GENETIC DIVERSITY ANALYSIS OF INDONESIAN RICE LANDRACES USING FLUORESCENTLY LABELED MICROSATELLITE MARKERS WITH CAPILLARY ELECTROPHORESIS

FATIMAH

GRADUATE SCHOOL
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2005
ABSTRAK

FATIMAH. Analisis Keragaman Genetik Padi Lokal Indonesia Menggunakan Penanda Mikrosatelit Berlabel Fluoresen dengan Elektroforesis Kapiler. Dibimbing oleh AGUS PURWITO dan MICHAEL J. THOMSON


Kata kunci: Keragaman genetik, penanda mikrosatelit, elektroforesis kapiler
ABSTRACT

FATIMA. Genetic Diversity Analysis of Indonesian Rice Landraces Using Fluorescently Labeled Microsatellite Markers with Capillary Electrophoresis. Under the direction of AGUS PURWITO and MICHAEL J. THOMSON

Generating a new crop variety with certain desirable traits requires a germplasm collection with wide genetic diversity. A survey of the genetic diversity among 253 traditional landraces of 20 provinces in Indonesia including cereh, guntil and bulu type, 13 International rice varieties and 22 wild relatives, was conducted using six multiplex panels of 30 fluorescent-labeled Simple Sequence Repeat (SSRs) markers. This study was conducted to evaluate the genetic variation within a diverse collection of wild rice species, with Indonesian traditional landraces among the provinces and modern cultivated varieties. Automated detection of fluorescently-labeled microsatellite markers using capillary electrophoresis proved to be a powerful and efficient technique for assessing genetic variation among closely related varieties. A total of 739 alleles, with an average of 25 alleles per locus and a Polymorphism Information Content (PIC) values at 0.75, were detected at the 30 SSR loci. Genetic variation was higher in indica than japonica rice, and higher in wild than cultivated rice, while lower in breeding lines than the local varieties. Genetic similarity, principle coordinate analysis and population structure analysis identified three major groups: group 1 corresponded to japonica, group 2 corresponded to indica and group 3 corresponded to wild rice. The geographical distribution of Indonesian rice accessions divided into two major subpopulations based on the genetic data. The first group was located in the Nusa Tenggara, Sulawesi, Maluku, and Irian Jaya provinces. The second group was located in the Sumatera, Jawa, Kalimantan, and Bali provinces.

Key words: Genetic diversity, microsatellite markers, capillary Electrophoresis
LETTER OF STATEMENT

This statement is to certify that the thesis titled Genetic Diversity Analysis of Indonesian Rice Landraces Using Fluorescently Labeled Microsatellite Markers with Capillary Electrophoresis was my research result with advisory committee and has never been published in any universities. The information sources that have been taken based on published papers have been mentioned in the text or in the References at the end of this thesis. I confirm that the information given above is correct.

Bogor, January 2005

Fatimah
P. 05502008.1
GENETIC DIVERSITY ANALYSIS OF INDONESIAN RICE LANDRACES USING FLUORESCENTLY LABELED MICROSATELLITE MARKERS WITH CAPILLARY ELECTROPHORESIS

FATIMAH

The Thesis Submitted to the Program Study of Biotechnology In Partial Fulfillment of the Requirements for the Degree of Master Science

GRADUATE SCHOOL
BOGOR AGRICULTURAL UNIVERSITY
BOGOR
2005
BIOGRAPHICAL SKETCH

The Author was born in Cirebon, April 23\textsuperscript{th} 1980. She is the 9\textsuperscript{th} among 9 children of Jauharul Arifin and Masnuah (both deceased). She had her primary school at Persatuan Umat Islam Elementary School, Cirebon. Her secondary school at SMP Muhammadiyah 1 Cirebon and graduated with "Best Student" award. She continued her school at SMU Negeri 1 Cirebon.

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In December 2004, author was accepted as a research staff at Indonesian Department of Agriculture.
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Bogor, January 2005

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INTRODUCTION

Background

Rice is the staple food on which nearly two-and-a-half billion people already depend. The number will increase by almost 50 % to more than 3.5 billion people in 2025. As the preferred staple food of many Indonesians, rice is the most important food crop of the country in terms of both production and consumption. Rice provides 49 % of the national calorie intake and 40 % of protein intake (IRRI, 2003).

Genetic diversity is a key factor in sustaining agricultural productivity. In order to preserve and make this diversity available for crop improvement, tremendous efforts have been made in the collection, maintenance and classical characterization of germplasm. Generating a new crop variety with certain desirable traits requires a germplasm collection with wide genetic diversity. The germplasm collection may be local, introduced, and bred varieties (Syam and Hermanto, 1995).

Indonesia has two groups of rice cultivars, cereh and bulu. The former is a local word meaning an awnless cultivar, and the latter refers to an awned cultivar. The two groups clearly differ from each other both morphologically and physiologically. Cereh belongs to Indica and bulu to Javanica. There exists a group known as “gundil” which has no awns but all other characters of bulu. This group is considered to be a variant of bulu (Kushibuchi, 1997).

Through the various exploration missions, about 3.500 accessions have been collected by the Genetic Resources Division at BB-Biogen, Bogor. They have a very important role as genetic resources. In the breeding program to create high yielding varieties of food crops, characterization and evaluation had been conducted to increase the utilization of genetic resources. Traits were computerized and arranged in a database and catalog of food crop germplasm. This makes it easier to exchange information about genetic resources to be used in research for high yielding varieties of food crops with better quality (Silitonga et al., 2001).
The value of collected and conserved rice germplasm has been demonstrated in many screening tests for useful economics traits. Hundreds of accessions have been screened for resistance to eight insect pests and five diseases of rice as well as tolerance to different abiotic stresses. Resistance to some pests and disease such as brown planthopper and rice blast is quite common in the cultivated rices (Silitonga et al., 2001).

The advent of molecular markers made it possible to map beneficial traits, such as yield, quality and disease resistance. Microsatellites are simple sequence repeats of 1-6 nucleotides. They appear to be ubiquitous in higher organisms, although the frequency of microsatellites varies between species. They are abundant, dispersed through out the genome and show a higher level of polymorphism than other genetic markers. This featured, coupled with their ease of detection, have made them useful molecular markers. Their potential for automation and their inheritance as a co-dominant manner are additional advantages when compared with other types of molecular markers (Holton, 2001).

Although a number of genetic diversity studies have used molecular markers on rice accessions, few have extensively analyzed Indonesian rice varieties. Glazmann (1987) surveyed 1688 traditional Asian rice varieties and identified six groups based on protein isozyme markers. Included in his study were 130 Indonesian varieties representing three well-known ecotypes: cereh, gundil and bulu. These three traditional Indonesian rice ecotypes fell into two isozyme groups (I and VI), which correspond to the indica and japonica classifications. Another study surveyed 511 rice accessions with isozyme markers, of which 12 were from Indonesia (Li and Rutger, 2000). The dataset of the 511 varieties supported the presence of two ecotypes within the japonica class: temperate japonica and tropical japonica. Varieties from both Indonesia and the Southern U.S. are largely considered to be tropical japonicas.

Single nucleotide resolution of DNA fragments requires the use of denaturing polyacrylamide gel electrophoresis (PAGE) or capillary electrophoresis. Simple Sequence Repeat (SSR) amplification products are detected in PAGE by silver staining or by fluorescence labeling (primer or PCR product). The use of fluorescently labeled primers, combined with an automated
capillary electrophoretic system, greatly simplifies the analysis of SSR allele sizes. The use of fluorescent primers enable PCR products to be detected without any post-electrophoresis treatments. Sample throughput can also be increased through multiplexing by the use of different fluorescent tags on different PCR products. PCR amplification can be performed separately for each SSR locus and then the PCR products run in the same lane. The use of internal size standard in each lane also allows for accurate automated sizing of bands. (Donini et al. 1998).

Although automated fluorescent detection of microsatellites is in common use for human genome studies (Jiang et al. 2002; Merci et al. 2002) and has been developed for several plant species such as *Theobroma cacao* (Saunders et al. 2001) and maize (Liu et al. 2003), this method has only recently been applied to the analysis of rice varieties (Blair et al. 2002; Ni et al. 2002; Coburn et al. 2002).

**Objectives of the Research**

1. to evaluate the genetic variation within a diverse collection of wild rice species, Indonesian traditional landraces, and modern cultivated varieties.

2. to determine differences in the patterns of diversity between the two rice subspecies (*indica* and *japonica*) and reveal genetic relationships within each subspecies.

3. to compare traditional slab gels and an automated capillary-electrophoresis based system

**Hypothesis**

1. These Indonesian landrace accessions contain a large amount of genetic diversity, represented by a diverse range of allelic variation

2. SSR fingerprinting could be used to differentiate the genotypes belonging to either the *japonica* or *indica* subspecies of cultivated rice.

3. *Indica* and *japonica* cultivars are genetically very distinct, and can be clearly classified using SSRs with capillary electrophoresis
Research Advantages

1. A detailed picture of the genetic range and structure of Indonesian rice biodiversity
2. Information about the genetic relationship between wild rices, traditional landraces, and modern cultivars
3. Description about the advantages and disadvantages of traditional slab gels and automated capillary electrophoresis.
LITERATURE REVIEW

Rice Genetic Diversity

Rice, *Oryza sativa* L., is one of the most important grain crops in the world, and a staple food for more than half the world population. As a self-pollinated crop species with extensive intra-specific variation, differentiation of *O. sativa* into two major subspecies, *indica* (Xian) and *japonica* (Geng), has been well documented (Oka, 1988). *Indica* is the predominant tropical subspecies. The *japonica* subspecies, consisting of temperate and tropical types, is widely grown in East Asia, North and South America, Australia, Mediterranean North Africa, and Europe, and accounts for about 20% of world rice production (Mackill, 1995). The genetic diversity of *japonica* rice is thought to be lower than for *indica* rice (Glazmann, 1987, Sun et al., 2001). The comparisons of the main characteristic of *Japonica* and *Indica*-type rice shown in Table 1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Character</th>
<th>Japonica-type rice</th>
<th>Indica-type rice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leaf shape and color</td>
<td>Narrow and dark green</td>
<td>Wide and light green</td>
</tr>
<tr>
<td>2</td>
<td>Angle and flag leaf and rachis</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>3</td>
<td>Culm length</td>
<td>Short</td>
<td>Long</td>
</tr>
<tr>
<td>4</td>
<td>Culm strength</td>
<td>Lethal and hard to break</td>
<td>Hard and easy to break</td>
</tr>
<tr>
<td>5</td>
<td>Lodging property of culm</td>
<td>Hard to lodge</td>
<td>Easy to lodge</td>
</tr>
<tr>
<td>6</td>
<td>Grain shape</td>
<td>Wide and thick and round cross section</td>
<td>Long, narrow and slightly flat</td>
</tr>
<tr>
<td>7</td>
<td>Shattering habit</td>
<td>Low shattering</td>
<td>High shattering</td>
</tr>
<tr>
<td>8</td>
<td>Awns</td>
<td>Mostly awnless, a few varieties with short awns</td>
<td>AWnns with a variation of length</td>
</tr>
<tr>
<td>9</td>
<td>Length and number of glume trichomes</td>
<td>Relatively dense and short</td>
<td>Not dense and relatively long</td>
</tr>
<tr>
<td>10</td>
<td>Lengthwise ratio of grains</td>
<td>2.5 or less</td>
<td>2.5 or more</td>
</tr>
<tr>
<td>11</td>
<td>Germination</td>
<td>Slow</td>
<td>Quick</td>
</tr>
<tr>
<td>12</td>
<td>Phenol reaction</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Potassium resistance chlorate</td>
<td>High</td>
<td>Susceptible</td>
</tr>
<tr>
<td>14</td>
<td>Low temperature tolerance</td>
<td>High</td>
<td>Susceptible</td>
</tr>
<tr>
<td>15</td>
<td>Drought resistance</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>16</td>
<td>Endosperm destruction by alkali</td>
<td>Easy</td>
<td>Hard</td>
</tr>
</tbody>
</table>

