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Duplication-degeneration as a mechanism of gene fission and the origin of new genes in *Drosophila* species

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Gene fission and fusion, the processes by which a single gene is split into two separate genes and two adjacent genes are fused into a single gene, respectively, are among the primary processes that generate new genes[1–4]. Despite their seeming reversibility[4,5], nothing is known about the mechanism of gene fission. Because the nucleotide sequences of fission genes record little about their origination process, conventional analysis of duplicate genes may not be powerful enough to unravel the underlying mechanism. In a survey for young genes in species of the *Drosophila melanogaster* subgroup using fluorescence *in situ* hybridization, we identified a young gene family, monkey king, whose genesis sheds light on the evolutionary process of gene fission. Its members originated 1–2 million years ago as retroposed duplicates and evolved into fission genes that separately encode protein domains from a multidomain ancestor. The mechanism underlying this process is gene duplication with subsequent partial degeneration.

To identify young genes in closely related *Drosophila* species (**Fig. 1a**), we used fluorescence *in situ* hybridization FISH analysis of polytene chromosomes with *D. melanogaster* cDNA probes. We selected the cDNAs that generated additional hybridization signals as candidates for further analysis. In this screening, we identified a gene family with up to three new members in the clade of *D. simulans*, *D. sechellia* and *D. mauritiana*, which have diverged in less than 1 million years[6]. We named this gene family monkey-king (*mkg*) after a mythical monkey king in ancient China who could transform his hair into many offspring.

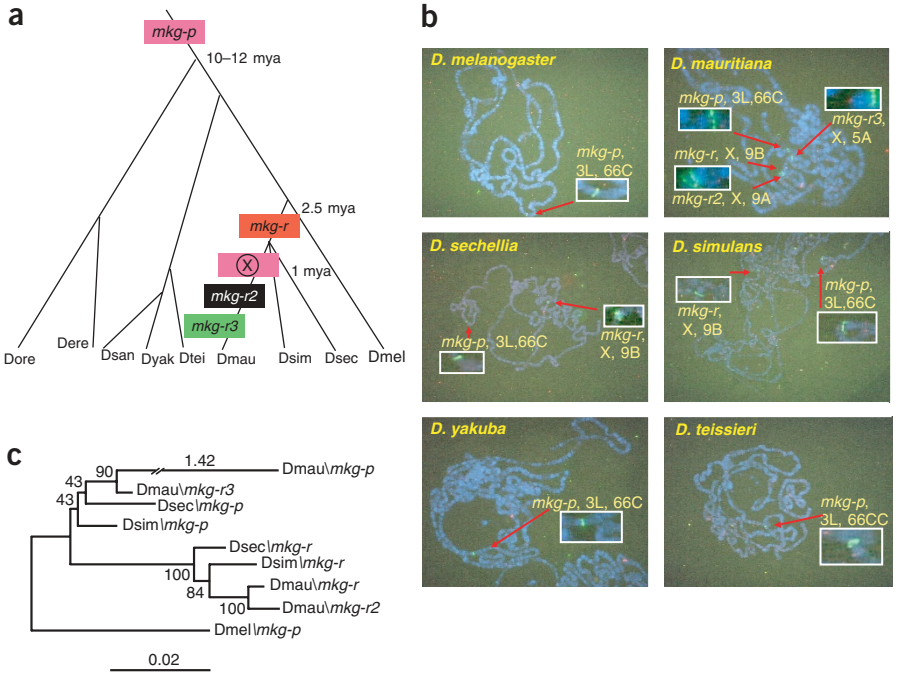

Figure 1 Origin of *mkg* gene family. (**a**) Origination events in the phylogenetic tree of the *D. melanogaster* species subgroup[6,27]: *D. melanogaster* (Dmel), *D. sechellia* (Dsec), *D. simulans* (Dsim), *D. mauritiana* (Dmau), *D. teissieri* (Dtei), *D. yakuba* (Dyak), *D. santomea* (Dsan), *D. erecta* (Dere) and *D. orena* (Dore). mya, million years ago. Pink bars indicate *mkg-p* genes; red, *mkg-r* genes; black, *mkg-r2* genes; and green, *mkg-r3* genes. The circular symbol in Dmau*mkg-p* indicates a degeneration event in the gene. (**b**) FISH detection of new genes in *D. melanogaster* subgroup using digoxigenin-labeled GH05885 cDNA as probe (the *D. erecta* data is not shown but is available on request). The cytological positions of the parental and new genes are given next to the insets that show the signals of the genes from FISH detection (green). (**c**) Neighbor-joining tree of members of the *mkg* family based on Kimura-2-parameter distance generated from the gene sequences. Bootstrap percentages are shown on the branches. Branch lengths are drawn to scale.

