in sequencing DNA at an unprecedented rate and, thereby, enabling impressive scientific achievements and novel biological applications (Schuster, 2008). We applied next generation sequencing (Roche 454 sequencing) and developed a large number of microsatellite markers by screening all the sequences with *in silico* mining. In general, tetranucleotide repeats tend to stutter less than the trinucleotide and dinucleotide repeats and are much more accurate and reliable (Edwards et al., 1991; Walsh et al., 1996). Therefore, primers of tetranucleotide microsatellites were designed and optimized in this study. Here, we first summarize the results of our 454 sequencing experiments and characterize the polymorphic tetranucleotide microsatellite loci. Moreover, we also assessed the genetic diversity of the *A. rufipectus* Laojunshan population (n=24) using these novel microsatellite markers. This study will be helpful in developing effective management strategies for this endangered species.

2. Materials and methods

2.1. Sample collection and DNA extraction

Muscle and blood samples from Sichuan Hill Partridges were obtained from the

Laojunshan Nature Reserve, Sichuan Province, China (muscle tissue=10, blood=14). The muscle tissue samples were collected from naturally dead individuals from 2000 and 2014. Blood samples were collected from live Sichuan Hill Partridges captured and released in the nature reserve in 2013. All the Sichuan Hill Partridges captures were treated carefully under ethical consideration with no harm to the birds, which were immediately released after the blood samples were taken.. The blood samples were carefully collected to avoid contamination and preserved in EDTA Vacutainers.

All samples were frozen at -20°C. Total genomic DNA was extracted from muscle tissue and blood samples using the commercially available Qiagen DNeasy Blood & Tissue Kit (the manufacturer's instructions were followed with some optimizations).

2.2. High-throughput sequencing and microsatellite discovery

In this study, the 454 Life Sciences/Roche GS-FLX genome sequence system (Roche Applied Science, Penzburg, Germany) (Margulies et al., 2005) was used for the identification of microsatellite sequences directly from microsatellite-enriched genomic DNA. Approximately 3 µg of genomic DNA was used to generate a shotgun library following the GS-FLX+ library preparation protocol (GS FLX Titanium General Library Preparation Kit/emPCR kit/sequencing kit, Roche Diagnostics, <http://www.roche.com>). The genomic DNA were then sequenced on 454 GS-FLX+ System at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The raw reads from the GS FLX454 were assembled using Newbler software version 2.3 (Roche Diagnostics). After that, the assembled sequences were scanned for perfect microsatellite repeats using the program MSEA v2.3 (<http://msdb.biosv.com/>) (Du et al., 2013). Detection criteria were restricted to identify perfect SSRs (i.e., those with uninterrupted repeats and compound

motifs) of 1-6 bp and a minimum repeat number of 12, 7, 5, 4, 4, and 4, for mono-, di-, tri-, tetra-, penta- and hexa-nucleotide microsatellites, respectively (Demuth et al., 2007). Repeats with unit patterns being circular permutations (e.g., AGC, GCA, and CAG) and/or reverse complements (e.g., AGC, GCT, CGT, and GCT) were considered as one type in this study (Jurka et al., 1995).

2.3. Development of tetranucleotidemicrosatellites

The flanking regions of microsatellites (100 bp either side) were extracted from the program output in order to design the primers for the microsatellite loci identified. These output sequences were further manually scanned and filtered according to the following criteria: (1) repeats should be tetranucleotide repeats, (2) the repeats number should be in the range of 10-22, and (3) the flanking sequences of microsatellites should not be single-copy sequences and must be long enough to design primers (e.g., more than 30bp). Primer 3.0 (Rozen et al., 2000) was used to design the primers to amplify the selected sequences. Primers were selected to be approximately 20 bp long, to have a GC content between 30% and 60%, and with a melting temperature between 57 and 63 °C (optimum 60 °C). The parameters were employed to design primers with an expected product size of 100 - 300bp. To examine SSR markers, newly-designed PCR primer pairs were tested for amplification using three Sichuan Hill Partridge samples.

