

# 1 Introduction

MultiSeq (shown in Fig. 1) is a unified bioinformatics analysis environment that allows one to organize, display, and analyze both sequence and structure data for proteins and nucleic acids. Special emphasis is placed on analyzing the data within the framework of evolutionary biology. MultiSeq was created to allow biomedical researchers to study the evolutionary changes in sequence and structure of proteins across all three domains of life, from bacteria to humans. The comparative sequence and structure metrics, and analysis tools introduced in the article by O'Donoghue and Luthey-Schulten <sup>1</sup> are part of MultiSeq. In par-

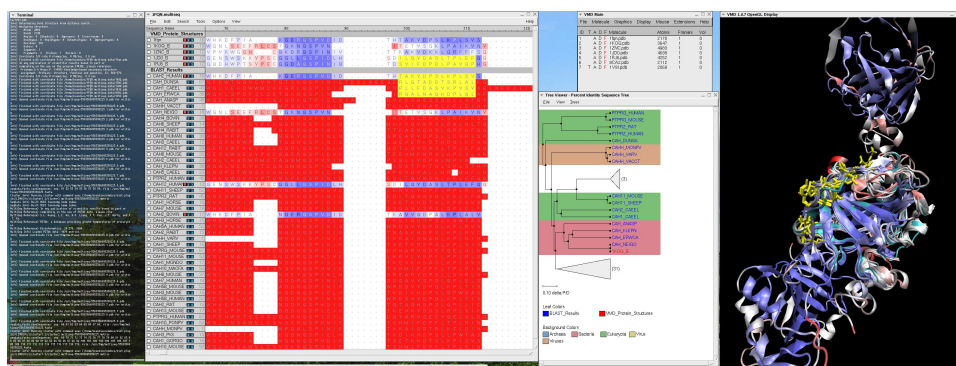


Figure 1: MultiSeq In VMD

ticular, the Luthey-Schulten group has included a structure-based measure of homology  $Q_H$  (see Appendix B), that takes into account the effect of insertions and deletions and has been shown to produce accurate structure-based phylogenetic trees. The STAMP structural alignment algorithm, kindly provided by our colleagues Russell and Barton, is included <sup>2</sup>. As a result, Multiple Alignment is an invaluable tool for relating protein structure to its function or malfunction.

In any publication of scientific results based completely or in part on the use of MultiSeq, please reference:

Elijah Roberts, John Eargle, Dan Wright, and Zaida Luthey-Schulten. MultiSeq: Unifying sequence and structure data for evolutionary analysis. BMC Bioinformatics, 2006, 7:382.

## 1.1 Installation

MultiSeq 3.0 is part of the VMD 1.8.7 release. You can download VMD from <http://www.ks.uiuc.edu/Research/VMD/>. Although BLAST is not necessary

<sup>1</sup>P. O'Donoghue and Z. Luthey-Schulten. "Evolution of Structure in Aminoacyl-tRNA Synthetases" MMBR, 67(4):550-73. December, 2003.

<sup>2</sup>R.B. Russell and G.J. Barton. "Multiple protein sequence alignment from tertiary structure comparison: assignment of global and residue confidence levels." Proteins: Struct. Func. Genet., 14:309-323. 1992.

for the overall function of MultiSeq, it is highly recommended to have BLAST installed locally (i.e. accessible through file browsing on your local computer). See <http://www.scs.uiuc.edu/~schulten/multiseq/> for links to tutorials with additional information on BLAST installation.

MultiSeq uses a collection of databases that need to be downloaded to your computer system. The first time you run MultiSeq you will be asked to create a folder to store these databases, and the databases will then be downloaded from our servers. When you subsequently run the plugin, it will check to insure that you have the most recent versions of the databases.

## 2 The MultiSeq Graphical Environment

MultiSeq is accessed as an extension within VMD. To begin MultiSeq, launch VMD and:

1. In the VMD main window, click on the Extensions Menu.
2. In Extensions, select Analysis → MultiSeq.

(alternatively, if you are a fan of command lines, you can type ‘multiseq’ into the VMD terminal window)

The main MultiSeq window (see Fig. 2) will appear (note that the first time you run MultiSeq, you will be prompted to download necessary databases before seeing the main window).

