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Effective DNA extraction method for fragment analysis using capillary sequencer of the kelp, *Saccharina* sp.

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Abstract

The DNA fragment analysis can become an effective tool to study genetic differences between not only species but also individuals on saccharinan kelp from which the little genetic diversity was reported. This time, extraction method of suitable DNA for use in the analysis with a capillary sequencer was examined on *Saccharina japonica* var. *diabolica* that contains polysaccharide abundantly. When AFLP was performed using genomic DNA extracted by seven different methods: (1) commercial kit, (2) original CTAB method, (3)-(5) three types of modified CTAB method, (6) modified SDS method, (7) combination of CTAB method and SDS method, high reproducible peak that was worth for the analysis was noticeable in the electropherogram in the experiment with the last combination method (7). It is considered that the pretreatment washing of polysaccharide and the subsequent purification for protein and RNA in SDS method and for polysaccharide in CTAB method are effective to obtain the high purity DNA.

Key words

AFLP, DNA extraction, DNA purification, fragment analysis, *Saccharina*

Introduction

In various locations globally, including the north of Japan, various saccharinan kelps form marine forests and play an important role in maintaining the marine ecosystem, such as in primary production, environmental control and provision of habitat for marine animals (e.g. Bartsch et al. 2008; Lobban and Harrison 1994). It is important to understand the genetic structure on component kelps to conserve these forests (e.g. Kusumo and Druehl 2000; Macaya and Zuccarello 2010). In the north of Japan, most of these kelps have been important sources of food for the Japanese for more than 1,000 years. The commercial value of a kelp product is strongly determined by not only the species but also the species variant from which it is derived. For this reason and because the morphological characteristics of Japanese *Saccharina* are known to be unreliable as a basis of identification across the genus, objective identification based on genetic information is indispensable (e.g. Yotsukura et al. 2008).

In the case of Japanese *Saccharina*, sequence comparison has been conducted on several DNA regions (e.g. ITS rDNA, 5S rDNA spacer, RuBisCo spacer, total mtDNA), and they were shown to exhibit little genetic divergence between species variants and populations (Yotsukura 2005; Yotsukura et al. 1999, 2006, 2010). Consequently, a high-resolution approach such as polymorphic analysis is required for differentiation between different species variants and populations.

In any polymorphic analysis, it is indispensable to obtain reproducible results. Therefore, it is very important to use high-quality DNA for all analyses. However, since saccharinan kelp contains a large amount of polyphenol and viscous polysaccharides such as alginic acid and fucoidan, the extraction of pure DNA from kelp samples is not easy because of interference by these substances. Amplified fragment length polymorphism (AFLP) is a powerful technique for elucidation of genetic diversity among the various types of polymorphic analysis because of its ability to identify a large number of genetic differences. However, for this method, DNA templates with extremely high purity are desirable. To date, for *Saccharina*, AFLP has been used to survey the genetic diversity and the phylogenetic relationships among cultivated strains and wild strains of *S. japonica* (Shan et al. 2010). In this previous study, the results were produced by comparison of electrophoresis profiles with large-size gel, not by comparison of electropherograms with a genetic analyzer (fluorescence-based capillary electrophoresis system), which is now a more widely utilized approach. The latter approach offers more abundant objective data but needs higher-quality DNA templates. Therefore, in this study, we attempted to establish a DNA extraction procedure that can be used for any polymorphic analysis with reproducible results by the AFLP method

with a genetic analyzer.

Materials and methods

An immature sporophyte of *S. japonica* (J.E. Areschoug) C.E. Lane, C. Mayes, Druehl & G.W. Saunders var. *diabolica* (Miyabe) Yotsukura, Kawashima, T. Kawai, T. Abe & L. D. Druehl was collected from Aikappu, Akkeshi (Hokkaido Island, Japan), on May 10, 2010, and taken to a laboratory immediately. The lamina was cut into small pieces with a razor (approximately 5.0 cm × 5.0 cm) and washed thoroughly with filtered sterilized seawater; it was subsequently stored in silica gel in a freezer (-80 °C) until DNA extraction.

DNA extraction

Fourteen microtubes were prepared. A small piece of a dry blade of the sporophyte (approximately 10–20 mg) was cut finely and placed in each microtube. Genomic DNA was extracted from the tissue fragment in the original tube by the following seven methods. For each method, two tests of extraction were attempted using one dry sample.

1. DNA extraction kit
The commercially available DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) was used. The procedure followed the manufacturer's instructions.

2. CTAB method
A basic method was followed using cetyl trimethylammonium bromide (CTAB), which was the same as that described by Shan et al. (2010).

3. Modified CTAB method (I)
The CTAB method was preceded by a removal of the alginic acid from the tissue according to Shiroma et al. (2007) with modification. The modification included a reduction by half of reaction times for softening of tissue with hydrochloric acid and for solubilization of alginic acid with sodium carbonate.

