

Disruption of FEM1C-W gene in zebra finch: evolutionary insights on avian ZW genes

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Received: 11 August 2008 / Revised: 8 December 2008 / Accepted: 9 December 2008 / Published online: 13 January 2009
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Abstract Sex chromosome genes control sex determination and differentiation, but the mechanisms of sex determination in birds are unknown. In this study, we analyzed the gene *FEM1C* which is highly conserved from *Caenorhabditis elegans* to higher vertebrates and interacts with the sex determining pathway in *C. elegans*. We found that *FEM1C* is located on the Z and W chromosome of zebra finches and probably other Passerine birds, but shows only Z linkage in other avian orders. In the zebra finch, *FEM1C-W* is degraded because of a point mutation and possibly because of loss of the first exon containing the start methionine. Thus, *FEM1C-W* appears to have degenerated or been lost from most bird species. *FEM1C-Z* is expressed in a cytoplasmic location in zebra finch fibroblast cells, as in *C. elegans*. *FEM1C* represents an interesting example of evolutionary degradation of a W chromosome gene.

Introduction

In birds, the female sex chromosomes are ZW and male ZZ. The ancestral chromosomes of avian sex chromosomes are

different from those of eutherian mammals and snakes (Fridolfsson et al. 1998; Nanda et al. 1999; Matsubara et al. 2006), although recent evidence indicates surprising shared gene content of the avian Z and monotreme X chromosomes (Rens et al. 2007). The gene content of the Z chromosome appears to have changed little during avian evolution, as shown by comparative painting analysis (Itoh and Arnold 2005; Nishida-Umehara et al. 2007; Tsuda et al. 2007), even in Ratites (Struthioniformes) which are considered to be an ancestral avian species whose sex chromosomes are morphologically relatively undifferentiated (Ogawa et al. 1998; Shetty et al. 1999). The sex determining system in birds is still unclear, although several candidate systems have been suggested. One possibility is that the W chromosome has a dominant ovary-determining gene, such as *ASW/Wpkci/HINT1W* (Hori et al. 2000; O'Neill et al. 2000) or *FET-1* (Reed and Sinclair 2002). Another is that the Z chromosome contains a gene that causes development of testes because of a higher expressed dose of the gene in ZZ males relative to ZW females. One such candidate is the Z-linked testis differentiation gene, *DMRT1*, which might induce testis differentiation because of higher expression in the ZZ male compared to ZW female (Raymond et al. 1999). It is also possible that Z and W genes both contribute to gonadal differentiation (Graves 2003).

The sex determination of *Caenorhabditis elegans* is controlled by a signal transduction pathway including the *fem-1* gene, which encodes an ankyrin repeat protein. Mutation of *fem-1* leads to feminization (male to hermaphrodite sex reversal). The FEM-1, FEM-2, and FEM-3 proteins form a complex with CUL-2 that regulates the activity of the TRA-1, a DNA-binding zinc finger transcription factor that regulates the DM domain gene *mab-3*, which in turn regulates male sexual development

Communicated by L. Comai

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(Sokol and Kuwabara 2000; Starostina et al. 2007; Kuwabara 2007).

The highly conserved *Fem1* gene family in vertebrates comprises three genes *Fem1a*, *Fem1b*, and *Fem1c* (Ventura-Holman et al. 1998, 2003). Human *FEMIC* maps to chromosome 5q22, and the exon–intron structure and expression pattern in adult tissue are conserved between mouse and human (Ventura-Holman et al. 2003). Both human *FEMIC* and mouse *Fem1c* transcripts show alternative splicing (Ventura-Holman et al. 2003). The 6.2-kb mRNA is ubiquitously expressed in all mouse tissues, and additional 3.8- and 2.8-kb transcripts are present in mouse testis. Humans have a ubiquitously expressed 6.2- and 3.8-kb mRNA and a testis-specific 2.8-kb transcript. Schlamp et al. (2004) reported a slight non-significant increase in the number of female pups in a mouse line with reduced expression of *Fem1c*, caused by insertion of the β Geo gene trap vector in the first intron of *Fem1c* gene, and concluded that the gene had no known effect on sexual development or fertility.

According to classical comparative mapping analysis, the chicken Z chromosome has large blocks of sequences that are syntenic with human chromosomes 5, 8, 9, and 18 (Schmid et al. 2000). In this study, we analyzed the sequence of human chromosome 5 to identify new sex-linked genes in birds. *FEMIC* gene, homologous to a *C. elegans* sex-determining gene, was cloned as a new sex-linked gene in birds and its linkage was confirmed by Southern blot and fluorescent *in situ* hybridization (FISH). Interestingly, an avian zoo blot analysis indicated that the coding region of *FEMIC* gene is Z-linked in most birds but also has W linkage in Passerines. Further analysis of the sequence of BAC (bacterial artificial chromosome) DNA shows evolutionary degradation of *FEMIC-W* in zebra finch, which provides us with an interesting example of sex chromosome gene evolution.

Materials and methods

Southern blot hybridization and cloning of zebra finch *FEMIC-Z* cDNA

We identified the *FEMIC* gene as a candidate chicken Z-linked gene because it is present on human chromosome 5q in the region syntenic with chicken Z genes such as *CHDI* or *HINT1*. The sex chromosome linkage of *FEMIC* was checked by Southern blot hybridization to male and female zebra finch genomic DNA as described (Itoh and Arnold 2005). Zebra finch total RNA was isolated from adult testis using Trizol Reagent (Invitrogen), and first-strand cDNA was synthesized by Superscript III reverse transcriptase

(Invitrogen). Polymerase chain reaction (PCR) was carried out in a 25- μ l mixture containing 0.2 mM each of dNTPs, 0.4 μ M each of primers, 10 ng of cDNA as a template, 0.25 μ l of 50 \times Titanium *Taq* polymerase (BD Biosciences), and one tenth volume of 10 \times Titanium *Taq* buffer (BD Biosciences). The primer sequences were designed to match highly conserved regions of human and mouse *Fem1c* sequences: FEMIC-3 (5'-GGG CAT ACT TGC TTG ATG AT-3', 5'-AGC ATC TTC ATG ATG TCC A-3') and FEMIC-4 (5'-TTG GAT ATG CAG CAG AGC AA-3', 5'-ATA AGG AGA TTC ATG ATG TC-3'). The PCR reaction was carried out at 95°C for 4 min before the cycling reaction and 40 cycles of 95°C for 45 s/52°C for 30 s/72°C for 1 min and then followed by single cycle reaction at 72°C for 7 min. The PCR product was separated by gel electrophoresis. PCR products were cloned into pGEM-T Easy vector (Promega).

For cloning longer cDNAs, we used reverse transcription PCR (RT-PCR), 5'- or 3'-RACE or screened a female zebra finch post-hatch day 1 brain cDNA library. Z or W assignment of cDNA fragments was evaluated by the sequence comparison to Z- or W-derived BAC sequences. BAC clone 297J07 (for *FEMIC-Z*, Genbank AC192094) and 197F15 (for *FEMIC-W*, Genbank AC192093) were sequenced by the Washington University Genome Sequencing Center (<http://www.genome.wustl.edu>).

Sequence analysis

The sequences were edited by Vector NTI sequence analysis software. The open reading frames of *C. elegans*, human, mouse, zebra fish, chicken, and zebra finch sequences were aligned using Clustal W. Values of dN/dS comparing zebra finch *FEMIC-Z* and the predicted *FEMIC-W* were estimated by MEGA3.1 (Kumar et al. 2004). Phylogenetic trees were generated by MEGA3.1 using the neighbor-joining method. The map of dN/dS ratios along the gene sequence was calculated in 120-bp length segments (one tenth of the entire region) with overlapping windows of 60 nucleotides.