## 3 Using and Managing Data

To begin analyzing proteins in MultiSeq, data from sequence<sup>3</sup> and structure<sup>4</sup> files is required. Import Data (from the File menu within MultiSeq) allows you to load structure and sequence files, both locally and via a network connection.

Various structure and trajectory files, such as PDB and PSI, can be loaded via the New Molecule function of the VMD Main window, but Import Data allows you to load sequence files as well. Additionally, Import Data has BLAST searching capabilities, if a local copy of BLAST is installed.

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<sup>3</sup>FASTA files.

<sup>4</sup>The ASTRAL database (<http://astral.stanford.edu>) is a compendium of protein domain structures derived from the PDB database. It divides each protein structure into its domain components. For example, AsprS is divided into three separate PDB files: one containing the catalytic domain, one with the insertion domain, and one for the anticodon binding domain. The names of the files contain the PDB extension, the letter a for ASTRAL, and a number, which corresponds to which domain it is in the original PDB file. The PDB is the single worldwide repository for the processing and distribution of 3-D structure data of large molecules of proteins and nucleic acids.

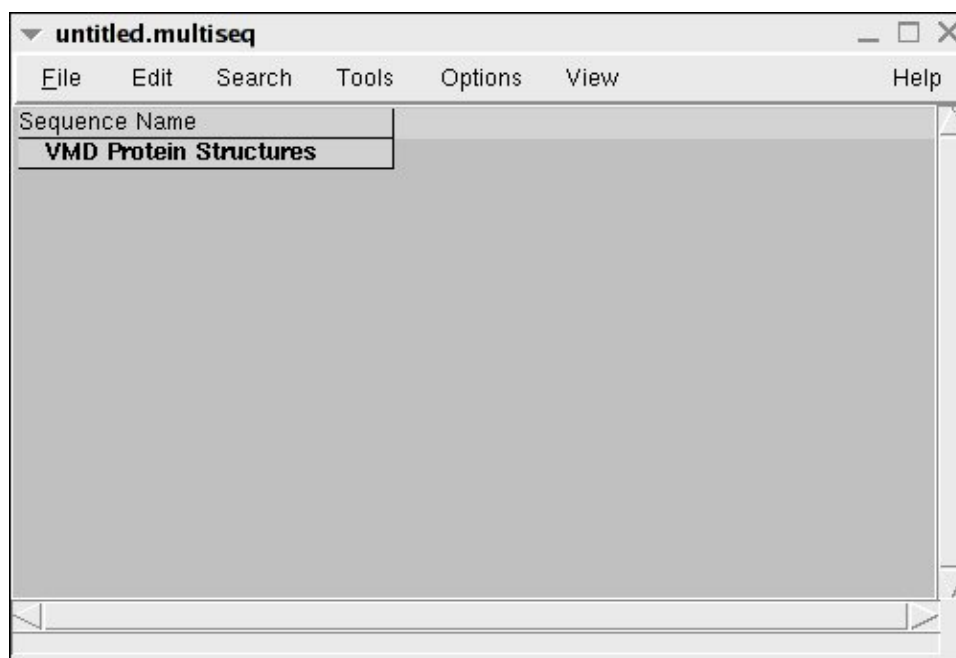


Figure 2: Main MultiSeq Window With No Structures Loaded

### 3.1 Importing from files

Structure<sup>5</sup> and Sequence files can be loaded into MultiSeq via **Import Data**. PDB files are structure files, whereas FASTA is a sequence file format. To load these files:

1. Make sure **From Files** is selected as a **Data Source**.
2. In the **Filenames:** dialogue, either type in the location of the file, or hit the browse button to locate the file. Another option is to simply type in the PDB or SCOP id. This option requires a network connection for your computer to obtain files from PDB or ASTRAL directly.
3. Hit the **OK** button.

