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APPENDICES

APPENDIX I

DNA Extraction protocol

DNA extraction protocol by using QIAamp® DNA Mini Kit

1. Place the end of the feather tip into1.5 ml microcentrifuge tube and add 180 µl of buffer ATL.
2. Incubate at 85°C for 10 minutes. Briefly centrifuge to remove drops from inside the lid.
3. Add 20 µl Proteinase K stock solution, mix by vortexing, and incubate at 56°C for 1 hour. Briefly centrifuge to remove drops from inside the lid.
4. Add 200 µl buffer AL to the sample, and mix thoroughly by vortexing, and incubate at 70°C for10 minutes. Briefly centrifuge to remove drops from inside the lid.
5. Add 200 µl Ethanol (96-100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.
6. Carefully apply the mixture from step 5 to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rmp for 1 minute. Place the QIAamp Spin Column in a clean 2 ml collection tube (provide), and discard the tube containing the filtrate.
7. Carefully open the QIAamp Spin Column and add 500 µl buffer AW1 without wetting the rim. Close the cap, and centrifuge at 8000 rmp for 1 minute. Place the QIAamp Spin Column in a clean 2 ml collection tube (provide), and discard the collection tube containing the filtrate.
8. Carefully open the QIAamp Spin Column and add 500 µl buffer AW2 without wetting the rim. Close the cap, and centrifuge at full speed 14,000 rmp for 3 minutes.
9. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube (not provide), and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 150 µl buffer AE or distilled water. Incubate at room temperature for 1 minute, and then centrifuge at 8000 rmp for 1 minute.
10. Store the DNA sample in microcentrifuge tube at -20°C until further needed.

APPENDIX II

Quality determination and size estimation can be calculated of DNA

1. Quality determination of DNA

Description previously was used follow:

1. An approximate amount of agarose was weighed out and heated to dissolve in an appropriate volume of 1X TBE buffer.
2. Malted agarose was poured into the gel mould (Mupid set electrophoresis) that the comb was already inserts to gel mould. When the gel completely set (the gel had cooled and solidified), the comb was gently removed.
3. The gel submerged in the gel chamber containing an enough of 1X TBE buffer that covered the gel to a depth about 1-2 mm. Each of extracted DNA was prepared for loading by mixing loading dye buffer and mixed well.
4. The samples were applied into the wells slowly that used Phi X 174 – λDNA digested with Hinf I was loaded into a well for served as a DNA standard.
5. A gel bath was connected to a power supply and turned on (the gel was run at 100 volt) then DNA migrated into the gel toward the anode. When bromophenol blue had migrated about three-fourths of a gel distance turned off the power supply and stain the gel with 0.25 µg/ml ethidium bromide.
6. The gel was destained in deionise distilled water for 5-10 minutes to leach out unbound ethidium bromide, placed on a long wavelength UV transluminator and photographed using Polaroid 667 film.

2. Size estimation of DNA

1. Set of 100 bp + 1.5 Kb DNA Ladder with stain was used as a DNA marker. An amount of standard marker is used that concentration was estimated 0.2 µg/µL.
2. The 100 bp + 1.5 Kb DNA Ladder has a number of proprietary plasmids which are digested to completion with appropriate restriction enzyme to yield 11 bands suitable for use as molecular weight standards for gel electrophoresis.
3. The recommended amount of size marker to load on a gel is 1 µL per lane.
4. The storage buffer composed of 10 mM Tris-HCl (pH 8.0), 50 mM NaCl and 1.0 mM EDTA and store at -20°C

APPENDIX III

Reagent preparation protocol

1. 1% Agarose

An enough amount of ingredients for a 100 ml gel composed of:

- Agarose	1.0	gm
- 1X TBE buffer	100.0	ml

How to apply the description previously is used follow:

1. Agarose powder about 1 gm is mixed into 1X TBE buffer 30 mL.
2. The agarose solution is cooked in microwave for 2 minutes.
3. Prepare gel mould for set the gel. When time is finished, the dissolved gel is transferred about 25-50 mL and is added with 0.2 µL of 1% ethidium bromide into gel. The gel is mixed.
4. The solution gel is poured into the gel mould, which the comb is already inserted to the gel mould.
5. When the gel has completely cooled and solidified, the comb was removed.
6. The gel is transferred into a gel chamber containing an enough of 1X TBE buffer that covered the gel to about 1-2 mm depth.