2.4. Polymorphism microsatellites isolation

After optimization, the forward primers of the available primers were labeled with fluorescent dyes (FAM or HEX) (Invitrogen Shanghai Sangon Biological Engineer Technology & Services, Shanghai, China) for fragment analysis on a Applied Biosystems 3100 Genetic Analyser. A total of 24 individuals were used to evaluate the ability of the

primer pairs to amplify polymorphic bands. PCR amplifications were carried out in 25 μ L reaction mixtures, comprising approximately 50ng of template DNA, 1.5-2 mm MgCl_2 (TaKaRa, Japan), 200 μ m of each dNTP, 15pmol of each primer, and 0.3 U of Ampli *Taq* DNA polymerase (TaKaRa, Japan). Amplifications were started with a denaturation step for 5 min at 95°C, followed by 35 cycles at 95°C for 45 s, 30 s at the locus-specific annealing temperature (55°C--65°C), 50 s at 72°C, and then given a final elongation for 10 min at 72°C. For genotyping, the PCR amplification products were separated by capillary electrophoresis using a denaturing acrylamide gel matrix on an ABI PRISM 377 Genetic Analyser (Applied Biosystems) using GeneScan Tarmara 350 internal size standard (ABI). Alleles were detected using the GeneScan/Genotyper software package of Applied Biosystems.

2.5. Statistical and genetic data analysis

Microsatellite variation, including the number of alleles per locus (A); observed heterozygosity (H_o); expected heterozygosity (H_e); and polymorphic information content (PIC) were computed for the Laojunshan Sichuan Hill Partridge population using CERVUS 3.0 software (Marshall et al., 1998). Tests for deviations from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium between pairs of loci were performed using GENEPOP 3.4 (Raymond and Rousset, 1995). The program Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004) was employed to examine the microsatellite data for evidence of null alleles whose frequency was estimated following Brookfield's method (Brookfield, 1996). Weir and Cockerham's version of Wright's F-statistics was calculated using FSTAT 2.9.3 program package (Goudet, 2001).

3.Results

3.1. Roche 454 sequencing results

A total of 7,106,406 reads with an average length of 251bp were generated from the Roche 454 GS FLX Titanium platform. The mean GC content was 43.6%. A total of 6,842,637 unique sequences with a length from 300bp to 500bp were identified after they were assembled. MSEA v2.3 (Du et al., 2013) identified 335,263 fragments containing perfect microsatellite motifs. In these different repeat types of microsatellites, mononucleotide (59.03%) was the most common motif followed by the tetra- (13.59%), tri- (8.39%), hexa- (7.55%), di- (6.49%), and penta-nucleotides (4.95%) (Table 1). Moreover, A, AGGGTT, AAAC, AC, AAAT, AAC, C, AG, AAAG, AAACA, AAT, AGG, AT, AGC, AAGG, and CCG, which occurred more than 2000 times, were the 16 predominant repeat types in the genome of *A. rufipectus* (Fig.1). From the total of 95,452 di- to tetra-nucleotide repeat types, we further extensively mined them and identified a total of 26,406 unique microsatellite-containing sequences with sufficient flanking regions. To summarize, there were a total of 6,280 di-nucleotides, 8,139 tri-nucleotides, and 11,987 tetra-nucleotides that met the criteria for primer design (Table 1).

3.2. Detection of microsatellite polymorphism and genotyping

Among 11,987 tetra-nucleotide microsatellite sequences, 234 sequences (repeats number in the range of 10 to 22) met our selection criteria (Table S1, Supporting Information). Thirty-five primer pairs for polymorphism tests were designed by Primer 3.0. After PCR amplification, 7 primer pairs produced more than one band, and 8 primer pairs yielded no products in the PCR. The remaining 20 primer pairs, which yielded satisfactory amplifications, were considered in the polymorphism assessment. All the forward primers of these 20 primer pairs were labelled with different fluorescent dyes

(HEX, TAMRA, FAM) and then used to genotype 24 DNA samples of *A. rufipectus*. Only two labelled primers were without polymorphism, and the remaining 18 showed polymorphism in this study (Table 2). These 18 sequences were added to the NCBI (GenBank accession numbers: KM388496 — KM388513). Table 2 presented the characteristics of the 18 tetra-nucleotide microsatellites.

