Isolation and Characterization of the *Xanthine Dehydrogenase* Gene of the Mediterranean Fruit Fly, *Ceratitis capitata*

R. J. Pitts and L. J. Zwiebel

Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee 37235

Manuscript received February 1, 2001
Accepted for publication May 23, 2001

ABSTRACT

Xanthine dehydrogenase (XDH) is a member of the molybdenum hydroxylase family of enzymes catalyzing the oxidation of hypoxanthine and xanthine to uric acid. The enzyme is also required for the production of one of the major Drosophila eye pigments, drosopterin. The XDH gene has been isolated in many species representing a broad cross section of the major groups of living organisms, including the cDNA encoding XDH from the Mediterranean fruit fly *Ceratitis capitata* (*Cc*XDH) described here. *Cc*XDH is closely related to other insect XDHs and is able to rescue the phenotype of the *Drosophila melanogaster* XDH mutant, *rosy*, in germline transformation experiments. A previously identified medfly mutant, termed *rosy*, whose phenotype is suggestive of a disruption in XDH function, has been examined for possible mutations in the XDH gene. However, we find no direct evidence that a mutation in the *Cc*XDH gene or that a reduction in the *Cc*XDH enzyme activity is present in *rosy* medflies. Conclusive studies of the nature of the medfly *rosy* mutant will require rescue by germline transformation of mutant medflies.

THE development of improved control strategies for insects that act as biological pests and disease vectors is vitally important for the prevention of the spread of human disease and for the alleviation of damage to economically important domestic animals and plant species. One of the most notorious agricultural pests is the Mediterranean fruit fly (medfly), *Ceratitis capitata*. The medfly has migrated from its origins in Africa throughout the Mediterranean region and into the Americas within the last 100 years (Haymer *et al.* 1997; Malacrida *et al.* 1998; Davies *et al.* 1999) and is responsible for the global annual loss of billions of dollars in fruit crop production. As a consequence, medflies are the subject of intense control efforts in many parts of the world, including Central America, Europe, and the United States (Kahn *et al.* 1990; Barinaga 1991; Carey 1991). The medfly is particularly destructive because it has a very wide host plant range, being able to infect some 200 fruit varieties, and because females puncture the fruit when laying eggs, allowing for larval and opportunistic microbial invasion (Saul 1986; Robinson 1989). It is hoped that a detailed understanding of the biology of the medfly will eventually lead to the development of the tools needed to effectively manage its populations. Germline transformation is one of the tools likely to contribute to the design of novel and effective control strategies.

To facilitate germline transformation, selectable or visible phenotypic markers are required to separate transformants from nontransformants. Many such visible markers have been used to great effect in the widely studied insect model system, *Drosophila melanogaster*. Currently, the two genetic markers most routinely used are the eye color genes *white* and *rosy* (Ashburner 1989). In Drosophila, *white* encodes an ABC family transporter that is responsible for import of eye pigments into photoreceptor cells (reviewed in Higgins 1992) while *rosy* encodes xanthine dehydrogenase (XDH), a member of the molybdenum-containing hydroxylase family of enzymes (Keith *et al.* 1987). The Drosophila *rosy*/XDH gene enzyme system has been studied in great detail, and it has long been established that XDH is required for the production of drosopterin eye pigment as well as for the conversion of purines into uric acid (reviewed in Chovnick *et al.* 1990; Wootton *et al.* 1991; Hille and Nishino 1995). The use of these genes in Drosophila transformation experiments has led to the suggestion of their similar application as phenotypic markers for germline transformation in the medfly and other insects (Zwiebel *et al.* 1995). Several medfly eye color mutants have been described, including *white*, *rosy*, *light eye*, and *Purple eye* (reviewed in Saul 1985, 1986) and, to date, *white* is the only one whose gene has been fully isolated from medfly (Zwiebel *et al.* 1995; Gomulski *et al.* 2001). As in Drosophila, the medfly *white* mutants completely lack eye pigmentation (Rossler and Koltin 1976; Rossler and Rosenthal 1992). Significantly, the medfly *white* gene is homologous to Drosophila *white*, and two naturally occurring mutations have been defined at the DNA level (Zwiebel *et al.* 1995; Gomulski *et al.* 2001).

Corresponding author: L. J. Zwiebel, Department of Biological Sciences, Box 82, Station B, Vanderbilt University, Nashville, TN 37235. E-mail: l.zwiebel@vanderbilt.edu

Indeed, partial phenotypic rescue of *white* mutants has been successfully carried out in medfly using the medfly *white* cDNA as a dominant marker for two transposon-based transformation vectors, *Minos* and *piggyBac* (Loukeris *et al.* 1995; Handler *et al.* 1998).

As is the case in Drosophila, the characterization of multiple molecularly defined phenotypic markers for transformation in the medfly will facilitate increased flexibility among different applications. As part of the effort to expand the range of germline transformation systems in this important agricultural pest insect, we have undertaken the isolation and characterization of the medfly XDH gene. By analogy to the *white* gene, *Ceratitis* XDH (*Cc*XDH) has the potential to be used as an additional phenotypic marker, but only if a corresponding medfly XDH mutant with an easily distinguishable phenotype can be identified. A previous study has isolated and characterized a *C. capitata* mutant, termed *rosy*, which phenotypically displays burgundy eyes as well as a sensitivity to purine-supplemented media (Saul 1982), two phenotypes that are characteristic of many of the *rosy* alleles of *D. melanogaster* (reviewed in Chovnick *et al.* 1990). This would suggest that the molecular basis for the *rosy* mutant may be a defect in the *Cc*XDH gene. Alternatively, these phenotypes could be the result of a mutation in a gene affecting *Cc*XDH function because the enzyme requires the actions of several cofactors and gene products for its normal function in Drosophila (Hughes *et al.* 1992; Hughes 1992). For example, the product of the *ma-l* gene is required for XDH activity (Forrest *et al.* 1956; Glassman and Mitchell 1959) where it is apparently responsible for the ability of XDH and other enzymes to incorporate sulfur (Wahl *et al.* 1982). Mutations in *ma-l* and other loci in Drosophila, including *low xanthine dehydrogenase* (*lxd*) and *cinnamon* (*cin*), also display a *rosy* eye phenotype and have a corresponding low level of XDH activity (reviewed in Kamdar *et al.* 1997). It will be critical to elucidate whether a lesion in XDH causes the medfly *rosy* mutant because it has the potential to be used in germline transformation.

To directly examine this issue we have cloned the full-length medfly *Cc*XDH cDNA and have tested the hypothesis that the medfly *rosy* phenotype is caused by a defect in *Cc*XDH. Our studies demonstrate that the *Cc*XDH gene is capable of functionally rescuing the Drosophila XDH *rosy* mutant and may therefore be useful as a marker for medfly germline transformation in a *Cc*XDH mutant background. However, we find no evidence for a defect in the XDH gene in the medfly *rosy* mutant at the levels of DNA, RNA, or enzyme activity *in vitro*. Final resolution of this question requires the use of *Cc*XDH cDNA in an attempt to rescue the medfly *rosy* mutant by germline transformation. We are prevented from conducting such studies due to the absolute quarantine against live medflies that is currently in place in the continental United States.

MATERIALS AND METHODS

Medfly strains: Two independently derived medfly laboratory strains were used in this study: Benakeion, which is associated with the *XDH*$^+$ allele and has wild-type eye color, and the *rosy* eye mutant strain, in the Wiedemann genetic background. The wild-type eye color strain Benakeion was originally established in the laboratory by P. A. Mourikis (Benakeion Institute of Phytopathology, Athens, Greece) with flies from the Southern Peloponnese (Greece) and Palermo (Italy; Rina and Savakis 1991). The *rosy* strain was established in the laboratory of Stephen Saul (Saul 1982).

***C. capitata* XDH cloning:** *Cc*XDH clones were isolated using degenerate PCR primers designed using the medfly codon bias values (Nakamura *et al.* 2000) against conserved domains of rat XDH and two available insect sequences, *D. melanogaster* and *C. vicina*. The primers were LJZVI 5′-ACI GCI TTY CGY GGY TTY GGI GGI CCW CAR GGI ATG-3′ (corresponding to the amino acid sequence TAFRGFGGPQGM) and LJZVIII 5′-YTG ICC RAT RTC IGC DGG RTT RAA IGA IGA ICC-3′ (corresponding to the amino acids QGSSLNPAIDIGQ). PCR generated an 860-bp product that was TOPO TA cloned (Invitrogen, San Diego) and sequenced to verify that it was XDH from medfly. This sequence corresponds to amino acids starting from position 1155 of the full *Cc*XDH peptide VGDD…etc. The 860-bp fragment was radiolabeled with [dATP]α^{32}P and used to isolate both genomic and cDNA clones from λ-phage libraries (Sambrook *et al.* 1989). Genomic clone 623 remains uncharacterized while λzap (Stratagene, La Jolla, CA) cDNA clone 114-1 was subcloned into the *Kpn*I and *Sal*I restriction sites of vector pSP73 (Promega, Madison, WI).