Northern blot hybridization

RNA of male and female adult zebra finch brain or gonads was isolated using Trizol (Invitrogen), then 8 μ g/lane samples were subjected to denaturing agarose gel electrophoresis and capillary transfer to a MAGNA nylon transfer membrane (Osmonics) in 10 \times SSC. The membrane was hybridized with a ³²P-labeled probe in the phosphate hybridization buffer (Itoh and Arnold 2005) at 68°C for 12 h then washed in 2 \times SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min twice, 1 \times SSC, 0.1%

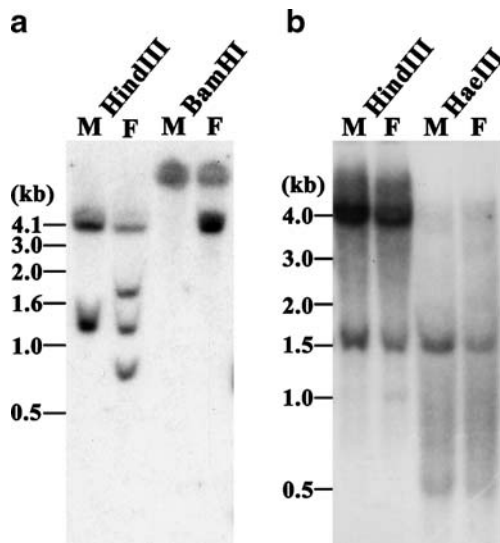


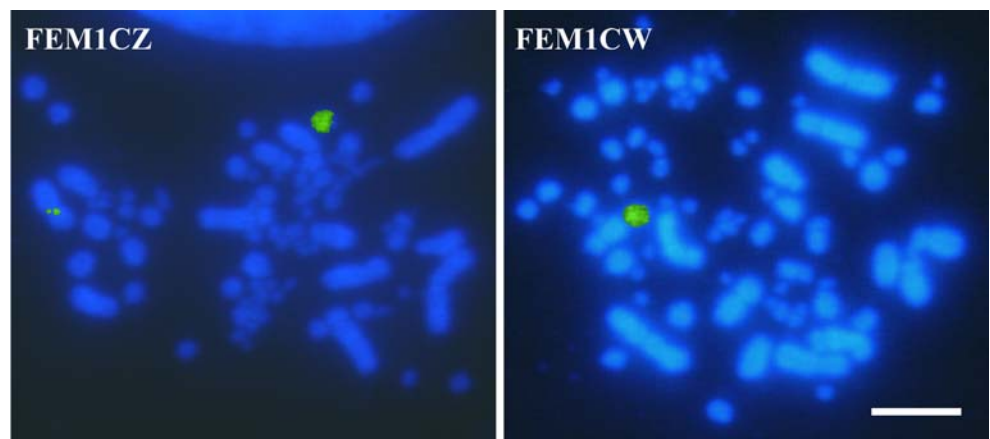
Fig. 1 Southern blot of hybridization genomic DNA, digested with *HindIII* or *BamHI*, from individual male (*M*) and female (*F*) of zebra finch probed with ^{32}P -labeled zebra finch *FEMIC-Z*. The probes were from two different coding region of *FEMIC-Z*: **a** *FEMIC-4*, C-terminal and **b** *FEMIC-3*, N-terminal region. The existence of bands in **a** showing greater hybridization in males suggests Z linkage, and the female-specific bands suggest W linkage

SDS at 37°C for 15 min twice, and 65°C for 15 min twice and subjected to autoradiography.

Fluorescence *in situ* hybridization

Zebra finch BAC clones were isolated from a zebra finch BAC library made by the Arizona Genomics Institute (<http://www.genome.arizona.edu>; Luo et al. 2006). BAC clones were identified by probing membranes spotted with the zebra finch clones and further confirmed by probing Southern blots of the identified clones after restriction digest. BAC clone 297J07 (for *FEMIC-Z*) and 197F15 (for *FEMIC-W*) were used as probes in FISH to metaphase

Fig. 2 Chromosome localization of BAC clone 297J07 encoding *FEMIC-Z* and 197F15 encoding *FEMIC-W* in zebra finch ZW female metaphase chromosome set. 297J07 BAC probe hybridized to both Z and W chromosomes because it includes repetitive sequences such as ZBM (Itoh et al. 2008). The position of *FEMIC-Z* on Z chromosome is $38.8 \pm 2.0\%$ from the p telomere. Scale bar, 10 μm



preparations of mitotic chromosomes prepared from zebra finch fibroblasts as described (Itoh and Arnold 2005).

GFP expression in fibroblasts

The coding region of *FEMIC-Z* was amplified by PCR and subcloned into pEGFP-N3 vector (Clontech). The zebra finch fibroblast cells were prepared from embryos and plated onto coverslips. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) and cells were cultured for 24 h. After washing with $1\times$ phosphate-buffered saline (PBS) twice, the cells were fixed with 4% paraformaldehyde / $1\times$ PBS at room temperature for 30 min. The nuclei were counterstained with DAPI and covered with VECTASHIELD mounting medium (Vector labs).

Results

Molecular cloning of zebra finch *FEMIC* gene

To search for novel avian ZW genes, we focused on genes encoded in a segment of human chromosome 5 homologous to chicken Z (Schmid et al. 2000), especially a block of genes including two known chicken chromosome Z genes, *CHD1* and *HINT1* (located at Hs 5q15-q21.1 and 5q23.2, respectively). In this segment, two genes drew our attention because of the expectation that Z genes might play special roles in sex determination or differentiation: *FEMIC* (5q22.3) and *HSD17B4* (5q23.1). Zebra finch *FEMIC* cDNA fragments were amplified by RT-PCR using the primers matching a highly homologous region in human and mouse. *HSD17B4* was from a zebra finch EST library (SB02032A1A09, Genbank CK313884). Southern blot analysis of male and female zebra finch genomic DNA using *FEMIC* and *HSD17B4* cDNAs as probes suggested the existence of *FEMIC-Z* and *-W* sequences

(FEM1C-4 probe; Fig. 1a), but no female-specific (W) hybridization of *HSD17B4* W-form (data not shown). Interestingly, although the C-terminal coding region of *FEM1C-Z* (FEM1C-4, 1,095- to 1,644-bp region of *FEM1C-Z*: Genbank EU825191) detected both Z and W sequence, the N-terminal region of *FEM1C* (FEM1C-3,

445- to 650-bp region of *FEM1C-Z*) did not show any clear sign of W linkage (Fig. 1a, b). We then localized the *FEM1C* sequence on Z and W chromosome by FISH mapping using zebra finch BAC clones as a probe (Fig. 2). Because of the existence of ZBM repetitive sequence (Itoh et al. 2008), the BAC clones derived from *FEM1C-Z* and

Fig. 3 Comparison of amino acid sequences of chicken (XM_428816), zebra finch (EU825191), human (NM_020177.2), mouse (NM_173423.3), zebra fish (NM_198145.2), and *C. elegans* (J03172) *FEM1C*. The positions of predicted ankyrin repeats (Table 2) are *underlined*