4. Modified CTAB method (II)
Before DNA extraction, mucilaginous polysaccharide including alginic acid in the tissue was removed as follows: The tissue fragment was suspended in 1ml of 0.1% β-mercaptoethanol, and stored at room temperature for 90 min. The solution was then

centrifuged at 13,000 × g for 1 min at room temperature, and the upper aqueous phase containing polysaccharide was removed. Subsequently, the precipitates were rinsed with 1 ml of distilled water ten times. Incubation time was room temperature for 1 min for the first five rinses and 65 °C for 3 min for the last five rinses. Subsequently, DNA was extracted by the above-mentioned basic CTAB method.

5. Modified CTAB method (III)

Before DNA extraction, polysaccharide was removed as described in the modified CTAB method (II). The DNA extraction was subsequently carried out by the applied CTAB method described by Barnwell et al. (1998) and Haque et al. (2004) with some modification. The precipitates were suspended in 700 µl of CTAB buffer (2% CTAB (w/v), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1% polyvinyl pyrrolidone (w/v), 1% β-mercaptoethanol (v/v), 10 µg/ml RNase A) and preserved at 65 °C for 60 min. After centrifugation at 1,500 × g for 20 min at room temperature, the supernatant was transferred to a fresh microtube, and an equal volume of chloroform/isoamylalcohol (CIA) (24:1) was added. The mixture was agitated and centrifuged at 1,500 × g for 20 min at room temperature. The CIA treatment was repeated two more times. Subsequently, a volume of CTAB buffer (10% CTAB (w/v), 0.7 M NaCl) equal to that of the aqueous solution was added and an equal volume of cold isopropylalcohol was also added to the mixture. After the mixture was stored at -20 °C for 20 min, the DNA was recovered at 13,000 × g for 10 min at 4 °C. The precipitates were washed twice with cold 70% ethanol. After drying in an evaporator, the DNA pellet was dissolved in 100 µl of 0.1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)).

6. Modified SDS method

Before DNA extraction, polysaccharide was removed as described in the modified CTAB method (II). The subsequent DNA extraction was carried out by the sodium dodecyl sulfate (SDS) method described by Dellaporta et al. (1983) with some modification. The precipitates were suspended in 750 µl of extraction buffer (100 mM Tris-HCl, 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol) and 50 µl of 20% sodium SDS solution (w/v), and were stored at room temperature for 30 min. After centrifugation at 20,000 × g for 20 min at 4 °C, the supernatant was transferred to a fresh microtube, and 0.7 volumes of cold isopropylalcohol were added. The mixture was agitated and stored at -20 °C overnight. The DNA pellet was recovered at 20,000 × g for 20 min at 4 °C and, after drying, was re-suspended in 750 µl of TE buffer at 65 °C

for 60 min. Subsequently, 0.1 volumes of 3 M NaAc and 0.7 volumes of cold isopropylalcohol were added. After the mixture was stored at -20 °C for 60 min, the DNA was recovered by centrifugation at 20,000 × g for 10 min at 4 °C. The precipitates were washed twice with cold 70% ethanol. After drying in an evaporator, DNA pellet was dissolved in 100 µl of 0.1 × TE buffer.

7. Combination of CTAB method and SDS method

Before DNA extraction, polysaccharide was removed as described in the modified CTAB method (II). The precipitates were suspended in 700 µl of the extraction buffer and 50 µl of 20% SDS solution (w/v), and were stored at 65 °C for 30–45 min. One-third volume of 5 M KAc was added, and the mixture was stored at -20 °C for 15 min. After centrifugation at 20,000 × g for 10 min at 4 °C, the supernatant was transferred to a fresh microtube, and 0.7 volumes of cold isopropylalcohol were added. The mixture was agitated and stored at -20 °C for 15 min. The DNA pellet was recovered by centrifugation at 20,000 × g for 10 min at 4 °C and washed twice with cold 70% ethanol. After drying in an evaporator, the DNA was re-suspended in 100 µl of 0.1 × TE buffer or TE buffer containing 1 µl of RNase A at room temperature overnight.

Subsequently, the DNA solution was suspended in 500 µl of CTAB buffer and stored at 65 °C for 30 min. Furthermore, 200 µl of 5 M KAc was added and the mixture was kept on ice for 15 min. After centrifugation at 20,000 × g for 10 min at 4 °C, the supernatant was transferred to a fresh microtube, and an equal volume of CIA (24:1) was added. The mixture was agitated and centrifuged at 20,000 × g for 10 min at room temperature. The CIA treatment was repeated two more times. Subsequently, 0.8 volumes of cold isopropylalcohol were added to the aqueous solution. After the mixture was stored at -20 °C for 20 min, the DNA was recovered by centrifugation at 20,000 × g for 10 min at 4 °C. The precipitates were washed twice with cold 70% ethanol. After drying in an evaporator, the DNA pellet was dissolved in 50–100 µl of 0.1 × TE buffer.

DNA purification

The extracted DNA was purified with GENECLEAN II (Bio 101 Inc., La Jolla, CA, USA) following the manufacture’s instruction.