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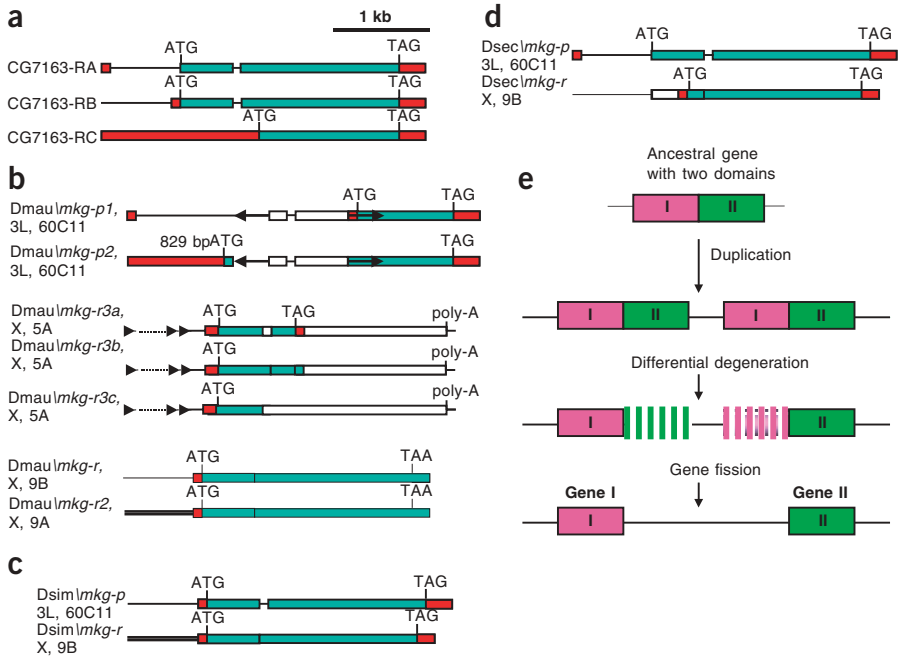


Figure 2 Gene structures of the members of *mkg* gene family. (**a**) Three transcripts of *D. melanogaster mkg* (*CG7163*). (**b**) The four members of *mkg* gene family in *D. mauritiana*. Different transcripts are shown for *mkg-p* and Dmau*mkg-r3*. (**c**,**d**) *mkg-r* and the parental copies of *mkg* in *D. simulans* and *D. sechellia*. The cytological positions are indicated. Green boxes indicate protein-coding regions, red boxes are untranslated regions, and white boxes show pseudoexon regions. Lines are introns or intergenic regions. Double lines indicate the indigenous flanking sequence at the new loci. Arrows show the inverted repeat in Dmau*mkg-p*. Black triangles indicate the repetitive sequence proximal to Dmau*mkg-r3*. The retained poly-A tract is indicated in Dmau*mkg-r3*. (**e**) A schematic model of gene fission by duplication and subsequent partial degeneration, based on findings from Dmau*mkg-p* and Dmau*mkg-r3*.

Two probes from cDNAs encoding zinc-finger protein–related sequences from the gene *CG7163* in *D. melanogaster* had identical hybridization patterns: three extra signals in *D. mauritiana* and one extra signal in both *D. simulans* and *D. sechellia* in addition to a common signal at cytological site 66C of chromosome 3 in all species (**Fig. 1b**). The three new signals in *D. mauritiana* were localized at regions 9A (Dmau*mkg-r2*), 9B (Dmau*mkg-r*) and 5A (Dmau*mkg-r3*) of the X chromosome. The new signal in *D. simulans* and *D. sechellia* was located at region 9B. Southern-blot hybridization experiments with digestion of genomic DNAs of the subgroup species by *Hin*dIII confirmed the FISH results (**Supplementary Fig. 1** online).

