African Journal of Biotechnology Vol. 10(57), pp. 12134-12141, 28 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1159
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

DNA-based species identification for faecal samples: An application on the mammalian survey in Mountain Huangshan Scenic Spot

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Accepted 4 July, 2011

Noninvasive methods using genetic markers have been suggested as ways to overcome difficulties associated with documenting the presence of elusive species. We presented and assessed the casework of species identification based on faecal samples of terrestrial mammals in well-known Mountain Huangshan Scenic Spot. By using a pair of universal primer (L2513/H2714), a 220-bp amplicon of mitochondrial 16S rRNA gene was sequenced and blasted in GenBank to search the species match. 162 out of a total number of 383 mammalian faecal samples were amplified and sequenced successfully. A total of 12 different mammalian species were detected consequently, some of which were endangered and listed as the protected species by the state, such as Chinese serow and clouded leopard. Our study shows that the universal primers are suitable for forensic applications on mammal's molecular identification. Results from molecular identification are the useful supplements for traditional field survey. Considering the sustainable development and ecological balance of resources, some suggestions on conservation and management of mammals distributed in Mountain Huangshan Scenic Spot were demonstrated in this paper.

Key words: Species identification, faecal DNA, 16S rRNA gene, terrestrial mammals.

INTRODUCTION

Mountain Huangshan Scenic Spot (MHSS) is located at the south of Anhui Province, eastern China. As one of China's best-known scenic spots, it has a worldwide reputation for its double world heritage wonders (UNESCO World Cultural and Natural Heritage). Biodiversity resources are a vital important part of scenic spot, which play a key role in maintaining ecological balance. Moreover, it is closely linked with the landscape quality and service quality (Yang et al., 2006). The mountainous area in Southern Anhui is always considered as one of the richest bio-diversity region in East China, since 83 (86.5%) of the total of Anhui's 96 known mammal species live in this region, which also occupies 14.3% of the total number of China's 583 recorded mammals (Wang, 1990; Wang et al., 2007; Song et al., 1998).

Previous studies on mammal fauna of MHSS indicated that about 70 mammalian species lived in this region, which belong to 8 orders and 21 families, respectively (Sheng et al., 1963; Wang, 1990). Nevertheless, few systematic researches on mammalian distribution in MHSS were reported after the 1990s. Meantime, in the past few decades, with the over-exploitation of tourism, the human's negative impact on the livelihood of animals inevitably runs deep in MHSS (Yang et al., 2006). Density and distribution are considered as key parameters for the conservation and management of most animal species (Wilson and Delahay, 2001). Documenting the presence and abundance of species is the first step in designing conservation plans for endangered species and in understanding population ecology (Palomares et al., 2002). To better understand the current living state and protect the rare or endangered mammal species, survey

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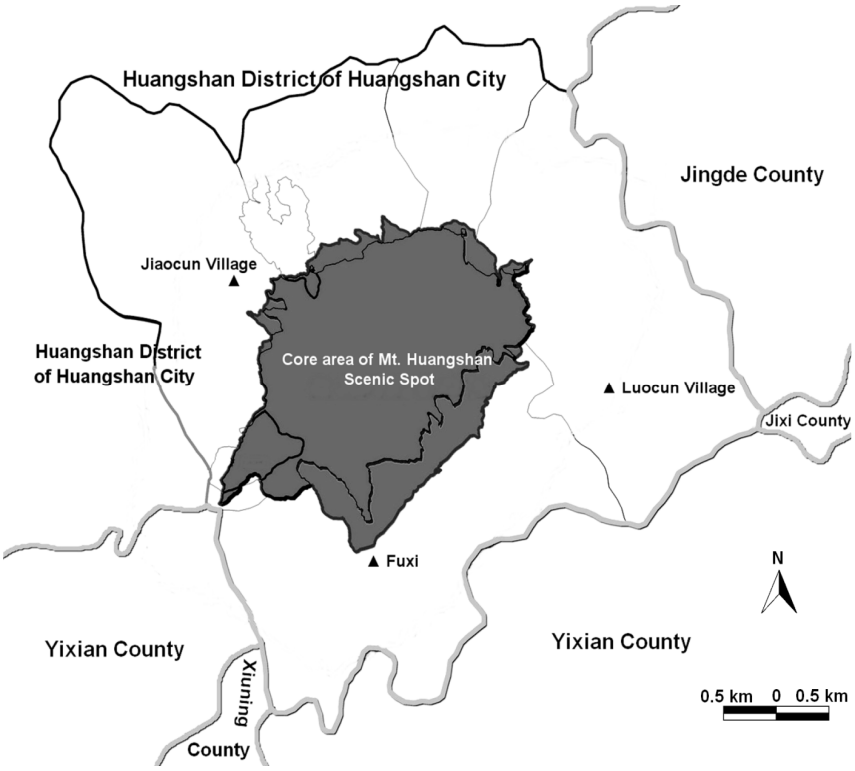

