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           L      K R  K              P
Danio_erio      1  1  ---MCFSVSVVPLLFILWMLVVRVFRQRSHRRTDGVAKENNKPEAVFPKLLHSLYKLFSTVSPSTISGPRSPLLLGNML  76
Tetraodon_nigroviridis  2  1  MSVEVLVAGVGAGFLVLLMLVKDMCKVKNHNL-----SFSAPPAVKRSLQLCGLLHRPSSPSPISGPPCRFLIGNMT  75
Fugu_rubripes   3  1  MVGKHLID-----LDRMCSEFLFCS-----SFSAPPAVKRNLPLSLWQLPLRSPSPPIGPPCRFLIGNMT  60
Gasterosteus_aculeatus  4  1  MVIEISFITAGAAFLVLL-MIFIGIFCQKQDQSV-----QGDHRGKAASGLLHLCRFLTRSSSPGLPGPPSLFLIGNMT  74
Oryzias_latipes  5  1  -----
Anguilla_japonica  6  1  MPVCMTFVVGAGTAVVLCVWLVVYVYPRQTDQK-----LCSQSN---VVGSTLLYPLNRLPHLTLSPSPLPPLGNNML  74
Homo_sapiens    7  1  MLL-----LGLLLLPL-----AGARLLWNNWKL-----RSLHPLPLAPGFLHL  41
Mus_musculus    8  1  MLL-----PGLLLLPL-----AGTRWLGWQWKL-----RKLHLPLAPGFLHFL  41

           H      R
1  77  DLAQDHLPPIHLTALAKCYGNIYRLNCGSTTMVVLNNSIIEALVKKWSDFAGRPYSYTGDIVSGGGRTISLGDFSEEWK  156
2  76  ELMQDHLPPIHLTDLAKRYGNIYRLKCGNTTMVVLSGGDVIREALVKKWSDFAGRVSYTADIVSGGGRTISLGDYTEEWK  155
3  61  ELMHDHLPPIHLTNLAKRYGNIYRLKCGNTTMIVLNSDIIREALVKKWSDFAGRAVSYTADIVSGGGRTISLGDYTEEWK  140
4  75  ELTHEHLPPIHLTNLARRYGNIYRLKCGNTTMVVLNSGEIIEALVKKWSDFAGRPVSYTGDIVSGGGHTISLGDYNEEWR  154
5  3   ELMHDHLPPIHLTSLAQRYGSIYRLKCGNTTMVVLNSGELIRETIVRKWSDFAGRPISYTGDIVSGGGRTISLGDYTEEWK  82
6  75  ELTRDHLPNHLTSLARHYGNIYRLYCGNTTIVVLSAEMIREALVKKWSDFAGRPHSYTGDIVSGGGRTISLGDYSEAWR  154
7  42  Q---PDLPIYLLGLTQKFGPIYRLHGLQDVVVLNSKRTIEEAMVKKWADFAGRPEPLTYKLVSRNYPDLSLGDYSLLWK  118
8  42  Q---PNLPYLLGLTQKLGPIYRIRLGMQDVVVLNSRRTIEEALIQWVDFAGRPHMLNGKMDL---DLSLGDYSLLMK  114

1  157  AHRRVTHSALQRCTDLSHSVIEKQAQHLCQVLRDYSKAVDLSDFTVASSNVITTLTFSKAYDKSSAELQKLEQCLNE  236
2  156  AHRRLVHSALQRCCQSLHDVIERQALQLRKVLINYQGGAVDLSDFTVASSNVITTLVFGKEYDKSSELQQLHSCLENE  235
3  141  ALRRLVHGALQRCCQKSLHNVIERQALQLRKVLVYRGGAVDLSDFTVASSNVITTLVFGKEYDKSSELQQLHSCLENE  220
4  155  AHRRLVHSALQRCCQSLHDVIERQALHREVLMYRSGSPVDLAEFTVAASNVIITLAFKEYDKSSELQQLHSCLENE  234
5  83   AHRRLVHGALQRCCQSLHSVIERQALHRLKVLMDYRSGSPVDLAEFTVAASNVIITLAFKEYDKSSELQQLHSCLENE  162
6  155  THRLAHSALQRCSAQCLHAVIQKALGLRQVLMYNETPVLDSEDFTVASSNVITTLVFGKEYDKSSELQQLHSCLENE  234
7  119  AHKKLRSALLLGRDSMEPVVEQLTQEFCEMRRAQPGTPVAIEEEFSLTCSIICYLTFGDKI-KDDNLMPAYKYCIQE  197
8  115  AHKKLRSALMLGMRDSMEPLIEQLTQEFCEMRRAQPGTPVAIHEKFSFLTCSSIIISCLTFGDK---DSTLVTQLDHCVD  191

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1  237  IVSLWGSPWISALDSFLLRFPNPPFSLRMKEVARRDELIGKHIEEFKVRQIVQIISTESCQKFSKGGTLTSSLLKCLE  316
2  236  IVALWGSAWISALDTFLLRFPNVPFSRLLREVTRDEIIRKHLNRYKVS-----QDNKSTDVITGSLLEGV  305
3  221  IVALWGSTWISALDTFLLRFPNVPFSRLLREVSRRDEIIRKHLNQFKVS-----EGHRTDVITGSLLEGV  290
4  235  IVALWGSSWISALDSFLLRRLKPNPVPFARLLKEVARDEIIREHLNXYK--S-----QNKTEADITGSLLEGLE  302
5  163  IVSLWGSSWISALDSFLLRRLKPNPVPFARLLKEVARRDEIIRKHLNFK--L-----QGHQSEDSITGSLQALD  230
6  235  IVSLWGSPWISALDSFLLRRLKPNPPFSLRLEKVARDDIIRSHIQEYK-----ELCQDEGTGGTITASLLQSLD  304
7  198  VLKTWSHWISQIVDVIPFLRFPNPLRRLKQAIERDHIIVEMQLRQKHESLV-----AGQWRDMMDYMLQGV  267
8  192  LLQAWNHSIQIILTIIPLLRFLPNPGLQKQIQRDHVIVKQQLKRHKDSL-----AGQWKMIDYMLQGV  261