<i>C. elegans</i>	(1)	MTPNGHHFRTVIYNAAAVGNLQRIKVFFTINSRNDRQWIIDCFNSDQDGRY
zebrafish	(1)	-----MDLKTAVFNAARDGKLLKLLQKLENNK--SDREVLKLMAEKTN NGAT
human	(1)	-----MDLKTAVFNAARDGKLRLLTKLLASK--SKEEVSSLISEKTN NGAT
mouse	(1)	-----MDLKTAVFNAARDGKLRLLTKLLASK--SKAEVSSLISEKTN NGAT
chicken	(1)	-----MDLKTAVFNAAREGKLRLLSKLLASK--TKEEVALLVSEKTD GAT
zebra finch	(1)	-----MDLKTAVFNAARDGKLRLLSKLLASK--TREEVAQLVSEKTN NGAT
<i>C. elegans</i>	(51)	<u>PLVIAARNGHANVVEYLLEIG-ADPSVRGVVEFDNENIQGT</u> PPLWAASAA
zebrafish	(44)	<u>PLLMASRYGHLELVEFLMECCAPVELGGSVNF</u> DGEVIEGAPPLWAASAA
human	(44)	<u>PLLMAARYGHLDMVEFLLEQCSASIEVGGSVNF</u> DGETIEGAPPLWAASAA
mouse	(44)	<u>PLLMAARYGHLDMVEFLLEQCSASIEVGGSVNF</u> DGETIEGAPPLWAASAA
chicken	(44)	<u>PLLVAAARYGHLDMVEYLLDHCASASIEVGGSV</u> SFDGETIEGAPPLWAASAA
zebra finch	(44)	<u>PLLMAARYGHLDMVEYLLDHCASASIEVGGSV</u> NFDGETIEGAPPLWAASAA
<i>C. elegans</i>	(100)	<u>GHIEIVKLLIEKANADVNOATNTRSTPLRGACYD</u> GHLDIVKYLLEKGDAP
zebrafish	(94)	<u>GHLKVVQSLLGHG-ASVNNNTLT</u> NSTPLRAACFDGHLDIVRYLVEHQADL
human	(94)	<u>GHLKVVQSLLNHG-ASVNNNTLT</u> NSTPLRAACFDGHLIEVKYLVEHKADL
mouse	(94)	<u>GHLKVVQSLLNHG-ASVNNNTLT</u> NSTPLRAACFDGHLIEVKYLVEHKADL
chicken	(94)	<u>GHLVVQSLLDHG-ASVNNNTLT</u> NSTPLRAACFDGHLIEVKYLVEHKADL
zebra finch	(94)	<u>GHLKVVQCLLDHG-ASVNNNTLT</u> NSTPLRAACFDGHLIEVKYLVEHKADL
<i>C. elegans</i>	(150)	<u>HTPNRHGHTCLMIASYNKVGIVEELLKTGIDV</u> NKKTERGNTALHDAEAS
zebrafish	(143)	<u>EVANRHGHTCLMISCYKGHREIAQFLLEKGA</u> DVNRKSVKGN TALHDCAES
human	(143)	<u>EVSNRHGHTCLMISCYKGHKEIAQYLLEKGA</u> DVNRKSVKGN TALHDCAES
mouse	(143)	<u>EVSNRHGHTCLMISCYKGHKEIAQYLLEKGA</u> DVNRKSVKGN TALHDCAES
chicken	(143)	<u>EVSNRHGHTCLMISCYKGHKEIAQYLLEKGA</u> DVNRKSVKGN TALHDCAES
zebra finch	(143)	<u>EVSNRHGHTCLMISCYKGHKEIAQYLLEKGA</u> DVNRKSVKGN TALHDCAES
<i>C. elegans</i>	(200)	<u>GNVEVVKILLKHGSVLMKDIQGVDP</u> LMGAALSGFLDVLNVLADQMP SGIH
zebrafish	(193)	<u>GSLEIMKMLLKFGASMEKDG</u> YGMTPLLSASVTGHTNIVDFLTAHPQTGLA
human	(193)	<u>GSLDIMKMLLMYCAKMEKDG</u> YGMTPLLSASVTGHTNIVDFLTHHAQTSKT
mouse	(193)	<u>GSLDIMKMLLMYCAKMEKDG</u> YGMTPLLSASVTGHTNIVDFLTHHAQTSKT
chicken	(193)	<u>GSLEIMNMLLKCYCAKMEKDG</u> YGMTPLLSASVTGHTNIVDFLTQHVTNKKA
zebra finch	(193)	<u>GSLEIMKMLLKCYCAKMEKDG</u> YGMTPLLSASVTGHTNIVDFLTQHVTNSKA
<i>C. elegans</i>	(250)	KRD-ALKLLGSTLLDKKMDAMSAMNCWKQSMVPLHADDLRLVREMETFF
zebrafish	(243)	ERISALELLGATFVDKKRDLGALQYWKRAMLRHSEADCMR-REEPLEP
human	(243)	ERINALELLGATFVDKKRDLGALKYWKKAMNMRYSDR TNII SKPVPQTL
mouse	(243)	ERINALELLGATFVDKKRDLGALKYWKKAMNMRYSDR TNII SKPVPQTL
chicken	(243)	DCINALELLGATFVDKKRDLGALKYWKRAMEMRYSDR TNII SKPVPQTL
zebra finch	(243)	ERINALELLGATFVDKKRDLGALKYWKRAMEMRYSDR TNII SKPVPQTL
<i>C. elegans</i>	(299)	EPQEVYEQREAQNIQAQVELLDGNI EAQRMQALVIRER IILGGAHTDVHYY
zebrafish	(292)	EP--AYDCSREVSTAEBELDGLIADPDDMRMQALLIRER IILGPAHPDTSYY
human	(293)	IM--AYDYAKEVNSAELEGLIADPDEM RMQALLIRER IILGSPHPDTSYY
mouse	(293)	IM--AYDYAKEVNSAELEGLIADPDEM RMQALLIRER IILGSPHPDTSYY
chicken	(293)	IM--AYAYAKEVNSSELEENLIADPDEM RMQALLIRER IILGSPHPDTSYY
zebra finch	(293)	IM--AYDYAKEVNSSELEENLIADPDEM RMQALLIRER IILGSPHPDTSYY
<i>C. elegans</i>	(349)	<u>LRFRGAVYCDMGQMNRCYDLWKHALELQ</u> QKHFAPLYGYTITTLQSFHETF
zebrafish	(340)	IRYRGAVYADSGNFERCIRLWKYALDMQ QSNLDPLSPMTASSLLSFAELF
human	(341)	IRYRGAVYADSGNFKRCINLWKYALDMQ QSNLDPLSPMTASSLLSFAELF
mouse	(341)	IRYRGAVYADSGNFKRCINLWKYALDMQ QSNLDPLSPMTASSLLSFAELF
chicken	(341)	IRYRGAVYADSGNFKRCINLWKYALDMQ QSNLDPLSPMTASSLLSFAELF
zebra finch	(341)	IRYRGAVYADSGNFKRCINLWKYALDMQ QSNLDPLSPMTASSLLSFAELF

Fig. 3 (continued)

