

## Development of Allozyme Markers in *Abies firma*

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### Introduction

The Japanese fir (*Abies firma* Sieb. et Zucc.) is one of the dominant endemic tree species in Japan, with a natural distribution ranging from Honshu-Island to the islands of Shikoku and Kyushu. *A. firma* is a coniferous evergreen species that is frequently found in warm-temperate zone mixed forests with the hemlock evergreen species, *Tsuga sieboldii* (SHIDEI, 1974), and many ecological studies have been performed on these mixed forests (KABAYA, 1975; KAJI, 1975; OKANO *et al.*, 1999). The forest area of *A. firma* has decreased due to logging for tree plantations, residential development, and other developments. These forests should be conserved as traditional Japanese landscape and as a genetic resource for the future.

The genetic diversity is essential for sustainability of species and populations. Therefore, we need to grasp the present genetic condition of the species or populations. But only one report on genetic diversity of *A. firma* using mtDNA markers (TSUMURA and SUYAMA, 1998) has been made. Moreover, the mating system in the population can be clarified by genetic tools, but no investigation has been made into *A. firma*. Many things are left to be studied through genetic approaches for the conservation of *A. firma* populations.

Many types of molecular markers are available for population genetic analyses, including isozyme, randomly amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), amplified fragment-length polymorphisms (AFLPs), and microsatellite loci. To perform population genetic analyses, the marker is desirable to be codominant for estimation of the exact allelic frequency. Among those molecular markers, allozymes, RFLPs and microsatellite are codominant markers. Recently, the DNA handling techniques have been so improved and many studies have been reported using DNA markers. Comparing these three molecular markers, allozyme analysis still needs the least time and the least expenses and provides useful data for population genetic analysis. Moreover to develop allozyme markers, we don't need any base-sequence information.

To perform population genetic analyses, it is desirable to employ codominant markers at a single locus. Isozyme variants can be encoded by several loci; therefore, before performing population genetic analyses it is first essential to confirm the mono-locus inheritance pattern of each isozyme. To address this issue, the inheritance patterns of several isozyme alleles have been investigated in many tree species (GURIES and LEDGI, 1978; MURILLO and HATTEMER, 1997; NA'IEM *et al.*, 1989; NEALE and ADAMS, 1981; SHIRAIISHI, 1988; TOMARU *et al.*, 1990; TSUMURA *et al.*, 1987). Haploid (n) megagametophytes in the seeds of conifer trees are useful for clarifying the inheritance of isozyme markers. In haploid megagametophytes, allozymes are expected to segregate into a 1 : 1 ratio of Mendelian inheritance (BARTELS, 1971), at each hetero allozyme locus. Therefore by analyzing haploid megagametophytes allozyme loci, the genotype of mother tree can be confirmed easily,

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Table 1. Investigated enzymes and their abbreviations used in this paper along with EC (Enzyme Commission) number.

No.	Enzyme	Abbreviation	EC No.
1	Shikimate dehydrogenase	ShDH	1. 1. 1. 25
2	Glycerate-2-dehydrogenase	G2D	1. 1. 1. 29
3	6-Phosphogluconate dehydrogenase	6PG	1. 1. 1. 44
4	Glucose-6-phosphate dehydrogenase	G6PD	1. 1. 1. 49
5	Glutamate dehydrogenase	GDH	1. 4. 1. 2
6	Diaphorase	DIA	1. 6. 4. 3
7	Glutamate oxaloacetate transaminase	GOT	2. 6. 1. 1
8	Phosphoglucomutase	PGM	2. 7. 5. 1
9	Esterase	EST	3. 1. 1
10	Leucin aminopeptidase	LAP	3. 4. 11. 1
11	Alanine aminopeptidase	AAP	3. 4. 11. 1
12	Aconitase	ACO	4. 2. 4. 3
13	Phosphoglucose isomerase	PGI	5. 3. 1. 9

using at least 6 seeds' megagametophytes.

In this study, we investigated the inheritance of isozyme alleles using haploid megagametophytes in seeds of *A. firma* whose isozyme marker has not developed yet. In practice, collecting cones of *A. firma* is difficult because it is very high tree, so we also analyzed the isozyme of diploid tissue, needle, and showed the zymograms.

