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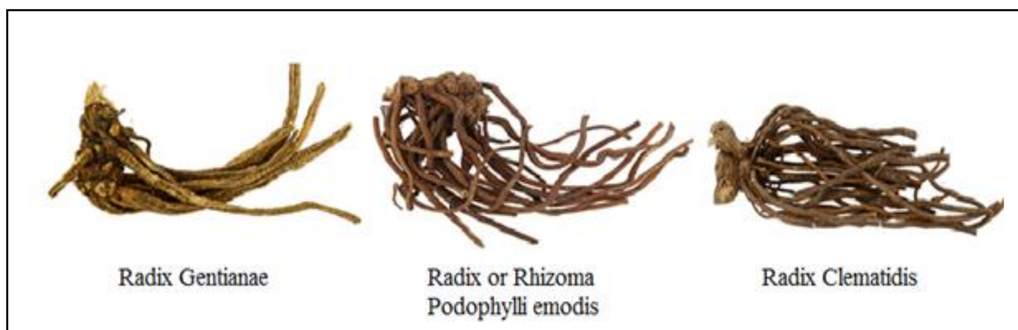
Identification of *Sinopodophyllum hexandrum* by polymerase chain reaction-restriction fragment length polymorphism

GCMTI Method Publications



Identification of *Sinopodophyllum hexandrum* by polymerase chain reaction-restriction fragment length polymorphism

1 Introduction

1.1 This method sets out the procedure for the identification of *Sinopodophyllum hexandrum* (Royle) Ying based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Note: *Podophyllum emodi* (Wall.) Ying is a synonym of *Sinopodophyllum hexandrum* (Royle) Ying.

1.2 *S. hexandrum* is a species of the genus *Sinopodophyllum*, family Berberidaceae. Sinopodophylli Radix et Rhizoma (桃兒七) is the dried root and rhizome of *S. hexandrum* according to the Hong Kong Chinese Materia Medica Standards (HKCMMS) and the Chinese Medicine Ordinance (Cap. 549).

Note: Radix or Rhizoma Podophylli emodis (鬼臼(桃兒七)) is also known as Sinopodophylli Radix et Rhizoma.

1.3 The high morphological similarity between Sinopodophylli Radix et Rhizoma and its confused species, including Clematidis Radix et Rhizoma (dried root and rhizome of *Clematis chinensis* Osbeck, *Clematis hexapetala* Pall. or *Clematis manshurica* Rupr.) and Gentianae Radix et Rhizoma (dried root and rhizome of *Gentiana scabra* Bge. or *Gentiana rigescens* Franch.), has resulted in the adulteration of Sinopodophylli Radix et Rhizoma and its confused species.

1.4 This method involves two tests with different primer pair for the following purposes:

1.4.1 Internal positive amplification control test (IPAC) is the internal control for providing assurance that the genomic DNA of tested sample is amplifiable. A partial chloroplast DNA region is amplified by polymerase chain reaction (PCR) to generate a single band profile for all tested plant species.

1.4.2 Differentiation test (DT) targets chloroplast DNA region ATP synthase CF0 subunit I (*atp*F), which has been commonly used for differentiation of plant species. The *atp*F region is first amplified by PCR and then digested with restriction enzyme *Eco*RI to generate a DNA band profile unique to *S. hexandrum*.

1.5 The analytical procedures of this method can be divided into the following steps: for both IPAC and DT, 1. Sample preparation and DNA extraction; 2. Amplification of target DNA region by

PCR; 3. Gel electrophoresis of PCR products; for DT, 4. Restriction digestion of PCR products; and 5. Gel electrophoresis of digested PCR products.

1.6 This method can be applied to raw herbal materials in their unprocessed 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 Safety Data Sheets (SDSs) 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 Reagents and materials

3.1 Chemicals or reagents involved shall be of molecular biology/PCR grade, if feasible. The water used in all steps shall be of 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 Ribonucleic acid (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 normalise 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 normalised DNA of samples, EPC and EBC. Suggested primer pairs 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 To determine the tested sample to be *S. hexandrum*, positive result shall be obtained from both Internal positive amplification control test (IPAC) (refer to Clause 6.1.1) and Differentiation test (DT) (refer to Clause 6.1.2). The tested sample cannot be determined as *S. hexandrum* if negative result is found with either one of the tests.

6.1.1 For IPAC, results are interpreted by visual inspection of DNA band profile of the PCR product in the PCR analysis:

	Resulting DNA band profile of PCR product in PCR analysis	**Result**
(a)	One band: ~130 bp	Positive
(b)	No band	Negative

6.1.2 For DT, results are interpreted by visual inspection of DNA band profiles of the digested PCR product. The differentiation between *S. hexandrum* and its confused species, including *C. chinensis*, *C. manshurica*, *C. hexapetala, G. scabra* and *G. rigescens*, is based on the resulting DNA band profiles of the digested PCR product in the restriction digestion analysis:

	Species	**Resulting DNA band profile of digested PCR product in restriction digestion analysis**	**Result**
(a)	(1) *S. hexandrum*	Two bands: ~180 bp and ~260 bp	Positive
(b)	(1) *C. chinensis* (2) *C. manshurica* (3) *C. hexapetala* (4) *G. scabra* (5) *G. rigescens*	One band: ~440 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 control parameters, 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. The control parameters shall exhibit the expected performance as described in

Clause 7. If any control parameter yields a result that differs from the expected result, the analysis shall be repeated.

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 PCR product of the anticipated amplicon size, ~130 bp for IPAC or ~440 bp for DT (refer to S6), is observed.

6.3.3 The amplification is considered negative when a PCR product of the anticipated amplicon size (refer to Clause 6.3.2) is absent.

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 normalised DNA (refer to Clause 5.2.2) added into the PCR master mix shall be diluted, and the PCR analysis shall be repeated; or the extracted DNA (refer to Clause 5.2.1) should be purified (refer to S3), and the PCR analysis shall be repeated with purified DNA after normalization.

6.3.4.2 the extracted DNA (refer to Clause 5.2) may be highly degraded/damaged, resulting in an amount of amplifiable normalised DNA that is below the limit of detection. In this case, the PCR analysis shall be repeated with an increasing amount of normalised DNA (refer to Clause 5.2.2) 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 ~180 bp and ~260 bp are observed.

6.4.3 The cleavage is considered negative when:

6.4.3.1 DNA bands of sizes ~180 bp and ~260 bp are absent while DNA band of size ~440 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 quality controls shall be performed for each batch of samples, or every 15 samples, whichever is the less.

7.1 Extraction Positive Control (EPC): At least one reference material of *S. hexandrum* shall be included, preferably two. EPC shall be processed along with the samples and undergo the entire analysis procedure to serve as a positive control.

Acceptance criteria: The expected observation of EPC 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): At least one extraction blank control shall be included, preferably two. EBC shall be processed along with the samples during DNA extraction to serve as an extraction negative control.

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

7.3 PCR Negative Control (PNC): At least one amplification blank control (prepared by PCR master mix with water instead of normalised DNA) shall be included, preferably two.

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

7.4 Restriction Digestion Negative Control (RDNC): At least one digestion blank control (prepared

by restriction digestion master mix with water instead of PCR product) shall be included, preferably two.

Acceptance criteria: The cleavage of RDNC in the restriction digestion analysis shall be negative. No DNA band shall be observed for RDNC in restriction digestion.

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

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 of the full set of samples shall be carried out until they meet the criteria. Otherwise, the analysis should be stopped. Identify and resolve the problems before restart the analysis.

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