Biotyping of Jatropha Curcas L. from Thailand, Laos and Tanzania by MALDI-TOF MS Technique

Thitiporn Ditsawanon, Rajabhat Rajanagarindra University, Thailand
Sittiruk Roytrakul, Genome Institute, Thailand
Janthima Jaresittikulchai, Genome Institute, Thailand
Worawut Chulalaksananukul, Chulalongkorn University, Thailand

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Abstract
It was shown that *Jatropha curcas* L. of the family *Euphorbiaceae* had significant economic importance for its seed oil, which could be used to substitute for diesel oil. It had been widely distributed in Thailand. This study was aimed at determining the biotype that could produce better oil content by using biotyping technique. It was found that among the biodiversity of 15 populations of *J. curcas*, Jc1 to Jc13 were obtained from different localities in Thailand, Jc 14 was obtained from Laos, and Jc15 was obtained from Tanzania. The biotyping technique, Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry method, was used for this purpose. The MALDI-TOF method was an inexpensive method that yielded rapidly with high accuracy results. Also, the results showed that the m/z spectrum pattern profiles of all populations were the same, and were confirmed with program ClinProTools 2.2. Therefore, it could be concluded that all populations (Jc1-15) were the same strain.

Keywords: Jatropha curcas L., Biotyping, MALDI-TOF, spectrum pattern

Introduction

Jatropha curcas, a biotype that belong to family *Euphobiaceae*, has attained significant economic importance due to its industrial uses and as very promising source of non-edible oil that can be used as feed stock for production of bio-diesel oil (Openshaw, 2000). The quality of biodiesel depends on the chemical composition of the fatty acids present in the oil. The fatty acids profile has a direct impact on ignition quality, heat of combustion and oxidative stability. An ideal biodiesel composition should have monounsaturated fatty acid such as oleic acid in high content. Jatropha oil fraction consists of two saturated fatty acids, i.e., 14.1-15.3% of palmitic acid and 3.7-9.8% of stearic acid, and several unsaturated fatty acids, i.e., 34.3-45.8% of oleic acid, and 29.0-44.2 % of linoleic acid (Berchmans and Hirata, 2008). It is a non-food crop with high quality of oil and it can grow on degraded soils. This plant, can be cultivated on non-agricultural land, and does not require a land that good for the production of food. Moreover, it can be used to control erosion and grown as a fence, especially for animal farms. Many parts of plant can be used as medicine, its leaves are a feed stock for silk worms, its flowers attract bees and then the plant has a honey production potential. The most important part is fruit that contain viscous oil that can be used for soap making in the cosmetics industry and as a diesel substitute (Openshaw, 2000). It is a tropical plant that can be grown in low to high rainfall areas. In several continents, i.e., America, Africa and Asia, it is cultivated mainly as a hedge to protect fields (Gübitz et al., 1999). The Portuguese merchants have introduced *J. curcas* in to Thailand for more than two centuries ago and now the crop is widely grown in various regions of Thailand (Sadakorn, 1984).

The biotyping technique is needed to classify plants into group individuals, which having the same genotype. The use of PCR-based molecular marker in *J. curcas* plant is a common method nowadays.

It was reported that 94.6% of similarity between toxic and non-toxic varieties using RAPD fingerprinting, whereas in recent study showed that the percentage of similarity was 84.91 by RAPD and 83.59 by AFLP fingerprinting (Sujatha et al., 2005; Pamidimarri et al., 2008). Not Only RAPD and AFLP but also SSRs were being widely employed in many studies because of high polymorphism of microsatellites.

Ranade and coworkers collected *J. curcas* leaves from different region more recently in India (2008), and assessed for the bio-diversity among any collections using SPAR, single-primer amplification reaction method. They found that the North East accessions were most dissimilar relative to other collections.

