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Establishing a Framework for Comparative Analysis of Genome Sequences

by

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Abstract

This paper describes a framework and a high-level language toolkit for comparative analysis of genome sequence alignment. The framework integrates the information derived from multiple sequence alignment and phylogenetic tree (hypothetical tree of evolution) to derive new properties about sequences. Multiple sequence alignments are treated as an abstract data type. Abstract operations have been described to manipulate a multiple sequence alignment and to derive mutation related information from a phylogenetic tree by superimposing parsimonious analysis. The framework has been applied on protein alignments to derive constrained columns (in a multiple sequence alignment) that exhibit evolutionary pressure to preserve a common property in a column despite mutation. A Prolog toolkit based on the framework has been implemented and demonstrated on alignments containing 3000 sequences and 3904 columns.
Keywords: Artificial intelligence, Conserved property, Genome sequence, Genome technology, Prolog, Protein, Sequence alignment, Sequence analysis, Parsimony, Phylogeny

1 Introduction

The interpretation of genetic sequence data represents one of the most interesting puzzles facing scientists today. This will almost certainly be the year in which we gain access to complete microbial genomes, and it is likely that hundreds of complete genomes will be obtained during the coming decade. Each such genome represents the complete blueprint of a living organism; that is, it holds the information characterizing a life form. Deepening our understanding of such genomes amounts to advancing our grasp of life itself. Such understanding will almost certainly drive many applications in areas ranging from medicine and agriculture to bio-remediation of the environment.

One fundamental approach toward understanding genetic sequence data is based on comparative analysis that uses phylogeny --- hypothesizing an evolution tree having common ancestral sequences. For example, the corresponding gene from a mouse and from a human look quite similar because they may have evolved from the same common ancestral genome. An essential step in performing comparative analysis is to form an alignment of similar sequences that are believed to be homologous (having a common ancestry). The alignment amounts to an assertion of correspondence between the characters in the sequences. For example, consider the fragments of similar protein sequences from five distinct organisms shown in Figure 1. The sequences have been "aligned"; that is, they have been positioned so that the characters in each column are believed to be derived from a single character in some common ancestral sequence. In the case of the last sequence, the alignment required introducing a ('-') as a spacer to maintain the hypothesized correspondence.

The construction, maintenance, and interpretation of such alignments will play a central role in the interpretation of genomes. This paper describes a framework and the corresponding toolkit of Prolog routines that allows users to gain efficient access to such alignments in a high-level language and to hypothesize the ancestral characters and mutation events by using a parsimonious analysis of phylogeny. This paper also describes an application of the framework to derive constrained columns that exhibit evolutionary pressure to preserve common properties despite mutations.

```
>Tuna
VQKCAQCHTVENGGKHKVGPNLWGLFGRKTGQAEGYSYTD
>Hippopotamus
VQKCAQCHTVEKGGKHKTGPNLHGLFGRKT GQS PGFSYTD
>Horse
VQKCAQCHTVEKGGKHKTGPNLHGLFGRKT GQAPGFTYTD
>Human
I MKCSQCHTVEKGGKHKTGPNLHGLFGRKT GQAPGY SYTA
>Yeast
KTRCAQCHT IE AGGPHKVGPNLHG IF SRHS GQAEG - SYTD
```

Figure 1: A multiple sequence alignment from different organism

The major contributions of this paper are

1. the development of a generic high level language library for complex analysis of multiple sequence alignment and phylogenetic tree;
2. the derivation of group of amino acids in homologous proteins that share some important common properties despite their differences; and
3. the identification of constrained columns (in an alignment of homologous sequences) which conserve some common properties despite mutations resulting into different types of amino-acids in the column.

Further application of the framework, when combined with heuristic biochemical knowledge [2], will facilitate understanding of the structure-function relationship of genome sequences.

The rest of the paper is as follows. Section 2 discusses some background necessary for computer scientists to understand standard biological terminology, and introduces definitions used to explain our concept. Section 3 discusses the multiple sequence alignment as an abstract data type. Section 4 describes parsimonious labeling of a phylogenetic tree. Section 5 describes heuristics to derive constrained columns that preserve a common property despite independent mutations. Section 6 briefly describes other applications of the tool. The last section concludes the paper.

2 Background and Definitions

In this section, some basic terminology is defined for genome analysis [1, 7] and Prolog [8], the target language for the development of analysis tools. Familiarity with terminology related to amino-acids and their classification is assumed [6, 9].

