Expansion and Evolution of the X-Linked Testis Specific Multigene Families in the *melanogaster* Species Subgroup

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Abstract

The testis specific X-linked genes whose evolution is traced here in the *melanogaster* species subgroup are thought to undergo fast rate of diversification. The **CK2**- and **NAC**-like genes encode the diverged regulatory β-subunits of protein kinase **CK2** and the homologs of β-subunit of nascent peptide associated complex, respectively. We annotated the **CK2**-like genes related to **CK2**-family in the D. *simulans* and D. *sechellia* genomes. The ancestor **CK2**-like genes preserved in D. *simulans* and D. *sechellia* are considered to be intermediates in the emergence of the D. *melanogaster* specific **Stellate** genes related to the **CK2**-family. The **CK2**-like genes are more similar to the unique autosomal **CK2**-genes than to **Stellate** genes, taking into account their peculiarities of polymorphism. The formation of a variant the **CK2**-gene **Stellate** in D. *melanogaster* as a result of illegitimate recombination between a NAC**tes** promoter and a distinct polymorphic variant of **CK2**-like ancestor copy was traced. We found a close nonrandom proximity between the dispersed defective copies of **DINE-1** transposons, the members of Helitron family, and the **CK2**- and **NAC**-genes, suggesting an involvement of **DINE-1** elements in duplication and amplification of these genes.


Editor: Dmitry I. Nurminsky, University of Maryland School of Medicine, United States of America

Received December 5, 2011; Accepted April 23, 2012; Published May 23, 2012

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Funding: This work was supported by the Molecular and Cellular Biology Program of Russian Academy of Sciences and the Russian Foundation for Basic Research grant (No. 11-04-00017-a). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The availability of genome sequences of related species permits to retrace the origination of new gene families [1]. New X-linked testis specific genes are thought to evolve frequently [2–4]. Recently, a role of the highly abundant transposable element **DINE-1** (also named **INE-1** and **DNAREP**) in the emergence of these genes in the *Drosophila* genomes has been suggested [5–7]. Using available data sets of genome sequences from FlyBase [8], we traced the origination and amplification in the *melanogaster* subgroup species of the X-linked testis specific genes related to two multigene families, **CK2**- and **NAC**-genes, encoding regulatory β-subunit of protein kinase **CK2** and β-subunit of protein nascent associated complex (NAC), respectively. **CK2** is a serine/threonine kinase that participates in a wide variety of cellular processes including cell differentiation, proliferation and survival [9–11]. The regulatory β-subunit ensures stability and specificity of **CK2**, and may also have functions distinct from **CK2** as a component of some other protein kinases [9,11]. Both conservative α- and β-subunits of NAC are known to contact with nascent polypeptide chains on the ribosome and contribute to the prevention of inappropriate interactions during the folding of nascent polypeptide [12]. The importance of NACβ in *vivo* function is emphasized by the early embryonically lethal *bicoid* phenotype of a NACβ mutant in *D. melanogaster* [13]. The testis specific functions of both **CK2**- and **NAC**-proteins remain elusive.

**D. melanogaster** contains several paralogous **CK2** protein kinase genes supposed to be involved in specification of **CK2** targeting in cells [14]. The single autosomal gene on chromosome 2 encodes protein kinase **CK2** regulatory β-subunit. The homologous amplified copies of the X-linked **Stellate** genes are normally silenced but have been shown to be expressed in the testes of *D. melanogaster* due to the absence of their Y-linked specific suppressors [14,15]. The unique autosomal **CK2**-genes are located in homologous regions in the *D. melanogaster*, *D. sechellia*, *D. yakuba*, and *D. erecta* genomes according to FlyBase [8], while its presence in *D. simulans* requires a much more detailed analysis of convincing sequencing results. The amplified **Stellate** genes are found only in *D. melanogaster*, their derepression in testes leads to male sterility or semi-sterility owing to the abnormality of chromosome condensation and nondonjunction of sex chromosomes [16,17]. Interest in **Stellate** genes has been inspired by the discovery of a RNA silencing mechanism of their repression [18]. The evolutionary significance of **Stellate** genes emergence remains an enigma, possibly their putative function is not limited to the modulation of protein kinase **CK2** activity, but is also related to chromatin assembly [19]. Actually, protein kinase **CK2** is predominantly a nuclear protein [9]. **Stellate** protein has been detected in both cytoplasm and nucleus, and an ability of lysine methylated **Stellate** to mimic epitope of H3K9me3 histone has been shown [19]. This observation suggests a capacity of **Stellate** protein to compete with some chromatin “readers” of histone H3K9me3 mark. The
The emergence of the CK2\textit{tes} family of Stellate gene has been driven by an acquisition of promoter from the \textit{NAC\beta\textit{tes}} gene [20].