(Source: Watanabe, 1997)
Indonesian Rice Diversity

Indonesia has two groups of rice cultivars *cereh* and *bulu*. The former is a local word meaning an awnless cultivar, and the latter refers to an awned cultivar. The two groups clearly differ from each other both morphologically and physiologically, as shown in Table 2. *Cereh* belongs to *indica* and *bulu* to *javanica* (Kushibuchi, 1997).

*Bulu* is a native rice in Indonesia and its eating quality is rated highly. However, since this group is weak in disease and insect resistance, it is now only grown in mountainous areas and fertile highlands where damage from stem borers is not very serious, and in some islands such as Bali and Lombok.

The growth period of recently bred cultivars is significantly shortened (110-120 days) and in addition, yield is improved, though moderately. As a result, the yield per day (efficiency of dry matter production) has noticeably increased. Furthermore, these cultivars mostly have combined resistance to several major diseases and insect pests. Nevertheless, these recently bred cultivars have some disadvantages concerning eating quality (Kushibuchi, 1997).

Germplasm characterization and evaluation, complemented by biosystematic studies using the wild species and molecular studies of genetic diversity, is generating the information base for more efficient use of these valuable genetic resources. Some of the molecular studies of genetic diversity conducted in Indonesia, include: a study of molecular marker linkage with a resistance gene to bacterial leaf blight (*xa-5*) in rice (Yunus, 1998), identification of the gene *Pi-2(t)* for blast resistance, characterizing the genetic diversity of Indonesian rice germplasm using STS and RAPD markers (Taslih et al., 2000), and a genetic diversity study of Indonesian rice germplasm for blast resistance using RGA primers (Suyono et al., 2002).

Microsatellite Markers

Microsatellites are tandem repeats of DNA sequences of only a few base pairs (1-6 bp) in length, the most abundant being the dinucleotide repeats. The technical efficiency and multiplex potential of SSRs makes them preferable for
many forms of high throughput mapping, genetic analysis and marker assisted plant improvement strategies.

Table 2 Characteristic of bulu and cereh rice groups

<table>
<thead>
<tr>
<th>Character</th>
<th>bulu</th>
<th>cereh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awn</td>
<td>Awned (There exist a group known as “gandil” which has no awn but all other characters of bulu. This group is considered to be a variant of bulu.)</td>
<td>Awnless</td>
</tr>
<tr>
<td>Leaf blade shape</td>
<td>Broad leaf blade with sparse hard pubescence</td>
<td>Narrow and long leaf blade with dense soft pubescence</td>
</tr>
<tr>
<td>Tillers</td>
<td>Few</td>
<td>Many</td>
</tr>
<tr>
<td>Culm thickness</td>
<td>Thick</td>
<td>Thin</td>
</tr>
<tr>
<td>Lodging</td>
<td>Resistant to lodging</td>
<td>Mostly susceptible to lodging</td>
</tr>
<tr>
<td>Shattering</td>
<td>Mostly difficult</td>
<td>Mostly easy</td>
</tr>
<tr>
<td>Disease and insect resistant</td>
<td>Susceptible to disease and insect</td>
<td>Resistant to disease and insect</td>
</tr>
<tr>
<td>Adaptableicity</td>
<td>Cultivable in favorable soil conditions</td>
<td>Cultivable even in less favorable and unfavorable soil conditions</td>
</tr>
<tr>
<td>Grain shape</td>
<td>Round and large</td>
<td>Long and small</td>
</tr>
<tr>
<td>Eating quality</td>
<td>Good—very good</td>
<td>Mostly poor</td>
</tr>
<tr>
<td>Photoperiodic sensitivity</td>
<td>Low</td>
<td>Mostly high</td>
</tr>
<tr>
<td>Natural crossing rate</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Dormancy</td>
<td>Relatively short</td>
<td>Mostly long</td>
</tr>
<tr>
<td>Minimum period to flowering</td>
<td>Long</td>
<td>Varied (usually smaller than bulu)</td>
</tr>
<tr>
<td>Yield</td>
<td>Relatively low</td>
<td>Relatively high</td>
</tr>
</tbody>
</table>

(Source: Takahashi, 1997)

The fact that SSR markers are co-dominant, multi-allelic and can be reliably used to analyse both indica and japonica germplasm as well as groups of AA genome Oryza species makes them attractive as genetic markers and facilitates the integration of results from independent studies. In addition, the highly polymorphic nature of many microsatellites is of particular value when analyzing closely related genotypes, as is often the case in breeding programs working with narrowly adapted gene pools. Thus the availability of a high-density SSR map is valuable as a public resource for studies aiming to interpret the functional significance of the rapidly emerging rice genome sequence information (McCouch et al., 2002).

Microsatellite markers have been developed and utilized in the study of cultivated rice, including variety identification and germplasm conservation.
(Olufowote et al., 1997) genetic diversity (Yang et al., 1994; Davierwala et al., 2000; Cho et al., 2000; Chen et al., 2002) and gene and quantitative trait locus analysis (McCouch, 1995; Xiao et al., 1996; Thomson et al., 2003a). They have also been applied to study the wild relatives of rice (Xiao et al., 1996; Thomson et al., 2003a; Ishii et al., 2001; Zhou et al., 2003).

Table 3 Comparative assessment of some of the salient characteristics of different molecular genetic screening techniques

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>RFLP</th>
<th>RAPD</th>
<th>SSR</th>
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<tr>
<td>Development cost (per probe)</td>
<td>Medium</td>
<td>Low</td>
<td>high</td>
</tr>
<tr>
<td>Level of polymorphism</td>
<td>Medium</td>
<td>Medium</td>
<td>high</td>
</tr>
<tr>
<td>Type of polymorphism</td>
<td>Single base</td>
<td>single base</td>
<td>variation in number of repeat motifs</td>
</tr>
<tr>
<td>Principle involved</td>
<td>DNA hybridization</td>
<td>blot</td>
<td>PCR amplification with random primer</td>
</tr>
<tr>
<td>Automation possible</td>
<td>no</td>
<td>Yes/no</td>
<td>Yes/no</td>
</tr>
<tr>
<td>Cost of Automation</td>
<td>high</td>
<td>Medium</td>
<td>high</td>
</tr>
<tr>
<td>Repeatability</td>
<td>high</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Level of training required</td>
<td>low</td>
<td>low</td>
<td>low/medium</td>
</tr>
<tr>
<td>Cost (per assay)</td>
<td>high</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>Radioactivity used</td>
<td>Yes/no</td>
<td>no</td>
<td>yes/no</td>
</tr>
<tr>
<td>Samples/day (without automation)</td>
<td>20</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Inheritance</td>
<td>codominant</td>
<td>dominant</td>
<td>codominant</td>
</tr>
<tr>
<td>Amount of DNA required</td>
<td>5-10 µg</td>
<td>10-25 ng</td>
<td>50-100 ng</td>
</tr>
<tr>
<td>Detection</td>
<td>Autoradiography, biotin labeling</td>
<td>Ethidium bromide, silver staining, fluorescence</td>
<td></td>
</tr>
</tbody>
</table>

(Source: Karp et al., 1997; Gupta et al., 1996)

Capillary electrophoresis

Capillary electrophoresis (CE) is an attractive alternative to slab gel electrophoresis and is rapidly becoming the dominant DNA technique in sequencing centers due to its capacity for full automation. (Merci et al., 2001, Saunders et al., 2002; Jiang et al., 2002). In CE, DNA separation is achieved in a fused-silica capillary, 25-100 µm in diameter. The high ratio of surface area to the volume of the small capillary tubes serves to efficiently dissipate the heat produced during electrophoresis, allowing the use of higher electric fields, which decreases the run time and improves DNA resolution. (Albarghouthi et al., 2000).
The principle behind the separation mechanism of capillary electrophoresis is similar to slab gel electrophoresis. Capillary electrophoresis occurs when an electric field is applied to an electrolyte solution (polyacrylamide) within a capillary, causing ions to migrate. This movement is referred to as electrophoretic migration. Having a negative net charge, DNA fragments are likewise affected by the applied field, moving toward the anode (+) (Figure 1). The sample plate containing labeled DNA fragments is automatically denatured and then separated by capillary electrophoresis. These dye-labelled primers will be detected by fluorescence and provide estimated sizes of DNA fragments (Beckman, 2002).