Poly(A)$^+$ RNA was isolated from adult medflies (*C. capitata*) and used to synthesize double-stranded cDNA followed by adapter ligation using the Marathon cDNA amplification kit protocol (CLONTECH, Palo Alto, CA). Adapter oligonucleotide primer AP2 5′-ACT CAC TAT AGG GCT CGA GCG GC-3′ was used in combination with oligonucleotide primer XDHRACE 5′-AGC ATA CAA CGC ACG GGT CTT C-3′ to PCR amplify the 5′ end of the XDH cDNA from the medfly rapid amplification of cDNA ends (RACE) library under the following conditions. A premix of 17.5 μl 10× Clontech RACE PCR buffer, 14 μl [10 mm] dNTP, 3.5 μl of Advantage Taq polymerase, and 119 μl dH_2O was mixed and kept on ice. Each reaction contained 21.5 μl of premix, 2.5 μl of a 1:200 dilution of adapter ligated RACE library, and 0.5 μl of each [10 μm] primer. A positive control reaction containing 2.5 μl of control cDNA and 0.5 μl of oligos AP1 5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′ and TFR3′ 5′-ATT TCG GGA ATG CTG AGA AAA CAG ACA GA-3′ produced the expected 2.9-kb product. Negative control reactions containing single primers produced no products. Reactions were carried out in a Perkin-Elmer (Norwalk, CT) 9700 thermal cycler as follows: 94° for 2 min; 5 cycles of 94° for 5 sec and 72° for 4 min; 5 cycles of 94° for 5 sec and 70° for 4 min; and 25 cycles of 94° for 5 sec and 68° for 4 min.

Digesting the 5′ RACE subclone with *Xho*I/*Bbs*I restriction endonucleases generated the full-length medfly cDNA clone (2.8 kb). This fragment was ligated into a *Sal*I/*Bbs*I-digested vector containing the 3′ end of XDH. The full-length cDNA, pSP73:*Cc*XDH, was sequenced in an ABI377 automated sequencer as described (Perkin-Elmer). *Cc*XDH was conceptually translated and alignments with similar peptides were performed using CLUSTAL W software (Thompson *et al.* 1994). The PAUP software package (Swofford 1991) was used to determine the phylogenetic placement of our medfly XDH sequence in relation to those of the full-length XDH genes previously sequenced and analyzed from other insect taxa (Komoto *et al.* 1999). These included sequences from two

different XDH loci from *Bombyx mori* as well as single copies from the drosophilid species *D. melanogaster*, *D. pseudoobscura*, and *D. subobscura*, and from the calliphorid *Calliphora vicina*. *B. mori* is the only insect reported to possess more than one XDH gene, each apparently serving a different current function (Yasukochi *et al.* 1998; Komoto *et al.* 1999). Our initial analyses used mouse XDH as an outgroup, while later analyses deleted mouse and used Bombyx sequences instead. Equally weighted XDH data sets were analyzed as both nucleotide and amino acid sequences using maximum parsimony, neighbor joining, and maximum likelihood methods. The bootstrap (Felsenstein 1985) was used to assess statistical support for relationships via branch and bound analysis of 100 pseudoreplicated data sets.

***D. melanogaster* transformation:** The Drosophila *P*-element vector, pP{CaSpeR-hs/act}(GenBank accession no. U60735; for vector map see http://www-hhmi.genetics.utah.edu/thummel/pelement.html), mini prep DNA (1 μg) was digested with *Not*I and *Bam*HI and pSP73:*Cc*XDH mini prep DNA (1 μg) with *Not*I and *Bgl*II. Digests were run on 0.7% agarose (TAE) gels. The 9.2-kb vector and 4.9-kb XDH insert sequences were excised from the gel with sterile razor blades and DNA was isolated from the gel slices using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). Ligations were set up as follows and were allowed to proceed overnight at 16°: 1 μl pP{CaSpeR-hs/act} fragment; 1 μl or 2 μl of pSP73:XDH *Not*I/*Bgl*II fragment; 1 μl T4 ligase buffer; 0.3 μl T4 ligase [400 units/μl]; dH_2O to 10 μl. Each ligation (2 μl) was transformed into XL1-Blue competent cells (40 μl) by electroporation (2.5 kV). Transformations were plated onto Luria broth (LB) + ampicillin (50 μg/ml) agar and incubated overnight at 37°. Fresh amp^r colonies were picked with a sterile toothpick and used to inoculate an overnight LB + $ampicillin^{50}$ culture (3 ml). Plasmid DNA was isolated by alkaline lysis mini prep and digested with *Sal*I to confirm XDH insertion.

A plasmid DNA prep (>100 μg) of one pP{CaSpeR-hs/act}:*Cc*XDH clone was sent to the laboratory of Nick Brown who performed injections into *D. melanogaster*. This vector was injected into true-breeding *white*, *forked* (*wf*) embryos of *D. melanogaster* (G0 generation). The injected adults were crossed back to *wf* adults, and individual w^+ G1 flies were crossed with *wf* adults to establish a G2 stock. Nine individual G2 transformed lines were established (letters A through I). W^+ G2 virgin females were crossed with *cn*/*cn* (II); *Ly* ry^{506}/TM3 *Sb* ry^{506} (III) males. W^+ G3 males with forked bristles and non-Lyra wings (and therefore *Sb* ry^{506}) were crossed to *BcElp*/*CyO* (II); ry^{506}/ry^{506} (III) virgin females. The G4 crosses were heat shocked every day for 1 hr at 37° until adult eclosion. G4 adults were scored for bristle type, wing type, and eye color. All G4 stubble flies were therefore homozygous for ry^{506}.

Genomic DNA analysis: Genomic DNA was isolated from wild-type *C. capitata* (Benakeion) adults or *rosy* pupae (Wiedemann) according to the protocol of Ish-Horowicz for Drosophila (protocol 47 in Ashburner 1989). Southern blots were carried out using Hybond N+ membrane according to the manufacturer's protocol (Amersham Pharmacia Biotech). A full-length XDH cDNA fragment was used to probe blot under low stringency conditions. PCR was performed as follows: ~100 ng of DNA, dNTPs [0.2 mm], 1× buffer (Perkin-Elmer) with $MgCl_2$ [0.15 mm], Taq polymerase, primers [0.2 μm], and dH_2O to 25 μl. Primer pairs started at the 5′ end of the gene and continued along the coding sequence to the 3′ end with each product overlapping the previous product. In this manner the entire coding region was examined. Primer combinations used for coverage were XDH for 5′-TAG ATA ACA GAA GCA TTT GGA-3′ and Xex1R 5′-ACC TTT TTC CCA TTG ACA AAA-3′ (223 bp); Xex2F 5′-TAT TGA TCC CAC ACC CGA T-3′ and XDH7 5′-AGC AAA TCT GAA AGC TCC AC-3′ (686 bp); XDH3 5′-CAC CAG AAC TGC ATT TAA AC-3′ and XDHRACE 5′-AGC ATA CAA CGC ACG GGT CTT C-3′ (1863 bp); XDH5 5′-CAC CGC GAG ATA GTG ATG AA-3′ and XDH1 5′-TTA CTT ATG CAC TCC TGC C-3′ (1245 bp); and XDH6 5′-CGT GCA TTA GGT ATA CCA AC-3′ and XDH3′end 5′-TTT GGC CAA TCC AAT CAG TT-3′ (1016 bp). No differences were detectable between PCR product sizes when 5 μl of each reaction were run side by side on 0.7% agarose gels.

RNA Analysis: *C. capitata* total RNA was isolated from 1-day-old pupae of the *rosy* (Wiedemann) mutant and from embryos, pupae, and adults of wild type (Benakeion), using the RNeasy RNA isolation kit (QIAGEN). RT-PCR was performed using the Titan One-Tube RT-PCR kit (Roche Molecular Biochemicals). The manufacturer's protocol was followed except that reactions were scaled down from 50 to 25 μl by using half the amount of each reagent. About 0.5 μg of each RNA sample and a 0.2-μm final concentration of XDHleft and XDH6 primers were used for each reaction. First-strand synthesis was performed at 50° for 30 min. This step was followed immediately by 10 cycles of 94° for 30 sec, 53° for 30 sec, and 68° for 45 sec, and then 30 cycles of 94° for 30 sec, 53° for 30 sec, and 68° for 45 sec + 5 sec per cycle. Reactions were concluded at 68° for 7 min. Five microliters of each reaction was analyzed on a 1.5% agarose gel.