Here we annotated in \textit{D. sechellia} and \textit{D. simulans} several paralogous genes related to CK2\textit{beta} family and designated as a new multigene family of CK2\textit{tes-like} genes. The estimation of a similarity of these genes to the unique autosomal CK2\textit{beta} genes and \textit{Stellate} genes in \textit{D. melanogaster} allowed us to consider a putative CK2\textit{tes-like} ancestor as an intermediate in the origination of \textit{Stellate} genes. Although only single copy of the \textit{NAC\beta\textit{tes}} gene is revealed in \textit{D. yakuba}, similar patterns of the X-linked amplifications of \textit{NAC\beta\textit{tes}} genes are detected in \textit{D. melanogaster} and sister \textit{D. simulans}/\textit{D. sechellia} species. The copies of amplified \textit{NAC\beta\textit{tes}} and CK2\textit{tes} gene families are localized in a restricted syntenic region (\~{}300–400 kb) in \textit{D. melanogaster} and \textit{D. simulans}/\textit{D. sechellia}.

Using available genomic data sets of FlyBase [8] we demonstrated the juxtaposition of the repeated young X-linked CK2\textit{tes-like} and \textit{NAC\beta\textit{tes}} genes to polymorphic fragments of \textit{DINE-1} transposable elements related to an enigmatic \textit{Helitron} type. A close nonrandom location of \textit{DINE-1}\textit{es} to these amplified copies hints for \textit{DINE-1}\textit{s} participation in the expansion of these protein-coding genes.

**Results and Discussion**

The structures of syntenic regions of the X-chromosomes of \textit{D. melanogaster}, closely related \textit{D. sechellia}/\textit{D. simulans} and \textit{D. yakuba} are presented in Fig. 1. These regions contain \textit{Stellate}, CK2\textit{tes-like} and \textit{NAC\beta\textit{tes}} genes. The synteny is clearly demonstrated by relative positions of gene \textit{bendless} (\textit{ben}) as well as CG12480/GM17653/GD17153/GE17116 and CG9400/GM17559/GD15853/GE16115. The annotation procedure allowed us to present orthologs CG18313/CG32601/CG32598/CG18157/GC13402 have been annotated earlier in \textit{D. melanogaster} as \textit{NAC\beta\textit{tes}} genes [20]. We have identified in the syntenic regions of the X-chromosomes in \textit{D. simulans} and \textit{D. sechellia} the CK2\textit{tes-like} genes related to autosomal CK2\textit{beta} gene (CG13591) in \textit{D. melanogaster}. We found the fragments of CK2\textit{beta} genes (\textit{y}\textit{CK2\beta\textit{tes}}) in \textit{D. simulans} and \textit{D. sechellia} at the same site where a cluster of \textit{Stellate} genes is known to be emerged in \textit{D. melanogaster}. The fragments of \textit{DINE-1} elements were localized in syntenic region of \textit{D. melanogaster}, \textit{D. simulans} and \textit{D. sechellia}.

The presented evolutionary tree of the representatives of the CK2\textit{beta} family. We traced the uprising of gene \textit{Stellate} as a result of illegitimate recombination between the \textit{NAC\beta\textit{tes}} promoter and a definite polymorphic variant of CK2\textit{beta-like} ancestor. At last we showed nonrandom associations of the remnants of \textit{DINE-1} elements with CK2\textit{beta-like}, \textit{Stellate} and \textit{NAC\beta\textit{tes}} genes.