AFLP analysis

The enzyme digestion and adaptor ligation template of AFLP involved the same constituents as described by Shan and Pang (2009) to the exception of addition of 0.05 M NaCl and incubation of the mixture for 8 hours at 37 °C. For preselective PCR, AFLP Ligation and Preselective Amplification Modules (Regular Plant Genome and Small Plant Genome) (Applied Biosystems, Foster, CA, USA) were used, and, for selective PCR with *Eco* RI selective primer (-ACC) and *Mse* I selective primer (-CAC), AFLP Selective Amplification Startup Modules (Regular Plant Genome and Small Plant Genome) (Applied Biosystems) were used. The profiles of preselective and selective PCR were set with reference to the protocol of Plant Mapping by Applied Biosystems. The selective PCR product was mixed with electrophoresis buffer (deionization formamide: blue loading dye:Gene Scan-500 ROX Standard = 5:1:1), and, subsequently, the solution was used for electrophoresis with a 3130 Genetic Analyzer (Applied Biosystems). On the basis of the obtained raw data, sizing and typing of each fragment were carried out with Gene Mapper software, ver. 4.1 (Applied Biosystems), and the electropherograms that have been obtained in two tests per DNA extraction method were compared. In the analysis, number of peaks in the electropherogram and total number of inconsistent peaks among the electropherograms were counted in certain extent of fluorescence intensity. The value of fluorescence intensity was decided considering the general standard (100 rfu) in AFLP analysis (e.g. Coyer et al. 2011) and the strict standard (20 rfu) that is minimum value that could finally distinguish peak from noise.

For the analysis, for which reproducibility of data had been obtained remarkably, reanalysis was attempted by using DNAs from nine different sporophytes. In the reanalysis, original selective primer pair E-AAC/M-CTC was tested.

Results

The obtained electropherograms varied according to the DNA extraction methods applied in this study (Table 1).

1. DNA extraction kit

With the criterion for acceptance of a peak in the electropherogram being set at a fluorescence intensity of superior or equal to 100 rfu, 17 and 19 peaks were confirmed in the two images (Fig. 1A). Among these peaks, only two were found in both images and even some peaks that showed strong fluorescence intensity in one of the images were not universally found.

2. CTAB method

Seventeen and eighteen peaks with a fluorescence intensity of superior or equal to 100 rfu were confirmed in the two images (Fig. 1B). Among these, four were found in one of the images, and this discrepancy involved a failure to detect the peak because of weak fluorescence intensity of either. When the criterion for acceptance of a peak was set at a fluorescence intensity of superior or equal to 50 rfu, 23 and 30 peaks were counted. Although there were eight discrepancies among them, all the differences involved a failure to detect the peak because of weak fluorescence intensity of either. Furthermore, when setting thresholds of superior or equal to 25 rfu and superior or equal to 20 rfu, 39 and 50 (13 discrepancies between them) peaks and 53 and 57 (15 discrepancies between them) peaks were counted, respectively. Most of the differences involved a failure to detect the peak because of weak fluorescence intensity of either, but, on 3 of 15 discrepancies with the threshold of superior or equal to 20 rfu, even the very small peak was not visible in one of the images (Fig. 2).

3. Modified CTAB method (I)

The specimen was disintegrated excessively by the acid treatment in the first stage of alginic acid extraction, and the level of successful genomic DNA recovery decreased remarkably.

Six and ten peaks with a fluorescence intensity of superior or equal to 100 rfu were confirmed, and only one of these peaks was found in both images; in the case of a threshold of superior or equal to 50 rfu there were 7 and 26 peaks, and only two of these peaks were found in both images (Fig. 3A). With the thresholds of superior or equal to 25 rfu and superior or equal to 20 rfu, 24 and 36 (22 discrepancies between them) peaks and 20 and 32 (27 discrepancies between them) peaks were counted, respectively. In these analyses, all the discrepancies involved presence versus absence of a peak.

4. Modified CTAB method (II)

The reproducibility of peaks in electropherograms by the modified CTAB method (II) was higher than that by the basic CTAB method.

Twelve and twenty-two peaks were confirmed with a fluorescence intensity of superior or equal to 100 rfu. Among these peaks, five were not found in both images; the discrepancy involved a failure to detect the peak because of weak fluorescence intensity of either (Fig. 3B). When the criterion for acceptance of a peak was set at a fluorescence intensity of superior or equal to 50 rfu, 13 and 16 peaks were counted. Although there were three discrepancies between two images, all the differences

involved a failure to detect the peak because of weak fluorescence intensity of either. With the thresholds of superior or equal to 25 rfu and superior or equal to 20 rfu, 23 and 24 (nine discrepancies between them) peaks and 28 and 29 (11 discrepancies between them) peaks were counted, respectively. Most of the discrepancies were just from differences in intensity, but, for 2 of 9 with the threshold of superior or equal to 25 rfu and 2 of 11 with the threshold of superior or equal to 20 rfu, the peak was not visible in one of the images.

5. Modified CTAB method (III)

In the modified CTAB method (III), results resembling those obtained by the basic CTAB method were detected (Fig. 3C). However, the number of peaks that appeared only in either one image or the other was greater than in the basic CTAB method.

