A phylogenetic study of the family Tephritidae (Insecta: Diptera) using a mitochondrial DNA sequence

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Achievements in tephritid taxonomy have greatly contributed to both basic research and pest management programmes. However, despite the large amount of taxonomic data available, the higher classification of the family Tephritidae is still a matter of debate. A molecular approach could help to provide a more accurate classification. A molecular study was therefore undertaken to gain insight into the phylogenetic relationships within the family Tephritidae. A DNA region of the mitochondrial cytochrome oxidase II gene was compared in species representing six genera of the family, namely Ceratitis, Rhagoletis, Dacus, Bactrocera, Anastrepha and Toxotrypana. A dendrogram was constructed using the neighbour-joining method with Liriomyza huidobrensis and Drosophila yakuba as outgroups. Two main clusters were obtained in the tree, the first grouping being the Ceratitis species, C. capitata, C. rosa, and C. cosyra, and the second showing two main branches, one for Dacus, Bactrocera and Rhagoletis, and the other for Anastrepha and Toxotrypana. The results are discussed in relation to published phylogenies.

INTRODUCTION
Among the most devastating of agricultural pests, the family Tephritidae, commonly known as fruit flies, includes more than 4000 species in some 500 genera distributed all around the world (White & Elson-Harris 1994). The enormous economic effort required in eradication programmes, together with great crop losses that these flies cause in fruit production, explains the increasing interest in the study of their biology.

Taxonomy is an essential foundation of biological research. Developments in tephritid taxonomy have greatly contributed to progress in areas of pure research and to pest management programmes. However, despite the large number of taxonomic data available, the higher classification of the family Tephritidae, based primarily on morphological data, is still a matter of debate (White 1996; Korneyev 2001). Many characters are difficult to interpret, and disagreements in the definition of groups have arisen owing to the relative importance that researchers place on these characters (Drew 1989).

In the last two decades the development of molecular techniques, based mostly on PCR and/or sequencing, has provided new and powerful tools to address the most diverse of biological problems. Taxonomy has profited much from them. A more accurate classification of the Tephritidae might be gained through the use of molecular techniques. Indeed, a number of recent studies using molecular data have contributed to a better understanding of the phylogenetic relationships within the Tephritidae family (Han & McPheron 1994, 1997, 2001; Malacrida et al. 1996; McPheron & Han 1997; Smith & Bush 1997; Morrow et al. 2000; Han 2000).

Specifically, mitochondrial DNA is a powerful material for phylogenetic studies. Its small size, different rates of evolutionary change, lack of recombination, and maternal inheritance make it suitable material for systematic studies at a wide variety of taxonomic levels (Simon et al. 1994; Han & McPheron 2001). Among the mitochondrial genes, cytochrome oxidase II, used by different authors to infer relationships within insect species (Sperling & Hickey 1994; Caterino & Sperling 1999; Gómez-Zurita et al. 2000, Durando et al. 2000), is particularly useful.

A molecular study was therefore undertaken to compare a DNA region of the mitochondrial cytochrome oxidase II gene from different tephritid species belonging to six different genera, in order to obtain an estimate of the phylogenetic relationships within some members of this dipteran family.

MATERIALS AND METHODS

Samples and DNA isolation
Insect samples included 16 species representing six genera in the family Tephritidae, namely, Ceratitis, Rhagoletis, Dacus, Bactrocera, Anastrepha and Toxotrypana, from representative areas of each taxon distribution (Table 1).

Total DNA from individual adults or pupae was
isolated applying a method specific for small samples, using SDS dissolution, phenol-chloroform extraction and ethanol precipitation (Reyes et al. 1997).

DNA amplification and sequencing

The polymerase chain reaction (PCR) was used to amplify a 259 bp segment of the mitochondrial cytochrome oxidase II gene. The primers used were CO2A (5’-GGACTACAAGATAGCCTC-3’) and CO2B (5’-CTTCAGTATCATTGATGACC-3’), provided by C. Fleming (The Queen’s University of Belfast, U.K.). The amplified fragment corresponds to positions 3124-3382 of the mitochondrial genome of Drosophila yakuba (Clary & Wolstenholme 1985).

Amplifications were performed in a PTC-100 MJ research thermocycler, in 100 µl reactions containing 1 µl of genomic DNA, 1 µM of each primer, 0.2 mM of each dNTP, 2 mM of MgCl₂, 10 µl of 10 × Eco Taq buffer (Ecogen), and 2.5 units of Eco Taq polymerase (Ecogen). PCR conditions involved an initial cycle of 5 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 55°C or 57°C (Bactrocera dorsalis), and 1 min 30 s at 72°C. The final cycle had an additional step of 6 min at 72°C. Negative controls were always included. PCR products were purified using a PCR purification kit (Boehringer).

Nucleotide sequences were determined directly from PCR fragments using the dideoxy chain termination method (Sanger et al. 1977) with fluorescently-labelled dideoxynucleotide terminators (Applied Biosystems). Sequencing reactions were analysed using an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Sequence alignment and phylogenetic analysis

Alignment of the sequences and the estimation of interspecific pairwise percentage sequence divergence was performed by the clustal method using the MegAlign programme included in the DNASTAR package (Lasergene System 1994).

A neighbour-joining (NJ) tree (Saitou & Nei 1987) was generated from the Jukes-Cantor distances (Jukes & Cantor 1969) using Drosophila yakuba and Liriomyza huidobrensis as outgroups (accession numbers 12918 and AF327292, GeneBank database).

The reliability of the tree was evaluated using 1000 bootstrap replicates (Felsenstein 1985), and by constructing a consensus tree from the 1000 bootstrapped trees obtained. Phylogenetic analysis was performed using the PHYLIP software package (Felsenstein 1993).

