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Original article

Kazutaka Kobayashi,[1] Junko Yoshikawa[2] and Mitsuo Suzuki[1, 3]: DNA identification of *Picea* species of the Last Glacial Age in northern Japan

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東北地方における最終氷期のトウヒ属化石のDNAによる種同定

Abstract Boreal coniferous forests in Japan were dominated by *Picea* during the Last Glacial Age. Extant *Picea* species have fairly similar morphology, and identification of fossil *Picea* species is often difficult even from its cone. We tried to identify *Picea* fossils using chloroplast DNA sequence. We collected fossil samples of the Last Glacial Age in the Tohoku District. DNA regions examined were two intergenic spacer regions about 200 base pairs selected by comparing DNA sequences among extant Japanese *Picea* species. Fossil DNA was sequenced by the polymerase chain reaction (PCR). We succeeded in sequencing five cones from Dekijima and one branchlet from Mameda, Aomori Prefecture. The sequences of five cones agreed with that of extant *P. glehnii*, and that of the branchlet with extant *P. koyamae* and *P. shirasawae*. Morphological identification of the five fossil cones resulted in two *P. glehnii* and one *P. maximowiczii* cones, and the remaining two not identifiable because of poor preservation. Although the fossil branchlet obtained from Mameda was not identifiable to species, cones collected from the same horizon were identified as *P. koyamae*. DNA and morphological identifications corresponded except for the cone identified as *P. maximowiczii*. This study showed a successful DNA analysis of fossil cones and needles and the value of DNA for identifying fossil species.
Key words: DNA identification, fossil DNA, Japan, Last Glacial Age, *Picea*

要　旨　最終氷期の日本列島にはトウヒ属の樹木が優占していたが，その種については様々な見解がある。トウヒ属の化石は主に毬果の形態で種が同定されるが，同定される種は研究者によって必ずしも一致してこなかった。そこで，葉緑体DNAの塩基配列によるトウヒ属化石の同定を試みた。試料は東北地方に分布する最終氷期の地層から採集したトウヒ属の毬果、針葉、材および種子の化石である。調査に用いたDNA領域は、日本に分布する現生トウヒ属7種2変種の塩基配列を比較して選定した葉緑体DNA上の2つの遺伝子間領域（各約200塩基対）である。PCR (polymerase chain reaction) 法を用いて、化石DNAの塩基配列を決定した。青森県西津軽郡木造町の出来島海岸で採集した毬果5個と同三戸郡新郷村の間明田で採集した針葉を付けた1枝で、その塩基配列を決定することができ、毬果5個は現生アカエゾマツと、針葉は現生ヤツガタケトウヒおよびヒメマツハダと塩基配列が一致した。毬果形態では、出来島の化石は2個がアカエゾマツ、1個がヒメバラモミに同定され、残り2個は風化が激しく同定が出来なかった。間明田については同一層準から採集した毬果がヤツガタケトウヒに同定された。形態形質による同定とDNAによる同定は、ヒメバラモミに同定された出来島の1毬果を除いて一致した。今回の結果は、毬果および針葉化石でのDNA解析の成功を示すとともに、植物化石種同定におけるDNAの有用性を示した。
キーワード：化石DNA，最終氷期，DNA同定，トウヒ属，日本

Introduction

Seven species and two varieties of *Picea* have been described for Japan: *Picea jezoensis* (Siebold et Zucc.) Carr., *P. jezoensis* var. *hondoensis* (Mayr) Rehder, *P. glehnii* (Fr. Schm.) Masters, *P. maximowiczii* Regel, *P. maximowiczii* var. *senanensis* (Nakai) Hayashi, *P. polita* (Siebold et Zucc.) Carr., *P. bicolor* (Maxim.) Mayr, *P. shirasawae* Hayashi and *P. koyamae* Shirasawa (Satake, 1989). *Picea shirasawae* is regarded as a synonym of *P. koyamae* based on both morphological (Shimizu, 1992) and isozyme criteria (Katsuki *et al*., 1995).

Boreal coniferous forests mostly comprising *Picea* species covered large areas of Honshu Island during

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the Last Glacial Age, as shown by numerous examples of fossil cones and woods from sediments of this age at many sites (Miki, 1957; Minaki, 1987b; Suzuki, 1991). Understanding how extensively *Picea* taxa dominated during the Last Glacial Age and migrated and evolved during the Postglacial Age, resulting in the present distribution patterns, is an important problem of the Japanese historical botany. Morphological and anatomical characteristics of needles and woods are fairly uniform within the genus *Picea*, allowing identification only to the level of genus, subgenus, or section. On the other hand, well-preserved fossil cones can be identified morphologically at the species level. Miki (1957) reported *P. bicolor*, *P. glehnii*, *P. koyamae*, *P. maximowiczii,* and *P. polita* from glacial Pleistocene sediments in Japan. Minaki reported *P. maximowiczii* and *P.* cf. *shirasawae* from the late Pleistocene in the Kanto District (Tsuji *et al*., 1984a, 1984b; Minaki, 1987a, 1987b). Suzuki (1991) studied fossil *Picea* cones of 46 Pleistocene sites from southern Hokkaido to central Honshu and identified them with extant *P. jezoensis*, *P. bicolor*, *P. glehnii*, *P. koyamae*, *P. shirasawae,* and *P. polita*, and extinct *P. pleistoceaca* Suzuki, *P. sohmae* Suzuki, and *P. tomizawaensis* Suzuki. Among these, *P. jezoensis*, *P. glehnii*, *P. pleistoceaca,* and *P. tomizawaensis* were common in the earlier half of the Last Glacial Age of southern Hokkaido and northern Honshu. The different identifications are probably due to the occurrence of several taxa during the same period, but also to the identification bias of respective researchers.

Recently, Konishi & Suzuki (1997) studied the morphological variation of cones and cone scales throughout the distribution range of *P. glehnii*. They found that the cone shape and size of *P. glehnii* were quite variable and that the variation ranges of some parameters overlapped partly with those of *P. koyamae* or *P. maximowiczii*. Suzuki (1991) distinguished *P. tomizawaensis* from *P. glehnii* by its smooth cone scale margin, but Konishi & Suzuki (1997) revealed that such a cone scale margin is common among extant *P. glehnii*. This suggests that the recognition of fossil *Picea* species based only on morphological characters can be misleading. Moreover, it is impossible to resolve the phylogenetic relationships between fossil and extant species just from morphology.

The availability of ancient DNA for identifying fossil species has been indicated recently. Although DNA decomposition begins immediately after an organism dies due to the release of DNases (Golenberg, 1994), DNA molecules could be preserved in well-preserved fossils under special conditions. One of the oldest records of fossil plant DNA was found in Miocene compression leaves of *Magnolia* (Golenberg *et al*., 1990). Since then, there have been several reports of ancient DNA, sometimes with negative arguments. There is one report of ancient plant DNA in Japan: Suyama *et al*. (1996) succeeded in reading the DNA