### Material and Methods

#### Haploid megagametophyte

Cones of *A. firma* were collected from nine open-pollinated mother trees in December 1999 in the University Forest in Chiba, the University of Tokyo. The cones were subsequently dried and seeds were extracted. All seeds were stored at 4°C until the time of the experiment. Seeds were soaked in a 3% solution of hydrogen peroxide for 24 hours, then stratified on moistened filter papers in petri-dishes at 4°C for more than one month to ensure that the enzymes were active (TSUMURA *et al.*, 1990).

Haploid megagametophytes were excised from the stratified seeds, and each megagametophyte was homogenized in 100  $\mu$ l of extract buffer. Ten  $\mu$ l of the extraction supernatant was loaded onto a vertical polyacrylamide gel electrophoresis slab for each enzyme system. Procedures for extraction, electrophoresis, and staining of the enzymes were as reported by TSUMURA *et al.* (1990). Thirteen enzyme systems were analyzed in this study (Table 1).

Inheritance of isozyme variants was postulated from the banding patterns observed in the megagametophytes of nine mother trees. Chi-square tests were used to examine the goodness of fit to the 1 : 1 ratio expected for single-locus genetic segregation in megagametophytes from heterozygous mother trees.

#### Diploid tissue

Fresh needles were collected from each mother tree from December 2000 to February 2001, and were brought to the laboratory under low temperature conditions, and stored them in a deep freezer at -80°C until the enzymes were extracted. One hundred mg of needle tissue was ground powdery under liquid nitrogen, homogenized in 1 ml extract buffer. Ten  $\mu$ l of the extraction supernatant was loaded onto a vertical polyacrylamide gel electrophoresis slab for each enzyme system. Procedures for extraction, electrophoresis,

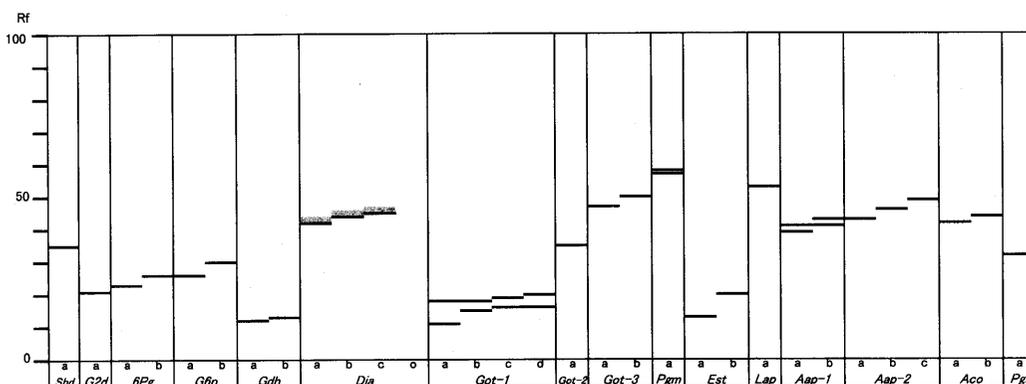


Fig. 1. Megagametophyte banding patterns and their allelic designations for 16 isozyme loci in *Abies firma*.

and staining of the enzymes were as reported by TSUMURA *et al.* (1990). Thirteen enzyme systems as same as haploid megagametophyte were analyzed in this study (Table 1).

## Results and Discussion

### Haploid megagametophyte

Isozymes of 13 enzyme systems were investigated in megagametophyte tissues from 9 mother trees. A total of 16 loci were identified for the 13 enzyme systems. The banding patterns of the isozyme variants that were detected and the Rf (the migrational distance of bands relative to that of the bromophenol blue front) value of each isozyme encoded by each allele in these loci are given in Fig. 1. Six of these loci (*Shd*, *G2d*, *Got-2*, *Pgm*, *Lap* and *Pgi*) had no variants. The inheritance of the other ten polymorphic loci were verified by checking observed segregation ratios for the expected 1 : 1 ratio (Table 2).

#### 1) Shikimate dehydrogenase (ShDH)

One invariant band was observed in all gels stained for ShDH. Two loci of ShDH with a single band in *Abies mariesii* (SUYAMA *et al.*, 1992), and one locus with a single band in *Abies sachalinensis* (NAGASAKA and KOONO, 1990), have been reported. We assume that the band observed in *A. firma* was encoded by a single locus (*Shd*).