**The family of the \textit{NAC\beta\textit{tes}} genes**

The \textit{NAC\beta\textit{tes}} genes in \textit{D. melanogaster} (CG13402, CG18157, CG32598, CG32601 and CG18313) are indicated according to our earlier published data [20]. \textit{D. melanogaster}, \textit{D. sechellia} and \textit{D. simulans} have several copies of highly homologous \textit{NAC\beta\textit{tes}} genes but the \textit{D. yakuba} genome contains only a single copy (GE17140). \textit{D. simulans} and \textit{D. sechellia} contain a pair of duplicated \textit{NAC\beta\textit{tes}} copies similar to those in \textit{D. melanogaster}, demonstrating their evolving in the common ancestor of these species. The \textit{NAC\beta\textit{tes}} genes may be considered the young ones, due to their presence in the \textit{melanogaster} subgroup species [20], but not in the \textit{D. pseudoobscura} taking into account available data sets of FlyBase. The \textit{NAC\beta\textit{tes}} pseudogenes are located adjacent to GM17553 and GD24509 in \textit{D. sechellia} and \textit{D. simulans}, respectively, but a complete sequence of \textit{D. simulans} pseudogene is not yet available (Fig. 1, Fig. S1). The duplicated copies of \textit{NAC\beta\textit{tes}} in \textit{D. sechellia} are located in the same region in \textit{D. melanogaster}, but in \textit{D. sechellia} these genes are flanked by CK2\textit{beta-like} copies (pair of genes GM17555/GM17556 and gene GM17552) (Fig. 1), forming a cluster of \textit{NAC\beta\textit{tes}} and CK2\textit{beta-like} genes.

**The family of the CK2\textit{beta-like} genes**

The CK2\textit{beta-like} copies comprise a new gene family represented by the variants of CK2\textit{beta} family genes that has been amplified in the \textit{D. sechellia}/\textit{D. simulans} lineage. The CK2\textit{beta-like} genes are homologous to the unique autosomal CK2\textit{beta} gene located in syntenic regions of the \textit{D. melanogaster}, \textit{D. sechellia} and \textit{D. yakuba} genomes. The precise genomic structure of homologous region in \textit{D. simulans} is not yet solved and only a single copy of CK2\textit{beta-like} (GD24508) is annotated here. However, some unannotated CK2\textit{beta-like} copies in \textit{D. simulans} may be also attributed to this region (Fig. 1). The tests specific transcription of a representative of this family, GD24508 in \textit{D. simulans}, was shown (Fig. S2). This observation allows us to consider this gene family as a tests specific one. \textit{D. yakuba} contains no CK2\textit{beta-like} genes on the X-chromosome and elsewhere in the genome.

Multiple alignment of amino acid residues of proteins and phylogenetic tree related to CK2\textit{beta} family genes (CK2\textit{beta}, CK2\textit{beta-like} and \textit{Stellate}) is shown in Fig. S3. The peculiarities of amino acid substitution patterns (Fig. S3A) as well as protein phylogenetic analysis (Fig. S3B) allow us to discriminate CK2\textit{beta-like} proteins as a distinct novel subfamily, and the phylogenetic tree demonstrates the origin of \textit{Stellate} genes from CK2\textit{beta-like} ancestor.

The CK2\textit{beta} subunit is remarkably conserved among species [21,22]. All CK2\textit{beta} subunits carry at their N-termini the site S2 of autophosphorylation known to be involved in CK2\textit{beta} stabilization [23]. All variants of CK2\textit{beta-like} subunits preserve zinc fingers with cysteines (Fig. S3) that are responsible for dimer CK2\textit{beta} formation and its association with catalytic subunit [10]. CK2\beta is reminiscent of cyclins that are regulatory subunits of cyclin-dependent kinases and has a motif involved in regulation of cyclin degradation. Significant similarity is observed in degradation motif DKENTGLN [9] in different CK2\textit{beta} subunits, the KFN1 sequence is preserved in CK2\textit{beta} subunits encoded by unique autosomal and amplified CK2\textit{beta-like} genes but not in \textit{Stellate}. The acidic loop of CK2\beta is involved in regulation of catalytic subunit activity by modulating polyanine binding [9]. The DPEFDNED motif of acidic loop is significantly varied in CK2\textit{beta} proteins: the number of acidic residues in duplicated X-linked CK2\textit{beta-like} subunits is reduced to two residues compared to four residues in autosomal CK2\textit{beta} subunits encoded by unique genes. Possibly, these differences may be related to the peculiarities of functional modulations of the activity of these proteins.