Ten and thirteen peaks were confirmed for a fluorescence intensity of superior or equal to 100 rfu, and five were not found in both images. However, in both cases, one peak was seen at every position even though it was small in size. In addition, 13 and 16 peaks were counted with a fluorescence intensity threshold of superior or equal to 50 rfu, and four were not found in both images. These differences were also related to their intensity. For the thresholds of superior or equal to 25 rfu and superior or equal to 20 rfu, 19 and 23 (7 discrepancies between them) peaks and 25 and 28 (7 discrepancies between them) peaks were counted, respectively. At the positions where a difference was detected, one of the peaks was not visible in two of seven images for superior or equal to 25 rfu and three of seven for superior or equal to 20 rfu.

6. Modified SDS method

Using the modified SDS method, almost the same number of peaks was obtained in the two electropherograms, even with a high threshold of fluorescence intensity.

Twelve and fifteen peaks were confirmed for a fluorescence intensity of superior or equal to 100 rfu (Fig. 3D). Among the discrepancies at four positions, only one involved a difference of presence versus absence of a peak. When the criterion for acceptance of a peak was set at a fluorescence intensity of superior or equal to 50 rfu, 15 peaks were counted in both images. Among the discrepancies at three of them, two involved presence versus absence of a peak. With a threshold of superior or equal to 25 rfu, 19 and 20 peaks were counted. Among these, five were not found in both images and two of them were seen in only one electropherogram. Moreover, with a threshold of superior or equal to 20 rfu, 25 and 28 peaks were counted. In both electropherograms, many very small peaks were detected and 7 of the 12 discrepancies involved presence versus

absence of a peak.

7. Combination of CTAB method and SDS method

By this combinatorial approach, results with extremely high reproducibility compared with those by the other methods were obtained (Figs. 3E).

Fourteen and fifteen peaks were confirmed for a fluorescence intensity of superior or equal to 100 rfu. Although one peak was not common in both electropherograms, a peak was found in both images at this position and the discrepancy depended on failure to detect a peak owing to low fluorescence. In a similar way, with a fluorescence intensity threshold of superior or equal to 50 rfu, there were 21 and 22 peaks, and one peak, albeit minor, could be found at a discrepant position by visual analysis. With fluorescence intensity thresholds of superior or equal to 50 rfu and superior or equal to 20 rfu, 37 peaks in both images (five discrepancies between them) and 41 and 42 (four discrepancies between them) peaks were counted, respectively. The discrepancies did not involve presence versus absence of a peak but rather a difference in the intensities of visible peaks.

As the result of reanalysis for which DNAs extracted from nine individuals was used, similar number (36-38) of peak with a fluorescence intensity of superior or equal to 100 rfu in any electropherogram and eight peaks that reflect polymorphic loci were detected (Fig. 4).

Discussion

Various methods of DNA extraction have been presented (e.g. Murray and Thompson 1980; Dellaporta et al. 1983; Barnwell et al. 1998; Haque et al. 2004; Wang et al. 2006; Ghosh et al. 2009); now, handy kits for DNA extraction are available and commonly used in general research (e.g. Drábková et al. 2002; Shepherd et al. 2002; Yotsukura et al. 2010). However, it is known that extraction of pure DNA is difficult for certain organisms, and new methods for these cases are being developed (Kejani et al. 2010; Jena 2010). In general, it is thought that DNA extraction from seaweed is not easy because it contains polysaccharide and polyphenol (Shivji et al. 1992; Kitade et al. 1996; Phillips et al. 2001). The polysaccharide increases the viscosity of the extraction solution, and so impedes agitation and pipetting and inhibits the activities of DNA polymerase and restriction enzymes (Crowley et al. 2003). The polyphenol is oxidized readily, and the oxide acts to prevent solubilization of extracted DNA pellet and to inhibit enzyme reactions (Porebski 1997; Khanuja et al. 1999).

This time, no reproducible electrophoretic profiles were obtained using a DNA

extraction kit that helps in sequencing analysis on kelp. One particular feature of the kit is that only a limited amount of extraction solution is used for DNA elution compared with the amount of sample. Because there are high levels of polysaccharides such as alginic acid and fucoidan in kelp, it is thought that, when only a small amount of extraction solution is used, the viscosity of the solution increases due to the polysaccharide and it is not uniformly mixed.