For convenience, sequences are represented within square brackets [...], a generic value is represented within angular brackets < ... >, elements of a set are represented within curly brackets {...}, union of sets is denoted by $\cup$, intersection is denoted by $\cap$, difference of two sets is denoted by -, membership is denoted by $\in$, forall is denoted by $\forall$, and abbreviated characters for various molecules are represented within parenthesis.

2.1 Terminology for Genome Analysis

The genome of an organism is encoded within molecules of DNA. A molecule of DNA is a sequence of four nucleotides: adenine 'A', cytosine 'C', guanine 'G', and thymine 'T'. Hence, a DNA molecule is represented by a sequence of characters from the alphabet {'A', 'C', 'G', 'T'}.

RNA molecules are similar to DNA molecules. The major difference relevant to this discussion is that uracil 'U' occurs rather than thymine. Thus, an RNA molecule is represented by a sequence of characters from the alphabet {'A', 'C', 'G', 'U'}.

The generated tools process both DNA and RNA sequence data. Hence, it is convenient to speak of nucleotides as characters from the alphabet {'A', 'T', 'G', 'U', 'C'}, denoted by $\mathcal{N}$.

A protein is a sequence of different types of molecules collectively known as amino acids. Commonly, the sequence of protein comprises Alanine 'A', arginine 'R', asparagine 'N', aspartic acid 'D', cysteine 'C', glutamic acid 'E', glutamine 'Q', glycine 'G', histidine 'H', isoleucine 'I', leucine 'L', lysine 'K', methionine 'M', phenylalanine 'F', proline 'P', serine 'S', threonine 'T', tryptophan 'W', tyrosine 'Y', and valine 'V'. The alphabet {'A', 'C', 'D', 'E', 'F', 'G', 'H', 'I', 'K', 'L', 'M', 'N', 'P', 'Q', 'R', 'S', 'T', 'V', 'W', 'Y'} is denoted by $\mathcal{A}$.

A genome sequence, denoted by $\mathcal{S}$, is of the form $[s_1, \ldots s_N]$ $(1 \le I \le N)$ where $s_I \in \mathcal{N}$ for DNA or for RNA and $s_I \in \mathcal{A}$ for protein. Two genetic sequences are similar, if there is a significant match between them after limited shifting of characters. Sequence alignment is the process of aligning similar sequences together in a way that asserts a correspondence between characters that are thought to derive from a common ancestral sequence. Aligning a set of sequences requires the introduction of spacing characters, which are referred to as indels. If the aligned sequences did indeed derive from a common ancestral sequence, then indels represent evolutionary events in which characters were either inserted or deleted.

A valid nucleotide alignment character is an element of $\mathcal{N} \cup \{\text{'-'}\}$. A valid protein alignment character is an element of $\mathcal{A} \cup \{\text{'-'}\}$. A multiple sequence alignment, denoted by $\mathcal{M}$, is of the form $[<\text{Label}_1, \mathcal{S}^A_1>, \ldots, <\text{Label}_M, \mathcal{S}^A_M>]$. Each $\mathcal{S}^A_I$ $(1 \le I \le M)$ is a transformed version of $\mathcal{S}_I$ such that $\text{length}(\mathcal{S}^A_I) = \text{length}(\mathcal{S}^A_J)$ and every element of $\mathcal{S}^A_I$ is a valid nucleotide alignment character; or every element is a valid protein alignment character. Each sequence in the alignment must have an associated unique identifier. A multiple sequence alignment is represented as two-dimensional matrix such that each row represents $\mathcal{S}^A_I$. Each column of $\mathcal{S}^A_I$ is denoted by C_J, where $1 \le J \le \text{length}(\mathcal{S}^A_I)$. Alternately, a multiple sequence alignment is represented as an association of columns,

denoted by $C_1 \oplus ... \oplus C_N$ (where N = length($\mathcal{S}^A_I$)) such that picking character at index I from every column gives the I_{th} sequence $\mathcal{S}_I$. For convenience, multiple sequence alignments will be referred to as alignments. A sub-alignment is a portion of alignment that is derived by selecting a subset of the rows and a subset of the columns.

In a conserved column the cumulative occurrence of other characters is insignificant compared with the occurrences of the most-occurring character. A set of columns $\{C_M,... C_N, ..., C_P\}$ are strongly related if the cumulative occurrence of other subsequences of characters in the set is insignificant compared with frequent subsequences.