We identified and sequenced all four *mkg* members in a *D. mauritiana* genomic library. From the sequences of the four copies with their flanking sequences, we observed extensive changes in the parental copy in *D. mauritiana* (Dmau*mkg-p*) compared with *D. melanogaster*, including a large insertion in exon 2 that contained an inverted repeat of a downstream coding segment (**Fig. 2a**,**b**; sequence alignment data stored in GenBank), a 1-bp deletion in exon 3 and numerous substitutions. These changes disrupted the previous reading frame in these exons. The three new genes are intronless, suggesting that retroposition was involved in their origination[7]. Using flanking copy-specific sequence probes, we mapped their cytological positions in related species by FISH. The specific probe for the *D. mauritiana* 9B copy (Dmau*mkg-r*) hybridized at the same cytological position as the 9B copy in *D. simulans* and *D. sechellia*, indicating that they are orthologs. Dmau*mkg-r2* at 9A seems to be derived from a single duplication event of Dmau*mkg-r*, as they are similar in both the retrosequence and the immediate flanking sequences (sequence alignment data stored in GenBank). Dmau*mkg-r3* at 5A, which contains an AT-rich repetitive sequence with a repeat unit of ~105 bp in length, seems to be a new processed retrosequence from the parental gene. This sequence is more closely related to the parental gene and has a poly-A tract and flanking short direct repeats (TATA/TATT) (data not shown). To our knowledge, this is the first example of new genes being repeatedly generated in a genome by retroposition from the same parent gene and then becoming fixed in natural populations within a short evolutionary period (<2 million years).

We constructed a neighbor-joining tree by defining the gene *CG7163* in *D. melanogaster* (Dmel*mkg-p*) as the outgroup (**Fig. 1c**). The tree supports the relationship deduced from molecular features described above; retroposed *mkg-r* in the three species share a common ancestor, Dmau*mkg-r2* is a duplicate of Dmau*mkg-r*, and Dmau*mkg-r3* is a recently retroposed gene from Dmau*mkg-p*.

Processed copies of gene duplicates are often called pseudogenes because these retroposed elements usually do not carry promoters and insert randomly in the genome. But retroposition can contribute to evolution by creating new functional genes[8–10]. To study whether these three new copies and the changed parental copy in the *mkg* family were expressed, we carried out RT-PCR and detected transcripts of Dmau*mkg-r*, Dmau*mkg-r3* and Dmau*mkg-p*, but not Dmau*mkg-r2* (**Fig. 3**). *mkg-r* in *D. simulans* (Dsim*mkg-r*) and *D. sechellia* (Dsec*mkg-r*) was expressed only in adult males, whereas Dmau*mkg-r* was detected in both sexes, with stronger signals in females (**Fig. 3**).

We characterized gene structures of all *mkg* members by 5′ and 3′ RACE (**Fig. 2b–d**). Each *mkg-r* copy in the three species had different fates concerning survival and exaptation as new genes. Dmau*mkg-r* retained a transcript similar to that of the original gene *CG7163* and is

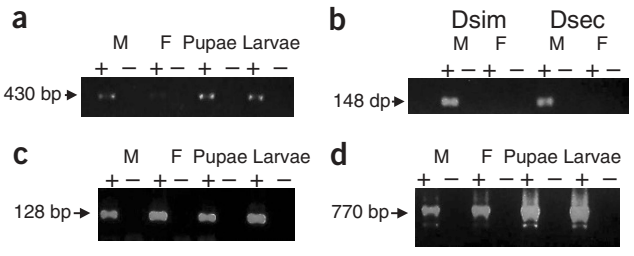


Figure 3 Expression patterns of *mkg* genes detected by gene-specific RT-PCR. (**a**) Dmau*mkg-r*. (**b**) Dsim*mkg-r* and Dsec*mkg-r*. (**c**) Dmau*mkg-r3*. (**d**) Dmau*mkg-p*. F, female; M, male; –, negative controls; sequence alignment data, AY572491–AY572499.

Table 1 K_a/K_s ratios between copies of *mkg* genes and lengths of homologous regions between copies

	Dmel*mkg-p*	Dsim*mkg-p*	Dsec*mkg-p*	Dmau*mkg-p*	Dsim*mkg-r*	Dsec*mkg-r*	Dmau*mkg-r*	Dmau*mkg-r3a* (exon 1)
Dmel*mkg-p*		4.72/11.89	4.62/13.23	4.68/13.51	6.87/11.18	4.89/9.26	6.21/13.50	4.98/11.48
Dsim*mkg-p*	1,926		1.28/3.57	1.60/8.04	4.60/5.09	3.03/4.53	4.02/6.69	0.74/2.27
Dsec*mkg-p*	1,851	1,851		1.40/6.44	4.82/7.91	3.31/7.84	4.18/9.77	0.37/2.75
Dmau*mkg-p*	879	828	753		3.76/10.22	1.84/6.08	1.84/10.58	–
Dsim*mkg-r*	1,314	1,314	1,314	216		1.92/2.21	2.07/3.82	4.20/5.47
Dsec*mkg-r*	1,314	969	969	216	1,314		1.71/4.74	–
Dmau*mkg-r*	1,314	1,314	1,314	216	1,314	969		4.20/5.42
Dmau*mkg-r3*	348	348	348	0	348	12	348	

