DNA sequence from a fossil pollen of *Abies* spp. from Pleistocene peat

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DNA was amplified from individual fossil pollen grains of *Abies* spp. (Pinaceae), which have been detected from Pleistocene peaty deposits (at least 150,000 years old). To identify the species of the fossil pollen by DNA analysis, the region indicating the species-specific sequence was searched among extant *Abies* species and the spacer region between *rrn5* and *trnR* in chloroplast DNA was sequenced for four grains of the fossil pollen. Three pollen samples produced the same sequence as extant *Abies* species. The sequence for the remaining sample differed from that of extant *Abies* by one substitution. This study showed not only a successful DNA analysis from a single grain of fossil pollen but also a new method to identify the species of fossil pollen for the pollen analysis field.

INTRODUCTION

Pollen grains of ancient plants often are preserved in Pleistocene sediment because their outside wall (exine) consists of a substance extremely resistant to chemical decomposition and biological decay. Fossil pollen has been utilized in pollen analysis to know the past vegetation by the discrimination of its taxon, and morphological characteristics of the pollen wall have been the primary focus of these studies. Since it is difficult to identify fossil pollen to the species level using morphology, a new technique for taxonomic identification of fossil pollen is needed.

On the other hand, technical advancement in molecular biology enable us to analyze ancient DNA in fossil plants. Recently, ancient DNA from well-preserved fossil plants has been extracted and sequenced (Golenberg et al., 1990; Soltis et al., 1992). Successful extraction of ancient DNA depends on the preservation state of the fossil; however, suitable materials are limited, and fossil pollen has not been focused as the material for ancient DNA.

Since the pollen grain is the male gametophyte in seed plants, it contains DNA. If DNA survives in fossil pollen it can be amplified using a polymerase chain reaction (PCR) and the ancient DNA can be analyzed. It has been suggested that plant fossils found in lake deposits may be the best sources for extraction of fossil DNA (Golenberg,

1994). The sturdy wall of fossil pollen and certain characteristics of lake or fluvial deposits where fossil pollen is found (e.g., low temperature and anoxic conditions) increase the potential for preservation of DNA. Staining of DNA with 4',6-diamidino-2-phenylindole (DAPI) has shown that DNA has survived in the fossil pollen of *Abies* spp. from Late Pleistocene peat (Kawamuro et al., 1995). This suggests that DNA from fossil pollen grains can be analyzed, however, no reports have ever tried to analyze DNA in fossil pollen.

In this study, we not only developed a method for DNA analysis from a single grain of fossil pollen but also suggested a novel procedure for identification of the species of fossil pollen by DNA analysis.

MATERIALS AND METHODS

Pollen samples. The material used in this study was recovered at a 44 m depth from a continuously drilled peat core collected at Kurota Lowland, Mikata, Fukui, Japan in 1991. Dating of the core sample is considered to correspond to the glacial period in Late Pleistocene (at least 150,000 years before present), just before the last interglacial period (Kawamuro et al., 1995). This determination was based on tephrochronology (Takemura et al., 1994) and pollen analysis (Takahara, 1994). The core sample was stored in a frozen state and a 10 g sample of peat was loosened with 50 ml of pure water. The sample then was passed successively through sieves of 0.21 and 0.063 mm hole diameters. The particles remaining on a 0.063 mm

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sieve were washed carefully with sterile water more than 10 times. After the particles were transferred to petri dishes, fossil pollen grains that were morphologically identified as *Abies* spp. and that bore no structural damage were collected with a Pasteur pipette under a stereo-dissecting microscope.

DNA extraction. Pollen grains were washed repeatedly and crushed in PCR tube with pipet tip under a optical microscope. Ten microliters of reaction buffer (10 mM Tris-HCl, pH 8.3 at 20°C; 1.5 mM MgCl₂, 50 mM KCl, 0.01% Proteinase-K, 0.01% SDS) were added into the PCR tube, overlaid with 30 µl of mineral oil, incubated for 60 min at 37°C, and heated for 10 min at 95°C. They were treated directly as template extract.

PCR amplification. Ninety microliters of reaction mixture containing 10 mM Tris-HCl (pH 8.3 at 20°C), 1.5 mM MgCl₂, 50 mM KCl, 0.1 mM each dATP, dCTP, dGTP and dTTP, 0.25 µM each 1st primers and 2.5 unit Taq DNA polymerase (Boehringer Mannheim) were added to template extract, and an initial denaturation of 94°C for 5 min is followed by 40 PCR cycles consisting of 94°C for 15 sec, 62°C for 15 sec and 72°C for 25 sec, followed by one extension period of 72°C for 5 min using Quick Thermo Personal

(Nippon Genetics Co. Ltd.). The 1st primers were 5'-TGG TGT CCC AGG CGT AGA G-3' and 5'-CGA CAC CGT GGT TCG TAG C-3'.

