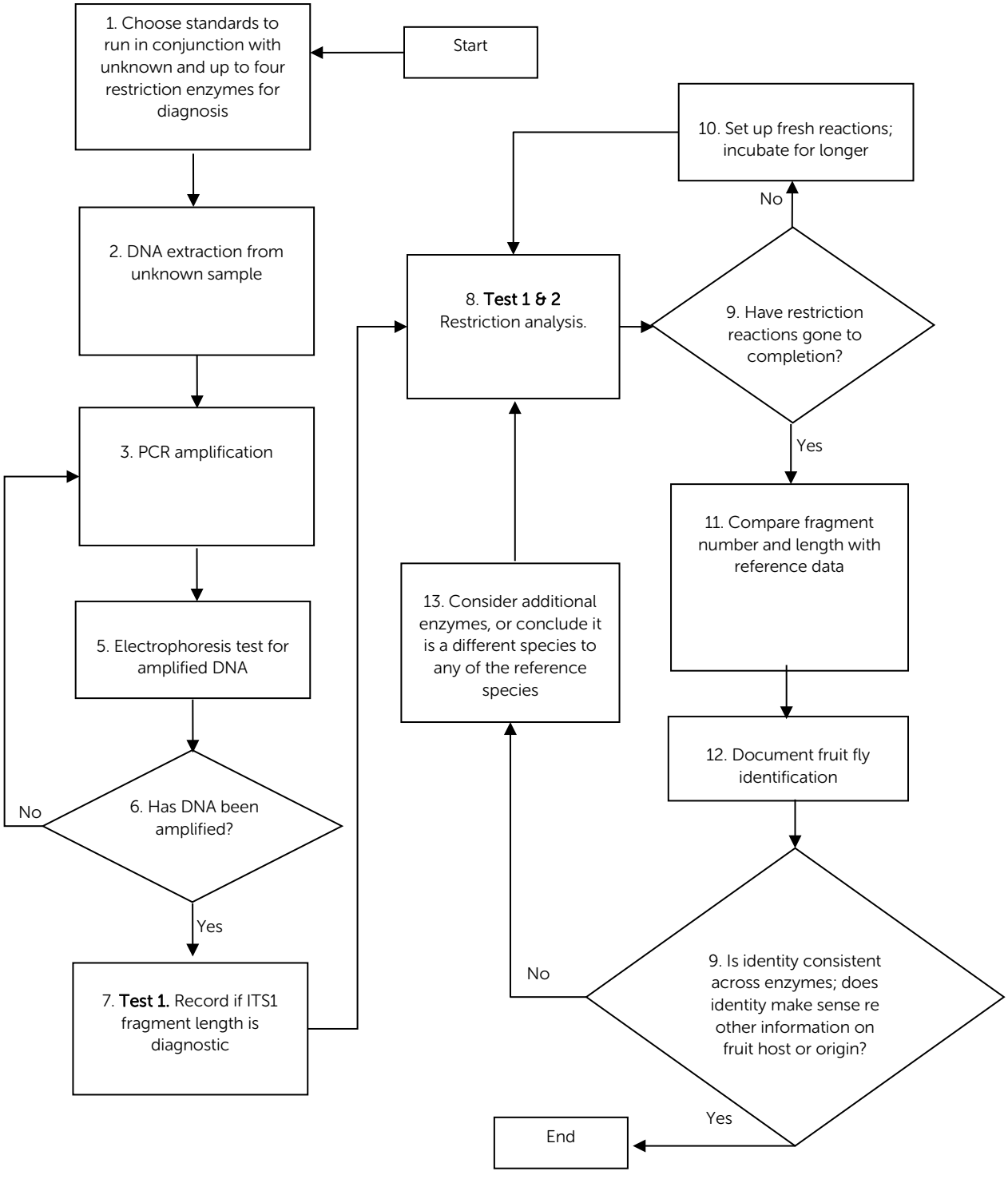
Fruit fly molecular diagnostics

METHOD: PCR-RFLP TEST 1

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:
  - baITS1f 5' GGA AGG ATC ATT ATT GTG TTC C 3', 10 µM
  - baITS1r 5' ATG AGC CGA GTG ATC CAC C 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)
Manufacturer's reaction buffer (10X)	1X	5
$MgCl_2$ (50 mM)	1.5 mM	1.5
dNTPs (2 mM)	200 µM	5
Forward primer (10 µM)	1 µM	5
Reverse primer (10 µM)	1 µM	5
H_2O		20.25
Taq polymerase enzyme (5U µL-1)		0.25
Total volume		42

3. Store Master Mix on ice in sterile 1.5 mL centrifuge tube.
4. Add 8 µL of sterile distilled H2O or DNA buffer to the first negative control tube.

