Simple and Rapid DNA Extraction from Milled Rice and Its Application to Thai Aromatic Rice (*Oryza sativa* L.) variety, Khao Dawk Mali 105

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Abstract

An aromatic variety, Khao Dawk Mali 105 (KDML 105), is one of the most important varieties of rice in Thailand. However, the increase of production is limited by adverse environmental conditions. As a result, varieties different from KDML 105 are often marketed under the name of KDML 105. Therefore, an efficient and objective method of cultivar identification is urgently needed. Molecular marker techniques based on the polymerase chain reaction (PCR), have provided objective methods of identification. To apply this technique to milled rice, high quality DNA from a single grain of milled rice is needed. DNA extraction method presented here is based on a freeze-and-thaw cycle that enables to extract purified high molecular weight grain DNA without the use of expensive equipment and/or time-consuming procedures. Microsatellite analysis method for cultivar identification was used for extracted DNA. The author analyzed rice samples obtained from Bangkok, Suwannaphum, and Kuala Lumpur markets, and selected varieties different from KDML 105 in Bangkok and Kuala Lumpur market rice samples. By this extraction procedure, we were able to obtain DNA for microsatellite analysis within 2hr and identification required 6hr in total.

Key words : cultivar identification, PCR, rapid extraction, genomic DNA

Introduction

A large quantity of rice is produced in the world presently. Rice farmers need to produce rice which can meet the demand of consumers. Aromatic rice, which has a stronger aroma than ordinary rice, has been very popular in Southeast Asia[5, 6] and has recently gained a wide acceptance in Europe and U.S.A. An aromatic variety, Khao Dawk Mali 105 (KDML105), is mainly produced in Northeast Thailand. Demand for this variety is increasing in both domestic and international markets, due to the recognition of its good quality. Although increase of production is urgently needed, the cultivation is limited due to infertile and drought-striken sandy soil. Erratic rainfall at the beginning of the rainy season and labor shortage for transplanting are other constraints on the production of KDML 105. As a result, varieties different from KDML 105 are often marketed under the name of KDML 105, because no objective method of evaluation has been developed for this variety.

In Japan, since rice is distributed as brown rice which can germinate, information about the varieties can be obtained. However, in Southeast Asia, rice is mainly distributed as milled rice which can not germinate. To identify varieties within milled rice mixed with varieties with a similar appearance, conventional methods like the determination of the length-width ratio are not suitable.

Molecular marker techniques based on the polymerase chain reaction (PCR)[10], have provided more objective methods for the identification of grain varieties. These techniques like microsatellite analysis, randomly amplified polymorphic DNA (RAPD)[12] and amplified fragment length polymorphism (AFLP)[7] could be very useful for plant-based identification.[4, 14] Microsatellite analysis is particularly useful for simple and rapid analysis. To apply this technique to milled rice, high quality DNA must be extracted from a single grain of milled rice. Preparation of DNA from single rice seedlings was reported[11], but milled rice cannot germinate.

Components of milled rice are mainly starch and protein. It is difficult to extract DNA, because the solubility of these components is the same as that of DNA. Especially, enzyme-inhibiting polysaccharides are often present in the "purified" DNA. Extraction procedures for simple and rapid identification of grain variety require that cell walls be broken, cell membrane be disrupted, DNA be protected from endogenous nucleases, and polysaccharides be removed. Murray & Thompson[8] reported a DNA extraction procedure from plant tissues using a detergent, CTAB (cetyltrimethylammonium bromide). This method has been widely used not only for plant tissues but for also grains.[3] However it takes 2 days to extract DNA from grain, and a large amount of sample is required. The author reported the use of a simple DNA extraction method[15], but this method needs harmful reagent such as benzyl bromide. The method presented here is based on a freeze-and-thaw cycle that enables to extract purified high moleclar weight grain DNA without the use of expensive equipment and/or time-consuming procedures.

