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RESEARCH ARTICLE

Cross-Species Amplification of Human Microsatellite Markers Using Noninvasive Samples From White-Handed Gibbons (*Hylobates lar*)

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Analysis of the population genetic structure and reproductive strategies of various primate species has been facilitated by cross-species amplification (i.e., the use of microsatellite markers developed in one species for analysis of another). In this study we screened 47 human-derived markers to assess their utility in the white-handed gibbon (*Hylobates lar*). Only eight produced accurate, reliable results, and exhibited levels of polymorphism that were adequate for individual identification. This low success rate was surprising given that human microsatellite markers typically work well in species (such as macaques) that are evolutionarily more distant from humans than are gibbons. In addition, we experienced limited success in using a set of microsatellite markers that have been reported to be useful in the closely-related *H. muelleri*, and applying our set of microsatellite markers to samples obtained from one *H. pileatus* individual. Our results emphasize the importance of extensively screening potential markers in representatives of the population of interest. Am. J. Primatol. 64:19–27, 2004. © 2004 Wiley-Liss, Inc.

Key words: microsatellites; gibbons; paternity; cross-species amplification; primate

INTRODUCTION

Genetic analysis of animals living in social groups in the wild has proven essential for uncovering patterns of paternity and relatedness among individuals, and understanding male and female reproductive strategies [Coltman et al., 1999; Di Fiore, 2003; Griffith et al., 2002; Vigilant et al., 2001; Worthington Wilmer et al., 1999]. For these purposes, individuals are usually characterized at a

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number of highly polymorphic microsatellite loci. These loci are genomic segments of a genome that contains tandemly repeated units (each 1–6 nucleotides long) flanked by nonrepetitive sequences. By amplifying a locus using the polymerase chain reaction (PCR), with primers placed in the segments flanking the repeats, one can characterize individual variations [Queller et al., 1993]. However, ascertaining microsatellite markers that are sufficiently polymorphic in a previously unexamined species of interest presents a challenge. The identification of novel microsatellite markers requires some technical expertise and an average of several months of work [Zane et al., 2002]. Therefore, the strategy of cross-species amplification (i.e., using loci characterized in one species to analyze representatives of another, usually closely-related species) has been widely used in primates [Bradley et al., 2000; Coote & Bruford, 1996; Kayser et al., 1996; Moore et al., 1991; Morin & Woodruff, 1992; Perelygin et al., 1996; Rogers et al., 2000; Smith et al., 2000a].

Gibbons have been termed the ''monogamous ape'' because the majority of social groups in this species consist of an adult male and female, and their presumed joint offspring. However, long-term field studies of white-handed gibbons (*H. lar*) in the Khao Yai National Park, Thailand, have revealed that most adult *H. lar* individuals engage in extrapair copulations [Reichard, 1995, 2003; Sommer & Reichard, 2000]. Palombit [1994] also observed extrapair copulations among siamangs (*H. syndactylus*) at Ketambe Research Station in Indonesia. During more than two decades of field observation of the Khao Yai white-handed gibbon population, researchers have frequently documented changes in the composition of social groups due to immigration and emigration, thus calling into question the simplistic view that socially paired gibbons also reproduce exclusively with each other [Reichard, 2003; Sommer & Reichard, 2000]. To date, only one study of genetic relationships in wild gibbon groups (*H. muelleri*) has been conducted [Oka & Takenaka, 2001]. In that study, it was found that three offspring could be attributed to the adults present in the social groups, and two older individuals were probably immigrants. To establish the basis for a study of the genetic relationships among multiple individuals in multiple groups of white-handed gibbons (*H. lar*), we conducted a large-scale search for species-specific microsatellite markers using a cross-species amplification strategy.

MATERIALS AND METHODS

Fecal samples weighing approximately 2–5 g were collected immediately upon defecation from known individuals in a wild population of white-handed gibbons in the Khao Yai National Park, Thailand. The genetic analysis presented here includes 49 individuals from 12 social groups. These gibbons have been the subjects of an ongoing behavioral study since 1992 [Reichard, 2003]. Samples collected between 1996 and 1997 were stored in 10–30 ml of ethanol, while those collected since 1998 were desiccated by storage in a tube containing silica gel beads [Bradley et al., 2000]. DNA was extracted with the use of a QIAamp DNA stool kit (Qiagen, Düsseldorf, Germany) as described in Bradley et al. [2000], with the modification that the feces were soaked overnight in lysis buffer from the kit, instead of being rehydrated in a glass chamber. All extractions were conducted in a separate, dedicated room, and two negative (no sample) controls were processed along with each set of 10–12 samples.

