Identification and characterization of polymorphic microsatellite loci in the red-crowned crane

L. Zhang[1], Z.H. Zhang[1], F.J. Shen[1], R. Hou[1], W.P. Zhang[1], Y.L. Liu[1], K.Y. Tu[1] and A.L. Yang[2]

[1]Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife, Chengdu Research Base of Giant Panda Breeding, Chengdu, China
[2]Southwest University for Nationalities, Chengdu, China

Corresponding author: L. Zhang
E-mail: lianne@panda.org.cn

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ABSTRACT. We isolated and characterized microsatellite loci for the red-crowned crane (*Grus japonensis*) from a microsatellite-enriched database, which was obtained using high-throughput sequencing technology. We designed primer sets for 445 microsatellite loci and after initial screening, 34 loci were genotyped in 31 red-crowned cranes. The number of observed alleles ranged from 3 to 10. Observed and expected heterozygosities ranged from 0.197 to 0.935 and 0.453 to 0.887, respectively; the mean polymorphic information content was 0.663. Loci Lia10943, Lia60455, Lia48514, Lia62171, Lia1059, and Lia5286 deviated from expectation of the Hardy-Weinberg equilibrium; however, significant linkage disequilibrium was not observed among the 34 loci. Using these 34 markers, we successfully completed parental identification for 19 cranes. The probability of exclusion for 7 selected loci (Lia271333, Lia3745, Lia11091, Lia45761, Lia16468, Lia21909, and Lia22355) was >0.9977 and analyses with more loci increased the combination efficiency. These 34 markers were also proven to be efficient for individual identification. We recommend that this

marker system be used in the systematic control of pedigree management and future genetic variation studies of red-crowned cranes.

Key words: Birds; Population analysis; Conservation; *Grus japonensis*

INTRODUCTION

The red-crowned crane is one of the most threatened avian species today. Currently, red-crowned cranes are classified as endangered species on the IUCN Red List (IUCN, 2013) and are listed in Appendix I of CITES (CITES, 2013). Its current range includes China, Japan, South Korea, North Korea, Mongolia, and the Russian Federation.

Similar to many other species, the primary threat to the red-crowned crane comes from extensive habitat loss occurring throughout its range. Human development, especially agricultural expansion, reed harvesting, river channelization, deforestation, and road building, has destroyed many of its historical breeding wetlands. Additional threats include fires that destroy nests, harassment by people, and poisoning from pesticide-treated grain (BirdLife International, 2013).

As of 2007, the global population of red-crowned cranes was estimated to be approximately 2750 (IUCN, 2013), and at the end of 2012, approximately 1022 individuals were distributed throughout 53 captive institutions in China (unpublished data, the Chinese Association of Zoological Gardens). Although the captive lineages were established in China, the records are vague and unreliable because of poor management, inaccurate documentation, and uncontrolled mating. As a result, the conservation and management of captive red-crowned cranes is problematic and in need of revision.

Microsatellite markers are very useful for determining parentage and evaluating the genetic diversity of populations (Luikart et al., 1999). In the past, researchers isolated microsatellite loci using the biotin capture method (Bloor et al., 2001). With the rapid development of DNA sequencing technology, it has become easier and less time consuming to obtain large numbers of loci. The Hiseq2000 Genome Sequencer platform (Illumina, USA) is one of the tools associated with the new wave of sequencing technologies, and its shotgun genome sequencing approach has been demonstrated to be very useful when dealing with short tandem repeat (STR) analyses. To date, 19 microsatellite loci of red-crowned cranes have been reported (Hasegawa et al., 2000; Zou et al., 2010). To increase the number of loci available for microsatellite analyses, Zou et al. (2010) also tried to use six loci from blue cranes (*Anthropoides paradiseus*) to genotype red-crowned cranes. The authors found that the markers were characterized in their study population (N = 26) of red-crowned cranes; however, no parental identification analysis was reported. Problems during the process of genotyping, including co-purifying contaminants, low amounts of DNA, false alleles, multiple alleles, and allelic dropout (Taberlet et al., 1996, 1997, 1999; Gagneux et al., 1997; Frantzen et al., 1998; Morin et al., 2001) may have impacted the accuracy of the subsequent results. Preliminary parental identification analyses that we performed on red-crowned cranes indicated that the 25 previously published loci may not meet the demands of exploring the genetic diversity of the species. In response, we isolated additional microsatellite loci to strengthen such analyses in the future.