Table 1 DNA samples obtained from living trees of the genera *Picea* and *Larix*

No.	Botanical name	Locality	Voucher No.	Collector	Accession No.			
					*rbc*L	*trn*L a-b	*trn*L c-d	*trn*L e-f
Pab	*Picea abies* (L.) Karst	Bot. Gard. Tohoku Univ.*	kk 0001	K. Kobayashi	AB045039	AB045053	AB045065	AB045076
Pbi1	*P. bicolor* (Maxim.) Mayr	Mt. Takahara, Tochigi	970001	J. & A. Yokoyama	AB045040	AB045054	AB045066	AB045077
Pbi2		Kiyosato, Yamanashi	WD729	T. Katsuki	AB045041	AB045055	AB045066	AB045077
Pgl1	*P. glehnii* (Fr. Schm.) Master	Hokkaido Univ. For. Uryu	TUS 169277	K. Kobayashi *et al.*	AB045042	AB045056	AB045067	AB045078
Pgl2		Hokkaido Univ. For. Uryu	TUS 169276	K. Kobayashi *et al.*	AB045042			
Pgl3		Hokkaido Univ. For. Uryu	TUS 169308	K. Kobayashi *et al.*	AB045042			
Pgl4		Mt. Hayachine, Iwate	TUS 169267	K. Kobayashi *et al.*	AB045042	AB045056	AB045067	AB045078
Pgl5		Mt. Hayachine, Iwate	TUS 169266	K. Kobayashi *et al.*	AB045042	AB045056	AB045067	AB045078
Pgl6		Mt. Hayachine, Iwate	TUS 169275	K. Kobayashi *et al.*	AB045042			
Pgl7		Enoura, Sakhalin	809008	M. Suzuki *et al.*	AB045042	AB045056	AB045067	AB045078
Pje1	*P. jezoensis* (Sieb. et Zucc.) Carr.	Hokkaido Univ. For. Uryu	TUS 167381	K. Kobayashi *et al.*	AB045043	AB045057	AB045068	AB045079
Pje2		Sedykh Lake. Sakhalin	807015	M. Suzuki *et al.*	AB045043	AB045057	AB045068	AB045079
Pje3		Krestonoshk L., Sakhalin	814004	M. Suzuki *et al.*	AB045044	AB045057	AB045068	AB045080
Pjh1	*P. jezoensis* var. *hondoensis* (Mayr) Rehder	Kamikochi, Nagano	518001	M. Suzuki	AB045045	AB045058	AB045069	AB045081
Pjh2		Nikko Bot. G. Univ. Tokyo	214003	K. Kobayashi *et al.*	AB045045	AB045058	AB045069	AB045082
Pko	*P. koyamae* Shirasawa	Mt. Nishidake, Nagano	TUS 169329	K. Kobayashi *et al.*	AB045046	AB045059	AB045070	AB045083
Pma	*P. maximowiczii* Regel	Kawakami-mura, Nagano	TUS 169285	K. Kobayashi *et al.*	AB045048	AB045061	AB045072	AB045085
Pms	*P. maximowiczii* Regel var. *senanensis* Hayashi	Kawakami-mura, Nagano	TUS 169286	K. Kobayashi *et al.*	AB045049	AB045062	AB045073	AB045086
Ppo1	*P. polita* (Sieb. et Zucc.) Carr.	Nikko Bot. G. Univ. Tokyo*	214005	K. Kobayashi *et al.*	AB045050	AB045063	AB045274	AB045087
Ppo2		Taima-cho, Nara	1001001	R. Hirano	AB045051	AB045063	AB045274	AB045088
Psh	*P. shirasawae* Hayashi	Mt. Nishidake, Nagano	TUS 169328	K. Kobayashi *et al.*	AB045047	AB045060	AB045071	AB045084
Lka	*Larix kaempferi* (Lamb.) Carr.	Kamikochi, Nagano	518002	M. Suzuki	AB045038	AB045052	AB045064	AB045075

*: cultivated.

sequences of four fossil *Abies* pollen grains of the Late Glacial Age. DNA from two pollen grains corresponded to extant *Abies firma*, one to extant *Abies veitchii* and *A. sachalinensis*, and the fourth to *A. firma* except for one base pair.

Picea species were most dominant in the Last Glacial forests of Japan, and there remain many extraordinarily well preserved fossil cones at many sites. In this paper, we will establish a molecular phylogenetic method to identify living species of *Picea* distributed in Japan and will try to identify fossil species from *Picea* macrofossils.

Materials

Living plant materials

Needle samples for DNA analysis of living taxa were collected from 20 individuals of seven species and two varieties of Japanese native *Picea* and one individual each of *P. abies* (L.) Karst and *Larix kaempferi* (Lamb.) Carr. (Table 1). *Larix kaempferi* was used as an outgroup referring to the result of Chase *et al.* (1993). All the voucher specimens are deposited in the Herbarium Tohoku Universitatis Sendaiensis (TUS).

Fossil plant materials

Fossil cones, branchlets, woods, and seeds of the Last Glacial Age were collected from seven localities in Tohoku District, northeast Japan (Table 2, Fig. 1). In total, 78 samples, were examined to extract ancient DNA: 57 cones from seven localities, 16 branchlets from two localities, three wood pieces from one locality, and two seeds from one locality. All fossil samples except for those from Mameda were deposited in peat. Fossil branchlets collected at Mameda were deposited in the volcanic ash of the Towada Hachinohe pumice fall (To-HP), just above a peat layer of the Upper Ofudo Formation. Samples were collected in the following four methods.

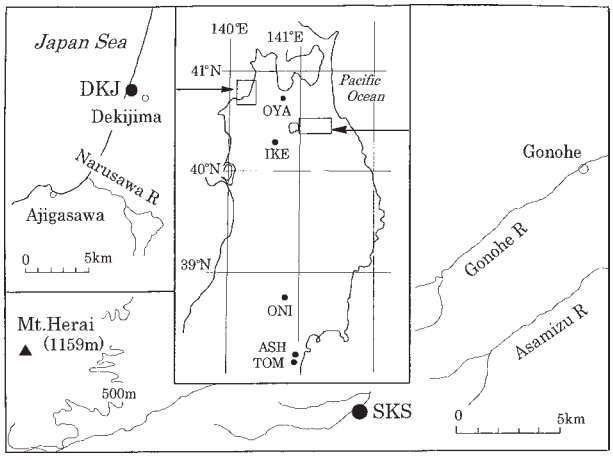


Fig. 1 Localities of fossil samples collected in northeastern Honshu. See Table 2 for abbreviations of locality names.

Method 1: Peat blocks containing fossil cones were dug out from the strata and were packed in polyethylene bags in the field. In the laboratory, fossil cones were then removed from the peat blocks.

Method 2: Fossil cones and needles were isolated in the field, placed, and preserved in a cooler box with dry ice.

Method 3: Fossil needles were isolated in the field and were placed in vinyl bags containing water.

Method 4: Fossil wood disks were cut from buried trunks in the field and were packed in vinyl bags.

Table 2 Fossil samples used in this study

Locality		Geological age (yr B.P.) (Code no.)	Sample number			
			cone	branchlet	wood	seed
DKJ	Dekijima, Kizukuri-machi, Nishitsugaru County, Aomori Pref.	21,160 ± 580[1] (Gak-6970)	15		3	
SKS	Mameda, Shingo-mura, Sannohe County, Aomori Pref.	12,660 ± 150/12,640 ± 150[2] (NUTA-2260/NUTA-2261)	9	11*		
OYA	Oyazawa-noda Site, Aomori City, Aomori Pref.	about 30,000[3]	15			
IKE	Ikenai Site, Odate City, Akita Pref.	about 13,000[4]	1			2
ONI	Onikobe, Narugo-cho, Tamatsukuri County, Miyagi Pref.	unknown	2			
TOM	Tomizawa Site, Sendai City, Miyagi Pref.	19,430 ± 400[5] (Gak-13861)	13	5		
ASH	Ashinokuchi Site, Sendai City, Miyagi Pref.	33,290 ± 2080[6] (Gak-15810)	2			
Total			57	16	3	2

1): Tsuji & Endo, 1978; 2): Noshiro *et al.*, 1997; 3): Tsuji *et al.*, 1998; 4): Terada & Tsuji, 1999; 5): Kigoshi, 1992; 6): Kigoshi, 1998.
* Seven samples were collected by K. Terada.

Each sample was divided into two pieces. One half was used to extract DNA. The remaining half was morphologically identified and was preserved as a voucher specimen in 50% ethanol. The morphological identification was based on the descriptions of fossil (Miki, 1957; Minaki, 1987a, 1987b; Suzuki, 1991; Tsuji *et al*., 1984a, 1984b) and extant *Picea* species (Satake, 1989; Shimizu, 1992).

