The *Bactrocera* (*Notodacus*) *xanthodes* (Broun) Species Complex (Diptera: Tephritidae): Comparison of 18S rRNA Sequences From Fiji, Tonga and Vanuatu Specimens Suggest Two Distinct Strains

P. HOEBEN[1], L. J. DANIEL[1], JING MA[1] and R. A. I. DREW[2]

[1]Centre for Molecular Biotechnology, School of Life Science, Queensland University of Technology, G.P.O. Box 2434, Brisbane, Qld 4001.
[2]Department of Primary Industries, Division of Plant Protection, Meiers Rd, Indooroopilly, Qld 4068.

ABSTRACT Preliminary data suggest that fly specimens from Fiji, Tonga and Vanuatu presently classified as *Bactrocera* (*Notodacus*) *xanthodes* (Broun) show significant divergence at the 18S ribosomal RNA gene level. Sequencing data indicate that the Vanuatu specimen may be considered a strain separate from that on Fiji and Tonga.

Introduction

Bactrocera (*Notodacus*) *xanthodes* (Broun) is a fruitfly that is a major pest throughout the South Pacific region, where it infests a wide range of commercial fruits. Recent host fruit surveys in this region undertaken under the Australian Centre for International Agricultural Research (ACIAR) fruit fly project, have revealed anomalous populations of flies on New Caledonia, Vanuatu, Tonga and Western Samoa. These anomalous populations are very similar to *B. xanthodes*, but do not infest commercial fruits. Present quarantine restrictions due to fruitflies severely reduce commercial fruit exports from these Pacific Island nations to countries such as Australia and New Zealand. If the anomalous population, the only morphotype occurring on Vanuatu, can be shown to be a non-pest strain or species, these restrictions may be lifted to allow export of fruit. To elucidate the taxonomic status of the new *xanthodes*-like population in addition to morphological studies we have amplified a region of the 18S (small) ribosomal RNA gene encompassing three variable regions (Hancock *et al.* 1988). The sequencing has revealed differential levels of homology between the 5′ end and the 3′ end of the amplified product. This suggests that *B. xanthodes* (Fiji) is more closely related to *B. xanthodes* (Tonga) than to the *B. xanthodes* Vanuatu morphotype.

Material and methods

Fly collection and storage. Adult *B. xanthodes* were collected from Fijian fruit plantations by Mr A. Allwood and from wild forest fruits in Vanuatu and Tonga by Mr D. Tau. Approximately five flies were stored in 20 mL of 100% absolute ethanol at room temperature.

Fly identification. The specimens from Fiji and Tonga were identified on morphology by one of us (R. A. I. D.) as *B. xanthodes* and those from Vanuatu as sp. near *xanthodes*, based on keys to species in Drew (1989).

DNA extraction (adapted from Xiong and Kocher 1991). Fruitfly specimens of both types were taken from ethanol storage and air dried for 5 min at room temperature. One specimen was pulverised in a pre-cooled mortar and the resulting powder was transferred to a 1.5 mL Eppendorf tube containing 400 µL of extraction buffer (100 mM TRIS/HCl pH 8; 20 mM EDTA pH 8; 100 mM NaCl; 1% w/v SDS; 50 mM DTT, and 0.4 mg/mL filter sterilised Proteinase K). After 2 h at 37 °C, the mixture was extracted with phenol, phenol/chloroform (1:1) and chloroform, respectively. The DNA was precipitated with 2.5 volumes of 100% ethanol and 1/10 of a volume of 3 M KAc pH 5.2. The DNA pellet was washed with 70% ethanol and air dried for 5 min at room temperature and subsequently dissolved in 100 µL of TE (10 mM TRIS/HCl pH 7.5; 1 mM EDTA). The final concentration was on average 20 ng/µL.

PCR. PCR primers and conditions were as follows: Primers 18SA: 5-'GAGGGAGCCTGA-GAAACGGCTAC-3', and 18SB: 5'-CCTTCCG-TCAATTCCTTTAAGTTTC-3' were designed on the basis of 100% sequence homology between the rat 18S rRNA gene sequence (Torczynski *et al.* 1983), the *Xenopus laevis* sequence (Salim and Maden 1981) and the *Saccharomyces cerevisiae* sequence (Rubstov *et al.* 1980). The 18SA primer starts at nucleotide position 440 and ends at position 463, whereas the 18SB primer runs from nucleotide position 1220 to position 1196, both in the rat sequence (Torczynski *et al.* 1983; see Fig. 3). Cycle conditions were initial denaturation, 5 min at 95 °C followed by 35 cycles of 30 s denaturation at 92 °C, annealing for 60 s at 59 °C and extension for 120 s at 72 °C. A Perkin Elmer Cetus Thermal DNA cycler model 480 was routinely used for the amplification reactions.

