Cloning of two *Bombyx* homologues of the *Drosophila rosy* gene and their relationship to larval translucent skin colour mutants

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Summary

To clone the *Bombyx* xanthine dehydrogenase (XDH) gene as a dominant marker for silkworm transgenesis, we performed nested reverse transcriptase–polymerase chain reaction (RT-PCR) using embryonic mRNA and primers designed from the conserved region of *Drosophila* and rat XDH genes. Sequencing of amplified 180 bp fragments showed that two different sequences were present in the fragments. Since both possessed striking similarity to XDH genes of other organisms, we considered these to be portions of silkworm XDH genes and designated them BmXDH1 and BmXDH2. Subsequently we cloned separately the entire region of the two cDNAs by PCR using phage DNA of an embryonic cDNA library and sequenced them. The two cDNAs were around 4 kb in size and possessed complete open reading frames. The deduced amino acid sequences of the two BmXDHs were very similar to each other and to those of other organisms. The expression pattern of wild-type larvae basically followed the tissue specificity of the enzyme and no significant difference was observed between the two XDH genes. The expression of both genes was detected in the XDH-deficient mutants, *oq* and *og*, but non-synonymous substitutions were specifically detected in the BmXDH1 of the *oq* mutant. In addition, a length polymorphism of the second intron of the BmXDH1 co-segregated with the *oq* translucent phenotype, suggesting that deficiency in BmXDH1 is the cause of the *oq* translucent phenotype.

1. Introduction

Construction of a system for transgenesis in the domesticated silkworm, *Bombyx mori*, is an important and urgent problem. This would be a powerful tool to study the mechanism of regulation of silkworm genes or to produce new races with specific characters, such as disease or high temperature resistance. To develop this system, cloning of a selectable marker gene to identify transgenic insects is crucially important and in this case visible marker genes would be highly preferable. For example, the *rosy* gene, which encodes the structural gene for xanthine dehydrogenase (XDH, EC 1.1.1.204) (Keith *et al.*, 1987; Lee *et al.*, 1987), is widely used as a selectable marker in *Drosophila* (Rubin & Spradling, 1983). However, no such marker gene has been cloned in the silkworm.

More than 320 mutant strains of silkworm are available, most of which carry morphologically visible mutations (Doira, 1978; Goldsmith, 1995). Among them three mutations were previously identified to be deficient in XDH activity, and this deficiency causes translucent skin in the mutant larvae (Tamura, 1977, 1983). Because uric acid is synthesized poorly or not at all, the larval skin, which normally accumulates uric acid granules and looks opaque and white, cannot accumulate the granules and becomes translucent (Tamura & Akai, 1990). Since the mutant larvae can easily be discriminated from wild-type larvae even at very early stages, we proposed that the combination of a normal XDH gene and such mutants would be useful to develop a marker-recipient system in the silkworm.

Thus, we decided to clone the XDH gene of *B. mori*. We report in this paper the isolation of two different XDH genes, designated BmXDH1 and BmXDH2. The sequences of the two full-length cDNAs indicate that they are similar but apparently different genes and similar to other known XDHs. Both partial sequencing of these genes of the XDH-deficient mutants and F_2 linkage analysis strongly suggested

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that mutations in BmXDH1 are the basis of the *oq* mutant phenotype.

2. Materials and methods

(i) *Silkworm stocks, mRNA purification and cDNA synthesis*

The *B. mori* strain, C108, was used in these experiments unless otherwise stated. This strain and the *og* mutant were obtained from the Laboratory of Genetic Resources, National Institute of Sericultural and Entomological Science, Tsukuba. The mutants, *og t* and *oq*, were generously provided by the Institute of Genetic Resources, Kyushu University. Purification of mRNA from embryos, fat bodies, midguts, malpighian tubes and silk glands was carried out with a QuickPrep micro mRNA purification kit (Pharmacia). The first-strand cDNA for polymerase chain reaction (PCR) was synthesized with a First-Strand cDNA Synthesis Kit (Pharmacia).

(ii) *PCR amplification to obtain fragments of the XDH gene*

The sequences of the oligonucleotides used as primers for PCR are shown in Table 1. First-strand cDNA synthesized from mRNA of 48 h embryos was used as a template for the first PCR reaction. The first PCR reaction was performed in a 25 μl reaction mixture containing 1 μl of synthesized embryonic cDNA, 1 μM primers, 1 U *Taq* polymerase (Promega), 0·2 mM of each of the dNTPs (Pharmacia), 50 mM KCl, 10 mM Tris-HCl (pH 9·0), 0·1 % Triton X-100 and 2·5 mM MgCl$_2$. The amplification was carried out with 10 min denaturation at 90 °C, followed by 10 cycles of denaturation (94 °C, 1 min), annealing (42 °C, 2 min) and extension (72 °C, 3 min), 10 cycles of [94 °C, 1 min; 52 °C, 2 min; and 72 °C, 3 min] and 10 cycles of [94 °C, 1 min; 55 °C, 2 min; and 72 °C, 3 min]. The second amplification was performed in the same reaction mixture as the first PCR except that 1 μl of the first PCR reaction was used instead of the first-strand cDNA reaction. This amplification was carried out with 2 min denaturation at 94 °C, followed by 30 cycles of [94 °C, 1 min; 42 °C, 2 min; and 72 °C, 3 min].

(iii) *Two-step PCR amplification to clone the 5′ and 3′ unknown region*

To clone the full-length XDH cDNA, an embryonic cDNA library was constructed from cDNA synthesized from mRNA of 48 h embryos using λgt11 (Stratagene). The library was divided into aliquots containing around 10^4 pfu, and transfected to *E. coli*, Y1088. After lysis, cell debris was removed by centrifugation. Then phage DNA was purified by phenol/chloroform extraction and ethanol precipitation and dissolved in 10-1 TE buffer. The PCR

Table 1. *Oligonucleotide primers used in this experiment*

Name	Sequence	Position of 5′ end on BmXDH1 and BmXDH2 cDNA sequences
xdhFA	TGYGGNACNAARYTNGGNTG	227(BmXDH1), 281(BmXDH2)
xdhFB	GCNGTNACNACNGTNGARGG	271(BmXDH1), 325(BmXDH2)
xdhRA	ATNGGNCKRTANCCNGTRCA	605(BmXDH1), 659(BmXDH2)
xdhRB	CANARRTTNCCYTGRAANGC	581(BmXDH1), 635(BmXDH2)
lamF	GGTGGCGACGACTCCTGGAGCCCG	lambda phage vector sequence
lamR	TTGACACCAGACCAACTGGTAATG	lambda phage vector sequence
xdh1RA	TACATGGACATTACGATTCC	512
xdh1RB	GGCGTACAGAAACCGCACTG	491
xdh2RA	GGCACGCATTAACCGCGATG	412
xdh2RB	CGAAACCATGACGGTACAAGCG	360
xdh2RC	CGTCCATTCCGGATCAGG	267
xdh1FA	GAAGGTGGATGTGGTGCTTG	271
xdh1FB	GGAATCGTAATGTCCATGTA	492
xdh2FA	GCACACCCGGAATAGTCA	538
xdh2FB	GAGGGTGCTTTACAAGGCA	609
xdh1QF	CCAGCCAACATCCCTCAGAGA	2479
xdh1QR	CCACGTTTTCTCCATCTATG	3176
xdh2QF	CGTGACGAGTCGATCTTGTATTG	2157
xdh2QR	CCGCCAAAGCCACCTCCA	2579
xdh1LF	GTGCGGAAGGTGGATGTGGT	266
xdh1LR	GTACGGTAAGAACGACGACT	783