Locality and stratigraphy of Dekijima and Mameda

Readable DNA sequences were obtained from samples collected at two localities: Dekijima (DKJ) and Mameda (SKS) (Fig.1, the corresponding strata in Fig. 2). DKJ (140°17′E, 40°51′N) is on the Dekijima coast (Endo & Tsuji, 1977) of the Tsugaru Peninsula, facing the Japan Sea, at Kizukuri-machi, Nishi-tsugaru County, western Aomori Prefecture (Fig.1). The Tateoka Formation (Fig. 2) is a glacial deposit from the Last Glacial Age consisting of peat intercalating two layers of fine white ash and one layer of white pumice (Fig. 2). The lower ash layer is identified as Aira-Tn Tephra (AT), the peat layer immediately below being radiocarbon dated at 21,160 ± 580 yr B.P. (Tsuji & Endo, 1978). The age of AT has been re-examined by AMS ^{14}C dating of planktonic foraminifera and is estimated to be 24,330 ± 225 yr B.P. (Murayama *et al*., 1993). The middle ash layer is identified as reworked AT. The upper pumice layer consists mainly of medium to fine white pumice and contains heavy minerals. The Tateoka Formation is directly overlain by the Dekijima Formation consisting of peat and dune sand. Fossil cones and woods were obtained from the peat just below AT in 1997.

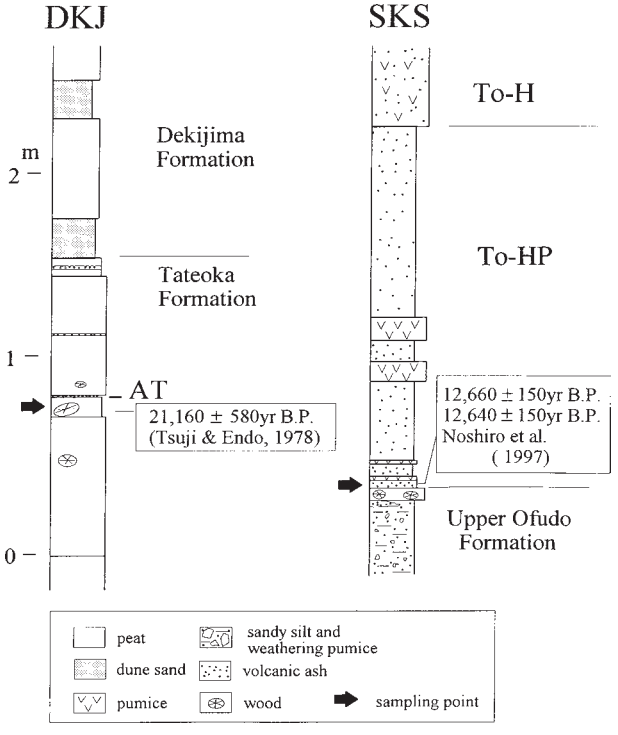


Fig. 2 Stratigraphy of the sampling localities at Dekijima (DKJ) and Mameda (SKS).

SKS (141°11′E, 40°25′N) is on the eastern slope of Towada Volcano at Shingo-mura, Sannohe County, eastern Aomori Prefecture (Fig.1). The Upper Ofudo Formation mainly consists of silt, sand, and gravel, and is overlain by 1–6 cm thick peat. The Upper Ofudo Formation is directly overlain by the Towada-Hachinohe tephra (To-HP, To-H): To-HP is a pumice fall about 2 m thick overlaid by To-H, a pyroclastic flow consisting of alternating fine ash and pumice. This tephra covers buried forests radiocarbon dated at 12,660 ± 150 and 12,640 ± 150 yr B.P. (Noshiro *et al*., 1997). Branchlets with needles were collected from the To-HP. They were green-brown when excavated, but immediately turned dark brown. Branchlets and cones were collected from the same point in 1997 including a branchlet collected by K. Terada in 1996.

Methods

Living plant materials

Target DNA regions were analyzed for their suitability as markers for phylogenetic relationships within the genus *Picea* including fossil specimens. Relatively short DNA regions were investigated for their ability to distinguish living species of *Picea*, because fossil DNA tends to be damaged and fragmented by oxidation and other factors (Golenberg, 1994). One coding and three non-coding regions on chloroplast DNA were chosen. The coding region is the large subunit of ribulose-1,5-bisphophate carboxylase/oxygenase (*rbc*L), and the non-coding regions are associated with the transfer RNAs for threonine (*trn*T), leucine (*trn*L), and phenylalanine (*trn*F). The non-coding regions are (i) an intergenic spacer between *trn*T and the *trn*L 5′ exon, (ii) the *trn*L intron, and (iii) another intergenic spacer between the *trn*L 3′ exon and *trn*F (hereafter referred to as *trn*L a-b, *trn*L c-d and *trn*L e-f region, respectively).

From each sample, four to five needles were cut and ground to powder in liquid nitrogen with a pestle and a mortar. Total DNA was extracted by the modified 2×CTAB method of Hasebe & Iwatsuki (1990).

Four target regions were amplified by the polymerase chain reaction (PCR) method (Saiki *et al*., 1988). Primers used in this study for the *rbc*L gene and three non-coding regions were constructed by Hasebe *et al*. (1994) and Taberlet *et al*. (1991), re-

spectively. The reaction mixture (50 μl) contained 100–200 ng extracted DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTP, 25 pmol of primer, and 1.25 unit rTaq DNA Polymerase (Takara Co.). Amplification was performed using a Quick Thermo Personal QTP-1 (NIPPON GENETICS Co.) or Thermal Sequencer TSR-300 (Iwaki Glass Co.). The PCR cycles were as follows: 1×(94°C for 2 min, 45°C for 2 min, 60°C for 3 min), 30×(94°C for 1.5 min, 45°C for 2 min, 60°C for 3 min), 72°C for 15 min. Amplified target DNAs were electrophoresed and purified using a GENECLEAN II Kit (BIO 101 Co.). Sequences were determined using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Co.) and two sequencers: ABI 373A (Ver. 2.10) DNA Sequencer (Perkin Elmer Co.)and ABI PRISM™ 310 Genetic Analyzer (Ver. 1.0J). The *rbc*L sequences were aligned in comparison with the three known *rbc*L sequences of *Picea pungens* Engelm. (GenBank Accession No. X58136), *Picea sitchensis* (Bong.) Carr. (No. X63660), and *Larix occidentalis* Nutt. (No. X63663). The sequences of the three non-coding regions were aligned with those of *Pinus thunbergii* Parlatore (No. D17510, Wakasugi *et al.*, 1994), whose chloroplast DNA sequence is completely known. Sequences have been deposited in the DDBJ/EMBL/GenBank database (see Table 1 for the Accession No.). Aligned sequences were then used to analyze the phylogenetic relationships of *Picea* species.

We performed heuristic search of the maximum parsimony method using PAUP ver. 3.1.1 (Swofford, 1993). In order to maximize the probability of discovering different islands of shortest trees (Maddison & Maddison, 1992), the analysis comprised 100 replicates with stepwise random taxon addition and the TBR (tree bisection-reconnection) branch swapping algorithm. Bootstrap analyses (Felsenstein, 1985) were performed with 100 replications to infer the relative support for each branch.