Each reaction consisted of 10 ng of template, 5 µL of 10* Taq Polymerase buffer (0.67 M TRIS, pH 8, 0.01 M $MgCl_2$, 0.098 M 2-Mercaptoethanol, 0.1% (v/v) Tween 20), 100 pmole of primers, 125 µM (final concentration) of dNTPs of 0.5 unit of

Tth Polymerase, water up to a volume of 50 μL and 50μL of mineral oil.

PCR product gel electrophoresis and purification. PCR products were analysed for size and purity in 2% agarose gels. For purification products were electrophoresed through 2% low melting temperature agarose and purified with Promega Magic kits (Manufacturer's instructions were followed). ΦX174 Hae III was routinely used as DNA size marker.

PCR product sequencing. Purified products were sequenced using either Sequenase kits and ^{35}S-dATP as label, or an ABI 373A automated DNA sequencer. Initial denaturation of the PCR products was done for 5 min at 95 °C, followed by immediate transfer to liquid nitrogen and subsequent thawing at room temperature for 20 min. From that point onwards the manufacturers' protocols were followed. The 18SA primer was used to sequence the 5′ end of the 780 bp PCR product. The 18SB primer was used to sequence the 3′ end of the amplified product. Final concentration of the primers was 1 pmole per reaction. Each product was sequenced completely in both directions. Only those regions with no inconsistencies were used for the sequence analyses. This resulted in approximately 600 bp sections of the 18S ribosomal gene for all specimens.

Sequence comparison. Sequences were compared using the "gap" and "clustal V" programs from the GCG group of programs available from the ANGIS database (Higgins and Sharp 1988). Each sequence was compiled from at least two sequences. For each species, one fly was analysed.

Results

The PCR product was a 780 bp long fragment (see Fig. 1) which was consistent with expectations. The amplified product ran from the relative nucleotide position of bp 441 to position 1220 in the *Tenebrio molitor* 18S ribosomal sequence (Hendricks *et al.* 1988), or nucleotide position 428 to position 1242 in the *Drosophila melanogaster* gene (Tautz *et al.* 1988). Fig. 2 shows the comparison of the sequences using Clustal V analysis. *Tenebrio molitor* was chosen as an outgroup. We sequenced approximately 600 bp of the 18S gene. This was a continuous section of the 18S ribosomal gene encompassed by the two primers 18SA and 18SB. Each sequence was compiled from three individual sequences and each of these was derived from the same fly. The actual percentage sequence homology for the 600 bp region between the various species is shown in Table 1. Tables 2 and 3 show the percentage homology for the V4 region (nucleotide position 645 to 945 in the *T. molitor* sequence), and V5 region (nucleotide positions: 1129 to 1199 in the *T. molitor* sequence), respectively (Hancock *et al.* 1988).