If you would like to load multiple files/structures/sequences at once, you can separate each with a comma.

### 3.2 Sequences and BLAST searching

You can conduct a BLAST search from within MultiSeq if you have the BLAST program installed on your computer. You will need to install BLAST if you

<sup>5</sup>See VMD Manual for supported formats

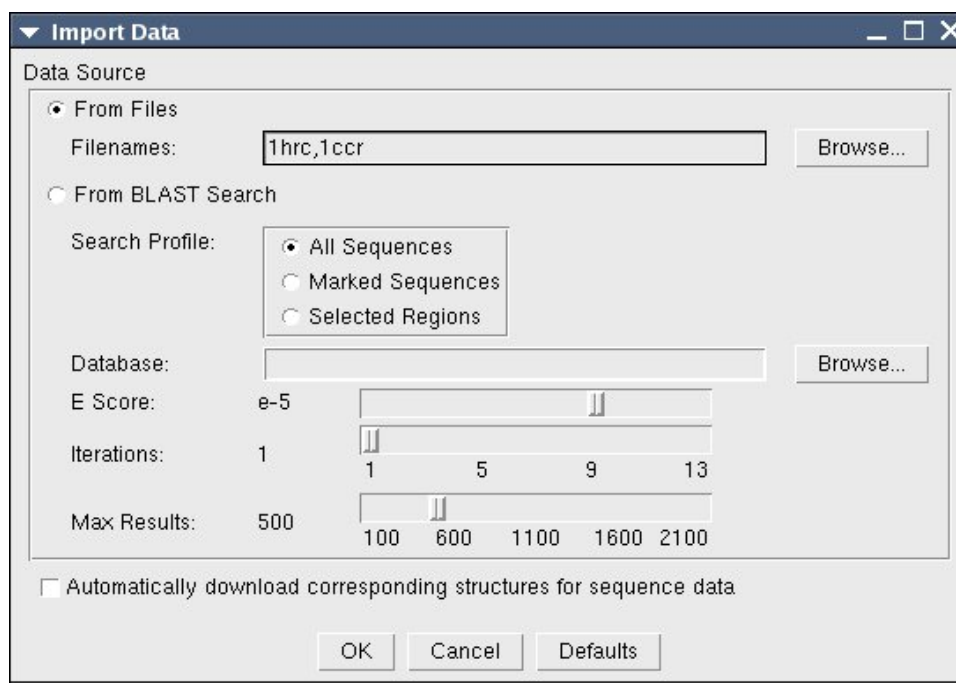


Figure 3: Import Data Window

haven't already done so, and you will have to configure MultiSeq to know where BLAST is installed (via File — Preferences — Software)

1. Before you open the **Import Data** window, you have the option of either selecting a set of sequences, or a region within a sequence.
2. Go to File and then **Import Data** and select **From BLAST Search**, and either **All Sequences**, **Marked Sequences**, or **Selected Regions**.
3. In the **Databases**, either type the location of the database, or use the **Browse** button to locate it. This could be something like a Swiss PROT database or otherwise. Once you give MultiSeq the name of a database, it will remember it for future searches.
4. Select the **E Score**, **Iterations**, and **Max Results**.
5. If you want MultiSeq to automatically download structure information for sequences found via the BLAST search, mark the checkbox for that.
6. Hit the **OK** button.

MultiSeq will then begin a BLAST search. This may take several minutes. When the search is done, a new window called **BLAST Search Results** will appear.