'Y' denotes T or C, 'R' denotes A or G, 'K' denotes G or T, and 'N' denotes A, C, G or T.
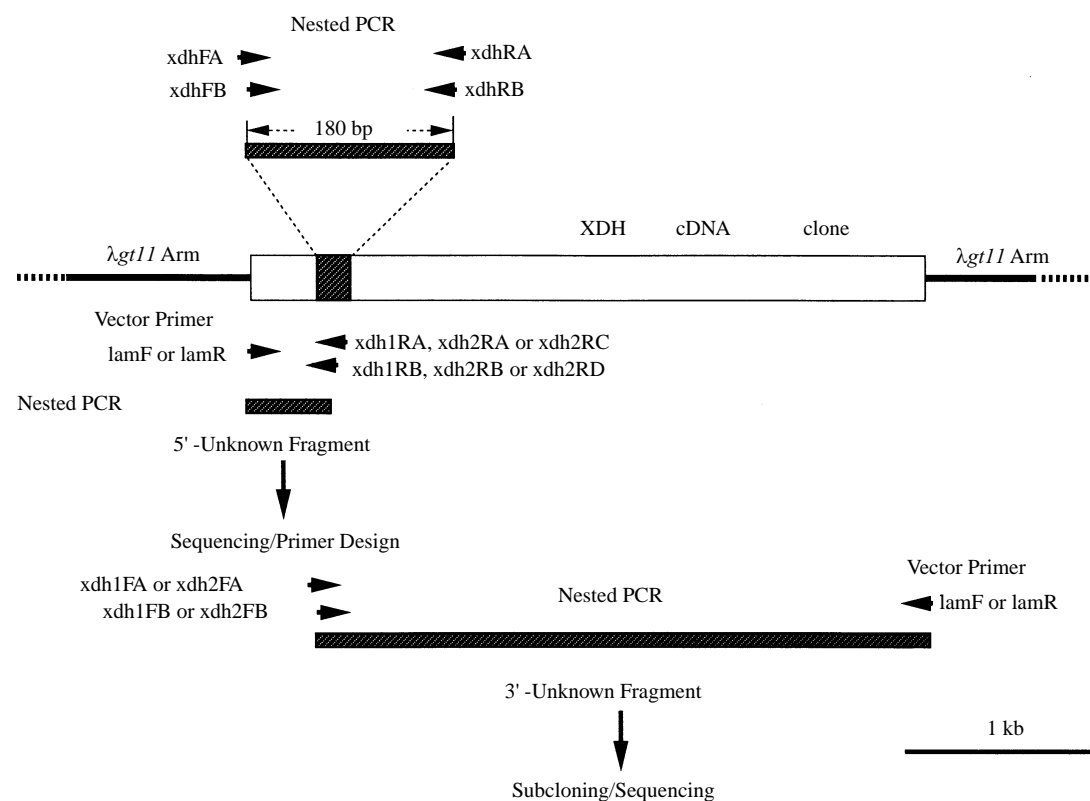All primers were synthesized with ABI Model 392 (Perkin-Elmer).

Fig. 1. Cloning strategy of *Bombyx* xanthine dehydrogenase (XDH) genes. Positions of the primers used and their names are shown as labelled arrowheads.

reaction was performed using this phage DNA solution as a template. The amplification of the 5′ region was performed under the same conditions as the second PCR described above. A long PCR was carried out for the cloning of the 3′ region. The first amplification was performed in a 25 μl reaction mixture consisting of 1 μl phage DNA, 1 μM primers, 1 U Takara Ex *Taq* polymerase (Takara shuzo, Japan) and 0·2 mM dNTPs in the accompanying buffer. The amplification was carried out with 2 min denaturation at 94 °C followed by 30 cycles of [94 °C, 30 s; 55 °C, 1 min; and 72 °C, 4 min]. The second PCR reaction was performed under the same conditions except for the substitution of 1 μl of the first amplified mixture for the phage DNA.

(iv) *Determination of nucleotide sequence*

Amplified PCR fragments were treated with T4 polynucleotide kinase (Nippon gene, Japan), blunted with Klenow fragment (Takara shuzo, Japan), and inserted into the *Sma*I site of pUC19. When fragments were too large for insertion, endonucleolytic digestion was performed with *Sau*3AI or *Rsa*I before ligation. DNA sequencing was carried out with these plasmid DNAs using an auto-sequencer (ABI 373A, Perkin-Elmer). The analysis of nucleotide sequence data, comparison of translated amino acid sequences and construction of phylogenetic tree were performed with

GENETYX-MAC software (v. 8·5; Software Development, Japan).

(v) *Detection of transcripts using RT-PCR*

Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to estimate the level of transcription. The first-strand cDNA synthesis was primed from random hexadeoxynucleotides and performed with a First-Strand cDNA Synthesis Kit (Pharmacia) in 10 μl reaction. Three hundred nanograms of poly-A RNA was used for each reaction and 0·33 μl of the completed reaction mixture was used for amplification. The amplification was carried out with 2 min denaturation at 94 °C, followed by 25, 27 or 29 cycles of [94 °C, 30 s, 55 °C, 1 min; and 72 °C, 2 min] using primers xdh1QF and xdh1QR for BmXDH1, and xdh2QF and xdh2QR for BmXDH2 (Table 1).

(vi) *Linkage screening of the BmXDH1 gene*

PCR primers, xdh1LF and xdh1LR, were designed to amplify the region containing the second intron of the BmXDH1 gene (Fig. 1, Table 1). The amplification was carried out with 2 min denaturation at 94 °C followed by 40 cycles of [94 °C, 1 min; 55 °C, 1 min; and 72 °C, 3 min]. Amplified products were isolated and checked by further PCR of an internal coding region.