#### 2) Glycerate-2-dehydrogenase (G2D)

One invariant single band was observed in all gels stained for G2D. One locus of G2D with a double-band pattern has been reported in *Abies mariesii* (SUYAMA *et al.*, 1992). We assume that the band observed in *A. firma* was encoded by a single locus (*G2d*).

#### 3) 6-Phosphogluconate dehydrogenase (6PG)

The gels stained for 6PG had a single zone of activity with two phenotypes represented by a single band present in each of two families. From the result of a chi-square test, the segregation of bands fitted the expected 1 : 1 ratio well (Table 2). In *Abies balsamea*, one locus of 6PG with a single band has been reported (NEALE and ADAMS, 1981). We assume the bands observed in *A. firma* were encoded by a single locus (*6Pg*).

#### 4) Glucose-6-phosphate dehydrogenase (G6PD)

G6PD gels had a single zone of activity with two phenotypes in one family. The results of chi-square tests showed that the segregation of bands fitted the expected 1:1 ratio (Table 2). The test indicated that the phenotypes were controlled by two alleles in a single locus, *G6p*. A single locus for this enzyme has been observed in *A. mariesii* (SUYAMA *et al.*, 1992), which is in agreement with our result.

Table 2. Segregation of isozyme variations in megagametophytes of heterozygous mother trees.

Locus	mother tree (Family)	Genotype	Segregation	Chi-square for 1 : 1 ratio	P
<i>6Pg</i>	8	a/b	15 : 15	0.00	—
	9	a/b	13 : 11	0.17	0.68
<i>G6p</i>	7	a/b	13 : 10	0.39	0.53
<i>Gdh</i>	1	a/b	14 : 16	0.13	0.72
	2	a/b	20 : 17	0.24	0.62
	5	a/b	37 : 17	7.41	0.01 <sup>1)</sup>
<i>Dia</i>	8	a/b	6 : 6	0.00	—
	7	a/c	13 : 9	0.73	0.39
	1	c/o	21 : 16	0.68	0.41
	2	c/o	15 : 15	0.00	—
<i>Got-1</i>	9	a/b	12 : 10	0.18	0.67
	1	a/d	10 : 13	0.39	0.53
	3	b/c	19 : 14	0.09	0.38
	4	b/c	18 : 13	0.73	0.37
	5	b/c	15 : 20	1.50	0.40
<i>Got-3</i>	8	a/b	13 : 12	0.08	0.84
<i>Est</i>	2	a/b	10 : 14	0.67	0.41
	3	a/b	15 : 9	1.50	0.22
<i>Aap-1</i>	1	a/b	12 : 12	0.00	—
<i>Aap-2</i>	2	a/b	20 : 19	0.03	0.87
	8	b/c	23 : 19	0.73	0.54
<i>Aco</i>	3	a/b	12 : 8	0.80	0.37
	4	a/b	17 : 17	0.00	—
	5	a/b	22 : 19	0.22	0.64

<sup>1)</sup> significant at 0.05 probability

##### 5) Glutamate dehydrogenase (GDH)

GDH gels had a single zone of activity with two phenotypes in three families. The results of the chi-square tests showed that the segregation of bands fitted the expected 1 : 1 ratio in two families (Table 2). In one family, however, the band segregation did not fit the expected 1 : 1 ratio. A similar distortion was observed in the Korean pine (*Pinus koraiensis*) by TOMARU *et al.* (1990). They noted that the observed distortion could have been caused by: (1) chance, (2) the effects of selection, (3) linkage with a lethal gene, or (4) accidental mix with other kindred. We assume that the distortion observed in the present study was caused by the same factors as those discussed for *P. koraiensis*. We suppose the possibilities of the factors of (2) and (3) are high among these 4 factors, because the mother tree, whose segregation of bands did not fit the expected 1:1 ratio, have unusual colored, red, flowers and it might have some unusual genetic characters. In *A. mariesii* (SUYAMA *et al.*, 1992), *A. balsamea* (NEALE and ADAMS, 1981) and *P. koraiensis* (Tomaru *et al.*, 1990), a single locus for this enzyme has been observed. We assume that the bands observed in *A. firma* were encoded by a single locus (*Gdh*).

