**PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments**

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**ABSTRACT**

PAL2NAL is a web server that constructs a multiple codon alignment from the corresponding aligned protein sequences. Such codon alignments can be used to evaluate the type and rate of nucleotide substitutions in coding DNA for a wide range of evolutionary analyses, such as the identification of levels of selective constraint acting on genes, or to perform DNA-based phylogenetic studies. The server takes a protein sequence alignment and the corresponding DNA sequences as input. In contrast to other existing applications, this server is able to construct codon alignments even if the input DNA sequence has mismatches with the input protein sequence, or contains untranslated regions and polyA tails. The server can also deal with frame shifts and inframe stop codons in the input models, and is thus suitable for the analysis of pseudogenes. Another distinct feature is that the user can specify a subregion of the input alignment in order to specifically analyze functional domains or exons of interest. The PAL2NAL server is available at http://www.bork.embl.de/pal2nal.

**INTRODUCTION**

An increasing body of research is based on the classification and evaluation of the rate of DNA evolution in coding regions. In many cases nucleotide substitutions are classified according to their impact on the encoded protein and the resulting classification can be used for a variety of analyses. Classification of synonymous (K_S) and non-synonymous (K_A) substitutions can be used to detect the presence or absence of selection (1) and the classification of substitutions according to codon position can be used with sophisticated evolutionary models to better reconstruct phylogenies. In both of these cases the programs that perform the analysis, such as PAML (2), require a codon alignment as input.

Owing to the degeneracy of the genetic code, where a single amino acid can be encoded by multiple codons, it is often preferable to align protein sequences rather than the underlying coding DNA as it increases sensitivity at longer evolutionary distances and prevents the introduction of frame shifts into an alignment. Thus the construction of a protein alignment first and then reverse translating this into a codon-based DNA alignment is invariably the optimal solution and provides reliable alignments to perform correct evolutionary analyses. In the ideal case where the protein and the corresponding DNA match perfectly, the conversion from a protein alignment into the corresponding codon alignment can be achieved by replacing each amino acid residue with three nucleotide residues, a procedure which is implemented in several tools, e.g. aa_to_dna_aln in the Bioperl toolkit (3), RevTrans (4), transAlign (5) and aa2dna (http://www.bio.psu.edu/People/Faculty/Nei/Lab/aa2dna.zip). However it often happens that the corresponding DNA sequence has mismatches when compared with its theoretical protein sequence, or contains untranslated regions (UTRs) and polyA tails. In such instances, the conversion process is much more complicated. Moreover the analysis of pseudogenes, which are an interesting subject of molecular evolution studies, requires dealing with frame shifts and inframe stop codons. These situations, which are rather frequent in large-scale analysis of sequenced genomes, cannot be solved by the programs mentioned above and therefore require additional solutions.

Here we describe a web server, PAL2NAL, which converts a protein sequence alignment into the corresponding codon alignment, despite the presence of mismatches between the protein and the DNA sequences, UTRs and polyA tails in the input DNA sequences, and frame shifts and inframe stop codons in the input alignment. Another useful feature of this server is that it is possible to obtain codon alignments for specific regions of interest, such as functional domains or...
A

**Figure 1.** An example of PAL2NAL input and output files. (A) The first input file: A multiple sequence alignment of human dihydrofolate reductase (GenBank accession no. BC070280) and its pseudogene in the CLUSTAL format with the notation used in GeneWise for frame shifts. Frame shifts and inframe stop codons in Figure 1.

![CLUSTAL W multiple sequence alignment](image1)

**B**

The first input file: A multiple sequence alignment of human dihydrofolate reductase (GenBank accession no. BC070280) and its pseudogene in the CLUSTAL format with the notation used in GeneWise for frame shifts. Frame shifts and inframe stop codons in Figure 1.