Figure 1. Map of Montain Huangshan Scenic Spot and sampling sites in this study. The grey region shows the core area of MHSS. Black triangles indicate the departure sites of survey, which are Jiaocun Village, Luocun Village and Fuxi, respectively.

on the presence and distribution of mammals in MHSS is greatly important and necessary.

Commonly, the ability to detect and analyze animal sign in the wild is an integral part of wildlife research and management (Foran et al., 1997). For some rare or elusive mammal species, they are frequently scarce and difficult to survey. Large home ranges and great mobility make them extremely difficult to detect and identify (Davison et al., 2002; Kindberg et al., 2009). Especially, in Mountain Huangshan Scenic Spot, due to the unique nature landscape (high forest coverage and steep high-latitude hill), it is not an easy task because individuals are difficult to identify and traditional research methods requiring the capture and handling of animals are not feasible.

Field collection of samples such as faeces or hair can provide some useful information, such as presence/absence and possibly a rough estimation of abundance (Piggott and Taylor, 2003). However, there are situations in which such samples may not be reliably identified to species level on the basis of morphology alone (Bulinski and McArthur, 2000). Fortunately, the development of new non-invasive sampling technique for extracting DNA from animal faeces, and the ability to identify individual animals from such DNA have opened up a new method of 'capturing individual animals' in field surveys (Wasser et al., 1997; Kohn et al., 1999; Kohn and Wayne 1997,; Taberlet and Luikart, 1999; Parsons, 2001). Within vertebrates, some conserved mtDNA sequences were commonly chosen as suitability for species identification on the basis of nucleotide diversity among species and the availability of nucleotide sequence data. The coding regions for cytochrome b and 12S and 16S ribosomal RNA (rRNA) ATPase subunits 8 and 6, and the noncoding D-loop region have shown their potential to be the targets for the species test (Parson et al., 2000; Imaizumi et al., 2007; Melton et al., 2007). In this study, as a useful supplement for traditional species survey, molecular identification for faecal DNA based on partial sequences of mitochondrial DNA (mtDNA), 16S rRNA gene was applied on the preliminary survey of terrestrial mammals in MHSS, which aimed at obtaining rounded baseline data of mammal species distribution and give some rationalization proposal on conservation management of mammal resources.

MATERIALS AND METHODS

Samples and total DNA extraction

A total of 384 mammalian faeces samples from Mountain Huangshan Scenic spot were collected randomly in December 2007 and April 2008, where mountain ranges twist toward the core region of scenic spot (Figure 1). According to characters, mammal

faeces samples with GPS records were collected along the mountain roads on the basis of morphology. The survey routes wriggled up to center of the core region within an altitude range of 240 to 1,310 m. Each sample was thoroughly mixed using 95% ethyl alcohol of 10 ml in a zip lock bag in the field. When brought back to the laboratory, they were stored at -20°C.

Faecal DNA from samples was extracted by the method of Zhang et al. (2006). The extracted total DNA was examined on 1% agarose gels stained with ethidium bromide (EB) and stored at -20°C.