K_a/K_s ratios (100) between copies of *mkg* genes are shown above the diagonal, and the lengths of homologous regions between copies are shown below the diagonal.

expressed ubiquitously, as detectable by RT-PCR. As the retroposed *mkg-r* sequence does not contain the parental promoter, the retrosequence was probably fortuitously inserted adjacent to a sequence with basal promoter function. Furthermore, the new promoters evolved rapidly: four new promoters were created in a short evolutionary time. Dsim*mkg-r* and Dsec*mkg-r* had new transcription initiation sites. Compared with Dmau*mkg-r*, Dsim*mkg-r* had a 139-bp deletion, a 1-bp deletion and several substitutions in the upstream region, which may be correlated with the new male-specific expression pattern (**Fig. 2c**). In Dsec*mkg-r*, the transcription start point was located in the center of the original exon 2 (**Fig. 2d**), suggesting that the former coding sequence had become a regulatory region. There were three substitutions within the 100 bp of sequence upstream of the new transcription start site of Dsec*mkg-r*, compared with the other *mkg-r* sequences. The finding that new promoters evolved in these new genes less than 1 million years ago supported the prediction that new promoters could originate and evolve rapidly[11].

We further investigated functionality of the *mkg* family. The ratio of K_a (nonsynonymous substitution rate) to K_s (synonymous substitution rate) is a simple and useful measurement of functional constraint on a protein-coding gene[12]. The K_a/K_s ratio should be 1 for pseudogenes, <1 for genes subject to functional constraint and >1 for genes subject to strong positive darwinian selection[13]. But because new genes have rapid changes in protein sequences[4,8], they may have more similar values of K_a and K_s than their parental genes, bringing the K_a/K_s ratio closer to 1. Therefore, the K_a/K_s ratio of a new gene may not be a powerful measure of functionality. Considering this peculiarity of new gene origination, we developed four independent lines of functionality analysis.

First, we found that all K_a/K_s ratios between expressed offspring copies, between expressed offspring and parental copies and between parental copies are <1 (**Table 1**). Under the null hypothesis that K_a/K_s ratios for pseudogenes would be randomly distributed around 1, the probability that all *mkg* members would have K_a/K_s ratios <1 is low ($P < 0.0156$). Second, a within-species variation analysis showed that all gene members, except one with only one polymorphism (Dsec*mkg-r*), have fewer replacement polymorphisms than synonymous changes (**Table 2**). Third, comparison of mutation patterns in coding and noncoding regions showed functional constraints among *mkg-r* genes. Although numerous deletions occur in the 3′ untranslated regions of *mkg-r* genes and different deletion patterns are associated with different species, no deletion was found in the coding regions (protein sequence data stored in GenBank), suggestive of a functional constraint on the coding regions of all *mkg* members. Fourth, these analyses showed that almost all members of the *mkg* family are expressed with tissue-specific patterns. Taken together, these results suggest that all *mkg* members are functional.

Further analysis of these new gene members identified a mechanism for gene fission: duplication was followed by subsequent partial degeneration to form complementary functions between Dmau*mkg-p* and Dmau*mkg-r3* (**Fig. 2e**). By BLAST search in the SWISS-PROT database, we found that the 659–amino acid product of the parental gene in *D. melanogaster* contains two domains (protein sequence data stored in GenBank). The first segment of 50 amino acids is homologous to the KRAB domain of zinc-finger protein 267 (ref. 14 and protein sequence data stored in GenBank), and the region of amino acids 100–400 is homologous to a group of proteins that contain a poly(A)-binding domain[15]. At first glimpse, the parental copy in *D. mauritiana* (Dmau*mkg-p*) seems to be a pseudogene because of extensive disruptions of the open reading frame in exons 2 and 3 (**Fig. 1a**). But RACE and RT-PCR experiments showed that this disrupted region had become an intron sequence and is spliced out together with the original intron 2 (**Fig. 2b**; sequence alignment data stored in GenBank) and that the parental copy in *D. mauritiana* actually encodes a protein starting from amino acid residue 290 of the ancestral protein, containing the poly(A) binding region (protein sequence data stored in GenBank).

