Non-Invasive Technique of Assessing the Ecology of the Japanese Macaque (*Macaca fuscata yakui*) in Yakushima: Focusing on their Feeding of Fig Fruits

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Monkey Team

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INTRODUCTION

A training course, aimed at assessing the ecology of Japanese macaque using non-invasive technique was held from 8th to 13th Sepetember at Yakushima and from 18th to 21st at Wildlife Research Centre at Kyoto. This is a report culminating from this exercise.

Non-invasive DNA sampling technique is considered a key for studying animals without disturbing or influencing their behavior in the wild (Broquet *et. al* 2007). Therefore we collected the feces of Japanese macaque in Yakushima (*Macaca fuscata yakui*) to extract DNA.

'Yakushima' is an island located in the south-southwest of Cape Sata on the Osumi Peninsula of Kagoshima Prefecture, Japan. It has about 500 km² in area and is circular in shape. It has its unique species in fauna and flora.

Wildlife Research Centre is one of the research centres in Division of Biological Science, Graduate School of Science of Kyoto University.

OBJECTIVES

The first objective was to identify the sex of the macaque. Sex identification is important to assess the composition of macaque groups. We can get this information by observation, however, this time we did both, observational and DNA analysis; to validate our data.

The second was to identify the species of the ficus fruits the macaques ate. Yakushima macaque is known to predominantly feed on ficus fruits species (Hill 1997, Otani *et. al* 2000). This study may help us to assess the preference of certain ficus fruit species over the others by macaques during this season.

METHODOLOGY

In order to understand and elucidate the ecology of macaques using a non-invasive DNA technique, feces was collected from macaques in the wild with minimal or no disturbance to their lives. Fecal DNA includes the DNA of animals, plants which the macaques digested, bacteria, virus, and parasites, which can provide valuable information on the ecology of wild animals. For this study, emphasis was laid towards identification of the sex of the macaque and the ficus species they ate.

The following steps were employed to identify the sex of the macaque and the species of ficus they fed on both *In-situ* and *Ex-situ*;

1) DNA collection from feces for sex identification

a. *Fecal samples, including epithelial cells from nine fecal samples and one blood sample of macaque, belonging to three groups, were collected and stored in lysis buffer.*
b. *Eight fecal samples' sexes were identified by direct observation before collection.*

We preserved the surface of feces or blood in 2ml tube with lyses buffer.

2) Seed collection from feces for species identification

Seeds, especially of ficus trees were collected from the feces of the macaques and directly from trees. The fecal samples were stored in plastic bags with infomation of individuals if available. These fecal samples were washed on a fine sieve and the solid insoluble-contents of the feces (hair, undigested animal and plant matter including the seed) were dried overnight at room temperature. Based on the morphology of the

seeds, they were categorized into different types (species). A total of 20 such seed samples were used for DNA analysis. Ten leaf samples from six species of ficus known to occur in the region were also collected and identified based on their morphology. These samples would be analyzed and used as controls for the seed samples.

3) Sex identification using monkey DNA

A. Isolation of DNA from the feces for sex identification

DNA was extracted from the ten samples using QIAamp DNA Stool Mini Kit according to manufacturer's instructions with minor modification.

B. PCR amplification

For sexing (with two positive controls)

We amplified two regions (ZFX and SRY) simultaneously. ZFX is a region on X and Y-chromosome, and SRY is a region on only Y-chromosome. Consequently, male samples yield two different size PCR products (445bp and 224bp), while female samples lack one of them (we find only 445bp bands). PCR products were checked by agarose gel electrophoresis, and then we can identify the sexes.

PCR was carried out in 10 µl reactions containing 5µl of AmpliTaq Gold master mix (Applied Biosystems), 0.5 µM of forward and reverse primers of ZFX (F: ATAATCACATGGAGAGCCACAAGCT , R:

GCACTTCTTTGGTATCTGAGAAAGT) and SRY (F: CCCATGAACGCATTCATTGTGTGG, R: ATTTTAGCCTTCCGACGAGGTCGATA) and 1μl DNA extract. After an initial incubation of 95 °C for 9 min, PCR amplification was conducted for 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min.

4) Species identification using plant DNA

A. Isolation of DNA from seeds and leaves for species identification.

Plant DNA was extracted from 20 seeds collected from 12 fecal samples and from 12 leaf samples as control.

First, 10-15 grains and 5 × 5 mm pieces of leaves were ground. Then we extracted DNA using DNeasy Plant Mini Kit according to manufacturer's instructions.

