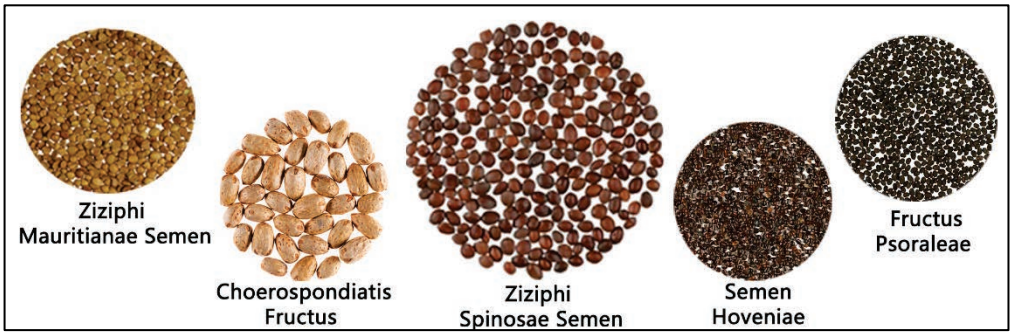
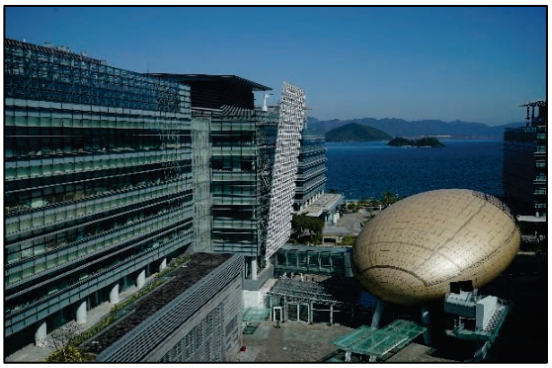
GCMTI RD-4:2024

Identification of *Ziziphus jujuba* var. *spinosa* by polymerase chain reaction-restriction fragment length polymorphism

GCMTI Method Publications





Identification of *Ziziphus jujuba* var. *spinosa* by polymerase chain reaction-restriction fragment length polymorphism

1 Introduction

1.1 This method sets out the procedure for the identification of *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

1.2 *Z. jujuba* var. *spinosa* is a species of the genus *Ziziphus*, family Ziziphaceae. Ziziphi Spinosae Semen is the dried ripe seed of *Z. jujuba* var. *spinosa* according to the Pharmacopoeia of the People's Republic of China.

1.3 The limited availability of Ziziphi Spinosae Semen has resulted in the use of morphologically similar herbs as substitutes, including Ziziphi Mauritianae Semen (the dried ripe seed of *Ziziphus mauritiana* Lam.), Choerospondiatis Fructus (the dried ripe fruit of *Choerospondias axillaris* (Roxb.) Burtt et Hill), Semen Hoveniae (the dried ripe seed of *Hovenia acerba* Lindl.), and Fructus Psoraleae (the dried ripe fruit of *Psoralea corylifolia* L.).

1.4 In this method, internal transcribed spacer 2 of nuclear ribosomal RNA (ITS2) is first amplified by polymerase chain reaction (PCR) using universal primers and then digested with restriction enzyme *Bfm*I to generate a DNA band profile unique to *Z. jujuba* var. *spinosa*.

1.5 The analytical procedures of this method can be divided into the following steps: (1) Sample preparation; (2) DNA extraction; (3) Polymerase chain reaction (PCR) analysis; and (4) Restriction digestion analysis.

1.6 This method can be applied to raw herbal materials in their whole or powdered form.

2 Safety precautions

2.1 This method involves the use of hazardous materials. It is the user's responsibility to apply appropriate precautions as described in the MSDS when handling such materials. Use eye and hand protection and where necessary carry out the work in a fume cupboard or biological safety cabinet.

3 Reagent and materials

3.1 Chemicals or reagents involved shall be of molecular biology/PCR grade, if feasible. The water used shall be molecular biology grade. Chemicals, reagents and water shall be autoclaved prior to use, if feasible. Operator shall wear powder-free gloves for all operations. The use of aerosol-protected pipette tips as protection against cross contamination is recommended. Preparation of PCR reagents should be performed in laminar flow hood to provide unidirectional flow of HEPA-filtered air over the work area to prevent contamination.

