

A Comparison of the Efficiency of Simple DNA Extraction Methods by Direct Amplification of *rbcl* gene in *Capsicum annuum* and *Capsicum chinense*

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Abstract

Chilli landraces of Thailand are an essential spice in Thai cuisine. Taxonomically, they belong to the family Solanaceae, genus *Capsicum*. This study aimed to (i) survey the biodiversity of chilli varieties in Tambon Saylampong, Amphoe Thatako, Nakhon Sawan Province, and (ii) compare the efficiency of two DNA extraction methods, a commercial kit and NaOH extraction, through the direct amplification of the *rbcl* gene. A line transect method was used for the biodiversity survey and sample collection, covering a distance of one kilometer. Young leaves from each chilli variety were collected and used for DNA extraction using the commercial kit and NaOH extraction method. The study found two chilli species comprising seven distinct varieties, *Capsicum annuum* consisted of five varieties: Chinda, Chee Fah, Khee Noo Si Moug, Khee Noo Khao, and Sai Pla Lai, and *Capsicum chinense* consisted of two varieties: Khee Noo Hom and Khee Noo Suan. DNA extracted using a commercial kit and NaOH extraction method were available to measure the quantity and purity by spectrophotometer. Regarding amplifying a partial sequence of the *rbcl* gene, DNA extracted using the commercial kit showed strong amplification in all samples. Some DNA samples of diluted DNA extracted using NaOH method were also successfully amplified, whereas all samples of non-diluted DNA extracted using NaOH method failed to amplify. These findings indicate that the commercial kit yields high-quality DNA suitable for polymerase chain reaction (PCR) applications. However, the NaOH extraction method presents a faster and more cost-effective alternative, though further optimization is needed to enhance its efficiency.

Keywords: DNA extraction, *rbcl* gene, NaOH, *Capsicum*, Chilli

1. Introduction

Chilli is the most widely used spice in food worldwide. It was classified as belonging to family Solanaceae, genus *Capsicum*. Five chilli species are widely domesticated for home use and economic purposes; *C. annuum*, *C. chinense*, *C. baccatum*, *C. frutescens* and *C. pubescens* (Popelka et al., 2017). The top five chilli-producing countries are China, Mexico, Turkey, Indonesia and Spain (Ferniah & Pujiyanto, 2017). In Thailand, chili is also an essential spice in Thai foods, especially in Thai curry paste. The collection of Thai chili landraces has been kept at the Department of Horticulture, Kasetsart University (Kamphaeng Saen Campus) for 250 accessions (Kethom et al., 2019). The pungency is the heat sensation of chilli when we consume it. Therefore, pungency is a unique characteristic of chilli caused by capsaicinoids, alkaloid compounds that are synthesized and accumulated in chilli fruit (Mazourek et al., 2009; Nelson, 1919). The pungent principle in chilli (*Capsicum*) was first isolated by Thresh and also assigned the name capsaicin. According to Micko's extraction method, the analysis and molecular weight determination of capsaicin indicated its formula $C_{18}H_{27}NO_3$ (Nelson, 1919). The heat measurement unit is the Scoville Heating Unit or SHU. It is the highest dilution of chilli extract at which heat can be detected or the dilution before it is no longer detected. Nowadays, the high-pressure liquid chromatography (HPLC) method can be used to determine the capsaicin content in chilli with more reliability and accuracy (Popelka et al., 2017). However, SHU is still a popular unit in many studies and reports. Due to the pungency, mammals do not like chilli and birds have no receptor for capsaicin, therefore, birds can eat chilli without heat feeling (Ferniah & Pujiyanto, 2017). The biological effects of capsaicin are dose-dependent and it has a spectrum

of effects in sensory neurons ranging from excitation to cell death. Neurotoxicity is induced by high doses of capsaicin. Most capsaicin fibers are polymodal nociceptors that respond to sensory stimuli, including noxious pressure, heat and chemical irritants. Thus, the most abundant class of capsaicin receptors is nociceptive fibers or nociceptive neurons (Mazourek et al., 2009; Winter et al., 1995).

DNA barcoding technique is a novel concept and is increasingly popular as an efficient tool for studying, categorizing and classifying species, including identification of new species, cryptic species and invasive species. DNA barcoding is a molecular tool that uses standardized genetic primers to classify species and enhance biodiversity assessment. It provides very useful genetic data for conservation strategies, conservation policies and conservation actions. However, the important role of DNA barcoding is the identification of species while the morphology has limitations. For precisely, DNA barcoding can be used in combination with the traditional taxonomic method for species classification (Farooq et al., 2020; Krishnamurthy & Francis, 2012).