##### 6) Diaphorase (DIA)

A single zone of prominent bands and several faint bands were observed on gels stained for DIA. The faint bands were omitted from genetic analysis. Four phenotypes in five families were observed. Three phenotypes were composed of single bands, each of

which had a faintly stained anodic side (Fig. 1). The fourth variant had a non-banded phenotype. In *A. mariesii* three variants were observed (SUYAMA *et al.*, 1992), and in *A. sachalinensis* one variant was observed for this enzyme (NAGASAKA and KOONO, 1990). In both species, one variant had a non-banded phenotype. The segregation of bands fitted the expected 1 : 1 ratio well in all families. We assume that the bands observed in *A. firma* were encoded by a single locus (*Dia*).

#### 7) Glutamate oxaloacetate transaminase (GOT)

There were three zones of activity on gels stained for GOT. We assume that the three zones of activity represented bands that were encoded by three different loci. Four double-banded isozyme variants were found in the *Got-1* locus. The *Got-2* locus had an invariant band on all gels and the *Got-3* locus had two single-banded alleles. Four phenotypes in five families were observed at the *Got-1* locus, and two phenotypes in one family were observed at the *Got-3* locus. The segregation of bands fitted the expected 1 : 1 ratio well at each locus. Three loci of GOT have also been observed in *A. mariesii* and *A. balsamea*, and are expressed in the same fashion (SUYAMA *et al.*, 1992; NEALE and ADAMS, 1981). This is in agreement with our results.

#### 8) Phosphoglucomutase (PGM)

A single zone of activity was observed on gels stained for PGM. The zone had an invariant double-banded phenotype. In *A. balsamea*, two active loci were observed for this enzyme, one of which had double-banded alleles (NEALE and ADAMS, 1981). In *A. sachalinensis* two loci with single-banded alleles were observed (NAGASAKA and KOONO, 1990). We assume that the bands observed in *A. firma* were encoded by a single locus (*Pgm*).

#### 9) Esterase (EST)

Several bands were observed on gels stained for EST. However, faint and inconsistent bands were omitted from the test. Consequently, we revealed one locus with two single-banded phenotypes in two families. The segregation of bands fitted the expected 1 : 1 ratio well at each locus. In *A. mariesii*, one locus with two single-banded phenotypes was observed (SUYAMA *et al.*, 1992). We assume that the bands observed in *A. firma* were encoded by a single locus (*Est*).

#### 10) Leucine aminopeptidase (LAP)

Two zones of active bands were observed on gels stained for LAP. However, the bands in one zone were faint whereas those in the other were clear. One invariant band was observed in the clear zone. In *A. mariesii* (SUYAMA *et al.*, 1992), *A. sachalinensis* (NAGASAKA and KOONO, 1990) and *A. balsamea* (NEALE and ADAMS, 1981), two loci encoding for LAP have been reported. We assume that the clear band observed in *A. firma* was encoded by a single locus (*Lap*).

#### 11) Alanine aminopeptidase (AAP)

Three zones of active bands were observed on gels stained for AAP. The bands in two zones were clear and those in the other zone were faint. There were two double-banded patterns in the slower-migrating clear zone, and three single-banded patterns in the faster clear zone. Segregation of these bands indicated the presence of two loci. The segregation of bands fitted the expected 1 : 1 ratio well. We assume that the bands were encoded by two loci (*Aap-1* and *Aap-2*).

#### 12) Aconitase (ACO)

Gels stained for ACO had a single zone of activity with two phenotypes in three families. The results of chi-square tests showed that the segregation of bands fitted the expected 1 : 1 ratio in all families (Table 2). Segregation of these bands indicated the presence of a single locus (*Aco*) with two alleles. This locus is thought to be expressed in

the same way in *A. mariesii* (SUYAMA *et al.*, 1992) and *A. sachalinensis* (NAGASAKA and KOONO, 1990).

### 13) Phosphoglucose isomerase (PGI)

One invariant band was observed in all gels stained for PGI. Two loci of PGI with a single-banded pattern have been reported in *A. balsamea* (NEALE and ADAMS, 1981) and *A. sachalinensis* (NAGASAKA and KOONO, 1990). We assume that the single band observed in *A. firma* was encoded by a single locus (*Pgi*).