Mass spectrometry has used and enabled great advantages to characterized proteins. Among the aforementioned techniques, the MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight mass spectrometry, technique was chosen as a biotype tool in this research because of several advantages, such as, great tolerance for impurities, a possibility of reanalyze the same sample, the optimal compatibility with simple technique and inexpensive time-of-flight (TOF) analyzers. This is a rapid, straightforward and high accuracy method to generate peak patterns from whole proteins (Monagas et al., 2010).

The results represent in mass to charge (m/z) spectrum, which specific to each protein. The MALDI-TOF MS technique has been applied to analyze bacterial cells such as grouping Myxococci (*Corallococcus*) strains, discrimination between wild-type and ampicillin-resistant strains of *Escherichia coli*, and phylogenetic classification of *Pseudomonas putida* strains. Furthermore, the MALDI-TOF MS method can be used to analyze mammalian cell lines (Stackebrandt et al., 2005; Johanna and Faith, 2007; Teramoto et al., 2007; Zhang et al., 2006). Several applications of MALDI-TOF MS analysis have been used. In the determination of the structure features of different plant pro-anthocyanidin types from food and non-food plants, an analysis of the N-linked glycan structures, and an analysis of condensed tannin in plant leaves and needles (Mokrzycki-Issartel et al., 2003; Karg et al., 2009; Behrens et al. 2003). In recently years, many researches of plants were carried out by this technique. Oszvald and team (2013) used MALDI-TOF-MS to identified molecular size of HMW glutenin subunit (HMW-GS) protein in rice endosperm, and found that expressed wheat HMW-GS showed positive effect on the functional properties of rice dough by significantly increasing the size distribution. In the same year, researchers from Japan reported that high –resolution MALDI-TOF MS showed that the structure of polyphenolic compounds from almond is a series of polyflavan-3-ol polymers composed of catechin/epicatechin units and gallocatechin/epigallocatechin units up to 11-mer with with several interflavanoid ether linkages. The results suggest almond seed skin contains highly polymerized polyphenols with strong α-amylase inhibitory activity, which retard absorption of carbohydrate (Tsujita et al., 2013). Morover, MALDI-TOF-MS analysis in apple leaves found interested proteins that located in chloroplast, and they play important roles in photosynthesis and stress resistance for plants (Ning et al., 2013).

About MALDI-TOF-MS in *J. curcas*, researchers from department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand reported that after purified seed coat of *J. curcas* by ammonium sulfate precipition and chromatography on DEAE SephacelTM and CM-cellulose columns and protein determined by MALDI-TOF-MS. The results suggested that Jc-SCRIP may be a potential natural antimicrobial and anticancer agent in medical application (Nuchsuk et al., 2013). However, this is the first report of a comparative analysis of *J. curcas* strains in Thailand, Laos and Tanzania using MALDI-TOF-MS.

The sample preparation is a crucial step in MALDI-TOF MS analysis, a careful optimization of the experimental parameters and sample preparation, i.e., matrix, concentration, solvents, and crystallization conditions. The use of internal standards for calibration and an averaging of multiple spectra are required in order to reduce the great variability of observed results (Szajli et al., 2008). In this study, proteins were extracted by SDS, and subsequently precipitated by cold acetone, and TCA. Sinapic acid (SA) matrice was used as matrix solution in this approach. Samples were mixed with matrix solution and analyzed directly by the MALDI-TOF MS.

Methodology

Plant material preparation

The final stage of full-developed fruits of fifteen local varieties of *J. curcas* were collected as shown in Table 1, where the varieties, Jc1 to 13, were obtained from

different localities in Thailand. The variety Jc 14 was collected from Laos, and the variety Jc 15 was obtained from Tanzania. Their seeds were stored at -80 ºC until used.