The amplification reactions (total volume=20 μL) were composed of 1X PCR buffer (Applied Biosystems, Darmstadt, Germany), 3.5 mM $MgCl_2$, 250 nM of

each primer, 250 μM of each dNTP, 1 U of Amplitaq Gold DNA polymerase (Applied Biosystems, Darmstadt, Germany), 8 μg of BSA, and a minimum of 25 pg of template DNA. The PCR conditions were as follows: 95°C for 5 min, followed by 45 cycles of 30 sec at 95°C, 30 sec at 48–55°C (depending on the primer set), and 30 sec at 72°C, with a final step of 30 min at 72°C. All sets of amplifications contained a human DNA positive control to confirm success of the PCR, and multiple negative controls to monitor contamination. Agarose gel electrophoresis of 2 μL of the reaction and visualization using ethidium bromide was used to evaluate whether the reactions were successful. The forward primers used in the PCR were fluorescently labeled. To further analyze all successful reactions, we determined allele sizes by genotyping the alleles on a genetic analyzer (model ABI310; Applied Biosystems, Germany) and comparing the results with an internal size standard. Since noninvasive samples typically yield low amounts of DNA, and hence tend to produce incorrect genotypes due to allelic dropout, we repeated the genotype determinations multiple times depending on the DNA concentration of the sample, as determined by quantitative PCR [Morin et al., 2001]. Specifically, both alleles of a heterozygote were typed twice before they were accepted, while apparently homozygous individuals were typed two (samples > 200 pg/μl) to seven times (samples = 25–100 pg/μl) before the genotype was accepted. For cases in which a single allele was observed at least twice, but too few times to be judged homozygous with confidence, the genotype was scored as x and ?, where x is the observed allele and ? indicates uncertainty regarding the second allele. Observed and expected heterozygosities were calculated with the use of CERVUS [Marshall et al., 1998]. The probability of identity was calculated as described in Waits et al. [2001].

RESULTS

We attempted to amplify 47 microsatellite markers (Table I) that were previously characterized in humans, and reported to amplify in nonhuman primates, using a ''test set'' of DNAs extracted from fecal samples of 13 individuals belonging to two social groups. We deemed it expedient to first test individuals from known social groups, since the ability to tell apart individuals that are presumably more closely related than members of the general population is of greatest interest. The two social groups used in this testing phase were selected because they offered large group membership and an abundance of available fecal samples. Most of the primers chosen for screening, and all of those eventually selected for further use have a tetranucleotide repeat motif that is thought to reduce the probability of typing error resulting from the stutter bands that are often associated with dinucleotide markers. Of the 47 loci screened, 39 were discarded for one or more of the following reasons: nonamplification in gibbons, poor amplification, or detection of only one or two alleles within the test set (Table I). Although DNA extracted from fecal samples may be copurified with inhibitory compounds, no evidence of such inhibition of the PCR was detected in experiments in which fecal extract DNA was added to positive control DNA. Eight loci amplified well, provided reproducible genotypes, and revealed multiple alleles in the test set of individuals. These loci were then used to genotype a total of 56 individuals from 12 social groups, with an average proportion of completed genotypes of 0.83. At each locus, a total of 43–52 individuals were characterized, and the number of alleles observed per locus ranged from three to 10, with an average of seven (Table II). A comparison of genotypes between individuals of known relationship