In this paper, simple and rapid DNA extraction from milled rice is described, and this procedure was applied to identification of KDML 105 and varieties with similar appearance by microsatellite analysis.

Materials and Methods

1) Samples

Authentic milled Thai rice varieties, Khao Dawk Mali 105, Chainat 1(CNT1), and RD 7 was obtained from Pathumthani Rice Research Center, Thailand. Two samples, "Khao Dawk Mali" and "Northeastern Khao Dawk Mali", were obtained from markets in Bangkok and Suwannaphum, Thailand, respectively. Malaysian sample, "Beras Wangi Thai", was obtained from market in Kuala Lumpur, Malaysia. For the market samples, 5 grains each were selected randomly and subjected to the identification.

2) Rapid DNA extraction method from single grain of milled rice (freeze-thaw method)

For each sample, a single grain of milled rice was heated at 99 ℃ with 70μl of ddH_2O. Heated sample was transferred to a fresh tube, and homogenized in 300μl of 1/10 diluted TE buffer (10mM Tris-HCl 1mM EDTA, pH8.0) with a pellet mixer (Treff 9.7339.9.01). A 400μl aliquot of phenol saturated with

TE buffer was immediately added, the solution was vigorously mixed and incubated in a freezer (-20 ℃) for 1h. The solution was thawed at room temperature. After centrifugation at 10,000*g* for 15min, the supernatant was transferred to a fresh tube. An equal volume of phenol : chloroform : 3-methyl-1-butanol (50:49:1 v/v/v) was added and the solution was mixed. After centrifugation at 10,000*g* for 10 min, the supernatant was subjected to DNA precipitation in 1/25 volume of 5M sodium chloride, and 2.5 volumes of ethanol. The DNA pellet was washed twice in 70%(v/v) ethanol, dried at room temperature and resuspended in 30μl of 1/10 TE buffer containing 10mg/ml RNAse.

The presence of genomic DNA was confirmed by electrophoresis on a 0.7%(w/v) agarose gel in TAE buffer (40mM Tris-Acetic acid, 1mM EDTA, pH8.0), stained with ethidium bromide and visualized under UV illumination as well as quantified. Quality was evaluated by absorbance at 260 and 280nm using a SHIMADZU UV-1200 spectrophotometer.

Table 1. Primer sequences used for cultivar identification of milled rice [1, 2, 9, 13]

Locus		Sequence
OSR6	Forward	CCAAGGGAAAGATGCGACAA
	Reverse	GTGGACGCTTTATATATGGG
OSR18	Forward	CCCCAAGGATAATATCAGGAG
	Reverse	TGCGTATGTTTGTTCCCAAG
OSR22	Forward	CTGAGTCTCCTGTCCTCATC
	Reverse	CTTGAATCTCTGCACTGCAC
OSR28	Forward	AGCAGCTATAGCTTAGCTGG
	Reverse	ACTGCACATGAGCAGAGACA
OSR29	Forward	GCTAGCAGCTATAGCTTAGC
	Reverse	AGACTGCCTGTGAGATCACA
OSR32	Forward	CTCCAGCTTCGGCAACGTCA
	Reverse	CTTCTTGATGCCCTCAATCGT
RM1	Forward	GCGAAAACACAATGCAAAAA
	Reverse	GCGTTGGTTGGACCTGAC
RM4	Forward	TTGACGAGGTCAGCACTGAC
	Reverse	AGGGTGTATCCGACTCATCG
RM10	Forward	TTGTCAAGAGGAGGCATCG
	Reverse	CAGAATGGGAAATGGGTCC
RM16	Forward	CGCTAGGGCAGCATCTAAAA
	Reverse	AACACAGCAGGTACGCGC
RM17	Forward	TGCCCTGTTATTTTCTTCTCTC
	Reverse	GGTGATCCTTTCCCATTTCA
RM19	Forward	CAAAAACAGAGCAGATGAC
	Reverse	CTCAAGATGGACGCCAAGA
RM122	Forward	GAGTCGATGTAATGTCATCAGTGC
	Reverse	GAAGGAGGTATCGCTTTGTTGGAC