MATERIAL AND METHODS

A female blood sample was collected from Chengdu Zoo (Chengdu, Sichuan, China)

and sent to the BGI Company, China. There, a genome survey (sequencing depth 45X coverage) was conducted using the Illumina HiSeq2000 Genome Sequencer Platform resulting in approximately 57 Gb of genomic DNA sequences. The total number of identified SSRs was 275,259, with more than 75% being monomers. We designed 445 random pairs of primers comprising trimers, pentamers, and quadmers using PRIMER3 (Rozen and Skaletsky, 2000). PCRs were performed using these primers on the DNA of six red-crowned cranes in a 10-µL reaction volume containing ~50 ng genomic DNA extracted from the blood, 0.2 µL 20 µM of each primer, 0.8-1.5 µL 25 mM $MgCl_2$, 0.2 µL 2.5 mM of each dNTP, and 0.2 U Taq polymerase. Following an activation step of 5 min at 95°C, the PCR mixture was subjected to 30 cycles of 95°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s, followed by 10 min at 72°C in a GeneAmp PCR system 9700 (Applied Biosystems, USA).The products were run on an agarose gel. Next, 70 primer pairs were chosen from the initial 445 primer pairs, and each of the forward primers was labeled with a fluorescent dye (6-FAM or HEX). Allelic variations at the 70 loci were examined on 16 red-crowned cranes. So far, after initial trials, 34 microsatellite loci (Table 1) were selected.

For the 34 selected loci, we typed 31 red-crowned cranes from 4 zoos (Chengdu, Shanghai, Chongqing, and Qinling Zoos). The PCR procedure was repeated at least 3 times for each individual for each locus. The PCR products were further tested by genotyping on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Fragment length was determined using the Genescan software (Applied Biosystems) with GeneScan-500 ROX as the internal size standard.

These 34 loci were successfully used to conduct parental identification on a group of 19 red-crowned cranes (Table 2), 18 adults (7 males and 11 females) and 1 subadult, from Chengdu, Chongqing, and Shanghai Zoos. Of the seven cranes in Chongqing Zoo, four were found to have originated from the Chengdu Zoo population.

We used the Cervus v3.0 software (Kalinowski et al., 2007) to characterize the polymorphic loci. Allele heterozygosity and polymorphism information content (PIC) for the 34 loci were also calculated. Deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were determined using GENEPOP v3.4 (Raymond and Rousset, 1995).

Probability of identity (PIsibs) was calculated using the software Genetic Identification with Multilocus Tags (GIMLET) v1.3.3 (Valiere, 2002). Parentage testing was performed by Cervus.

RESULTS

The genotyping of 31 individual red-crowned cranes showed high levels of polymorphism within a total of 209 alleles. The number of alleles ranged from 3 (Locus Lia10419) to 10 (Lia271333 and Lia48514), with a mean of 6.15 alleles per locus (Table 1).

PIC ranged from 0.410 to 0.868, with a mean PIC of 0.663. The values of observed heterozygosity and expected heterozygosity varied from 0.197 to 0.935 and 0.453 to 0.887, respectively. Loci Lia10943, Lia60455, Lia48514, Lia62171, Lia1059, and Lia5286 deviated from the expectations of Hardy-Weinberg equilibrium (Table 1); however, significant linkage disequilibrium ($P \leq 0.001$) was not observed among these loci.

Our results demonstrate that the microsatellites selected have considerable power of exclusion (Figure 1), given that we were able to reach 0.9977 exclusion using 7 loci (Lia271333, Lia3745, Lia11091, Lia45761, Lia16468, Lia21909, and Lia22355) and analyses with additional loci increased the differentiation power.

Table 1. Characterization of 34 polymorphic microsatellite loci in red-crowned cranes.