TABLE I. Microsatellite Markers Tested in White-Handed Gibbons

Locus	Repeat motif	Result	Reported use in non-human primate(s)
D1S533	Tetra	Poor amplification	Goosens et al. [2000]
D1S548	Tetra	Polymorphic	Goosens et al. [2000]; Rogers et al. [2000]
D1S550	Tetra	Dimorphic	Bradley et al. [2000]; Goossens et al. [2000]
D1S1656	Tetra	Poor amplification	Morin et al. [1998]
D1S1675	Tetra	Dimorphic	Morin et al. [1998]
D2S367	Di	Poor amplification	Oka and Takenaka [2001]
D2S434	Tetra	Monomorphic	Morin et al. [1998]
D2S1326	Tetra	Monomorphic	Morin et al. [1998]; Bradley et al. [2000]
D2S1329	Tetra	Polymorphic	Morin et al. [1998]; Bradley et al. [2000]
D2S1777	Tetra	Monomorphic	Oka and Takenaka [2001]
D3S1766	Tetra	Polymorphic	Goosens et al. [2000]; Rogers et al. [2000]
D3S2459	Tetra	Polymorphic	Zhang et al. [2001]; Rogers et al. [2000]
D4S1627	Tetra	Poor amplification	Morin et al. [1998]; Bradley et al. [2000]
D4S2366	Tetra	Monomorphic	Launhardt et al. [1998]
D4S2408	Tetra	Poor amplification	Zhang et al. [2001]
D5S807	Tetra	Poor amplification	Oka and Takenaka [2001]
D5S1470	Tetra	Poor amplification	Kayser et al. [1996]; Bradley et al. [2000]
D5S1457	Tetra	Polymorphic	Goosens et al. [2000]
D5S1475	Tetra	Poor amplification	Goosens et al. [2000]
D5S1505	Tetra	Poor amplification	Zhang et al. [2001]
D6S265	Di	Monomorphic	Clisson et al. [2000]
D6S501	Tetra	Poor amplification	Morin et al. [1998]; Zhang et al. [2001]
D7S817	Tetra	Poor amplification	Bradley et al. [2000]; Goossens et al. [2000]
D7S2204	Tetra	No amplification	Bradley et al. [2000]
D8S1106	Tetra	No amplification	Morin et al. [1998]; Bradley et al. [2000]
D9S302	Tetra	Monomorphic	Oka and Takenaka [2001]
D9S910	Tri	Poor amplification	Bradley et al. [2000]
D10S1432	Tetra	Polymorphic	Morin et al. [1998]; Bradley et al. [2000]
D11S1984	Tetra	Monomorphic	Oka and Takenaka [2001]
D11S2002	Tetra	Poor amplification	Morin et al. [1998]; Bradley et al. [2000]
D12S66	Tetra	Poor amplification	Kayser et al. [1996]; Bradley et al. [2000]
D13S321	Tetra	Polymorphic	Zhang et al. [2001]; Rogers et al. [2000]
D13S765	Tetra	Poor amplification	Zhang et al. [2001]
D13S788	Tetra	Poor amplification	Oka and Takenaka [2001]
D14S255	Di	Dimorphic	Kayser et al. [1996]; Oka and Takenaka [2001]
D14S306	Tetra	Monomorphic	Goossens et al. [2000]; Oka and Takenaka [2001]
D16S2624	Tetra	Monomorphic	Bradley et al. [2000]
D17S804	Di	Monomorphic	Oka and Takenaka [2001]
D18S537	Tetra	Poor amplification	Goosens et al. [2000]
D20S206	Tetra	Polymorphic	Oka and Takenaka [2001]
D22S684	Tetra	Poor amplification	Morin et al. [1998]; Zhang et al. [2001]
DQCar	Di	Monomorphic	Clisson, et al. [2000]
MIB	Di	Monomorphic	Clisson, et al. [2000]
MOG C	Di	Dimorphic	Clisson, et al. [2000]
MOG E	Tetra	Poor amplification	Clisson, et al. [2000]
TNF	Di	Poor amplification	Clisson, et al. [2000]
vWF	Tetra	No amplification	Bradley et al. [2000]

TABLE II. Microsatellite markers selected for genotyping of white-handed gibbons

Locus	No. Individuals	Annealing temp. (°C)	No. alleles	Allele sizes	He	Ho
D1S548	47	48	7	160–188	0.777	0.957
D2S1329	52	50	7	188–216	0.700	0.865
D3S1766	47	48	8	248–280	0.804	0.787
D3S2459	46	50	3	246–262	0.490	0.739
D5S1457	47	50	7	130–154	0.704	0.787
D10S1432	45	55	10	152–198	0.857	0.844
D13S321	45	50	9	215–251	0.808	0.822
D20S206	43	48	5	159–175	0.633	0.721

He, expected heterozygosity; Ho, observed heterozygosity.