PCR and sequencing analysis

In PCR reactions, partial sequences (about 220 bp) of mitochondrial DNA 16S rRNA gene were obtained with the universal primers L2513 (5'- GCC TGT TTA CCA AAA ACA TCA C-3') and H2714 (5'-CTC CAT AGG GTC TTC TCG TCT T-3') (Kitano et al., 2007). The amplification was carried out in the PTC-200 thermal cycler (MJ Research, USA) in a 30 µl reaction mixture containing 10 mM Tris-HCl (Ph 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 150 µlM of each dNTP, 10 pM of each primer, 20 to 50 ng template DNA, and 1U Taq DNA polymerase (Takara Co.). The amplification cycle consisted of a 4 min denaturation at 95°C followed by 30 cycles of 95°C denaturation for 40 s, annealing for 40 s and 72°C extension for 1 min. Cycling was concluded with a 10 min extension at 72°C. Annealing temperature ranged from 52 to 58°C. In addition, blank control was used to detect the possible amplicon contamination during the procedure of PCR, which consisted of all reagents used but contained no sample DNA.

The amplified products were detected on 1% EB-agarose gels and examined under ultraviolet light to confirm the success of amplification. The PCR products were purified by Cycle-pure kit (Invitrogen Co., Hangzhou) and sequenced using ABI 3730 Genetic analyzer of bio-technology company (Sangon Co., Shanghai). The authenticity of the sequences was conducted by Blast program in GenBank. Any match with an E-value < 0.001 and a Max Identify > 92% can be reliably confirmed. Furthermore, as the negative control, faecal DNA from a domestic dog (*Canis lupus familiaris*) was used to monitor the possible contamination during the procedure of DNA extraction and PCR.

RESULTS

DNA extraction and PCR

In this study, valid total DNA of 291 samples was collected from 384 faeces samples. According to the presence of a 220-bp amplicon from mtDNA 16S rRNA gene, the existence of high quality DNA was demonstrated. Of the 291 faecal samples, a total of 162 samples were amplified and sequenced successfully by the primers L2513 and H2714. Moreover, both amplicons from the positive control and blank control had none, which indicated there was no forensic DNA contamination in the PCR reactions. The percentage of positive amplification reached 58.4% and 129 faecal samples failed to obtain amplified products. The possible reason was the absence of high quality DNA, which was caused by the lowness of fresh degree in some faecal samples. Since the shed target cells degrade like all that are exposed to the elements (Kohn et al., 1995), more fresh samples should be collected in the field to avoid degrading as much as possible.

Data analysis

During the blasting of sequences, single nucleotide substitutions between the query and the subject sequence can be attributed to individual variation (Parson et al., 2000). Consequently, 162 amplicons out of a total of 291 samples on the basis of partial 16S rRNA gene were sequenced successfully and blasted in GenBank. The amplification success rate of 58.4% was obtained. In addition, no product was amplified from the positive blank control, which shows that forensic DNA contaminations were avoided. Accordingly, a total of 12 mammal species of origin were detected from 162 valid DNA samples, which belong to 4 orders and 7 families, respectively (Table 1).

In Table 2, of a total of 162 faecal samples, the origin of 78 samples (*Macaca thibetana*), and 49 samples (*Macaca mulatta*) were from rhesus macaque, which held 48.15 and 30.25% of all faecal samples, respectively. Considering the randomicity of sampling, the amount of macaques and its wide distribution in MHSS was the possible reason. Furthermore, Table 2 indicates that Tibetan macaque was detected in all the three sampling sites, and rhesus macaque was found in two sites except for the site of Fuxi.

