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# **Genome-wide acceleration of protein evolution in flies (Diptera)** Joël Savard\*<sup>1</sup>, Diethard Tautz<sup>1</sup> and Martin J Lercher<sup>2,3</sup>

Address: <sup>1</sup>Institut für Genetik der Universität zu Köln, 50674 Köln, Germany, <sup>2</sup>Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK and <sup>3</sup>European Molecular Biology Laboratory, 69012 Heidelberg, Germany

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Email: Joël Savard\* - savard@uni-koeln.de; Diethard Tautz - tautz@uni-koeln.de; Martin J Lercher - lercher@embl.de

\* Corresponding author

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#### **Abstract**

**Background:** The rate of molecular evolution varies widely between proteins, both within and among lineages. To what extent is this variation influenced by genome-wide, lineage-specific effects? To answer this question, we assess the rate variation between insect lineages for a large number of orthologous genes.

**Results:** When compared to the beetle *Tribolium castaneum*, we find that the stem lineage of flies and mosquitoes (Diptera) has experienced on average a 3-fold increase in the rate of evolution. Pairwise gene comparisons between *Drosophila* and *Tribolium* show a high correlation between evolutionary rates of orthologous proteins.

**Conclusion:** Gene specific divergence rates remain roughly constant over long evolutionary times, modulated by genome-wide, lineage-specific effects. Among the insects analysed so far, it appears that the *Tribolium* genes show the lowest rates of divergence. This has the practical consequence that homology searches for human genes yield significantly better matches in *Tribolium* than in *Drosophila*. We therefore suggest that *Tribolium* is better suited for comparisons between phyla than the widely employed dipterans.

#### **Background**

Understanding the causes of rate variation in protein evolution is central for many fields including molecular evolution, comparative genomics and structural biology. A widely accepted principle is that more important proteins evolve more slowly [1]. However, evolutionary rate variation not only exists between different proteins, but also between lineages [2]. This was already observed in the earliest comparative studies, both within [3-5] and across animal phyla [6].

Following the initial observation that Drosophilids are fast evolving [6], it was shown that the rate of synonymous substitutions in *Drosophila melanogaster* is approxi-

mately two times higher than in rodents and ten times higher than in primates [7]. However, the estimated acceleration seemed to depend on the types of proteins examined, with another study reporting only a 3-fold difference between *Drosophila* and mammalian rates [8]. A study of the relative rates of ribosomal RNA evolution in insect lineages showed that there was an episodic substitution rate increase of about 20-fold in the stem lineage of dipterans [9], suggesting that high evolutionary rates may be characteristic of the whole dipteran order. However, it is currently unclear whether the observed rate accelerations encompassed the whole nuclear genome, or whether they were restricted to certain classes of genes.

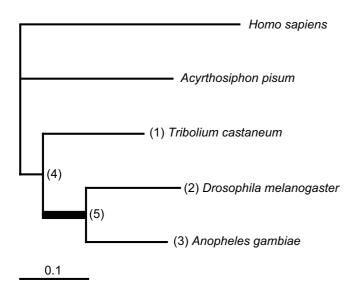


Figure I
Phylogenetic tree topology and maximum likelihood branch length estimates. Numbers in parentheses indicate nodes used to measure distances in Table I. The thick branch between nodes 4 and 5 represents the dipteran stem lineage.

In this study, we assess the genome-wide variation of evolutionary rates between insect lineages, by comparing the beetle *Tribolium castaneum*, and the dipterans *Drosophila melanogaster* and *Anopheles gambiae*. We test (i) whether there is an acceleration of protein evolution in dipterans that is observable on a genome-wide scale, (ii) whether this acceleration is confined to an episodic burst of changes at the base of the dipteran lineage, and (iii) whether this acceleration affects all genes to a similar extent.