The degree of nucleotide similarity between coding region of CK2\textit{beta-like} pairs GM17552/GM17570, GM17555/GM17552 and GD15860/GD24508 of paralogs approximates 83–86%. The extent of interspecific similarity between pair of orthologous copies GD13590/GM17570 and GD24508/GM17552 approximates 93% and 95%, respectively. Two paralogs, GM17552 and GM17556, in \textit{D. sechellia} as well as the ortholog GD24508 in \textit{D. simulans} are characterized by quite similar patterns of nucleotide substitutions (Fig. 1, Fig. S4). This similarity may be explained by duplication of the ancestor gene GM17552 and formation of a new copy GM17556 in \textit{D. sechellia}. We found two practically identical CK2\textit{beta-like} copies in \textit{D. sechellia} (GM17557a, GM17557b) separated by a sequence containing \textit{DINE-1} fragments (Fig. 1, Fig. S4). We also detected a fragment of CK2\textit{beta-like} gene in \textit{D. sechellia} and a vestige of its presence in \textit{D. simulans} in a
Figure 1. Scheme of syntenic X-chromosome regions comprising the CK2ßtes and NACßtes multigene families in Drosophila species.

The synteny is demonstrated by vertical dashed lines indicating positions of orthologous genes. The sizes of regions are ~400 kb in D. melanogaster (X:13890387..14275449), ~280–350 kb in D. simulans (X:10696104..10968610)/D. sechellia (scaffold_20:553142..877095) and ~330 kb in D. yakuba (X:8186055..8516953). Positions of genes related to gene families are depicted by pentagons indicating direction of transcription. Yellow pentagons designate NACßtes copies, blue pentagons - CK2ßtes-like copies, light blue pentagons - Stellate genes. Promoters are indicated by small rectangles fused to these signs: light yellow rectangles depict homologous Stellate and NACßtes promoters, blue rectangles depict CK2ßtes-like ones. Blue rectangles designate the remnants of CK2ßtes-like sequences (D. melanogaster X:14189495..14189605 [-], D. sechellia scaffold_20: 574563..574 704[-], D. simulans X:10724910..10724990[-]). A remnant of CK2ßtes-like gene represented by the ORF for 37 amino acids is designated in intron of gene CG9400 in D. melanogaster. Lilac and rose arrowheads designated earlier annotated and newly detected DINE-1 elements, respectively. Orientations of arrowheads correspond to predicted direction of transcription. Positions of some orthologous genes are depicted by black arrows. In D. simulans several CK2ßtes-like copies (GD24508:chrX_Mrandom_708:8043..8830[-], GD24510: chrX_Mrandom_706:885-1556[-]), and orthologs GD15860/GM17570. This sequence is missed in all species. (Fig. S3, Fig. S4).

Origenation of gene Stellate, a new variant of the CK2ßtes gene family

The coding region of testis specific Stellate genes in D. melanogaster are homologous to the unique autosomal CK2ßtes gene [14,15], but Stellate precursor has acquired a promoter region from the NACßtes gene [20]. A careful comparison of nucleotide sequences of Stellate and CK2ßtes-like genes in D. sechellia and D. simulans revealed the shared diagnostic sequence stretch between Stellates and orthologs GD15860/GM17570. This sequence is missed in all the other CK2ßtes-like copies (Fig. 2). This observation allows us to consider the ancestor GD15860/GM17570-like copy to be a partner of illegitimate recombination with NACßtes gene (Fig. 2). The CK2ßtes-like genes in D. simulans/D. sechellia (GD15860/ GM17570) and NACßtes (CG13402) in D. melanogaster are located precisely at the same sites adjacent to orthologs GD17153, GM17553 and CG12400, respectively (Fig. 1). We suppose that the ancestor genome contained the juxtaposed CK2ßtes-like and NACßtes genes at this site and such an arrangement allowed for recombination between these genes ensuring the emergence of the Stellate precursor copy.

The location of the CK2ßtes-like pseudogene in D. sechellia coincides with the site of the emergence of tandemly repeated Stellate cluster (Fig. 1). We propose that evolutionary diversification of genes related to CK2ßtes family has been occurred specifically in this specific region of the ancestor genome. These events appear to be quenched in D. simulans/D. sechellia lineage, but have led to the formation of Stellate cluster in D. melanogaster. The similarity of the tandemly repeated ORFs of novel young Stellate genes (2.5% divergence), which may be maintained by an unknown mechanism of homogenization [24,25], is significantly higher than the extent of similarity of the homologous more ancient CK2ßtes-like copies in D. sechellia/D. simulans (Fig. S3, Fig. S4). We detected an expansion of genes CK2ßtes and NACßtes by duplications. The usual fate of a gene duplicate is pseudogenization, but that has not occurred for most amplified NACßtes and CK2ßtes-like copies. Only one of six NACßtes copies in D. melanogaster is a pseudogene, located on the X-chromosome outside of this syntenic region, and only one CK2ßtes-like pseudogene of six undamaged CK2ßtes-like genes in D. sechellia is observed. Thus most duplicate copies remain functional.
To summarize the obtained data, we present a chronology of the events of the NACβtes and CK2βtes-like genes amplification as well as Stellate origination related to the evolutionary tree of melanogaster group species (Fig. 3). It is evident that amplification events of NACβtes genes and insertion of a precursor of CK2βtes-like/Stellate genes on the X-chromosome have been occurred in the common ancestor of D. melanogaster, D. simulans and D. sechellia. The CK2βtes-like and NACβtes genes recombination that has led to the emergence of the Stellate genes is supposed to be proceeded in an immediate ancestor of D. melanogaster. Amplification of the CK2βtes-like genes has been originated in the common ancestor of D. simulans and D. sechellia.