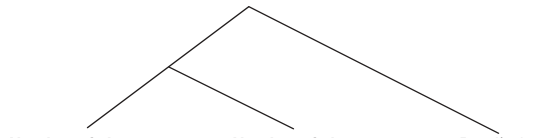
We detected three short transcripts of Dmau*mkg-r3* (**Fig. 2b**). All of them contain intact coding sequence for the KRAB domain of parental zinc-finger proteins and are polyadenylated at 5′ premature positions. Dmau*mkg-r3* is expressed as ubiquitously as the parental copy (**Fig. 3**), probably providing a KRAB-domain function that has been lost in Dmau*mkg-p*. It is conceivable that, in its early stage, this new duplicate was redundant with the parental gene and that subsequent complementary degenerations resulted in the current compensatory pattern. The result was that the ancestral *mkg-p* gene in *D. melanogaster*, *D. yakuba* and *D. teissieri* was split into two loci in *D. mauritiana*, a typical case of gene fission.

The idea that these new genes were subject to functional partitions and evolution was also supported by evolutionary analysis. A relative rate test[13,16] for these genes using Dmel*mkg-p* as the outgroup showed that evolution rates were significantly higher in the *mkg-r* and *mkg-r2* genes than in the parental genes (**Fig. 4**), except for Dmau*mkg-r3*, which has a small number of changes with a limited statistical power

Table 2 Summary of within-species variation

		Dsim*mkg-r*	Dmau*mkg-r3b*	Dmau*mkg-p*	Dmau*mkg-r2*
Replacement sites:	π	0.00532	0.00138	0.00520	0.00183
	θ	0.00544	0.00145	0.00390	0.00230
Synonymous sites:	π	0.00930	0.00423	0.00604	0.00589
	θ	0.01039	0.00625	0.00668	0.00595
Number of alleles		11	13	13	12

Only one polymorphism was found in eight Dsec*mkg-r* alleles.



Number of changes	Number of changes	Dmel*mkg-p* Probability
(1) Dsim*mkg-p*	Dsim*mkg-r*	
Total sites (1,521 bp)		
13	75	0.000
(2) Dsec*mkg-p*	Dsec*mkg-r*	
Total sites (1,190 bp)		
20	47	0.001
(3) Dsim*mkg-p*	Dmau*mkg-r*	
Total sites (1,314 bp)		
13	44	0.000
(4) Dsim*mkg-p*	Dmau*mkg-r2*	
Total sites (1,314 bp)		
13	48	0.000

Figure 4 The results of relative rate tests. Dmel*mkg-p* was defined as the outgroup in all tests. Four different ingroups are shown. *P* values are shown after each group. Total number of substitutions in each lineage were used to do the tests.

(data not shown). Dmau*mkg-p* was not included in the comparison because of its evolved gene structure. This suggests possible functional divergence of *mkg-r* and *mkg-r2* from the parental genes, although population genetic tests[16–20] did not detect recent selection on these genes in *D. mauritiana* and *D. sechellia*.

Although gene fission is a common process in prokaryotes[2] and has been reported in eukaryotes[4,21], the mechanism involved was unknown. The process by which *mkg* originated identified the first such mechanism: duplication followed by complementary partial degeneration. This also provides a mechanism to generate new introns in the degenerate region of a previously intronless gene by creating new splice signals[22] (**Fig. 2b**). The molecular origins of the *mkg* family are reminiscent of the multifunctional gene model[23] that proposes specification of protein functions in duplicate copies and the later subfunctionalization model[24] in which gene duplicates are maintained by a complementary expression pattern. But the *mkg* family shows that two or more distinct genes can be derived from different domains of an ancestral protein through a fission process whose mechanism differs entirely from that of its inverse, gene fusion[3–5,25].

METHODS

FISH analysis of polytene chromosomes. To generalize mechanisms of new gene origination, we searched for young genes by using *D. melanogaster* cDNAs from the *Drosophila* Gene Collection (Research Genetics, Invitrogen) for *in situ* hybridization on the polytene chromosomes of species in the *D. melanogaster* subgroup[26]. There are nine species in the *D. melanogaster* subgroup: *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. teissieri*, *D. yakuba*, *D. santomea*[27], *D. erecta* and *D. orena* (**Fig. 1a**). We amplified the cDNA inserts and labeled them by PCR using the vector primers (T7 and PM001). We labeled the probes with digoxigenin or biotin (Roche Molecular Biochemicals). By comparing hybridization signals in the polytene chromosomes of these species, we could detect new homologs that were duplicated to new cytological sites by retroposition or other possible processes.