RESULTS AND DISCUSSION

Nucleotide sequences of 259 bp were obtained for the cytochrome oxidase II region studied.

Interspecific pairwise percentage sequence differences ranged from 0.8 to 22.8% (Table 2), with a mean of 16%. The lowest value observed was for comparisons between Bactrocera dorsalis and Bactrocera cucumis (0.8%). The highest interspecific divergence was found between Anastrepha suspensa and Bactrocera latifrons (22.8%). The mean of 16% for the interspecific sequence differences is close to the 11% found by Han & McPheron (1997) in representative species of the family Tephritidae (based on the analysis of a fragment of mitochondrial 16S ribosomal DNA). The sequence divergence values obtained in the present work were generally low (20% or less). Comparisons of these results with those reported by others authors show that they would normally be thought to correspond to related species. In the case of Bactrocera dorsalis and Bactrocera cucumis, a divergence of 0.8% falls in the range of values generally found at the intraspecific levels. This close relationship between these species agrees with recent classifications which place them both in the 'Bactrocera dorsalis complex' (White & Elson-Harris 1994).

This is a group of morphologically inseparable but biologically distinct species whose members may be referred to as sibling species (White 1996).

In the genus Rhagoletis, comparisons among members of the pomonella and cingulata groups

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Table 1. Species used and origin of the samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anastrepha obliqua</td>
<td>Mexico</td>
</tr>
<tr>
<td>Anastrepha striata</td>
<td>Mexico</td>
</tr>
<tr>
<td>Anastrepha suspensa</td>
<td>U.S.A. (Florida)</td>
</tr>
<tr>
<td>Bactrocera cucumis</td>
<td>Australia</td>
</tr>
<tr>
<td>Bactrocera cucurbitae</td>
<td>U.S.A. (Hawaii)</td>
</tr>
<tr>
<td>Bactrocera dorsalis</td>
<td>U.S.A. (Hawaii)</td>
</tr>
<tr>
<td>Bactrocera latifrons</td>
<td>U.S.A. (Hawaii)</td>
</tr>
<tr>
<td>Bactrocera oleae</td>
<td>Spain</td>
</tr>
<tr>
<td>Bactrocera zonata</td>
<td>Mauritius Island</td>
</tr>
<tr>
<td>Ceratitis capitata</td>
<td>Spain</td>
</tr>
<tr>
<td>Ceratitis cosyra</td>
<td>South Africa</td>
</tr>
<tr>
<td>Dacus ciliatus</td>
<td>Réunion Island</td>
</tr>
<tr>
<td>Dacus demmurezi</td>
<td>Réunion Island</td>
</tr>
<tr>
<td>Rhagoletis cerasi</td>
<td>Switzerland</td>
</tr>
<tr>
<td>Rhagoletis pomonella</td>
<td>U.S.A. (New York)</td>
</tr>
<tr>
<td>Toxotrypana curvicauda</td>
<td>U.S.A. (Florida)</td>
</tr>
</tbody>
</table>
showed sequence divergences below 0.5% (McPheron & Han 1997). These groups are complexes of sympatrically distributed sibling species that differ in host-plant choice.

Figure 1 shows a phylogenetic tree for the species in the present work, with *Liriomyza huidobrensis* and *Drosophila yakuba* as outgroups, constructed using the neighbour-joining method based on Jukes-Cantor distances. Two major clusters can be seen. The first comprises the *Ceratitis* species *C. capitata*, *C. rosa* and *C. cosyra*, while the second has two main branches, one for species of the genera *Dacus*, *Bactrocera* and *Rhagoletis*, and the other for species of the genera *Anastrepha* and *Toxotrypana*.

Recent classifications of the family Tephritidae, based mainly on morphological characters (White & Elson-Harris 1994; Korneyev 2001), place species of the genera *Ceratitis*, *Bactrocera* and *Dacus* in the subfamily Dacinae, whereas the species of the genera *Anastrepha*, *Toxotrypana* and *Rhagoletis* are included in the subfamily Trypetinae. Similarly, a close relationship between members of the tribes Ceratitini (including the genus *Ceratitis*)
and Dacini has been reported, based on morphological (Hancock 1986; Foote et al. 1993; Norrbom et al. 1999) and molecular data (Han & McPherson 1997, 2001; Malacrida et al. 1996). However, the results of the present work show the Ceratitis species to be clearly separated from the rest of the studied genera, and places the species of the tribe Dacini (genera Bactrocera and Dacus) with members of the subfamily Trypetrinae (genus Rhagoletis). This relationship between Bactrocera and Rhagoletis species is consistent with the results obtained by Han & McPherson (1994) and Malacrida et al. (1996), based on the analysis of nuclear ribosomal DNA and allozyme data, respectively.

A closer relationship between Anastrepha and Toxotrypana has been suggested (Kitto 1983; Norrbom & Foote 1989; Han & McPherson 1994; 1997, 2001). The present analysis supports close affinities between members of this group, currently placed in the tribe Toxotrypanini (Foote et al. 1993; White & Elson-Harris 1994; Korneyev 2001).

In summary, the present data agree with those of previous investigations using different characters (morphological and molecular) in terms of the close affinity between Anastrepha and Toxotrypana, and in terms of the relationships between Bactrocera and Rhagoletis. However, the data also revealed Ceratitis species to be equally separated from all other genera studied.

Thus the classification – especially the higher classification – of the family Tephritidae, and the phylogenetic relationships of its members, remain unsolved. Both the morphological and the partial molecular approaches are severely limited. In future, the addition of more data from different sequences and species will probably contribute to the resolution of the relationships among the species of this important pest group.

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REFERENCES


FELSENSTEIN, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.