3.2 General chemicals and reagents for this method are listed in the Supplementary Information.

4 Apparatus

4.1 General laboratory equipment and apparatus for this method are listed in the Supplementary Information.

5 General procedure

The complete experimental steps are listed in the Supplementary Information. Controls shall be run in parallel with test samples as described in Clause 7 in order to detect DNA contamination of the analytical reagents or sample-to-sample contamination.

5.1 Sample preparation

5.1.1 Prepare the test samples, Extraction Positive Control (EPC, refer to Clause 7.1) and Extraction Blank Control (EBC, refer to Clause 7.2). Proceed to Clause 5.2.

5.2 DNA extraction

5.2.1 To obtain DNA extracts that has sufficient quality for PCR analysis, it is advisable to remove the following compositions from the herbal materials:

5.2.1.1 RNA;

5.2.1.2 polysaccharides such as cellulose, starch;

5.2.1.3 proteins;

5.2.1.4 Lipid fractions; and

5.2.1.5 Pigments such as phenolics compounds.

5.2.2 Determine the quantity of DNA extracts by spectrophotometric analysis and normalize

DNA extracts. Proceed to Clause 5.3.

5.3 PCR analysis

5.3.1 Using PCR Negative Control (PNC, refer to Clause 7.3) as a control, perform PCR on the template DNA of samples, EPC and EBC. Suggested primers and PCR conditions are stated in the Supplementary Information.

5.3.2 After PCR, check the sizes and number of bands of PCR products by gel electrophoresis.

5.4 Restriction digestion analysis

5.4.1 Using Restriction Digestion Negative Control (RDNC, refer to Clause 7.4) as a control, perform restriction digestion on the PCR products of samples and EPC. The suggested restriction enzyme and restriction digestion condition are stated in the Supplementary Information.

5.4.2 After restriction digestion, check the sizes and numbers of bands of restriction digestion products by gel electrophoresis.

6 Results interpretation

6.1 Results are interpreted by visual inspection of DNA band profiles of the restriction digestion analysis. The differentiation between *Z. jujuba* var. *spinosa* and its commonly confused species, including *Z. mauritiana*, *C. axillaris*, *H. acerba* and *P. corylifolia*, is based on the resulting DNA band profiles of the restriction digestion analysis:

	Species	**Resulting DNA band profile of restriction digestion analysis**	**Report result**
(a)	(1) *Z. jujuba* var. *spinosa*	Two bands: ~200 bp and ~300 bp	Positive
(b)	(1) *Z. mauritiana* (2) *C. axillaris* (3) *H. acerba* (4) *P. corylifolia*	One band: ~ 500 bp	Negative

6.2 Quality control

6.2.1 Inconsistent results in duplicates in the PCR analysis or the restriction digestion analysis

indicate that only few DNA targets are present in the samples, potentially reaching the limit of detection. For these inconclusive findings, the analysis shall be repeated to confirm the results.

6.2.2 The system controls, including EPC, EBC, PNC, RDNC, shall give the expected amplification in PCR analysis (refer to Clause 6.3) and cleavage of PCR product in restriction digestion analysis (refer to Clause 6.4) so as to ensure that the test findings are valid. System controls shall exhibit the expected performance as described in the table below. If any system control yields a result that differs from the valid value, the analysis shall be repeated.

System control	**Expected amplification in PCR analysis**	**Expected cleavage of PCR product in restriction digestion analysis**
Extraction Positive Control (EPC)	Positive	Positive
Extraction Blank Control (EBC)	Negative	N/A
PCR Negative Control (PNC)	Negative	N/A
Restriction Digestion Negative Control (RDNC)	N/A	Negative[1]

[1] No DNA band shall be observed for RDNC in restriction digestion analysis (refer to Clause 6.4.3.2).

6.3 Amplification in PCR analysis

6.3.1 The amplification shall be assessed by visual examination of the resulting DNA band profiles obtained from the PCR analysis.