The DNA extraction method using CTAB developed by Murray and Thompson (1980) has been introduced in various studies, and applied methods have been reported to be effective for DNA extraction from samples containing a large amount of polysaccharide because CTAB binds to nucleic acid in the aqueous layer and selectively precipitates the polysaccharide under high NaCl concentration (e.g. Ghosh 2009; Jena et al. 2010). In laminarial algae, some AFLP analyses with extracted DNA by the modified CTAB method were carried out and successful results were reported (Kusumo and Druehl 2000; Shan and Pang 2009; Shan et al. 2010). However, in all the analyses, polyacrylamide gel was used for electrophoresis and unclear bands were specifically excluded. Meanwhile, in AFLP analysis with a capillary sequencer, even bands with weak fluorescence appear as clear peaks in the electropherogram. To date, little AFLP research using extracted DNA by the modified CTAB method with a capillary sequencer has been carried out on seaweed (e.g. Donaldson et al. 1998, 2000; Niwa et al. 2004). In the limited research on brown seaweed: *Postelsia* (Kusumo et al. 2004) and *Fucus* (Coyer et al. 2011), the electropherograms were not reported, but only peaks with high fluorescence intensity (100 rfu $\leq$) were accepted in the study by Coyer et al. (2011). High-intensity peaks in the study showed a certain level of reproducibility, though there were few peaks, which limits the reliability of the analysis.

The pretreatment involving extraction of alginic acid was expected to reduce the influence on viscosity of polysaccharide binding to DNA. Hence, reproducible results were not obtained by both modified CTAB methods (I) and (II) in this study. The reason for this is thought to be that DNA was degraded by the long-term acid treatment needed to liberate alginic acid. Despite this, alginic acid that flowed out to the surface of tissue and hydrosoluble polysaccharide in the tissue, such as fucoidan, seemed to be removed to some extent by repeated washing of the sample. It is now thought that washing with distilled water imparts little damage to the cell. However, it is generally known that DNase is activated in the presence of Mg^{2+} (Adams et al. 1999), and furthermore it is reported that a rice species (*Oryza sative*) has 13 kinds of DNase and each of them is activated in the presence of a different metal ion (Sodmergen et al. 1991). Therefore, the reason for the existence of peaks only in one of two images seems to be that the

washing improves the activity of DNase and promotes the degradation of DNA.

There is a commonly-held view that protein adsorbs extracted DNA and, accordingly, it influences the result of PCR. It is known that high purity DNA is isolated by formation of complex with positive-charged protein and SDS that is anionic surfactant agent, under the potassium ion existence (Dellaporta 1983; Niu et al. 2008). In the DNA extractive process with SDS, the surfactant is added when extracted DNA is dissolved because it inhibits the activity of RNase. Hence, RNA is resolved for sufficient time, since it takes time to dissolve dry DNA. On the contrary, it was reported that further purification in this method is necessary for organisms for which DNA extraction is difficult (Weising et al. 2005). In the present study, DNA extraction by the combination of CTAB method and SDS method after warming treatment and washing of polysaccharide on the surface of lamina with distilled water could remove the protein, the RNA and the polysaccharide effectively and an analytical result with high reliability could be obtained. Incidentally, SDS inhibits the enzyme reaction (e.g. Goldenberger et al. 1995; Davis et al. 1980) and, therefore, it is effective to perform CTAB method after SDS method to prevent SDS remained in the DNA solution.

As described before, binding of polyphenol to the extracted DNA causes decreased reproducibility in AFLP analysis. It is known that polyvinylpyrrolidone (PVP) adsorbs the polyphenol and β-mercaptoethanol shows an antioxidative effect (e.g. Porebski et al. 1997; Dempster et al. 1999; Khanuja et al. 1999; Brunner et al. 2001; Biss et al. 2003; Bharmauria et al. 2010; Kejani et al. 2010). In the present study, the extraction solution with the modified CTAB method contained PVP and β-mercaptoethanol, and it is suggested that this DNA extraction technique is effective both to remove the polyphenol and to curb the oxidization of polyphenol.

As a whole, from the confirmation of high reproducibility of the results even in the analysis by strict standard (20 rfu≤) and the detection of polymorphic locus in the reanalysis by general standard (100 rfu≤), it was shown that high purity genomic DNA extracted by combination of CTAB method and SDS method was useful as the template for AFLP analysis.

In this study, reproducible results were obtained from genomic DNA extracted by the method prioritizing the removal of polysaccharide. However, the method requires considerable time from extraction to purification, including for preprocessing. Furthermore, there is a risk of cross-contamination during the extraction because a procedure involving the replacement of DNA solution twice or more is necessary. However, there is a benefit that the method does not require hazardous chemicals such as phenols, and nor is enzyme processing such as using proteases or

super-centrifugation necessary. Generally, in view of the frequent need to treat a large number of samples in DNA fragment analysis, it is thought that the method established in this study is an extremely effective way to extract pure DNA to obtain reproducibility in the analysis of seaweed with abundant polysaccharide, such as kelp, with a capillary sequencer.