3) Microsatellite analysis (PCR)

Selected primers, flanking the tandem repeats[1, 2, 9, 13] shown in Table 1, were used for amplification of genomic DNA fragments. PCR amplifications were performed in a total volume of 20μl containing 3μl of extract, 0.1mmol of each primer, 200mM each of dNTP, 50mM KCl, 10mM Tris-HCl (pH8.3), 0.01% gelatin, 3mM $MgCl_2$ and 0.5unit *rTaq* DNA polymerase (TOYOBO). PCR was initiated by a denaturation step at 94°C for 2min, followed by 35 cycles of 55°C for 1min, 72°C for 1min, 94°C for 1min and a final extension at 72°C for 5min. PCR was performed on a Corbett Research PC-960G. Amplification products were resolved by electrophoresis on a 3% agarose gel in TAE buffer, stained with ethidium bromide and visualized under UV illumination.

The reactions were repeated at least 3 times to confirm the reproducibility of the results.

Results and Discussion

1) Rapid DNA extraction from single grain of milled rice (freeze-thaw method)

The purity of the DNA, determined from the A_{260}/A_{280} ratio averaged > 1.78 for all the samples. There was no evidence of degrading of DNA during preparation (Fig. 1). The yield of DNA ranged from 200 to 300 ng/single grain for all the samples.

Since a sample of milled rice was once heated to break the cell wall and to denature endogenous nucleases in the method (Fig. 1). Polysaccharides, inhibiting the enzyme reaction, were removed efficiently during the freezing and thawing cycle.

2) Identification of variety by using microsatellite analysis

The primer set RM17 was particularly useful for discriminating the 3 varieties (Fig. 2). The market samples were analyzed by RM17.

Fig. 3 shows the results of microsatellite analysis of market samples on agarose gel. "Beras Wangi Thai", sampled in a Kuala Lumpur market, and "Khao Dawk Mali", in a Bangkok market, did not correspond to Khao Dawk Mali 105 variety. Khao Dawk Mali 105 was identified only from "Northeastern

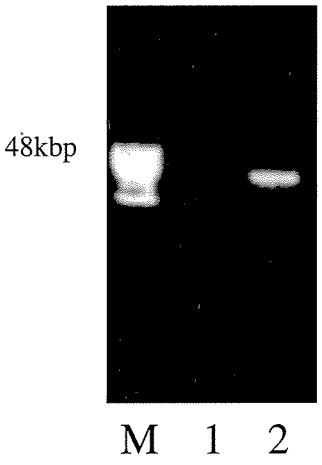
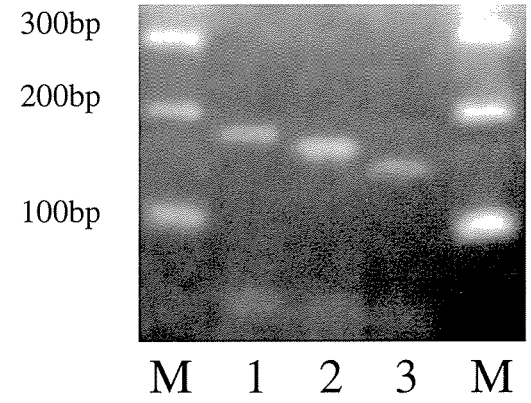


Fig. 1.
Agarose gel electrophoresis of DNA from milled rice with/ without heating (KDML 105)

1:milled rice without heating
2:milled rice with heating
M:1 mix (Fermentas)

Fig. 2.
Agarose gel electrophoresis of DNA fragments amplified from authentic samples by PCR using primer set RM17
1:RD 7
2:KDML 105
3:CNT 1
M:100bp ladder
(New England Bio Lab.)