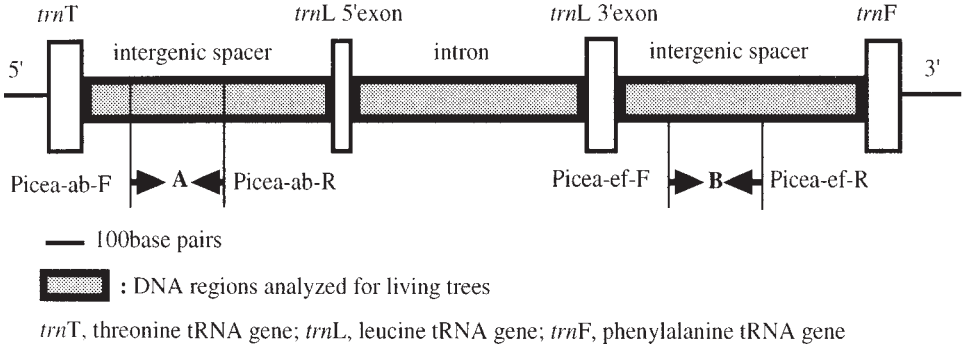
Table 3 Sequences of four primers designed for fossil DNA amplification

Name	Sequence 5' → 3'
Picea-ab-F	TGTAGATTGTAGATTCCTTC
Picea-ab-R	CCTTCTCTCGCCATCTCTAT
Picea-ef-F	GATAGATGATCCACATAGAT
Picea-ef-R	TGCTCTACCAACTGAGCTAT

Fossil plant materials

DNA was extracted from fossils by the same methods as for living materials. The halved fossil cones and small wood blocks had their surface scraped off with a metal knife to remove soil, were frozen in liquid nitrogen, and were ground to powder using a pestle and a mortar. Ten to twenty needles cut from fossil branchlets were ground in the same way. The pestle and mortar used for fossil DNA extractions were sterilized with an autoclave and were never used for living plant DNA analyses. Extraction and PCR negative controls containing reaction mixture without any samples were used in parallel to monitor contamination.

Four PCR Primers, Picea-ab-F, Picea-ab-R, Picea-ef-F, and Picea-ef-R, were newly designed in this study based on extant *Picea* sequences (Fig. 3, Table 3). These primer sequences were well conserved among extant Japanese *Picea* species. These two pairs of primers, (Picea-ab-F, Picea-ab-R) and (Picea-ef-F, Picea-ef-R), amplified approximately 200 base pairs (bp) in the *trn*L a-b and *trn*L e-f regions, which were named regions A and B respectively. PCR amplification for fossil DNA comprised two steps. In the first amplification, the reaction mixture (10 μl) contained template DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl,



*trn*T, threonine tRNA gene; *trn*L, leucine tRNA gene; *trn*F, phenylalanine tRNA gene

Fig. 3 Organization of DNA regions analyzed in the present study. Arrows indicate the orientation and approximate position of primer sites.

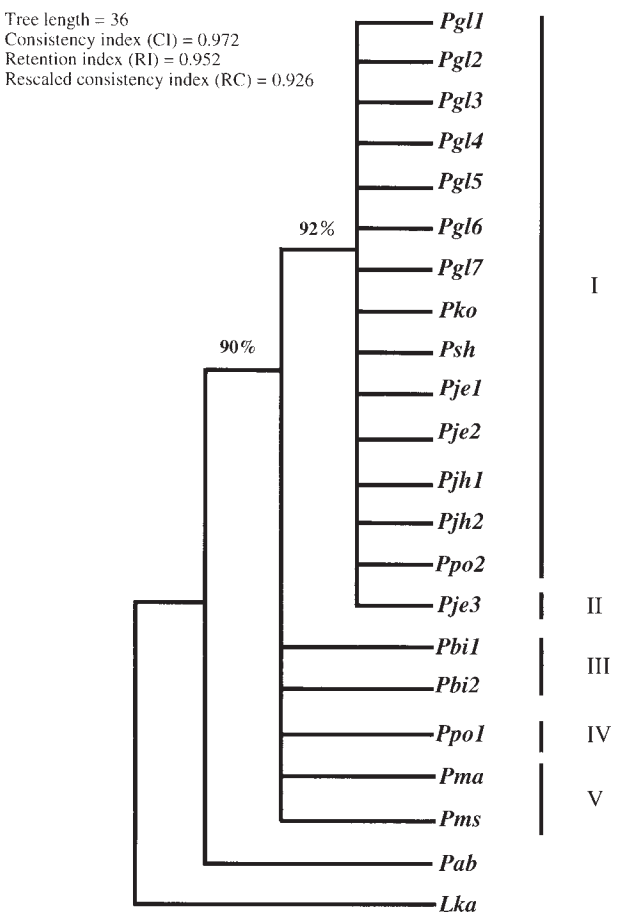


Fig. 4 The strict consensus tree obtained from the six most parsimonious trees using sequence analysis of *rbc*L. The upper numbers at each node indicate the bootstrap value as a percentage based on 100 replicates. See Table 1 for abbreviations of materials. Roman numbers and vertical bars show recognized grouping.

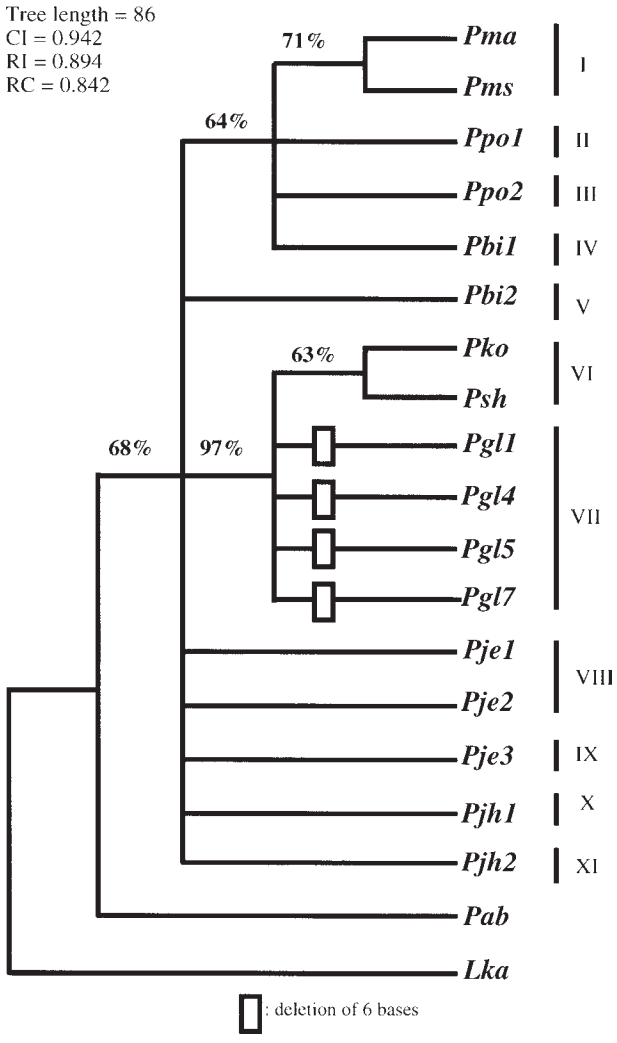


Fig. 5 The strict consensus tree obtained from the 37 most parsimonious trees using sequence analysis of three non-coding regions between the *trn*T and *trn*F. The upper numbers at each node indicate the bootstrap value as a percentage based on 100 replicates. See Table 1 for abbreviations of materials. Roman numbers and vertical bars show recognized grouping.

1.5 mM $MgCl_2$, 10 mM dNTP, 1.25 pmol of each of the two appropriate primers, and 0.25 unit rTaq DNA Polymerase (Takara Co.). PCR cycles were 95°C for 2 min, 20×(94°C for 1 min, 45°C for 4 min, 60°C for 2 min) and 72°C for 15 min ("Booster PCR": Ruano *et al.*, 1989). The second amplification was performed using a 5 μl aliquot of the first PCR product in the same cycles as for the living plant analysis. The PCR products were electrophoresed, purified, and sequenced as for the living plant analysis.

Results

Screening of target regions for fossil DNA amplification

The four regions on the chloroplast DNA (*rbc*L and the three non-coding regions) were compared among extant *Picea* species and were investigated for the kind and number of mutations.