### Results Genomic rate estimates confirm an acceleration in

To estimate genomic evolutionary rates of insect lineages, we formed 439 protein clusters derived from cDNA or EST

data from beetle (*Tribolium castaneum*), fly (*Drosophila melanogaster*), mosquito (*Anopheles gambiae*), aphid (*Acyrthosiphon pisum*), and human (*Homo sapiens*). Concatenation of these orthologous sequences resulted in a single, well-defined alignment of 64,134 amino acids. Given the known tree topology in Figure 1[10], branch lengths were estimated using a maximum likelihood method [11] (Table 1). The dipteran branches are much longer than the *Tribolium* branch, indicating accelerated evolution in the Diptera. Since their last common ancestor, *Drosophila* has accumulated 36% (95% CI: 31–42%) more amino acid substitutions compared to *Tribolium*, while the corresponding increase for *Anopheles* compared to *Tribolium* is 23% (95% CI: 18–29%).

In order to assign absolute rates of evolution, we combined the sequence distances in Figure 1 with absolute dates obtained from palaeontological estimates. Based on the oldest coleopteran fossil [12] and the timing of the primary radiation of holometabolous insect orders [13], the divergence of dipterans (Drosophila and Anopheles) from coleopterans (Tribolium) has been estimated to the early Permian (the Artinskian; about 284.4-275.6 Mya). The divergence between the Brachycera (Drosophila) and the Nematocera (Anopheles) lineages has been estimated to the Middle Permian (the Anisian; about 245.0-237.0 Mya) [14]. Combining these dates with the maximumlikelihood estimates of branch lengths, we obtained the absolute evolutionary rates listed in Table 1. This shows that the 39 My time interval starting with the separation of coleopterans and dipterans and culminating in the radiation of Diptera was characterised by an episodic increase of evolutionary rate, averaging 3.07 times the mean rate found for the Tribolium lineage (95% CI: 2.39-4.34).

#### Deviations from clock-like evolution

The accuracy of absolute rate estimates depends on the completeness of the known fossil record. Thus, the uncertainty assigned to the dates in Table 1, which reflects the uncertainty in the dating of the strata where fossils were

Table 1: Relative evolutionary rates of *Tribolium castaneum*, and the dipterans *Anopheles gambiae* and *Drosophila melanogaster*. Amino acid distances, divergence time estimates, and substitution rates for the holometabolous insect branches and the base of Diptera are shown

Branch (from nodeto node)	Distance [aa subs/site]	Time <sup>a</sup> [My]	Rate [10 <sup>-3</sup> aa subs/site/My]	Relative rate <sup>b</sup>
Tribolium (14)	0.180 ± 0.003	280.0 ± 4.4	0.643 ± 0.015	
Anopheles – base (34)	0.222 ± 0.003	280.0 ± 4.4	0.793 ± 0.016	1.233 ± 0.038
Drosophila – base (24)	0.245 ± 0.003	280.0 ± 4.4	0.875 ± 0.017	1.361 ± 0.041
Anopheles (35)	0.145 ± 0.002	241.0 ± 4.0	0.602 ± 0.013	0.936 ± 0.030
Drosophila (25)	0.168 ± 0.002	$241.0 \pm 4.0$	0.697 ± 0.014	1.084 ± 0.033
Base of Diptera (45)	0.077 ± 0.002	39.0 ± 5.9	1.974 ± 0.303	3.071 ± 0.477

<sup>&</sup>lt;sup>a</sup> Age of geological stage as defined in "A Geologic Time Scale" [39] (see text for details).

<sup>&</sup>lt;sup>b</sup> Relative rate = Rate/(Tribolium Rate)

Table 2: Maximum likelihood estimates of different molecular clock models for concatenated sequences. The models are compared to the model without a clock via likelihood ratio tests.

Clock model	<b>Parameters</b>	In(L)	$2[\ln(L_{nc})-\ln(L)]^a$	df⁵	P
No clock	8	-459144	-	-	-
Global	5	-459296	302.89	3	$2 \times 10^{-65}$
Base of Diptera	6	-459229	169.93	2	$1 \times 10^{-37}$
Drosophila and Anopheles	7	-459203	117.57	1	$2 \times 10^{-27}$
Tribolium and Drosophila	7	-459146	2.69	I	0.1
Tribolium and Anopheles	7	-459150	10.60	1	0.001
Tribolium and Diptera	7	-459170	52.21	I	5 × 10-13
Tribolium and base of Diptera	7	-459170	52.21	1	5 × 10 <sup>-13</sup>
Tribolium and tips of Diptera	7	-459170	52.21	1	5 × 10 <sup>-13</sup>