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BMXDH1    1:M-GLLNAEEDP-NKICTELVFYVNGKKVIESSPDDPEWTLLWYLRKKLRLTGTKLGCAEGGCGACTVMVSKYNRQENKIIHLAVNACLAPVCAMHGLAVTTVEGIGSTKTKLHPVQERIAK 118
BMXDH2    1:M-DAIN-EVVP-RNAETALVFFVNGKKVLESNPDDPEWTLLFYLRKKLKLTGTKYGCGEGGCGACTVMVSKYLKNEDRINHIAVNACLISVCAMHGLAVTTVEGIGSTQDRLHPVQERIAK 117
CALVI     1:MTQEHNAAVLDLNPTFSTLIFFVNGKKVIDTNPDPECTLLTYLREKLRLCGTKLGCGEGGCGACTVMISRIDTLTNRIKHIAVNACLTPVCAMHGSAVTTVEGIGSTRTRLHPVQERLAK 120
DROME     1:MS----------N--SVLVFFVNGKKVTEVSPDPECTLLTFLREKLRLCGTKLGCAEGGCGACTVMVSRLDRRANKIRHLAVNACLTPVCSMHGCAVTTVEGIGSTKTRLHPVQERLPK 107
RAT       1:-------------TADELVFFVNGKKVEKNADPETTLLVYLRRKLGLCGTKLGCGEGGCGACTVMISKYDRLQNKIVHFSVNACLAPICSLHHVAVTTVEGIGNT-QKLHPVQERIAR 105
CHICKEN   1:---------MAPPETGDELVFFVNGKKVVEKDVDPETTLLTYLRRKLGLCGTKLGCGEGGCGACTVMISKYDPFQKKILHHTANACLFPICALHHVAVTTVEGIGNTKSRLHPAQERIAK 111
           *  * *** ***       *** *** **  *** *** *** ****  ***  * * **  ***********   ** **  ****  ** * ********* * *** ***

BMXDH1  119:AHGSQCGFCTPGIVMSMYTLLRSCKNIQYSDLEVAFQGNLCRCTGYRAIIEGYKTFIEDWE----TQR----IVKNG--P-QNGTC-AMGKDCCKNKSDSCEEADSESQYIFDKSSFLPY 226
BMXDH2  118:SHGSQCGFCTPGIVMSMYALLRNNIKIAYEDIEGALQGNLCRCTGYRPIIEGFKTFMEGWE-----N-----VYSTG----GN-MC-RMGENCCRIKKET-EH-D----ILFDPSAFRPY 215
CALVI   121:AHGSQCGFCTPGIVMSMYALLRNLSQPSMKDLEIAFQGNLCRCTGYRPILEGYKTF-TK-E-----F-----GCAMG----DK-CC-KVNGNKC-G-EGMENGGDMVDDKLFEKSEFVPF 220
DROME   108:AHGSQCGFCTPGIVMSMYALLRNAEQPSMRDLEVAFQGNLCRCTGYRPILEGYKTF-TK-E-----F-----ACGMG----EK-CC-KVSGKGC----GTDAE---TDDKLFERSEFQPL 202
RAT     106:SHGSQCGFCTPGIVMSMYTLLRNQPEPTVEEIENAFQGNLCRCTGYRPILQGFRTFAKD---------G-GCC--------GG--SGN-N-PNCCMNQTKDQTVSLSPSLFNPEDFKPL 202
CHICKEN 112:SHGSQCGFCTPGIVMSMYTLLRNKPKPKMEDIEDAFQGNLCRCTGYRPILEGYRTFAVDSNCCGKAANGTGCCHSKGENSMNGGCCGGKANGPGCCMNE-KENVTMMSSSLFDSSEFQPL 230
           ****************** *** *  **  * ***********  **   *              *    *            *       *  *           *  *     *  *

BMXDH1  227:DSSQEPIFPPELKLSSIYDSQYVIYRGKQTTWYRPTNIETVLSLKDKFPNAKVVVGNSEVGVEVKFKRCVYPIIIMPNCVPELNNITENEHGLTVGASVTLNDIEKTFREYIKKLPPYKT 346
BMXDH2  216:DPTQEPIFPPELKLENEYSTSYLVFRGENVIWLRPRNLKELVLVKSRIPDSKVVVGNTEIGVEMKFKKKFYPVLISPTIIGEVNYCSIENDGILVGAAVTLTELQIFLKSFIVEHPS-KS 334
CALVI   221:DPSQEPIFPPELQLNKDWDSQTLVYKGERATWYRPGNLEDLLKIKAQFPEAKLVVGNTEIGVEVKFKHFLYPVLVNPTKVKEMIDVQELEDSIYFGASVSLMDIDRILRSSIEKLPEHQT 340
DROME   203:DPSQEPIFPPELQLSDAFDSQSLIFSSDRVTWYRPTNLEELLQLKAKHPAAKLVVGNTEVGVEVKFKHFLYPHLINPTQVKELLEIKENQDGIYFGAAVSLMEIDALLRQRIELLPESET 322
RAT     203:DPTQEPIFPPELLRLKDTPQKKLRPEGERVTWIQASTMEELLDLKAQHPDAKLVVGNTEIGIEMKFKNMLFPLIVCPAWIPELNSVVHGPEGISFGASCPLSLVESVLAEEIAKLPEQKT 322
CHICKEN 231:DPTQEPIFPPELMTQRNKEQKQVCFKGERVMWIQPTTLQELVALKSQYPNAKLVVGNTEVGIEMRLKNMLYPVILAPAWIPEMNAVQQTETGITTGAACTLSSVEEVLRKAVAELPSYKT 350
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BMXDH1  347:RVLTTIVEMLNWFAGKQIRNVAAIGGNVMTGSPISDLNPILMSLKVNLNLLSQENG---HRTVLMDETFFTGYRKNVVKSNEILLSIEIPFSTKFQYLKAIKQAKRREDDISIVTSAVNV 463
BMXDH2  335:KIFFKAVNAMLHWFAGKQVRNVASLTGNIVTASPISDLNPILMPCSAVLNVYSTTNG---SRQITIDENFFKGYRKTILEDDEVVISIKLPFSTNDQYFKSYKQARRRDDDISIVTAAFNV 451
CALVI   341:RFFQCAVNMLHYFAGKQIRNVASLGGNIMTGSPISDMNPVLMAGAVKFKVAKYVEVCMASGFFTGYRKNVIEPTEILVGLYPPKTLEHQYVVAFKQAKRRDDDIAIVNAAINV 460
DROME   323:RLFQCTVDMLHYFAGKQIRNVACLGGNIMTGSPISDMNPVLSAAGAQLEVASFVDGKLQKRSVHMGTGFFTGYRRNVIEAHEVLLGIHFRKTTPDQYIVAFKQARRRDDDIAIVNAAINV 442
RAT     323:EVFRGVMEQLRWFAGKQVKSVASIGGNIITASPISDLNP-V---FMASGAKLTLVSRGTRRTVRMDHTFFPGYRKTLLRPEEILLSIEIPYSKEGEFFSAFKQASRREDDIAKVTSGMRV 438
CHICKEN 351:EIFQAALEQLRWFAGPQIRNVAALGGNIMTASPISDLNP-V---LMASGSKLTLISMEGKRTVMMDEKFFTGYRKTIVKPEEVLLSVEIPYSKEGEYFSAFKQAYRREDDIAIVTCGMRV 466
             * ***  ** **   *    ***** **  *   **        *          *        **  ***       *         **** *** **** *  *