3.3. Genetic diversity of the Laojunshan *A. rufipectus* population

The 18 polymorphic loci were used to assess the genetic diversity of the *A. rufipectus* population from Laojunshan, Sichuan (n=24). The microsatellite loci showed high variation in their polymorphism (see Table 3). A total of 165 alleles were identified in this population, and the number of alleles per locus ranged from 5 (AR-32) to 13 (AR-13). The observed and expected heterozygosity varied between 0.292 and 0.917, and between 0.722 and 0.909, respectively. The PIC ranged from 0.661 to 0.880 with an average of 0.786, and the F_{IS} values ranged from -0.150 (AR-32) to 0.624 (AR-30), showing an average of 0.141. After a sequential Bonferroni correction, thirteen loci conformed to the Hardy–Weinberg equilibrium; whereas, the other five (AR-6, AR-9, AR-17, AR-25, AR-30) showed significant heterozygote deficiency ($P < 0.05$, Table 3). The analysis with the program Micro-Checker showed evidence of null alleles in these five loci, with frequencies ranging from 0.164 to 0.286. No significant linkage disequilibrium was detected between loci.

4. Discussion

4.1 Mining genomic SSR loci using the 454 pyrosequencing technology

As one of the characteristic species of the Chinese Subtropical Forest Endemic Bird Area (Stattersfield et al., 1998), *A. rufipectus* is in decline. The study of its genetic

background in the Laojunshan population is the first step to evaluate the resource's state and to develop conservation and management strategies. Nevertheless, until now, genetic markers for population genetic assessment of *A. rufipectus* were not available. In this work, we isolated microsatellite markers for *A. rufipectus* for the first time.

The traditional methods of microsatellite development used a library-based approach for targeted SSR repeat motifs, which was time consuming and expensive, with low-throughput (Castoe et al., 2010). The identification of SSRs from genomic DNA using the 454 pyrosequencing technology was relatively advanced. In the present study, we used SSR-enriched sequencing technology to generate 7,106,406 *A. rufipectus* genomic reads, of which a total of 335,263 SSRs were identified from the assembled sequences. This was a very positive result, as shown by the high ratio of SSR-containing sequences and the large number of putative SSRs obtained. These SSR sequences provide substantial resources for the development of microsatellite markers for *A. rufipectus*. Among the SSRs, mono- (59.03%) and tetra-nucleotide (13.59%) repeat motifs dominated in the *A. rufipectus* genomic sequences, similar to findings in *Gallus gallus* (Huang et al., 2012). Without taking into account the mononucleotide repeats, it is generally reported that di-nucleotides are the most common microsatellites in many organisms (Li et al., 2002). There may be a distribution bias in favor of tetra-nucleotides in birds.

4.2 The development of the polymorphic loci for *A. rufipectus*

The 18 microsatellite loci characterized for *A. rufipectus* in this study showed a high level of genetic variation ($PIC > 0.50$). The number of alleles ranged from 5 for locus AR-32 to 13 for locus AR-13. These values were similar to other phasianid species such

as *Syrnaticus humiae* (4–13) (Bei et al., 2013), *Bambusicola thoracica* (4–13) (Huang and Liao, 2010), and *Syrnaticus ellioti* (6–11) (Lin et al., 2011) but lower than the values found in *Chrysolophus pictus* (2–17) (He et al., 2009). On the other hand, the observed heterozygosity in this population ranged between 0.292 and 0.917, indicating considerable genetic variation in this population. This result is in agreement with the variability levels of this type of markers in other phasianid species (Huang and Liao, 2010; Mukesh et al., 2011; Bei et al., 2013).

4.3 Genetic diversity of *A. rufipectus* from Laojunshan

The genetic diversity of the Laojunshan *A. rufipectus* population is surprisingly rich in genetic variability. Nevertheless, heterozygote deficits relative to Hardy–Weinberg equilibrium were observed in five loci after sequential Bonferroni correction in this study. Heterozygosity present in populations reflects the genetic variation of the population at different loci, which can be used as an optimal parameter to evaluate genetic diversity at population and species levels (Nei et al., 1975). There have been many reports of heterozygote deficiency in natural populations of phasianid species analyzed with microsatellites (Huang and Liao, 2010; Mukesh et al., 2011; Bei et al., 2013). Theoretically, the heterozygote deficiencies could be attributed to the Wahlund effect (presence of population substructure), inbreeding, selection, and the null alleles (Zouros and Foltz, 1984; Lade et al., 1996). The presence of null alleles creates false homozygotes and increases the estimated levels of homozygosity (Nantón et al., 2014). Since Micro-Checker software detected the presence of null alleles at all loci deviating from Hardy–Weinberg equilibrium, these alleles are most likely the cause of the heterozygote deficiency observed in our study. Moreover, all of these deficiencies are