XDH enzyme assay: Crude extracts were prepared by homogenizing single medfly pupae or five Drosophila adult flies in 1.5 ml Eppendorf tubes in 80 μl cold buffer: 100 mm Tris, 1 mm EDTA, 0.5 mm NAD, and 0.05% 2-mercaptoethanol (pH 7.5). Each homogenate was centrifuged for 5 min at 13,000 rpm and 4°. Supernatant was transferred to a new tube and centrifuged as before. Protein concentrations of each extract were measured using the bicinchoninic acid method and according to the manufacturer's protocol (Pierce Chemical). Five microliters of each extract were loaded onto cellulose acetate gels that had been pretreated in a running buffer of 61.4 mm Tris, 4 mm EDTA, and 13.6 mm citric acid (pH 7.5). Gels were run at 100 V for 20–30 min. Gels were stained in Tris buffer with 1.4 mm hypoxanthine, 2.4 mm NAD, 0.4 mm phanazine methosulfate (PMS), and 1.2 mm nitro blue tetrazolium (NBT) (or xanthine:PMS:cytC or xanthine:NAD+).

RESULTS

***C. capitata* cDNA:** The full-length *Cc*XDH cDNA sequence is 4397 bp in length, including the 5′ and 3′ untranslated sequences (UTR; GenBank accession no. AY014961). The coding region spans 4041 bp, with the ATG translational start codon located at position 223 and the TGA stop codon located at position 4264. The ATG at position 223 is presumed to be the correct translational start because it is the first methionine following several cryptic stop codons in the 5′ UTR, including one that is just eight codons upstream in the same reading frame. Furthermore, this start site facilitates the longest possible open reading frame that is consistent with the sizes of closely related XDH sequences while the next potential start point lies 65 codons downstream. Within the 3′ UTR, a potential polyadenylation signal sequence, AATACA, precedes the observed polyadenylation site of the cDNA by 20 bp.

The cDNA encodes a peptide of 1347 amino acids when conceptually translated (Figure 1A). Highly con-

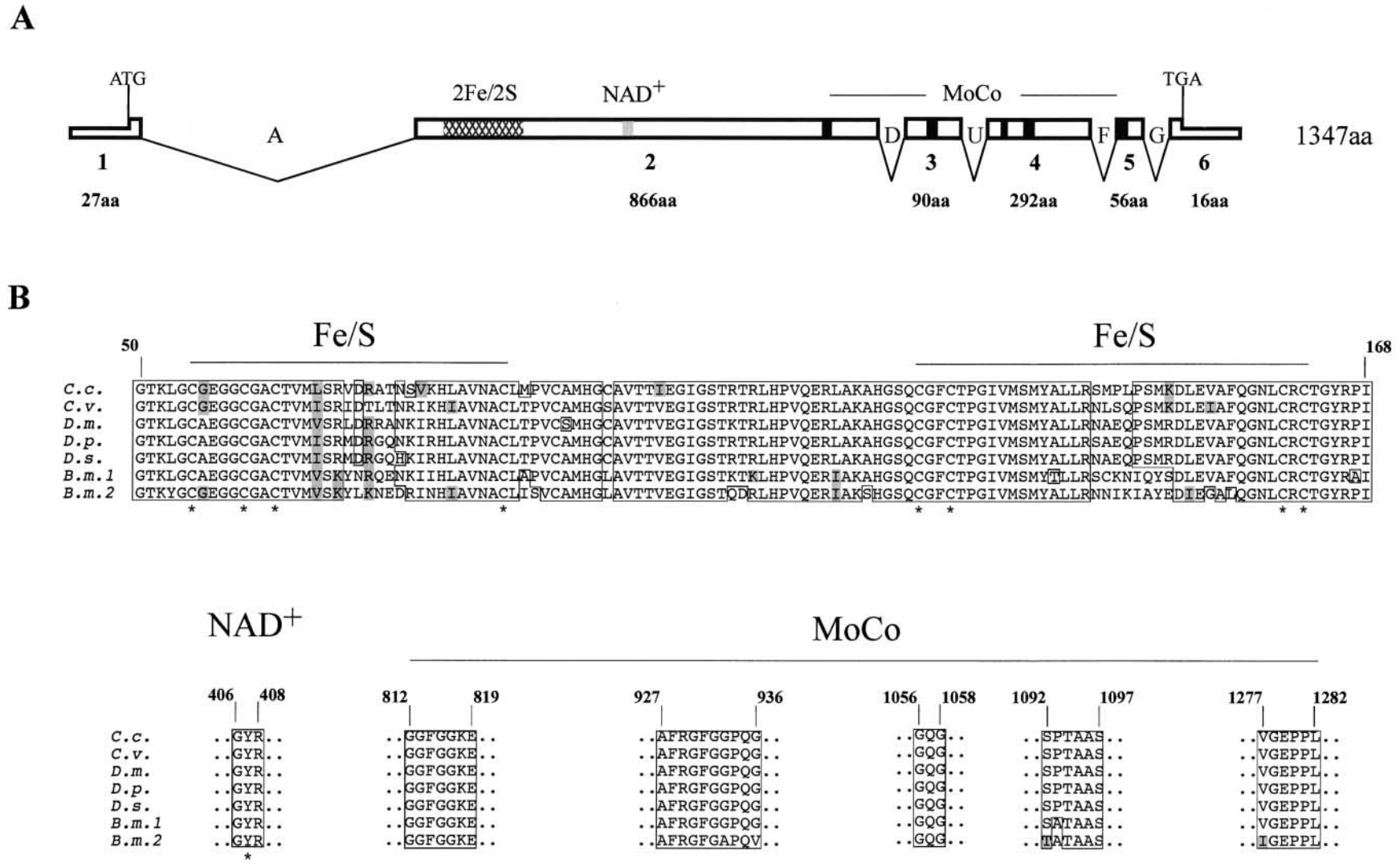

FIGURE 1.—Medfly XDH gene. (A) Structure of the *Cc*XDH gene. Boxes represent exons 1 through 6 of indicated size and thin lines denote introns A through G. Conserved functional areas of enzyme are 2Fe/2S, iron-sulfur domains (stippled box); NAD^+, nucleotide binding site (shaded box); and MoCo, molybdenum cofactor (solid boxes). (B) Partial amino acid sequence alignments of highly conserved domains. Identical residues are boxed while conserved residues are shaded. Asterisks denote the eight cysteines and the single tyrosine implicated in XDH function.

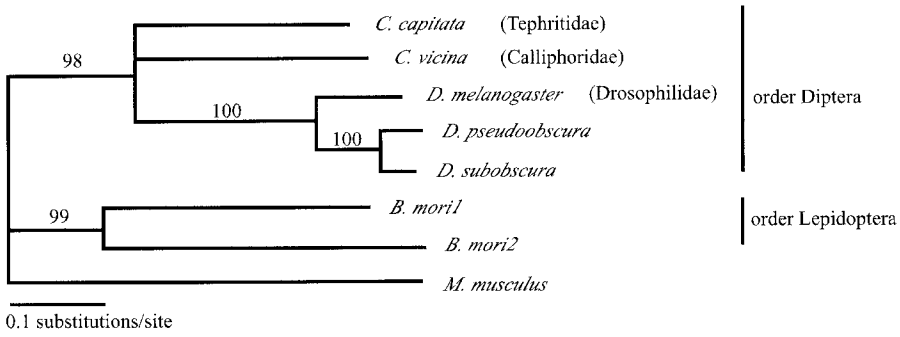

FIGURE 2.—XDH phylogram. Nucleotide sequences of insect XDH genes were aligned and phylogenetic relationships were assigned as described in MATERIALS AND METHODS. Numbers indicate bootstrap values. The XDH gene from *Mus musculus* was used as an outgroup.