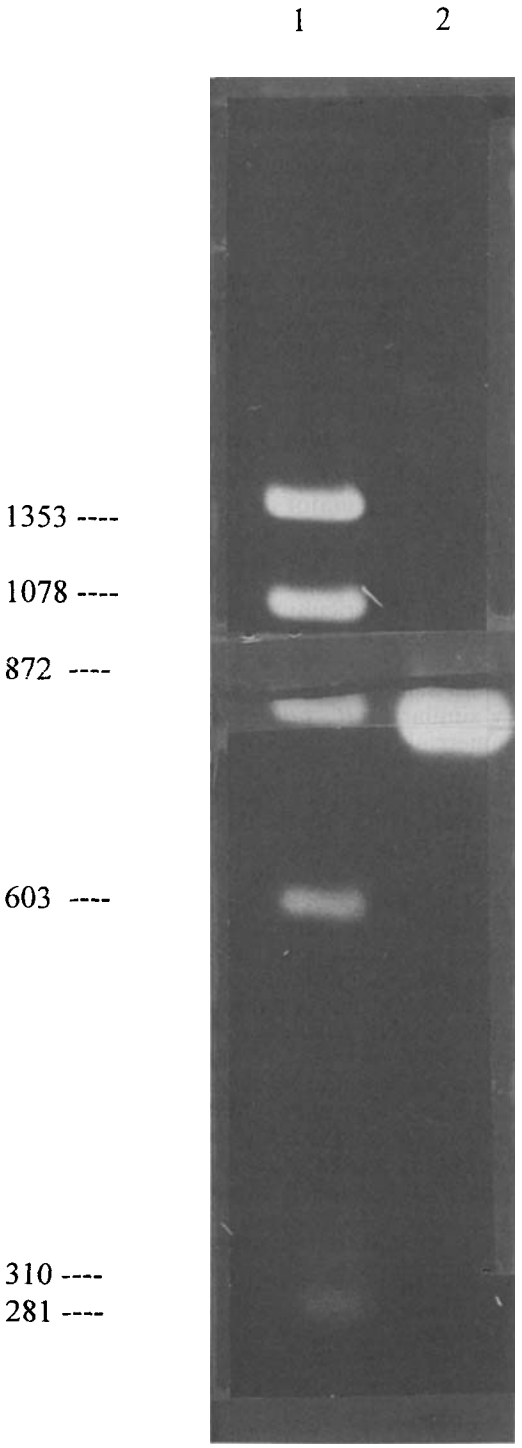

Fig. 1. 2% Agarose gel showing the 780 bp PCR product. Lane 1:ΦX174 Hae III size marker; Lane 2: PCR product.

14406055, 1996, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/j.1440-6055.1996.tb01362.x by Research Information Service, Wiley Online Library on [04/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Table 1. Percentage sequence homology between the indicated species. Abbreviations: BT: *B. xanthodes* from Tonga; BF: *B. xanthodes* from Fiji; BV: *B. xanthodes* from Vanuatu; TM: *Tenebrio molitor.*

Species	TM	BT	BF
BT	76.8	100	—
BF	77.2	99.3	100
BV	75.9	98.7	98.6

Table 2. Percentage sequence homology between the indicated species for the V4 region. Abbreviations: BT: *B. xanthodes* from Tonga; BF: *B. xanthodes* from Fiji; BV: *B. xanthodes* from Vanuatu; TM: *Tenebrio molitor.*

Species	TM	BT	BF
BT	60.5	100	—
BF	60.5	100	100
BV	60.5	99	99

Table 3. Percentage sequence homology between the indicated species for the V5 region. Abbreviations: BT: *B. xanthodes* from Tonga; BF: *B. xanthodes* from Fiji; BV: *B. xanthodes* from Vanuatu; TM: *Tenebrio molitor.*

Species	TM	BT	BF
BT	78.5	100	—
BF	80.3	95.8	100
BV	74.2	94.3	95.8

Discussion

Ribosomal RNA genes are found in all living organisms. The 18S ribosomal RNA genes have been extensively used in phylogenetic studies (Torczynski *et al.* 1983; McClintock Turbeville *et al.* 1991; Carmean *et al.* 1992). The 18SA and 18SB primers amplify a 780 bp region of the