**DINE-1 transposons and expansion of the CK2βtes and NACβtes genes**

Most genes from the CK2βtes-like and NACβtes families are flanked by DINE-1 copies (Fig. 1). It has been reported that the evolution of new genes in Drosophila genomes is often associated with the abundant DINE-1 transposons [6,7,26] related to the enigmatic Helitrons family of transposable elements [27–34]. Our results support this view, providing examples of nonrandom DINE-Is localization near the amplified members of multigene families evolved in the course of evolution of the melanogaster subgroup genomes. The estimation of association of paralogs with DINE-1 elements in D. melanogaster argues in favor of this view: 1180 genes grouped in 344 paralog families are known in D. melanogaster, and the fraction of paralogs having at least one DINE-1 within 3 kb flanking sequences is significantly higher than can be expected by chance (243 vs. 156, P-value<0.005).

DINE-1 transposons are thought to have invaded the Drosophila genome before the diversification of the melanogaster subgroup [27,35]. It seems that DINE-1 has gone through multiple independent cycles of activation and suppression [26]. These elements were suggested to be active and then silenced in the common ancestor of melanogaster subgroup species. D. yakuba is the only species showing evidence of a second, recent transpositional burst [35]. D. melanogaster and D. sechellia/D. simulans contain highly polymorphic DINE-1 copies represented by the remnants of parent copies. The absence of nearly identical Helitrons at different loci in one genome indicates that these elements have been silenced for a long time and have undergone significant disruption processes [35]. Nevertheless, the analysis of the generalized structures of DINE-1 sequences from 12 Drosophila genomes allowed the authors to discriminate some consensus regions including 5’- and 3’-subterminal inverted repeats, a core, and a 3’-terminal region containing a stem-loop structure that is supposed to be involved in the termination of DINE-1 replication [26]. Using this consensus we were able to detect several profoundly damaged DINE-1 copies in D. melanogaster, D. sechellia and D. simulans, adjacent to genes related to two studied multigene families (Fig. 1).

Alignment of nucleotide sequences of DINE-1 copies and D. melanogaster consensus sequence [26] is shown in Fig. 4A. Although there are no extended shared regions between some copies (for example, between INE2976 and INE2978), their relation to DINE-1 is clearly traced by a comparison with the consensus sequence [26]. The relation of simINE_m telo to DINE-1 is validated by its comparison to the earlier version of DINE-1 consensus [36] (Fig. 4B). The vestiges of DINE-1s flanking NACβtes duplications are detectable in both D. sechellia and D. simulans (Fig. 4A), confirming the presence of DINE-Is in the common ancestor of D. melanogaster and D. sechellia/D. simulans. The CK2βtes-like solo copies (GM17570 and GD15860) as well as the duplicated ones are located adjacent to damaged DINE-1 sequences in D. simulans/D. sechellia (Fig. 1, Fig. 4, Table S1) at the distances not exceeding...
200–1000 bp. Interestingly, the \( \kappa NAC \) beta (CR42877) located at a distance of 1 Mb from the studied region in D. melanogaster is also juxtaposed to a DINE-1 copy. Two non-homologous fragments of DINE-1 flank the Stellate cluster (Fig. 1, Fig. 4A). The nucleotide sequence of the cluster including the distal marginal Stellate copy (CG33247), which is distinct in its 3'-noncoding region from the adjacent homogeneous tandem Stellate repeats, is identical to the "Stellate orphan" (Ste12D OR) located near the ben gene (Fig. 1). The observed identity of Ste12D OR and marginal Stellate copy (CG33247) in cluster (Fig. S3) allows us to propose the role of DINE-1s in duplication of Ste12D OR followed by its local amplification to generate the Stellate cluster. While the sequences of the orphon and marginal Stellate copies are identical to each other, the adjacent DINE-1 copies (INE1972 and INE2968) contain similar 3'-stem-loop sequences, but have been deeply disrupted in the rest of the DINE-1 sequence. We propose that diverged DINE-1 copies may participate in the ancestor genomes causing non-allelic recombination that is capable to ensure reshuffling of protein coding genes. Alternatively, DINE-1 sequences may be prone to breakages followed by illegitimate recombination [6]. Thus DINE-1 participation in evolution of multigene families remains to be mysterious.