served structural genes for XDH have been cloned from a wide range of species including bacteria, fungi, plants, insects, birds, and mammals (KEITH *et al.* 1987; HOUDE *et al.* 1989; RILEY 1989; AMAYA *et al.* 1990; TERAO *et al.* 1992; ICHIDA *et al.* 1993; GLATIGNY and SCAZZOCCHIO 1995; SATO *et al.* 1995; BERGLUND *et al.* 1996; COMERON and AGUADE 1996). Of these, *Cc*XDH is most similar in size and sequence to other insect XDH peptides. Overall, it is 74–75% identical to XDH peptides from *C. vicina*, *D. melanogaster*, *D. pseudoobscura*, and *D. subobscura*, all of which are dipteran flies. The medfly peptide is somewhat less similar to the two XDH peptides identified in the silkworm moth, *B. mori* (order Lepidoptera), displaying 58 and 52% identity to the *B. mori* 1 and *B. mori* 2 peptides, respectively. A partial alignment (Figure 1B) of the seven peptides from the six available insect species shows that XDH is highly conserved within the regions thought to bind iron-sulfur (2Fe-2S), flavin adenine dinucleotide, and molybdenum (MoCo) cofactors (HUGHES *et al.* 1992; SATO *et al.* 1995; DOYLE *et al.* 1996). Notably, there are eight completely conserved cysteine residues, four in each iron-sulfur domain, that are likely to participate in the binding of iron-sulfur cofactors and a conserved tyrosine residue at position 407 that is likely to bind NAD^+ (asterisks, Figure 1B). The cysteine residues correspond positionally with cysteines of the aldehyde oxido-reductase (Mop) from *Desulfovibrio gigas* that, on the basis of crystal structure analysis, have been directly implicated in iron-sulfur binding (ROMAO *et al.* 1995; ROMAO and HUBER 1997). The tyrosine corresponds to the same residue of the chicken xanthine dehydrogenase that is reported to participate in NAD binding (NISHINO and NISHINO 1989).

Genomic DNA Southern blot analysis indicates that *Cc*XDH is a single copy gene in the medfly (Figure 4A), as is the case in Drosophila (KEITH *et al.* 1987). Furthermore, all phylogenetic analyses provide statistical corroboration of the findings of KOMOTO *et al.* (1999) where Drosophila XDH sequences formed a monophyletic grouping within a more inclusive dipteran clade, with the two Bombyx moth sequences being united in a separate clade. While our medfly sequence was firmly placed inside the dipteran clade, the relationships among medfly, *C. vicina*, and Drosophila XDH sequences varied among analyses. Some estimates of dipteran phylogeny suggest that the Tephritidae (including the medfly) are more closely related to the Drosophilidae than either is to the Calliphoridae (MCALPINE 1989). However, more recent estimates made by comparing the sequences of two dipteran genes, glucose-6-phosphate dehydrogenase, *G6pdh* (SOTO-ADAMES *et al.* 1994), and *white* (GOMULSKI *et al.* 2001), suggest that the Tephritidae are more closely related to the Calliphoridae than to the Drosophilidae. In this study, XDH from the calliphorid *C. vicina* was observed to group with either the Drosophila or with *C. capitata* in certain analyses. Nonetheless, the relationships among these three lineages were not strongly supported by bootstrap in any single analysis and are presented here as unresolved (Figure 2).

PCR analysis indicates that at least four introns are found within the *Cc*XDH gene, and evidence is described for a fifth. In these studies, genomic DNA was used as a PCR template for a series of primers covering the XDH coding region, and products that were larger than cDNA control fragments were subcloned and sequenced. In this manner, four small introns (introns 2 through 5) were identified, ranging in length from 59 to 85 bp (Figure 1A and Table 1). The positions of three of these introns, D, F, and G (KOMOTO *et al.* 1999), are absolutely conserved when compared with the positions of introns of other insect species (Table 1), all of which are bounded by gt-ag splice site consensus sequences (BREATHNACH and CHAMBON 1981). We confirm the results of TARRIO *et al.* (1998), who discovered that one of these four introns (intron U in Figure 1A) is absent in all other reported insect XDH genomic sequences (TARRIO *et al.* 1998). This unique medfly intron does not correspond to the position of intron E (KOMOTO *et al.* 1999) and it is believed to be a duplication of intron D because their sequences are very closely related in the medfly (TARRIO *et al.* 1998).

There are also considerable lines of evidence suggesting the existence of a large first intron in medfly. Many of the introns within insect XDH genes are positionally conserved and all of the known insect XDH genes contain a commonly located intron A as their first, and largest, intron (KOMOTO *et al.* 1999). The size range of the first intron varies from 815 bp in *D. melanogaster* to 15 kb in *C. vicina* (KEITH *et al.* 1987; HOUDE *et al.* 1989). Furthermore,

TABLE 1

XDH gene intron sizes

	Conserved intron positions							
	A	B	C	D	U	E	F	G
C. cap	>5kb	—	—	65	82	—	85	59
C. vic	15kb	—	—	—	—	—	67	51
D. mel	815	—	—	281	—	—	—	65
D. pse	1024	—	—	62	—	—	67	67
D. sub	1535	—	—	528	—	—	62	68
B. mor1	4488	1198	75	77	—	897	454	2040
B. mor2	1398	395	455	76	—	165	1247	847

Conserved intron positions A through G are lettered according to Komoto *et al.* (1999). The size of each intron is listed in base pairs except where indicated in kilobase pairs. Hyphens denote the lack of a particular intron within a species. Intron U is unique to *C. capitata* (Tarrio *et al.* 1998).

Southern blots of medfly genomic DNA, probed with PCR products derived from cDNA spanning the putative splice site, hybridize with large molecular weight bands, indicating an intron size of >5 kb (data not shown), and several attempts to PCR amplify the first intron region of the XDH gene from wild-type genomic DNA failed. Nonetheless, genomic PCR reactions designed to amplify the exons immediately surrounding the putative intron site gave products of the expected length for cDNA and therefore indicate that these regions themselves are uninterrupted by introns (data not shown).

Rescue of Drosophila *rosy* mutant: To demonstrate that *Cc*XDH encodes a functional xanthine dehydrogenase enzyme *in vivo*, phenotypic rescue experiments were carried out using the well-established *P*-element transformation protocols available in *D. melanogaster* (Rubin and Spradling 1982; Ashburner 1989). Prior to transformation, and as an indication that the *Cc*XDH cDNA could be fully translated, a peptide of ~150 kD corresponding to the expected size for the full-length XDH monomer (Edwards *et al.* 1977; Keith *et al.* 1987) was observed in an *in vitro* rabbit reticulocyte translation system (data not shown). The full-length *Cc*XDH cDNA was subsequently cloned into the *P*-element transformation vector, pP{CaSpeR-hs/act}, which utilizes a *white* mini gene for transformation selection, a 5′ heat-shock promoter to drive expression of the insert, and 3′ Act 5C sequences to promote transcript stability. This construct was introduced into the germline of *white, forked* recipient *D. melanogaster* flies that were then crossed into an appropriate ry^- background. Ultimately, two transformed lines, B and I, each having X chromosome insertions, were chosen for detailed rescue analyses. The results from one such experiment with line B are presented in Figure 3 and are representative of repeated analyses with both lines B and I. In these studies, all G4 females inherit the *Cc*XDH transgene and those with stubble bristles are homozygous for the *ry* mutation (see materials and methods). Under heat-shock conditions, all these flies have wild-type eye pigmentation, indicating that the *Cc*XDH transgene was both functional and apparently able to fully complement the *ry* mutant phenotype (Figure 3D). In contrast, the G4 stubble males, which do not carry the X-linked XDH transgene and are also *ry* homozygotes, retained the *rosy* eye mutant phenotype under the same heat-shock conditions and served as an internal negative control (Figure 3C). This experiment establishes the *Cc*XDH as the functional ortholog of the Drosophila XDH.

Medfly *rosy* mutant: To initially address the possibility that the medfly *rosy* mutant carries a defect in the XDH gene, we carried out comparative Southern blot and PCR analyses between genomic DNA prepared from wild-type and *rosy* medflies. Inasmuch as identical bands on Southern blots and identical PCR products were

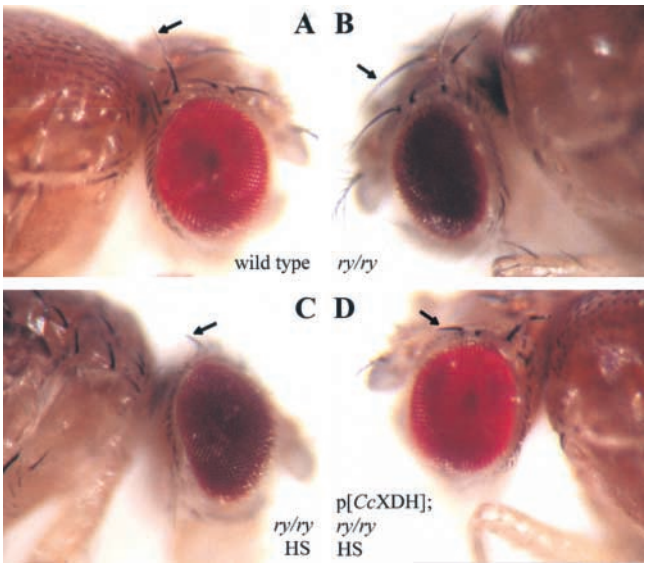

Figure 3.—Rescue of Drosophila *rosy* with *Cc*XDH. Eye color phenotypes of (A) Oregon-R (OR) wild type, (B) ry^{506} homozygous mutant, (C) G4 *ry*/*ry* males, and (D) G4 *ry*/*ry* transgenic females. Arrows denote bristle phenotype: wild type in A and B and Stubble in C and D, indicating the presence of the *ry* balancer chromosome.

