

Microarray based diagnostics of pest fruit flies

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Quarantine fruit flies

Fruit flies include many agronomically relevant pest species. On the list of quarantine plant pest species published by the European and Mediterranean Plant Protection Organization (EPPO), a total of **19 out of 89 quarantine insects and 83 percent of all dipteran species belong to the family of fruit flies (Tephritidae)**. As their name implies, fruit fly maggots mainly attack fruits and are responsible for heavy losses in crop production. The danger of fruit flies to be spread to other countries through commercial trade routes is high. Consequently, export and import are highly restricted and phytosanitary measures are of major concern. Some of the fruit fly species are morphologically very similar and the identification of adult flies is difficult even for professional taxonomists. This is even truer for larvae extracted from traded fruit.

It would be of great value to have a diagnostic tool which is independent of morphological traits and developmental state and which could be used to identify all species of fruit flies that may occur at the border of a country. Ideally, this tool should be based on a simple technique that could be applied directly at the customs office. Our case study demonstrates the potential of microarray technology for the reliable molecular identification of quarantine species using a chip for identification of nine Tephritid species

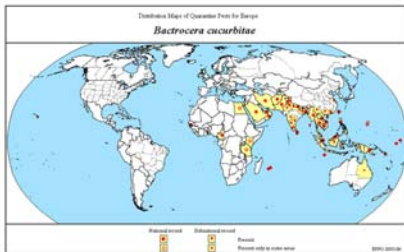


Fig. 1

Bactrocera cucurbitae, one of the quarantine fruit flies in Europe and its distribution world wide.

Species on the chip

For the diagnostic chip presented here we included nine species from two different genera of fruit flies, five of them are listed by EPPO as quarantine insects: *Rhagoletis mendax*, *Rhagoletis pomonella*, *Rhagoletis cingulata*, *Bactrocera cucurbitae*, *Bactrocera tryoni*, *Rhagoletis cerasi*, although a significant pest, is not a quarantine species as it naturally occurs in Switzerland and other European countries. *Bactrocera umbrosa* and *Bactrocera papayae* are mentioned on the list of pest fruit flies of the world but are not considered an immediate threat within the EPPO region. Finally, *Bactrocera nigrotibialis* was also included on our chip.



Fig. 3
Injured apple and adult fly of *Rhagoletis pomonella*



Fig. 4
Damage on Walnut, Larva and adult fly of *Rhagoletis completa*

Chip preparation

We used sequences of two different mitochondrial genes, **cytochrome oxidase I (COI)** and **cytochrome b (cytb)**, as markers to design a total of four probes per species. To enhance the specificity of the probes we included as much information from other species as possible. We sequenced a total of **37 specimens from 21 species** of Tephritids that were identified and provided to us by tephritoid taxonomists. In addition, we used all sequences of Tephritids that were available on GenBank in February 2005 i.e., **18 sequences from 9 species for cytb and 93 sequences from 56 species for COI**. We used the program "probe search" for a pre-selection of possible species-specific probes with a length of 25 bp and a GC content of more than 50% if possible. The proposed probes were then visually selected according to other criteria such as amount and place of mismatches. The probes finally used had a GC content of 36-66% and a melting temperature between 68 and 80°C. Each of the selected probes was supplemented with a C6-aminolinker and a 15 T spacer to decrease interferences with the surface.

Tephritidae-Chip

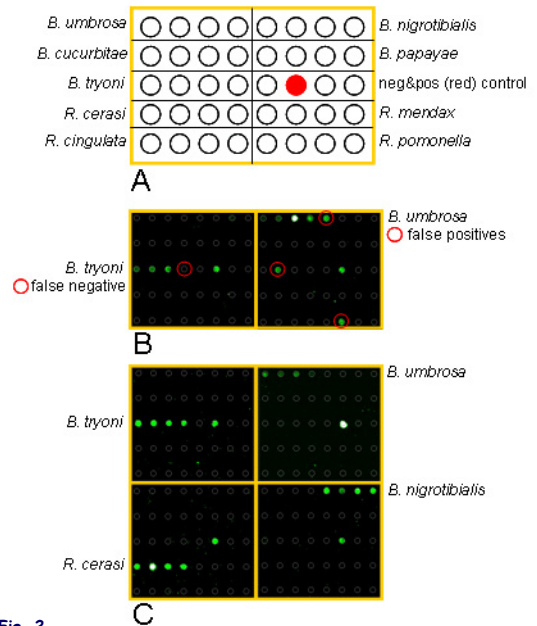


Fig. 2

Hybridisation of DNA of different Tephritidae to a microarray chip.

(A) Schematic organization of a fruit fly chip with 9 species included
(B) Left: One of three fragments was not amplified, leading to a false negative signal (red circle). Right: Cross-hybridisations of the PCR fragment to other probes through non-specific hybridisation, leading to false positives (red circle).
(C) Four examples for positive identification of *Bactrocera tryoni*, *B. umbrosa*, *B. nigrotibialis* and *Rhagoletis cerasi*.

Results & Difficulties

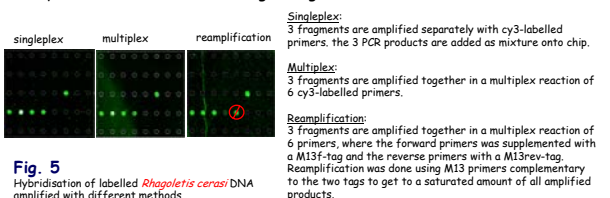
The first tests that we made look promising (see Fig. 2). However, to validate the chip it is necessary to test with organisms from a different lab or population than the ones that were used to develop the probes. This is a much more serious problem than we thought, as it is very hard to get enough of these quarantine specimen.

A special challenge in designing diagnostic tools for fruit flies is the very close relationship between some of them. For example, *Bactrocera papayae* belongs to the oriental fruit fly species complex *B. dorsalis*, to which many major fruit pests belong. We found it to be impossible to design four different probes that distinguished this species from any other species within the complex. Among complete COI and partial cytb (total 2'235 bp), only 3 nucleotides distinguished *B. papayae* from its sympatric sister species *B. carambolae*, and we do not know whether these mutations are species specific. Therefore, our mitochondrial markers are unable to differentiate reliably between these two species. In such cases it is necessary to include a marker with higher variation on the chip (e.g. actin gene BdA1, Naeole & Haymerhave, 2003).

The same problem occurred with *Rhagoletis mendax* and *R. pomonella* where we found only 7 bp differences on COI and 1 bp on cytb. The two species belong to the *Rhagoletis pomonella* species complex that is thought to have undergone recent speciation, and they are primarily distinguished by host preference or, on a molecular basis, for example by allozyme frequencies.

Singleplex-Multiplex-Reamp ?

The 3 different gene fragments can be amplified in singleplex or in multiplex PCR reactions. We also tried a reamplification protocol using tagged primers to equalize the amount of the single fragments.



Preliminary results suggest that, if the primers fit and DNA is fresh, the multiplex reaction works as well as singleplex. However, with reamplification, false positives occur that don't show up in the other methods.