Figure 1 Capillary electrophoresis (Source: Beckman, 2002)

Figure 2 Aluminum Backed PAGE Sequencing System (Owl)

Figure 3 Capillary Electrophoresis Equipment (Source: Beckman, 2002)

1. Upper buffer Chamber (UBC)
2. U-shaped silicone gasket
3. Floating aluminum heat sink
4. Lower buffer chamber (LBC)
5. Removable lightweight bottle
6. Clamps slide

1. Sample Access Cover (extended)
2. Capillary access Cover (extended)
3. Status Indicator
4. Plate holder & Sample Transport
5. Capillary Temperature Control Cover
6. Rubber Latches
7. Manifold Access Cover
8. Gel Waste Bottle
9. Power Switch
10. Gel pump
11. Gel Cartridge Access Cover
Capillary electrophoresis has many advantages over slab gel electrophoresis (Behr et al, 1999):

- CE offers a very high resolution, which is due to two effects:
  - Because of the very small inner diameter, the temperature gradient within the tube is small, leading to very low convection and therefore a negligible disturbance of the bands/peaks.
  - Because of the small dimensions, capillaries offer a good heat dissipation which allows us to apply rather high electric fields (up to 1000 V/cm and more). The high electric fields allow a very reduced analysis time which means less diffusion of the bands.
- CE offers higher speed (about one order of magnitude faster) than slab gel techniques
- Capillaries are available in a variety of inner diameters (about 10 to 300 um) making them useful for many different needs.
- Their length can be chosen in a wide range (from a few centimeters to several meters), offering many choices according to the task (e.g. fast separation, high resolution separation). Further miniaturization ("microchips") is also possible.
- CE can be easily automated as it offers on-line detection. Pouring of gels and time consuming gel loading is not necessary.
- CE systems can inject from a variety of sample formats (Eppendorf tubes, microtitre plates, etc.) and even from single cells.
- CE is compatible with a variety of detection methods, such as absorption, LIF, MS, chemiluminescence, voltammetry etc.
- The sample volume can be very small (e.g. 1 ul).
MATERIAL AND METHOD

Time and Place

This research was held at the Laboratorium of Molecular Biology at the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB-Biogen) in Bogor, Indonesia, from October 2003 until October 2004.

Plant Material

Genetic diversity study, in this germplasm collection, currently screening 248 Indonesian rice accessions collected across all 20 provinces in Indonesia as representative varieties chosen from all the traditional classification groups of Indonesian rice including Cereh, Gundil and Bulu and five breeding lines. Accessions were obtained from the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB-Biogen) Indonesia. Twenty two wild rice species were included originally from the International Rice Research Institute (IRRI, Philippines). Thirteen international cultivated varieties to be use as controls to help determine accurate allele sizes were obtained from Cornell University (Appendix 1).

DNA Extraction

DNA was extracted from young leaf of 21 days, by a modification of the chloroform microprep method used by the McCouch lab at Cornell, and updated by Thomson (2003b). Fresh and young leaf tissue was collected into 1.5–2 ml tubes held in racks suspended above liquid nitrogen. Frozen tissue was crushed in each tube using a wooden stick before adding 800 μL extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM NaCl, 1.25% SDS (w/v), 8.3 mM NaOH, 0.38 g Sodium bisulfite per 100 ml of buffer). Tubes were mixed well by vortexing and placed tubes in 65°C water bath in a tube holder for at least 20 minutes. Tubes were removed and filled with 800 μL chloroform mix (24:1 mixture of chloroform and isoamylalcohol) and then vortexed or inverted vigorously for 5 minutes. After mixing, tubes were centrifuged for 15 minutes at maximum speed in a microcentrifuge. The upper aqueous layer was removed to a fresh tube. Usually
this means taking approx. 500 μL supernatant. 1 mL ethanol or 350 μL isopropanol was added, tubes were placed in –20°C for 1 hour, centrifuged for 15 minutes at maximum speed (13,000 rpm). Solution was discarded and the pellet was washed with 800 μL ice-cold 70 % ethanol, and dried. The pellet was resuspended in 50 μL TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA) in a 65°C water bath.

**Checking DNA Quality and Quantity on Agarose Gel Electrophoresis**

A quality check of the DNA was performed using agarose gel electrophoresis, and 3μl undigested DNA samples were loaded and compared to various amounts of bacteriophage lambda DNA (Invitrogen).

Neutral Agarose gel electrophoresis (Helentjaris., 1998) with minor modification. Agarose (1 %) was added to the proper amount of 0.5 X TBE gel buffer. Agarose was melted in a microwave oven and cooled after boiled. Agarose was poured into a tray to solidify and insert combs (20-30 min). Tray was placed in gel rig filled with 0.5 X TBE gel buffer. Combs were removed to load samples. Samples were run into gel at 15-20 mA and 100 volt. Tray was removed from rig and stained in 1 μg/ml, Ethidium bromide (100 μL of 10 mg/ml ethidium bromide in 1000 ml dH₂O) for 15 minutes with gentle shaking. The gel was rinsed in dH₂O for 20 min. The gel was Photographed using The Gel Doc™ EQ, Bio-Rad’s Quantity One®.

**Microsatellite Genotyping using Silver-Stained PAGE**

In the study for comparing microsatellite genotyping techniques, four unlabeled SSRs (Table 4) were run on 6 accessions (Table 5) using silver-stained PAGE gels on an Aluminum Backed Sequencing System (Owl model S3S).

**PCR Reaction**

Polymerase Chain Reaction (PCR) analysis followed procedures with minor modification. It was performed in 15.0 μL of a mixture containing 1.2 μL (1:200 dilution) DNA template of each sample, 1 μL of both primers (forward & reverse 5 μM), 0.5 μL of dNTP mix (10 μM) and 0.075 μL Promega Taq™ DNA Polymerase in Storage Buffer B. (20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.1 mM
EDTA, 1 mM DTT, 50 % glycerol, 0.5 % Nonidet®-P40, 0.5 % Tween®-20), 0.9 μL MgCl₂, and 1.5 μL 10X buffer.

The PCR machine of DNA Engine Tetrad MJ Research PTC-225 Peltier Thermal Cycler was run as follows: (i) an initial denaturation step of 2 min at 94°C, (ii) 30 cycles of 45 second at 94°C, 45 second at 55/61/63°C (depend on primer annealing temperature), 1 min at 72°C and (iii) a final extension step for 5 min at 72°C.

Table 4 List of Microsatellite Markers used in the Silver-Stained PAGE Study

<table>
<thead>
<tr>
<th>Prior</th>
<th>Locus</th>
<th>Chr</th>
<th>Size range (bp)*</th>
<th>Motif</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RM125</td>
<td>7</td>
<td>91-151</td>
<td>(GCT)8</td>
<td>aacagccctgagcaggccgacc</td>
<td>aagggatcatgigcccgaaggcc</td>
</tr>
<tr>
<td>2</td>
<td>RM250</td>
<td>2</td>
<td>143-187</td>
<td>(CT)17</td>
<td>ggtcaaccaggccgatca</td>
<td>gatggagaaccttcacgcaagcag</td>
</tr>
<tr>
<td>3</td>
<td>RM452</td>
<td>2</td>
<td>193-210</td>
<td>(GTC)9</td>
<td>cgtacagagagctggaagggg</td>
<td>gggtacaaaccacgtttcag</td>
</tr>
<tr>
<td>4</td>
<td>RM454</td>
<td>6</td>
<td>251-295</td>
<td>(GCT)8</td>
<td>ctcagcttgacgctgcag</td>
<td>ggtgctacgccattagccag</td>
</tr>
</tbody>
</table>

Table 5 List of Samples used in the Silver-Stained PAGE Study

<table>
<thead>
<tr>
<th>No.</th>
<th>No. Acc.</th>
<th>No. Reg.</th>
<th>Name</th>
<th>Origin</th>
<th>Group</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C142</td>
<td>JC1</td>
<td>India</td>
<td>Aroma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C601</td>
<td>Kadam</td>
<td>Japan</td>
<td>Aus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>Park</td>
<td>Sulu</td>
<td>Javanica</td>
<td>Bulu</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>42</td>
<td>Serad</td>
<td>Jubar</td>
<td>Javanica</td>
<td>Bulu</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>269</td>
<td>Pokol</td>
<td>Bali</td>
<td>Javanica</td>
<td>Bulu</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>K.Berasan</td>
<td>Kaiteng</td>
<td>indica</td>
<td>Cere</td>
<td></td>
</tr>
</tbody>
</table>

Protocols for Microsatellite Genotyping using Silver-Stained PAGE (Tai, 1998; modified by Thomson, 2003c):

Preparation of glass plates:

Each gel was poured in a mold consisting of two glass plates, a large plate and a smaller, notched plate, which were separated by spacers. The gel matrix was poured between the plates and allowed to polymerize before being used for electrophoretic separations.

Glass plates were cleaned thoroughly with water and detergent. Plates were rinsed with deionized water and dried by leaving in a rack or wiping with paper towels. The sides of the plates that were to be treated were subjected to cleaning with 95% ethanol 3 times and the ethanol was removed with soft tissue (e.g. Kimwipes®).
Preparation of small (notched) glass plate:

Fresh binding solution was prepared in a chemical fume hood by adding 3 ul of bind silane (γ-methacryloxypropyltrimethoxysilane, Sigma M-6514). This mixture was dispensed onto the surface of the notched plate using a pipetman and wiped using Kimwipe® tissue. The binding solution was allowed to dry for 5 minutes. In order to remove the excess binding solution, the small glass plate was cleaned with about 2 ml of 95% ethanol using a Kimwipe® tissue.

Preparation of large glass plate:

In a chemical fume hood, 1 ml of Sigmacote® (Sigma) was applied to the ethanol-cleaned surface of a new large glass plate with a dry Kimwipe® tissue and let dry about 5 minutes. Any excess Sigmacote® was removed by gently wiping the surface with a dry Kimwipe® tissue.

Assembling and pouring the gel:

Spacers were placed along the bottom and side edges on the bind silane-treated surface. The large glass plate was put on the small one (in a sandwich-like fashion). The glass plates and spacers were held in position by using metal binder clips (4 clips on each side and 3 to 5 clips on the bottom of the glass plates). The assembly was turned over so that the small plate was facing up and the notched side was closest.

Acrylamide solution (4%) was poured into a dispensing bottle. For each 60 ml solution, add 60 µl of TEMED (N,N,N', N'-Tetraethyl-ethylenediamine) and 500 µl of 10% APS (ammonium persulfate) to the acrylamide solution, and mix gently by swirling.

The acrylamide solution was poured into the space between the glass plates starting at the lower corner slowly. When the acrylamide solution nears the top of the gel, stop pouring and position the gel horizontally; the acrylamide should fill in the remaining space. The straight side of a sharkstooth comb was inserted into the space between the plates at the top of the gel. The top of the gel was covered with plastic wrap and then the comb was secured with two or three metal clips. Allow polymerization to proceed for at least 1 hour.
Setting up gel assembly in electrophoresis apparatus:

The clamps were removed from the polymerized acrylamide gel assembly and the outside of the glass plates were cleaned. TBE 0.5X (400 ml) was added to the bottom chamber of the electrophoresis apparatus. The comb and bottom spacers were removed and also any excess polyacrylamide from the top of the glass plates. Assembly was taken over to the apparatus. Top of assembly was placed where small (notched) side facing downward. While the plates were holding, the air space was filled left with 0.5X TBE buffer using transfer pipet. The plate was raised into an upright position. To the upper chamber of the electrophoresis apparatus 0.5X TBE (600 ml) was added. The air bubbles were removed using transfer pipet The gel was pre-run until approximately 45 to 50°C (about 45 to 60 minutes), 40 cm polyacrylamide gel: 2000 V (maximum), 50 mA, 75 W.