The plant's DNA barcoding has been established as shown in many studies. Their results indicated that the phylogenetic gene markers in land plants are involved in very limited variation and not enough to determine the species limit or not enough to differentiate the species. To solve this problem, using a single chloroplast region or a combination of different regions was suggested. Most DNA barcode genes in plants are contained in the chloroplast DNA (cpDNA) such as *rbcL*, *matK*, and *trnH-psbA*, and it has been used with a success rate of 87.1-92.7% in plant DNA barcoding (Farooq et al., 2020). The *rbcL* and *matK* genes were proposed to be the "Core Barcode" at the Consortium for the Barcode of Life (CBOL) in 2009 by a plant working group and using supplemented with additional regions were required (CBOL Plant Working Group, 2009; Hollingsworth, 2011). The *rbcL* gene is a gene that encodes the protein ribulose-1,5-biphosphate carboxylase-oxygenase (RuBisCO), which contributes to carbon fixation in the photosynthesis process. The total length of the *rbcL* gene is about 1,400 base pairs (bp) and provides many characters for phylogenetic studies in plants (Basith et al., 2021; CBOL Plant Working Group, 2009).

In this study, we aimed i) to survey the biodiversity of domesticated chilli variety in Tambon Saylampong, Amphoe Thatako, Nakhon Sawan Province and ii) to compare the efficiency of simple DNA extraction methods by evaluating direct amplification of the *rbcL* gene using a commercial kit and the NaOH extraction method.

2. Materials and Methods

2.1 Survey method and sample collection

The survey area covered fifteen villages in Tambon Saylampong, Amphoe Thatako, Nakhon Sawan Province. Line transect was used in this study for one kilometer on the main road (left and right sides) in each village. The name of chilli variety was recorded by interviewing the owner in each house and the young leaves (small, light-green leaves at the shoot tips) were collected by cleaning with 70% alcohol and kept in a plastic zip-lock. All samples were kept at -20°C until used for DNA extraction.

2.2 Genomic DNA extraction

A hundred milligrams of young chili leaf tissue was used for genomic DNA (gDNA) extraction. The sample was homogenized using an autoclaved plastic pestle and extracted using either a commercial kit or the NaOH extraction method. The plant DNA extraction kit (GenUP™ Plant DNA Kit) was purchased from Apsalagen (Germany), and the extraction was performed according to the manufacturer's protocol. The NaOH extraction method was adapted from He et al. (2009) with modifications. The young chili leaf sample was homogenized in 50 mL of 0.5 M NaOH, incubated at room temperature for 5 minutes, and the process was repeated twice. The sample was then centrifuged at 8,000 rpm for 5 minutes. Five microliters of the supernatant were transferred to a 1.5 mL eppendorf tube as the non-diluted DNA extract, while another portion was diluted in 200 µl of sterile distilled water to obtain the diluted DNA extract.

2.3 Quantity and purity of gDNA examination

The extracted chilli gDNA was examined for quantity and purity by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). The optical density of nucleic acids was measured at 260 nm (A260), while protein contamination was measured at 280 nm (A280). The concentration of extracted chili gDNA was shown in

ng/ μ L, and its purity was determined by the A260/A280 ratio. A high-quality DNA sample was indicated by an A260/A280 ratio within the range of 1.8–2.0.

2.4 Validation of gDNA by using PCR amplification

Two microliters of extracted chili gDNA were used to amplify a partial *rbcL* gene by PCR with universal *rbcL* primers. The forward primer used was *rbcL-af* (5'-ATGTCACCACAAACAGAAAC-3'), and the reverse primer was *rbcL-724R* (5'-TCGCATGTACCTGCAGTAGC-3') (Basith et al., 2021; Fay et al., 1997). The PCR conditions included an initial preheating step at 95°C for 5 minutes, followed by 35 amplification cycles consisting of denaturation at 95°C for 45 seconds, annealing at 60.8°C for 45 seconds, and extension at 72°C for 45 seconds. A final extension step was performed at 72°C for 10 minutes. The *rbcL* gene amplicon was analyzed using 1% agarose gel electrophoresis with a 100 bp DNA ladder marker (GeneDireX X-DM001-R500, Bio-Helix). The gel was stained with ethidium bromide, and the expected *rbcL* gene amplicon size was approximately 519 bp.