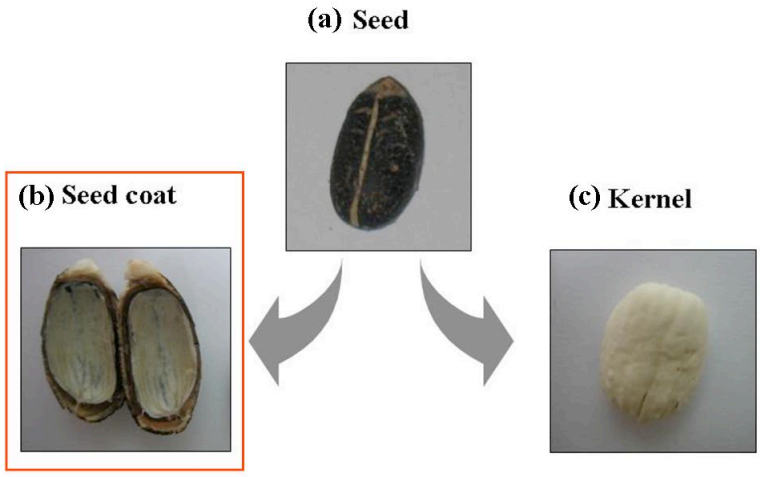
Table 1 Details of fifteen *J. curcas* plants (Jc1-15) from different localities in Thailand, Laos and Tanzania

No. of samples	Local source	A part of Thailand
Jc 1	Nakhon Ratchasima 1	Northeast
Jc 2	Nakhon Nayok	Central
Jc 3	Bangkok 1	Central
Jc 4	Nakhon Sawan 1	North
Jc 5	Chiang Mai	North
Jc 6	Chon Buri	East
Jc 7	Nakhon Sawan 2	North
Jc 8	Chanthaburi	East
Jc 9	Suphan Buri	Central
Jc 10	Satun	South
Jc 11	Prachin Buri	East
Jc 12	Nakhon Ratchasima 2	Northeast
Jc 13	Bangkok 2	Central
Jc 14	Laos	-
Jc 15	Tanzania	-

Protein extraction and precipitation

Seed coat tissue (Figure 1) from each sample was ground by mortar and pestle with liquid nitrogen. A 250 mg of a fine powder sample was transferred into a microtube, added 1 ml of 0.1% SDS and then shaking vigorously with vortex for 1 hour. A 1 ml of cold acetone was added into a supernatant in a ratio of 2:1 between acetone and sample, mixed the mixture vigorously and finally frozen overnight at -20 ºC. The frozen sample was thawed and centrifuged at 10,000 rpm for 15 min, and the supernatant was discarded. The pellet was resuspended directly in 1,100 µl 0.15% DOC (deoxycholic acid), mixed well and let it settled for 15 min. A 100 µl 72% TCA (trichloroacetic acid) was added, mixed, and frozen overnight at -20 ºC. The frozen sample was thawed and centrifuged at 10,000 rpm for 15 min, then kept the pellet and resuspended in 0.1% TFA (trifluoroacetic acid).

Figure 1 Components of *J. curcas* seed: a) seed b) seed coat and c) kernel. Seed kernels were used to protein prepared.



Matrix solution preparation

Sinapic acid, the matrices that most commonly used in MALDI-TOF-MS, was freshly prepared daily as saturated solution in a solvent mixture. The mixture ratio between 0.1% Trifluoroacetic acid and 100% Acetonitrile was 2:1.

Analysis of the preparation

Sixteen dilutions of analyzed mixtures, i.e., 1X, 5X, 10X, 25X, 50X, 100X, 250X, 500X, 1000X, 1500X, 2000X, 2500X, 3000X, 3500X, 4000X and 5000X, were tested with the Jc 5. The analyzed mixture was prepared on a stainless steel template. The dilution mixture at 1X concentration was spotted in 1 µl for a sample, 1 µl for the matrix solution, and the others used matrix solution as a diluent and were spotted in 2 µl. ProteoMass^{TM} Peptide & Protein MALDI-MS Calibration Kit (Sigma Aldrich Co., USA) was used to analyze the spectra.

The dilution of the mixture that showed the most obvious peak spectrum was used in all samples for 8 repeats each.

