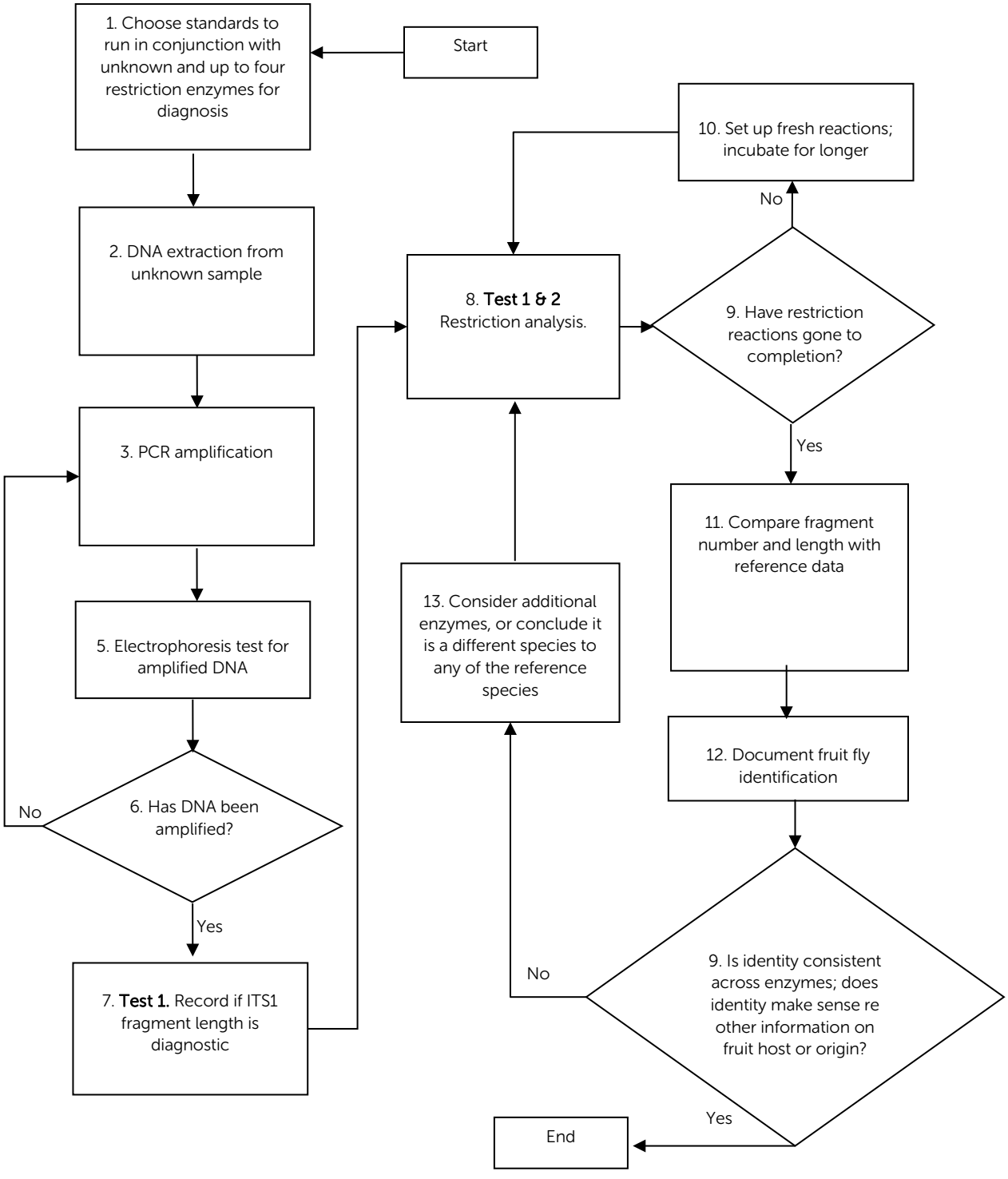
Fruit fly molecular diagnostics

METHOD: PCR-RFLP TEST 2

WORKFLOW



PART A. PCR

Equipment

- Pipettors and tips
- Sterile disposable microcentrifuge tubes
- Microcentrifuge
- Gel tank and power pack
- Latex or Nitrile gloves
- Microwave
- UV transilluminator with camera
- Thermocycler
- Personal protective equipment including lab coat, eye protection, gloves

Reagents

See Composition and preparation of reagents for reagent compositions.