A control-sequence is used to mark columns in an alignment. Markers are assigned to the subset of columns corresponding to nonindels in the control sequence. Thus, one might speak of ''E. coli position 413'' meaning ''the column in the alignment in which the 413th nonindel character in the sequence with identifier E.coli occurs.''

Alignments are stored in files in FASTA format. In this format, the file contains a sequence of alignment entries. Each alignment entry begins with a line containing ">" immediately followed by the identifier of the sequence. The remainder of the alignment entry is composed of one or more lines containing the characters of the sequence itself.

A phylogenetic tree depicts a hypothesized evolutionary history of the objects at the leaves of the tree. If the leaves are organisms, then the tree shows a set of ancestral organisms, along with the points at which distinct lineage diverged. If the leaves are homologous sequences, then the nodes of the tree correspond to ancestral sequences. This paper is concerned with phylogenetic trees in which the leaves are sequences from an alignment. A phylogenetic tree asserts a hypothesized evolutionary history for the sequences of an alignment; that is the leaves of the tree are identifiers from an alignment. Now consider the characters that occur in a specific column C_I of the alignment, and suppose that the characters of C_I are added to the appropriate leaves of the tree. What can be inferred about the ancestral values corresponding to the column? There are different approaches to inferring ancestral values.

One approach is to label the internal nodes (assign values to the ancestral sequences) in a way that minimizes the number of evolutionary events required to explain the values of the leaves; that is, one chooses a parsimonious labeling. Often, there are several equivalent parsimonious labelings.

Example 1

A protein alignment is shown in Figure 2. The sequence identifiers are P25112, P33285, P33286, P02380, and P34789. Each block of protein contains ten characters. The alignment contains five rows and 69 columns. The columns C_{26}, C_{57} and C_{69} are equal to [Q, G, G, G, Q], [T, V, V, V, T], and [R, R, R, R, R], respectively. The column C_{69} is a conserved column, and the columns C_{26} and C_{57} are strongly related.

The corresponding phylogenetic tree is given in Figure 3. The leaf nodes are marked with sequence identifiers P25112, P34789, P33285, P33286, P02380; internal nodes are labeled as N_0, N_1, and N_2; and the arcs are marked with the numeric values corresponding to approximate evolutionary distances such as .11540 (between P25112 and N_1) and .16202 (between N_1 and N_0)

```
1                11                21      26        31                41                51        57      61          69
> P25112
MDTSRVQPIK  LARVTKVLGR  TGSQG Q CTQV  RVEFMDDTSR  SIIRN VKGPV   REGDVL T LLE  SEREARRL R
>P33285
- - MDTKTPVT  LAKVI KVLGR  TGSRG G VTQV  RVEF LEDTTR  TIVRNVKGPV   REGD IL V LME  SEREARRL R
>P33286
- - MDS KTPVT  LAKV I KVLGR  TGSRG G VTQV  RVEF LEDTTR  TIVRNVKGPV   REGD IL V LME  SEREARRL R
>P02380
- - MDNKTPVT  LAKV I KVLGR  TGSRG G VTQV  RVEF LEDTSR  TIVRNVKGPV   REND IL V LME  SEREARRL R
>P34789
- - - - MDSQIK  HAVVVKVMGR  TGSRG Q VTQV  RVKF TDSD- R  FIMRNVKGPV   REGDVL T LLE  SEREARRL R
```