MALDI-TOF MS analysis

All dilutions of analyzed mixtures were air dried at room temperature, then the template was conducted in UltraFlex III TOF/TOF (Bruker Daltonik GmbH, Germany) equipped with N_2 laser. All spectra were recorded in linear, positive ion mode. Laser shot was controlled by FlexControl 3.0 (Bruker Daltonik GmbH, Germany), which the laser was randomly shot until it accumulated to 3,000 shots across a spot. Mass range of 1,000-20,000 m/z (Da) was used for analysis.

The peak of MALDI-TOF MS spectra were detected and listed as mass using FlexAnalysis 3.0 (Bruker Daltonik GmbH, Germany). Spectra analysis generated data that included both peak position and the intensity. All data samples were compared by ClinProTools 2.2 (Bruker Daltonik GmbH, Germany) and peak spectrum were analyzed using statistics.

Results, discussion and conclusion

Comparison of optimal dilution mixtures

The comparison of MALDI-TOF MS profiles in the mass range of interest (1,000-20,000 m/z) using 16 dilution mixtures revealed the appropriate dilution was 250X that showed the most obvious and more peak spectrums. Then, the 250X dilution was chosen for all samples.

MALDI-TOF MS analysis of 15 *J. curcas* samples

The experiments were repeated for 8 times for each sample to confirm the results with 120 (15x8) spots totally. It was interesting to note that after peaks detected by FlexAnalysis software, all samples produced highly similar characteristic spectra pattern as demonstrated in figure 2. The mass spectra of whole protein exhibited a large number of peaks in the m/z range between 1,000-20,000 Da. Many of the major peaks observed in all samples are common, such as, the peak at 1978.92, 3495.33 and 5744.11 Da. All 21 spectra peak masses that found in all samples and their standard deviation are shown in Table 2.

Figure 2 The representative spectra of the MALDI-TOF MS spectra peak patterns of the Jc 1-15 where whole proteins ranging from 1000 to 20000 Da