Loading and running samples in a PAGE apparatus:

PCR samples was prepared by adding 3X STR buffer. It was denatured by heating at 85°C for 4 minutes and immediately chilling on ice. After the pre-run, the sharkstooth comb teeth was inserted into the gel so that the teeth were touching the surface of the gel. The samples were loaded using 3 μl (96 well) using 8 Hamilton syringes. The gel was run using the same conditions as the pre-run step.

Fixing and silver staining PAGE gels:

After electrophoresis, the buffer chamber was emptied and the gel clamps were carefully loosened. The gel was removed from the apparatus. The gel was placed on a flat surface. The comb and the side spacers was removed and the two glass plates were separated. The gel (the small plate) was placed in a shallow plastic tray and proceed with the following steps (Table 6). The gel was positioned upright and dry overnight.

Microsatellite Genotyping Using Fluorescent Capillary Electrophoresis

The genetic diversity study used the CEQ 8000 Genetic Analysis System (Beckman Coulter 608305). This capillary electrophoresis-based genetic analysis
process included gel preparation, sample denaturation, sample loading, and data collection and analysis (Beckman, 2002).

Table 6 Protocol Fixing and silver staining PAGE gels

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution (2 L)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fixing</td>
<td>Fix/stop solution</td>
<td>20 minutes</td>
</tr>
<tr>
<td>2. Wash</td>
<td>dH2O</td>
<td>2 minutes</td>
</tr>
<tr>
<td>3. Wash</td>
<td>Repeat step 2 twice</td>
<td>2 x 2 minutes</td>
</tr>
<tr>
<td>4. Staining</td>
<td>Staining solution</td>
<td>30 minutes</td>
</tr>
<tr>
<td>5. Rinse</td>
<td>dH2O</td>
<td>5 to 10 seconds</td>
</tr>
<tr>
<td>6. Developing</td>
<td>Developer solution (4 to 10°C)</td>
<td>2 to 5 minutes</td>
</tr>
<tr>
<td>7. Stopping</td>
<td>Fix/stop solution</td>
<td>5 to 6 minutes</td>
</tr>
<tr>
<td>8. Wash**</td>
<td>dH2O</td>
<td>2 to 3 minutes</td>
</tr>
</tbody>
</table>

Microsatellite Fragment sizing on the CEQ 8000 (BB-Biogen Standard Operating Procedure series; Thomson, 2004a)

Thirty rice microsatellite primers were chosen to represent the entire rice genome (Table 7). Oligos labeled with the cyanine-based WellRED fluorescent dyes and LightSabre dyes absorb and emit in the near-infrared region.

PCR amplification of microsatellite marker DNA CEQ™ Fragment Analysis System

Individual Polymerase Chain Reaction (PCR) reaction analysis followed procedures recommended by the manufacturer (CEQ™ 8000 Series Genetic Analysis Systems, Beckman Coulter) with minor modification.

It was performed in 20.0 µL of a mixture containing 3 µL (5 ng/µL) DNA template of each sample, 1.2 µL (5 µM) CEQ-compatible fluorescently-labeled primer pairs (forward-labeled, reverse unlabeled), 0.6 µL of dNTP mix (10 mM), 2 µL 10X PCR buffer and 0.15 µL Roche FastStart Taq DNA Polymerase kit 5 units/µL (200 mM Tris-HCl pH 8.4, 500 mM KCl, and 20 mM MgCl₂).

The PCR machine of DNA Engine Tetrad® 2 Peltier Thermal Cycler MJ Research was run in CEQ55_TD program following touchdown procedure: (i) an initial denaturation step of 4 minute at 95°C, (ii) 13 cycles touchdown -0.5°C increments, 45 second at 95°C, 45 second at 61.5°C decrease by 0.5°C every cycle, 30 second at 72°C, followed by 27 cycles 45 second at 95°C, 45 second at 55°C, 30 second at 72°C and (iii) a final extension step for 5 min at 72°C.
Table 7 Fluorescently-labeled microsatellite marker used in six multiplex PCR reactions

<table>
<thead>
<tr>
<th>Panel</th>
<th>Dye</th>
<th>Color</th>
<th>Dye Type</th>
<th>Marker</th>
<th>Chr</th>
<th>Motif</th>
<th>Range</th>
<th>Amount of PCR product used (µL)</th>
<th>Amount of 1:30 dil (µL)</th>
<th>SLS/Std mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D2</td>
<td>Black</td>
<td>WellRED</td>
<td>RM65</td>
<td>1</td>
<td>(GA)14</td>
<td>84-149</td>
<td>0.12</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D2</td>
<td>Black</td>
<td>WellRED</td>
<td>RM433</td>
<td>8</td>
<td>(AG)13</td>
<td>215-239</td>
<td>0.2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM555</td>
<td>3</td>
<td>(GA)17</td>
<td>213-242</td>
<td>0.16</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>D4</td>
<td>Blue</td>
<td>WellRED</td>
<td>RM215</td>
<td>9</td>
<td>(CT)16</td>
<td>126-161</td>
<td>0.04</td>
<td>1.2</td>
<td>22.6</td>
</tr>
<tr>
<td>1</td>
<td>D4</td>
<td>Blue</td>
<td>WellRED</td>
<td>RM514</td>
<td>3</td>
<td>(AC)12</td>
<td>233-275</td>
<td>0.06</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>D2</td>
<td>Black</td>
<td>WellRED</td>
<td>RM214</td>
<td>7</td>
<td>(CT)14</td>
<td>79-168</td>
<td>0.28</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM111</td>
<td>7</td>
<td>(GA)17</td>
<td>115-151</td>
<td>0.18</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>D3</td>
<td>Green</td>
<td>LightSable</td>
<td>RM144</td>
<td>11</td>
<td>(ATT)11</td>
<td>208-290</td>
<td>0.8</td>
<td>4.8(1.6)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>D4</td>
<td>Blue</td>
<td>WellRED</td>
<td>RM217</td>
<td>1</td>
<td>(CT)18</td>
<td>113-156</td>
<td>0.05</td>
<td>1.5</td>
<td>18.1</td>
</tr>
<tr>
<td>2</td>
<td>D4</td>
<td>Blue</td>
<td>LightSable</td>
<td>RM171</td>
<td>10</td>
<td>(GATG)5</td>
<td>306-352</td>
<td>0.06</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>D2</td>
<td>Black</td>
<td>LightSable</td>
<td>RM133</td>
<td>6</td>
<td>(CT)8</td>
<td>224-254</td>
<td>0.08</td>
<td>2.4</td>
<td>22.6</td>
</tr>
<tr>
<td>3</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM287</td>
<td>11</td>
<td>(GA)21</td>
<td>87-119</td>
<td>0.12</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM259</td>
<td>1</td>
<td>(CT)17</td>
<td>144-192</td>
<td>0.2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>D4</td>
<td>Blue</td>
<td>LightSable</td>
<td>RM250</td>
<td>2</td>
<td>(CT)17</td>
<td>143-187</td>
<td>0.06</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>D4</td>
<td>Blue</td>
<td>LightSable</td>
<td>RM907</td>
<td>5</td>
<td>(AAGA)7</td>
<td>231-266</td>
<td>0.12</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D2</td>
<td>Black</td>
<td>WellRED</td>
<td>RM161</td>
<td>5</td>
<td>(AG)20</td>
<td>116-199</td>
<td>0.27</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM283</td>
<td>1</td>
<td>(GA)18</td>
<td>104-168</td>
<td>0.12</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D3</td>
<td>Green</td>
<td>LightSable</td>
<td>RM124</td>
<td>4</td>
<td>(TC)10</td>
<td>261-304</td>
<td>0.32</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D4</td>
<td>Blue</td>
<td>WellRED</td>
<td>RM162</td>
<td>6</td>
<td>(AC)20</td>
<td>200-250</td>
<td>0.07</td>
<td>2.1</td>
<td>15.4</td>
</tr>
<tr>
<td>4</td>
<td>D4</td>
<td>Blue</td>
<td>LightSable</td>
<td>RM277</td>
<td>12</td>
<td>(GA)11</td>
<td>108-126</td>
<td>0.04</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>D2</td>
<td>Black</td>
<td>WellRED</td>
<td>RM431</td>
<td>1</td>
<td>(AG)16</td>
<td>226-262</td>
<td>0.3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM154</td>
<td>2</td>
<td>(GA)21</td>
<td>106-234</td>
<td>0.15</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>D3</td>
<td>Green</td>
<td>LightSable</td>
<td>RM484</td>
<td>10</td>
<td>(AT)9</td>
<td>289-305</td>
<td>0.14</td>
<td>4.2</td>
<td>18.2</td>
</tr>
<tr>
<td>5</td>
<td>D4</td>
<td>Blue</td>
<td>LightSable</td>
<td>RM105</td>
<td>9</td>
<td>(CT)16</td>
<td>63-149</td>
<td>0.05</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>D4</td>
<td>Blue</td>
<td>WellRED</td>
<td>RM536</td>
<td>11</td>
<td>(CT)16</td>
<td>206-248</td>
<td>0.07</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>D2</td>
<td>Black</td>
<td>LightSable</td>
<td>RM125</td>
<td>7</td>
<td>(GCT)8</td>
<td>91-151</td>
<td>0.08</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>D2</td>
<td>Black</td>
<td>LightSable</td>
<td>RM19</td>
<td>12</td>
<td>(ATC)10</td>
<td>195-255</td>
<td>0.06</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>D3</td>
<td>Green</td>
<td>WellRED</td>
<td>RM541</td>
<td>6</td>
<td>(TC)16</td>
<td>132-199</td>
<td>0.14</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>D4</td>
<td>Blue</td>
<td>WellRED</td>
<td>RM413</td>
<td>5</td>
<td>(AAG)1</td>
<td>70-112</td>
<td>0.05</td>
<td>1.5</td>
<td>27.7</td>
</tr>
<tr>
<td>6</td>
<td>D4</td>
<td>Blue</td>
<td>LightSable</td>
<td>RM474</td>
<td>10</td>
<td>(AT)13</td>
<td>195-280</td>
<td>0.08</td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

(Source: Thomson, 2004b)

**Sample preparation for loading into the CEQ**

1. A small amount of the PCR reaction was required to run on the CEQ.
2. The samples (3 µL PCR product) were diluted in 12 µL dH₂O (1:5 dilution) for each well.
3. Amount of 2.5 µL of PCR/dH₂O mixture were diluted in 12.5 µL SLS (1:6 dilution) for total 1:30 dilution to each well.
4. Make SLS/DNA size standard dilution mix. On one panel, a total of 1:30 dilution (PCR/dH₂O/SLS) samples volume was subtracted from a final
injection volume (40μL). The volume was a total volume of SLS, including 0.5 μL CEQ 400 size standard.