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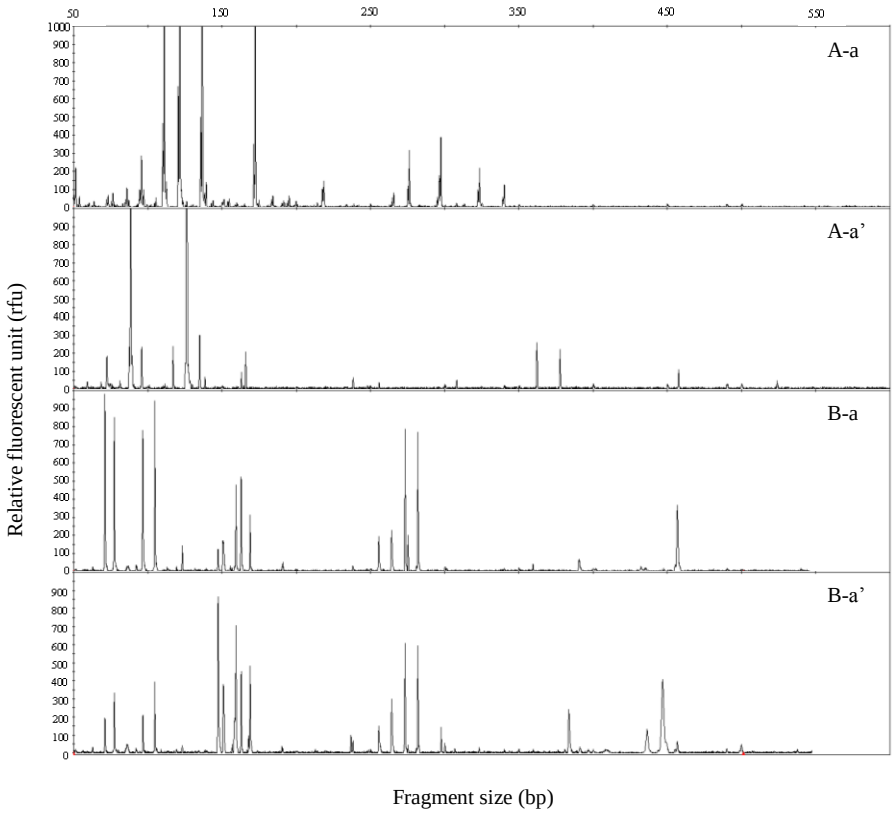
Fig. 1 Comparison of electrophelogram in the AFLP analysis with primer pair E-ACC/M-CAC on two samples that were from one individual of S. *japonica* var. *diabolica* from Aikappu, Akkeshi (a & a’). The template DNA was extracted by DNA

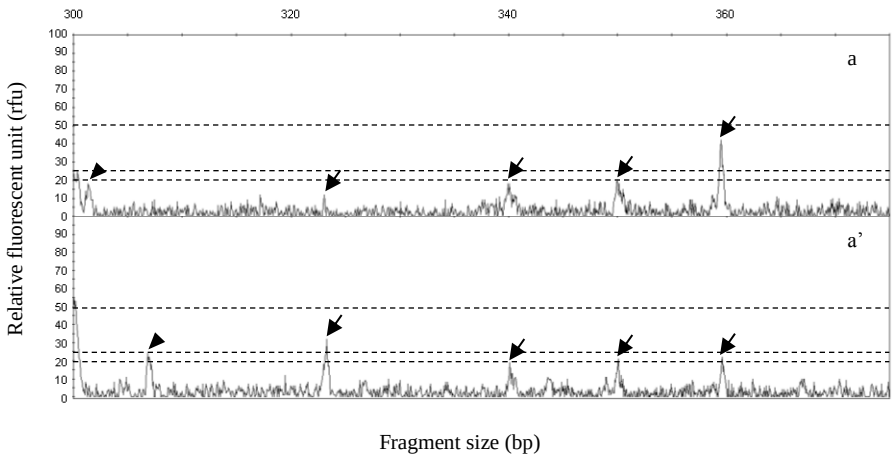
extraction Kit (A) and CTAB method (B). Preselecive amplification was performed with preselective amplification primer pairs for Small Plant Genome (Applied Biosystems, Foster, CA, USA). The horizontal scale represents the size of fragments in base pairs (bp), while the vertical scales represents the fluorescence intensity of a fragment (rfu) in a run.