Interestingly, clouded leopard (Carnivora: Felidae) were detected from three faecal DNA samples (HS7003, HS7007 and HS7009), in which samples were collected at a 700-meter altitude near Fuxi site. Clouded leopard is an endangered species and listed as the first class protected by state in China. According to a report on special survey for clouded leopard in 2000 (Fang et al., 2006), there were about less than 150 individuals of clouded leopard in southern Anhui. The only distribution of clouded leopard in Anhui is centered in southern mountains, including MHSS (Wang, 1990). To verify whether the 3 faecal samples identified are from the same individual of clouded leopard, 3 sequences (HS7003, HS7007 and HS7009) and a sequences of GenBank (No. DQ257669) were aligned by ClustalX 2.0 (Larkin et al., 2007). According to the aligned result, variable sites and genetic distance among the 3 samples was calculated by MEGA 4.0 (Tamura et al., 2007). Accordingly, in Table 3, genetic distance between HS7003 and HS7009 was 0, which may indicate that H7003 and H7009 share the same origin of individual, but H7007 was from the other individual of clouded leopard. By using the program of MEGA, a UPGMA tree was constructed based on 4 sequences of 16S rRNA gene with 1000 bootstrap replicates (Figure 2). From Figure 2, it is clear that two individuals (HS7003 and HS7009) were located at the same branch on the top of tree closely.

In China, Chinese serow is an endangered species and listed as the second class protected by the state.

Table 1. List of mammal species identified from 162 faecal samples in the study.

Identified species	Taxonomic status (Order: Family)	Species name	Number of sample	Percentage in 162 sequenced samples (%)	IUCN red list categories
Tibetan macaque	Primates: Cercopithecidae	*Macaca thibetana*	78	48.15	NT
rhesus macaque	Primates: Cercopithecidae	*Macaca mulatta*	49	30.25	LC
Edward's rat	Rodentia: Muridae	*Rattus edwardsi*	1	0.62	-
Sladen's rat	Rodentia: Muridae	*Rattus tanezumi*	1	0.62	-
brown Rat	Rodentia: Muridae	*Rattus norvegicus*	5	3.09	-
formosan yellow-throated marten	Carnivora: Mustelidae	*Martes flavigula*	4	2.47	LC
Siberian weasels	Carnivora: Mustelidae	*Mustela sibirica*	2	1.23	LC
domestic cat	Carnivora: Felidae	*Felis catus*	1	0.62	-
chinese serow	Artiodactyla: Bovidae	*Capricornis sumatrensis*	9	5.56	VU
wild pig	Artiodactyla: Suidae	*Sus scrofa*	7	4.32	LC
black muntjac	Artiodactyla: Cervidae	*Muntiacus crinifrons*	2	1.23	VU
clouded leopard	Carnivora: Felidae	*Neofelis nebulosa*	3	1.85	VU

In the table, the capital Roman letter indicates the protection class set by state, and the lowercase letter indicates the protection class set by Anhui province. Transverse line means the species is not listed as the protected wildlife by any local government.

Table 2. Collection sites of 162 valid faecal samples in the study.

Species	Number of sample	Number in Fuxi site	Number in Jiaocun site	Number in Luocun site
Macaca thibetana	78	33	22	23
Macaca mulatta	49	0	3	46
Rattus edwardsi	1	0	0	1
Rattus tanezumi	1	0	0	1
Rattus norvegicus	5	2	0	3
Martes flavigula	4	1	1	2
Mustela sibirica	2	2	0	0
Felis catus	1	1	0	0
Capricornis sumatrensis	9	2	2	5
Sus scrofa	7	1	2	4
Muntiacus crinifrons	2	0	0	2
Neofelis nebulosa	3	3	0	0

Table 3. The matrix of genetic distance among 3 faecal samples.

Parameter	HS7007	DQ257669	HS7003	HS7009
HS7007				
DQ257669	0.005			
HS7003	0.003	0.008		
HS7009	0.003	0.008	0.000	

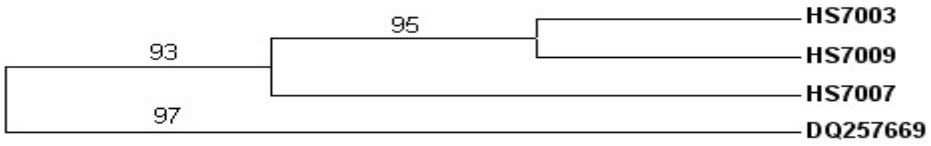

Figure 2. UPGMA tree of the three samples constructed by the MEGA program based on partial sequences of 16S rRNA gene with 1000 bootstrap replicates.