Locus name	Sequence (5'-3')	Fluorescent label	Repeat motif	Size range (bp)	Ta (°C)	N_A	N_g	H_O	H_E	PIC	P value	GenBank accession No.
Lia2794	F: GACCAATAATGAAAGGGTAAGC R: ACATCATGGAGCCAGAAATAAG	R:FAM	$ATC_{(14)}$	100-150	53	4	31	0.339	0.453	0.427	0.1231	KM226806
Lia4342	F: CTACCTCGAGCTTAGTGGTTCAA R: CACAGGAAGGTTTGGAAAACTAC	F:HEX	$AAAAC_{(9)}$	110-180	60	9	31	0.870	0.835	0.810	0.1832	KM226807
Lia10943	F: GTAGACATAAATGGGTCGTATAACAC R: ATTCAATTTCAATGGTGCCAG	R:FAM	$ATG_{(14)}$	120-140	56	4	31	0.197	0.541	0.467	0.0014	KM226808
Lia47103	F: CTGATTCCCTGACTGAAAACAAG R: GGACAGCAAGAAACTTTTTGAGA	F:FAM	$AAAAC_{(9)}$	100-140	56	5	31	0.680	0.710	0.652	0.4420	KM226809
Lia66563	F: GCGTGAGGACATGAACATTT R: CCTGTTGGCCTTGTATTATC	F:HEX	$AGAAA_{(35)}$	180-240	53	6	31	0.764	0.844	0.817	0.8402	KM226810
Lia9623	F: TGGGGACCACAGAAGCTAAT R: CTAAGGAAATGCCACTGATGTT	F:FAM	$TAATAC_{(7)}$	120-160	53	4	31	0.645	0.525	0.426	0.0849	KM226811
Lia31298	F: GAGTTATGAAGTGACATCAAAGT R: AGTGTAACAAAGAACTGAGAATAA	F:HEX	$TTCTT_{(9)}$	110-200	53	8	31	0.465	0.700	0.661	0.0382	KM226812
Lia31570	F: CTTCTTGGATGGAGTAGC R: TGTGACTCGTCTGTTGGTA	R:HEX	$TTCT_{(27)}$	180-200	53	5	29	0.690	0.684	0.613	0.2428	KM226813
Lia5286	F: ACTTTAGTCTCCCCTGGATTGC R: GAATCGCTTGCTCTGTCAGTAGTA	F:FAM	$GAGAA_{(11)}$	120-160	60	5	31	0.871	0.731	0.673	0.0063	KM226814
Lia5754	F: AGATTCAAACTCATCCATCCTTAGA R: TAGATCATCACTGTTGGTCCCTTC	R:HEX	$ATAGG_{(9)}$	80-130	56	5	31	0.733	0.770	0.728	0.6574	KM226815
Lia1771	F: TGCTGTCTTAAGGATCTCTTCAC R: CACTGCACTAAAACATAACCAAA	F:HEX	$TTTC_{(14)}$	130-150	55	5	30	0.533	0.606	0.526	0.3650	KM226816
Lia654	F: TGTTCGTTTGTTCTTCCTTTC R: TCAGGAGCAAGTGGGGAG	R:FAM	$TTCC_{(31)}$	110-140	56	6	31	0.548	0.710	0.650	0.3099	KM226817
Lia16468	F: TACCACTGTATGATGGGCAATG R: AAAACTCTGCTTTGGATGGGT	R:HEX	$TTCC_{(13)}$	120-160	56	9	31	0.806	0.844	0.810	0.8424	KM226818
Lia271333	F: AGCATCCATCTATCACATTCCAA R: CTCCAGAAGCAGAGCAAATGTT	F:HEX	$TAT_{(14)}$	130-170	56	10	31	0.859	0.887	0.868	0.5494	KM226819
Lia20751	F: GGTCCCTTCCAACCAAAA R: TAGCCTCTACGGAAAACAG	F:FAM	$TATTC_{(24)}$	130-180	56	6	31	0.736	0.781	0.742	0.7574	KM226820
Lia60455	F: GGAGGGAGTGGTGTTATGAGTT R: AGAAAGGCTGTTCACAAAGGAT	R:HEX	$CTTT_{(13)}$	100-130	56	8	31	0.838	0.801	0.767	0.0024	KM226821
Lia10419	F: GTTTTTGGTTTTCTTTCCCT R: AATCCAGGACAGGACTTGAC	F:FAM	$CCTT_{(11)}$	110-150	53	3	31	0.516	0.592	0.492	0.7651	KM226822
Lia29562	F: AATCCACCCAAATTAGAGCAAG R: GGAAATGAAGGTCCAAAGAGAG	F:HEX	$ATA_{(14)}$	120-150	60	7	31	0.731	0.777	0.738	0.8820	KM226823
Lia17261	F: CATAATGCCCTACAGACAGA R: GAGAACCCTCATCATAAAATAA	F:HEX	$GAT_{(16)}$	100-130	53	8	31	0.742	0.745	0.704	0.7418	KM226824
Lia3745	F: ACAGCAAAGAAGTCTGGAGTAA R: GCAGTCAATCCCAACTATGTC	R:FAM	$GGAA_{(11)}$	120-170	56	9	31	0.903	0.860	0.828	0.1626	KM226825
Lia11091	F: CTGTCAAGCCTCCACATTTT R: CGTTCAGTGGTATGTTCAAGTAA	R:HEX	$CTTTTC_{(8)}$	100-150	55	7	31	0.742	0.820	0.779	0.2622	KM226826

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Table 1. Continued.