<sup>&</sup>lt;sup>a</sup> L<sub>nc</sub> is the maximum-likelihood estimate of the model without a molecular clock.

found, is an underestimate of the true uncertainty. To confirm that the dipteran rate acceleration is not an artefact of erroneous palaeontological estimates, we performed additional analyses that directly compared relative evolutionary rates. To this end, we implemented several models involving global or local molecular clocks [15]. Because these models are nested, they can be compared via likelihood-ratio tests [16]. Considering the branch length differences in Table 1, it is not surprising that the model employed above (without a clock assumption) fits the data much better than a model with a global clock, which enforces a constant rate of evolution across all lineages (P < 10<sup>-66</sup>, Table 2). Is the clock assumption violated only at specific (local) branches? Table 1 assigns the strongest rate acceleration to the base of the Diptera. However, an evolutionary model involving a local clock for this branch (with all other branches evolving at the same rate) still fits the data significantly worse than the model without a clock ( $P < 10^{-12}$ , Table 2); the same is indeed true for all models involving a single local clock (data not shown). Thus, a burst of accelerated evolution at the base of the dipterans is not sufficient to explain the rate increase found for dipterans as a whole.

Further comparison of increasingly complex models of evolution show that only models involving separate local clocks for the *Tribolium* and *Drosophila* lineages can fit the data as good as the model without a clock assumption (P = 0.1, Table 2). All other combinations of local clocks between the *Tribolium*, *Drosophila* and *Anopheles* lineages were found to be inferior to the model without the clock (P < 0.01, Table 2 and data not shown). Thus, although the accelerated evolution of dipterans was most pronounced during a burst of changes that occurred at the base of the order (Table 1), a sustained increase is also detected in the branches separating *Drosophila* from *Anopheles*. Remarkably, the need for a coleopteran local clock also demonstrates that the *Tribolium* lineage has gen-

erally evolved much slower than most of the other taxa considered here.

It should be noted that Table 2 contains multiple statistical comparisons, thereby decreasing the overall specificity of the statistical test. A conservative strategy to control for this is to divide the *P*-value cutoff for significance by the number of comparisons (Bonferroni-correction), i.e., to use  $P_0 = 0.05/8 = 0.0063$ , or – if accounting for all comparisons in data not shown –  $P_0 = 0.05/41 = 0.0012$ . This correction does not affect our conclusions.

### The dipteran acceleration affects the majority of individual genes

By analysing concatenated amino acid sequences, we have demonstrated accelerated evolution in dipterans, compared to a slower rate in the *Tribolium* lineage. Is this rate change confined to certain genes, or does it extend across the genome as a whole? To analyse this issue, we calculated branch lengths for 1199 orthologous groups comprising sequences from *Tribolium* and *Drosophila*, using human as an outgroup. In Figure 2, we compare the amino acid distances of *Tribolium* and *Drosophila* proteins to their last common ancestor. There is a very strong relationship between these *a priori* unrelated distances (Spearman's rank correlation coefficient  $r_s = 0.718$ ,  $P < 10^{-3}$ ).

When calculated from single gene distances, the mean evolutionary rate of the *Drosophila* lineage is significantly larger than the one of the *Tribolium* lineage (0.284 > 0.245; two-tailed Mann-Whitney *U*-test,  $P < 10^{-3}$ ). The corresponding constant of proportionality is 1.31, in close agreement with the relative rate increase of 1.36 obtained from the concatenated sequences (Table 1). Thus, although evolutionary rates among genes of a genome can vary by several orders of magnitude, these rates are nevertheless correlated between species even after more than 280 My of independent evolution.

b df = degrees of freedom

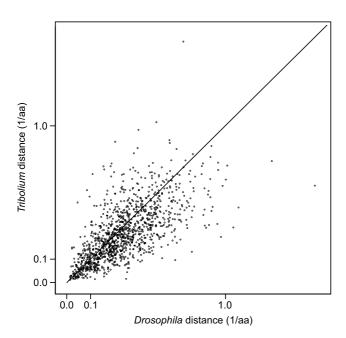


Figure 2
Amino acid distances of I 199 individual *Tribolium* and *Drosophila* genes to their last common ancestor.
Comparison to the identity line expected under equal rates demonstrates that the acceleration is a genome-wide effect.