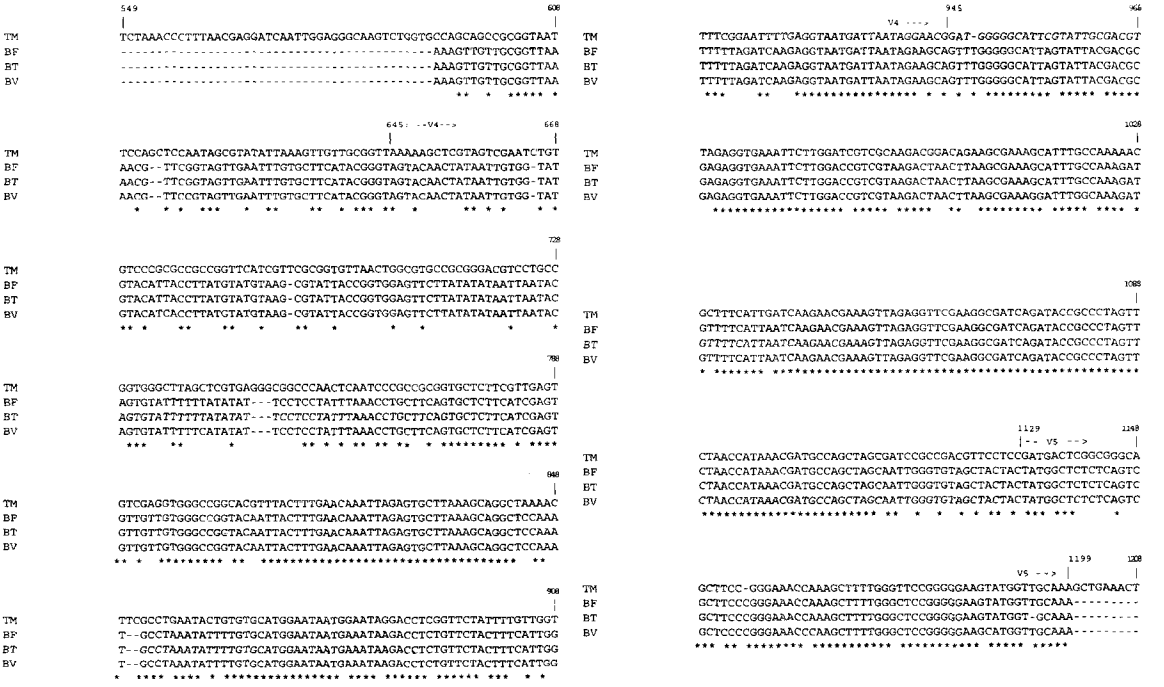

Fig. 2. Clustal V sequence comparison of the 600 bp sections of the 18S ribosomal gene. * denote 100% homology between the sequences. Numbers indicate the relative nucleotide positions in the *T. molitor* 18S ribosomal gene sequence. Abbreviations: BT: *B. xanthodes* from Tonga; BF: *B. xanthodes* from Fiji; BV: *B. xanthodes* from Vanuatu; TM: *Tenebrio molitor*; V4: Variable region #4 and V5: Variable region #5, according to the terminology in Hancock *et al.* (1988).

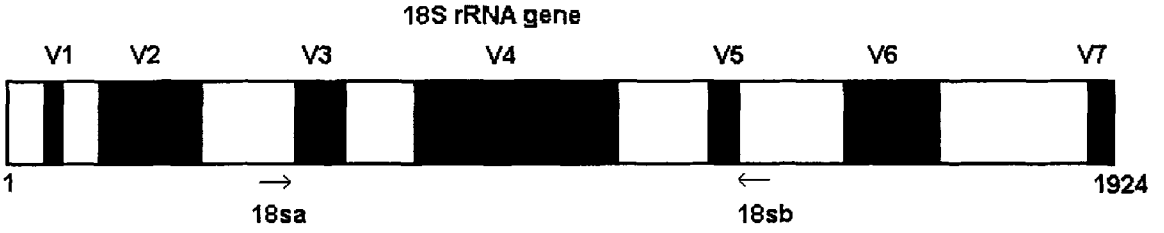

Fig. 3. Schematic diagram representing the *Drosophila melanogaster* 18S ribosomal RNA gene with the 7 variable regions (shaded) and the relative positions of the primers used to amplify (Tautz *et al.* 1988).

14406055, 1996, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/j.1440-6055.1996.tb01362.x by Research Information Service, Wiley Online Library on [04/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

ribosomal RNA gene. This region encompasses three variable (expansion) regions of the gene, and core elements which show extreme conservation across species and taxonomic divisions (see Fig. 3). This combination makes the region an excellent candidate for taxonomic and phylogenetic studies. Analysis of the 18S ribosomal RNA gene has been used to resolve the phylogenetic relationships of holometabolous insects. Carmean *et al.* (1992) compared morphological data with molecular data. Their findings showed little variation within families with average, pairwise-corrected substitution rates among hymenopteran families of 1.8% (±0.6%). However they also reported that the Diptera showed 2-3 times higher rates of substitution when compared to other Holometabola. Our results show levels of variation comparable to the hymenopteran families. When concentrating on the 5′ end of the amplified product, i.e. from nucleotide position 645 to 945 in the *T. molitor* sequence which encompasses major variable region #4 (V4; Fig. 3 and Table 2), *B. xanthodes* (Fiji) shows 100% homology to *B. xanthodes* (Tonga). For the same region the level of homology decreases to 99% when compared to the Vanuatu specimen. All three *xanthodes* specimens show the same level of homology compared to *T. molitor*: 60.5%. At the 3′ end of the amplified product which contains region #5 (V5, nucleotide positions: 1129 to 1199 in the *T. molitor* sequence; see Fig. 3 and Table 3), the situation is more complex. *B. xanthodes* (Fiji) shows 95.8% homology in this region compared to the Tonga and Vanuatu specimens. Whereas the Tonga specimen shows only 94.3% when compared to the Vanuatu specimen. On average the homology between the *xanthodes* group and *T. molitor* in this region is around 77.7%. Both the V4 and V5 regions confirm, however, that the Vanuatu specimen had the lowest homology to *T. molitor* which is consistent with the other findings. The intermediate region between V4 and V5 shows an average level of 84.7% homology. Within the *xanthodes* group the lower levels of homology occur in the V5 region rather than the V4 region. This is despite the greater size of the latter which would allow for a higher level of divergence. This could have implications for the rate of evolution in these regions. Overall homology shows that both the Fiji and Tonga specimens are closer related to the *T. molitor* sequences than is species near *B. xanthodes* (Vanuatu). The very high degree of homology between the Tongan and Fijian specimens, and the higher degree of divergence between this group and the Vanuatu specimen suggests the existence of two distinct strains. This is corroborated by the occurrence of distinct morphotypes on Fiji and Vanuatu.