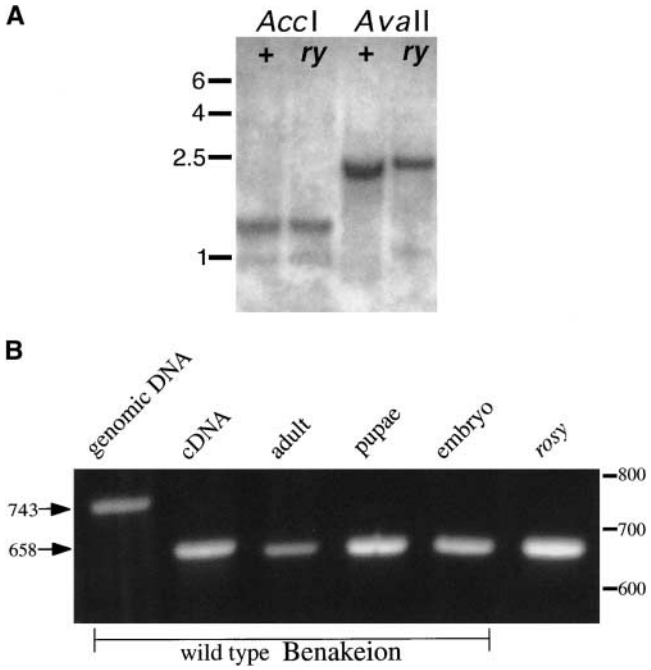

FIGURE 4.—XDH gene analysis in medfly. (A) Southern blot displaying single copy nature of medfly XDH gene. + and *ry* indicate Benakeion and *rosy* genomic DNAs, respectively, while numbers indicate sizes in kilobase pairs. Bands of 0.9 kb and 1.3 kb were expected for *Acc*I digests while a band of 2.5 kb was expected for *Ava*II digests. (B) RT-PCR analysis of RNA isolated from three developmental stages of wild type and one stage of the *rosy* mutant all show XDH transcript expression. Oligonucleotide primers spanning intron F result in products of 743 and 658 bp in genomic and cDNA controls, respectively.

observed in a series of side-by-side reactions covering the entire coding region of the gene, these comparisons show the medfly *rosy* XDH gene to be indistinguishable from the wild-type gene (Figure 4A; PCR data not shown). Furthermore, RT-PCR comparisons demonstrated the presence of indistinguishable XDH transcripts in RNA isolated from various life cycle stages of wild-type and *rosy* medfly pupae, the only life cycle stage of the *rosy* mutant at our disposal (Figure 4B). Finally, because some of the primers used in these RT-PCR studies were located near the 3′ end of the gene where mRNA instability is likely to be the greatest, the *rosy* mutant most likely produces a full-length transcript.

Given that the XDH gene of the medfly *rosy* mutant was indistinguishable from the wild type at the DNA and RNA levels within the limits of our studies, and in the absence of appropriate antisera that might be used for Western or immunohistochemical analyses, we tested wild-type and *rosy* medflies for XDH enzyme activity using a cellulose acetate gel technique (MEERA KHAN 1971; MALACRIDA *et al.* 1992). In these studies, crude extracts were prepared from wild-type and *rosy* mutant pupae, subjected to cellulose acetate gel electrophoresis, and subsequently stained for XDH activity using either xanthine or hypoxanthine as a reducing substrate. Figure 5A shows a typical gel from this study, in which extracts from medfly *rosy* and wild-type pupae appear to have similar levels of XDH activity. Importantly, when similar amounts of protein were loaded in each lane, control extracts from *D. melanogaster* OR wild type have high XDH activity, while extracts from ry^{506}, an XDH null mutant (GELBART *et al.* 1974; CLARK *et al.* 1986), lack any visible activity (Figure 5A). Replicate experiments suggest that the difference in migration observed in Figure 5A between wild-type and *rosy* medfly extracts is the result of slight gel loading variation in the absence of predefined sample wells. While no attempts were made to quantify XDH activity assayed here, no differences in enzyme activity between wild-type and *rosy* medfly extracts were detected on cellulose acetate gels using three different assay systems: hypoxanthine: NAD^+: PMS:NBT; xanthine:NAD^+; or xanthine:PMS/cyt*C* under similar conditions.

DISCUSSION

XDH cloning: The XDH gene of *C. capitata* shares considerable nucleotide and amino acid sequence identity with XDH genes of other insect species (Figure 1B). Comparison of the known, complete XDH nucleotide sequences produces the phylogenetic tree in Figure 2. Not surprisingly, the *Cc*XDH protein sequence is most closely related to other sequences from dipteran species and less similar to the sequences of the two XDH genes of the lepidopteran, *B. mori*. Given that the dipteran XDHs are all single copy genes, their relationships might reasonably be considered orthologous, or all derived from a common ancestor gene by speciation in the insect lineages. Further evidence for XDH orthology was presented by KOMOTO *et al.* (1999), who noted that upstream of *Bm*XDH2 is a region homologous to l(3)12s, a gene that is similarly located upstream of the XDH genes of *D. melanogaster*, *D. pseudoobscura*, and *D. subobscura* (RILEY 1989; DUTTON and CHOVNICK 1991; COMERON and AGUADE 1996). The apparent XDH duplication event within *B. mori* thus occurred after the divergence of the dipteran and lepidopteran orders (KOMOTO *et al.* 1999).

As indicated for the *white* genes of several insect species (GOMULSKI *et al.* 2001), the evolutionary relationships of the reported species are also reflected by the conservation of intron positions within the XDH gene (KOMOTO *et al.* 1999). For example, the intron positions A, B, F, and G of the *Cc*XDH gene are conserved when compared with the intron positions for several other insect species (KOMOTO *et al.* 1999). It is interesting to note that the medfly gene lacks introns B, C, and E, introns that may have been lost during the evolution of the XDH gene. This is reminiscent of the loss of introns that has been suggested for introns B, C, E, and F of the XDH gene of *D. melanogaster* (KOMOTO *et al.* 1999). Alternatively, those introns may have been gained later

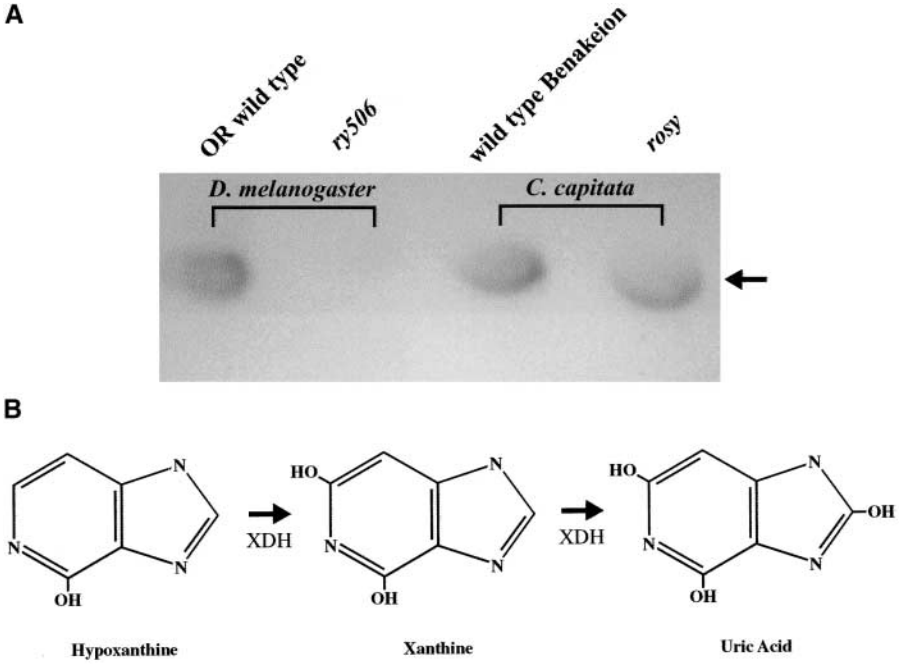

FIGURE 5.—XDH enzyme activity. (A) Extracts from wild-type and *rosy* mutant medflies display XDH enzyme activity when hypoxanthine or xanthine are used as a substrates. Similarly prepared Drosophila extracts serve as controls. (B) The chemical reactions catalyzed by XDH include the conversion of hypoxanthine to xanthine and xanthine to uric acid.

in XDH evolution and therefore persist in the genes of *B. mori* and mammals (KOMOTO *et al.* 1999). Recent evidence supports the introns-gained hypothesis through the comparison of several partial XDH sequences (TARRIO *et al.* 1998).