#### **Discussion**

Using 439 nuclear transcripts, we find that the dipteran lineage (represented by Drosophila and Anopheles) has experienced an episodic increase in evolutionary rate when compared to the coleopteran lineage (represented by Tribolium). This rate subsequently dropped in the diversifying dipteran lineage, but remained above average in Drosophila. This is consistent with previous findings from studies of ribosomal RNAs [9,17]. The rate increases found in our genome-scale analysis of protein sequences are lower than those reported for rRNAs, with an average increase of only 1.3-fold during dipteran evolution versus an at least threefold average increase for rRNAs [9]. However, our analysis is necessarily biased towards parts of genes that are sufficiently conserved to be detected in the outgroup (human) and that could be unequivocally aligned. Still, Figure 2 demonstrates that the averaged rate increase is representative for a large range of genes.

The Neutral Theory of Evolution [1,18,19] predicts that the accumulation of molecular changes is only driven by the mutation rate and the degree of purifying selection. Accordingly, genome-wide variation in the rate of protein evolution between species might be caused by physiological and ecological factors that affect the mutation rate (e.g. metabolic rate [20,21], temperature [20]), or by differences in the efficiency of selection (notably variation in effective population size [22-24] or the level of outbreed-

ing [25]). In the case of the rRNA comparisons, we could indeed show that there has been a mutational bias towards incorporating more A/T nucleotides than G/C nucleotides at the base of the Diptera [9], which has probably caused the episodic acceleration of evolutionary rates. Such a mutational bias is likely to have affected the whole genome. However, the GC content of the *Tribolium* genes in this study (46.0%) is very close to that of the *Drosophila* orthologs (47.1%), suggesting that mutational biases are unlikely to be responsible for the continued acceleration in dipterans. This interpretation is supported by the fact that it is the *Tribolium* proteins which show a slight bias towards AT-rich amino acids when compared to the *Drosophila* orthologs (28.1% AT-rich amino acids for *Tribolium*, compared to 27.2% for *Drosophila*).

We observe a strong correlation between evolutionary rates in the Tribolium and the Drosophila lineages (Figure 2). This indicates that the selective forces acting on the majority of these genes have been similar between the two lineages, consistent with a neutral model. The correlation would be expected to become weaker or even disappear if positive selection would occur episodically among these genes. Our finding that most proteins diverge in a clocklike fashion, with clock speeds that differ among lineages only due to genome-wide effects, suggests that episodic changes are an exception, although such changes are known to occur in some cases [26,27]. However, we note that the class of fast evolving genes could not be analysed here, as they diverge too fast to allow identification as orthologs in distant comparisons [28]. In a dedicated study of such genes between closely related Drosophila lineages, we found that there are indeed a significant number of genes that must have undergone episodic changes in substitution rates on a gene by gene basis [29]. Thus, we emphasize that the above conclusions relate only to relatively conserved (or non-orphan) genes, while the generalized evolutionary patterns of fast evolving (or orphan) genes will need further study.

#### **Conclusion**

We reported here the analysis of evolutionary rate variation of a large number of orthologous genes between insect lineages. Variation in the rate of evolution has genome-wide effects, and is correlated between orthologous genes over very long evolutionary time scales.

Because *Drosophila melanogaster* is among the best studied animal model organisms, the peculiar evolutionary pattern confirmed here has practical implications. In BLAST searches of human sequences against *Tribolium* and *Drosophila*, *Tribolium* sequences are found in 70% of cases to be more similar to human than *Drosophila* genes (N = 1221,  $P = 10^{-50}$  from sign test). Thus, when attempting to link human genes to their *Drosophila* homologs, data from

*Tribolium* will be helpful to provide a more conservative reference sequence. This approach has been used, e.g., to resolve the evolutionary relationship between the *Drosophila zen* gene and human HOX3 genes [30]. The slowly evolving beetle *Tribolium castaneum* is thus likely to play a significant role for comparisons between phyla.