6.3.2 The amplification is considered positive when a DNA band of size ~500 bp is observed.

6.3.3 The amplification is considered negative when a DNA band of size ~500 bp is not observed.

6.3.4 A negative amplification in samples and EPC indicates:

6.3.4.1 inhibitory substances in the sample matrix may have been co-extracted in the DNA extraction process. In this case, the template DNA added into the PCR

master mix shall be diluted, and the PCR analysis shall be repeated; or

6.3.4.2 the isolated DNA may be highly degraded/damaged, resulting in an amount of amplifiable template DNA that is below the limit of detection. In this case, the PCR analysis shall be repeated with an increasing amount of template DNA added to the PCR master mix.

6.3.5 If negative amplification persists despite following the actions as stated in Clause 6.3.4.1 or 6.3.4.2, the analyses shall be terminated at this stage. Rectify the problems and restart the analysis.

6.4 Cleavage of PCR product in restriction digestion analysis

6.4.1 The cleavage of PCR product shall be assessed by visual examination of the resulting DNA band profiles obtained from the restriction digestion analysis.

6.4.2 The cleavage is considered positive when DNA bands of sizes ~200 bp and ~300 bp are observed.

6.4.3 The cleavage is considered negative when:

6.4.3.1 DNA bands of sizes ~200 bp and ~300 bp are not observed while DNA band of size ~500 bp is observed; or

6.4.3.2 No DNA band is observed.

6.4.4 If no DNA band is observed (Clause 6.4.3.2) for sample and EPC, this may indicate that the input amount of PCR products is below the limit of detection. In this case, the restriction digestion analysis shall be repeated by increasing the amount of PCR product added to the restriction digestion master mix.

7 Quality control parameters

The analytical performance of each run is evaluated against the quality control criteria to ensure the results of analysis are acceptable and meet the objective of the method. To ensure compliance with the QC plan, the user should determine an appropriate number of samples in a batch that allows for reasonable handling capacity for operator conducting DNA analysis. The following system controls shall be performed for each batch of samples, or every 15 samples, whichever is the less.

7.1 Extraction Positive Control (EPC): Reference materials of *Z. jujuba* var. *spinosa* shall be

included to serve as a control for the entire analytical process.

Acceptance criteria: The expected observation shall be:

7.1.1 positive amplification in the PCR analysis; and

7.1.2 positive cleavage in the restriction digestion analysis.

7.2 Extraction Blank Control (EBC), preferably two, shall be performed. Such controls shall be processed along with the samples during DNA extraction.

Acceptance criteria: The amplification in the PCR analysis shall be negative.

7.3 PCR Negative Control (PNC), which acts as an amplification blank, shall be included. The PNC is prepared by adding water instead of template DNA in the PCR analysis. Preferably two PNC shall be performed.

Acceptance criteria: The amplification in the PCR analysis shall be negative.

7.4 Restriction Digestion Negative Control (RDNC), which acts as a digestion blank, shall be included. The RDNC is prepared by adding water instead of PCR products in the restriction digestion analysis. Preferably two RDNC shall be performed.

Acceptance criteria: The cleavage in the restriction digestion analysis shall be negative.

7.5 At least one random sample duplicate control shall be included. The control shall undergo the entire analysis procedure.

Acceptance criteria: Consistent results shall be obtained from duplicate analysis.

7.6 If the quality control results fail to comply with the above specified acceptance criteria, re-test shall be carried out until they meet the criteria. Otherwise, the analysis should be stopped. Identify and resolve the problems before restart the analysis.

8 References

8.1 Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China Volume 1, 2020 ed. China Medical Science Press.

8.2 Chen SL, Yao H, Han JP, Liu C, Song JY, et al. (2010) Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE 5(1): e8613.

8.3 Glass fiber plate DNA extraction. (n.d.). CCDB protocols. Retrieved from https://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_DNA_Extraction.pdf

8.4 Yao H, Song JY, Liu C, Luo K, Han JP, Li Y, Pang XH, Xu HX, Zhu YJ, Xiao PG, Chen SL (2010) Use of ITS2 Region as the Universal DNA Barcode for Plants and Animals. PLoS ONE 5(10): e13102.