For species identification of plants

We extracted DNA from the fig seeds in the feces and leaves of figs. We amplified rbcL and internal transcribed spacer (ITS) regions (Kita and Ito 2000, Azuma et al. 2010, Li et al. 2012). Those regions are two of the common markers for plant DNA barcode system. RbcL was located in chloroplast genome and ITS was a nucleic region. Only ITS region can distinguish the species well, but are difficult to amplify because the copy number of nucleic DNA is quite smaller than chloroplast DNA. So, if we succeed amplifying ITS region, we will sequence this region. If we succeed amplifying only rbcL region, we will sequence this region. In the latter case, detailed species identification may be difficult.

PCR was carried out in 15 μl reactions containing 0.75 U of Ex Taq enzymes (Takara), 1μM of forward and reverse primers, 2mM of each dNTP, PCR Buffer and 1.5μl of DNA extracts. Primers for RbcL were followings; rbcL1F: ATGTCACCACAAACAGAAAC and 724R: TCGCATGTACCTGCAGTAGC. Primers for ITS were ITS-Y5: TAGAGGAAGGAGAAGTCGTAACAA and ITS-Y4: CCCGCCTGACCTGGGGTCGC. After an initial incubation of 94 °C for 5 min, PCR amplification was conducted for 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 48 °C (rbcL) or 50 °C (ITS) for 30 min, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min.

After checking PCR products using agalose gel electrophoresis, the products were cleaned up using ExoSap-IT, sequence reaction was conducted using BigDye Terminator ver3.1, and then sequence run was performed in ABI 3130xL.

Sequence results were analyzed using MEGA ver 4.0.2 and ficus species were identified by using BLAST in NCBI database. Yakushima has six species of ficus and all of which have registered ITS sequences. Neighbor joining phylogenetic tree was then constructed using MEGA ver 4.0.2.

RESULTS

1. Sex identification in macaques

DNA was extracted from fecal samples and a blood sample of ten macaques. We performed agarose gel electrophoresis and analyzed them to identify the sex of

macaques. We compared the sex of macaques between the results obtained from direct observation and DNA analysis. We tested this experiment by using markers (ZFX and SRY regions). We observed the DNA bands. As a result; we identified the sex of macaques and found out that five among ten samples were females. The result also matched the direct observation of known individual by a hundred percent.