Figure 2: A multiple sequence alignment for homologous proteins

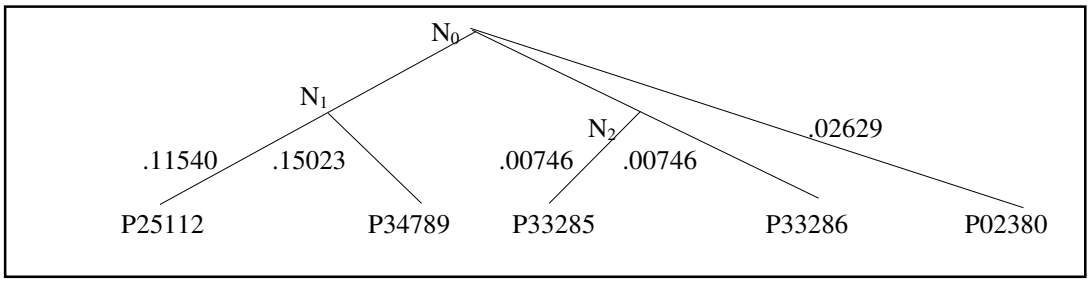

Figure 3: A phylogenetic tree for the alignment

2.2 Prolog

Prolog is a popular high level declarative AI language [8] based on the logic programming paradigm. Prolog facilitates programming because of the natural manipulation of high level data structures such as lists, sets, and trees. Prolog supports rapid prototyping, heuristic reasoning, and an elegant programming style. However, in order to reduce any performance penalty associated with use of a high-level language, a small kernel of functions (necessary for overall performance) has been implemented in the language C. The result is a high-level language library that facilitates rapid code development and reasonable performance.

3 Alignment as Abstract Data Type

In this section alignment is described as an abstract data type, and a kernel set of abstract commands is described. These abstract commands (or functions) have been used to read large alignments (often alignments contain millions of characters), verify alignments for the presence of invalid characters, perform statistical computation on alignments, and integrate analysis of the alignment with processing of an associated phylogenetic tree. Sequences from different genome banks may have nonstandard

characters: characters representing ambiguity between amino acids at a specific position, characters representing an unidentifiable amino acid at a specific position, and erroneous characters introduced during information processing. Such characters necessitate validating the characters in a sequence.

During alignment analysis there is a need for conversion from DNA sequence to RNA sequence and vica versa, and conversion of ASCII representation to internal representation for fast counting and statistical computation.

Cumulative count of different valid characters in a column is needed for statistical computation, to identify a set of conserved columns, and to identify strongly related columns.

During sequence alignment, the actual characters in a sequence are shifted as a result of presence of indels. A control sequence is used to refer to positions in an alignment: a position in the alignment is referred as a pair of the form (position of the last valid character of control sequence, number of indels following the character).

Abstract Operations on Multiple Sequence Alignments

read_alignment(input: File; output: Alignment)

reads an alignment from a File (in FASTA format), constructs an internal representation, and binds the alignment to a ‘‘handle’’ for easy access.

display_alignment(input: Alignment)

displays an alignment.

size_of_alignment(input: File; input-output: Rows, Columns)

reads a multiple sequence alignment from a file (in FASTA format), and returns the number of sequences and number of columns in the alignment.

alignment_columns(input: Alignment; input-output: Column)

returns (or verifies) the number of columns in an alignment.

alignment_rows(input: Alignment; input-output: Rows)

returns (or verifies) the number of rows in an alignment

alignment_type(input: Alignment; input-output: Type)

returns (or verifies) the type of an alignment.

save_alignment(input: Alignment; output: File)

saves an alignment in a file using FASTA format.

free_alignment(output: Alignment)

removes an alignment from the environment.

copy_alignment(input: Alignment; output: Alignment_copy)

creates a new copy of an alignment, and is useful when the original version has to be preserved.

normalize_nucleotide_alignment(input-output: Alignment)
converts ASCII version of valid nucleotide characters of a nucleotide alignment to internal representation for efficient statistical computation.

normalize_protein_alignment(input-output: Alignment)
converts ASCII version of valid protein characters of a protein alignment to internal representation for efficient statistical computation

transform_to_ascii(input: Internal_characters; output: ASCII_characters)
transforms the internal representation of characters to ASCII representation for user interface.

dna_to_rna(input-output: Alignment)
converts all occurrences of 'T' by 'U'.

rna_to_dna(input-output: Alignment)
converts all occurrences of 'U' by 'T'.

validate_nucleotide_alignment(input: Alignment; output: Characters)
returns the set of invalid nucleotide characters in an alignment.

validate_protein_alignment(input: Alignment; output: Characters)
returns the set of invalid protein characters in an alignment.

column_characters(input: Alignment, Column; input-output: Sequence)
returns (or verifies) the sequence of characters for the specified column of an alignment.

row_characters(input: Alignment, Row; input-output: Sequence)
returns (or verifies) the sequence of characters for the specified row of an alignment.

sub_alignment(input: Alignment, Rows, Columns; output: Sub_alignment)
returns a subalignment by selecting the set of sequences in an alignment, and then selecting the characters from the given columns. A subalignment is also treated like an alignment, and all abstract operations are applicable on subalignments.

identifier_to_row(input: Alignment; input-output: Identifier, Row)
returns (verifies) the row number for an identifier in an alignment.