We find 35 polymorphic nucleotides between our sequence and the partial sequence of 2085 bp reported by TARRIO *et al.* (1998), which would lead to eight amino acid substitutions. These differences probably reflect XDH allelic differences within the same laboratory strain, since both studies used Benakeion DNA, a fact that is not surprising given that several XDH allozymes have already been described in the medfly (MALACRIDA *et al.* 1992). Furthermore, multiple XDH alleles have been identified in a laboratory population of *C. vicina* (ROCHER-CHAMBONNET *et al.* 1987; HOUDE *et al.* 1989) and a high level of heterozygosity has been observed at the XDH locus in *D. pseudoobscura* (SINGH *et al.* 1976).

The XDH gene of *C. capitata* was shown to encode an active XDH enzyme by its ability to rescue the eye color phenotype of the *D. melanogaster* mutant, ry^{506}, which lacks any detectable XDH activity (for review see CHOVNICK *et al.* 1990). Homozygous ry^{506} Drosophila that carried a heat-shock-driven *Cc*XDH cDNA had normal, bright red eyes, while those lacking the *Cc*XDH had the deep red eyes characteristic of the *rosy* mutant (Figure 3, C and D) even under heat-shock conditions. This result demonstrates that the *Cc*XDH cDNA encodes a functional enzyme that is also biologically active in heterologous species separated by over 100 million years of evolution (BEVERLY and WILSON 1984). Heterologous rescue of the Drosophila *rosy* mutant has been previously shown using a chimerical XDH containing the C-terminal portion of the *C. vicina* XDH gene and an N-terminal portion of the *D. melanogaster* XDH including the 5′ UTR (TIVERON *et al.* 1991). These results indicate that *Cc*XDH has the necessary functionality for use as a marker for germline transformation within the medfly.

***rosy*-like mutant:** The most likely *C. capitata* XDH mutant isolated to date, *rosy*, displays a deep red eye color and a sensitivity to purine supplemented growth media, both characteristic of Drosophila *rosy* (*i.e.*, XDH) mutants (SAUL 1982). Although red drosopterin pigments have not yet been identified in wild-type medflies (ZIEGLER and FERON 1965), it remains unclear as to whether or not they are present in medfly eyes. Indeed, the existence of the medfly *rosy* mutant provides strong, albeit circumstantial, evidence for their presence. Further study of eye pigments in the medfly may also lend insight into the nature of the medfly *rosy* mutant. For example, because the Drosophila *rosy* mutant specifically lacks isoxanthopterin and accumulates 2-amino-4-hydroxyterin, the pteridine product and substrate of the XDH enzyme, respectively (REAUME *et al.* 1991), a similar pattern might be expected to occur in *rosy* medflies. It is also formally possible that the medfly lacks drosopterin pigments and the *rosy* phenotype is the result of a defect in a gene unrelated to XDH.

Interestingly, the medfly XDH gene has been genetically mapped by allozyme analysis to the same position as the medfly *rosy* mutant on linkage group D, the genetic element that has subsequently been renamed chromosome 2 (SAUL and ROSSLER 1984; SAUL 1986; MALACRIDA *et al.* 1990, 1992). In fact, cytological mapping by *in situ* hybridization to polytene chromosomes of the *Cc*XDH cDNA reported here has further refined its location to section 4C of the long arm of chromosome 2 (A. R. MALACRIDA and C. TORTI, personal communica-

tion). As more genetic markers become available in medfly, finer mapping studies of *rosy* should be possible, either confirming or rejecting its potential correlation to XDH. Given the similar phenotypes of medfly *rosy* and Drosophila *rosy*, as well as the correlation of *Cc*XDH with the *rosy* mutant on the medfly genetic map, we examined the medfly *rosy* mutant for potential abnormalities at the XDH locus.

While we were unable to detect gross DNA differences such as large deletions, insertions, or chromosomal rearrangements between the wild type and *rosy* mutant medflies in our genomic analyses (Figure 4A), we could not eliminate the possibility that point mutations, small deletions, or small insertions may lie within the XDH coding region of the *rosy* mutant. This is especially relevant since the *rosy* mutant was generated by formaldehyde treatment of medfly eggs, a process thought to cause small DNA mutations (Saul 1982). To further compare the XDH gene products, we carried out a reverse transcription/PCR analysis of wild-type and *rosy* RNAs. In these studies, indistinguishably sized XDH transcripts could be amplified from several developmental stages of wild-type medflies as well as *rosy* pupae (Figure 4B). These data show a similar developmental pattern of XDH transcript expression in wild-type medflies as in wild-type Drosophila (Covington *et al.* 1984). Furthermore, the XDH expression that was detected in *rosy* pupae occurs at a time that is relevant for eye color development in Drosophila and is therefore likely to be equally important for the medfly (Barrett and Davidson 1975). Lastly, the primers used for RT-PCR expression studies were located near the 3′ end of the gene and as such indicate that the transcript is likely to be full length and stable. Taken together, these data suggest that the *rosy* mutant might not be the result of the alteration or loss of XDH transcripts. Again, as with the DNA analysis, these data neither rule out the possibility that small, mutagenic changes exist with the *Cc*XDH mRNA and are responsible for the mutant phenotype, nor eliminate the possibility that the transcript is otherwise improperly translated but the protein remains functional *in vitro*.

In the absence of a functional antiserum against medfly XDH that might be used to directly assay protein levels, we attempted to address the possibility that the medfly *rosy* mutant lacks or has aberrant levels of XDH enzymatic activity. In these studies, we examined the ability of crude extracts from wild type as well as *rosy* medflies to reduce xanthine or hypoxanthine *in vitro* and to produce a colorimetric reaction product on cellulose acetate gels. Importantly, we were able to take advantage of the availability of well-characterized Drosophila wild-type and ry^{506} strains for the preparation of extracts to serve as positive and negative controls for XDH activity, respectively. These data (Figure 5A) clearly demonstrate that the medfly *rosy* mutant retains considerable, if not wild-type, levels of XDH enzymatic activity *in vitro*. Whether or not the mutant has XDH activity *in vivo* has not been directly examined. In Drosophila, XDH is synthesized in the fat bodies surrounding the eye and must be transported into the eye for normal pigmentation to develop (Barrett and Davidson 1975; Reaume *et al.* 1989, 1991). It is possible that the medfly *rosy* mutant may produce an enzyme *in vivo* that is either inactive or not expressed in the proper tissues or at the proper time during development. It is also formally possible that our assay system using crude extracts cannot detect specific mutations, particularly point mutations, within the XDH enzyme that would alter, but not eliminate, XDH activity or localization. Several detailed Drosophila studies have been carried out that define functional domains of the XDH peptide where known single amino acid changes in *rosy* mutants affect XDH activity (Hughes *et al.* 1992; Hughes 1992; Doyle *et al.* 1996). In those experiments, extracts from some *rosy* mutants, such as G800E and G1011E, show relatively high levels of *in vitro* activity in some assays, especially following mild oxidation (Hughes *et al.* 1992; Hughes 1992; Doyle *et al.* 1996). Therefore we cannot rule out the possibility that the *rosy* mutant of medfly may be the result of a point mutation in XDH that is not discernible in our enzyme activity assays or that mild oxidation of XDH occurs during the extraction process.

On the basis of our examinations of the *Cc*XDH locus at the levels of DNA, RNA, and enzyme activity, we cannot support, nor conclusively rule out, the possibility that the medfly *rosy* phenotype is caused by a mutation at the *Cc*XDH locus. Definitive evidence would best be obtained by performing medfly transformation rescue experiments using *rosy* medflies and an appropriate expression construct with the functional, wild-type cDNA reported here along with either the *Minos* or *piggyBac* vectors that have been previously used in the generation of transgenic medflies (Loukeris *et al.* 1995; Handler *et al.* 1998). However, because of the absolute quarantine against live medflies in the continental United States, we are prevented from carrying out this final test. Such an experiment would help define the potential of the *rosy* mutant to be used in combination with the wild-type cDNA as a marker for germline transformation experiments in medfly. Aside from its proposed function as a visible marker, the wild-type *Cc*XDH may be useful as a selectable marker if it can restore purine resistance alone in the medfly *rosy* mutant and may thereby contribute to population control strategies.