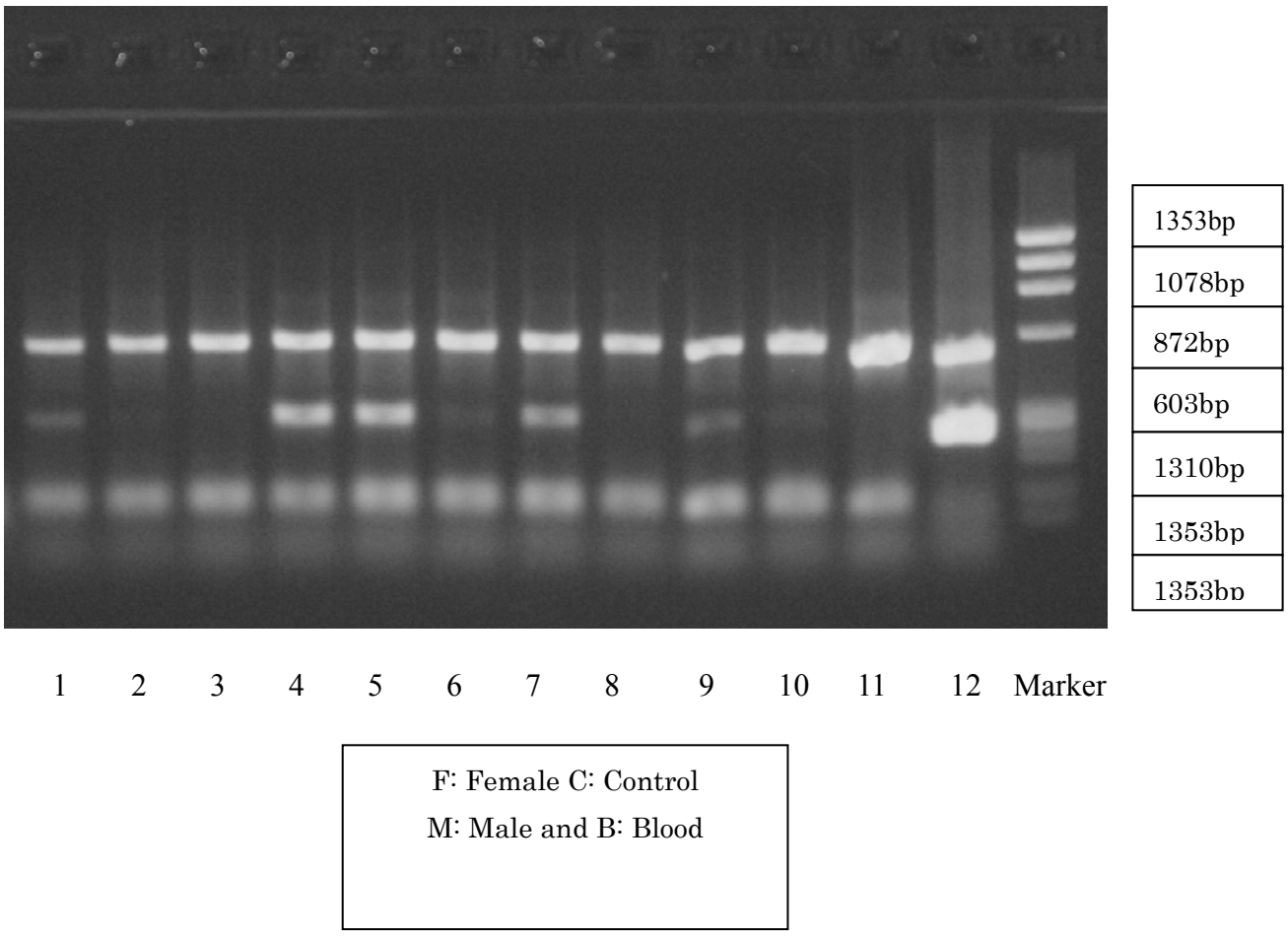

Figure 1: Agarose gel electrophoresis of DNA from Japanese macaques. (1), (4), (5) (7) and (9) are male macaques. (2), (3), (6) female macaques.

2. Identification of ficus species from macaque feces

Plant DNA was extracted from seeds collected from macaque feces to identify the ficus species fed by them. We analyzed the sequences and constructed a neighbor joining phylogenetic tree using the best matches to our sequences obtained from the National Centre for Biotechnology Information (NCBI) nucleotide database by using Basic Local Alignment Search Tool (BLAST). A series of registered internal transcriber spacer (ITS) sequences of six ficus species were used as references. Figure 2 shows the phylogenetic tree. As a result the genetic analysis shows that macaques fed mostly on *Ficus superba* and *F. thunbergii* among the six ficus species. However, a clear assignment of the seeds to either *F. superba* or *F. thunbergii* was not possible.