BMXDH1  464:EFEENTNVIKYINLAFGGMAPVTKIATNTGNVLKGLKWNENMLEKAYSLLIDELPLDPSAPGGNIQFRRALTMSLFLKSY---L-AIGKAMSSDYVYGDLIESYYGSGADSFHGNVPKSS 579
BMXDH2  452:QFEGN-KVIK-SKLCYGGMGPTTLLASKSSKMLLGKHWNHETLSTVFHSLCEEFNLEFSVPGGMAEYRKSLCLSLFFKFY---L-NVKDKLDISNGESSTRPPKLSCGDET-RGE-PSSS 563
CALVI   461:FIDPRSITVDKVYMAFGGMAPTTVLATRTADIMVKQQWNKVLMERVVENLCAELPLAPSAPGGMIAYRRSLVVSLFFKAY---LTITQQLIKSGILPQDSLPQEELSGSVFHTPALKSA 577
DROME   443:RFEEKSNIVAEISMAFGGMAPTTVLAPRTSQLMVGQEWSHQLVERVAESLCTELPLAASAPGGMIAYRRALVVSLFFKAY---LAISLKLSKSGITSSDALPPEERSGAETFHTPVLKSA 559
RAT     439:LFKPGTIEVQELSLCFGGMADRTISALKTTPKQLSKSWNEE-LQ-----------LAPDAPGGMVEFRRTLTLSFFFKFYLTVLQKL--GRADLEDMCGKLDPTFASATLLFQKDPPANV 544
CHICKEN 467:LFQBGTSRVQEVKLSYGGMAPTTILALKTCRELAGRDWNEKLLQDACRLLAGEMDLSPSAPGGMVEFRRTLTLSFFFKFYLTVLQKLSKDQNGPNNLCEPVPPNYISATELFHKDPIAST 586
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BMXDH1  580:QYFELVGEKQLKSDAVGRPIQHMSAYKQATGEAIYCDDMPIAEGELYLAFVLSSKAHAKLISVDAKKALAEPGVIAFYSAKDLTKEQNSIGPIFHDEELFARDKVLSQGQTIGVIVAVDQ 699
BMXDH2  564:QYFEIRNSGEV--DALGKPLPHASAMKHATGEAIYCDDLPRIDGELFLTLVLSSESHAKIKSIDTTAALSIPGVVAFFCAKDLEVDRNIWGSIIKDEEIFCSTYVTSRSCIVGAIVATSE 681
CALVI   578:QLFEKVSNKQSECDPIGRPKIHASALKQATGEAIYCDDMPRMENELYLALVLSTRAKIKSIDASEALAMDHVGPVHAFFSSKDITQHENEVGPVFHDEEVFASDMVYCQGQVIGAIAANDH 697
DROME   560:QLFERVCSDQPICDPIGRPKVHAAALKQATGEAIYTDDIPRMDGEVYLAFVLSTKPRAKITKLDASEAIALDGVHQFFCYKDLTEHENEVGPVFHDEHVFAAGEVHCYGQIVGAIAADNK 679
RAT     545:QLFQEVPKDQSEEDMVGRPLPHLAANMQASGEAVYCDDIPRYENELSLRLVTSTRAHAKITSIDTSEAKKVPGFVCFLTAED-VPNSNATG-LFNDETVFAKDEVTCVGHIIGAVVADTP 662
CHICKEN 587:QLFQEVPRGQLVEDTVGRPLVHLSAAKQACGEAVYCDDIPHYENELYLTLVTSTQAHAKILSIDASEAQSVPGFVCFVSAKD-VPGSNITG-IANDETVFAEDVVTCVGHIIGAVIADTQ 704
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BMXDH1  700:ATAQAAARMVKVEYEEIQPIIVTIEDAIKYNSFYPQFPKTIKRGNVKAVFDDKNNIIIEGRCRMGGQEHFYLETHAAFAIPKKEDDELQIFCSSQHPSEIAKLVSHILHVPMNRIVARVK 819
BMXDH2  682:IVAKKARDLVSITYERLQPVIVTLEDAIEHNSYFENYPQTLSQGNVDEVF-SKTKFTVEGKQRSGAQEHFYLETISAYAI-RKE-DELEIICSSQSPSEIASFVSHTLGIPQHKVIAKVK 798
CALVI   698:NFSSKTARKVTIEYEDIKPVIITIEQAIEHKSYFPDYPRFTEIGDVEKA-FSEADHVYEGSCRMGGQEHFYETHASLAVP-RDSDEIEIFCSTQHPSEVQKLVAHVLSTSAHRVVCRAK 815
DROME   680:ALAQRAARLVKVEYEELSPVIVTIEQAIELKSYFPDYPRFVTKGNVEEA-LSQADHTFEGTCRMGGQEHFYLETHAALAVP-RDSDELELFCSTQHPSEVQKLVAHVTALPAHRVVCRAK 797
RAT     663:EHAQRAARGVKITYE-DLPAIITIQDAINNNS-FYGSEIKIEKGDLKKGF-SEADNVVSGELYIGGGQEHFYLETNCTIAVPKGEAGEMELFVSTQNTMKTQSFVAKMLGVPDNRIVVRVK 779
CHICKEN 705:EHSRRAAKAVKIKYE-ELKPIVTIQEAIEQQS-FIKPIKRIKKGDVNKGF-EESDHILEGEMHIGGQEHFYLETHCTLAVPKGEDGEMELFVSTQNLMKTQEFTASALGVPSNRIVVRVK 821
                * **      **  * ***    *          *           *  *        * *  ********  ** *   *   *  *   *      * *** *** ***