related to the highest values of F_{IS} , indicating inbreeding is one reason for heterozygote deficit in this population. This is because of the small effective population sizes leading to mating between relatives and consequent genetic drift. Analyzed samples might have belonged to closely related individuals. Increased samples may also provide an insight into the existing genetic diversity in the population and inbreeding level estimates.

Habitat loss and fragmentation, poaching, and human disturbance have all contributed to the dramatic decreases in wild populations of pheasants in China (Zhang et al., 2003). Nature reserve managers have devoted considerable energy and resources to restore habitat and reduce human disturbance in their reserves, and, in the Laojunshan Nature Reserve, there is some evidence that the Sichuan Hill Partridge population has increased (with counts of 35 birds in 2000 and 130 in 2002; Liao et al., 2008). However, the heavy fragmentation of Sichuan Hill Partridge habitat still presents considerable concern. Although there are high levels of population genetic diversity in this species, great attention should be paid to possible inbreeding in this population. Habitat fragmentation poses a threat by facilitating inbreeding, which leads to loss of genetic variation. Therefore, we suggest that habitat management plans incorporating this new information can now focus more effectively on increasing the connectivity among remnant patches and fragments in order to increase the opportunity of greater gene flow among the fragmented populations of this threatened and special endemic species--the Sichuan Hill Partridge.

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Tables and figures

Fig.1. Distribution of the predominant microsatellite motifs of *Arborophila rufipectus*.

Table 1. Distribution of microsatellites with respect to motif length in genome of *Arborophila rufipectus*

Table 2. Locus names, repeat motifs, primer sequences, annealing temperatures (T_m), size ranges, and GenBank accession numbers for the 18 loci discovered in this study.

Table 3. Characteristics of the 18 microsatellite loci identified in the Sichuan Hill Partridge (*Arborophila rufipectus*), including number of individuals (N), number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content (PIC), P-value for Hardy–Weinberg equilibrium test, estimated frequency of null alleles (Null freq.), and inbreeding coefficient (F_{IS}).

Fig.1. Distribution of the predominant microsatellite motifs of *Arborophila rufipectus*

Table 1 Distribution of microsatellites with respect to motif length in genome of

Arborophila rufipectus

Motif length (bp)	Mono-	Di-	Tri-	Tetra-	Pentra-	Hexa-	Total
No	197,913	21,746	28,131	45,575	16,591	25,307	335,263
Percent of each repeat(% ^a)	59.03	6.49	8.39	13.59	4.95	7.55	
The number and percent of the microsatellite-containing sequences with sufficient flanking region for primer design	-	6280 (28.88%)	8139 (28.93%)	11987 (26.30%)	-	-	

%^a = no./total no. of microsatellites.

Table 2 Locus names, repeat motifs, primer sequences, annealing temperatures (Tm), size ranges, and GenBank accession numbers for the eighteen loci discovered in this study.