In a laminar flow hood or PCR cabinet:

5. add the Taq polymerase to the Master Mix
6. aliquot 42 µL Taq Master Mix to each PCR tube
7. add 8 µL of DNA extract/control to each sample tube as appropriate
8. PCR amplify the DNA in a thermal cycler using the following program:

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

9. Place reaction products on ice or freeze until ready to analyse.
10. Mix 3 µL of each PCR sample with 2 µL loading dye.
11. Load samples and 100 bp DNA ladder onto separate wells of 1% (w/v) agarose gel in 1X TBE.
12. Electrophorese in 1X TBE buffer at 100 V for around 40 min.
13. Stain the gel in ethidium bromide or other stain, according to local Standard Operation Procedure.
14. Visualise bands and capture image using the Gel Documentation System.

DIAGNOSTIC USE OF ITS1 AMPLICON SIZE

The expected size of the amplified product is between 500 and 1000 bp, depending on the species (see table below).

For some species the size can be an additional diagnostic character. However, relying on the resolution available on a gel to distinguish the majority of these amplicon sizes is not advisable as the only diagnostic character, but is useful in conjunction with restriction analysis as described below.

Flies producing fragments of less than 700 bp or greater than 900 bp are segregated and then restriction enzymes are used in series to differentiate the species.

Sizes are given as a range to reflect that sizing is approximate when using low-resolution gel electrophoresis systems as here.

The approximate size of the ITS1 amplicon is given for each species. Potential diagnostic size ranges are contained in a yellow or white coloured block.

Species fragment size range	
ITS1 amplicon approximate size range (bp)	Potential species
500-520	*D. pornia*
590-610	*B. cucurbitae*
640-680	*A. ludens*
650-690	*A. obliqua*
670-700	*B. xanthodes*
740-760	*A. serpentina*
740-780	*R. pomonella*
750-780	*B. umbrosa, B. facialis*
760-770	*B. cucumis*
760-780	*B. latifrons*
770-790	*B. musae*
770-800	*B. endiandrae*
780-800	*B. psidii*
790-840	*B. bryoniae, B. tryoni* sp. complex
800-820	*B. moluccensis*
800-840	*B. dorsalis sp., B. jarvisi*
810-840	*B. passiflorae*
820-850	*B. zonata*
830-860	*B. carambolae, B. curvipennis, B. frauenfeldi*
840-860	*B. albistrigata, B. kirki*
890-900	*C. capitata*
1000-1040	*C. rosa*

PART B. RESTRICTION DIGESTION OF PCR PRODUCT

The use of a combination of enzymes, in series, allows definitive identification of the majority of the species. This also eliminates the reliance on discrete restriction sites and minimises the likelihood of false negatives that may arise through a rare recombination event.

Restriction endonucleases used are VspI, HhaI, SspI, HinfI, BsrI, SnaBI and/or Sau3aI. During the development of this test standard enzymes purchased from New England Biolabs were used but other brands would work equally well. Enzymes were also selected based on the requirement for differences in fragment sizes to be easily detected by visual examination of an agarose gel.

If the likely species can be narrowed down (e.g. by fruit or geographic region), then a reduced number of enzymes could be used. However, for robust diagnoses, it is recommended that at least four enzymes are used, even if there is a diagnostic pattern for one enzyme that distinguishes a species from all others.

Equipment

- Pipettors and tips
- Sterile disposable microcentrifuge tubes
- Microcentrifuge
- Dry heating block, water bath or similar
- Gel tank and power pack
- Latex or nitrile gloves
- Microwave
- UV transilluminator with camera and image capture and analysis software
- Personal protective equipment including lab coat, eye protection, gloves

Reagents

- Sterile distilled water
- Bovine serum albumin (BSA, 10 µg/µL) (comes supplied with NEB enzymes)
- Restriction enzymes VspI, HhaI, SspI, HinfI, BsrI, SnaBI, and Sau3aI
- Restriction buffer supplied with enzyme
- Ethidium bromide solution, 800 ng/µL final concentration, or non-toxic options such as Syber Safe, or Red Safe according to manufacturer's instructions