BMXDH1  820:RMGGGFGGKESRGMLVALPVALAAHKLNRPVWCMLDRDEDMQMTGTRHPFLIKYKAAATKEGKIVGAVVNIYNNGGYSTDLSGPVVERAMFHFENAYYIPNCEVTGYVCRTNLPSNTAFR 939
BMXDH2  799:RIGGGFGGKETRSSSSLALPVAIAAYILKKPVRSVLDRDEDIQMSGYRHPFLTKYKVAFDENGKIAGAVFDVFANGGFSMDLSCALIERSTFHVDNCYSIPNIKINAYVCKTNLPSNTAFR 918
CALVI   816:RLGGGFGGKESRAIAVALPVALACHRLRRPIRCMLDRDEDMMITGTRHPFLKYKIAFTSEGRLTGCYIECYNNAGWSMDLSFSVLERAMFHFENCYKIPNIKVGGWVCKTNLPSNTAFR 935
DROME   798:RLGGGFGGKETRGISVALPVALAAYRMGRPVRCMLDRDEDMLITGTRHPFLFKYKVGFTKEGLITACDIECYNNAGWSMDLSFSVLERAMFHFENCYRIPNVRVGGWVCKTNLPSNTAFR 917
RAT     780:RMGGGFGGKETRSTVVSTALALAAHKTGRPVRCMLDRDEDMLITGGRHPFLAKYKVGFMKTGTVVALEVAHFSNGGNTEDLSRSIMERALFHMDNAYKIPNIRGTGRICKTNLPSNTAFR 899
CHICKEN 822:RMGGGFGGKETRNTILTTVVAVAAFKTGRPVRCMLDRDEDMLISGGRHPFLGRYKVGFMKNGKIKSLEVSYYSNGGNSADLSHGVMDRALLHLDNSYNIPNVSIMGFICKTNLSSNTAFR 941
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BMXDH1  940:GFGGPQGMFGAGNMVREIAHRLGKSPEEISRLNLYRGNNTTHYGQVLTYCTLQRCWDECVQNSNLAERKLKIKEFNKQHRWRKRGISIIPTKFGIAFTEKLLNQAGALVLVYVDGSVLLS 1059
BMXDH2  919:GFGAPQVMLAAESMIRQIASTLGKSYEEIVEVNIYKEGSVTYYNQLLTYCTLSRCWNQCIDSSRYIARKKAVNDFNRSNRWKKKGIALVPTKYGISFQTDVLMQAGALLLVYNDGAVLLS 1038
CALVI   936:GFGGPQGMFAGEHIIRDVARILGKDYLEIMKQNFYKEGDITHYQQKLDNFPIEKCFYDCLQQSNYYQKRKEIEEFNRNHRWRKRGISLVPTKYGIAFGVSHLNQAGALINIYADGSVLLS 1055
DROME   918:GFGGPQGMYAGEHIIRDVARIVGRDVVDVMRLNFYKTGDYTHYHQQLEHFPIERCLEDCLKQSRYDEKRQDIARFNRENRWRKRGMAVVPTKYGIAFGVMHLNQAGSLINIYGDGSVLLS 1037
RAT     900:GFGGPQGMLIAEYWMSEVAITCGLPAEEVRRKNMYKEGDLTHFNQKLEGFTLPRCWDECIASSQYLARKRKVEVEKFNRENCWKKRGLCIIPTKFGISFTLPFLNQGGALVHVYTDGSVLLT 1019
CHICKEN 942:GFGGPQGMMIAECWMSDLARKCGLPEEVRKINLYHEGDLTHFNQKLEGFTLRRCWDECLSSSNYHARKKLIEEFNKQNRWKKRGMCIIPTKFGISFTVPFLNQAGALVHVYTDGSVLLT 1061
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BMXDH1 1060:HGGTEMGQGLHTKMIQVATRALGIDVSKIHISETSTDKVPNTSATAASAGSDLNGMAVLEACEKIMKRLKPYIDKNPDGKWENNVVSAAYVDRVSLSATGFHATPDI-GFDFKTTSGKPFN 1178
BMXDH2 1039:IGGIEMGQGLFTKMIQIASKALEIGQSRIHISEAATDKIPNSTATAASMSSDLYGMAVLNACNTLNQRLKPYKTKDPNGKWEDWVSEAYVDRVCLFATGFYSAPKI-EYNRNTNSGRLFE 1157
CALVI  1056:HGGVEIGQGLHTKMIQCCARALQIPIEFIHISETATDKVPNTSPTAASSGSDLNGMAVLDACEKLNKRLAPIKEANPNGSWTEWINKAYFERVSLSATGFYRMPDIGIGDPVQNPNALMYN 1175
DROME  1038:HGGVEIGQGLNTKMIQCAARALGIPSELIHISETATDKVPNTSPTAASVGSDLNGMAVLDACEKLNKRLAPIKEALPGGTWKEWINKAYFDRVSLSATGFYAMPGIGYHPETNPNARTYS 1157
RAT    1020:HGGTEMGQGLHTKMVQVASRALKIPTSKIHISETSTNTVPNTSPTAASASADLNGQGVYEACQTILKRLEPFKKKKPTGPWEAWVMDAYTSAVSLSATGFYKTPNLG-YSFETNSGNPFH 1138
CHICKEN 1062:HGGTEMGQGLHTKMIQVASRSLGIPTSKIYISETSTNTVPNTSPTAASVSADINGMAVHNACQTILKRLEPIKQSNLKGSWEDWIKTAYENCISLSATGFYRIPDVG-YNFETNKGKPFH 1180
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BMXDH1 1179:YFTYGVACTEVEIDCLSGDHQVLRTDIVMDLGESLNPAIDIGQIEGGFIQGYGLFTIEELIYSPTGTLYSRGPGAYKIPGFGGYSSEFNVSLLKGAPNPRAVYSSKAVGEPPLFLASSAY 1298
BMXDH2 1158:YFTYGVACSEVIIDCLTGDHEVLRTDIVMDVGESINPAIDIGQIEGAFMQGYGFLTMEEVVFSANGETLSRGPGTYKIPTLSDIPKEFNVSLLKGAPNPRAVYSSKAIGEPPLFLAASVF 1277
CALVI  1176:YFTNGVGSSIVEIDCLTGDHQVLSTDIVMDIGSSLNPAIDIGQIEGAFMQGYGLFTLEEMISYDQCLQSNYYQKREIDEFNVTILTGAANPRAVYSSKAVGEPPLFIGCSVF 1295
DROME  1158:YYTNGVGVTVVEIDCLTGDHQVLSTDIVMDIGSSLNPAIDIGQIEGAFMQGYGLFTLEELMYSPQGMLYSRGPGMYKLPGFADIPGEFNVSLLTGAPNPRAVYSSKAVGEPPLFIGSSAF 1277
RAT    1139:YFSYGVACSEVEIDCLTGDHKNLRTDIVMDVGSSLNPAIDIGQVEGAFVQGLGLFTMEELHYSPEGSLHTRGPSTYKIPAFGSIPIEFRVSLLRDCPNKRAIYASKAVGEPPLFLASSIF 1258
CHICKEN 1181:YFSYGVACSEVEIDCLTGDHKNIRTDIVMDVGTSLNPAIDIGQIEGAFVQGIGLFTMEELRYSPEGNLYTRGPGMYKIPAFGDIPTEFYVSLLRDCPNSKAIYSSKAVGEPPLFLSASVF 1300
           *  **   **** *** ****** * ****** *  ********* ** **** * **   *** *  *  * *      ***  *   * * *** *** ****  *

BMXDH1 1299:FAIHEAIKAAR---ADAGVPLEFDMEAPATSARIRMACEDHITKKLDKPDPNSF-VPWNVVP 1356
BMXDH2 1278:FAIKEAIMAAR---SDSGVPVEFELDAPATCERIRMSCEDDITLQV-KPTVKRIGVPWNNVIA 1335
CALVI  1296:FAIKEAITSAR---LMNGLSEDFKLESPATSARIMACQDEFTNLIEQP-PAGSYVPWNIVP 1353
DROME  1278:FAIKEAIAAAR---EDQGLSGDFPLEAPSTSARIRIACQDKFTELLEIP-EPGSFTPWNIVP 1335
RAT    1259:FAIKDAIRAARAQHGD-NAKQLFQLDSPATPEKIRNACVDQFT-TLCVTGVPENCKSWSVRI 1318
CHICKEN 1301:YAIKDAIYSAR-EDSGV-TEP-FRLDSPATPERIRNACVDTFT-KMCPSAEPGTFKPWSVRA 1358
           ** ** *        *     *      *   *

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Fig. 2. Comparison of XDH amino acid sequences. The amino acid sequences of BmXDH1, BmXDH2 and XDHs of *D. melanogaster* (Keith *et al.*, 1987), *Calliphora vicina* (Houde *et al.*, 1989), rat (Amaya *et al.*, 1990) and chicken (Sato *et al.*, 1995) were compared. The identical residues among them are shown with asterisks at the bottom. Conserved 2 Cys residues, putative 2Fe/2S-type redox centres, are underlined. Shaded regions indicate intermediate fragments of proteolytic digestion (Sato *et al.*, 1995).