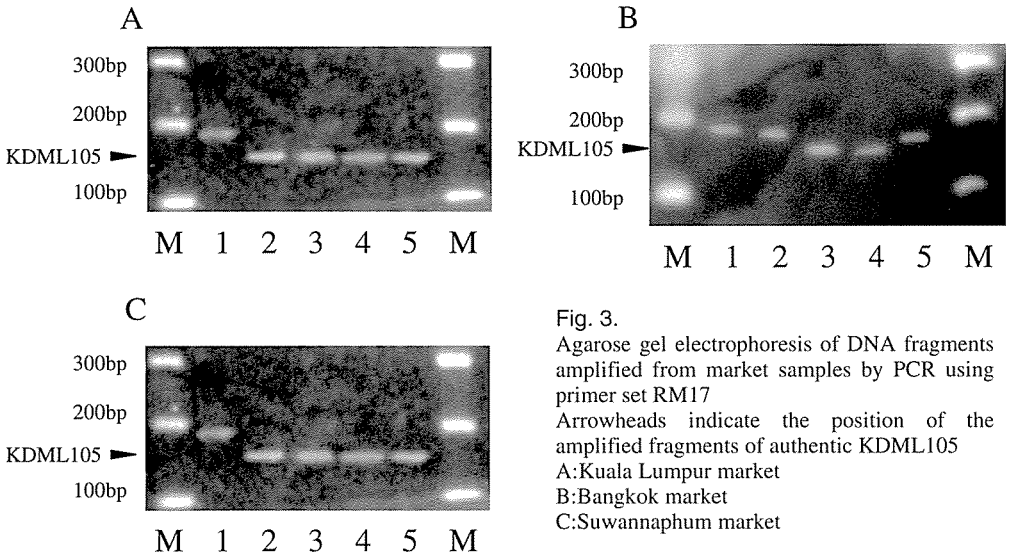


Fig. 3.
Agarose gel electrophoresis of DNA fragments amplified from market samples by PCR using primer set RM17
Arrowheads indicate the position of the amplified fragments of authentic KDML105
A:Kuala Lumpur market
B:Bangkok market
C:Suwannaphum market

Khao Dawk Mali", sampled in a Suwannaphum market.

A simple and rapid method of cultivar identification of milled rice is illustrated in Fig.4. By applying the improved method, we were able to obtain DNA for microsatellite analysis within 2hr and identification required 6hr in total.

The efficiency, speed and the lack of expensive instruments or toxic chemicals in the present method are an attractive alternative to the existing methods of rice grain genomic DNA. These results show that DNA extracted in this simple, low-cost, rapid and safe protocol is of high quality and can be used in PCR-based techniques for cultivar identification even in laboratories with a moderate level of technology.