(i.e., mother–offspring) did not reveal any deviations from the expected Mendelian inheritance pattern. The alleles observed at each locus fell within a limited size range, as is typically observed for a single species, and for four of the eight loci the observed range of allele sizes in gibbons differed from the predicted minimum allele size in humans (data not shown). There were no instances in which more than two alleles were consistently observed, which suggests that replicable amplification of contaminating mammalian, bacterial, or fungal DNA was not occurring [Bradley & Vigilant, 2002]. The average observed heterozygosity was 0.815, with a range of 0.721–0.957, and in several cases the observed heterozygosity exceeded the expected heterozygosity. The distribution of alleles at D3S2459 was found to differ from Hardy-Weinberg expectations ($P<0.001$), while all other loci conformed to expectations. This departure and the variations in heterozygosity levels may be explained by the facts that individuals from a structured population were sampled, and a conservative approach was used to score the homozygous loci. There was no evidence for the presence of null alleles at this or any other of the eight loci, as judged by allele frequencies and by comparison of genotypes from known mother–offspring pairs. Within the population characterized, this set of markers is powerful enough to be used for parentage analysis. Specifically, the average probability of exclusion is 0.966 for cases in which neither parent is known. For cases in which one parent is already known, the average exclusion probability for the second parent increases to 0.997. We estimate that the expected probability of identity is 1.1×10^{-8}, which indicates that two individuals drawn at random from this population have a very low chance of having the same genotype at these loci.

DISCUSSION

Of the 47 loci tested in this study, eight (17%) fulfilled the criteria of amplifying well, providing reproducible genotypes, and exhibiting levels of polymorphism adequate to distinguish individuals. This is a surprising result given that the use of human microsatellite markers for analysis of rhesus macaques, a more distantly related species, usually results in higher rates of success (Table III). Among the markers assessed here were 10 that were previously used in an analysis of 15 wild Bornean gibbons (*H. muelleri*) [Oka & Takenaka, 2001]. It is interesting to note that only one of those primers

TABLE III. Success of cross-species amplification of microsatellite markers originally identified in humans

Cross-amplified species	No. tested	No. amplified (%)	No. polymorphic (%)	Reference
Baboon (*Papio hamadryas*)	>1,300	nd	325 (25%)	Rogers et al. [2000]
Rhesus macaque (*Macaca mulatta*)	24	19 (79.2)	12 (50.0)[a]	Kayser et al. [1996]
Rhesus macaque (*Macaca mulatta*)	72	nd	13 (18.0)	Morin et al. [1997]
Rhesus macaque (*Macaca mulatta*)	51	37 (72.5)	23 (45.1)[a]	Nürnberg et al. [1998]
Rhesus macaque (*Macaca mulatta*)	34	26 (76.5)	18 (52.9)	Smith et al. [2000a]
Rhesus macaque (*Macaca mulatta*)	400	116 (29.0)	76 (19.0)	Hadfield et al. [2001]
Vervet monkeys (*Chlorocebus aethiops*)	55	43 (78.2)	14 (25.5)	Newman et al. [2002]
Langur (*Presbytis entellus*)	32	22 (68.8)	11 (34.3)	Launhardt et al. [1998]
Squirrel monkey (*Saimiri boliviensis*)	76	41 (53.9)	6 (7.9)	Witte and Rogers [1999]
Bornean gibbon (*Hylobates muelleri*)	38	nd	10 (26.3)	Oka and Takenaka [2001]
White-handed gibbon (*Hylobates lar*)	47	24 (51.1)	8 (17.0)	This study

[a]Loci were considered polymorphic with two or more alleles, for all other studies polymorphic loci have 3 or more alleles.
nd, not specified.

(D20S206) amplified repeatably and was polymorphic enough to be of use in our analysis of white-handed gibbons. This result might have been improved upon had greater attention been given to primer optimization or redesign, but economy of time and money suggested that the better strategy was to screen more primers rapidly, rather than devote significant resources to a few primers that did not give an early indication of utility. Another factor in this decision was the necessary use of DNA from fecal samples for marker assessment, due to the lack of availability of numerous high-quality DNA samples from captive white-handed gibbons.

It is increasingly apparent that cross-species amplification using common microsatellite markers may pose a greater challenge than was previously believed [Primmer & Merilä, 2002; Smith et al., 2000b]. A key difficulty is that while cross-species amplification may be possible for a sizable percentage of the markers tested, differences in allele numbers and frequencies in the new population of interest mean that only a few of these cross-amplified markers contribute as much statistical power to the analysis of the new species as they did to the first [Morin et al., 1998]. This emphasizes the importance of conducting pilot studies to establish the utility of the planned genotyping system before large-scale projects are initiated. The increasing efficiency of the microsatellite discovery process [Zane et al., 2002], and the poor success rate of cross-species amplification, particularly in New World primates [Ellsworth & Hoelzer, 1998] (L. Muniz, personal communication), mean that the establishment of species-specific primers should be considered a viable alternative to the time-consuming process of screening human-derived markers.

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