In the *rbc*L region, 1309 bp were sequenced. Seven base substitutions were observed among Japanese *Picea* species. Based on these mutations, Japanese *Picea* species were classified into the five groups sharing the same *rbc*L sequence (Fig. 4): group I consisting of *P. glehnii*, *P. jezoensis* (Pje 1, 2), *P. jezoensis* var. *hondoensis*, *P. koyamae*, *P. shirasawae* and *P. polita* (Ppo 2); group II of *P. jezoensis* (Pje 3); group III of *P. bicolor* (Pbi 1, 2); group IV of *P. polita* (Ppo 1); and group V of *P. maximowiczii* and *P. maximowiczii* var. *senanensis*. *Picea jezoensis* and *P. polita* showed variations within species: Pje 1 and Pje 2 from

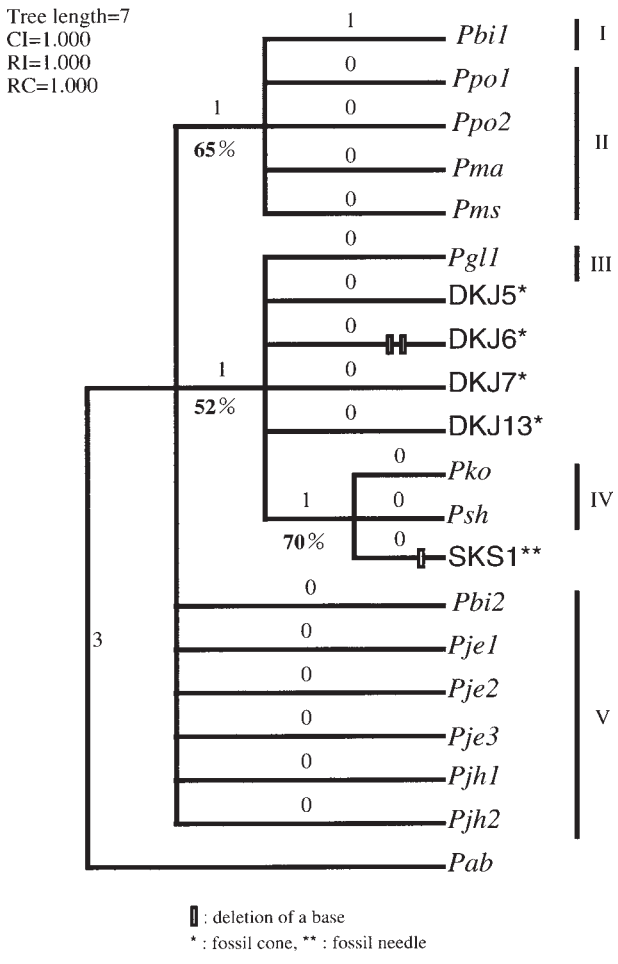


Fig. 6 The most parsimonious tree using sequence analysis of region A for living and fossil *Picea* species. The upper and lower numbers at each node indicate base substitutions per site and bootstrap value as a percentage based on 100 replicates. Roman numbers and vertical bars show recognized grouping.

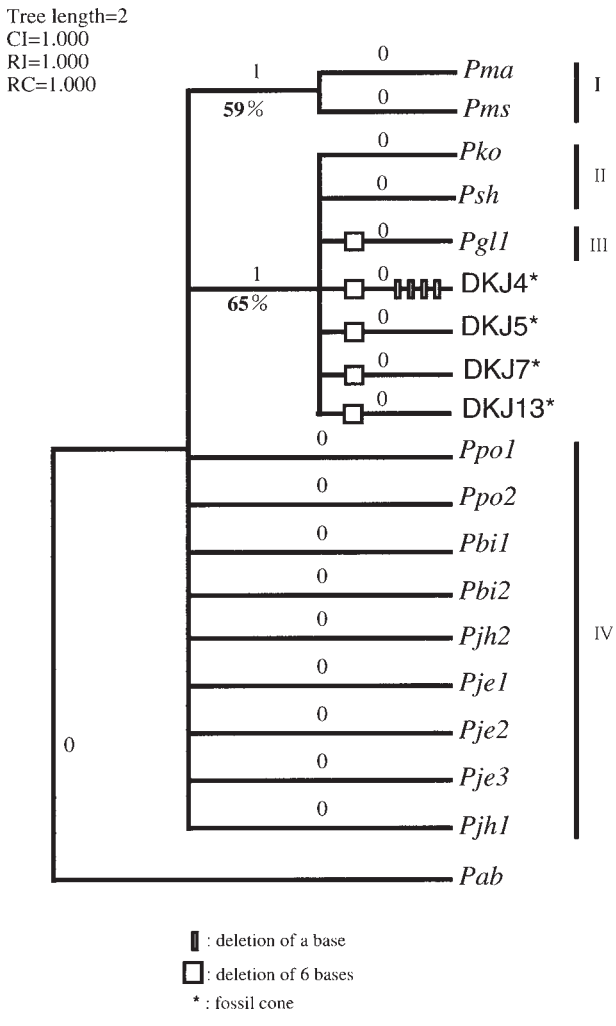


Fig. 7 The most parsimonious tree using sequence analysis of region B for living and fossil *Picea* species. The upper and lower numbers at each node indicate base substitutions per site and bootstrap value as a percentage based on 100 replicates. Roman numbers and vertical bars show recognized grouping.

Pje 3 in 2 bp, and Ppo 1 from Ppo 2 in 5 bp.

In the three non-coding regions, 1179 bp were sequenced: 426 bp in the *trn*L a-b region, 400 bp in the *trn*L c-d region, and 353 bp in the *trn*L e-f region. Fourteen base substitutions and six bp deletions were observed among Japanese *Picea* species: seven substitutions in the *trn*L a-b region, one in the *trn*L c-d region, and six base substitutions and six bp deletions in the *trn*L e-f region. Based on these mutations, Japanese *Picea* species were classified into 11 groups sharing the same sequence (Fig. 5): group I consisting of *P. maximowiczii* and *P. maximowiczii* var. *senanensis*; group II of *P. polita* (Ppo 1); group III of *P. polita* (Ppo 2); group IV of *P. bicolor* (Pbi 1); group V of *P. bicolor* (Pbi 2); group VI of *P. koyamae* and *P. shirasawae*; group VII of *P. glehnii*; group VIII of *P. jezoensis* (Pje 1, 2); group IX of *P. jezoensis* (Pje 3); group X of *P. jezoensis* var. *hondoensis* (Pjh 1); and group XI of *P. jezoensis* var. *hondoensis* (Pjh 2). Pgl 2, 3, and 6 were considered to have the same sequence as other *P. glehnii* samples because seven *P. glehnii* samples had no variations in the *rbc*L region, then these three samples were not sequenced.

Based on these results, the two short regions (regions A and B) were chosen from the *trn*L a-b and *trn*L e-f regions. These regions had more variations in their short sequences than other regions investigated in this study. Region A grouped the extant Japanese species into five groups (Fig. 6) and region B into four groups (Fig. 7).

Fossil DNA extraction and amplification

In the process of DNA extraction from fossil samples, no precipitate was observed. Extracted DNA was electrophoresed, and 25 of the 78 samples showed about 20 kbp fragments. Among 25 samples having 20 kbp fragments, DNA amplification was observed in two samples. Two pairs of primers, (Picea-ab-F and Picea-ab-R) and (Picea-ef-F and Picea-ef-R), amplified about 200 bp fragments in 14 and 17 of the 78 samples, respectively. These 31 PCR products were treated as the template for sequencing, and both strands were sequenced. We succeeded to read the