#### Methods Sequences

Drosophila melanogaster, Anopheles gambiae and Homo sapiens peptides were obtained from Ensembl [31]. For Tribolium castaneum, EST data available through NCBI dbEST [32] were assembled into contigs using phrap and manually curated to ensure high quality of the data set. For the pea aphid Acyrthosiphon pisum, a publicly available EST-based unigene set was obtained from the INFOBIOGEN GnpSeq database [33]. Tribolium and Acyrthosiphon pisum contigs were then searched against all Drosophila melanogaster proteins using BLASTx. The reading frame from the best hit was assumed to be the correct reading frame. We then chose the longest run of peptides uninterrupted by a stop codon as the peptide corresponding to each EST contig.

#### Identification of orthologs

We performed BLASTp searches of all proteome pairs among *Tribolium castaneum*, *Drosophila melanogaster*, *Anopheles gambiae*, *Acyrthosiphon pisum* and *Homo sapiens*. Orthologs were selected based on reciprocal best blast hits [34] using an e-value cut-off of 10<sup>-10</sup>. A group of sequences with exactly one member in each species was accepted as an orthologous family if each sequence had each of the other family sequences as the best BLASTp hit in the respective proteome. This requirement of all-against-all reciprocal best hits is very stringent, and thus gives good confidence in the inferred orthology.

#### Alignments and distance estimation

Multiple sequence alignments were performed with MUS-CLE [35] using default settings. Resulting alignments were purged from putatively misaligned positions as well as gap positions with Gblocks using default settings [36].

Branch lengths were calculated with the maximum likelihood model by Goldman and Yang [11] as implemented in the PAML package [37]. We used the empirical transition matrix compiled by Jones et al. [38]. The distribution of evolutionary rates across sites was approximated by a discrete  $\Gamma$ -distribution, with the shape parameter as an additional free parameter. When calculating rates for individual genes, we assumed a uniform rate across sites. Clusters containing genes with zero branch length were discarded from further analysis.

#### List of abbreviations

df - degrees of freedom

My - million years

Mya - million years ago

rRNA - ribosomal RNA

#### **Authors' contributions**

JS and MJL designed the study, carried out sequence comparisons, performed the statistical analysis, and drafted the manuscript. DT conceived the study, and participated in its design and coordination.

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#### References

- Smith AB, Lafay B, Christen R: Comparative variation of morphological and molecular evolution through geologic time: 28S ribosomal RNA versus morphology in echinoids. *Philos Trans R Soc Lond B Biol Sci* 1992, 338(1286):365-382.
- Bromham L, Rambaut A, Harvey PH: Determinants of rate variation in mammalian DNA sequence evolution. J Mol Evol 1996, 43(6):610-621.
- Adachi J, Cao Y, Hasegawa M: Tempo and mode of mitochondrial DNA evolution in vertebrates at the amino acid sequence level: rapid evolution in warm-blooded vertebrates. J Mol Evol 1993, 36(3):270-281.
- Catzeflis FM, Sheldon FH, Ahlquist JE, Sibley CG: DNA-DNA hybridization evidence of the rapid rate of muroid rodent DNA evolution. Mol Biol Evol 1987, 4(3):242-253.
- Gu X, Li WH: Higher rates of amino acid substitution in rodents than in humans. Mol Phylogenet Evol 1992, 1(3):211-214.
- Britten RJ: Rates of DNA sequence evolution differ between taxonomic groups. Science 1986, 231(4744):1393-1398.
- Moriyama EN: Higher rates of nucleotide substitution in Drosophila than in mammals. Jpn J Genetics 1987, 62:139-147.
- Sharp PM, Li WH: On the rate of DNA sequence evolution in Drosophila. | Mol Evol 1989, 28(5):398-402.
- Friedrich M, Tautz D: An episodic change of rDNA nucleotide substitution rate has occurred during the emergence of the insect order Diptera. Mol Biol Evol 1997, 14(6):644-653.
- The Tree of Life web project [http://tolweb.org/tree/phylog eny.html]
- Goldman N, Yang ZH: Codon-based model of nucleotide substitution for protein-coding DNA sequences. Mol Biol Evol 1994, 11:725-736.
- Ponomarenko AG: Superorder Scarabaeidea Laicharting, 1781. Order Coleoptera Linné, 1758. The Beetles. In History of Insects Edited by: Rasnitsyn AP, Quicke DLJ. Dordrecht, Kluwer Academic Publishers; 2002:164-176.
- Rasnitsyn AP: Cohors Scarabaeiformes Laicharting, 1781. The Holometabolans. In History of Insects Edited by: Rasnitsyn AP, Quicke DLJ. Dordrecht, Kluwer Academic Publishers; 2002:157-159.
- Blagoderov VA, Lukashevich ED, Mostovski MB: Order Diptera Linné, 1758. The True Flies. In History of Insects Edited by: Rasnitsyn AP, Quicke DLJ. Dordrecht , Kluwer Academic Publishers; 2002:227-241.
- Yoder AD, Yang ZH: Estimation of primate speciation dates using local molecular clocks. Mol Biol Evol 2000, 17(7):1081-1090.