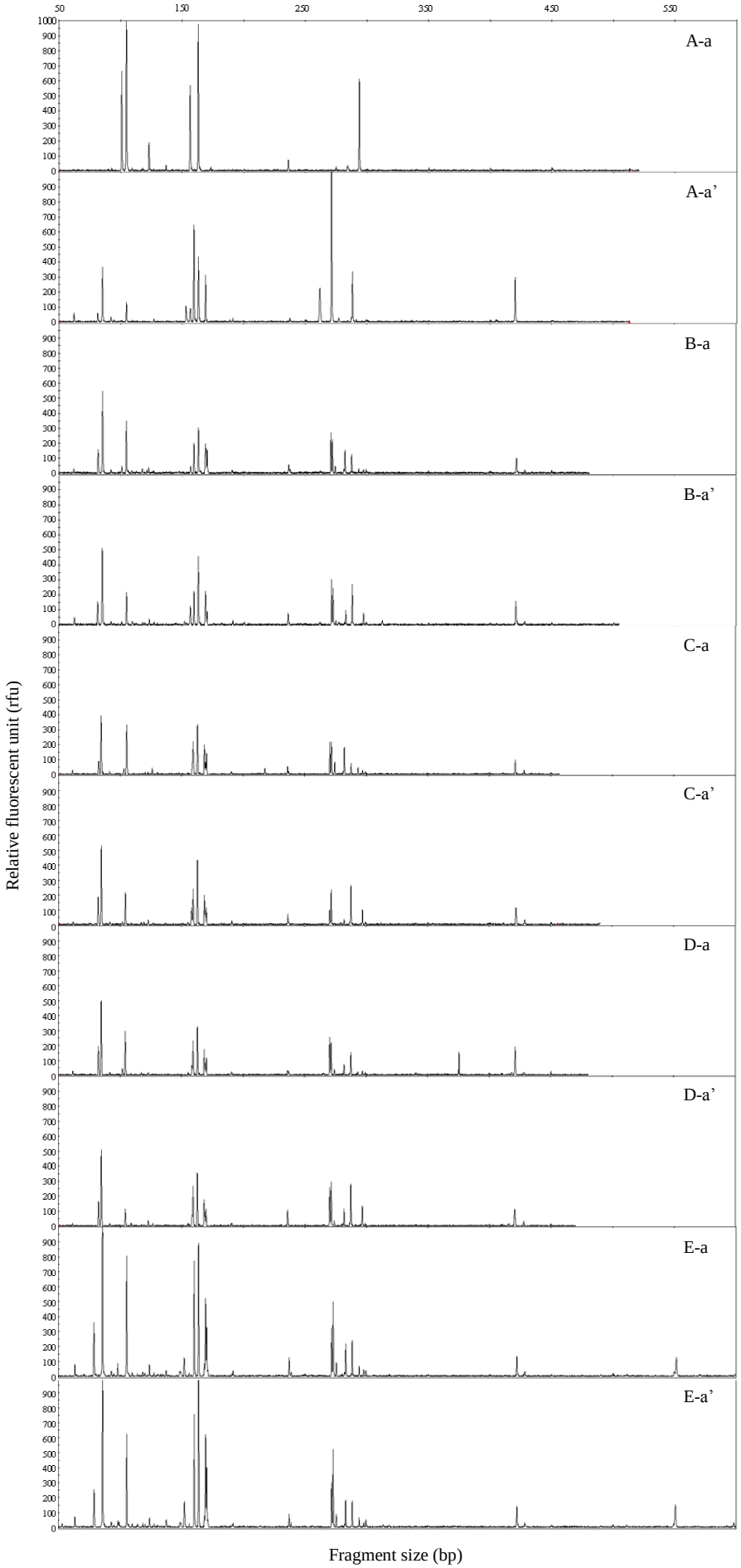
Fig. 2 Partial enlargement of elactrophelogram in the AFLP analysis with primer pair E-ACC/M-CAC on two samples that were from one individual of S. *japonica* var. *diabolica* from Aikappu, Akkeshi (a & a'). The template DNA was extracted by CTAB method. Preselecive amplification was performed with preselective amplification primer pairs for Small Plant Genome (Applied Biosystems, Foster, CA, USA). The horizontal scale represents the size of fragments in base pairs (bp), while the vertical scales represents the fluorescence intensity of a fragment (rfu) in a run. The arrows indicate peaks to which fluorescent strength is weak in either (a) or (a'). The arrow heads indicate peaks detected in either (a) or (a').

Fig. 3 Comparison of electrophelogram in the AFLP analysis with primer pair E-ACC/M-CAC on two samples that were from one individual of *S. japonica* var. *diabolica* from Aikappu, Akkeshi (a & a') The template DNA was extracted by Modified CTAB method (I) (A), Modified CTAB method (II) (B), Modified CTAB method (III) (C), Modified SDS method (D), Combination of CTAB method and SDS method (E). Preselective amplification was performed with preselective amplification primer pairs for Small Plant Genome (Applied Biosystems, Foster, CA, USA). The horizontal scale represents the size of fragments in base pairs (bp), while the vertical scales represents the fluorescence intensity of a fragment (rfu) in a run.

Fig. 4 Comparison of electrophelogram in the AFLP analysis with primer pair E-AAC/M-CTC on nine samples that were from different individuals of S. *japonica* var. *diabolica* from Aikappu, Akkeshi (a-i). The template DNA was extracted by combination of CTAB method and SDS method. Preselecive amplification was performed with preselective amplification primer pairs for Small Plant Genome (Applied Biosystems, Foster, CA, USA). The horizontal scale represents the size of fragments in base pairs (bp), while the vertical scales represents the fluorescence intensity of a fragment (rfu) in a run. The heavy gray lines indicate positions of polymorphic loci.