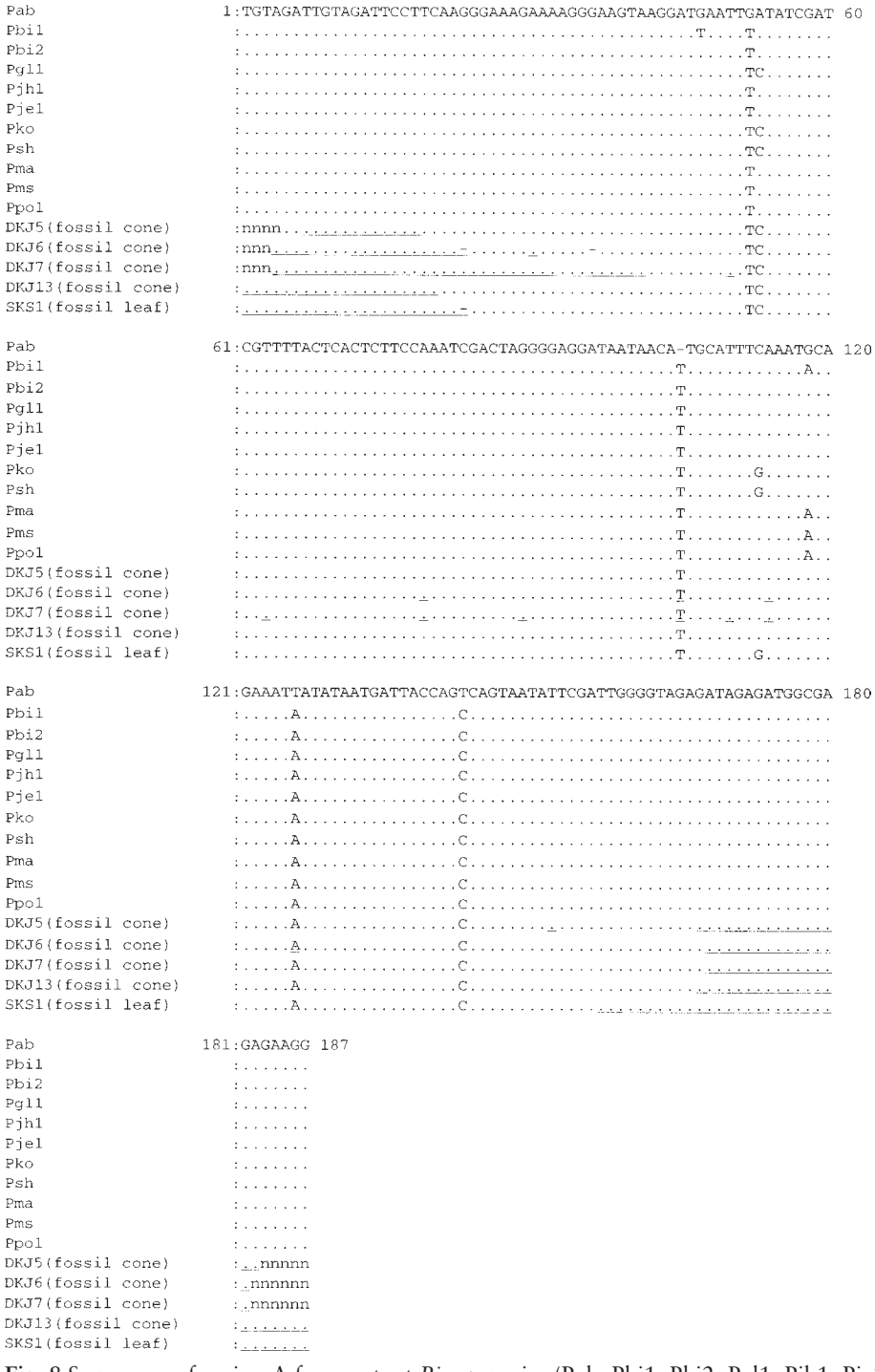


Fig. 8 Sequences of region A from extant *Picea* species (Pab, Pbi1, Pbi2, Pgl1, Pjh1, Pje1, Pko, Psh, Pma, Pms, Ppo1) and fossil samples (DKJ5, DKJ6, DKJ7, DKJ13, SKS1). Dots (.) indicate the same nucleotide as that of Pab; dashes (-) indicate insertion/deletion; (n) indicates an unreadable nucleotide; underlines indicate sequence supported by a single strand.

sequence of nine PCR products, in which the two amplified samples of 20 kbp fragments were not included. Other PCR products had not enough quantity of DNA or were not amplified in the Dye terminator sequencing.

Identification of fossils by DNA sequences

We succeeded to sequence the two regions of fossil DNA in five cones (DKJ4 to 7 and DKJ13) collected at Dekijima and a branchlet (SKS1) collected at Mameda. Of these, successful sequencing of region A was achieved for DKJ5 to 7, DKJ13, and SKS1 (Fig. 8), and of region B for DKJ4, DKJ5, DKJ7, and DKJ13 (Fig. 9). The sequences of regions A and B of DKJ5, DKJ7 and DKJ13 corresponded to that of extant *P. glehnii*. Though the sequences of region A of DKJ6 and SKS1 and that of region B of DKJ4 had deletions specific to each sample, DKJ4 and DKJ6 had a sequence characteristic of *P. glehnii,* and SKS1 had a sequence characteristic of *P. koyamae* and *P. shirasawae*. Amplification of region A of DKJ4 and region B of DKJ6 and SKS1 were not observed. In the amplification of fossil DNA sequence, no DNA amplification was observed in the negative controls. As a supple-

```
Pab                    1:GATAGATGATCCACATAGATGAAATCATTTGGAAATTATTCAGTCGCAGTCCATTTTTTC 60
Pbi1                     :............................................................
Pgl1                     :.....................................------.....A
Pje1                     :............................................................
Pjh1                     :............................................................
Pko                      :...........................................................A
Psh                      :...........................................................A
Pma                      :............................................................
Pms                      :............................................................
Ppo1                     :............................................................
DKJ4(fossil cone)        :nnnnnn..............................------.....-A
DKJ5(fossil cone)        :nnnnnnnnnnnnnnn.....................------.....A
DKJ7(fossil cone)        :nnn.....................------.....A
DKJ13(fossil cone)       :.....................------.....A

Pab                   61:TCATATTAGTGACTTCCAGATTGAAAATAAGAAAGATCATTCTCAAAACTGGAAAAATAG 120
Pbi1                     :............................................................
Pgl1                     :............................................................
Pje1                     :............................................................
Pjh1                     :............................................................
Pko                      :............................................................
Psh                      :............................................................
Pma                      :............................................................
Pms                      :............................................................
Ppo1                     :............................................................
DKJ4(fossil cone)        :............................................................
DKJ5(fossil cone)        :........................_....................................
DKJ7(fossil cone)        :............................................................
DKJ13(fossil cone)       :............................................................

Pab                  121:TTTTTTCCTTATTTTTAGTTGACACAAGTGAAAACCCTGTACCTGGATGATCCACAGGGA 180
Pbi1                     :............................................................
Pgl1                     :............................................................
Pje1                     :............................................................
Pjh1                     :............................................................
Pko                      :............................................................
Psh                      :............................................................
Pma                      :.....................T.....................................
Pms                      :.....................T.....................................
Ppo1                     :............................................................
DKJ4(fossil cone)        :......-.....................-.....................-.
DKJ5(fossil cone)        :......................................................_______
DKJ7(fossil cone)        :............................................................
DKJ13(fossil cone)       :............................................................

Pab                  181:AGAGCCGGGATAGCTCAGTTGGTAGAGCA 209
Pbi1                     :.............................
Pgl1                     :.............................
Pje1                     :.............................
Pjh1                     :.............................
Pko                      :.............................
Psh                      :.............................
Pma                      :.............................
Pms                      :.............................
Ppo1                     :.............................
DKJ4(fossil cone)        :........._____________nnnnn
DKJ5(fossil cone)        :...._____nnnnnnnnnnnnnnnnnnnnnn
DKJ7(fossil cone)        :.........nnnnnnnnnnnnnnnnnnnn
DKJ13(fossil cone)       :........._____________nn
```

Fig. 9 Sequences of region B from extant *Picea* species (Pab, Pbi1, Pgl1, Pje1, Pjh1, Pko, Psh, Pma, Pms, Ppo1) and fossil samples (DKJ4, DKJ5, DKJ7, DKJ13). Dots (.) indicate the same nucleotide as that of Pab; dashes (-) indicate insertion/deletion; (n) indicates an unreadable nucleotide; underlines indicate sequence supported by a single strand.

mental information, *P. glehnii*, *P. koyamae,* and *P. shirasawae* were not distributed and cultivated near the sampling sites or around the laboratory.