- 16. Huelsenbeck JP, Rannala B: Phylogenetic methods come of age: testing hypotheses in an evolutionary context. Science 1997, **276(5310):**227-232.
- Carmean D, Kimsey LS, Berbee ML: 18S rDNA sequences and the holometabolous insects. Mol Phylogenet Evol 1992, 1(4):270-278.
- Ohta T: Very slightly deleterious mutations and the molecular clock. J Mol Evol 1987, 26:1-6.
- 19. Kimura M, Ohta T: On the rate of molecular evolution. J Mol Evol 1971, 1:1-17.
- 20. Gillooly JF, Allen AP, West GB, Brown JH: The rate of DNA evolution: effects of body size and temperature on the molecular clock. Proc Natl Acad Sci U S A 2005, 102(1):140-145.
- 21. Martin AP: Substitution rates of organelle and nuclear genes in sharks: implicating metabolic rate (again). Mol Biol Evol 1999, **16(7):**996-1002.
- 22. Jordan IK, Rogozin IB, Wolf YI, Koonin EV: Microevolutionary genomics of bacteria. Theoretical Population Biology 2002, 61(4):435-447.
- 23. Eyre-Walker A, Keightley PD, Smith NG, Gaffney D: Quantifying the slightly deleterious mutation model of molecular evolution. Mol Biol Evol 2002, 19(12):2142-2149.
- 24. Gu Z, David L, Petrov D, Jones T, Davis RW, Steinmetz LM: Elevated evolutionary rates in the laboratory strain of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 2005, 102(4):1092-1097.
- 25. Bustamante CD, Nielsen R, Sawyer SA, Olsen KM, Purugganan MD, Hartl DL: The cost of inbreeding in Arabidopsis. Nature 2002, 416(6880):531-534.
- Ayala FJ: Molecular clock mirages. Bioessays 1999, 21(1):71-75. Rodriguez-Trelles F, Tarrio R, Ayala FJ: Erratic overdispersion of three molecular clocks: GPDH, SOD, and XDH. Proc Natl Acad Sci USA 2001, **98(20):**11405-11410.
- 28. Schmid KJ, Tautz D: A screen for fast evolving genes from Drosophila. Proc Natl Acad Sci USA 1997, 94(18):9746-9750.
- 29. Domazet-Loso T, Tautz D: An evolutionary analysis of orphan genes in Drosophila. Genome Res 2003, 13(10):2213-2219.
- Falciani F, Hausdorf B, Schroder R, Akam M, Tautz D, Denell R, Brown S: Class 3 Hox genes in insects and the origin of zen. Proc Natl Acad Sci USA 1996, 93(16):8479-8484.
- Project Ensembl [http://www.ensembl.org/]
- 32. Expressed Sequence Tags database [http:// www.ncbi.nlm.nih.gov/projects/dbEST/]
- 33. INFOBIOGEN GnpSeq database [http://urgi.infobiogen.fr/data/ gnpSeq/run.php]
- Tatusov RL, Koonin EV, Lipman DJ: A genomic perspective on protein families. Science 1997, 278(5338):631-637.
- Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. 32(5):1792-1797. Nucl Acids Res 2004,
- Castresana J: Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 2000, 17(4):540-552.
- 37. Yang ZH: PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci 1992, 8:275-282.
- Jones DT, Taylor WR, Thornton JM: The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 1992, 8(3):275-282.
- 39. Gradstein F, Ogg J, Smith A: A Geologic Time Scale 2004. Cambridge University Press, UK; 2004.

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