5) Load the mixture of SLS/DNA size standard dilution mix and 1:30 dilution (PCR/dH2O/SLS) samples in each well based on panel dilution (Table 9) to reach a final injection volume of approximately 40μL to each well.

6) Add 1 drop mineral oil to each sample.

7) Add 10-12 drop separation bufer to each well of bufer plate or approximately ¼ from total volume of each well.

---

Figure 4 Sample multiplexing preparation scheme for loading into the CEQ.

Protocol for Running CEQ 8000

Computer was turned on and wait for Windows to load. After that CEQ is turned on. Double-click on “CEQ System” icon on desktop.

Choose DATABASE icon from CEQ main menu to open Data Manager window. A New user can create a new database and project as follows: (i) File/New Database [enter name] and select “Set as working database” then OK (ii) Highlight [database name] then File/New then highlight New project then File/Rename [enter name] and press Enter and then File/Exit. A returning user selects previous database as “working database” (appears bold) and then choose File/Exit.

Choose SETUP icon from CEQ main menu to open Sample Set-up window: (i) Click Create new sample plate or Use existing sample plate and click OK (ii) Highlight cells and enter samples names (one cell at a time, or multiple cells at once) (iii) Assign method to each sample column (if using the DNA Size standard 400, then select Frag-1 from drop-down menu below column), then choose File/
Exit (iv) Choose Parameter system dye spectra for Analysis. Print and Export sections can be left alone.

Choose RUN icon from CEQ main menu to open CEQ Run window. Select File/System Preferences and enter Operator name, Project name, etc.

1. Wetting tray

The wetting tray is where the capillary rests when installed on the CEQ but not in use. The wetting tray should be filled with approximately ⅔ full of deionized water. The wetting tray should be changed after each run.

Under Direct Control click on the “wetting tray” area in the picture to release the wetting tray. Once the “Event Type” says “Idle” then remove wetting tray, the remove the lid and fill with ultrapure water up to “fill line” then dry the outside and put back in place (close locks).

![Figure 5 Wetting tray (Source: Beckman, 2002)](image)

2. Capillary Array

The capillary array and array fitting are extremely fragile and must be handled with care. When handling the capillary array, always hold the tab on the electrode block. Handling the array near the capillaries may result in breakage.

Select Replenish/Release Manifold Plug or Install Capillary Array from Run menu (depending on which is currently in the machine). Open “Sample Access Cover” and lift to vertical locking position. Open “Capillary Access Cover” and lift to vertical locking position. Unlatch two rubber latches and lift “Temperature Control Cover” to vertical position. Loosen screws for “Plenum Assembly”, carefully pull Plenum straight back from the instrument (slowly) and set it aside. Lift the “Eject Lever” to release “Array Fitting” (or
“Manifold Plug”). If removing array, touch array fitting to base plate and let gel stand dry first. Grasp “Electrode Block” tab and pull out of the instrument, and put array in container.

Select correct option (Replace Array or Install Manifold Plug) and click OK. To install new array, insert the “Array Fitting” into CEQ while holding the tab (facing downwards) and push all the way in, until it is secure. Insert the “Electrode Block” by aligning the pins (and by holding the tab). Carefully route the capillaries through the hole in the short plenum. Replace Plenum (2 screws), then Access Cover (1 screw), and three covers. In Install Capillary Array box, click Set to New if new array, otherwise enter proper numbers for serial numbers, number of runs or days on the instrument, click Done.

If brand new array is installed, then under the Direct Control Menu, choose Manifold Purge, use the default settings and click OK. And after that is complete, under the direct control Menu and choose Gel Capillary Fill and use the default settings and click OK.

![Figure 6](image_url) Remove or install Capillary Array (Source: Beckman, 2002)

3. Gel Cartridge

Select Replenish/Release Gel Cartridge or Install Gel Cartridge from Run Menu. Open “Gel cartridge Access Cover” by gentle pushing the top (it will spring open). Pull the cartridge Locking Lever and swing barrel 90° from its locked position. Grasp wings of the gel cartridge or gel pump plug and pull it out of the barrel. Note the lot number and hours on the instrument for the gel cartridge.
If installing a gel cartridge, remove air bubbles first (by pressing plunger) until a small amount of gel appears at the top of the gel cartridge. Insert into the barrel and press all the way in (by aligning the wings). Push the barrel back into its locked position in the instrument. Select the correct option (Install Cartridge or Install Plug) and Set to New or enter the lot number and hours on machine of a previously used gel cartridge, click Done. Note that the gel cartridge should not be used if it has been on the CEQ for > 72 hours. Close access cover and click Done, and wait for “Event Type” to say “Idle”.

![Figure 7 Replace and install gel cartridge](Source: Beckman, 2002)

4 Install sample plate and sample set up

Prepare buffer plate with 250-300 L separation buffer, and have sample plate ready to go. Select RUN/Start sample plate then select [project] and [plate] and click OK. Confirm configuration and then load plates. Click load and wait until “Even Type” says “Idle” and then click Start.

![Figure 8 Loading the sample plate](Source: Beckman, 2002)
The CEQ 8000 should do everything automatically from this point, the data being collected is watched by clicking the Data tab. When the sample plate is finished running the “Event Type” will then say “Idle”.

Proceed to replace the array with the array fitting, replace the gel cartridge with the gel plug rinse and empty the wetting tray, and remove the sample and buffer plates (see instructions above). Remember to record the data for the array and gel cartridge (runs, hours). Turn off the machine, and then exit the software and turn off the computer.

**SSR Fragment Analysis**

PCR product was analyzed in an automated CEQ 8000 Genetic Analysis System, Beckman Coulter. Fragment lengths were estimated using internal size standards by The Fragment Analysis Module software. The number of alleles per locus was based on an evaluation of the 288 rice accessions.

Based on these alleles sizes, statistical analysis software, (i) program NTSYSpc 2.11p (Exeter Software, Setauket, USA) was used for SSR analysis. The presence or absence of alleles at each locus was coded in binary form 1 or 0, respectively. The Dice Coefficient (SIMQUAL) and UPGMA method (Unweighted Pair Group Method Arimatic) used to cluster the varieties and visualize their genetic relatedness to each other. Principal Coordinate Analysis was conducted to create a scatter plot showing genetic relationship among accession (ii) Genetic distance was calculated using Dc (Cavalli-Sforza and Edwards, 1967) Phylogenetic reconstruction was based on the Neighbor-joining method (Saitou and Nei, 1987) implemented in PowerMarker V3.0 (http://www.powermarker.net), by Jack Liu (powermarker@hotmail.com) and (iii) The inferred ancestry of the rice accessions was calculated using Bayesian Model-based analysis method (Pritchard et al., 2000) using “Structure v2.1” (http://pritch.bsd.uchicago.edu). The posterior probabilities were estimated assuming prior values of k between 2 and 8. The posterior probabilities were estimated using the Markov Chain Monte Carlo method (MCMC). The result presented in this study were based on burnin period length =10,000; MCMC iteration after burnin = 100,000; where alpha was constant.
RESULT AND DISCUSSION

Comparing traditional slab gels and automated capillary-electrophoresis

Automated detection of fluorescently-labeled microsatellite markers using capillary electrophoresis proved to be a powerful and efficient technique for fingerprinting rice accessions and assessing genetic variation among closely related varieties. The use of multiple fluorophores increased the multiplexing potential compared to manual detection using silver staining in this diverse collection of germplasm. Automated allele detection using within lane size standards and specialized software also improve the accuracy and efficiency of the analysis by reducing the time required to estimate the size of each allele.

Capillary instruments offer several significant advantages over slab gel scanners (Table 8). The most obvious advantage of multicapillary systems is the elimination of gel plate assembly and gel casting, because a flowable separation matrix is automatically injected into each capillary just before loading new samples. Some care must be taken to ensure that air bubbles are not introduced into the matrix, because their presence in a capillary causes noise from laser light diffraction and instability in the electrophoretic current.

Figure 9 shows the peak height and dye signal of the 400 DNA Size Standard and the microsatellites from Panel 5 amplified for Popot rice from East Kalimantan Province. The size standard was effective for estimating molecular weights for all the PCR products amplified across all the multiplex panels, which ranged from 60 to 420 nt long. Each individual microsatellite had a typical fragment pattern with a characteristic number of stutter-peaks, cross talk between the different color channels, relative peak height, slope threshold that could easily be recognized. Because only the most intense fragment was evaluated for molecular weight, the stuttering did not interfere with allele calling.

Figure 10 shows polyacrylamide gel electrophoresis (traditional slab gel) patterns revealed from four microsatellite of six rice accessions. The size marker in the edge of the plate to estimate the molecular weight of each allele manually.
Figure 9  Picture of the fluorescent signals from the Beckman CEQ8000 machine. The dye signal is on the Y-axis and the DNA size is on the X-axis (in nucleotide, nt). The evenly-spaced peaks (in red) are the DNA size standards (60 nt up to 420 nt). The other peaks represent microsatellite (SSR) markers (in blue, green, and black colors). For this rice variety from East Kalimantan (named Popot), five SSR marker peaks are visible.

Figure 10  Polyacrylamide gel electrophoresis (traditional slab gel) patterns revealed from four microsatellite of six rice accessions: lane 1, size marker; lane 2, K. beranakan; lane 3, Pokal gambrong; lane 4, Sereh; lane 5, Pre Pulung Cina; lane 6, Kasalath; lane 7, JC1. Next lane repeated of each primer.
Table 8  Comparing traditional slab gels and automated capillary-electrophoresis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Slab Gels</th>
<th>Capillary Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
<td>Chemistry and hardware</td>
<td>Chemistry, hardware and software</td>
</tr>
<tr>
<td>Treatment</td>
<td>Pre (gel plate assembly and gel casting, pouring of gels, gel loading) and Post (fixing and silver staining) Treatment</td>
<td>Automated (Sample Set-up, Remove or install Capillary array, Replace gel cartridge Install sample plate, Install buffer plate)</td>
</tr>
<tr>
<td>Amount of PCR reaction required</td>
<td>3-12 µl</td>
<td>3 µl</td>
</tr>
<tr>
<td>Resolution</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Data &amp; Picture Collection</td>
<td>Manual</td>
<td>Automated (store in a database and also be exported)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Multiplexing</td>
<td>Moderate (Combination of different primer size)</td>
<td>High (Combination of different primer size and colour)</td>
</tr>
<tr>
<td>Contamination</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Critical factors</td>
<td>Contamination</td>
<td>Excess salt</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>Impurities in sample matrix</td>
</tr>
<tr>
<td></td>
<td>Voltage</td>
<td>Sample loading amount</td>
</tr>
<tr>
<td></td>
<td>Fixing and silver staining</td>
<td>Capillary temperature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Voltage</td>
</tr>
<tr>
<td>Time consuming</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Labor force</td>
<td>High (manual)</td>
<td>Low (automated)</td>
</tr>
<tr>
<td>Toxicity</td>
<td>High (Unpolymerized Polyacrylamide)</td>
<td>Low (Polymerized Polyacrylamide)</td>
</tr>
<tr>
<td>Safety</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Development cost</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

Allele Scoring with Fluorescent-labeled microsatellites

Six multiplex panels were optimized for the evaluation of 288 rice accessions. This study is the first to use microsatellite markers to analyze the genetic diversity among 253 Indonesian rice accessions. Using 30 microsatellite markers, a total of 739 alleles were detected among the 288 rice accessions.