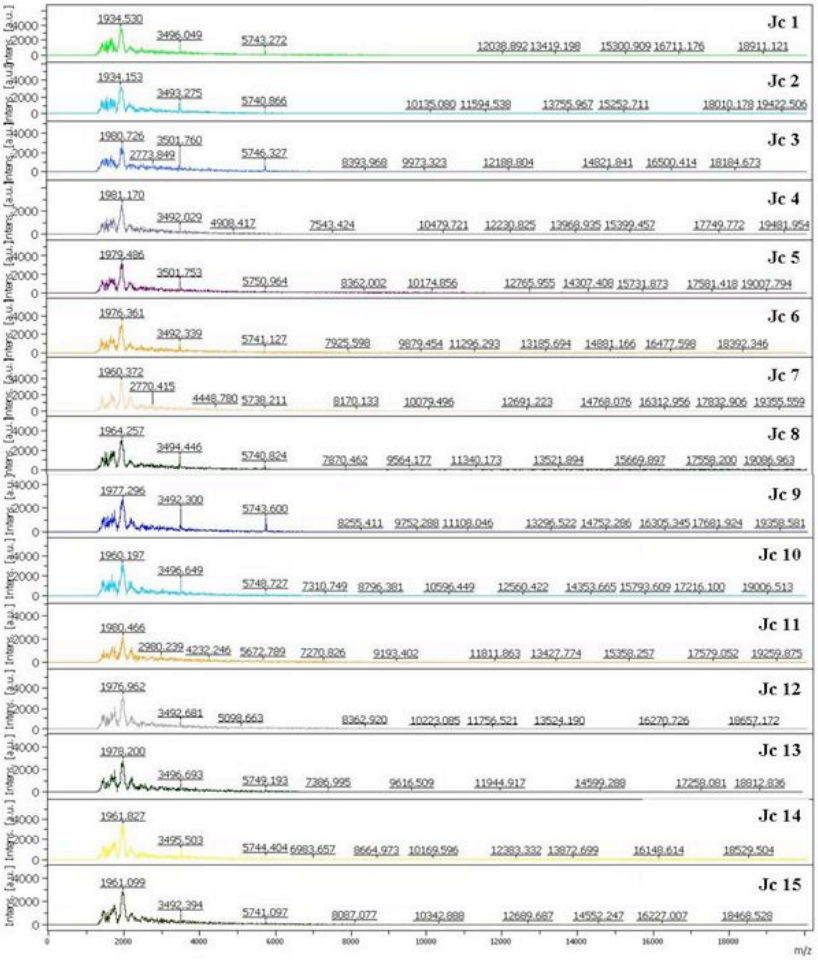


Table 2 Peak statistic table show the mass averages and their standard deviation

Index	Mass	StdDev
1	1350.19	0.71
2	1367.14	0.57
3	1428.48	2.05
4	1450.61	2.42
5	1467.64	2.64
6	1487.77	1.47
7	1497.81	1.73
8	1528.99	1.94
9	1544.93	1.48
10	1574.29	2.68
11	1591.93	2.5
12	1737.02	3.87
13	1753.76	3.46
14	1769.44	2.94
15	1938.53	3.88
16	1945.64	3.43
17	1961.38	4.24
18	1978.92	3.28
19	1994.53	2.4

ClinProTool 2.2 was used to compare peak patterns of all samples in order to determine the reliability of the data. According to Figure 3, each gray stripe represented of each peak, the dark one meant high intensity peak. From the results, it was observed that the stripe patterns of all samples were highly similar and the average peaks intensity was shown in figure 4.

Figure 3 Photography of gel view showing the spectra of 15 *J. curcas* samples with 120 spots. The x-axis records the m/z value from 1000 to 10000 Da. The left y-axis displays the running spectrum number originating from subsequent spectra loading. The peak intensity is expressed by color intensity.

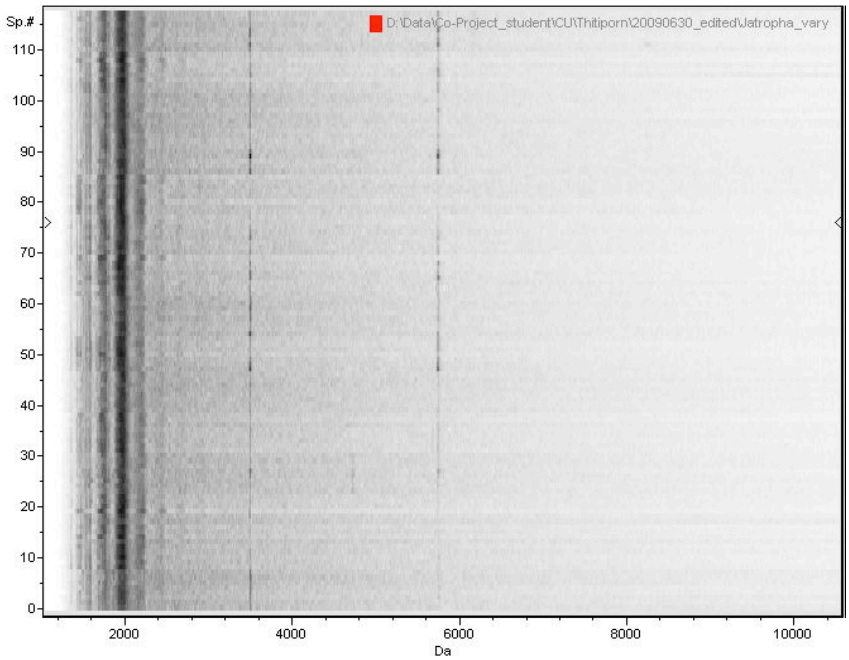
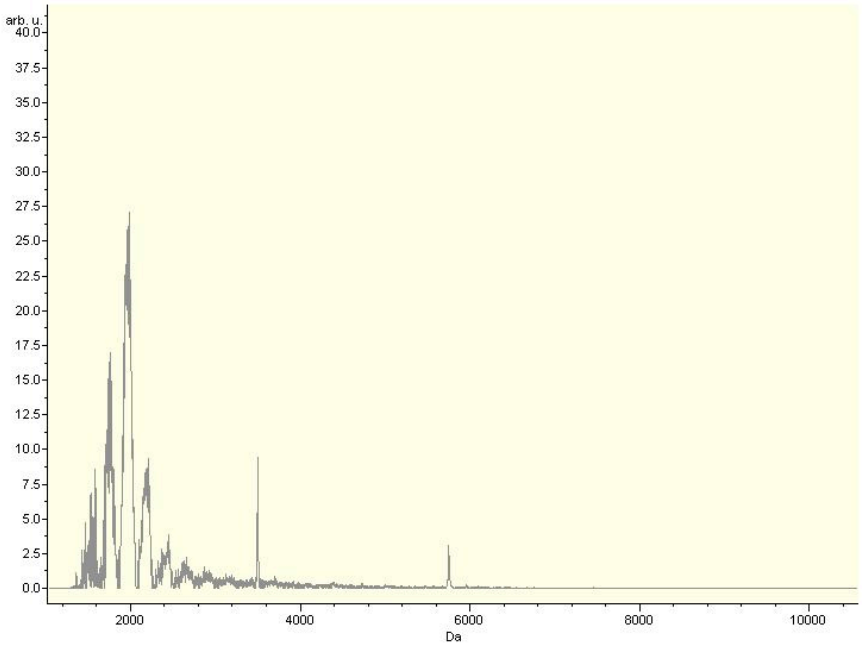