We assume three loci which encoded double-banded pattern as *Got-1*, *Pgm* and *Aap-1*. The double-bands may be produced by modification of glucose or phosphate residue etc, or by the segregation. The multiple-bands are difficult to be made out, especially in diploid tissue. But the analysis of inheritance of isozymes described above helps us to make out the genotypes.

The zymograms of allozyme of *A. firma* are compared with those of *A. mariesii* (SUYAMA *et al.*, 1992), *A. balsamea* (NEALE and ADAMS, 1981), *A. sachalinensis* (NAGASAKA and KOONO, 1990), *Pinus koraiensis* (TOMARU *et al.*, 1990) and *P. densiflora* (NA'ITEM *et al.*, 1989). The ratio of loci whose banding patterns and the Rf values are similar to those of *A. firma* are 43% (6/14), 86% (6/7), 56% (5/9), 38% (3/8) and 46% (6/13), respectively. The zymograms of *A. balsamea* is most similar to those of *A. firma*. When it talks especially about the banding pattern, the ratio of similar loci is 79% (11/14), 100% (7/7), 78% (7/9), 50% (4/8) and 62% (8/13), respectively. It suggests that the banding pattern of isozyme is similar within the same genus. Therefore, when making out the genotypes from the banding pattern, we can make reference to the pattern of other species, especially within the same genus.

As described above, 13 enzyme systems with clear banding zones were observed. Genetic variations were observed for 8 of the 13 enzyme systems. It was estimated that the observed enzyme systems were under the control of 16 loci with 31 alleles in total. All alleles of the polymorphic loci, except for *Gdh* in one family, segregated at the expected 1 : 1 ratio. By characterizing the inheritance pattern and levels of variation at these 16 loci, genetic analyses of *A. firma* will now be possible using haploid megagametophyte.

### Diploid tissue

Needles of mother trees were used for analysis of isozymes in diploid tissue. Thirteen enzyme systems were analyzed and clear bands were observed at 9 loci of 8 enzyme systems that were identified in haploid megagametophyte. No clear band was observed for 3 enzyme systems of 13 enzyme systems (G6PD, DIA and EST) and at *Got-1* locus and *Aap-1* locus encoding 2 enzyme systems (GOT and AAP). Clear bands were observed for GDH and ACO, but no varieties could be recognized even in heterozygotes. Six enzyme systems, SHDH, G2D, GOT, PGM, LAP and PGI, showed activities and clear bands were observed at six identified loci (*Shd*, *G2d*, *Got-2*, *Pgm*, *Lap*, *Pgi*), but they had no varieties. We found 3 polymorphic loci (*6Pg*, *Got-3*, *Aap-2*) encoding 3 enzymes as follows. The zymograms are shown in Fig. 2.

#### 1) *6Pg*

Two phenotypes were observed; one had only 1 band and the other had 3 bands, representing the homozygotes and heterozygotes, respectively. This shows that the enzyme encoded by *6Pg* locus is a dimeric enzyme and that is agreement with the report on *Carpinus laxiflora* (KITAMURA *et al.*, 1992b) and on *Fagus crenata* and *F. japonica* (KITAMURA *et al.*, 1992a).

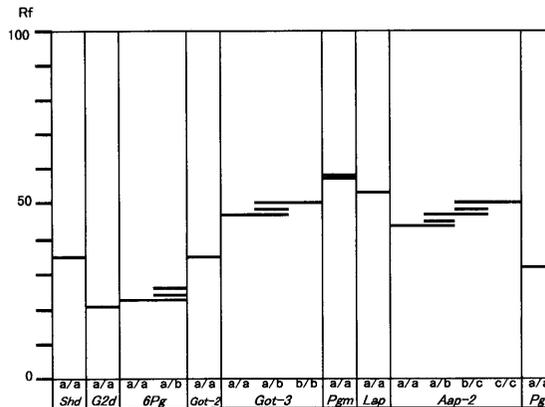


Fig. 2. Needle tissue banding patterns and their allelic designations for 9 isozyme loci in *Abies firma*.

### 2) *Got-3*

Three phenotypes were observed; two had only 1 band and the other had 3 bands. One-banded patterns are for the homozygotes and 3-banded pattern is for heterozygotes. This shows that the enzyme encoded by *Got-3* locus in *A. firma* is a dimeric enzyme as in *Carpinus laxiflora* (KITAMURA *et al.*, 1992b) and in *Fagus crenata* and *F. japonica* (KITAMURA *et al.*, 1992a).