These data have been derived from PCR amplified products so the danger of contamination must be considered. The sequences were compiled from one fly from each species and sequenced at least twice in both the forward and reverse directions. The amplification of the 18S ribosomal gene was performed on separate days for each species and positive displacement pipettes were used to reduce contamination. And finally, DNA extraction and storage of DNA samples was in a physically separate location from the area where PCR amplifications were carried out.

Presently, studies are under way to sequence the same 18S region of *B. xanthodes* from Samoa, New Caledonia and other Pacific Island nations. To corroborate these data ITS2 (Internal Transcribed Spacer regions, found between the 5.8S and 28S ribosomal genes), mitochrondrial cytochrome b and D loop sequences will be analysed for all the specimens.

References

Carmean, D., Kimsey, L. S. and Berbee, M. L. (1992). 18S rDNA sequences and the holometabolous insects. Molec. Phylogenet. Evol. 1: 270-278.

Drew, R. A. I. (1989). Tropical fruitflies (Diptera: Tephritidae: Dacinae) of the Australasian and Oceanic regions. Mem. Qd Mus. 26: 1-512.

Hancock, J. M., Tautz, D. and Dover, G. A. (1988). Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. Molec. Biol. Evol. 5: 393-414.

Hendricks, L., de Baere, R., van Broeckhoven, C. and de Wachter, R. (1988). Primary and secondary structure of the 18S ribosomal RNA of the insect species *Tenebrio molitor*. FEBS Lett. 232: 115-120.

Higgins, D. G. and Sharp, P. M. (1989). CLUSTAL: a package for performing multiple sequence alignments on a micro computer. Gene 73: 237-244.

McClintock Turbeville, J., Pfeifer, D. M., Field, K. G. and Raff, R. A. (1991). The phylogenetic status of Arthropods, as inferred from 18S rRNA sequences. Molec. Biol. Evol. 8: 669-686.

Rubtsov, P. M., Musakhanov, M. M., Zakharyev, V. M., Krayev, A. S., Skryabin, K. G. and Bayev, A. A. (1980). The structure of the yeast ribosomal RNA genes 1. The complete nucleotide sequence of the 18S ribosomal RNA gene from *Saccharomyces cerevisiae*. Nucl. Acid. Res. 8: 5779-5794.

Salim, M. and Maden, B. E. H. (1981). Nucleotide sequence of *Xenopus laevis* 18S ribosomal RNA inferred from gene sequence. Nature 291: 205-208.

Tautz, D., Hancock, J. M., Webb, D. A., Tautz, C. and Dover, G. A. (1988). Complete sequences of the rRNA genes of *Drosophila melanogaster*. Molec. Biol. Evol. 5: 366-376.

Torczynski, R., Bollon, A. P. and Fuke, M. (1983). The complete nucleotide sequence of the rat 18S ribosomal RNA gene and comparison with the respective yeast and frog genes. Nucl. Acid. Res. 11: 4879-4890.

Xiong, B. and Kocher, T. D. (1991). Comparison of mitochondrial DNA sequences of seven morphospecies of black flies (Diptera: Simuliidae). Genome 34: 306-311.

(Accepted 7 August 1995)

14406055, 1996, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/j.1440-6055.1996.tb01362.x by Research Information Service, Wiley Online Library on [04/04/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License