50
150
250
350
450
550
A-a
A-a'
B-a
B-a'
Relative fluorescent unit (rfu)
Fragment size (bp)


300
320
340
360
a
a'
Relative fluorescent unit (rfu)
Fragment size (bp)


50
150
250
350
450
550
A-a
A-a'
B-a
B-a'
C-a
C-a'
D-a
D-a'
E-a
E-a'
Relative fluorescent unit (rfu)
Fragment size (bp)

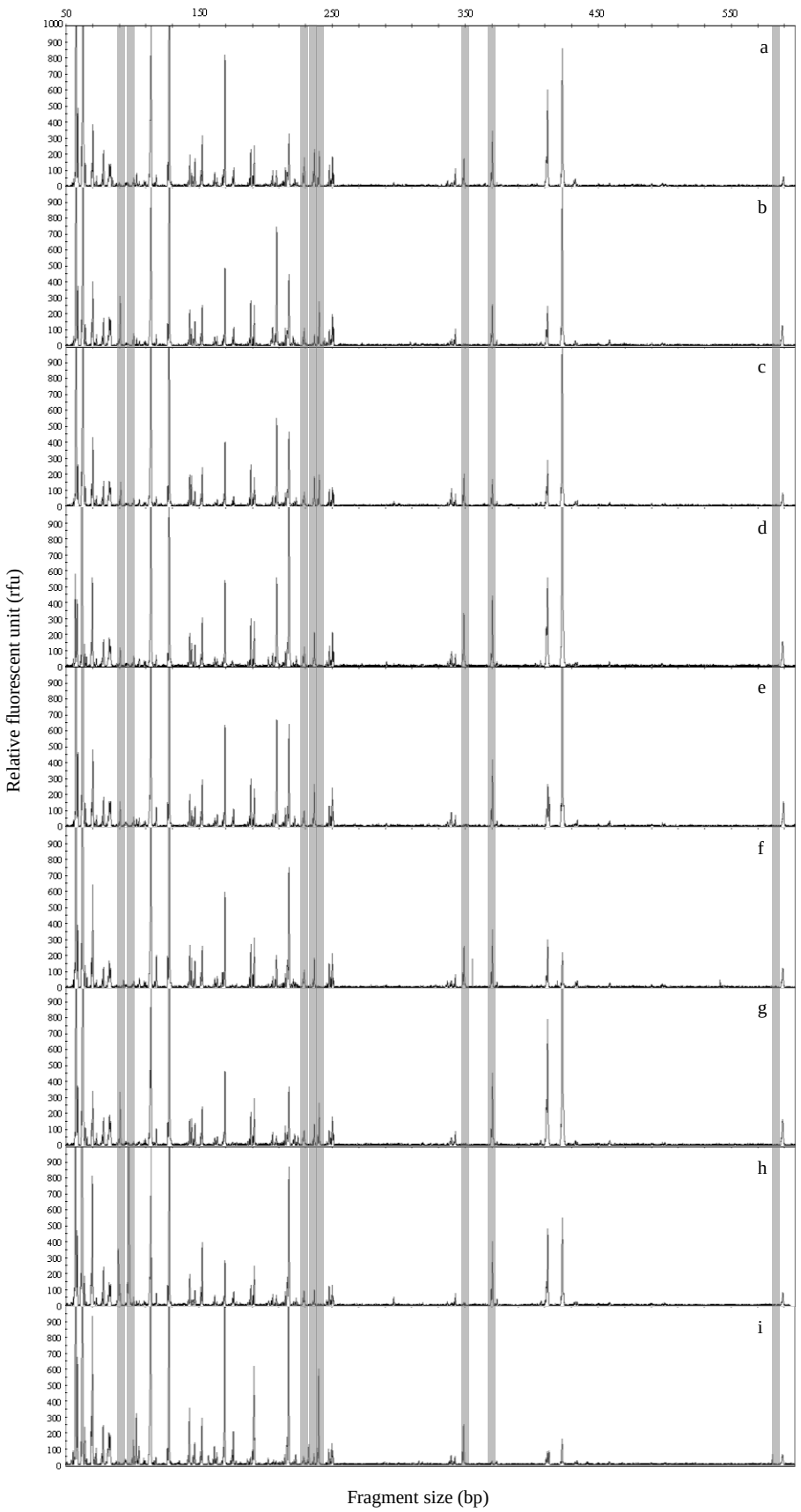

50
150
250
350
450
550
a
b
c
d
e
f
g
h
i
Relative fluorescent unit (rfu)
Fragment size (bp)

Table 1 Cmparison of number of peaks detected in setup condition of fluorecence intensity in AFLP analysis. NP indicates the number of peaks in a elecrtropherogram. ND indicates the nuber of discrepancies between in two electropherograms.

DNA extraction method	100 rfu ≤		50 rfu ≤		25 rfu ≤		20 rfu ≤	
	NP	ND	NP	ND	NP	ND	NP	ND
DNA extraction kit	17 19	17	- -	-	- -	-	- -	-
CTAB method	17 18	4	23 30	8	39 50	13	53 57	15
Modified CTAB method (I)	6 10	15	7 26	31	24 36	22	20 32	27
Modified CTAB method (II)	12 22	5	13 16	3	25 20	9	23 24	11
Modified CTAB method (III)	10 13	5	13 16	4	19 23	7	25 28	7
Modified SDS method	12 15	4	15 15	3	19 20	5	25 28	12
Combination of CTAB method and SDS method	14 15	1	21 22	1	37 37	5	41 42	4

rfu: relative fluorescence unit