3. Results and Discussion

3.1 Biodiversity of chilli

Two species and seven varieties of chilli were recorded from fifteen villages in Tambon Saylampong, Amphoe Thatako, Nakhon Sawan Province. Five varieties of *Capsicum annuum* consisted of Chinda, Chee Fah, Khee Noo Si Moung, Khee Noo Khao, and Sai Pla Lai (Figure 1). Two varieties of *Capsicum chinense* consisted of Khee Noo Hom and Khee Noo Suan (Figure 2). These two species of chilli were classified to family Solanaceae, Genus *Capsicum*.

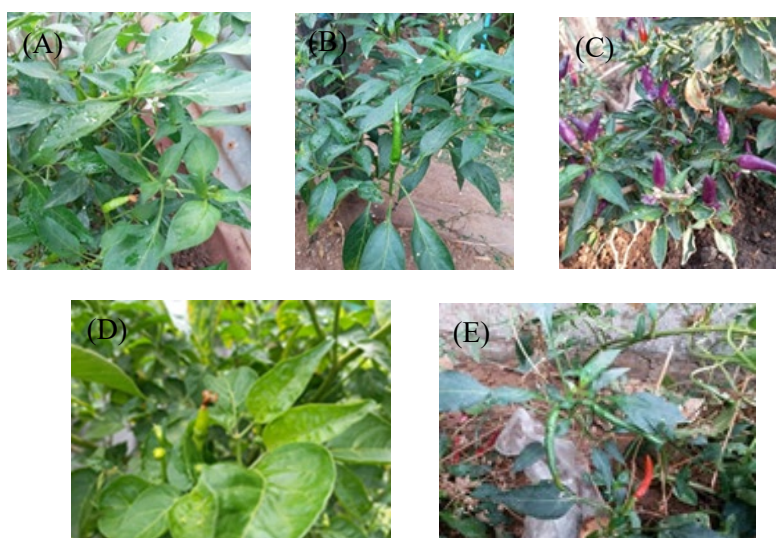


Figure 1. Five varieties of *Capsicum annuum*; Chinda (A), Chee Fah (B), Khee Noo Si Moung (C), Khee Noo Khao (D) and Khee Sai Pla Lai (E).



Figure 2. Two varieties of *Capsicum chinense*; Khee Noo Hom (A) and Khee Noo Suan (B).

Chilli (*Capsicum annuum* L.) was originated from Mexico and introduced to Europe, Africa and Asia. The unique characteristic of chilli is pungency which induces a burning or heat sensation after consumed (Kethom et al., 2019). In Thailand, chilli is an essential spice for Thai cuisine, including fresh consumption, chilli paste and sauce. Therefore, we can find chilli around Thailand, especially in the village which is planted for home usage. There are five main domesticated species of chilli, *C. annuum* L., *C. chinense* Jacq., *C. baccatum* L., *C. frutescens* L. and *C. pubescens* Ruiz & Pavon, because of their economic value. The selected characteristics of chilli for planting consisted of the shape, color, flavor, degree of pungency, non-pungent, size of fruit and fruit wall thickness. Therefore, the diversity of chilli can be analyzed by its qualitative and quantitative morphology. The morphological variation is affected by the environment and leads to the genetic relationship among the cultivars (Ferniah & Pujiyanto, 2017). This study found seven varieties of chilli in Tamboon Saylampong, belonging to *Capsicum annuum* and *Capsicum chinense*. Among them, five varieties are classified under *C. annuum*; Chinda, Chee Fah, Khee Noo Si Moung, Khee Noo Khao, and Sai Pla Lai. The remaining two varieties, Khee Noo Hom and Khee Noo Suan, belong to *C. chinense*. The primary purpose of chilli cultivation is for household use, particularly cooking. As a result, some varieties, such as Chinda, Chee Fah, Khee Noo Hom, and Khee Noo Suan, are well-known in the commercial market, while others, including Khee Noo Si Moung, Khee Noo Khao, and Sai Pla Lai, are not commercially recognized. The preference for each type of chilli is a personal choice, varying from person to person depending on their tolerance for spiciness.

3.2 Quantity and purity of the isolated gDNA between commercial kit and NaOH extraction method

This study prepared gDNA from young leaves of seven chilli varieties of *C. annuum* and *C. chinense*. The concentration and purity of extracted gDNA were determined by NanoDrop 2000 spectrophotometer as shown in Table 1. The concentration of gDNA ranged between 18.3-89.7 ng/μl and the purity ranged between 1.32-9.29 by commercial kit. The concentration of gDNA ranged between 49.0-246.6 ng/μl and the purity ranged between 1.59-2.12 by diluted NaOH extraction method. The concentration of gDNA ranged between 1,951.8-10,529.9 ng/μl and the purity ranged between 1.51-1.91 by non-diluted NaOH extraction method. The yield of gDNA by commercial kit and diluted NaOH extraction showed a similar concentration yield, while gDNA extracted by non-diluted NaOH extraction showed an extremely high concentration. The A260/280 ratios of all extracted gDNA samples indicated good purity, except for the Khee Noo Khao sample, which showed an unusually high ratio of 9.29 when extracted using the commercial kit, suggesting possible contamination or measurement error.