Identification of fossils by morphology

Fossils from DKJ

The five fossil cones from DKJ whose DNA sequences were read (DKJ4, 5, 6, 7, and 13) were classified into three taxa based on morphology (Table 4). DKJ5 (Fig. 10-2) and DKJ6 (Fig. 10-3) were identified as *P. glehnii*. DKJ13 (Fig. 10-5) was identified as *P. maximowiczii*. DKJ4 (Fig. 10-1) and DKJ7 (Fig. 10-4) were poorly preserved and could not be identified. They are here called unidentifiable cones.

Fossils from SKS

Among 11 branchlets and nine cones from SKS, only one branchlet yielded a readable DNA sequence. All branchlets from SKS had the same morphology: needles linear, 1.0–1.5 cm long and 0.15–0.30 cm wide (Fig. 10-7), rhomboidal in cross-section with four stomatal zones on each face. This type of *Picea* needle is characteristic of section *Picea* subsection *Picea* (Farjon, 1990), including *P. glehnii*, *P. maximowiczii*, *P. maximowiczii* var. *senanensis*, *P. polita*, *P. bicolor*,

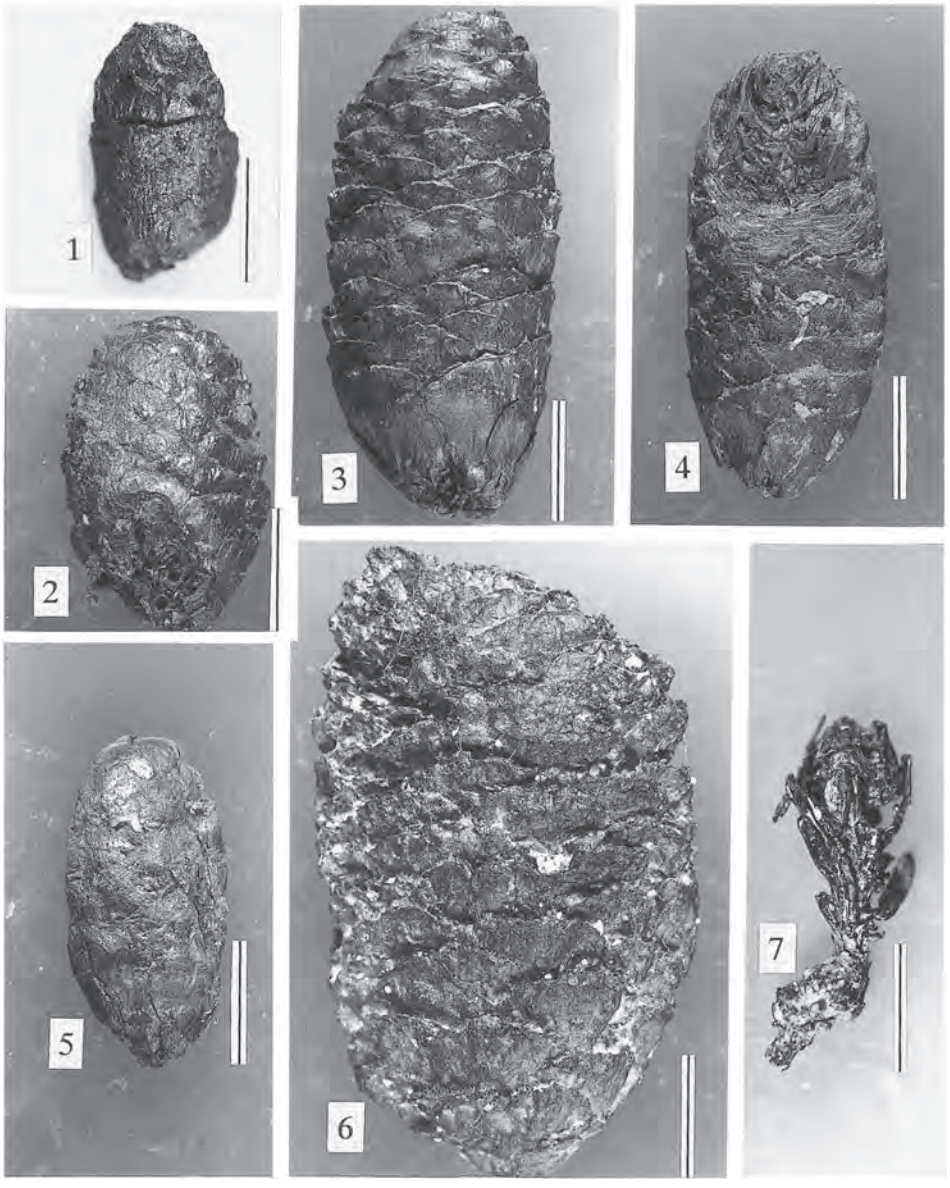


Fig. 10 Fossil cones and a branchlet of *Picea* (collected at localities DKJ and SKS) from which DNA was extracted. — 1: DKJ4, unidentifiable. — 2: DKJ5, *P. glehnii*. — 3: DKJ6, *P. glehnii*. — 4: DKJ7, unidentifiable. — 5: DKJ13, *P. maximowiczii*. — 6: SKSa, *P. koyamae*. — 7: braSKS1, section *Picea* subsection *Picea* branch. Scale bar = 1 cm.

Table 4 Morphological characters of *Picea* fossil cones at DKJ and SKS

Loc.	Sample No.	Cone (mm)			Cone scale (mm)				Morphological identification
		Shape	Length	Width*	Shape	Length	Width	Exposure ratio**	
DKJ	DKJ5, DKJ6	cylindrical	30+	15	rhombic apical margin waved some lines on the surface	15.0	12.0	0.24	*P. glehnii*
DKJ	DKJ13	obovate	35	14	rhombic to fan shaped apical margin rounded no lines on the surface	10.3	8.7	0.48	*P. maximowiczii*
SKS	SKSa	oblongly cylindrical	50+	24	rhombic to fan shaped apical margin waved and thin many lines on the surface	11.5	11.5	0.35	*P. koyamae*
DKJ	DKJ4, DKJ7	weathered	_	_	weathered	_	_	_	Unidentifiable

*circumference of cone / π, **exposed length / cone scale length.

P. shirasawae, and *P. koyamae* (Shimizu, 1992). Fossil cones collected at SKS (SKSa, Fig. 10-6) were all identified as *P. koyamae* (Table 4).

Discussion

Molecular phylogeny of Japanese *Picea*

Among the extant Japanese species of *Picea* examined in this study, there were five types of sequence in the *rbc*L region and 11 in the three non-coding regions (Figs. 4, 5). The agreement of sequences in the four regions in Pko and Psh suggest that *P. koyamae* and *P. shirasawae* are the same species, supporting the results of morphological comparison (Shimizu, 1992) and isozyme analysis (Katsuki *et al.*, 1995). In spite of a fairly wide distribution range and a wide variation in cone morphology (Konishi and Suzuki, 1997), four samples of *P. glehnii* across the distribution range did not show any differences among their sequences. On the other hand, intraspecific variations in DNA sequence were observed for *P. bicolor*, *P. jezoensis*, *P. jezoensis* var. *hondoensis*, and *P. polita*. These species are widely distributed (Hayashi, 1969), and probably possess intraspecific differentiation among populations.

Despite the presence of these intraspecific variations in DNA sequences in Japanese *Picea* species, the topology of the phylogenetic tree derived from the sequences of the three non-coding regions corresponded well to that from the RFLPs of cp DNA reported by Sigurgeirsson & Szmidt (1993). They studied 31 species of *Picea* including six Japanese species and recognized three phyletic groups: the first group including *P. jezoensis*, the second group including *P. polita*, *P. maximowiczii,* and *P. bicolor,* and the third including *P. glehnii* and *P. koyamae*. The first group corresponds to groups VIII to XI in the present study, the second group to groups I to V, and the third group to groups VI and VII (Fig. 5). Based on this correspondence of phylogenetic trees from two different DNA sequence studies, the sequenced regions used in the present study can be considered to reflect phylogenetic relationship in Japanese *Picea* species.