Table 4. The matrix of genetic distance among the eight samples.

Parameter	HS9028	HS9034	HS9036	HS9056	HS9121	HS9156	FJ	HS9157	HS8006
HS9028									
HS9034	0.000								
HS9036	0.000	0.000							
HS9056	0.000	0.000	0.000						
HS9121	0.000	0.000	0.000	0.000					
HS9122	0.000	0.000	0.000	0.000	0.000				
HS9156	0.000	0.000	0.000	0.000	0.000	0.000			
FJ	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
HS9157	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	
HS8006	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.047	0.020

Moreover, Chinese serow is listed as the level of VU by IUCN Red List. By the same approach, genetic distance among 9 samples was obtained, which were blasted and identified as Chinese serow (*Capricornis sumatrensis*). In Table 4, it is shown clearly that the genetic distances among the 7 samples (HS9028, HS9034, HS9036, HS9056, HS9121, HS9122 and HS9156) were 0. In addition, genetic distances among the 7 samples and FJ sequence of GenBank (No. FJ207534) were 0, too. Consequently, it indicates that the origin of the 7 faecal samples may be the same serow individual.

DISCUSSION

Reliability of identification by faecal DNA

Due to the development of molecular technology, nowadays faecal samples can be used to provide vital information on wildlife distribution (Palomares et al., 2002; Kovach et al., 2003), abundance (Bellemain et al., 2005; Mondol et al., 2009), diet (Parsons et al., 2005; Deagle et al., 2005), species identification (Reed et al., 1997; Kurose et al., 2005; Fernandes et al., 2008) and field monitoring (Ernest et al., 2000; Adams et al., 2003). However, faecal sample is likely to contain relatively low concentrations of target DNA, most of which will be degraded (Kohn et al., 1995). In addition, some unknown strong PCR inhibitors are included (Kohn et al., 1997). Some approaches of faecal DNA extraction have been reported in previous studies, such as the guanidinium thiocyanate-silica method (Reed et al., 1997), phenol–chloroform method (Ernest et al., 2000), the Chelex-100 method (Walsh et al., 1991), the commercial product of QIAamp DNA Stool Mini Kit (Qiagen Co.) and so on.

Therefore, a suitable protocol must extract as much DNA as possible from each sample, remove any potential PCR inhibitors and yet be quick and simple. In this study, a novel approach was used to isolate the faecal DNA.

Despite the challenge from old faecal samples, our result confirmed that it is a reliable, quick and cheap DNA extraction method. As compared with the known QIAamp DNA Stool Mini Kit, cost of each sample by this method is about one fifth of the former within a similar time (Zhang

et al., 2006). As a valid supplement for traditional species survey, this method is reliable and suitable for more forensic applications.

On the other hand, a satisfactory success rate of PCR in the study is due in part to the small mtDNA amplified fragment. For medium and long amplicons, success rates decreased slightly (Frantzen et al., 1998). The suitable primer pair is an effective method to purifying faecal DNA during amplification (Reed et al., 1997). In this study, by using a pair of universal primer L2513 and H2714, a short 220-bp fragment from partial 16S rRNA gene of mtDNA was obtained. It is suggested that regions residing in the 12S and 16S rRNA loci in the mitochondrial genome among mammals are more strictly conserved than the region in the cyt b locus (Kitano et al., 2007). It was also confirmed by our pre-experiment. For more complex field-collected faecal samples, primers L2513/H2714 have great applicability on field species survey. Moreover, control samples from known species allow the surveyor to predict what size of amplicons will be generated from target species. It is useful for reliable identification at the species level on the basis of morphology alone.