Table 1. Comparison of the concentration and purity of gDNA extracted from commercial kit and NaOH method.

Chili variety	Commercial kit		NaOH extraction method			
			Diluted (D)		Non-diluted (N)	
	DNA conc. (ng/μl)	DNA purity (A _{260/280})	DNA conc. (ng/μl)	DNA purity (A _{260/280})	DNA conc. (ng/μl)	DNA purity (A _{260/280})
<i>Capsicum annuum</i>						
Chinda	41.4	1.32	125.8	1.59	7,454.3	1.51
Chee Fah	39.6	1.87	67.5	2.00	3,551.3	1.78
Khee Noo Khao	54.7	9.29	171.4	1.79	10,529.9	1.41
Khee Noo Si Moung	28.6	1.55	49.0	2.12	1,951.8	1.91
Sai Pla Lai	89.7	1.76	87.2	2.00	4,016.0	1.74
<i>Capsicum chinense</i>						
Khee Noo Hom	18.3	1.87	246.6	1.77	8,912.6	1.58
Khee Noo Saun	35.9	3.31	147.7	1.93	9,554.4	1.60

Nowadays, genetic analysis is the source of information about genetics and genomics in organisms. The sufficient of DNA with adequate quality will be indicated the success of genetic analysis by higher techniques such as PCR, DNA sequencing, and/or related techniques. Therefore, the success of DNA isolation is the first important step for molecular genetics research. The main step of nucleic acid (DNA & RNA) isolation methods

can be divided into four steps: organic extraction method (phenol/chloroform), inorganic extraction method (salting out), solid phase extraction method (solid matrix) and nucleic acid purification (El-Ashram et al., 2016). In recent years, many publications have tried to develop simple, inexpensive, and efficient novel nucleic acid extraction methods for PCR applications and other techniques. In plants, DNA samples are typically extracted from fresh, young and soft organs such as leaves, buds, flowers, and shoots, which serve as a good source of DNA. The quantity and purity of the extracted gDNA were measured using a spectrophotometer. The gDNA extracted using a commercial kit provided a good quantity and purity of DNA in this study; it might be the use of the proteinase K enzyme to denature various proteins in cytoplasm after cell lysis and a few times of washing steps, while the gDNA extracted using NaOH method was not use proteinase K during the extraction process (to reduce the cost). The quantity and purity of DNA extracted using NaOH method were able to measure by spectrophotometer. The non-diluted gDNA extracted using the NaOH method yielded more DNA than the diluted gDNA extracted using the same method. The non-diluted gDNA extracted using NaOH method showed the highest concentration of DNA when compared to diluted gDNA extracted using NaOH method and the commercial kit. This may be attributed to the presence of secondary metabolites and polysaccharides that interfere with the absorbance value. Plant species often produce secondary metabolites such as alkaloids, flavonoids, phenolic compounds, gummy polysaccharides, terpenes and quinine. These secondary metabolites can interfere with the success of DNA isolation and other techniques in molecular biology (Amani et al., 2011). Polyphenols are especially powerful oxidizing agents that can reduce the yield and purity of extracted DNA (Porebski et al., 1997). These secondary metabolites might be diluted in diluted gDNA extracted using NaOH method, therefore, some of their samples could amplify the *rbcL* gene by PCR amplification. While non-diluted gDNA extracted failed to amplify in all samples.

3.3 Amplification of the partial part of *rbcL* gene

The direct amplification of the *rbcL* gene was used to assess the efficiency of each gDNA extraction method. All seven chilli varieties produced strong PCR products of the *rbcL* gene when gDNA was extracted using the commercial kit (Figure 3). Among the samples extracted with diluted NaOH, five varieties, Chee Fah (2D), Khee Noo Khao (3D), Sai Pla Lai (5D), Khee Noo Hom (6D), and Khee Noo Suan (7D), successfully yielded *rbcL* gene amplicons. However, the remaining gDNA samples, including those extracted with non-diluted NaOH, failed to amplify (Figure 3).