Secondary amplification was performed on a 0.5 µl aliquot of the 1st PCR products in a new PCR reaction (99.5 µl) containing 2nd primers for 25 cycles during which the annealing temperature was 55°C. The 2nd primers were 5'-TCT ACT GCG GTG ACG ATA C-3' and 5'-CAC GTG CTC TAA TCC TCT G-3'.

DNA sequencing. PCR products and the 2nd primers were used for sequencing using DyeDeoxy Terminator Cycle Sequencing Kit and 373A DNA Sequencing System (Perkin Elmer).

RESULTS

DNA staining. Before DNA amplification from the fossil pollen, the contents of some grains were stained with DAPI to examine the presence of DNA. The methods for DNA staining have been described previously (Kawamuro et al., 1995). As a result, the luminous parts indicating the existence of DNA were identified in the fossil pollen contents (Fig. 1). The luminous parts were not found in the samples treated with DNase. It appears that DNA

Fig. 1. Epifluorescence micrograph of a fossil pollen of *Abies* spp. from Pleistocene peaty deposits (at least 150,000 years old) stained with DAPI. Scale bar represents 20 µm.

survived in the fossil pollen at least 150,000 years.

Screening of a target for DNA amplification. Next, a target for DNA amplification from the fossil pollen was selected. Since ancient DNA is thought to be fragmented and degraded, it would be difficult to amplify a long segment from a single pollen grain. Additionally, sequence data from related species is necessary to design PCR primers. Furthermore, it is necessary to search for the region indicating the species-specific sequence among Japanese *Abies*, because that enables identification of the species of fossil pollen. Based upon the above considerations, the targets were set on spacer regions (non-coding regions) several hundred-bp long in chloroplast DNA. Eight sets of 19–20 mer PCR primers that correspond to coding regions of both sides of eight chloroplastic spacer regions were designed based on the chloroplast DNA sequence from *Pinus thunbergii* (Sugiura 1994; accession No. D17510) and *Nicotiana tabacum* (Shinozaki et al., 1986) (not shown). The inter-specific variation among five *Abies* species (*A. firma*, *A. homolepis*, *A. veitchii*, *A. mariesii* and *A. sachalinensis*) distributed currently in Japan was investigated using the eight primer sets. DNA of the extant species was extracted from their needle tissue by a modification of the method used by Wagner et al. (1986). In the preliminary investigation, the greatest variation was detected in the spacer region between *rrn5* and *trnR* (ca. 220-bp), in which the species-specific sequences were observed except between *A. veitchii* and *A. sachalinensis*. To evaluate the intra-specific variation, this region also was investigated for 10 samples each of the five *Abies* species from five to ten regions of the present natural distribution. As a result, no variant was observed in the following three species: *A. firma*, *A. veitchii* and *A. sachalinensis*. On the other hand, there was one variant in each of the remaining two species of *Abies* examined. Those variants differed from the typical sequence by one substitution. Besides, there is no macrofossils that shows the existence of nonextant *Abies* from Middle to Late Pleistocene of Japan (Minaki, 1989). These results suggested that species of the fossil pollen of Japanese *Abies* from Late Pleistocene could be identified by the sequencing of this region.

DNA amplification from a single pollen. In the next step, a method for DNA amplification from a single grain of extant pollen was developed. The *rrn5-trnR* spacer region was amplified and sequenced from the extant pollen of *A. homolepis* and *Pinus thunbergii*. The sequences from one pollen grain were consistent with those from DNA samples extracted from their needle tissue. The results showed that DNA analysis from a single pollen grain is possible.

DNA amplification and sequencing from fossil pollen. Finally, the DNA fragment from the fossil pollen was amplified using the same method as for extant pollen. Each pollen grain was washed repeatedly with sterile water on a siliconized slide under a microscope, because contamination is a serious problem in the analysis of ancient DNA. Furthermore, in order to certify that the amplified DNA fragment did not come from material surrounding the fossil pollen, the water samples used for the last washing of each fossil pollen grain were treated in parallel, as amplification-negative controls. Primers for the 1st amplification were designed from positions 91604–91622 in the *rrn5* gene and 91974–91992 in the *trnR* gene of *Pinus thunbergii* (Sugiura 1994; accession No. D17510). Amplification was attempted on 125 fossil pollen grains and success was achieved for four of these grains. The first amplification produced a ca. 390-bp fragment (Fig. 2).

The positive amplification products from four samples were used for the 2nd PCR with the 2nd primer set designed for the inside of the 1st PCR products. These 2nd primers correspond to bases 91660–91678 and 91955–91973 of the *P. thunbergii* chloroplastic sequence. The second amplification in all of the four samples also was successful. The PCR products were treated as the template for sequencing and each strand was sequenced twice. As a result, three kinds of sequences were provided from the four samples. Results of two of the four samples corresponded to the extant *A. firma* sequence. The third one differed from the extant *A. firma* sequence by one substitution. The other pollen sample gave the same sequence as in *A. veitchii* and *A. sachalinensis*. The sequences from fossil pollen and extant *Abies* species are shown in Fig. 3a and a parsimonious network of them is shown to visualize the relationships in Fig. 3b.