<i>C. elegans</i>	(399)	SMSLNDFVNNHHANRNLVRSSWVKYVFNGVCLLELERAAWTGPALLEDT
zebrafish	(390)	SFMLQDRAKG-----LLGAGVSFQQLMEILCRSVLEIE
human	(391)	SFMLQDRAKG-----LLGTTVTFDDLMGILCKSVLEIE
mouse	(391)	SFMLQDRAKG-----LLGTTVTFDDLMGILCKSVLEIE
chicken	(391)	SFMLQDRAKG-----LLGTTVTFDDLMGILCKSVLEIE
zebra finch	(391)	SFMLQDRAKG-----LLGTTVTFDDLMGILCKSVLEIE
<i>C. elegans</i>	(449)	ECCGKERQCQHATEESEYKLVYVAVHLVNVLERLSLPSAHIDDSDEEKEP
zebrafish	(423)	RAVKQPRSPDPDPAQLSKLLSIIHLICLLEKLPSCS---PEQDQHKKET
human	(424)	RAIKQTQCP--ADPLQLNKALSIIHLICLLEKVPCT---LEQDHFKKQT
mouse	(424)	RAIKQTQCP--ADPLQLNKALSIIHLICLLEKVPCT---VEQDHFKKQT
chicken	(424)	RAMKQTQCP--PDPIQLNKALAIHLICLLEKVPSCS---SEQEYFKKQT
zebra finch	(424)	RAMKQTQCL--PDPIQLNKALSIIHLICLLEKVPSCS---SEQEHFKKQT
<i>C. elegans</i>	(499)	KADVRLMIVCHELHIIPLLHHTLEERIPDSNSAELGLPKAAVLEQLLELD
zebrafish	(470)	IYRFLKLQACGRNGFSPLHLAVDHNTTCVGRYPVCKFPSLQVAVSLLCEG
human	(469)	IYRFLKLHPRGKNNFSPLHLAVDKNTTCVGRYPVCKFPSLQVTAILECG
mouse	(469)	IYRFLKLHPRGKNNFSPLHLAVDKNTTCVGRYPVCKFPSLQVTAILECG
chicken	(469)	IYKFLKLQPRGKNNFSPLHLAVDNSTTCVGRYPVCKFPSLQVTAILECG
zebra finch	(469)	IYKFLKLQPRGKNNFSPLHLAVDNNTTCVGRYPVCKFPSLQVTAILECG
<i>C. elegans</i>	(549)	LDVNATDKNNDTPMHILLRAREFRKSLVRALLVVRTWLFARNRHGDVVLN
zebrafish	(520)	ADVNSRDLDNNSPLHVAASN--NHPDIMKLLISGGTHFDSFNALQQTACD
human	(519)	ADVNVDRSDNNSPLHIAALN--NHPDIMNLLIKSGAHFDATNLHKQTASD
mouse	(519)	ADVNVDRSDNNSPLHIAALN--NHPDIMNLLIKSGAHFDATNLHKQTASD
chicken	(519)	ADVNVDRSDNNSPLHIAALN--NHPDIMNLLINSGSHFDATNSHRKTASD
zebra finch	(519)	ADVNVDRSDNNSPLHIAALN--NHPDIMNLLIKSGSHFDATNSCKQTASD
<i>C. elegans</i>	(599)	VMKRMKALNHANFDDIPLGRHITLAGLVANAMRVKYPKPFVGVVEQMPLE
zebrafish	(568)	LLDHTQLAKSL----IQPINHTTLQCLAARAIIRHR----LVYRGSIPER
human	(567)	LLDEKEIAKNL----IQPINHTTLQCLAARVIVNHR----IYYKGIPEK
mouse	(567)	LLDEKEIAKNL----IQPINHTTLQCLAARVIVNHR----IYYKGIPEK
chicken	(567)	LLDDKEIAKNL----IQPINHTTLQCLAARVVVNRN----IRYAGHIPEK
zebra finch	(567)	LLDEKEIAKNL----IQPINHTTLQCLAARVIVNHN----ISYAGHIPEK
<i>C. elegans</i>	(649)	LRRFYLAH-
zebrafish	(610)	LEAFVLLHR
human	(609)	LETFSVSLHR
mouse	(609)	LETFSVSLHR
chicken	(609)	LENFILLHR
zebra finch	(609)	LEKFVLLHR

-*W* both stained the entire *W* chromosome. The physical position of *FEMIC-Z* was $38.8 \pm 2.0\%$ from p-terminal of *Z* chromosome.

With RT-PCR, 5'- or 3'-RACE and screening for female zebra finch post-hatch day 1 brain cDNA library, we isolated a 3,108-bp zebra finch *FEMIC-Z* cDNA clone (Genbank EU825191). Despite considerable effort (RT-PCR using female RNA, screening of a female brain cDNA library, 5'- or 3'-RACE using several specific primers for *FEMIC-W* genomic sequence), we were not able to isolate a zebra finch *FEMIC-W* cDNA clone and therefore have no direct evidence for expression of *FEMIC-W*.

Characterization of zebra finch *FEMIC-Z* gene

We compared *FEMIC* gene amino acid sequences among chicken (XM_428816), zebra finch, human (NM_020177), mouse (NM_173423), zebra fish (NM_198145), and *C.*

elegans (J03172; Fig. 3). The *FEM1C* amino acid sequence was highly conserved among *C. elegans* and higher vertebrates (consensus similarity: human–mouse, 99.7%; human–chicken, 94.5%; human–zebra fish, 86.1%; human–*C. elegans*, 46%; chicken–zebra finch, 96.8%), suggesting that it serves important functions. There were eight ankyrin repeat motif regions found by MOTIF search (<http://motif.genome.jp/>) for BLOCKS database (Fig. 3 and Table 1). Interestingly, vertebrates have three more ankyrin repeat sequences in the C-terminal half region relative to *C. elegans*. Assuming that the *C. elegans* form of *fem1c* represents an ancestral form based on the single *fem1* gene in that species, the addition of several whole ankyrin repeats in the lineage leading to the vertebrates suggests adaptive evolution of this region. The chicken does not have one of the ankyrin repeats, located at 482–494aa, as a result of one amino acid substitution.

Southern blot hybridization of the *FEM1C-4* probe, encoding the C-terminal region of *FEM1C-Z*, to an avian

Table 1 Ankyrin repeats located in *FEMIC*. Chicken, zebra finch, human, mouse, and zebra fish *FEMIC* amino acid sequence was analyzed against BLOCKS database (<http://motif.genome.jp/>). The score value represents the quality of the ankyrin repeat, and a score above 1,000 is judged to be a better match to the model repeat

Species	Location (aa)	Sequence	Score
<i>C. elegans</i>	48...60	GRYPLVIAARNGH	1,169
Zebra fish	41...53	GATPLLMAARYGH	1,189
Human	41...53	GATPLLMAARYGH	1,239
Mouse	41...53	GATPLLMAARYGH	1,239
Chicken	41...53	GATPLLVAARYGH	1,257
Zebra finch	41...53	GATPLLMAARYGH	1,239
<i>C. elegans</i>	89...101	GTPPLWAASAAGH	1,082
Zebra fish	83...95	GAPPLWAASAAGH	1,076
Human	83...95	GAPPLWAASAAGH	1,076
Mouse	83...95	GAPPLWAASAAGH	1,076
Chicken	83...95	GAPPLWAASAAGH	1,076
Zebra finch	83...95	GAPPLWAASAAGH	1,076
<i>C. elegans</i>	123...135	RSTPLRGACYDGH	1,073
Zebra fish	116...128	NSTPLRAACFDGH	1,112
Human	116...128	NSTPLRAACFDGH	1,112
Mouse	116...128	NSTPLRAACFDGH	1,112
Chicken	116...128	NSTPLRAACFDGH	1,112
Zebra finch	116...128	NSTPLRAACFDGH	1,112
<i>C. elegans</i>	156...168	GHTCLMIASyrnk	1,072
Zebra fish	149...161	GHTCLMISCYKGH	1,141
Human	149...161	GHTCLMISCYKGH	1,141
Mouse	149...161	GHTCLMISCYKGH	1,141
Chicken	149...161	GHTCLMISCYKGH	1,141
Zebra finch	149...161	GHTCLMISCYKGH	1,141
<i>C. elegans</i>	189...201	GNTALHDAAESGN	1,230
Zebra fish	182...194	GNTALHDAESGS	1,145
Human	182...194	GNTALHDAESGS	1,145
Mouse	182...194	GNTALHDAESGS	1,145
Chicken	182...194	GNTALHDAESGS	1,145
Zebra finch	182...194	GNTALHDAESGS	1,145
<i>C. elegans</i>	N/A	N/A	N/A
Zebra fish	214...226	GMTPLLSASVTGH	1,086
Human	214...226	GMTPLLSASVTGH	1,086
Mouse	214...226	GMTPLLSASVTGH	1,086
Chicken	214...226	GMTPLLSASVTGH	1,086
Zebra finch	214...226	GMTPLLSASVTGH	1,086
<i>C. elegans</i>	N/A	N/A	N/A
Zebra fish	483...495	GFSPLHLAVDHNT	1,122
Human	482...494	NFSPLHLAVDKNT	1,053
Mouse	482...494	NFSPLHLAVDKNT	1,053
Chicken	N/A	N/A	N/A
Zebra finch	482...494	NFSPLHLAVDNNT	1,064
<i>C. elegans</i>	N/A	N/A	N/A
Zebra fish	529...541	DNSPLHVAASNNH	1,196
Human	528...540	DNSPLHIAALNNH	1,192
Mouse	528...540	DNSPLHIAALNNH	1,192
Chicken	528...540	NNSPLHIAALNNH	1,196
Zebra finch	528...540	NNSPLHIAALNNH	1,196

zoo blot detected both Z and W sequence in DNA of the common finch (Fig. 4a). Although the snowy owl, which is interestingly classified in the same “land birds” clade as zebra finch according to recent phylogenetic analysis (Hackett et al. 2008), had a very faint female-specific signal, other birds (except for ostrich) only had a Z-linked signal pattern. With the N-terminal region of *FEMIC* probe (FEM1C-3), no signal was detected in domestic duck and the signals in Japanese quail and snowy owl were faint (Fig. 4b), which would suggest poor conservation of this region in birds. The hybridization to ostrich was similar in the two sexes, suggesting that *FEMIC* is present in the large pseudoautosomal region common to the Z and W chromosome of that species, although minor divergence is not ruled out.