We estimated the probability that all individuals are homozygotic for particular locus by the formula $p = (1-r)^{2n}$, where p is probability, r is recombination frequency and n is number of individuals.

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers: D38159 for BmXDH1 and D43965 for BmXDH2.

3. Results

(i) *Amplification of XDH homologous sequence*

The complete cloning strategy for the silkworm XDH gene is shown in Fig. 1. When comparing the *Drosophila* and rat XDH genes, many regions of amino acid sequence are well conserved (Keith *et al.*, 1987; Amaya *et al.*, 1990). From these conserved regions, we designed four degenerated primers listed in Table 1. To obtain the first short fragment of the *Bombyx* XDH gene, we performed nested PCR using the primers xdhFA and xdhRA for the first amplification, and xdhFB and xdhRB for the second PCR. As a result, a fragment of about 180 bp consistent with our expectations was amplified from the second PCR and inserted into the plasmid pUC19. Sequencing of several of these pUC19 clones showed that two apparently different types of insertions were present. Since these two types of sequences possessed striking similarity to the sequences near the 5′ end of the XDH genes of other organisms (data not shown), the cloned fragments were regarded to be the corresponding sequences of independent XDH genes of *B. mori*. Therefore, we named these two genes BmXDH1 and BmXDH2.

(ii) *Determination of 5′ and 3′ unknown sequences*

We failed to obtain XDH clones from the embryonic cDNA library using the amplified fragments as probes. This is probably because the size of the fragments was too small. To obtain the full-length cDNA sequences, we performed direct PCR of phage DNA extracted from the cDNA library as shown in Fig. 1, and primers were designed from the sequenced region and both arms of λgt11.

With regard to the cloning of the 5′ unknown region of BmXDH1, we used the primers lamF and xdh1RA for the first amplification and then lamF and xdh1RB for the second PCR. In this PCR, we were able to clone the region containing the putative initiation codon. Except for BmXDH2, the amplified products were too short. Therefore, upstream primers (xdh2RC) were newly synthesized and similar PCR was performed. Finally, the fragment including the putative initiation codon was obtained and sequenced.

The 3′ unknown regions were too large for ordinary PCR. However, long PCR for amplifying more than 10 kb was recently reported (Barnes, 1994; Chen *et al.*, 1994). We used this method to obtain the 3′ regions using the same strategy. When using ordinary *Taq* polymerase in the protocols for the amplification of short fragments, we could find no specific products after performing nested PCR of xdh1FA and lamR, followed by xdh1F2 and lamR or xdh2FA and lamR, and then xdh2FB and lamR. In contrast, 3′ regions of BmXDH1 and BmXDH2 were successfully amplified with a specially prepared *Taq* polymerase for long PCR in its accompanying buffer by using the same nested PCR and primers.

Both amplified products of BmXDH1 and BmXDH2 were around 3·5 kb in size. This is too large to sequence directly; therefore, we digested the fragments with *Sau*3AI or *Rsa*I and inserted the digested fragments into plasmids. These clones were sequenced, and the gaps between clones were filled with the sequences obtained from the fragments amplified directly from cDNA or genomic DNA.

Partial genomic sequencing of this 5′ portion revealed the positions of two introns in BmXDH1 (nucleotide positions between 182 and 183, 336 and 337), and five introns in BmXDH2 (nucleotide positions between 234 and 235, 387 and 388, 2499 and 2500, 2808 and 2809, 3142 and 3143). The position of the first intron was conserved among BmXDH1, BmXDH2 and known Diptera XDH genes, but the second intron of the *Bombyx* genes was located just downstream from the first intron, unlike in Diptera.

Beginning with identical putative initiation codons, we confirmed the largest open reading frames of 4068 bp in BmXDH1 and 4005 bp in BmXDH2 (data not shown), respectively. We found that these two open reading frames possessed significant similarity throughout the region with other organisms, but similarity between BmXDH1 and BmXDH2 was not so remarkable (57·3% nucleotide identity and 55·8% amino acid identity) as compared with other organisms (49·9–58·8% amino acid identity). The phylogenetic tree showed that the divergences of XDH genes are generally consistent with the known phylogeny of mammals, birds, Diptera and Lepidoptera (Fig. 3).

(iii) *Tissue specificity of BmXDH1 and BmXDH2 gene expression*

Since BmXDH1 and BmXDH2 were cloned from mRNA of embryos 48 h after egg laying, both were transcribed in embryos at this stage. To examine whether there is tissue specificity for these two genes,
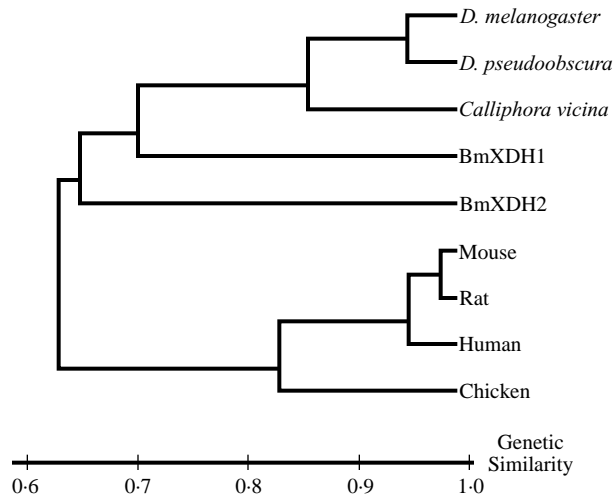
Fig. 3. Phylogenetic tree of the XDH genes. Amino acid sequences of mouse (Terao *et al.*, 1992), human (Wright *et al.*, 1993) and *D. pseudoobscura* (Riley, 1989) were obtained from GSDB, DDBJ, EMBL and NCBI databases. Cluster analyses were conducted on similarity estimates using the unweighted pair-group method for arithmetic average (UPGMA).

RT-PCR was carried out on poly-A RNA extracted from fat body, midgut, malpighian tubes and silk glands (Fig. 4). As expected, expression of both XDH genes was observed in fat body, midgut and malpighian tubes, but was quite low in silk glands. It seemed that the level of transcription was higher in malpighian tubes and lower in the midgut (lanes 1–9). This expression pattern was in basic agreement with the observation of the distribution of enzyme activity in larval tissues (Hayashi 1961*a*, *b*; Tamura, 1983). We found no significant difference in tissue specificity of BmXDH1 and BmXDH2 at the resolution of our assay.