Technical problems with fossil DNA study

There are some serious problems for fossil DNA studies, especially in material sampling and DNA sequence reading. When fossils were dug out from sediment matrix and were exposed to air, their DNA would decompose rapidly. Branchlets recovered from the tuffceous matrix at Mameda changed color from greenish to brownish within several seconds, suggesting a sudden and rapid oxygenation, perhaps indicative of rapid DNA decomposition. Therefore, to obtain fossil DNA, it is manifestly important to develop methods to minimize DNA decomposition.

Among the four collection methods used in the present study, only methods 1 and 3 were successful. Successfully sequenced fossil cones (five out of 15) were extracted from large peat blocks transported to the laboratory (Method 1). One sequenced branchlet was transported to the laboratory in a vinyl bag with water (Method 3). No successful sequencing was achieved for 40 samples treated with Methods 2 and 4. Though some of these 40 fossil materials may no longer have preserved DNA, successes achieved in DKJ and SKS suggest that the employed methods for obtaining and preserving fossil DNA in the field must have been inadequate. Since low temperatures and anoxic conditions are essential for the preservation of DNA, future collections should utilize preservation in liquid nitrogen in the field.

Comparison of molecular and morphological identifications of fossil *Picea*

The successfully sequenced fossil cones had a sequence specific to extant *P. glehnii* in both regions A and B, and the branchlet had a sequence specific to extant *P. koyamae* and *P. shirasawae* in region A (Figs. 6–9). One base deletion detected in region A of DKJ6 and SKS1 and region B of DKJ4 would be decomposition after the death or an amplification error in PCR or could be a variation in *P. glehnii*, *P. koyamae,* and *P. shirasawae*. Fossil cones identified by DNA as *P. glehnii* morphologically corresponded to two species, *P. glehnii* (DKJ5 and 6) and *P. maximowiczii* (DKJ13) (Fig. 10, Table 4). *Picea maximowiczii* has been considered a distinct species by its small obovate cones and cone scales with a smooth entire margin (Hayashi, 1969; Satake, 1989). However, the present study indicates that these characters are included within the variation range of *P. glehnii*, and that fossil identification based only on the current standards of cone morphology may misidentify small cones of *P. glehnii* with smooth cone scales as *P. maximowiczii* (cf. Konishi & Suzuki, 1997). These two species have widely disjunct distribution ranges and can be distinguished easily in the field, but misidentification is possible when dealing with fossil cones. To avoid such misidentification, a more detailed analysis of the morphological variation of extant species using a large number of specimens is required (cf. the study on *P. glehnii*; Konishi & Suzuki, 1997).

The branchlet from Mameda (SKS1) was identified as *P. koyamae* from DNA sequence, treating *P. shirasawae* as its synonym. All fossil cones from the buried forest below To-HP were morphologically identified as *P. koyamae*, and branchlets with needles were identified as section *Picea* subsection *Picea* which includes *P. koyamae*. Thus DNA identification of the branchlet agreed with the morphologically identified species at Mameda.

Meaning of DNA identification *Picea glehnii* and *P. koyamae*

The present identification fossil *Picea* species from two localities is significant for the phytogeography and evolution of *Picea* species during the Last Glacial Age in Japan. *Picea glehnii* is presently distributed in southernmost Sakhalin, Hokkaido (except in the southwest, Hakodate area), and Mt. Hayachine in northern Honshu (Konishi & Suzuki, 1997). *Picea glehnii* cone fossils from the Last Glacial Age are common throughout northern Honshu and Hokkaido (Suzuki, 1991), but its distinction from *P. maximowiczii* and *P. tomizawaensis* is obscure because of their overlapping morphological variations. The present study confirmed occurrence of *P. glehnii* in Aomori during the Last Glacial Age.

The distribution of *P. koyamae* is presently restricted to southeastern Nagano Prefecture. Fossil cones identified as this species and its allies (*P. koyamae*, *P. shirasawae,* and *P.* cf. *shirasawae*) are fairly common in Tohoku and Kanto districts north to Fukushima Prefecture (Miki, 1957; Tsuji *et al*., 1984a, 1984b; Minaki, 1987a, 1987b; Suzuki, 1991). The occurrence of this taxon in Aomori Prefecture revealed in the present paper clarified its quite wide distribution range extending almost to the northern end of Honshu during the Last Glacial Age.

In this study, we showed that DNA analysis of fossil cones and needles is possible and that regions specific to taxa are valuable for identifying species of fossils. DNA identification of fossil species applied to morphologically similar taxa thus contribute to the vegetation historical studies, clarifying past existence of species unidentifiable by conventional methods.

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（辻　誠一郎・鈴木三男・能城修一・山口義伸・太田正文・伊藤昭雄．1998．青森市横内川遊水地の埋没林調査中間報告．12 pp．青森県埋没林調査委員会，青森．）
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書　評：岩槻邦男・加藤雅啓，編．2000．多様性の植物学．1 巻　植物の世界． v + 323 pp. ISBN 4-13-064237-5. **2 巻　植物の系統．** v + 318 pp. ISBN 4-13-064238-3. **3 巻　植物の種．** v + 287 pp. ISBN 4-13-064239-1. 東京大学出版会．各巻 4600 円 + 消費税.

遺伝子の塩基配列を読むことが遺伝学の専門家でなくても可能となったこの10年ほどの間に，植物分類系統学は大きく進展し，周辺領域との結びつきが一層緊密になった。それまでは形態を厳密に比較するしか系統を判断する手だてがなかったものが，現在では遺伝子の進化と形態の進化とを比較して論じる状況になったばかりでなく，形態形成そのものも遺伝子の発現との関連で分子遺伝学的に解析されるようになってきている。本シリーズはそのような植物分類系統学の最先端の状況を周辺領域との関連を踏まえて，包括的に紹介するものである。

本シリーズでは，こうした系統進化学の最先端を紹介する一方で，やはりこの10年ほどのあいだに世界的に問題となってきた生物多様性の危機も中心的な課題として取り上げている。そしてアジアを中心として，植物の多様性が現時点でどの程度まで解明され，まだ何が明らかになっていないかを具体的に描いている。植物相あるいは植物地理の研究はある意味ではひじょうに古典的な学問領域であるが，それすらも未完成なまま，早くも多くの生物がこの地球上から姿を消しており，そういう現状の中で我々は研究を行っているということは認識しておくべきであろう。さらに人間による生育環境の破壊や撹乱によって，単に植物群が失われるだけでなく，現存の植物群の遺伝的な組成にも多大な影響が及ぶことが随所で指摘されている。

この他，植物の種の実態が，近年より具体的に明らかになってきたことも紹介される。やはりこの方面でも遺伝情報を容易に扱えるようになったことが大きな進展に結びついており，集団遺伝学的解析にもとづいた種分化の解明や，植物と昆虫との共進化，送粉様式の多様性の解析，性の進化に対する実証的な研究などが紹介されている。

このように本シリーズは，もっとも現代的な植物学がえがきだす植物像を手っとり早く把握するのには絶好のものといえる。章によって，かなり難易にばらつきがあり，いくつかの章は，その分野の専門知識をそれなりに必要とするが，そうした章は現在，何が研究されているのかの概要をつかめば十分であろう。

植生史の研究を行っていると，とかく分類群に名前をつけ，それから過去の植物群を復原し，その変遷を押さえれば，それで済んだように思いがちである。しかし植物は，それぞれの時代に，現代と同じく，同種の他個体からはじまって，他の植物群や動物群と様々な関わりをもって生きていた。以前に恐竜学がわずかの研究者で，ほとんど骨の化石のみに基づいて多様な成果をあげていることを紹介したが，植生史研究においても，現代の植物学の成果を踏まえて，過去の植物のわくわくするような生き様を解明していきたいものである。

（能城修一）