SSR Polymorphism in the Entire Sample

Number of alleles per locus

The number of alleles per locus averaged 25 and ranged from 11 (RM507, RM133, RM484) to as many as 48 (RM474). Those with tri-repeats had the greatest number of alleles (av.=33) compared to those with other di-nucleotide (av.=27) and GA-repeats (av.=23). RM 171 (chromosome 10) and RM507 (chromosome 5) had a (GATG)\textsubscript{5} MOTIF and (AAGA), motif, respectively and detected the lowest number of alleles (11 and 15) for a tetra-nucleotide SSR.
The number of alleles per locus was not significantly correlated with the different repeat motifs (P<0.05). The gene diversity, allele frequency and PIC value was significantly correlated with the different repeat motifs (P<0.05).

Allele size range

The size variation between the smallest and the largest allele at a given SSR locus was correlated with the number of alleles per locus (r=0.27). RM105 presented the smallest allele size range (13 bp) and had 17 alleles/locus while RM514 had the largest allele size range (137 bp) and a total of 18 alleles.

PIC Values

Polymorphism Information Content (PIC) values, a reflection of allele diversity and frequency among the varieties, were very high for most of the microsatellites, averaging 0.754 and ranging from a low of 0.46 (RM507) to a high of 0.91 (RM259, RM19, RM541). PIC values showed a significant positive linear correlation with the number of alleles (r=0.67) but negative linear correlation with allele size range (r=0.06) for microsatellite evaluated in this study.

High Frequency Alleles

An average of 36.6% of the 288 accessions shared a common allele at any given locus. RM507 was present the highest frequency allele up to182 out of 288 (63.3%) of the accessions. RM19 and RM259 showed the lowest frequency alleles, 14.6% and 19.7% respectively. The number of alleles per locus and the frequency of the most common allele at that locus were negatively correlated (r=0.47).

Rare Alleles

An allele that was observed in <16 of the 288 genotypes (<5%) was considered a rare allele. Rare alleles were observed at all of the SSR loci in one or more of the 288 accessions. In general, markers detecting a greater number of alleles/locus detected more rare alleles. The number of rare alleles per locus was positively correlated with the number of alleles (r=0.898). RM19 and RM144, base on tri-nucleotide repeat, had the highest frequency of rare alleles (97.1% and
97.5% respectively). While poly(GA)n detected the highest rare alleles (88%) compared to other di-nucleotide, tri- and tetra-nucleotide of SSR motifs. Rare alleles are highly informative for fingerprinting analysis.

**Multiplex Panels**

Multiplex panels of rice microsatellites providing whole genome coverage have recently been designed to facilitate diversity analysis of rice germplasm (Coburn et al. 2002). Panels of compatible microsatellites for multiplex fluorescent detection are useful for studies of cultivar diversity and high-throughput genotyping in this important crop species. These panels have been evaluated and optimized to confirm their efficiency and compatibility using a wide range of diverse accessions. For the current study, a set of 30 SSRs were designed into six panels.

Markers in Panel 6 had the largest number of alleles (172) but small average allele size range (50 bp) followed by Panel 2 (134 alleles, range of 53.6 bp), Panel 4 (115 alleles, range of 45.6 bp), Panel 3 (112 alleles, range of 47.8 bp), Panel 5 (107 alleles, range of 45.6 bp), and Panel 1 (99 alleles, range 55.2 bp). It is of interest that Panel 1 and 4 contained all di-nucleotide repeats, Panel 3, 5, and 6 contained a mixture of di- and tri- nucleotide SSR motifs while Panel 2 contained all a mixture of di-, tri-, and tetra- nucleotide SSR motifs.

The size range of alleles affects the degree of multiplexing that can be accommodated in a single lane. It is a concern that the wider allele size range might cause problems of overlapping alleles from different microsatellites within a single dye color. However the markers were not observed to overlap with each other. This occurred because this study allowed for a 40-bp "buffer" between markers in the design of the multiplex panel and alleles falling outside of the predicted size-range did not exceed the buffer zone. Therefore it seems that the multiplex panel can be confidently designed (Cho et al, 2000; Blair et al, 2002).
### Table 9  Allele size range, number of alleles, polymorphism information content (PIC), and mean allele sizes found among 288 rice accessions.

<table>
<thead>
<tr>
<th>Panel</th>
<th>Marker</th>
<th>min Size (bp)</th>
<th>max Size (bp)</th>
<th>Allele No</th>
<th>Gene Diversity</th>
<th>Allele Frequency</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>RM5</td>
<td>95</td>
<td>133</td>
<td>21</td>
<td>0.8471</td>
<td>0.285</td>
<td>0.8321</td>
</tr>
<tr>
<td>1</td>
<td>RM433</td>
<td>207</td>
<td>307</td>
<td>20</td>
<td>0.7507</td>
<td>0.3345</td>
<td>0.713</td>
</tr>
<tr>
<td>1</td>
<td>RM55</td>
<td>153</td>
<td>238</td>
<td>16</td>
<td>0.6354</td>
<td>0.5448</td>
<td>0.5938</td>
</tr>
<tr>
<td>1</td>
<td>RM215</td>
<td>111</td>
<td>225</td>
<td>24</td>
<td>0.775</td>
<td>0.3333</td>
<td>0.7438</td>
</tr>
<tr>
<td>1</td>
<td>RM514</td>
<td>234</td>
<td>371</td>
<td>18</td>
<td>0.7736</td>
<td>0.3566</td>
<td>0.7447</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>95</td>
<td>371</td>
<td>20</td>
<td>0.7563</td>
<td>0.3708</td>
<td>0.6273</td>
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<tr>
<td>2</td>
<td>RM214</td>
<td>79</td>
<td>168</td>
<td>35</td>
<td>0.7388</td>
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<tr>
<td>2</td>
<td>RM11</td>
<td>113</td>
<td>149</td>
<td>25</td>
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<td>2</td>
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<td>0.2411</td>
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<tr>
<td>2</td>
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<td>141</td>
<td>19</td>
<td>0.7649</td>
<td>0.4299</td>
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<td>RM171</td>
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<td>347</td>
<td>15</td>
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</tr>
<tr>
<td>Mean</td>
<td></td>
<td>79</td>
<td>347</td>
<td>27</td>
<td>0.7943</td>
<td>0.3833</td>
<td>0.8923</td>
</tr>
<tr>
<td>3</td>
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<td>190</td>
<td>232</td>
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<td>0.6227</td>
<td>0.4599</td>
<td>0.551</td>
</tr>
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<td>RM287</td>
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<td>0.8715</td>
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<td>0.8597</td>
</tr>
<tr>
<td>3</td>
<td>RM259</td>
<td>116</td>
<td>179</td>
<td>31</td>
<td>0.9167</td>
<td>0.1976</td>
<td>0.9116</td>
</tr>
<tr>
<td>3</td>
<td>RM250</td>
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<td>193</td>
<td>27</td>
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<td>0.5754</td>
<td>0.6446</td>
</tr>
<tr>
<td>3</td>
<td>RM507</td>
<td>217</td>
<td>303</td>
<td>11</td>
<td>0.523</td>
<td>0.6333</td>
<td>0.4618</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>64</td>
<td>303</td>
<td>22</td>
<td>0.7176</td>
<td>0.4227</td>
<td>3.4286</td>
</tr>
<tr>
<td>4</td>
<td>RM161</td>
<td>70</td>
<td>182</td>
<td>27</td>
<td>0.7853</td>
<td>0.4211</td>
<td>0.7709</td>
</tr>
<tr>
<td>4</td>
<td>RM283</td>
<td>128</td>
<td>193</td>
<td>28</td>
<td>0.7765</td>
<td>0.3449</td>
<td>0.7458</td>
</tr>
<tr>
<td>4</td>
<td>RM124</td>
<td>257</td>
<td>298</td>
<td>16</td>
<td>0.8367</td>
<td>0.2544</td>
<td>0.8164</td>
</tr>
<tr>
<td>4</td>
<td>RM162</td>
<td>185</td>
<td>244</td>
<td>29</td>
<td>0.8232</td>
<td>0.3576</td>
<td>0.8091</td>
</tr>
<tr>
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<td>RM277</td>
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<td>133</td>
<td>15</td>
<td>0.7313</td>
<td>0.3895</td>
<td>0.6889</td>
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<td></td>
<td>70</td>
<td>298</td>
<td>23</td>
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</tr>
<tr>
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<td>238</td>
<td>272</td>
<td>18</td>
<td>0.767</td>
<td>0.4231</td>
<td>0.7457</td>
</tr>
<tr>
<td>5</td>
<td>RM154</td>
<td>148</td>
<td>202</td>
<td>32</td>
<td>0.8617</td>
<td>0.3214</td>
<td>0.8534</td>
</tr>
<tr>
<td>5</td>
<td>RM484</td>
<td>292</td>
<td>325</td>
<td>13</td>
<td>0.6234</td>
<td>0.5374</td>
<td>0.5704</td>
</tr>
<tr>
<td>5</td>
<td>RM105</td>
<td>97</td>
<td>110</td>
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</tr>
<tr>
<td>5</td>
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<td>27</td>
<td>0.8194</td>
<td>0.3207</td>
<td>0.8007</td>
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<td>Mean</td>
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<td>21</td>
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<td>0.3685</td>
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<td>0.7599</td>
<td>0.364</td>
<td>0.7254</td>
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<td>6</td>
<td>RM19</td>
<td>208</td>
<td>260</td>
<td>35</td>
<td>0.9207</td>
<td>0.1466</td>
<td>0.9155</td>
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<tr>
<td>6</td>
<td>RM541</td>
<td>132</td>
<td>199</td>
<td>41</td>
<td>0.9234</td>
<td>0.2021</td>
<td>0.9195</td>
</tr>
<tr>
<td>6</td>
<td>RM413</td>
<td>56</td>
<td>112</td>
<td>21</td>
<td>0.7688</td>
<td>0.3841</td>
<td>0.7409</td>
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<td>6</td>
<td>RM474</td>
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<td>306</td>
<td>48</td>
<td>0.8277</td>
<td>0.3947</td>
<td>0.8224</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>56</td>
<td>306</td>
<td>34</td>
<td>0.8401</td>
<td>0.2983</td>
<td>4.1236</td>
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<tr>
<td>Total</td>
<td>Mean</td>
<td>56</td>
<td>371</td>
<td>25</td>
<td>0.781</td>
<td>0.3662</td>
<td>0.7574</td>
</tr>
</tbody>
</table>

### Clustering of Rice Accessions with Microsatellite Markers

#### Genetic similarity among accessions

Genetic similarity values between the various rice accessions were used to produce an UPGMA dendrogram (cluster tree analysis, Figure 11) which clearly explained the relationship among 288 rice accessions. Using 10% dissimilarity,
as the threshold for UPGMA clustering three major groups were observed (Figure 11). Group 1 represent *indica* accessions, Group 2 represent *japonica* varieties and Group 3 contained wild relatives.