(iv) *Non-synonymous substitutions detected in BmXDH1 of oq*

We also analysed XDH gene expression in *og* and *oq* mutants. The level of the two BmXDH gene transcripts was assayed by RT-PCR of poly-A RNA prepared from fat body of homozygotes and heterozygotes of *og*, *oq* and *og*[t], an allele of *og*. The transcriptional level of the XDH genes in these mutants was not so different from that of wild type at the resolution of our assay (data not shown). However, we often found aberrant products when BmXDH1 cDNA was amplified from mRNA of the *oq* mutants (data not shown), suggesting the possibility that some mutations might have occurred in BmXDH1 of the *oq* mutants. Consequently, we sequenced the 5′ portion of the cDNA (nucleotide positions between 1 and 1679) from *oq* homozygotes and heterozygotes. Sequences of heterozygotes showed a clear dimorphism and one sequence was coincident with that of the homozygotes. Sequencing of an extended region confirmed the dimorphism of heterozygotes. We found three non-synonymous and 21 synonymous substitutions in the homozygous type sequence and one non-synonymous and nine synonymous substitutions in the other type. We also performed partial sequencing of the BmXDH1 and BmXDH2 genes from *og* and *og*[t] mutants, but there were no non-synonymous substitutions (data not shown). These results suggested that the BmXDH1 gene is concerned with the *oq* mutation.

(v) *Polymorphism in length of the BmXDH1 second intron co-segregated with the oq phenotype*

To determine the relationship between the BmXDH1 gene and the *oq* mutation, we performed an F_2 linkage
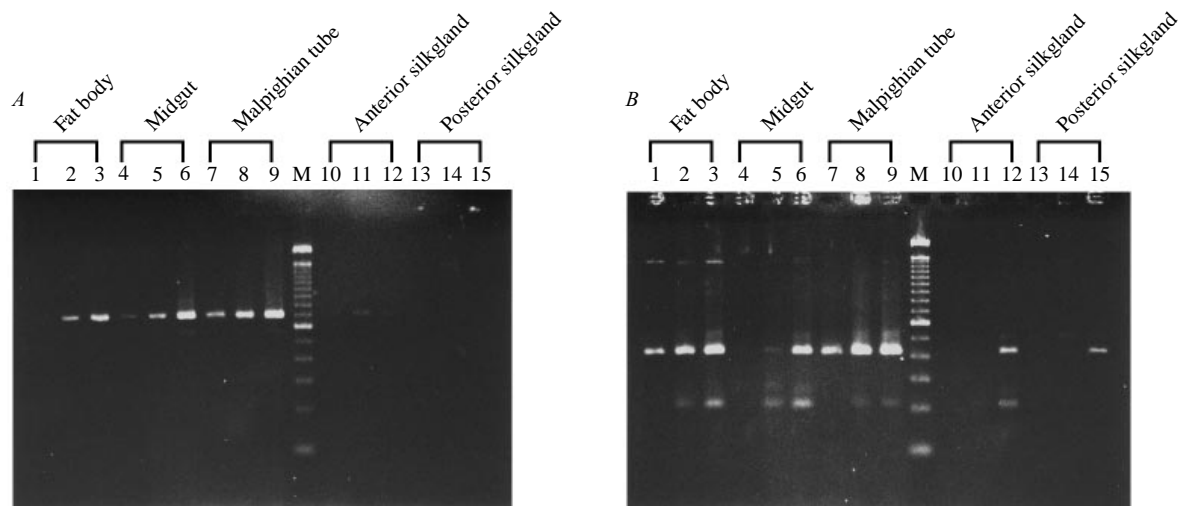
Fig. 4. Expression of BmXDH1 (*A*) and BmXDH2 (*B*) in various tissues. RT-PCR amplification was stopped at the 25th, 27th or 29th cycle. Lanes 1, 4, 7, 10 and 13, PCR products of 25 cycles; lanes 2, 5, 8, 11 and 14, 27 cycles; lanes 3, 6, 9, 12 and 15, 29 cycles.
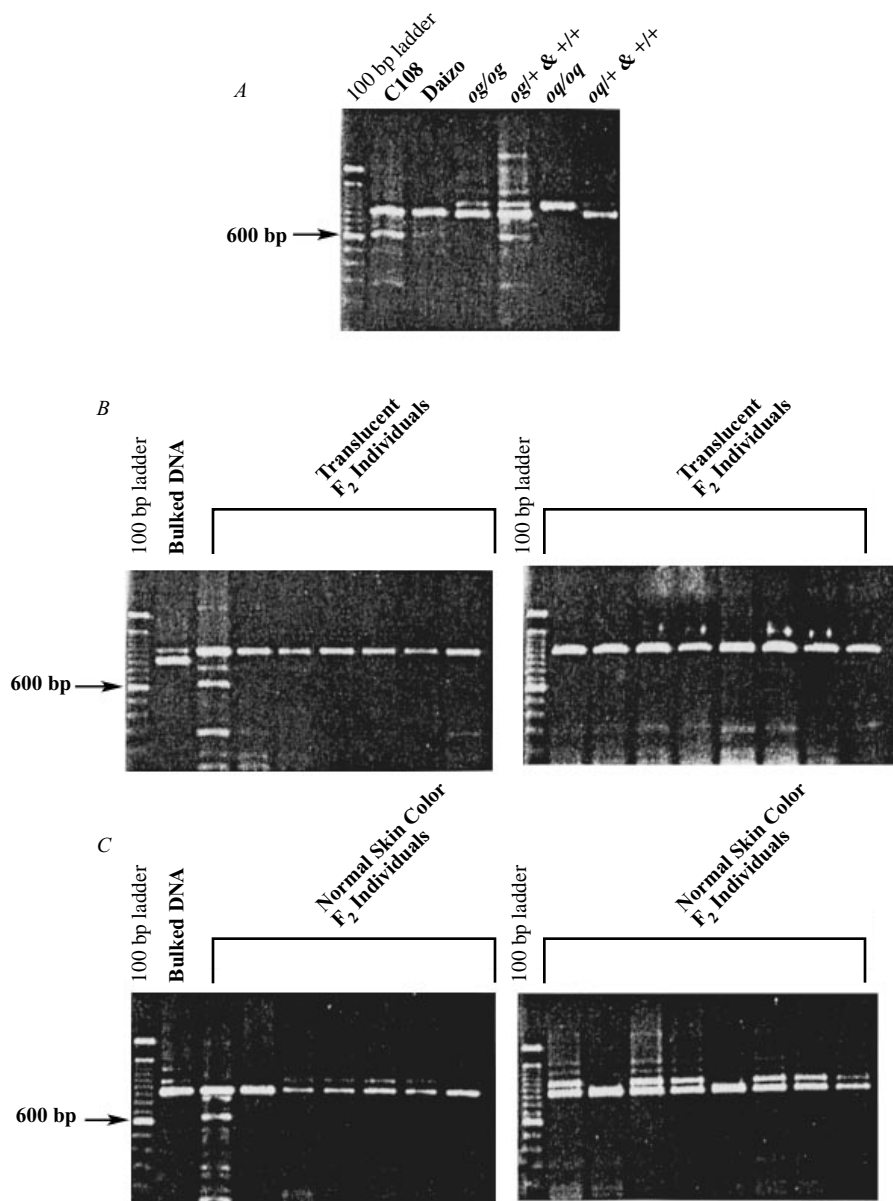
Fig. 5. Co-segregation of a BmXDH1 polymorphism and *oq* phenotype. Primers xdh1LF and xdh1LR were designed to amplify the second intron inserted at the nucleotide position between 336 and 337 of BmXDH1 cDNA. (*A*) Polymorphism in the length of the second intron in the BmXDH1. Template DNAs were prepared from 50 larvae per strains. (*B*), (*C*) Amplification of the polymorphic fragment from genomic DNAs of 15 translucent (*B*) and normal skin colour (*C*) F₂ individuals. Bulked DNA indicates a product generated from genomic DNA of 50 normal skin colour F₂ insects.