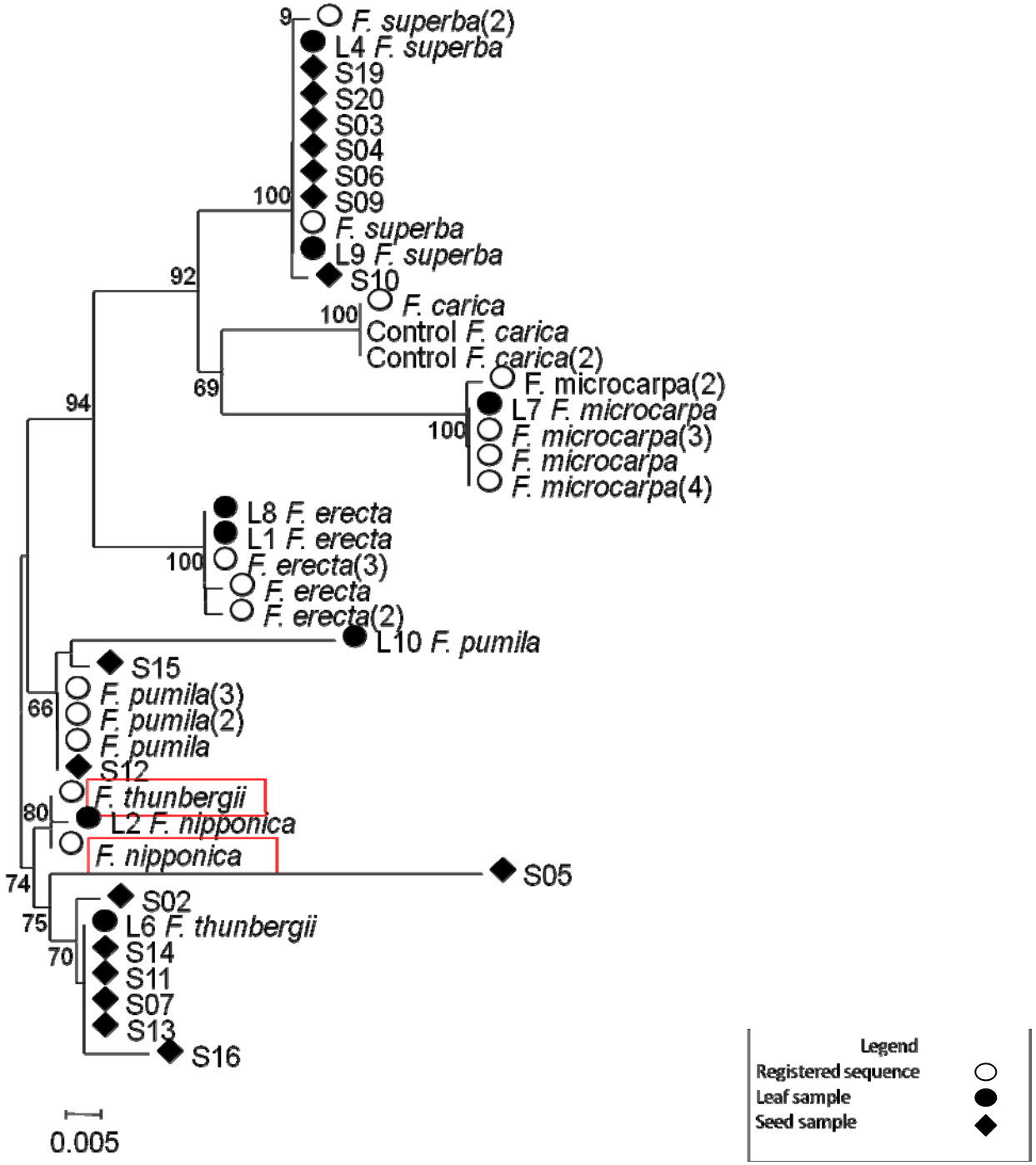

Figure 2: Phylogenetic tree

DISCUSSION

We made an attempt to study the feeding behavior of Yakushima macaques by using molecular technique. Major advantage of non–invasive methods is that, we can understand the given species without disturbing them. However we need long term observations, to completely understand their feeding ecology.

Our first objective was to carry out molecular sexing of the individuals that we had observed. Though, we can identify sex of the individual by visual observations, this technique is very much useful in validating the collected data and in areas where observations are difficult to carry out. Totally we collected nine fecal samples and a blood sample. DNA was extracted followed by agarose gel electrophoresis. With the help of amplified regions ZXF which is present on both X and Y-chromosome and SRY is the region present only on Y-chromosome. From the collected samples, five of them were from females and rest of them was from males. By running Agarose gel electrophoresis, we can differentiate whether the given sample is from male or male. Male samples yielded two different size PCR products (445bp and 224bp), while female samples lack one of them (445bp bands).

Main objective of the study was to understand the macaques feeding behavior. That can be easily understood by examining the feces of macaques. We collected seeds from their feces and leaves of fig trees. The latter was used as a control. For DNA sequencing, two amplified regions were used they are, rbcL and internal transcribed spacer (ITS) regions (Kita and Ito 2000, Azuma *et. al*., 2010 and Li *et. al.,* 2012). These regions are the most common markers used for plant DNA barcode system. RbcL is located in chloroplast genome and ITS is present in nucleic region. Later sequences were analyzed using MEGA 4.0 software. The final sequence was compared with the

sequence obtained from the NCBI nucleotide database with the help of BLAST, which has registered ITS sequences of many plant and animal species. We mainly looked for six ficus species, which are widely distributed in Yakushima Island and monkeys predominantly feed upon.

We found that Yakushima macaques mainly feed on *Ficus superba* and *Ficus thunbergii.* Apart from ficus, we observed, monkeys eating mushrooms and insects (mostly Beetles, sometimes other small insects). By using this little information, we constructed phylogenetic tree which explains distance between each species sequence from one another.

CONCLUSION

Monkeys are believed to be generalist feeders. To prove this we might need long term observations on different individuals, in different seasons, in different altitudinal ranges.

Non invasive techniques greatly help to understand certain valuable information about species which can't be obtained from simple observations. To understand Yakushima macaques feeding ecology, long term observation is strongly recommended.

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