Group 1, *indica* cluster, consisted of 177 Indonesian rice accessions. Based on morphological characteristic including 129 *Cereh*, 17 *Bulu*, 5 *Gundil*, 5 breeding lines (Cisadane, Sintanur, Cihang, IR64-60, Widaj), 21 unknown and 3 international rice varieties (Popot-165, IR64, Binulawan). One Subgroup consisted of *Aus* varieties (DhalaShaitta, DV85, Kasalath) and aromatic varieties (JC1) (Appendix 2 & 3).

Figure 11 Dendogram showing three major groups from cluster analysis (NTSYS-PC) of 288 accessions using genetic diversity data for 739 alleles at 30 SSR loci.
Group 2, the *japonica* cluster, consisted of 76 Indonesian rice accessions. Based on morphological characteristic including 32 *Cereh*, 22 *Bulu*, 4 *Gundil*, 18 unknown and 6 International rice varieties including 4 Tropical *Japonica* (Sinampaga selection, Maintmolotsy 1226, Moroberekan, Trembese), and 2 Temperate *Japonica* (Caloro, Koshihikari) (Appendix 4).

Group 3, wild cluster, consisted of 2 satellites that included in either Group 1 (*indica*) or Group 2 (*japonica*). Group 1, 8 wild relatives (*O. rufipogon*, *O. nivara*, *O. longiglumis*) and Group 2, 14 wild relatives (*O. glaberrima*, *O. australiansis*, *O. alta*, *O. latifolia*, *O. minuta*, *O. punctata*, *O. malampuzhaensis*, *O. officinalis*, *O. rhizomatis*, *O. grandiglumis*) (Appendix 4).

The grouping identified by PCoA (Figure 12) were similar to those identified by the UPGMA tree cluster analysis (Figure 11). One hundred seventy seven of the 288 accessions formed a distinct cluster in the right of the diagram corresponding to *indica* rice, well separated from the 78 accessions corresponding to *japonica* clusters in the left of the diagram. Twenty two of the wild relatives clusters in the middle of the diagram. The remaining 11 accessions falling outside these groups include one *aromatic* rice (JC1) and 10 Indonesian rice accessions (Ingsa Bondo, Ketan Siam, Jonoko, Popot, Ketan Putih, Superwin, Nggondo, Pulut Putih, Engkoran, Sasak Jalan).

![Diagram showing clusters of rice varieties](image)

**Figure 12** The two dimensional scaling resulting from PCoA analysis (NTSYS-PC) of 288 accessions using genetic diversity data for 739 alleles at 30 SSR loci. Oval area represent the three major clusters of germplasm; *japonica* (left oval), wild (middle circle), *indica* (right oval).
Genetic similarity values between the various rice accessions were used to produce a phylogenetic reconstruction based on the Neighbor-joining method (Figure 13). The grouping identified by Neighbor-joining tree cluster analysis were similar to those identified by the UPGMA tree cluster analysis (Figure 11). The NJ unrooted cluster observed three major groups Group 1 contained indica accessions, Group 2 represent japonica varieties and Group 3 contained wild relatives.

Group 1, indica cluster, consisted of 182 rice accessions consisted of 174 Indonesian rice accessions including 5 breeding lines (Cisadane, Sintanur, Ciheraing, IR64-60, Widas) and 3 International rice varieties (POPOPOT-165, IR64, Binulawan). Group 2, the japonica cluster, consisted of 82 rice accessions consisted of 76 Indonesian rice varieties and 6 International rice varieties including 4 Tropical Japonica (Sinampaga selection, Maintmolotsy 1226, Moroberekan, Trembese) and 2 Temperate Japonica (Caloro, Koshikihari). Group 3, wild cluster, consisted of 3 Aus varieties (DhalaShaitta, DV85, Kasalath) and 21 wild relatives.

In addition, no Indonesian varieties have been identified with the aus or aromatic groups.

Figure 13 Neighbor Joining tree showing three major groups from cluster analysis (Powermarker) of 288 accessions using genetic diversity data for 739 alleles at 30 SSR loci.
According to UPGMA and NJ clusters, it is of interest that some breeding lines close together either with breeding lines or Indonesian local varieties. Sintanur with Cisadane, IR64 (from IRRI) with LonengA from West Kalimantan (bulu), Widus with IR64-60 (from Indonesia), and Ciberang with Pulut Mute from East Kalimantan (cereh). It is believed that LonengA and Pulut Mute may have arisen via hybridization with the breeding lines.

While molecular fingerprinting can be used to distinguish varieties from one another, it can also suggest the possibility that two varieties with the same names may be genetically different. To give examples, Toliwang II closely related with Keser B while Toliwang IV with Keser A (Table 10). Also possible that two varieties with the same names and genetically similar. Examples, Ndangan Cantik 1 and Ndangan Cantik 2 from South Sulawesi, Popot from East Kalimantan and Popot from Cornell, Porosi A and Porosi B from Central Sulawesi, Pulut Putih 1 and Pulut Putih 2 from North Sumatera, and Pare Dangang A and Pare Dangang B from South Sulawesi. This may be a case where the same variety is being cultivated under different names.

Table 10 List of the same accessions name that genetically different

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Accession no.</th>
<th>Type</th>
<th>Origin</th>
<th>Closely with</th>
<th>Origin</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jula-JulI</td>
<td>4224</td>
<td>Cereh</td>
<td>East Java</td>
<td>Hoing</td>
<td>East Java</td>
<td>cereh</td>
</tr>
<tr>
<td>Jula-JulI 2</td>
<td>4259</td>
<td>Cereh</td>
<td>East Java</td>
<td>Kail</td>
<td>Kalimantan</td>
<td>unknown</td>
</tr>
<tr>
<td>Keser A</td>
<td>20824</td>
<td>Cereh</td>
<td>North Maluku</td>
<td>Toliwang IV</td>
<td>North Maluku</td>
<td>cereh</td>
</tr>
<tr>
<td>Keser B</td>
<td>20825</td>
<td>Cereh</td>
<td>North Maluku</td>
<td>Toliwang II</td>
<td>North Maluku</td>
<td>cereh</td>
</tr>
<tr>
<td>Lemo 1</td>
<td>20513</td>
<td>Unknown</td>
<td>Kalimantan</td>
<td>Syair</td>
<td>Aceh</td>
<td>cereh</td>
</tr>
<tr>
<td>Lemo 2</td>
<td>4403</td>
<td>Cereh</td>
<td>West Kalimantan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lokal Buntu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sangala 1</td>
<td>20905</td>
<td>Cereh</td>
<td>South Sulawesi</td>
<td>Sianak Bogor</td>
<td>North Sumatera</td>
<td>cereh</td>
</tr>
<tr>
<td>Lokal buntu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sangala 2</td>
<td>20906</td>
<td>Cereh</td>
<td>South Sulawesi</td>
<td>Rencong</td>
<td>West Java</td>
<td>cereh</td>
</tr>
<tr>
<td>LonengA</td>
<td>3981A</td>
<td>Bulu</td>
<td>West Kalimantan</td>
<td>IR64</td>
<td>IRRI</td>
<td>Breeding lines</td>
</tr>
<tr>
<td>LonengB</td>
<td>3981B</td>
<td>Bulu</td>
<td>West Kalimantan</td>
<td>Padi Sudora</td>
<td>Bengkulu</td>
<td>cereh</td>
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<tr>
<td>Pako I</td>
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<td>Cereh</td>
<td>North Maluku</td>
<td>Kayowa</td>
<td>North Maluku</td>
<td>unknown</td>
</tr>
<tr>
<td>Pako II</td>
<td>20827</td>
<td>Cereh</td>
<td>North Maluku</td>
<td>Pae Dae Ulee-ule</td>
<td>SouthEast Sulawesi</td>
<td>Bulu</td>
</tr>
<tr>
<td>Pilihan Putih I</td>
<td>20846</td>
<td>Cereh</td>
<td>North Sulawesi</td>
<td>Klara Super</td>
<td>North Sulawesi</td>
<td>cereh</td>
</tr>
<tr>
<td>Pilihan Putih II</td>
<td>20849</td>
<td>Gundil</td>
<td>North Sulawesi</td>
<td>Bengkongang</td>
<td>Central Java</td>
<td>Bulu</td>
</tr>
<tr>
<td>Toliwang II</td>
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<td>Cereh</td>
<td>North Maluku</td>
<td>Keser B</td>
<td>North Maluku</td>
<td>cereh</td>
</tr>
<tr>
<td>Toliwang IV</td>
<td>20831</td>
<td>Cereh</td>
<td>North Maluku</td>
<td>Keser A</td>
<td>North Maluku</td>
<td>cereh</td>
</tr>
</tbody>
</table>
Population genetic structure and differentiation

The inferred ancestry from a population of 288 accessions could also be divided into three major subpopulations using Pritchard's structure analysis (Figure 14). The natural log (ln) probability of the data, which is proportional to the posterior probability, is maximized at k=3 subpopulations (-26877.4), converged well and showed comparable or higher likelihoods than k= 4-8 among multiple runs of the program.