By northern blot analysis, the *FEMIC* is equally expressed in males and females (Fig. 5), but in gonads, the expression in testis is higher than in ovary. The ratio of *FEMIC* to *GAPDH* expression is not obviously different in testis vs.

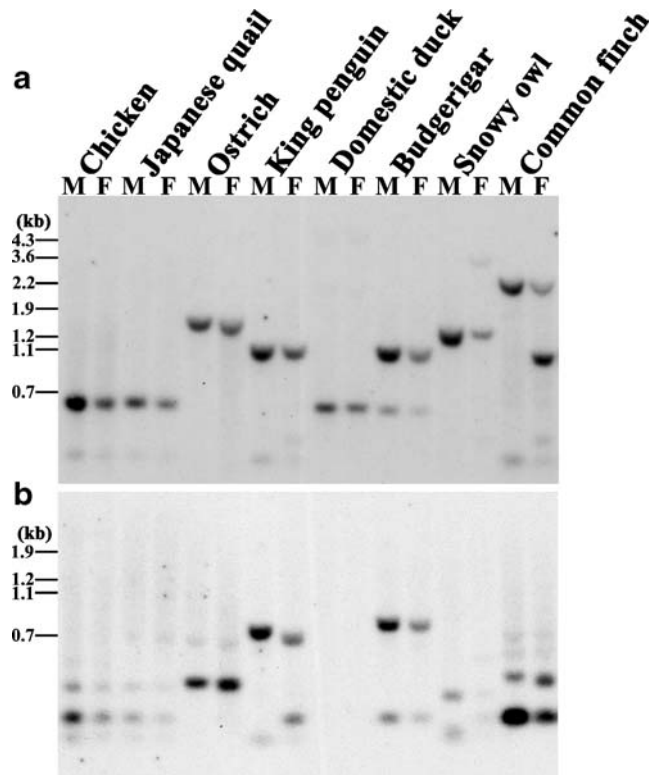
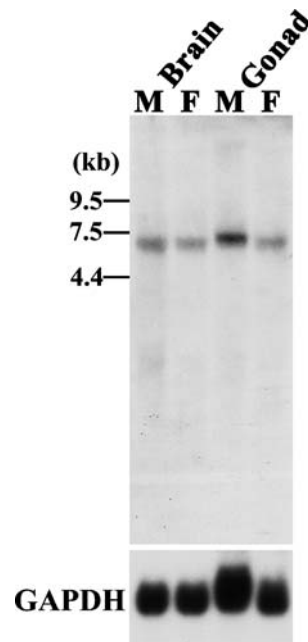


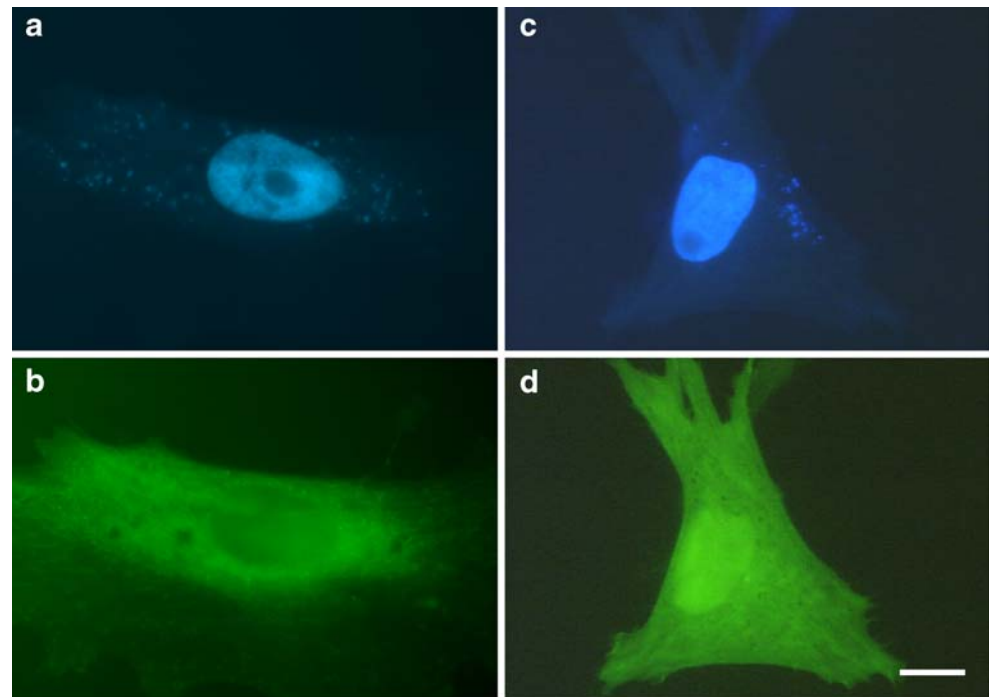
Fig. 4 Zoo Southern blot analysis for Passerine (finch) and non-Passerine birds. Two *FEMIC*-Z probes were used: **a** probe FEMIC-4 encoding the C-terminal region which recognizes both Z and W in zebra finch, **b** probe FEMIC-3 encoding the N-terminal region which only recognizes Z in zebra finch. Four micrograms of *Hae*III digested male or female genomic DNA was electrophoresed in each lane. Species include the chicken (*Gallus g. domesticus*), Japanese quail (*Coturnix c. japonica*), ostrich (*Struthio camelus*), king penguin (*Aptenodytes patagonica*), domestic duck (*Anas platyrhynchos domestica*), budgerigar (*Melopsitacus undulatas*), snowy owl (*Nyctea scandiaca*), and common finch (*Lonchura striata* var)

Fig. 5 Northern blot analysis of zebra finch adult brain and gonad total RNA with zebra finch *FEM1C-Z* probe (*FEM1C-4*) and *GAPDH* as a control



ovary, but the use of a loading control such as *GAPDH* is problematic because of the fundamental differences in cell types in the two tissues. It is not clear if the higher expression in testis suggests testis-specific function, particularly because most *Z* genes are not dosage-compensated and are thus expected to be expressed higher in males than females (Itoh et al. 2007; Ellegren et al. 2007; Arnold et al. 2008). The northern blot offers no strong evidence for alternative splicing in testis as occurs in mouse and human (Ventura-Holman et al. 2003).

Fig. 6 The localization of zebra finch *FEM1C-Z*-GFP fusion protein in the cell body (**b**). The nucleus of the same cell is stained with DAPI in **a**. As a control, the pEGFP-N3 vector was also transfected in zebra finch fibroblast (**c**, **d**). Unlike pEGFP-N3 vector control, *FEM1C-Z*-GFP fusion protein was excluded from nucleus, which suggests cytoplasmic localization of this protein



To test *FEM1C* function further, we analyzed the cellular localization of green fluorescence protein (GFP) fusion protein for zebra finch *FEM1C-Z*. Interestingly, in zebra finch embryonic fibroblast cells, GFP-*FEM1C-Z* fusion protein was localized in cytoplasm (Fig. 6a, b), and this pattern was not observed in cells transfected with the EGFP protein expression as a control (Fig. 6c, d).