### 3) *Aap-2*

Four phenotypes were observed; two had only 1 band and two had 3 bands. One-banded pattern is for the homozygotes and 3-banded pattern is for heterozygotes. This shows that the enzyme encoded by *Aap-2* locus is a dimeric enzyme.

No clear band was observed at 5 loci which showed clear bands in the megagametophyte. It may be because the inhibitor of enzyme activity is contained in needle more than in megagametophyte, or the two tissues are different in the enzyme activity. This inhibition and the difference in the enzyme activity among tissues are problems in allozyme analysis. Development of stable detection technique for each species and tissue and unification of the tissue are necessary for the effective allozyme analysis.

One clear band was observed but no varieties could be recognized even in heterozygotes at two loci. The bands of the variations observed at each locus in megagametophyte were near by each other. It suggests that the allozymes are very similar and they did not separate clearly on the gel in diploid tissue.

As described above, 8 enzyme systems with clear banding zones were observed. Genetic variations were observed for 3 of the 8 enzyme systems. It was estimated that the observed enzyme systems were under the control of 9 loci with 13 alleles, 15 phenotypes in total. Genetic analyses of *Abies firma* using diploid tissue of needle will now be possible.

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### Summary

Megagametophyte tissues of seeds collected from 9 mother trees of *Abies firma* Sieb. et Zucc were subjected to polyacrylamide vertical slab gel electrophoresis and their isozymes were analyzed to find the marker genes of this species. Of the 13 enzyme systems, 16 loci were identified and 31 allele were found. Diploid tissues and needles of mother trees were analyzed at the 16 loci found in megagametophyte analysis and 13 allele at 9 loci for 8 enzyme systems were found. These results indicate that the techniques described herein would allow the genetic analysis of this species using megagametophyte or needles.

**Key words:** *Abies firma*, isozyme, inheritance, megagametophyte, needle

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## モミのアロザイムマーカの開発

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### 要 旨

モミの標識遺伝子を検出することを目的として9母樹から採取した種子の雌性配偶体を用いて、平板ポリアクリルアミドゲル垂直電気泳動法を用いて、アイソザイムの遺伝分析を行った。その結果13酵素種を支配する16遺伝子座を明らかにし、31対立遺伝子を得ることができた。また、二倍体である針葉を用いてアイソザイム分析を行い、雌性配偶体で明らかにした16遺伝子座のうち、8酵素種9遺伝子座で13対立遺伝子が判別可能であった。ここで得られた遺伝子座を利用することにより、雌性配偶体または針葉を用いてモミの遺伝的な解析が可能となった。

**キーワード:** モミ, アイソザイム, 遺伝, 雌性配偶体, 針葉

## Development of Allozyme Markers in *Abies firma*

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Megagametophyte tissues of seeds collected from 9 mother trees of *Abies firma* Sieb. et Zucc were subjected to polyacrylamide vertical slab gel electrophoresis and were analyzed to find the marker genes of this species. Of the 13 enzyme systems, 16 loci were identified and 31 allele were found. Diploid tissues and needles of mother trees were analyzed at the 16 loci found in megagametophyte analysis and 13 allele at 9 loci for 8 enzyme systems were found. These results indicate that the techniques described herein would allow the genetic analysis of this species using megagametophyte or needles.

## Geology and Stratigraphy of the Sorachi and Yezo Groups in the Tokyo University Forests in Hokkaido, Japan

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The upper Jurassic to lower Cretaceous Sorachi Group is distributed along the western part of the Tokyo University Forests in Hokkaido, Japan. The Sorachi Group in this area consists mainly of mafic and intermediate igneous rocks, with subordinate volcanogenic sedimentary rocks. The group is divided into the Gokurakudaira Formation, Chikushidake Micromonzonite, Nunobe Formation, Ogurose Formation, and Rokugo Formation, in ascending order. An unconformity is found between the Chikushidake Micromonzonite and the Nunobe Formation, and the basal conglomerate on the unconformity contains oolite limestone blocks. These facts suggest that the Sorachi basin rose above the sea level, and ooids were formed around the shallow coast of an emerged basin.