Locus	Repeat motif	Primer sequence(5'-3')	Tm(°C)	Size rang (bp)	GenBank accession No.
AR-6	(GAAA)22	F:TCGGCGGTAGAGATCAATTATT R:ACAGAGACAAAGACAAATACAGC	58	210-262	KM388496
AR-7	(GAAA)22	F:TGGAAGCCACTATATTGCCT R:CAGCATCACTATTATTAAGGCCA	58	206-258	KM388497
AR-8	(GAAA)21	F:AGGATGGAGAGGTTTGTGTG R:CCTGCATGACAAGAGATGGA	57	141-193	KM388498
AR-9	(GAAA)20	F:CCAAACTTACAGGGGAAAAGA R:GTGATACCAAGAGCATTTC	58	186-274	KM388499
AR-10	(GAAA)-20	F:TGCTAATGACATACGGGAAAGT R:AGAAGAAACCCAAAGATGAGGA	58	203-247	KM388500
AR-11	(GAAA)20	F:GGGAGAAAGGGAGAAGGAAA R:TGTGCTGTCAATAGTCTCT	58	248-280	KM388501
AR-13	(AAAG)20	F:CTGTGTTCTCAGGCAGTGA R:TGTTGTTTGCCTCCTTTG	60	181-265	KM388502
AR-16	(TTCT)-19	F:CCTCTAGCAAATAAGGCACAG R:GGTCCCACTGAGAATAGGT	58	295-327	KM388503
AR-17	(GAAA)19	F:TTCTTGCGCTTAGTGAGATGT R:CCCACAAACAAACAAACAAACA	58	199-243	KM388504
AR-18	(GAAA)19	F:GCCTTTCTTTTCCCTCCTAA R:AAACACCCTAGCTTACCTTC	58	195-227	KM388505
AR-20	(TCTT)19	F:GGGCAAGCAGATATCCAGAT R:TCCATGAGACCAGCTTTTCA	58	162-202	KM388506
AR-21	(CTTT)18	F:GAAGGGAGGAAGGACAGAAG R:GTGTGTGTGTGAGAGAGAGA	60	184-248	KM388507
AR-25	(AGAA)18	F:TTAGATGACTTTGGCACCGA R:ACTGGAGGCAGAGAATAGGA	58	222-258	KM388508
AR-29	(TTCT)17	F:CCACCTTGATGAATGATGGGT R:AGCTCAGTGATTTAACCCTTCT	58	203-259	KM388509
AR-30	(GAAA)17	F:AAACACAGAAGTTGCAAGGT R:TCCTTTCCACCTCTCAAACA	57	165-197	KM388510
AR-31	(CTTT)17	F:TGATAGGTAGATTACTAGGCCAA R:AAAAGTTTGGGTGGTTTCTTT	57	181-209	KM388511
AR-32	(GAAA)17	F:AAGCAGAAGTAGACCTCCTC R:CCCATTACCAAGCACTGAAA	57	198-218	KM388512
AR-33	(GAAA)17	F:GAGAAAAGACAGCGCAGAAG R:GTGGTTGATGCCTCTGTTTT	57	174-206	KM388513

Table 3 Characteristics of the 18 microsatellite loci identified in the Sichuan Hill Partridge (*Arborophila rufipectus*), including number of individuals (N), number of alleles (Na), observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content (PIC), P-value for Hardy–Weinberg equilibrium test, estimated frequency of null alleles (Null freq.), and inbreeding coefficient (F_{IS}).

Locus	N	Na	H_o	H_e	PIC	P-value	Null freq.	F_{IS}
AR-6	24	9	0.542	0.850	0.813	0.0006	0.164	0.368
AR-7	23	10	0.739	0.827	0.783	0.2973	-	0.108
AR-8	23	10	0.783	0.832	0.791	0.4718	-	0.060
AR-9	24	10	0.500	0.847	0.808	0.0012	0.196	0.415
AR-10	24	11	0.917	0.887	0.855	0.2212	-	-0.035
AR-11	24	9	0.917	0.855	0.818	0.1329	-	-0.073
AR-13	24	13	0.875	0.909	0.880	0.2145	-	0.038
AR-16	24	8	0.792	0.833	0.791	0.3325	-	0.051
AR-17	24	9	0.458	0.871	0.836	0.0000	0.224	0.479
AR-18	24	8	0.750	0.801	0.754	0.1888	-	0.065
AR-20	24	8	0.750	0.743	0.694	0.4834	-	-0.010
AR-21	24	13	0.917	0.909	0.880	0.6581	-	-0.009
AR-25	24	8	0.375	0.722	0.683	0.0000	0.236	0.486
AR-29	24	12	0.875	0.895	0.864	0.3263	-	0.023
AR-30	24	7	0.292	0.766	0.719	0.0000	0.286	0.624
AR-31	23	8	0.783	0.811	0.767	0.2838	-	0.035
AR-32	24	5	0.833	0.727	0.661	0.1418	-	-0.150
AR-33	24	7	0.750	0.801	0.753	0.5041	-	0.064
Average		9.12	0.714	0.827	0.786	0.237		0.141