row_to_identifier(input: Alignment; input-output: Identifier, Row)
returns (verifies) the identifier associated with a row in an alignment.

row_identifiers(input: Alignment; output: Identifier_list)

returns the list of identifiers for all the sequences in an alignment. The function is used to test whether the given sequence is a member of an alignment.

character(input: Alignment, Row, Column; input-output: Character)

returns (or verifies) a character present in a given row and column of an alignment.

set_character(input: Row, Column, Character; input-output: Alignment)

writes a character in the position identified by row and column.

count_sequence_characters(input: Alignment, Rows; output: Counts)

returns a sequence of pairs of the form [$<s_1, c_1>$, ..., $<s_N, c_N>$] for the given set of sequences, where s_I is a valid nucleotide (or protein) character for nucleotide (or protein) sequence, and c_I is the count.

count_column_characters(input: Alignment, Columns; output: Counts)

returns a sequence of pairs of the form [$<s_1, c_1>$, ..., $<s_N, c_N>$] for the given set of columns, where s_I is a valid nucleotide (or protein) character for nucleotide (or protein) sequence, and c_I is the count. This function is used to identify conserved columns and strongly related columns in an alignment.

map_control_sequence(input: Control_sequence; input-output: Alignment)

builds up the markers for each column position, and stores the markers in a vector of triples of the form <column, position, markers>.

column_reference(input: Alignment, Column; input-output: Index + Offset)

returns the marker for the a column in the form (index of last valid character of control sequence + Offset), where offset is the number of indels after the last valid character.

4 Labeling of a Phylogenetic Tree

Parsimony labeling has been superimposed on phylogenetic tree to derive the possible set of characters at the ancestral nodes, and to derive mutation events during evolution. Parsimony has been imposed by using following two principles:

1. Majority rule is used to identify the value of the parent for the given values of children,
2. Probability of mutation is equal for every child.

These two principles ensure minimum cumulative mutation count and have been cited in literature [3, 4]. This paper describes a two-pass algorithm for labeling. The first phase generates a set of all possible labels in a bottom-up manner by using majority rule at every internal node. The second pass uses a top-down approach to restrict to one value, using a random selection among equally probable characters, and identifies the mutation arcs by comparing the dissimilar values at a parent node and the children nodes.

The rules for value restriction during second pass is summarized in Table 1. For convenience, restricted values of the parent nodes are denoted as V^P, the value of the child node derived during the first pass is denoted as V^C_1, and the restricted value of child node during second phase is denoted as V^C_2. Note that V^C_1 could be either a single value or a set of equally probable values.

Example 2

Figure 4 demonstrates the parsimonious analysis of the phylogenetic tree given in Figure 3 for the column 57. The result after pass 1 is given in Figure 4a, and both the possibilities of parsimonious labeling after pass 2 is given in Figures 4b and 4c. The arcs with a circle mark the mutation events.

Table 1: Rules for parsimonious labeling of a phylogenetic tree

V^C_1 Type	Relation	V^C_2	Mutated
singleton	$V^P = V^C_1$	V^P	no
singleton	$V^P = V^C_1$	V^C_1	yes
set	$V^P \in V^C_1$	V^P	no
set	$V^P \in V^C_1$	$\in V^C_1$	yes

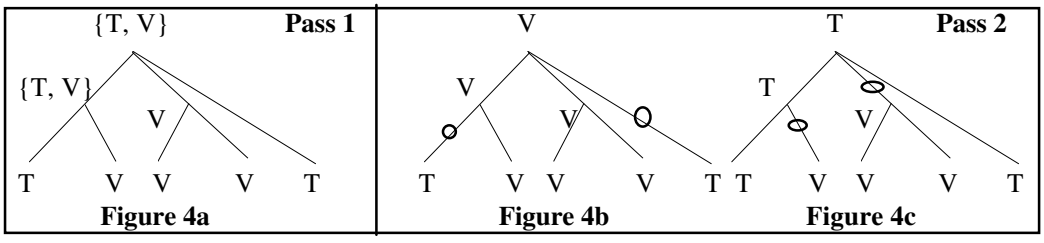

Figure 4: Labeling a phylogenetic tree

Abstract Commands for Labeling

parsimonious_labels(input: Tree, Alignment, Column; output: Labeled_tree)

builds a labeled phylogenetic tree for a column of an alignment and the corresponding phylogenetic tree. Every internal node of the labeled tree carries the set of equally probable values derived after pass 1, the randomly selected value derived during pass 2, and total mutation count (based on the selected value) for the subtree rooted at the node.

min_mutations(input: Labeled_tree, output: Mutations)

returns the total count of mutations for the labeled phylogenetic tree.

pars_values(input: Labaled_tree, output: Node_labels)

returns the set of equally probable values (derived during pass 1) for an internal node of the labeled phylogenetic tree.