DISCUSSION

In General, studies of ancient DNA have always involved the pitfall of contamination by modern DNA and artifact through the amplification. In this study, it is hardly recognized that a chance artifact of PCR could produce the identical sequence with extant Japanese *Abies*. Additionally, the probability that the amplified DNA has originated from another species such as *Pinus* will be very low. Because, for example, the sequence of *rrn5-trnR* spacer region of *Pinus thunbergii* is quite different from that of *Abies* species (not shown). The main concern possibility to make error in this study is contamination of modern DNA from extant Japanese *Abies*. To monitor the contamination in this work, we treated the samples with amplification-negative controls in parallel. As shown in Fig. 2, their amplification-negative controls did not contain any template DNA. It cannot be proved directly that the amplified DNA surely originate from fossil pollen, but it gives authenticity to the results that DNA

Fig. 2. Amplification product of DNA recovered from the fossil pollen using the 1st PCR primers, showing the ca. 390-bp fragment of the region between *rrn5* and *trnR* in chloroplast DNA. M indicates molecular weight marker (ϕ X174 digested with *HincII*). Lanes 1, 3, 5, 7, 9, 11 and 13 are amplification products from fossil pollen with a positive result in lane 9. Lanes 2, 4, 6, 8, 10, 12 and 14 are amplification-negative controls (all amplification reagents with sterile water used for the last washing of each fossil pollen grain). Lane 15 is a positive control (amplification product from extant pollen of *Abies homolepis*). Lane 16 is a negative control (all amplification reagents).

	1	41
A.f	GATAAAAAGCTTA-----TCTTATTCTCATTAAAAGATCG	TCTTATTCTGAATTGAAACCCCATTTCTTACATCTTTCT
A.v	.....	.....
A.s	.....	.....
A.h	.....	.....
A.m	.....ATGCC.....T.....	.....
F.1	.....	.....
F.2	.....	.....
F.3	.....	.....
F.4	.....	.....
	81	121
A.f	TTCCCACTCCATACTTTACTTTGAAACGCAATACCTGCGC	TCGCTCATAGCGAGGAACCTCGCTTCTGCATCCTCTTACCC
A.v	.....	.....
A.s	.....	.....
A.h	.....	.....(T).....
A.m	.(A).....	.....
F.1	.....	.....
F.2	.....	.....
F.3	.....	.....
F.4	.....	.....
	161	201
A.f	CGGCTGAATAGAGG----CGTCCCTTTTGGGAGGGACGGA	CTCCTGGGAACGAATCCAATGGAGACG
A.v	.....AATC.....	.....
A.s	.....AATC.....	.....
A.h	.....AATC.C.....	.....
A.m	.....AATC.C.....	.....
F.1	.....	.....G.....
F.2	.....	.....
F.3	.....	.....
F.4	.....AATC.....	.....

Fig. 3a. Sequences of the spacer region between *rrn5* and *trnR* in chloroplast DNA from extant *A. firma* (A.f), *A. veitchii* (A.v), *A. sachalinensis* (A.s), *A. homolepis* (A.h), *A. mariesii* (A.m) and fossil pollen grains (F.1 to 4). A dot indicates that base is identical to that of extant *A. firma*, a dash indicates an insertion/deletion and the base in parentheses indicate their variation type.

Fig. 3b. A parsimonious network of extant *A. firma* (□), *A. veitchii* (○), *A. sachalinensis* (◇), *A. homolepis* (△), *A. mariesii* (▽) and fossil pollen (●) based on the sequence of the spacer region between *rrn5* and *trnR* in chloroplast DNA. Each symbol indicates an individual sample. Crossed bars and numbers drawn in each branch show the mutations and their position of the mutation (see Fig. 3a).

staining indicated the existence of DNA in fossil pollen.

Although one of the four sequences from the fossil pollen gave a sequence that was not detected from the investigation of extant Japanese *Abies*, it is considered to be a variation type of *A. firma* or it could be a amplification error of PCR. The results of this work shows the fossil pollen grains from peat deposits of 150,000 years old would originate from *A. firma* and *A. veitchii* or *A. sachalinensis* of that time period. However, it would be better to analyze plural independent regions or more variable region of more pollen grains to apply this method to the field of pollen analysis.

In this study, the contents of fossil pollen were analyzed

and it was shown that DNA analysis from a single grain of fossil pollen is possible. Furthermore, it was possible to identify the species of fossil pollen by DNA analysis of a specific region. This is an advancement in technology and an innovation in the field of pollen analysis. As DNA amplification from older fossil pollen grains is anticipated in the future, the information gained will be valuable for palaeobotany, plant evolution and systematics research. Fossil pollen may be considered as a time capsule of ancient DNA.

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