2. 8% polyacrylamide gel

An enough amount of ingredients for a 120 ml gel composed of:

- 7M urea	50.4	gm
- 10X TBE buffer	12.0	ml
- 30% stock acrylamide solution (Bio-red®, acrylamide monomer : bis- acrylamide = 29 : 1)	32.0	ml
- distilled water for added up to	120.0	ml

How to use the description previous is used follow:

1. The dissolved ingredients are added 10% APS 500 µL (fresh prepared) and TEMED 100 µL before used.
2. Then the ingredients are poured into the gel apparatus, the comb is inserted into the upper and pointing out for marking a sharp cut edged of the gel.
3. The polymerization process is allowed to complete for 3 hours.
4. The comb is then removed that urea and small pieces of gel are flushed out of the wells prior to loading PCR products.

3. 10X Tris Boric EDTA buffer (10X TBE)

An enough amount of ingredients for a 1000 ml gel composed of:

- Tris aminomethane	108	gm
- Boric acid	50.4	gm
- EDTA	7.44	gm

The solution is prepared as follow:

1. Tris, Boric and ADTA are mixed into volumetric bottle 1000 mL.
2. Double distilled water is added up to 1000 mL.
3. Solutions stirred until completely dissolve.
4. Store in room temperature and use 10X TBE for acrylamide, while use 1X TBE for running electrophoresis.

4. 40% Methanol

The solution was prepared as follow:

Methanol is poured about 400 mL into 1000 mL cylinder. Double distilled water was added up to 1000 mL, and mixed gently.

5. 1 M Nitric acid

The solution was prepared as follow:

1. 2 N of Nitric acid is prepared for stock solution and poured into 500 mL cylinder about 62.9 mL for 1 M working solution (prepared in hood).
2. Deionized water is added up to 500 mL.
3. Mixed gently and kept at room temperature.

6. 0.2% Silver nitrate

The solution was prepared as follow:

Silver nitrate 0.2 gm is prepared for 100 mL total volume working solution in 500 mL bottle (freshly prepared and mixed gently)

7. Developer solution (3% Sodium carbonate and 40% formaldehyde)

The solution was prepared as follow:

1. Sodium carbonate 3 gm is prepared and added double distilled water about 100 mL.
2. The solution is mixed gently for dissolve.
3. 40% formaldehyde about 50 μL is added into the solution, before use.

8. Stop solution (0.1 M Citric acid or 20% acetic acid)

The solution was prepared as follow:

0.1 M Citric acid

1 M Citric acid is prepared for stock solution and used 0.1 M for working stop reaction.

20% acetic acid

10 mL of glacial acetic acid is poured into 500 mL cylinder and added double distilled water up to 500 mL. Mixed gently.

APPENDIX IV

Silver nitrate staining method

Silver nitrate staining

How to use the description previously was used as follow:

1. Transfer the gel that covering PCR products into the staining chamber.
2. Then the gel was fixed in 40% methanol for 12 minutes. When finished, discard solution.
3. The gel was rinsed in double distilled water (DDW) and discard.
4. Prepared suitable physical condition of the gel by soaked in nitric acid for 5 minutes. When finished, discard solution.
5. The gel was soaked in DDW for 4 minutes and discard.
6. The gel was soaked in 0.2% silver nitrate for 16 minutes that this solution was fresh prepared. When finished, discard solution.
7. The gel was rinsed in DDW and discard.
8. Then the gel was soaked in developer solution in developer solution 3% sodium carbonate, 37% formaldehyde 50 µL was added before used. When PCR products band were occurred, discard solution.
9. Then stop reaction, the gel was soaked in 0.1 M citric acid or 0.2% acetic acid for 1 minute, discard solution and soaked the gel in DDW for 5 minutes, discard solution.
10. Wrap the gel by cellophane and air-dried for over night. Labeled the gel and take photography.

APPENDIX V

Wilcoxon sign rank test

Table comparison of mean number of allele (A), mean effective of allele (a_e) and mean expected heterozygosity (H_E) between two investigated varieties by Wilcoxon sign rank test. The significant level at 0.05

Parameters	P-value
A	0.317
a_e	0.498
H_E	0.889

Biography

Mister Pirach Thawonwan was bone on the 9th of June 1983 in Nakhon Pathom Province. He graduated his bachelor's degree of Science in Biology (2nd Class Honours) in 2005 from Department of Biology, Faculty of Science, Chulalongkorn University. He continued his graduated study for a master's degree of Science in Zoology at Chulalongkorn University in 2009