![CLUSTAL W multiple sequence alignment](image2)

**C**

# WARNING: pseudogene pepAlnPos 11: V does not correspond to GAT

E

D

| Accession | Description |
|-----------|-------------|
| BC070280  | Dihydrofolate reductase (human) |
| BC070280  | Dihydrofolate reductase pseudogene (human) |
| BC070280  | --- |

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z
particular exons by selecting the positions in the input protein sequence alignment.

METHODS AND IMPLEMENTATION

The server requires a multiple sequence alignment of proteins and the corresponding DNA sequences as input. The internal action of the program can be divided into three main steps: (i) upload the protein sequence alignment and DNA sequences, (ii) reverse translation, i.e. conversion of the protein sequences into the corresponding DNA sequences in the form of regular expression patterns and (iii) generation of the codon alignment. In the second step, each protein sequence is converted into DNA sequence of a regular expression. For example, a short peptide sequence, MDP, is reverse-translated into a regular expression pattern of the DNA sequence as \((A(U[T]G) \{GA(U[T])[C|Y] \} \{CC. \})\). For frame shifts, we adapted the notation used in GeneWise (6): if an insertion or deletion is found in the coding region, it is represented by the number of nucleic acid residues at that site instead of an amino acid code. For example, M2P indicates that there is 1 nt deletion between methionine and proline. With this notation, it is easy to convert the peptide sequence into a regular expression pattern, in this case \((A(U[T]G) \ldots(CC. \})\). After converting into a regular expression pattern, the input DNA sequence is searched with the pattern to obtain the corresponding coding region. Unmatched DNA sequence regions are discarded. The pattern matching has been designed to be tolerant of mismatches. This was achieved by extending 10 amino acid regular expression matches in both directions until the entire coding region of the input DNA sequence is covered. The regions between the extended fragments and those not covered by the extension are taken as mismatches, and reported, if any, in the output. In the third step, the protein sequence alignment is converted into the corresponding codon alignment by replacing each amino acid residue with the corresponding codon sequence.

There are six options in the PAL2NAL server. (i) *Codon tables*. The users can select a codon table: either ‘universal’ (default) or ‘vertebrate mitochondrial’. (ii) *Remove gaps and inframe stop codons*. This option excludes codons with gaps and inframe stop codons from the output. This option should be selected if the codon alignment is to be further analyzed by codeml to calculate \(K_S\) and \(K_A\) since the PAML package does not accept codon alignments containing gaps or inframe stop codons. (iii) *Calculate \(K_S\) and \(K_A\)*. If the second option (*Remove gaps and inframe stop codons*) is selected and the input is a pair of sequences, this option also allows to calculate \(K_S\) and \(K_A\) values by the codeml program (8) included in the PAML package. The calculation of \(K_S\) and \(K_A\) is only performed for sequence pairs because the computationally demanding construction of phylogenetic topologies would be required for alignments with more than two sequences. (iv) *Remove mismatches*. If there are mismatched codons between the protein and the DNA sequences, the users can either remove or retain such codon sites by this option. (v) *Use only selected positions*. With this option, only the codon alignment corresponding to the regions marked by ‘\#’ in the input alignment is generated. This option is very useful because it allows the construction of codon alignment for a certain exon or a domain or conserved blocks, for example those identified automatically by Gblocks (9). (vi) *Output format*. There are three output formats: CLUSTAL, PAML and FASTA. The output can be modified by combining these options (Figure 1D).

CONCLUSION

The PAL2NAL server (http://www.bork.embl.de/pal2nal) is useful for constructing codon multiple alignments, which are required in many molecular evolutionary analyses, such as the calculation of \(K_S\) and \(K_A\) values. For a large-scale analysis, the distribution version of the PAL2NAL script, which is written in Perl and works in command line, is also available for download. We successfully applied the distribution version of PAL2NAL, for example, to the detection of human pseudogenes (10) and to the annotation of genes in human chromosomes 2 and 4 (11).

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Conflict of interest statement. None declared.

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