Accessions belonging to the major groups shared at least 80% ancestry with other group members (Table 11). Seventy nine rice accessions comprised more than 90% shared ancestry. This first Group corresponding to japonica cluster, consisted of 73 Indonesian rice accessions and 6 International rice varieties including 4 Tropical Japonica (Sinampaga selection, Maintmolotsy 1226, Moroberekan, Trembese).and 2 Temperate Japonica (Caloro, Koshihikari) (Table 10). The largest group has 175 rice accessions showed more than 90% shared ancestry. This second group corresponding to indica cluster, consisted of 167 Indonesian rice accessions, 5 breeding lines (Cisadane, Siantanur, Cihergang, IR64-60, Widia), and 3 International rice varieties (POPOT-165, IR64, Binulawan). The third group has 23 rice accessions showed more than 90% shared ancestry corresponding to wild cluster, consisted of 20 wild relatives and 3 Aus accessions (DhalaShaita, DV85, Kasalath). The remaining 11 rice accessions have <80% (45-78%) membership in any one group and were assigned to a admixtures group that was identical to the outlying accessions identified by PCoA analysis.

Figure 14  Graph Showing proportion of shared ancestry among the 288 accessions based on allele molecular size data from 30 SSR loci using Pritchard's model-based method Structure. The grey areas represent admixtures
<table>
<thead>
<tr>
<th>Group</th>
<th>Accessions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Japonica</strong></td>
<td>Maintmolotys1226, Mororoberekan, Sinampanga Selection, Koshihikari, Caloro, Trembese, Pare Lambar, Pae Daya Indololye, Gandis, Laka, Asemadi, Lambow Putih, Pare leleng, Pare Pulang Cina, Pare Pulhungta, Jamudin, Sereh, Ganggoi, Reko, Pae Daya Ule-ule, Lokal tulem, Talum ungu, Talum putih, Ketan berasanakan, Pare pulut bampo, Huma gatog, Limar, Pakol, Pare, Pare-Eja3, Toraja, Padi Merah, Garu, Empat bulanDarah, Tiung, Taring menjanggan, Buleng, Pare tambongh, Ndangan Camitik, Ndangan CamitikZ, Ase Dungan, Ase Bakacan, Pare Bulan, Kembang Ading, Rumbay, Padi Java, Genjah Rawe, Cempa Hulut, Ketan Hitam, Kulit Bawang, Sawah Duku, Uyun, Pulut latu, Tholo, Koyak, Selasi, P.Pasir, Lemunyau, P.Gogo, Siame Putih, Dupa, Cempaka, Burung Samih, Tagalobo, Porosia, Porosia, Pare Pulut Bolong, Pae wita, Pae Biyu Anggolopu, Baur, KeserA, Keser, Pakoli, ToliwangIl, Toliwang IV, Kayowa, Mama, Laka Tesam, Rangkat, Ngondo.</td>
</tr>
<tr>
<td><strong>Wild</strong></td>
<td>O.rhiotrichatis23, O.grandishumus21, O.minuta17, O.punctata13, O.affinis12, O.glaberrima4, O.affinis11, O.glaberrima5, O.rufigogon6, O.rufigogon7, O.mivara_10 O.mivara_11, O.punctata14, O.malayapseudoalis15, O.australis16, O.minuta18 O.lata19, O.latifolia20, O.longisili23, O.rufigogon8, DV85, Dhalastra, Kasalath</td>
</tr>
<tr>
<td><strong>Admixtures</strong></td>
<td>O.rufigogon9, 9C1, Ingga Bondo, Ketan putih, Rigen, Jonoko, Raden Ratu, Sasak Jalan, Popen, Ketan Siam, SuperWin</td>
</tr>
</tbody>
</table>

Note: The 288 rice accessions in this study are listed with the groups from the STRUCTURE analysis. Accessions in the admixtures group show <80% membership for any group.

**Comparison of the genetic diversity of the indica and japonica subspecies of cultivated rice.**

As a control, international cultivated varieties had fewer indica (3) than japonica (6) included in this study, the average number of alleles observed in indica (4.2) was higher to that in japonica (2.7). The indica group had greater PIC value (0.63) than the japonica group (0.43). The evaluation of gene diversity in
the *indica* group (0.68) was greater than that for the *japonica* group (0.49) (Table 12). It suggests that *indica* has a higher level of genetic variation than *japonica* rice, and is in accordance with previous reports (Mackill, 1995; Yang et al., 1994).

**Comparison of the genetic diversity of Breeding lines and local varieties**

The average number of alleles observed in *cereh* (14.8), *bulu* (8.2), *gundil* (4.5), was higher than the breeding lines (2.03). The *bulu* group had the highest average PIC value (0.70), followed by *cereh* (0.69), *gundil* (0.63) and the breeding lines (0.28). The evaluation of gene diversity in *bulu* group was highest (0.74), followed by *cereh* (0.72), *gundil* (0.68) and the breeding lines (0.34) (Table 12). It suggests that *cereh* has a higher level of genetic variation than the other groups. Therefore, the breeding lines have a lower level of genetic variation than the local varieties. This result argues that plant breeders could capture additional diversity by working with local varieties. It is likely that the landraces contain numerous agronomically useful alleles not represented in the breeding lines.

**Comparison of the genetic diversity of wild relatives and cultivated rice**

The wild relatives had a higher level of genetic variation as observed in the average number of alleles (12), PIC value (0.85) and gene diversity (0.86) than to the other cultivated rice groups (Table 12). The result of this study suggests that much of the genetic diversity of wild rice was already lost in cultivated rice. All of these facts indicate that there is great potential for the discovery and utilization of wild rice and local varieties in rice breeding.

**Table 12** Allele frequency, number of allele, gene diversity and PIC values among eight groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Allele Frequency</th>
<th>Number of allele</th>
<th>Gene Diversity</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aro</em></td>
<td>0.9833</td>
<td>1.0333</td>
<td>0.0167</td>
<td>0.0125</td>
</tr>
<tr>
<td><em>Ass</em></td>
<td>0.6500</td>
<td>2.1333</td>
<td>0.3949</td>
<td>0.3373</td>
</tr>
<tr>
<td>breeding</td>
<td>0.7267</td>
<td>2.0333</td>
<td>0.3388</td>
<td>0.2863</td>
</tr>
<tr>
<td><em>Bulu</em></td>
<td>0.3690</td>
<td>8.2000</td>
<td>0.7424</td>
<td>0.7038</td>
</tr>
<tr>
<td><em>Gundil</em></td>
<td>0.4222</td>
<td>4.5667</td>
<td>0.6845</td>
<td>0.6341</td>
</tr>
<tr>
<td><em>Indica</em></td>
<td>0.4138</td>
<td>4.2000</td>
<td>0.6834</td>
<td>0.6331</td>
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<tr>
<td><em>Japonica</em></td>
<td>0.5883</td>
<td>2.7667</td>
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<td>0.4363</td>
</tr>
<tr>
<td><em>Cereh</em></td>
<td>0.4326</td>
<td>14.8000</td>
<td>0.7194</td>
<td>0.6905</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.3871</td>
<td>8.1000</td>
<td>0.7406</td>
<td>0.7070</td>
</tr>
<tr>
<td>Wild</td>
<td>0.2263</td>
<td>11.9333</td>
<td>0.8629</td>
<td>0.8483</td>
</tr>
</tbody>
</table>
Geographical distribution of Rice Accessions

The inferred ancestry 288 accessions could also be divided into three major subpopulations using Pritchard's structure analysis (Figure 15). The natural log (ln) probability of the data, is maximized at k=3 subpopulations (-26881.2) showed comparable or higher likelihoods than k= 4-8 among runs of the program.

Accessions belonging to the major groups shared at least >50% ancestry with other group members. The first group corresponded to wild, has 34 rice accessions consisted of 24 international wild and 3 aus (Bangladesh, Japan, India). The second group has 84 accessions located from Nusa Tenggara, Sulawesi, Maluku, and Irian Jaya. The third group has 182 rice accessions located from Sumatera, Jawa, Kalimantan, and Bali including 6 International rice varieties (Madagascar, Guinea, Philippines, Japan, California).

Figure 15 Inferred ancestry across 20 provinces of Indonesia among the 253 accessions based on allele molecular size data from 30 SSR loci using Pritchard's model-based method Structure.

However, because this study included only a few from Sulawesi (52), Maluku (10) and Irian Jaya (Papua) (5) therefore represents a small sampling of their wide geographic range, do not represent the full diversity of rice. It is possible that different genomic regions and sampling will offer different views of both the frequency and the distribution of polymorphism.
CONCLUSION

Automated detection of fluorescently-labeled microsatellite markers using capillary electrophoresis proved to be a powerful and efficient technique for assessing genetic variation among closely related varieties. The multiplex panels of 30 SSRs developed in this study will also be useful for future studies of Indonesian rice germplasm for genetic diversity analysis and variety identification. Among 288 accessions, the genetic variation was higher in indica than japonica rice, higher in wild relatives than cultivated rice, while the breeding lines had less genetic variation than the local varieties. Genetic similarity, principle coordinate analysis and population structure analysis identified three major groups. Group 1 corresponded to japonica, group 2 to indica and group 3 to wild rice. The geographical distribution of rice accessions divided into three major subpopulations. The first group was international wild, the second group was located in the Nusa Tenggara, Sulawesi, Maluku, and Irian Jaya (Papua) provinces. The third group was located in the Sumatera, Jawa, Kalimantan, and Bali provinces.
REFERENCES


## Appendix 1  List of rice germplasm accessions used for SSR analysis

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Accession no.</th>
<th>Source</th>
<th>Origin</th>
<th>Accession number</th>
<th>Abbrev.</th>
<th>Morphological Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IRRI 103410</td>
<td>IRRI</td>
<td>Sri Lanka</td>
<td>O. sativa</td>
<td>IR103410</td>
<td>RM-9</td>
</tr>
<tr>
<td>2</td>
<td>IRRI 103580</td>
<td>IRRI</td>
<td>Brazil</td>
<td>O. glaberrima</td>
<td>IR103580</td>
<td>RM-11</td>
</tr>
<tr>
<td>3</td>
<td>IRRI 104415</td>
<td>IRRI</td>
<td>Philippines</td>
<td>O. sativa</td>
<td>IR104415</td>
<td>RM-15</td>
</tr>
<tr>
<td>4</td>
<td>IRRI 104694</td>
<td>IRRI</td>
<td>Ghana</td>
<td>O. glutinosa</td>
<td>IR104694</td>
<td>RM-13</td>
</tr>
<tr>
<td>5</td>
<td>IRRI 100491</td>
<td>IRRI</td>
<td>Thailand</td>
<td>O. officinalis</td>
<td>IR100491</td>
<td>RM-103</td>
</tr>
<tr>
<td>6</td>
<td>IRRI 100158</td>
<td>IRRI</td>
<td>Guinea</td>
<td>O. glaberrima</td>
<td>IR100158</td>
<td>RM-99</td>
</tr>
<tr>
<td>7</td>
<td>IRRI 102166</td>
<td>IRRI</td>
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