While the precise testis specific functions of the members of both multigene families remain unknown, positive selection has been shown for \( \kappa NAC \) beta genes [37]. At the same time, the involvement of DINE-1 in duplication of the testis specific \( kep1 \) gene followed by formation of a young gene implicated in regulation of the Y-linked male fertility genes has been demonstrated [7]. The elucidation of CK2beta and \( \kappa NAC \) beta genes functions in testes will help to understand whether there is an evolutionary benefit to their expansion and coupled evolution in Drosophila species.

Materials and Methods

The gene annotation of D. melanogaster (v.5.35), D. sechellia (v.1.3), D. simulans (v.1.3) and D. yakuba (v.1.3) is according to FlyBase (http://flybase.org/). The degree of nucleotide similarity between coding regions of \( \kappa NAC \) beta family genes was evaluated by BLAST (v. 2.2.26) [38]. All alignments were performed by ClustalW implemented in Vector NTI program (Invitrogen).

The identification of novel DINE-1s in the D. simulans/D. sechellia genomes was performed by BLAST (v. 2.2.21) [38] using the DINE-1 consensus sequences [26,36] as queries. The found candidate fragments of DINE-1s copies were additionally reverse BLASTed against D. melanogaster genome assembly to check if they are matched to known INE-1 repeats only. The evolutionary history of proteins related to CK2beta family was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [39]. All positions containing gaps and missing data were eliminated. The resulted tree is a bootstrap consensus tree inferred from 500 replicates [40]. Evolutionary analyses were conducted in MEGA5 [41]. The list of D. melanogaster paralogs was fetched from Homo-loGene NCBI database (http://www.ncbi.nlm.nih.gov/homologene). The expected number of paralogs with nearby DINE-1s was calculated as a possibility to find the DINE-1 near the gene (total number of DINE-1s located within 3 kb of RefSeq gene flanks divided to the total number of all RefSeq genes) magnified to the total number of paralogs. Statistical significance of difference between the expected and observed numbers of paralogs were checked by Chi-square test. The genes and DINE-1s on chromosomes U and Uextra were not taken into account.
RT-PCRs were carried out using RNA from testes, heads and carcasses of adult flies of *D. simulans* (stock 199 from Bloomington Stock Center). Total RNA was extracted by Trizol reagent (Invitrogen), and first strand cDNA synthesis was performed by using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). Sequences of the used primers were 5'-GGCTTGTAACGACGTCTTCAAGC-3' (GD24508_F) and 5'-ATTGCC-AATCAGGAGACTCCG-3' (GD24508_R). The PCR products were sequenced for verification of their specificity.

**Supporting Information**

![Figure S1 Pair alignment of the NAC\textsubscript{\beta}tes gene and pseudogene sequences of \textit{D. sechellia}. \$\psi$NAC\textsubscript{\beta}tes is localized in \textit{D. sechellia} scaffold\textunderscore 20:807538\textunderscore 808222.](EPS)

**Figure S2 RT-PCR validation of testis expression of \textit{CK2}\textsubscript{\beta}tes-like GD24508 gene in \textit{D. simulans}.** Lanes 1, 100 bp marker; 2, total DNA; 3, 4 and 5, RNA from testes, heads, and carcasses of adult males, respectively. Specificity of PCR products was confirmed by sequencing. Designated primers flank the 100 bp marker; 2, total DNA; 3, 4 and 5, RNA from testes, heads, and carcasses of adult males, respectively. Specificity of PCR products was confirmed by sequencing. Designated primers flank second small intron (\textasciitilde 50 nt).

![Figure S3 Analysis of proteins related to \textit{CK2}\textsubscript{\beta}tes family.](PDF)

**References**