BAC sequence analysis of zebra finch *FEM1C-W*

We found no evidence for expression of a *W* form of *FEM1C*, but could have missed it if expression is restricted to a short developmental period or specific tissue. The absence of a *W* form in most bird species measured in the zoo blot (Fig. 4) supports the idea that a *W* form is not functionally important in birds. To clarify the sequence of *FEM1C-W*, we analyzed the sequences of two BAC clones encoding *FEM1C-Z* and *-W*. Unfortunately, the first one or two exons of *FEM1C* were missing in each clone. Converting these BAC-derived *FEM1C* sequences to amino acid sequences, the predicted *FEM1C-W* sequence aligned closely to that of *FEM1C-Z*, except for two stop codons detected inside the coding region (Fig. 7). Thus, at most, a truncated form of *FEM1C-W* is likely to be expressed.

Table 2 shows the *dN*, *dS*, and *dN/dS* values for the comparison among human, mouse, chicken, and zebra finch. Higher *dN/dS* values denote either adaptive evolution or relaxed constraint. High conservation (low *dN/dS*) was observed between human and mouse, suggesting an

Fig. 7 Comparison of zebra finch FEM1C-Z amino acid sequence and predicted FEM1C-Z and W derived from BAC DNA sequences (clones 297J07 and 197F15). Two underlined asterisks show the stop codons in the predicted FEM1C-W sequence

Predicted Fem1c-W (1)	-----GNTALHDCAESGS*EIMKM
Predicted Fem1c-Z (1)	-----GNTALHDCAESGSLEIMKM
Fem1c-Z cDNA (151)	TCLMISCYKGHKEIAQYLLLEKGDVNRKSVKNTALHDCAESGSLEIMKM
Predicted Fem1c-W (19)	LLKYCAKMEKDGYGMPLLSASVTGHTNIVDFLTQHEQTSKIECINALEL
Predicted Fem1c-Z (20)	LLKYCAKMEKDGYGMPLLSASVTGHTNIVDFLTQHVTQSKAERINALEL
Fem1c-Z cDNA (201)	LLKYCAKMEKDGYGMPLLSASVTGHTNIVDFLTQHVTQSKAERINALEL
Predicted Fem1c-W (69)	LGATFVDKRRDLLCALKYWKQAMEMRYNDRISILNRPVPQTLIMAYDYAK
Predicted Fem1c-Z (70)	LGATFVDKRRDLLGALKYWKRAMEMRYSDRTNILLSKVPVQTLIMAYDYAK
Fem1c-Z cDNA (251)	LGATFVDKRRDLLGALKYWKRAMEMRYSDRTNILLSKVPVQTLIMAYDYAK
Predicted Fem1c-W (119)	EVNSFEELNLIADPDEMRMQUALLIRERILGSPHPDTSYYIRYRGAVYAD
Predicted Fem1c-Z (120)	EVNSFEELNLIADPDEMRMQUALLIRERILGSPHPDTSYYIRYRGAVYAD
Fem1c-Z cDNA (301)	EVNSFEELNLIADPDEMRMQUALLIRERILGSPHPDTSYYIRYRGAVYAD
Predicted Fem1c-W (169)	SGNFKRCINLWKYALDMQOSNLDPLSPMTDSSLLSFAELFSFMLQDKAKG
Predicted Fem1c-Z (170)	SGNFKRCINLWKYALDMQOSNLDPLSPMTASSLLSFAELFSFMLQDRAKG
Fem1c-Z cDNA (351)	SGNFKRCINLWKYALDMQOSNLDPLSPMTASSLLSFAELFSFMLQDRAKG
Predicted Fem1c-W (219)	LLGATVTFDDLMGILCKSVLEIERAMKQIQCPDPPIQLNKALSIIHLHIC
Predicted Fem1c-Z (220)	LLGTTVTFDDLMGILCKSVLEIERAMKQTQCLDPPIQLNKALSIIHLHIC
Fem1c-Z cDNA (401)	LLGTTVTFDDLMGILCKSVLEIERAMKQTQCLDPPIQLNKALSIIHLHIC
Predicted Fem1c-W (269)	LLEKVPCTSEQEHFKKQTIYKFLKLPQKGNFNSPLHLAVDKNNTTCVGRY
Predicted Fem1c-Z (270)	LLEKVPCTSEQEHFKKQTIYKFLKLPQKGNFNSPLHLAVDKNNTTCVGRY
Fem1c-Z cDNA (451)	LLEKVPCTSEQEHFKKQTIYKFLKLPQKGNFNSPLHLAVDKNNTTCVGRY
Predicted Fem1c-W (319)	PVCKFPSLQVTAAILVECGANINVRSDNNSPLHIAALNNHGLIMNFLIES
Predicted Fem1c-Z (320)	PVCKFPSLQVTAAILVECGADVNRSDNNSPLHIAALNNHPDIMNLLIKS
Fem1c-Z cDNA (501)	PVCKFPSLQVTAAILVECGADVNRSDNNSPLHIAALNNHPDIMNLLIKS
Predicted Fem1c-W (369)	GSHFDATNFHKQTARDLLDEKKIAKNLIHPINHTTL*CLAACFIVNHNIC
Predicted Fem1c-Z (370)	GSHFDATNSCKQTASDLLDEKEIAKNLIQPINHTTLQCLAARVIVNHNIS
Fem1c-Z cDNA (551)	GSHFDATNSCKQTASDLLDEKEIAKNLIQPINHTTLQCLAARVIVNHNIS
Predicted Fem1c-W (418)	YAGHIPEKLEKVFLLHR
Predicted Fem1c-Z (420)	YAGHIPEKLEKVFLLHR
Fem1c-Z cDNA (601)	YAGHIPEKLEKVFLLHR

important functional role for *Fem1c* in mammals. In contrast, the dN/dS value was markedly higher when comparing chicken vs. zebra finch not because of a lower dS which is about the same (~0.325) in both comparisons but because of a higher dN value (Table 2). This observation suggests higher conservation of *Fem1c* gene in mammals. In the comparison of zebra finch FEM1C-Z and -W, the dN/dS value was higher than chicken Z vs. zebra finch Z (Table 2) because of lower dS (0.271) and higher dN (0.036) relative to the chicken Z vs. zebra finch Z comparison (dS=0.325, dN=0.024). The phylogenetic tree of avian FEM1C genes (Fig. 8) suggested a closer relationship of zebra finch FEM1C-Z and -W than either with chicken FEM1C based on both nucleotide substitution and synonymous mutations (Fig. 8).

The divergence of the nucleotide sequences of FEM1C-Z and -W varies considerably along their lengths (Fig. 9). Although specific regions of the gene showed high or low dN/dS in the mammal–bird comparisons, there was no remarkable peak observed in the mammal to mammal (human to mouse) comparison (Fig. 9a). The pattern of peaks and valleys was quite similar when comparing mammal to either chicken or zebra finch (Fig. 9b). In bird

to bird comparison (chicken to zebra finch), the same pattern was recognizable (Fig. 9c). These observations suggest FEM1C sequences in birds evolved mainly after divergence of birds and mammals. Moreover, evolutionary

Table 2 The dN/dS values comparing FEM1C in human, mouse, chicken, and zebra finch

	Chromosome	dN	dS	dN/dS
Human/mouse	A/A	0.002	0.325	0.007
Human/chicken	A/Z	0.053	0.592	0.089
Mouse/chicken	A/Z	0.056	0.635	0.088
Human/zebra finch	A/Z	0.039	0.594	0.066
Mouse/zebra finch	A/Z	0.042	0.678	0.061
Chicken/zebra finch	Z/Z	0.024	0.326	0.074
Chicken/zebra finch*	Z/Z	0.024	0.325	0.073
297J07/197F15**	Z/W	0.036	0.271	0.133

*Comparison of chicken FEM1C-Z to zebra finch sequences derived from Z BAC clone 297J07. The dN, dS, and dN/dS values are quite similar to full length comparison.