Locus name	Sequence (5'-3')	Fluorescent label	Repeat motif	Size range (bp)	Ta (°C)	N_A	N_g	H_O	H_E	PIC	P value	GenBank accession No.
Lia5699	F: TGAGATGGGAAGATTGAAAG R: AGTGAGCAGTACAACTGGATG	R:HEX	$TCATA_{(9)}$	90-110	56	4	31	0.839	0.647	0.568	0.0191	KM226827
Lia21909	F: ATCACTCTGCCTTCCTCTCC R: TGGGTTGAGAAGTCATTATTCG	F:FAM	$GCA_{(14)}$	110-150	56	8	31	0.645	0.821	0.783	0.0178	KM226828
Lia62171	F: AAGCCTGAAAGATAGAACCA R: ATCTGTGATTTGTAGGGAGC	F:HEX	$CCTT_{(12)}$	80-120	54	7	31	0.710	0.608	0.565	0.0050	KM226829
Lia48514	F: CATTATCCTGCCACACAAAAAT R: AAAGGAGAGGAAGGGGACAG	F:HEX	$TTCC_{(47)}$	170-210	54	10	31	0.935	0.817	0.779	0.0049	KM226830
Lia8655	F: TGAAAGTTGAGATGTGGAAAT R: TCCTCTAGATATGAAATGCCA	F:FAM	$ATGTA_{(11)}$	110-170	56	8	31	0.742	0.771	0.723	0.9736	KM226831
Lia22355	F: GAATAATCCCACCTGTTTTG R: GTAGCCTGGAATTTCATCTT	F:HEX	$AGAA_{(11)}$	130-160	54	5	31	0.774	0.780	0.730	0.5670	KM226832
Lia35642	F: CTTTCTTACTACTTGTTTCTTGA R: GCTGTAGAAATGTAGTTAGTGTG	R:HEX	$TATCTA_{(10)}$	150-200	54	4	31	0.355	0.471	0.410	0.5067	KM226833
Lia65948	F: AGAAGAAGAAAATGGACACG R: ACGTCAGCTCCCTACAAAG	F:HEX	$AGGAA_{(12)}$	100-150	56	7	31	0.742	0.695	0.648	0.1729	KM226835
Lia19150	F: ATAGAAGTGGAGGTGTGGTT R: AAGTCTGTATTCAGCAGTAAG	F:HEX	$TATT_{(11)}$	90-120	53	4	31	0.677	0.731	0.669	0.9138	KM226836
Lia45761	F: GCTGGAAAAGTCAGGTGAC R: GCTCTTCATTCAGAAAATCG	F:HEX	$TCTT_{(15)}$	100-130	55	5	30	0.800	0.775	0.723	0.0931	KM226837
Lia52857	F: GAGGGTAGGCAGTTCCATTT R: ACTTGCCTGCTCTGGTTTAT	F:HEX	$AAAT_{(11)}$	120-150	55	4	31	0.613	0.688	0.615	0.5383	KM226838
Lia1059	F: AATGGGACAGACAAGGACGA R: TTGGGCGGTGAGAAATTGT	R:FAM	$GAA_{(22)}$	120-140	54	5	31	0.226	0.628	0.566	0.0063	KM226840
Lia20367	F: GACCCAAACAATTAAAAGCT R: CACCATGATTCTCACCTACC	R:FAM	$AAAG_{(11)}$	130-160	53	5	31	0.774	0.685	0.614	0.1818	KM226841

Ta = annealing temperature; N_A = number of alleles observed; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content; P value of test for deviations from Hardy-Weinberg equilibrium.

Table 2. Parental identification of red-crowned crane using 34 microsatellite loci.

Offspring	Dam	Candidate fathers	Confirmed father
Chengdu Zoo			
Q01-0826	Q01-0827	Q01-0824	Q01-0824
Q01-0825	Q01-0827	Q01-0824	Q01-0824
0281D005	Q01-0827	Q01-0824, Q01-0826	Q01-0824
T1669	Q01-0827	Q01-0824	Q01-0824
0281D004	Q01-0827	Q01-0824, Q01-0826, T1670	Q01-0824
T1668	Q01-0827	Q01-0824	Q01-0824
T1670	Q01-0827	Q01-0824, Q01-0826	Q01-0824
Chongqing Zoo			
Q01-0814	Q01-0827	Q01-0824	Q01-0824
Q01-0815	Q01-0827	Q01-0824	Q01-0824
Q01-0816	Q01-0827	Q01-0824	Q01-0824
Q01-0817	Q01-0827	Q01-0824	Q01-0824
Shanghai Zoo			
SH20	Sh33	Sh34, Sh35	Sh34
Sh21	Sh33	Sh34	Sh34
Sh22	Sh33	Sh35	Sh34