Southern-blot hybridization. We extracted genomic DNAs of *D. melanogaster*, *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. teissieri*, *D. yakuba*, *D. erecta* and *D. orena* using the Puregene DNA isolation kit (Gentra Systems). We digested DNAs with *Hin*dIII, separated them on an agarose gel and transferred them to a nylon membrane (Roche Molecular Biochemicals) by Southern blotting. We hybridized the GH05885 probes to the membrane to confirm the copy numbers in different species as detected by FISH experiments.

Screening genomic DNA library and sequencing positive clones. To obtain sequences of all the *mkg* copies in *D. mauritiana*, we screened a λ phage genomic library of *D. mauritiana* (constructed and provided by C.-T. Ting, University of Chicago). All four copies, including the parental one, were identified and sequenced.

Characterization of gene structures and expression patterns. We used the RACE (rapid amplification of cDNA ends) assay and RT-PCR to detect possible transcripts of each copies. The gene structure of each copy was deduced by comparing the obtained cDNA and genomic DNA sequences. We examined expression patterns in adult females, adult males, second and third instar larvae or pupae. We carried out 5′ RACE using the FirstChoice RLM-RACE kit (Ambion). For 3′ RACE, we used adapter-linked oligo dT primers (Life Technologies) to synthesize first-strand cDNA.

Polymorphism data and statistical analyses. We generated polymorphism data of genes using population samples of *D. simulans*, *D. mauritiana* and *D. sechellia*. The worldwide *D. simulans* sample contained 11 strains. We used 13 *D. mauritiana* strains: lines 72, 75, 105, 197, 207, g23, g35, g62, g74, g122, g130, g193 and G122. We used eight *D. sechellia* strains: lines 4, 15, 21, 22, 24, 25, 81 and 034. These strains were provided by J. Coyne, S.-C. Tsaur and M.-L. Wu (University of Chicago). We extracted total DNA from a single male of each strain using Puregene DNA extraction kit (Gentra Systems). We did not collect polymorphism data for Dmau*mkg-r* because it is difficult to specifically amplify this gene in many *D. mauritiana* strains. In *mkg-r2*, all 12 alleles showed significantly stronger preference for synonymous polymorphism, whereas one allele (w136) contained a stop codon, probably a transient mutant, and was excluded from analysis. Alleles of *mkg-r2* also had a preference for synonymous substitution (K_s) over nonsynonymous substitution (K_a) in divergence analysis in all comparisons, except for one with *mkg-r3*. Thus, the sequence analyses at two levels of variation, polymorphism and divergence, suggest that *mkg-r2* is subject to functional constraint.

We calculated K_a, K_s and two statistics that describe within-species variation, π (nucleotide diversity) and Watterson's θ, with DNAsp 3.5 (ref. 16) and K-estimator[28]. We also carried out Tajima's *D* test, the McDonald-Kreitman test, Fu-Li test and Fay-Wu test[17–20] with DNAsp 3.5. We created a neighbor-joining tree of genes and carried out a relative rate test using Mega 2.0 (ref. 29).

We used sign tests[30] to test the null hypothesis that new genes and parental genes are pseudogenes. If they were pseudogenes, some new genes should have K_a/K_s ratios <1 and some others should have K_a/K_s ratios >1. Under the simple assumption that the probability that the K_a/K_s ratio for a pseudogene is <1 is equal to the probability that the ratio is >1, the binomial distribution on the assumption of $p = q = 0.5$ can be used to calculate the probability of the null hypothesis, $p = n!/(m!k!).(1/2)^n$, where $n = m + k$, m = the number of the ratios >1 and k = the number of the ratios <1. For a conservative test, we used the number of independent comparison ($n = 6$, considering that Dmel*mkg-p*, Dsim*mkg-p*, Dsec*mkg-p*, Dsim*mkg-r*, Dsec*mkg-r* and Dmau*mkg-r* contain two domains whereas Dmau*mkg-p* and Dmau*mkg-r3* contain single different domains) when computing this probability.

GenBank accession number. *mkg* genes, AY562976–AY562984; sequence alignment data, AY572491–AY572499.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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