**Comparison of zebra finch FEM1C-Z and -W based on the sequences from Z (297J07) and W BAC clones (197F15). The sequences from the BAC clones lack N-terminal sequences found in FEM1C-Z cDNA sequence

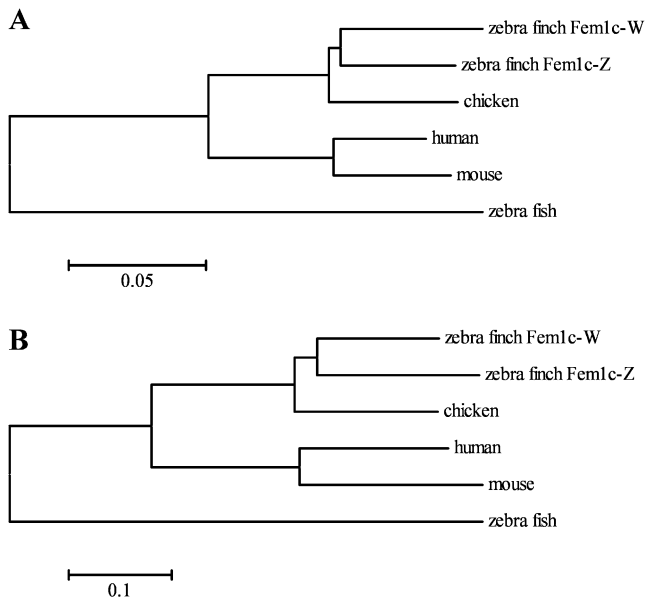


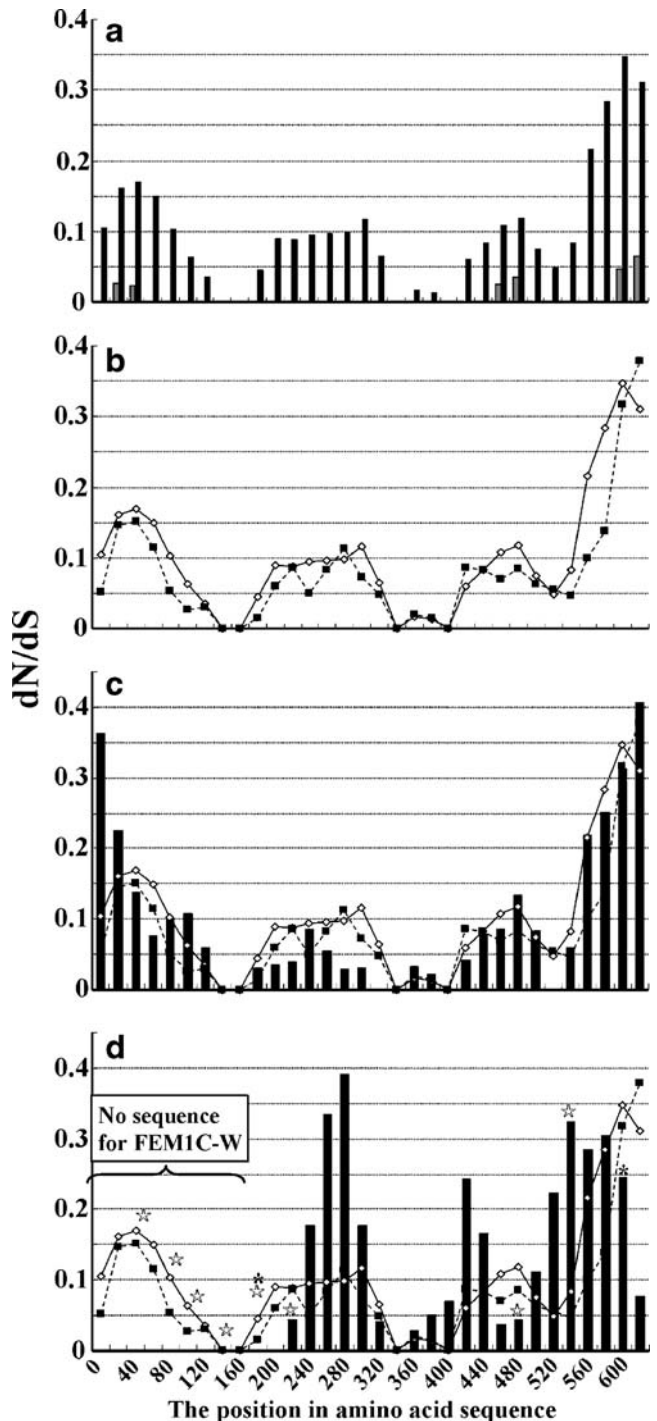
Fig. 8 Neighbor-joining phylogenetic trees of the *Fem1c* gene family based on the currently available coding sequences. Branch lengths indicate relative relatedness. **a** p-distance for nucleotide substitutions resulting in any nucleotide substitutions. **b** p-distance for synonymous changes only. *Bar*, substitutions per nucleotide site

pressure was not equal along the sequence, but different bird species (chickens and zebra finches) show a common divergence from mammals, suggesting that the mammal–bird difference preceded the galliform–passerine split. Zebra finch *FEMIC-Z* and *-W* diversion was more significant compared to human–birds (Fig. 9d). The peaks occurred at three general positions, although the locations were slightly different from human–birds, one of which contains a stop codon. The other stop codon is outside the peak at amino acid position 195 in a region of evolutionary

Fig. 9 The dN/dS values as a function of position along *FEMIC* genes. **a** The comparison of human–chicken (mammal–bird, *black*) and human–mouse (mammal–mammal, *gray*). *FEMIC* gene is highly conserved along the entire open reading frame between human and mouse. **b** Mammal–bird comparison: human–chicken Z (*open square, solid line*) and human–zebra finch Z (*closed square, dashed line*). The positional dN/dS values of chicken and zebra finch have similar pattern along the entire sequence. **c** Bird–bird comparison: chicken Z vs. zebra finch Z. The *histograms* show comparison of the two birds, and the *line graphs* of the mammal–bird comparison (**b**) are superimposed as a reference. The valleys and peaks of bird–bird dN/dS values followed the pattern of mammal–bird values, suggesting the region-specific changes occurred after divergence of mammals and birds. **d** The comparison of *FEMIC-Z* and predicted *FEMIC-W* in zebra finch. The graph of mammal–bird comparison (**b**) is shown as a reference. The valleys and peaks of zebra finch *FEMIC-Z* and *-W* have a pattern roughly similar to that of the mammal–bird comparison, but the values are larger. *Asterisks* are the positions of stop codons in *FEMIC-W* and *open stars* are the ankyrin repeat sites. The numbers on the *abscissa* are the amino acid positions on the zebra finch *FEMIC-Z* with the start Met as position 1

conservation (Figs. 7 and 9d). This pattern suggests that the nonsense mutations occur randomly relative to divergence on the Z and W sequences. There is no obvious relation between the positions of the valleys/peaks and ankyrin repeats.

The zebra finch repetitive sequence ZBM (Itoh et al. 2008) was found in the *FEMIC-W* BAC clones. This ZBM repeat sequence is found at high density on the W



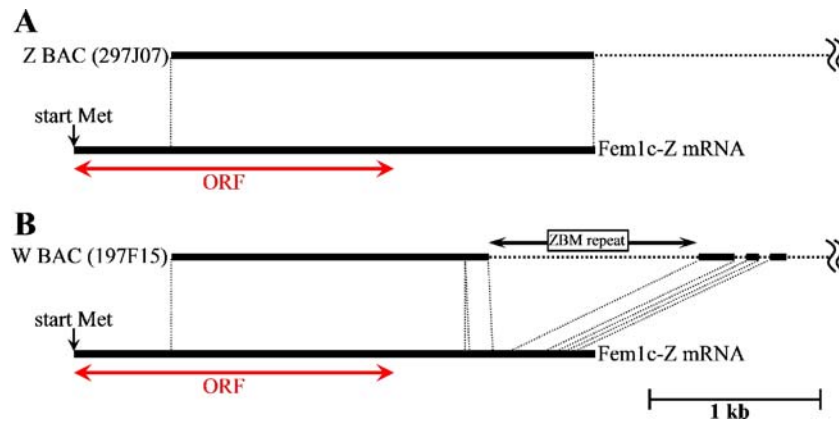


Fig. 10 Comparison of *FEMIC-Z* mRNA sequence and Z BAC 297J07 (a) and W BAC 197F15 (b). The sequences homologous to *FEMIC-Z* mRNA sequence are shown as a *thick solid line* in both Z and W BAC sequences and non-homologous genomic sequences as a *dashed line*. In both 297J07 and 197F15 BAC sequences, at least one exon covering the region from the start Met site to 543 bp is missing.

chromosome but at lower density on the other chromosomes and tends to avoid gene coding regions (Itoh et al. 2008). The ZBM repetitive sequence is inserted into in 3'-UTR region of the predicted *FEMIC-W* sequence (Fig. 10).