- Manufacturer's polymerase enzyme buffer 10X
- Taq polymerase enzyme (5U µL-1)
- Primer (McKenzie et al. 1999) are:
  - NS15 5' CAATTGGGTGTAGCTACTAC 3', 10 µM
  - ITS6 5' AGCCGAGTGATCCACCGCT 3', 10 µM
- dNTPs (2 mM)
- $MgCl_2$ (50 mM)
- Sterile water
- 1X TBE buffer
- 1% (w/v) agarose gel: 1 g DNA grade agarose per 100 mL 1X TBE
- 6X Loading dye
- DNA molecular weight marker (aka 100 bp ladder)
- Gel staining solution, e.g. ethidium bromide (final concentration 800 ng/µL), or non-toxic options such as Syber Safe, or Red Safe according to manufacturer's instructions.

Method

In a pre-PCR cabinet:

1. label sterile 0.2 ml PCR tubes
2. make a Reagent Master Mix for the total number of samples to be analysed according to the table below, all reagents except for the polymerase. (Note: recommend adding an extra volume for more than 10 samples to allow for any loss of solution via adherence to the outside of the pipette tip.)

Reagent	Final concentration	Vol per reaction (µl)
Double-distilled H2O		30.6
Expand High Fidelity polymerase buffer	1 X	5
dNTPs (2.5 µM)	200 µM	4
Forward primer 10 µM (NS15)	0.5 µM	2.5
Reverse primer 10 µM (ITS6)	0.5 µM	2.5
Expand Hi Fidelity Taq Polymerase	2 U	0.4
Total		45

3. Store Master Mix on ice.
4. Vortex DNA extractions for 5 seconds.
5. Aliquot 5 µL of each unknown DNA template to labelled 0.2 mL tubes, plus at least one positive control DNA and one negative control (water or the buffer in which the DNA is suspended).
6. Aliquot 45 µL of Master Mix to each 0.2 mL tubes (containing 5 µL of template DNA).
7. Mix product and reagents well (or vortex briefly) and centrifuge for 3-5 seconds.
8. Place samples in PCR machine and program the following temperature profile (based on Armstrong and Cameron 1998):

Cycle 1	denature 94°C	2 min
Cycles 2 to 35	denature 94°C	15 sec, anneal 60°C 30 sec, extend 68°C 2 min
Cycle 36	extend 72°C	5 min

9. Add 1 µL of loading dye to 5 µL of PCR product
10. Load onto a 1.5% agarose gel, together with a 100 bp molecular ladder at either side and electrophorese according to Composition and preparation of reagents. If product visible at 1.5-1.8 kb then proceed to Section B, Restriction digestion of PCR product.

PART B. RESTRICTION DIGESTION OF PCR PRODUCT

Equipment

As above.

Reagents

- Restriction enzymes, on ice.
  - Choose at least four of the enzymes listed in Restriction enzyme haplotype chart, according to which combination will provide the best discrimination for the potential species; the letters represent RFLP patterns in Diagnostic restriction patterns.
  - In the specific circumstance of distinguishing only *B. tryoni* complex and *C. capitata,* the enzyme combination of AluI, DdeI and RsaI (10 U/µl) and SspI (5 U/µl) is diagnostic.
- Restriction enzyme buffers, 10X
- Sterile nuclease free H_2O

Method

1. Prepare Master Mix (following recipe below) for each enzyme; multiplying volumes plus one for the number of reactions required.

Adapted from Armstrong and Cameron (1998)

Reagent	Final concentration	Vol per reaction (µl)
Double-distilled	H_2O	5.6*
10X Buffer	1X	1.0
Enzyme†	4 U	0.4
Total		7.0

* volume of water can be varied to accommodate any change to DNA volumes added, see below
† Standard stock concentrations are usually 10 U/µl; if not, this volume will change accordingly

2. Aliquot 7 µL of each master mix into labelled tubes
3. Add 2-3 µL (100-200 ng) PCR product into each tube depending on how strong or weak the PCR products are; adjust water in reaction mix accordingly
4. Flick to mix reagents and PCR product, centrifuge briefly for 3-5 s.
5. Place samples in incubator at 37°C 2-3 h unless otherwise recommended by the enzyme manufacturer
6. Prepare a 2-3% agarose gel according to Composition and preparation of reagents, at least 10 cm in length, to visualise fragment pattern and use a 100 bp ladder for determining fragment sizes
7. Compare results with positive controls and fragment patterns in Diagnostic restriction patterns
8. Species diagnosis is considered positive if all four enzyme patterns agree.

ADDITIONAL DOCUMENTS

Composition and preparation of reagents

Restriction enzyme haplotype chart

Diagnostic restriction patterns