5 Identifying Constrained Columns

In this section, I describe a heuristics to identify a significant portion of the sequence, using the notion of mutationally independent nodes. Two nodes of a labeled phylogenetic tree are mutationally independent if the nodes have the same character which is different from the background character - character occurring at the root node of the labeled phylogenetic tree, and the root node of the minimal subtree covering the nodes has background character, and there are only two or three types of characters in a column.

Since there are multiple possible parsimonious labeling for a pair of phylogenetic tree and multiple sequence alignment, choice of independent nodes depends upon the choice of the labeled tree. However, the presence of independent nodes is not affected by the choice of the labeled trees.

Example 3

Consider the labeled phylogenetic trees in Figure 4b and 4c for the phylogenetic tree in Figure 3 and multiple sequence alignment in Figure 2. In Figure 4b, the background character is V; the nodes P25112 and P02380 are independent nodes, since the node N_0 has amino acid character V, and both P25112 and P02380 have characters T. In Figure 4c, the background character is T; and the nodes P34789 and N_2 are independent. Note that the number of mutational events between the independent nodes is two.

The constrained columns possibly play a major role in marking the portions necessary for structure or function of a genome (or protein) sequence, since the presence of the same character despite independent mutations exhibits evolutionary pressure to preserve a common property necessary for stabilization of the molecule. Moreover, the difference in the properties of two amino acids (or nucleotides for genes) at mutationally independent nodes may be responsible for function-preserving variations in physical and chemical properties between homologous proteins

The algorithm for identifying constrained columns is given in Figure 5. In the algorithm, function *parsimonius_labels* generates a labeled phylogenetic tree, the function *make_string* concatenates all the label in a set, the function *character* returns the parsimonious value at the node.

The algorithm was coded in Prolog and executed on various homologous protein sequence alignments in protein data banks available at Argonne National Laboratory. The corresponding unrooted phylogenetic tree for every protein alignment was created by using a software at Argonne National Laboratory.

Results and Discussions

Table 2 exhibits the pairs of amino acids and their relative occurrence. The strong

```
Algorithm constrained_columns;
Input:  1. A multiple sequence alignment M;
        2. A phylogenetic tree T;
Output: 1. S^1 - A set of constrained columns;
        2. S^2 - A set of  group of substitutable characters in all the constrained columns.
begin
        1.S^1 = Φ; S^2 = Φ; /* initialize */;
        2. Let the set of columns with two or three types of characters be S^C .
        3. ∀ C ∈ S^C   /* process each column containing two or three types of character */
        do
          4. T^M = parsimonius_labels(T^M);  /* generate a labeled phylogenetic tree */
          5. Let the background character in the column be B;
          6.∀ internal node n ∈ T^M  such that character(n) has value B
                /* pick a node with background character */
                do
                7. Let the set of characters in left subtree be S^L;
                8. Let the set of characters in right subtree be S^R;
                9. S^M = S^L ∩ S^R - B; /* get the characters different from the background character */
                10. if S^M ≠ Φ then  /* non-empty set of characters */
                        11. S^1 = S^1 ∪ {C};  /* mark the columns as constrained*/
                        12. S^2 = S^2 ∪ {make_string({B} ∪ (S^M)};  /* include in the set */
                    endif;
                od ;
        od;
end.
```

Figure 5: Identifying constrained columns

substitutability between two amino acids is related to specific common properties being preserved. Simple rules of equivalence and transitivity do not work in this domain, since two amino acids may share more than one common property. For example, D is substituted by E and by N quite regularly. However, E is rarely substituted by N.