Discussion

We have identified a *FEMIC* gene homologue in chicken by comparative analysis of the human genome database and confirmed its sex chromosome linkage in zebra finch. The *FEMIC* sequences were located on Z and W chromosome in snowy owl, common finch, and zebra finch, but only on the Z chromosome in several other non-ratite bird species. The *FEMIC-W* sequence in zebra finch contained two stop codons in the sequence we analyzed. Together with the absence of W sequences in other bird species, the insertion of stop codons in the zebra finch W sequence suggests that *FEMIC-W* gene in birds has or is undergoing degradation. If that is the case, however, it is surprising that much of the protein sequence of *FEMIC-W* is conserved in zebra finch. If the W form is totally non-functional because of the presence of stop codons, one would have expected the accumulation of other non-synonymous mutations in

In the Z chromosome BAC sequence, there was no intron sequence found in the region corresponding to the 544- to 3,108-bp region of *FEMIC-Z* cDNA sequence (Genbank: EU825191). The zebra finch W chromosome repetitive ZBM repeat sequence (Itoh et al. 2008) was found in the predicted 3'-UTR of *FEMIC-W*

the coding sequence. Similar W degradation has been reported in *ATP5A1* gene in parrots, but unlike *FEMIC-W*, the majority of species possesses a highly conserved *ATP5A1W* gene (de Kloet 2001). *C. elegans fem-1* genes are critical for male sex determination; thus, if the avian

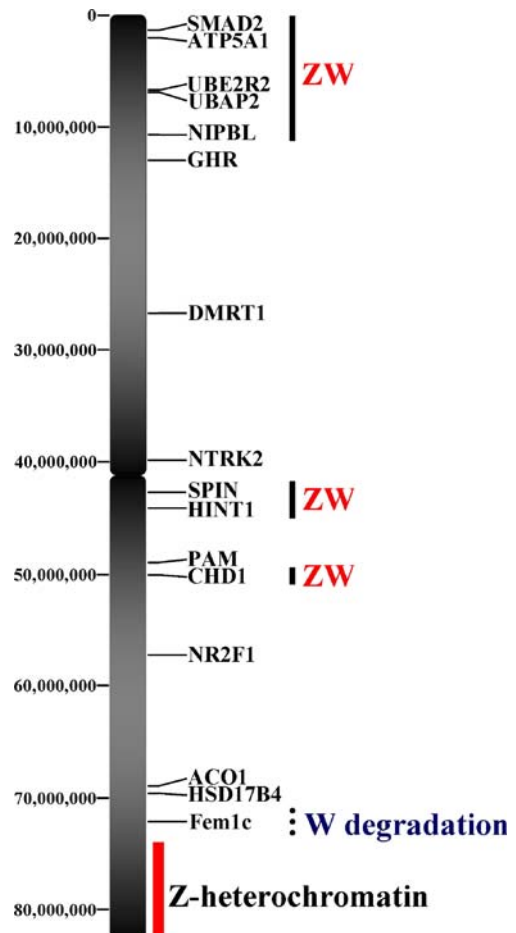


Fig. 11 The chicken *FEMIC-Z* gene is localized around the long arm terminal heterochromatic region of Z chromosome. The gene positions are based on chicken genome assembly 2 (May 2006, www.ensembl.com). The other ZW genes are clustered on the terminal region of short arm and the centromeric region of the long arm. The Zq heterochromatin was identified by Hori et al (1996), but is apparently absent from the current chicken Z sequence. Thus, the length of Z chromosome shown here is larger than the published chicken Z chromosome sequence

FEMIC gene is also involved in a similar signaling cascade, the elimination of *FEMIC-W* might be advantageous to females. We did not detect any sex difference in expression of *FEMIC-Z* in brain, but the testis appeared to have slightly higher expression than the ovary. The cytoplasmic localization of *FEMIC-Z* gene in zebra finch fibroblast is similar to that of the *C. elegans fem-1* gene. The presence of *FEMIC* on the Z chromosome is compatible with its potential role as a male-benefit testis gene (Vallender and Lahn 2004), but our results do not establish a function for *FEMIC*.

Birds diverged from mammals about 310 Mya (million years ago), and the monotreme platypus diverged from the other mammals about 210 Mya. In platypus, there are five X and five Y sex chromosomes, both of which form chains during the meiosis. One of the candidate avian sex determination genes, *DMRT1*, is localized on chromosome X5 in platypus. In human, the *FEMIC* gene is localized on human 5q22.3, close to the *DMXL1* locus (5q23.1; Rens et al. 2007). *FEMIC* and *DMXL1* have remained in a syntenic block during mammalian and avian evolution. The *DMXL1* gene is on platypus chromosome X5 near *DMRT1* (Rens et al. 2007), but *FEMIC* is not found in the platypus genome database (<http://www.ensembl.org>). Although both *DMRT1* and *FEMIC* relate to the sex determination in fly and nematode, their role in birds and platypus are unknown even though they are situated on the sex chromosomes where a testis-determining role could be favored. The other *Fem1* homologous genes, *Fem1a* and *Fem1b*, are found on chicken chromosome 28 and 10, respectively, and other components of the *C. elegans* sex-determining pathway (e.g., *tra-1*, *fem-2*, *elc-1*, and *cul-2*) show no conservation in the chicken genome so far.

Avian ZW chromosome genes reported so far appear to be conserved widely across species: *SMAD2* (Mizuno et al. 2002), *ATP5A1* (Nanda et al. 2000), *UBE2R2* (Scholz et al. 2006), *UBAP2* (Handley et al. 2004), *NIPBL* (Berlin and Ellegren 2006), *SPIN* (Itoh et al. 2001), *HINT1* (Hori et al. 2000; O'Neill et al. 2000), *CHD1* (Ellegren 1996). ZW genes are clustered on the chicken Z chromosome at the terminal end of short arm and the centromeric region of long arm (Fig. 11). *FEMIC* was located nearly at the terminal end of long arm in chicken genome database near what we believe to be the boundary of the Zq terminal heterochromatic region (Hori et al. 1996). The *FEMIC-Z* gene is therefore located outside of the other clusters of ZW genes. Based on reported evolutionary strata of Z chromosome using the divergence ratio between Z and W gene (Handley et al. 2004), the terminal region of short arm (including *ATP5A1*) differentiated about 58–85 Mya, and the centromeric region of Zq that includes *SPIN*, *HINT1*, and *CHD1* is older and ceased recombination about 102–170 Mya. However, as

we mentioned above, the recent chicken Z chromosome map suggests that ZW genes are restricted to specific regions although there is a large area unanalyzed because of lack of information on ZW genes. It is also possible that this region lacks ZW genes. At the terminal region of long arm where *FEMIC-Z* gene located, the homologous W sequence has completely diverged from Z in chicken, but this divergence has not been completed in zebra finch. These species comparisons suggest that it will be useful to examine the sequence of other species Z chromosome to analyze evolutionary strata and estimate the degree of ZW divergence.

Acknowledgments This work was supported by NIH grant DC000217 to A.P. Arnold and a Yamada Science Foundation grant to Y. Itoh. BAC sequences were determined by the Washington University Genome Center under the supervision of Dr. Wes Warren. We thank Klaus-Peter Koepfli and Robert Wayne for the helpful discussions.

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