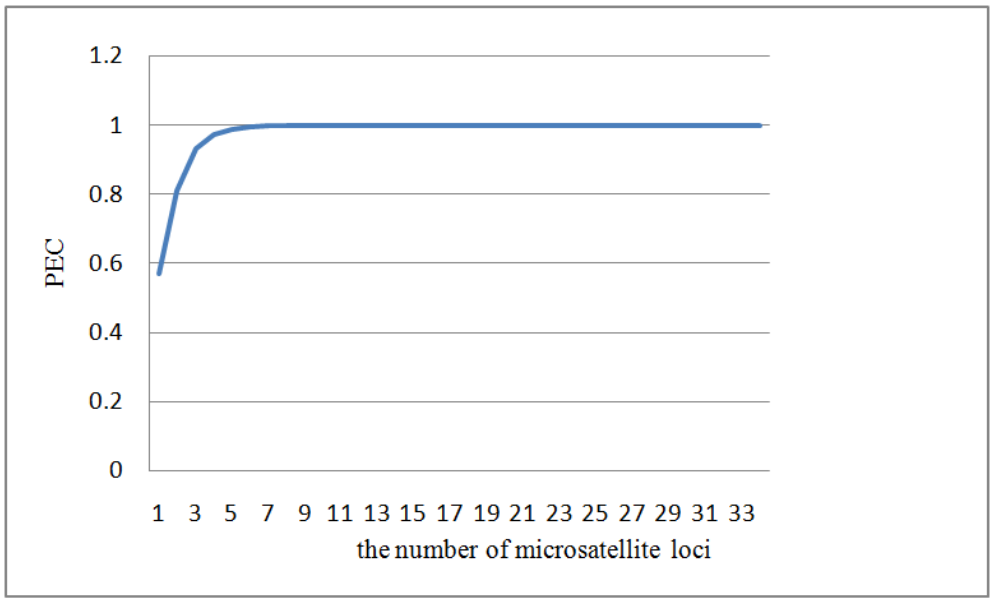

Figure 1. By minimized seven selected loci (Lia271333, Lia3745, Lia11091, Lia45761, Lia16468, Lia21909, and Lia22355), the power of exclusion (PEC) can reach to 0.9977.

We found numerous STR loci using DNA sequencing technology, but flanking sequences in most of these were too short to design effective primers. In addition, nonspecific amplification was very common in the first round of screening of the initial 445 loci identified. Following this first round of screening, 70 loci remained. Additional screening was performed through genotyping and we found that multiple amplifications were the primary reason for the elimination of loci. Mutations were also observed in a small number of loci (3 of 70) within or between populations, and these loci were abandoned.

DISCUSSION

The use of microsatellite typing for individual identification, parentage control, and solving problems of questionable maternity or paternity is a routine procedure within some animals, such as horses (Sereno et al., 2008), giant pandas (Zhang et al., 2003), and dogs (Binns et al., 1995). No successful paternity identification of red-crowned cranes has been reported to date because of the previously discussed deficiencies of the microsatellite loci (Hasegawa et al., 2000; Zou et al., 2010).

We consider the 34 microsatellite marker system reported here to be highly useful for parentage identification of the red-crowned crane. In the experiment of parentage identification of the red cranes from the Chengdu, Chongqing, and Shanghai Zoos, analysis using GIMLET suggests that 7 markers could form a minimum system. However, the group of 7 loci may need to be adjusted when performing parentage analyses on different populations or for populations of different sizes.

We also observed that the 34 selected molecular markers were effective for individual identification. Using this system, we successfully identified 3 randomly selected individuals from the total study population (N = 31). In the future, individual identification and genealogical control of captive populations of red-crowned cranes based on this panel of genetic markers would be extremely helpful for the preservation program being applied to these populations.

Conservation of red-crowned cranes is inherently linked to our ability to monitor both captive and wild populations. Unfortunately, the threats facing this species, particularly the loss of their wetland habitats due to population growth and resource exploitation, pose a serious and imminent threat to their continued existence. The microsatellite loci reported here have proven to be highly stable and are likely to be effective tools for detecting genetic structures of populations and for aiding investigations of genetic variation among red-crowned crane populations in the future. Such applications will help to strengthen the current conservation efforts being put forth, and ultimately improve the status of red-crowned cranes throughout their range.

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