The analysis shows that a large percentage of constrained columns are covered by three amino acids--- isoleucine, leucine, and valine--- which form the nonpolar hydrophobic region in proteins. Some frequently occurring pairs are 'AG', 'AP', 'AS', 'AT', 'AV', 'DE', 'DN', 'EQ', 'FL', 'GS', 'GN', 'IL', 'IV', 'KQ', 'KR', 'LM', 'LV', 'NS', 'QR', 'ST'. Many of these pairs belong to similar subclass of amino acids. For example, the pairs 'AG[1]', 'IL', 'IV', 'LV', and 'AV' are aliphatic amino acids and exhibit hydrophobicity; the amino acids 'DE' are acidic and have a fully ionized second carboxyl group; the amino-acids 'KR' are positively charged basic amino acids; and the amino acids 'GS', 'GN', and 'ST' are uncharged polar hydroxyamino acids. Similarly, the amino acid 'N' is an amide of 'D', and the amino acid 'Q' is an amide of 'E'. Although, some of the pairs have amino-acids from different sub classes such as 'AS', 'AT', 'LM', 'FL', and VT. However, these pairs share a common property. For example, 'S' is the hydroxyl version of the 'A'; 'T' is a hydroxyl version of 'V'; 'and both 'LM' and 'FL' have nonpolar hydrophobic side-chain. Some of the less frequent pairs such as 'KQ' have a common

[1] G is a border line case between non-polar aliphatic amino-acids and uncharged polar amino-acids.

potentiality of hydrogen acceptance [9]. A further study of relaxing the constraints to allow three different types of character in a column exhibited that there are constrained columns with triples such as 'ILV' a situation that is to be expected because all three are highly hydrophobic aliphatic acids.

Table 2: Relative frequency of correspondence of amino acids for constrained columns

	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Total	Probability in %
A	X	11	5	11	2	102		2	14	8	2		37	1	4	176	51	51		477	8.8
C	11	X			2	1		1		2		1			2	14		2		36	0.6
D	5		X	165		12	6				2	72	1	2	2	11	3	1		282	5.2
E	11		165	X		6	1		15	1	1	3	5	43	1	2	1	3		258	4.7
F	2	2			X		3	8		118	3		1			3		5	7	152	2.8
G	102	1	12	6		X			6			11	4	2	5	31	5	3	1	189	3.5
H			6	1	3		X		3	1	1	9		17	11	3	1			56	1
I	2	1			8			X		1	213	25	2		1	1	16	582	1	853	15.8
K	14			15		6	3	1	X	4	2	14	2	23	198	6	6	3		297	5.5
L	8	2		1	118		1	213	4	X	81		13	4	6	8	4	87		550	10.2
M	2		2	1	3		1	25	2	81	X			2		1		10		130	2.4
N		1	72	3		11	9	2	14			X		5	5	28	9			159	2.9
P	37		1	5	1	4			2	13			X	5	3	15	9	2		97	1.8
Q	1		2	43		2	17		23	4	2	5	5	X	20		2	1		127	2.3
R	4	2	2	1		5	11	1	198	6		5	3	20	X	2	2		4	266	4.9
S	176	14	11	2	3	31	3	1	6	8	1	28	15		2	X	118			419	7.7
T	51		3	1		5	1	16	6	4		9	9	2	2	118	X	20	1	248	4.6
V	51	2	1	3	5	3			582	3	87	10		2	1		20	X			14.3
W					7	1		1							4		1			14	0.2

6. Other Applications

The tool has also been used to derive conserved columns and strongly related group of columns. For example, conserved columns are derived by using the function count_columns and by identifying the character with maximum count with a percentage value above a threshold value. Strongly related columns are identified by the iterative derivation of the counts of character-groups for a set of columns, using the function count_columns such that the set of character-groups is restricted to very few possibilities from Cartesian product $\mathcal{N}$ X ... X $\mathcal{N}$ for nucleotides and $\mathcal{A}$ X ... X $\mathcal{A}$.

7. Conclusion

In this paper, a high level language framework for comparative analysis of homologous sequence alignment and the corresponding toolkit have been described. The toolkit is suitable for complex analysis of large multiple sequence alignments and large phylogenetic trees, and the integration of information derived from multiple sequence alignment and phylogeny. The framework has been applied to identify a new set of constrained columns which exhibit some evolutionary pressure to preserve a common property despite mutations resulting into different amino acids The set of these constrained columns is significant because their common properties characterize the portion of homologous protein sequences.

Currently, the properties of different amino-acid groups (based on their chemical structure) are being studied in detail, and strongly related constrained columns are being

studied in order to integrate this information with secondary and tertiary structures predicted using other techniques.

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