We thank Dr. F. C. Kafatos and the entire faculty and staff at The European Molecular Biology Laboratory (Heidelberg, Germany) where this work was initiated. We are very grateful to Dr. Anna Malacrida and Cristina Torti for providing unpublished XDH *in situ* mapping information as well as helpful discussions of XDH function (Universita di Pavia, Italy). We thank Dr. C. S. Thummel (University of Utah, Salt Lake City) for Drosophila transformation vector pP{CaSpeR-hs/act}, Dr. N. H. Brown (Wellcome/CRC Institute, Cambridge, UK) in whose laboratory the Drosophila transformations were carried out, and Dr. D. E. McCauley (Department of Biology, Vander-

bilt University) for instruction in the use of cellulose acetate gels. Special thanks to Dr. D. J. Funk (Department of Biology, Vanderbilt University) and Dr. L. M. Gomulski (Universita di Pavia) for critical review of the manuscript with particular regard to XDH phylogeny. We also thank Richie Lin and Clay Ross for technical support as well as A. N. Fox, C. E. Merrill, and other members of the Zwiebel laboratory for comments on the manuscript. This research was supported by the U.S. Department of Agriculture-CSRS (92-37302-8237) to L.J.Z. and the National Science Foundation (US)-NATO fellowship (9255297) to L.J.Z.

LITERATURE CITED

Amaya, Y., K. Yamazaki, M. Sato, K. Noda and T. Nishino, 1990 Proteolytic conversion of xanthine dehydrogenase from the NAD-dependent type to the O2-dependent type. Amino acid sequence of rat liver xanthine dehydrogenase and identification of the cleavage sites of the enzyme protein during irreversible conversion by trypsin. J. Biol. Chem. **265:** 14170–14175.

Ashburner, M., 1989 *Drosophila: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Barinaga, M., 1991 A new buzz in the medfly debate. [news] Science **253:** 1351.

Barrett, D., and N. A. Davidson, 1975 Xanthine dehydrogenase accumulation in developing Drosophila eyes. J. Insect Physiol. **21:** 1447–1452.

Berglund, L., J. T. Rasmussen, M. D. Andersen, M. S. Rasmussen and T. E. Petersen, 1996 Purification of the bovine xanthine oxidoreductase from milk fat globule membranes and cloning of complementary deoxyribonucleic acid. J. Dairy Sci. **79:** 198–204.

Beverly, S. M., and A. C. Wilson, 1984 Molecular evolution in Drosophila and the higher Diptera. II. A time scale for fly evolution. J. Mol. Evol. **21:** 1–13.

Breathnach, R., and P. Chambon, 1981 Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50:** 349–383.

Carey, J. R., 1991 Establishment of the Mediterranean fruit fly in California. [see comments] Science **253:** 1369–1373.

Chovnick, A., S. H. Clark, F. L. Dutton, Jr. and A. G. Reaume, 1990 The rosy locus and xanthine dehydrogenase in *Drosophila melanogaster*. Prog. Clin. Biol. Res. **344:** 1–18.

Clark, S. H., M. McCarron, C. Love and A. Chovnick, 1986 On the identification of the *rosy* locus DNA in *Drosophila melanogaster*: intragenic recombination mapping of mutations associated with insertions and deletions. Genetics **112:** 755–767.

Comeron, J. M., and M. Aguade, 1996 Synonymous substitutions in the Xdh gene of Drosophila: heterogeneous distribution along the coding region. Genetics **144:** 1053–1062.

Covington, M., D. Fleenor and R. B. Devlin, 1984 Analysis of xanthine dehydrogenase mRNA levels in mutants affecting the expression of the *rosy* locus. Nucleic Acids Res. **12:** 4559–4573.

Davies, N., F. X. Villablanca and G. K. Roderick, 1999 Bioinvasions of the medfly *Ceratitis capitata*: source estimation using DNA sequences at multiple intron loci. Genetics **153:** 351–360.

Doyle, W. A., J. F. Burke, A. Chovnick, F. L. Dutton, J. R. Whittle *et al.*, 1996 Properties of xanthine dehydrogenase variants from rosy mutant strains of *Drosophila melanogaster* and their relevance to the enzyme's structure and mechanism. Eur. J. Biochem. **239:** 782–795.

Dutton, Jr., F. L., and A. Chovnick, 1991 The *l(3)S12* locus of *Drosophila melanogaster*: heterochromatic position effects and stage-specific misexpression of the gene in *P*-element transposons. Genetics **128:** 103–118.

Edwards, T. C., E. P. Candido and A. Chovnick, 1977 Xanthine dehydrogenase from *Drosophila melanogaster*: a comparison of the kinetic parameters of the pure enzyme from two wild-type isoalleles differing at a putative regulatory site. Mol. Gen. Genet. **154:** 1–6.

Felsenstein, J., 1985 Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:** 783–791.

Forrest, H. S., E. Glassman and H. K. Mitchell, 1956 Conversion of 2-amino-4-hydroxypteridine to isoxanthopterin in *Drosophila melanogaster*. Science **124:** 725–726.

Gelbart, W. M., M. McCarron, J. Pandey and A. Chovnick, 1974 Genetic limits of the xanthine dehydrogenase structural element within the *rosy* locus in *Drosophila melanogaster*. Genetics **78:** 869–886.

Glassman, E., and H. K. Mitchell, 1959 Maternal effect of *ma-l+* on xanthine dehydrogenase of *Drosophila melanogaster*. Genetics **44:** 153–162.

Glatigny, A., and C. Scazzocchio, 1995 Cloning and molecular characterization of *hxA*, the gene coding for the xanthine dehydrogenase (purine hydroxylase I) of *Aspergillus nidulans*. J. Biol. Chem. **270:** 3534–3550.

Gomulski, L. M., R. J. Pitts, S. Costa, G. Saccone, C. Torti *et al.*, 2001 Genomic organization and characterization of the *white* locus of the Mediterranean fruitfly, *Ceratitis capitata*. Genetics **157:** 1245–1255.

Handler, A. M., S. D. McCombs, M. J. Fraser and S. H. Saul, 1998 The lepidopteran transposon vector, *piggyBac*, mediates germline transformation in the Mediterranean fruit fly. Proc. Natl. Acad. Sci. USA **95:** 7520–7525.

Haymer, D. S., M. He and D. O. McInnis, 1997 Genetic marker analysis of spatial and temporal relationships among existing populations of the Mediterranean fruit fly. Heredity **79:** 302–309.

Higgins, C. F., 1992 ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:** 67–113.

Hille, R., and T. Nishino, 1995 Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. FASEB J. **9:** 995–1003.

Houde, M., M. C. Tiveron and F. Bregegere, 1989 Divergence of the nucleotide sequences encoding xanthine dehydrogenase in *Calliphora vicina* and *Drosophila melanogaster*. Gene **85:** 391–402.

Hughes, R. K., 1992 Xanthine dehydrogenase from *Drosophila melanogaster*: purification and properties of the wild-type enzyme and of a variant lacking iron-sulfur centers. Biochemistry **31:** 3073–3083. [erratum: Biochemistry **33**(27): 8384].

Hughes, R. K., W. A. Doyle, A. Chovnick, J. R. Whittle, J. F. Burke *et al.*, 1992 Use of rosy mutant strains of *Drosophila melanogaster* to probe the structure and function of xanthine dehydrogenase. [see comments] Biochem. J. **285:** 507–513.

Ichida, K., Y. Amaya, K. Noda, S. Minoshima, T. Hosoya *et al.*, 1993 Cloning of the cDNA encoding human xanthine dehydrogenase (oxidase): structural analysis of the protein and chromosomal location of the gene. Gene **133:** 279–284.

Kahn, E., R. J. Jackson, D. O. Lyman and J. W. Stratton, 1990 A crisis of community anxiety and mistrust: the Medfly eradication project in Santa Clara County, California, 1981–82. Am. J. Public Health **80:** 1301–1304.

Kamdar, K. P., J. P. Primus, M. E. Shelton, L. L. Archangeli, A. E. Wittle *et al.*, 1997 Structure of the molybdenum cofactor genes in Drosophila. Biochem. Soc. Trans. **25:** 778–783.