Widespread distribution of macaques

In the study, a total of 127 samples were identified as macaques, including the origin of 78 *M. thibetana* and 49 *M.mulatta*, respectively. With the random sampling in the field, the primate faeces held 78.4% of 162 valid faecal samples, which may suggests that there is wide distribution of macaques in MHSS. In Table 2, the data demonstrated that there was a relative uniform-distribution for Tibetan macaques over all the 3 sampling sites. Distribution of rhesus macaques, however, only focused on the Luocun site. The possible reasons are presented as follows: On one hand, Tibetan macaque is a typical arboreal animal at high altitude, but rhesus macaque's distribution is wide at altitude. Meanwhile, all faecal samples were collected along the route, of which altitude ranges from lowness to highness. Features from distribution and survey route may have caused such high yield on Tibetan macaque in this study. On the other hand, there are just two wild primates that live in Anhui province, namely *M. thibetana* and *M. mulatta* (Wada et al., 1987; Wang, 1990). As far as the population size is concerned, population number of rhesus macaque was more than that of Tibetan macaque (Li et al., 2006). According to a survey report from Department of Forestry of Anhui Province in 2006, about 500 Tibetan macaques from 8 populations and only 300 rhesus macaques from 5 populations live in the region of Mountain Huangshan and surrounding area (Li et al., 2006). Moreover, an increase trend of wild populations was revealed in 1994 (Wang et al., 1994). There is a wide distribution of macaques in MHSS, the phenomena of which is in accordance with the status based on the field survey simultaneously. Accordingly, more attentions should be focused on population dynamic of macaques in MHSS.

Suggestions on wildlife conservation and management

According to previous studies, there are a total of 70 mammals from 8 orders and 21 families, respectively (Sheng et al., 1963; Wang, 1990) but our result shows that 12 mammals from 4 orders and 7 families were confirmed consequently. Meanwhile, wide distribution of macaques in MHSS was found in this study. Therefore, our study supports the viewpoint that mammalian diversity decreased in the past few decades (Wang, 1990; Yang et al., 2006). It is well known that home range and territory play an important role in survival of mammalian species. The greatest challenge for wildlife conservation, however, is habitat destruction, which is the leading cause of species extinction (Pimm and Raven, 2000). Hence, stricter planning of land use and land control should be formulated and implemented in the scenic region. Long-term ecological effects from tourism exploitation, such as large-scale building planning on highway or ropeway, should be encouraged and evaluated. Rich landscape diversity in Mountain Huangshan provide alot of suitable habitats for wildlife's survival, in addition, it is the development basis of MHSS.

In consideration of the priority of species conservation, determination of a keystone species was valuable in the management and conservation of an ecosystem (Mills et al., 1993). The keystone species (keystone predator) preferentially can consume and control the abundance of prey species depending on low individuals, which play an important role in maintaining the ecological balance in different ecosystems. According to the result of this study, MHSS clouded leopard is suggested to be protected as the keystone species. Considering the ecological balance and tourism security of tourists, a reasonable plan containing buffer zone and core zone should be formulated on the basis of data from clouded leopard's distribution and population dynamic in the future.

Furthermore, in terms of the balance between tourism economics and wider species conservation, flagship species should be defined to strengthen conservation and management (Walpole and Leader-Williams, 2002). As the protected species with the second level by the state in China, we think Tibetan macaque is the best suitable species in MHSS. Nowadays, a known scenic spot named 'Wild Monkey Valley' located at Fuxi scene attract a lot of tourist every year. Such wildlife-based tourism attractions can be useful surrogates for biodiversity conservation because local people have the potential to generate funds and public support that benefit the protected areas. However, human-macaque interactions caused by human disturbance should be paid more attention in practical management (Berman and Li,

2002). Food provision and restricting wild macaque range have potential harmful consequences (Berman et al., 2007). Managers of scenic spot therefore should be more concerned about population dynamic of Tibetan macaque.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Found of China (No. 30470244), the research fund of the State Forestry Administration of China (No. 2130211) the special Fund for Excellent Creative Research Team of Animal Biology in Anhui Normal University and Provincial Key Laboratory of Biotic Environment and Ecological Safety in Anhui.

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