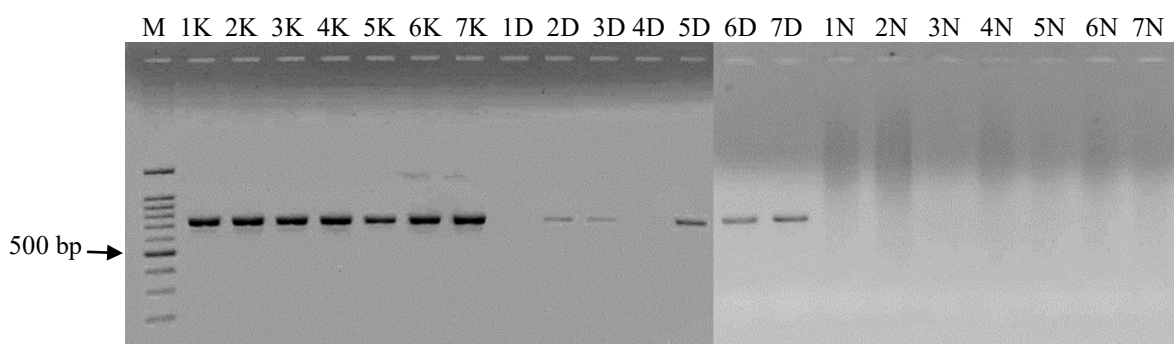


Figure 3. Direct PCR amplification of *rbcL* gene using gDNA isolated from commercial kit and NaOH extraction method in seven varieties of chilli. (M = 100 bp ladder; 1 = Chinda, 2 = Chee Fah; 3 = Khee Noo Kwao; 4 Khee Noo Si Moun; 5 = Sai Pla Lai; 6 = Khee Noo Hom; 7 = Khee Noo Suan; K = Commercial kit; D = diluted NaOH extraction; N = non-diluted NaOH extraction)

Plant DNA fingerprinting involves the use of molecular marker techniques to identify cultivars. Therefore, developing a DNA isolation protocol that yields high-quality DNA in sufficient quantity is essential for PCR techniques and sequencing. The DNA extraction is the basic method and an important step for molecular biology research. Using the commercial DNA extraction kit can provide good quality and quantity of DNA extracted. However, the main problem of commercial DNA extraction is its high cost. Alternative methods, such as the

cetyltrimethylammonium bromide (CTAB) method, also require substantial time, other chemicals and other laboratory equipment. The sodium hydroxide (NaOH) DNA extraction method in plant was initially described by Wang et al. (1993) and has been modified in various studies since then (He et al., 2009; Osmundson et al., 2013; Satya et al., 2013; Werner et al., 2002). These studies demonstrated the effectiveness of DNA extracted using the NaOH method for PCR applications. Sodium hydroxide (NaOH) is an alkaline solution, and the principle of this method relies on the selective alkaline denaturation of high-molecular-weight chromosomal DNA, which is why it is called alkaline extraction method (Tan & Yiap, 2009). However, the extracted DNA by NaOH method was contaminated with secondary metabolites, which may interfere with the PCR process. To reduce the concentration of these metabolites, we diluted the DNA extracted using the NaOH method. The results of *rbcL* gene amplification showed successful amplification in some samples using direct PCR, whereas non-diluted DNA failed to amplify the *rbcL* gene in all samples.

4. Conclusions

Seven varieties of chilli were cultivated in Tambon Saylampong, Amphoe Thatako, Nakhon Sawan Province, comprising five varieties of *C. annuum* and two varieties of *C. chinense*. Young leaves of these seven chilli varieties were used to extract DNA using a commercial kit and NaOH extraction method (diluted and non-diluted extracted gDNA). The quantity and purity of all extracted gDNA samples were able to measure by NanoDrop 2000 spectrophotometer. All of the extracted gDNA samples using the commercial kit were successfully amplified for the *rbcL* gene. While some diluted extracted gDNA samples using NaOH method were successfully amplified for *rbcL* gene, on the other hand, all non-diluted extracted gDNA samples using NaOH method failed to amplify *rbcL* gene. These findings suggested that the commercial kit provides high-quality DNA suitable for polymerase chain reaction (PCR) applications. Nevertheless, the NaOH extraction method offers a fast and inexpensive protocol for isolation of sufficient amounts of DNA of adequate quality for PCR-based techniques; however, further optimization is necessary to improve its efficiency for molecular biology research.

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Conflict of Interest

This research received no specific grant from any funding agency, and the authors have no conflicts of interest relevant to the content of this research article.

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