analysis based on PCR. We used bulked genomic DNAs prepared from 50 larvae per strain for screening of polymorphisms. Primers xdh1LF and xdh1LR, encompassing the second intron of the BmXDH1 gene, showed a clear length polymorphism between the *oq* mutants and two wild-type strains, C108 and Daizo (Fig. 5*A*). Compared with wild-type strains, which had a single 920 bp fragment, the *oq* homozygotes had a longer single 1200 bp fragment. Both fragments were observed in the F₂ progenies showing normal skin colour, a mixture of *oq*/+^{oq} and +^{oq}/+^{oq}.

Similar heterozygosity was also detected in the *oq* mutants, but there was no difference between mutant and normal skin colour populations (Fig. 5*A*).

Genomic DNAs were isolated from 30 larvae of an intercross of *oq*/+^{oq}. Fifteen of them were translucent and the rest were of normal skin colour. Following identical PCR amplification, all the translucent individuals had single longer fragments without exception (Fig. 5*B*). The probability of this event was 18·4% when BmXDH1 was 5 cM from the *oq* locus, and 54·5% when BmXDH1 was 2 cM from it. If bulked

DNA from 50 larvae were taken into consideration, these values were 0·0652 and 7·23%, respectively. In contrast, five of 15 normal skin colour insects had a single shorter fragment, and the rest had both fragments. The ratio was identical to 1:2, the expected ratio of $+^{oq}/+^{oq}$ to $oq/+^{oq}$ (Fig. 5*C*). These results strongly suggested that BmXDH1 and the *oq* locus are closely linked.

4. Discussion

We have determined the full-length cDNA sequence of two *Bombyx* xanthine dehydrogenase genes (XDH) from embryonic mRNA. This is the first report of cloning and sequencing of XDH in lepidopterous insects. XDH plays very important roles in nitrogen metabolism. The final nitrogenous excretory product in most Lepidoptera is uric acid, and the enzyme catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid (Bursell, 1967; Cochran, 1975). The enzyme activity and amount of uric acid are influenced by nutritional conditions in the silkworm (Ito & Murakami, 1964; Horie & Watanabe, 1983). Purification of this enzyme from *Manduca sexta* reveals that there are specific cellular localizations and levels of enzyme during development (Buckner *et al.*, 1993). Cloning of the XDH gene would facilitate studies on these physiological roles and regulation mechanisms in Lepidoptera.

In our study we found two *Bombyx* XDH genes. Both genes possessed significant similarity with those of other organisms, including flies (Keith *et al.*, 1987; Lee *et al.*, 1987; Houde *et al.*, 1989; Riley, 1989), chicken (Sato *et al.*, 1995) and mammals (Amaya *et al.*, 1990; Terao *et al.*, 1992). Sato and colleagues proposed that XDH were composed of three domains: the N-terminal iron–sulphur centres, the intermediate FAD associating domain, and the C-terminal molybdenum centres (Sato *et al.*, 1995). Both *Bombyx* genes have putative 2Fe/2S-type redox centres (Fig. 2, underlined 2 Cys residues). They also reported that the hinge region of the N-terminal and the intermediate domains were highly sensitive to subtilisin digestion (Sato *et al.*, 1995). It coincides with very low similarity in the region (Fig. 2).

No organisms have been reported to date to have two or more functional XDH genes. A phylogenetic tree of XDH genes suggested that the divergence of BmXDH1 and BmXDH2 was later than that of vertebrates and invertebrates but earlier than that of Lepidoptera and Diptera (Fig. 3). BmXDH1 seems to be a member of the previously known Diptera *rosy* gene family and BmXDH2 is fairly remote from them (Fig. 3). However, this cannot explain the fact that no other insects have been reported to have multiple XDH genes or pseudogenes. One speculation is that they exist but have just not been discovered yet. Alternatively, BmXDH1 and BmXDH2 diverged after Lepidoptera and Diptera were differentiated but functional differences between them make genetic similarity lower than expected. Further studies on other insects will be required to solve this problem.

We could not find a functional difference between BmXDH1 and BmXDH2 so long as we only examined tissue specificity in wild-type larvae. Both BmXDHs were highly expressed in malpighian tubes, fat body and midgut, but not in the silk glands (Fig. 4); similarly, transcription of BmXDH1 and BmXDH2 were observed in fat bodies from *og* and *oq* homozygotes (data not shown). However, expression levels of the BmXDH1 mRNA in *oq* homozygotes are reported to be lower than in normal skin colour insects (N. Komoto *et al.*, unpublished data).

Our primary interest is to develop a marker-recipient system for transgenesis in the silkworm. For this purpose, identifying a visible mutation and defining its underlying basis is the most important objective. Upon examining RT-PCR products in more detail, we found that none of the sequences was inserted or deleted. Instead, we detected an evident dimorphism in sequences of clones from *oq* heterozygotes, one of which was identical to that found in *oq* homozygotes. Moreover, polymorphism in the length of the second intron of BmXDH1 was co-segregated with the *oq* phenotype (Fig. 5). These results strongly suggest that some deficiency in the region around the BmXDH1 gene is the cause of the *oq* translucent phenotype.

However, it remains unclear whether the base substitutions and the intron length polymorphism reported in this experiment are the result or the cause of the mutation. Most base polymorphisms were located on the hinge and intermediate domain where the functional requirements seemed to be relatively low (Sato *et al.*, 1995). We did not succeed in detecting any alternative splicing related to the second intron. Additional studies are needed, including sequencing extended regions of mutants and site-directed mutagenesis.

Further analyses are required to characterize these mutations; however, our results suggest that a combination of functional BmXDH1 together with the *oq* mutant is a promising candidate for a marker-recipient transgenic system for the silkworm. We are now trying to isolate intact clones of BmXDH1 and BmXDH2 and to construct a transfer vector to introduce into mutant silkworms by a microinjection or particle delivery system.

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