Figure 4 The average peak intensity of all samples.

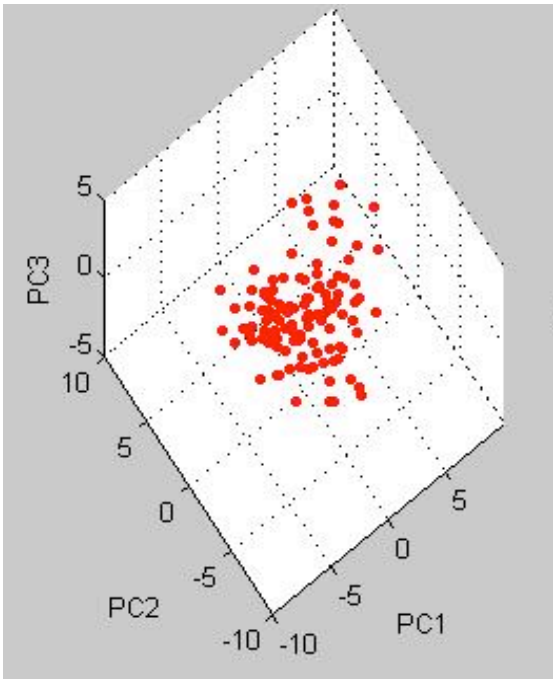


Moreover, to show an obvious result, ClinProTool was used to integrate a statistic data and shown in terms of principle component analysis (PCA). In the case of mass spectra, the variables are represented by the intensity at defined masses. According to the resolution, the number of these variables can be very high. The PCA reduces the number of dependent variables contained within the spectra set replacing groups of variables by a single new variable. By this, a set of new variables, so called principle

components will be generated. Each principle component (PC) is a linear combination of the original variables. All principle components are orthogonal to each other, so there is no redundant information.

As demonstrated in figure 5, the obtained data shown that majority plotted spot concentrated together in the centre and only about 20 spots or approximately 10% were disperse around. The PCA result implied that the set of data have little variances. The result indicates that all samples of *J. curcas* are the same strain. This conclusion was related to the report of Sadakorn in 1984 that *J. curcas* in Thailand was introduced by the Portuguese merchants more than two centuries ago and now the crop is widely grown in various regions of Thailand. It is possible, if *J. curcas* from different part in Thailand are the same strain.

Figure 5 A statistic data shown in terms of principle component analysis (PCA)



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Contact email: thitiporn.boo@gmail.com