Method

1. Label microcentrifuge tubes, including one for the positive control.
2. To each centrifuge tube add:

Water	2.3 µL
10X buffer	2 µL
BSA (10 µg/µL)	0.2 µL
PCR product	5 µL
Restriction enzyme	0.5 µL

3. Mix reagents and place tubes in a water bath preheated to 37oC for 2 h.
4. Store tubes on ice or at -20oC until ready to load on agarose gel.
5. Add 3 µL of 6X loading buffer to each tube.
6. Load the entire volume of each sample (23 µL) into a lane of a 2% (w/v) high resolution agarose gel.
7. Load 100 bp DNA molecular weight marker into one or two wells of the gel.
8. Analyse products by electrophoresis at 100 V for 50 min.
9. Stain the gel with ethidium bromide, or alternative non-toxic stain.
10. Visualise fragments using a UV transilluminator.
11. Capture gel image using gel documentation system.

ANALYSIS OF RFLP PRODUCTS

For diagnostic purposes, RFLP bands under 100 bp and over 1500 bp in size are disregarded due to difficulty in accurate sizing. The molecular weights of the restriction fragments are estimated with reference to the DNA molecular weight standard loaded on the same gel.

The documents Restriction enzyme haplotype chart and Diagnostic restriction patterns summarise the expected fragment lengths for the six restriction enzymes used in this method.

All species listed can be differentiated from each other, with the exception of those within the *B. tryoni* complex. **Care should be taken with fragments produced for *B. albistrigata* and *B. kirki***, which are very similar for all six restriction enzymes and potentially difficult to confidently distinguish on an electrophoresis gel.

ADDITIONAL DOCUMENTS

Composition and preparation of reagents

Restriction enzyme haplotype chart

Diagnostic restriction patterns

Table. Analysis of RFLP products from ITS1 fragments from fruit flies

Species	ITS1*			HinfI		VspI		HhaI		SspI		BsrI		SnaBI		Sau3aI	
	<700	700-900	>900	DNC	Cuts*	DNC	Cuts	DNC	Cuts	DNC	Cuts	DNC	Cuts	DNC	Cuts	DNC	Cuts
A. ludens	X				550		550	X		X		X		X		X	
A. obliqua	X				450, 270		550	X			550, 150	X		X			450, 200
A. serpentina		X		X			420, 250	X		X		X		X			530, 200
B. albistrigata		X		X		X			670, 180		620, 180	X		X			450, 400
B. aquilonis		X			770	X			640, 190		570, 180		600, 200	X			415
B. bryoniae		X			760	X			620, 200		560, 180		600, 230	X			400
B. carambolae		X		X			480, 350		680, 200	X			650, 250		530, 350		450, 400
B. cucumis		X		X		X			550, 180	X		X		X		X	
B. cucurbitae	X			X		X			400, 180	X		X		X		X	
B. curvipennis		X		X		X			620, 170		550, 200		570, 250	X			420
B. dorsalis sp. complex		X			770	X			650, 190	X			650, 260		540, 320	X	
B. facialis		X		X		X			600, 180	X			600, 200	X			390
B. frauenfeldi		X		X		X					620, 180	X		X			450, 400
B. jarvisi		X			770	X			640, 180		700		600, 250	X			420
B. kirki		X		X		X			680, 190		620, 180	X		X			450, 400
B. latifrons		X		X		X			600, 190	X			600, 200	X		X	

B. musae	X	X		X	635, 220	X		600, 250		520, 320	X	
B. neohumeralis	X		770	X	640, 190		570, 180	600, 200	X			420
B. passiflorae	X		770	X	650, 190		750	650, 270	X		X	
B. psidii	X	X		X	640, 190		570, 250	X	X		X	

The length of the ITS1 fragment and the response of each to seven restriction enzymes (HinfI, VspI, HhaI, SspI, BsrI, SnaBI, Sau3aI) are indicated for each of the target species. ITS1 fragment length is scored as one of three classes (approximate length in bp). Enzyme responses are measured in two classes - either does not cut (DNC) or cuts.

*Cuts – this column shows the length of each fragment in bp. Highlighted boxes denote diagnostic RFLP patterns, which are more than 20 bp different to other fragment produced by that restriction enzyme.