Keith, T. P., M. A. Riley, M. Kreitman, R. C. Lewontin, D. Curtis *et al.*, 1987 Sequence of the structural gene for xanthine dehydrogenase (*rosy* locus) in *Drosophila melanogaster*. Genetics **116:** 67–73.

Komoto, N., K. Yukuhiro and T. Tamura, 1999 Structure and expression of tandemly duplicated xanthine dehydrogenase genes of the silkworm (*Bombyx mori*). Insect Mol. Biol. **8:** 73–83.

Loukeris, T. G., I. Livadaras, B. Arca, S. Zabalou and C. Savakis, 1995 Gene transfer into the medfly, *Ceratitis capitata*, with a *Drosophila hydei* transposable element. [see comments] Science **270:** 2002–2005.

Malacrida, A. R., G. Gasperi, L. Baruffi and R. Milani, 1990 The contribution of formal genetic studies to the characterization of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.), pp. 85–89 in *Research Co-ordination Meeting of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture (1988)*. International Atomic Energy Agency, Vienna.

Malacrida, A., G. Gasperi, A. Zacharopoulou, C. Torti, E. R. Francos *et al.*, 1992 Evidence for a genetic duplication involving alcohol dehydrogenase genes in *Ceratitis capitata*. Biochem. Genet. **30:** 35–48.

Malacrida, A. R., F. Marinoni, C. Torti, L. M. Gomulski, F. Sebastiani *et al.*, 1998 Genetic aspects of the worldwide colonization process of *Ceratitis capitata*. J. Hered. **89:** 501–507.

McAlpine, J. F. (Editor), 1989 *Manual of Nearctic Diptera*. Vols. 1–3. Canadian Government Publishing Centre, Quebec.

Meera Khan, P., 1971 Enzyme electrophoresis on cellulose acetate

gel: zymogram patterns in mgh-mouse and man—Chinese hamster somatic cell hybrids. Arch. Biochem. Biophys. **145:** 470–483.

Nakamura, Y., T. Gojobori and T. Ikemura, 2000 Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res. **28:** 292.

Nishino, T., and T. Nishino, 1989 The nicotinamide adenine dinucleotide-binding site of chicken liver xanthine dehydrogenase. Evidence for alteration of the redox potential of the flavin by NAD binding or modification of the NAD-binding site and isolation of a modified peptide. J. Biol. Chem. **264:** 5468–5473.

Reaume, A. G., S. H. Clark and A. Chovnick, 1989 Xanthine dehydrogenase is transported to the Drosophila eye. Genetics **123:** 503–509.

Reaume, A. G., D. A. Knecht and A. Chovnick, 1991 The *rosy* locus in *Drosophila melanogaster*: xanthine dehydrogenase and eye pigments. Genetics **129:** 1099–1109.

Riley, M. A., 1989 Nucleotide sequence of the Xdh region in *Drosophila pseudoobscura* and an analysis of the evolution of synonymous codons. Mol. Biol. Evol. **6:** 33–52.

Rina, M., and C. Savakis, 1991 A cluster of vitellogenin genes in the Mediterranean fruit fly *Ceratitis capitata*: sequence and structural conservation in dipteran yolk proteins and their genes. Genetics **127:** 769–780.

Robinson, A. S. E. (Editor), 1989 *Genetic sexing mechanisms in the Mediterranean fruit fly Ceratitis capitata (Wiedemann)*. Plenum Press, New York.

Rocher-Chambonnet, C., P. Berreur, M. Houde, M. C. Tiveron, J. A. Lepesant *et al.*, 1987 Cloning and partial characterization of the xanthine dehydrogenase gene of *Calliphora vicina*, a distant relative of *Drosophila melanogaster*. Gene **59:** 201–212.

Romao, M. J., and R. Huber, 1997 Crystal structure and mechanism of action of the xanthine oxidase-related aldehyde oxidoreductase from *Desulfovibrio gigas*. Biochem. Soc. Trans. **25:** 755–757.

Romao, M. J., M. Archer, I. Moura, J. J. Moura, J. LeGall *et al.*, 1995 Crystal structure of the xanthine oxidase-related aldehyde oxido-reductase from *D. gigas*. Science **270:** 1170–1176.

Rossler, Y., and Y. Koltin, 1976 The genetics of the Mediterranean fruitfly, *Ceratitis capitata*: three morphological mutations. Ann. Entomol. Soc. Am. **69:** 604–608.

Rossler, Y., and H. Rosenthal, 1992 Genetics of the Mediterranean fruit fly (Diptera:Tephritidae): morphological mutants on chromosome five. Ann. Entomol. Soc. Am. **85:** 525–531.

Rubin, G. M., and A. C. Spradling, 1982 Genetic transformation of Drosophila with transposable element vectors. Science **218:** 348–353.

Sambrook, J., E. F. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sato, A., T. Nishino, K. Noda and Y. Amaya, 1995 The structure of chicken liver xanthine dehydrogenase. cDNA cloning and the domain structure. J. Biol. Chem. **270:** 2818–2826.

Saul, S. H., 1982 Rosy-like mutant of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera:Tephritidae), and its potential for use in a genetic sexing program. Ann. Entomol. Soc. Am. **75:** 480–483.

Saul, S. H., 1985 Two new eye color mutants in the Mediterranean fruit fly, *Ceratitis capitata*. Proc. Hawaiian Entomol. Soc. **25:** 125–129.

Saul, S. H., 1986 Genetics of the Mediterranean fruit fly (*Ceratitis capitata*) (Wiedemann). Agric. Zool. Rev. **1:** 73–108.

Saul, S. H., and Y. Rossler, 1984 Genetic markers of the autosomal linkage groups of the Mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). Ann. Entomol. Soc. Am. **77:** 323–327.

Singh, R. S., R. C. Lewontin and A. A. Felton, 1976 Genetic heterogeneity within electrophoretic "alleles" of xanthine dehydrogenase in Drosophila pseudoobscura. Genetics **84:** 609–629.

Soto-Adames, F. N., H. M. Robertson and S. H. Berlocher, 1994 Phylogenetic utility of partial DNA sequences of *G6pdh* at different taxonomic levels in Hexapoda with emphasis on Diptera. Ann. Entomol. Soc. Am. **87:** 723–736.

Swofford, D. L., 1991 PAUP: Phylogenetic Analysis Using Parsimony (and other methods), Version 4. Sinauer Associates, Sunderland, MA.

Tarrio, R., F. Rodriguez-Trelles and F. J. Ayala, 1998 New Drosophila introns originate by duplication. Proc. Natl. Acad. Sci. USA **95:** 1658–1662.

Terao, M., G. Cazzaniga, P. Ghezzi, M. Bianchi, F. Falciani *et al.*, 1992 Molecular cloning of a cDNA coding for mouse liver xanthine dehydrogenase. Regulation of its transcript by interferons in vivo. Biochem. J. **283:** 863–870.

Thompson, J. D., D. G. Higgins and T. J. Gibson, 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Tiveron, M. C., M. Houde, C. Vola, R. Hipeau-Jacquotte, P. Berreur *et al.*, 1991 Inter-species complementation of a rosy deficiency in *Drosophila melanogaster*. Biochim. Biophys. Acta **1088:** 390–394.

Wahl, R. C., C. K. Warner, V. Finnerty and K. V. Rajagopalan, 1982 *Drosophila melanogaster ma-l* mutants are defective in the sulfuration of desulfo Mo hydroxylases. J. Biol. Chem. **257:** 3958–3962.

Wootton, J. C., R. E. Nicolson, J. M. Cock, D. E. Walters, J. F. Burke *et al.*, 1991 Enzymes depending on the pterin molybdenum cofactor: sequence families, spectroscopic properties of molybdenum and possible cofactor-binding domains. Biochim. Biophys. Acta **1057:** 157–185.

Yasukochi, Y., T. Kanda and T. Tamura, 1998 Cloning of two Bombyx homologues of the Drosophila *rosy* gene and their relationship to larval translucent skin colour mutants. Genet. Res. **71:** 11–19.

Ziegler, I., and M. Feron, 1965 Quantitative Bestimmung der hydriertin Pterine und des Xanthommatins in den Augen von *Ceratitis capitata* Wied. Dipt. Trypetidae. Zeitschrift fur Naturforschung, Part C. Bioscience **20:** 318–322.

Zwiebel, L. J., G. Saccone, A. Zacharopoulou, N. J. Besansky, G. Favia *et al.*, 1995 The white gene of Ceratitis capitata: a phenotypic marker for germline transformation. [see comments] Science **270:** 2005–2008.

Communicating editor: T. C. Kaufman