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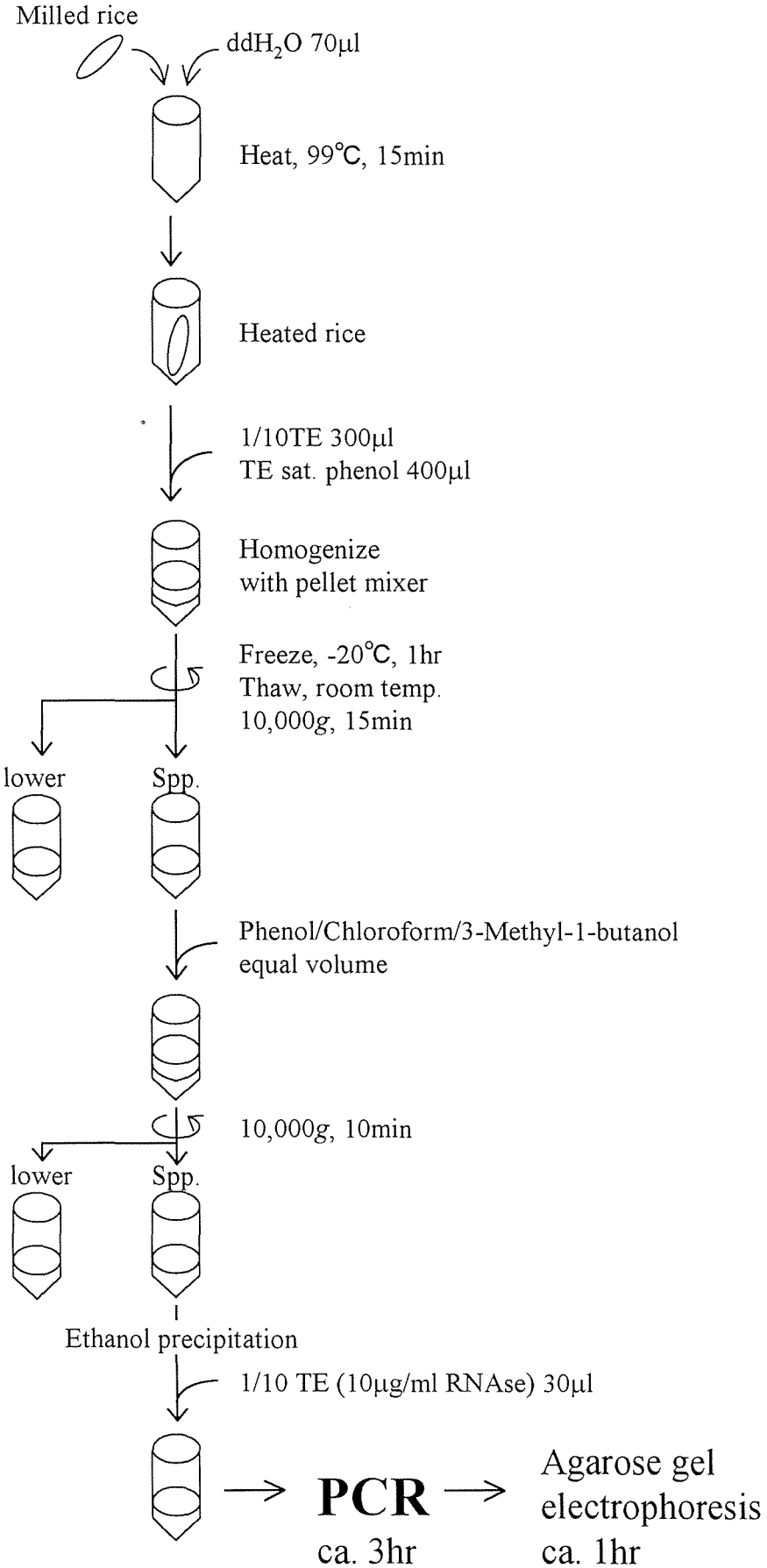

Fig.4. Scheme of rapid DNA extraction and cultivar indentification of milled rice.

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精米からの簡易迅速 DNA 抽出法の開発と、そのカオドマリ 105 への応用

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摘　要

香り米品種カオドマリ 105 はタイにおける主要な品種である。しかしながら、生産量の拡大はカオドマリの種々の特性により困難である。さらに、客観的な評価法がないため不正な取引が野放しになっている。そのため、客観的な品種判別技術の開発が必要とされている。PCR 法をベースとした分子マーカー法は客観的な品種判別法として有用であるが、その適用には、高分子量の DNA を精米１粒１粒から抽出する必要がある。筆者は凍結・融解を利用し、迅速かつ高価な機器が必要でない高分子量の DNA の抽出法を開発した。抽出した DNA のマイクロサテライト分析を行うことにより、品種判別を行った。バンコク・スワナプン・クアラルンプールそれぞれの市場のサンプルを分析することにより、バンコク・クアラルンプール市場品より、カオドマリ 105 ではない品種を検出した。本抽出法を品種判別に適用することにより、DNA 抽出まで２時間、品種判別まで６時間以内に判別することが可能となった。

キーワード：品種判別、PCR、迅速抽出、ゲノム DNA