           M      H
1  317  PQQGAANHTTLDTHVHMTVDLLIGGTETIAALLNWTVAFLHRPEVQDKVYEEELCVLDVR---YPQYSDRHRKLPYL  392
2  306  KHR-KDHVVDLTDVHVMATVDLLIGGTETTAAWLNWTVAFLLHRPEIQTKVYEELCTVLEGR---YPKYSDRHRLPVL  380
3  291  RHQ-TEPGVVLTDVHVMATVDLLIGGETTAAWLNWTVAFLLHRPEIQTKVYEELCTVLEGR---YPKYSDRQRLPVL  365
4  303  RNNNTDHKVRLTDIIVHVMATVDLLIGGTETTAAWLNWTVAFLLHRPEVQAKVYEEELCTVLEGR---YPKYSDRRLPVL  378
5  231  KHQNTKEGQLTDVHVMATVDLLIGGTETTAAWLNWTVAFLLHRPEIQDKVYEELCTVPEGR---YPKYSDRQRLPVL  306
6  305  QPLGEKERVALTETHVHMTVDLLIGGTETTAAWLWTVAFLLHRPEVQCRVYSELCTVLDTR---YPQYSDRKLPL  380
7  268  PXM-EEGSQLLEGHVHMAVDLLIGGTETTANTLSWAVVFLHHPPIQRLQEELDHLELPGASSRVPYKDRARLP  346
8  262  QRD-GKDEERLHEGHVHMSVVDLFIGGTETTATLSWAVAFLLHHPPIQRLQEELDKLPGF---SLLYRNRMQLPLL  337

1  393  CALISEMLRLRPVAPLAVPHRAIRNSSIAGHFIKNTIIIPNLYGAHHDPEVWDDPYSFKP--ERFLEGGGSLRSLIPF  470
2  381  CSLVHEVRLRLRPVAPLAVPHKAVRDSIAGYFIKNTIIPNLFAGHHDPAVWDPYFVKPQERFLEGGGSPRALVPF  460
3  366  CSLIHEVRLRLRPVAPLAVPHKAIRDSSIAGYFIPRNTIIPNLFAGHHDPEVWSDPYSFKP--ERFLEGGGSTRALIPF  443
4  379  CSLINEVRLRLRPVAPLAVPHRAVRDSIAGYFIKNTVIIPNLFAGHHDPAVWSDPYSFRP--ERFLEGGESSTRALMPF  456
5  307  CALVNEVRLRLRPVAPLAVPHRAIRNSSIAGYFIPKNSVIIPNLFAGHHDPAVWSEYFVKP--ERFLEGGGSTRALVPF  384
6  381  SALINEVRLRLRPVAPLAVPHRAIRDSSIAGYRIPKDTVIIPNLYGAHHDPAWIDPYAFKP--ERFLDGGRASSTRALLP  458
7  347  NATIAEVRLRLRPVPLALPHRTTRPSSISGYDIPGETVIIPNLQGAHLDETVWERPHEFWP--DRFLEP--GKNSRALAF  422
8  338  MATIAEVRLRLRPVPLALPHRATRASSISGYDIPKDMVIIPNIQGANLDEMWWELPSKFWP--DRFLEP--GKNPRTPSF  413

           A
1  471  GGGARLCLGEAVAKMEMFLFTAYLLREFKFLPASKEEPLPELRGV--ASVVLKVKPYTVIAHPR--EQ-----  534
2  461  GGGARLCLGETVAKMELFLFTAYLLRDFCFCLPDVEAALPDLRGV--ASVVLKVKAFYVWARPRIRPLS-----  527
3  444  GGGARLCLGETVAKMELFLFTAYLLRDFCFVLPDSEAPLPDLRGV--ASVVLKIKSFTVIARPR-----  505
4  457  GGGARLCLGESVAKMELFLFTAYLLRDFHFIPENEASLPDLRGV--ASVVLKVSXYVTARPR--PLT-----  521
5  385  GGGARLCLGESVAKMELFLFTAYLLRDFQFIPESNVFPDLRGI--ATVVLKIKPKYKVIARPR--PAIGP----  451
6  459  GGGARLCLGESIAKMEFLFTAYLLRDFQFLPSGNKETLPDLTGV--ASVVLKVKPFQVIVRPR--PLT-----  523
7  423  GCGARVCLGEPLARLELVVLRLLQAFT--LLPSGD--ALPSLQPLPHCSVILKMFPQVRLQPRGMGSAHSPGQSQ  495
8  414  GCGARVCLGEPLARLELVVLRLLQAFT--LLPPPDTLPSLQPPYAGINLPIPPFQVRLQPRNLAPDQGERP  487

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Figure S3 Constraint-based multiple-alignment of 21-hydroxylase (cyp21a2). The cyp21a2 orthologs of *Danio rerio* (zebrafish), *Tetraodon nigroviridis* (green spotted pufferfish), *Fugu rubripes* (Japanese pufferfish), *Gasterosteus aculeatus* (stickleback),

Oryzias latipes (medaka), *Anguilla japonica* (Japanese eel), *Homo sapiens*, and *Mus musculus* are aligned for comparison. The male allele of *Danio rerio* identified within the cross is aligned at the top, with concurrent amino acid changes of the female allele designated above. Highlighted positions are allele changes at residues conserved among fish (in blue), and additionally among humans and mice (in yellow).