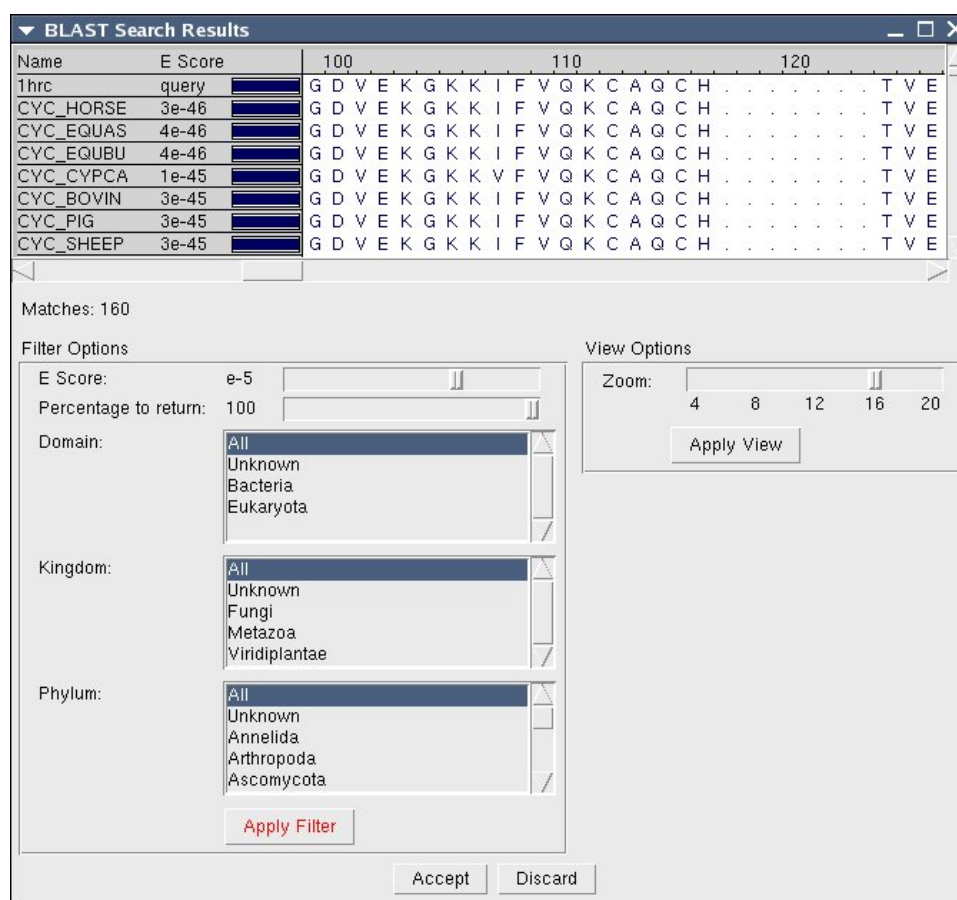


Figure 4: BLAST Search Results

The results do not immediately appear in the main MultiSeq window, because you can apply further filters on the retrieved sequences. The BLAST Search Results window is divided into three main parts: the sequence viewer, Filter Options, and View Options.

The sequence viewer is a read-only display of the sequences that match your BLAST search. The number of matches is listed below the sequence viewer.

You can use the **Zoom** to change how much of each sequence you see. You can change the zoom level and **Apply View** and you will see fewer or more sequences in the sequence viewer portion of the window.

In the **Filter Options** you can tweak the parameters to reduce or expand the number of sequence matches. Once you have changed a parameter you can hit **Apply Filter** and see which sequences match.

Once you have a collection of sequences that you want to import, you can hit the **Accept** button at the bottom and they will be added to the MultiSeq

window.

## 4 Working in the Environment

MultiSeq provides a unique working environment for the analysis of proteins.

### 4.1 Grouping

While working with the Sequence Viewer in MultiSeq, you may notice certain patterns or trends. As a result you would like to put certain sequences closer to others to analyze such motifs. MultiSeq allows such grouping based on taxonomy or you can customize the groupings. Right clicking on a group name (such as VMD Protein Structures will bring up a context menu where you can manage groups.

### 4.2 Info Viewer

Whenever you load a sequence or structure into MultiSeq an ‘i’ box will appear next to the protein’s ID. If you click on this box, a new window will appear called the **Info Viewer** (See Fig. 5). Within this window information regarding the species the protein is from will appear. If you have PSIPred installed and configured, you can predict the secondary structure at the bottom of the Info window.

### 4.3 Selecting vs. Marking

As you browse the menus of MultiSeq you will notice options for **Selected Sequences** or **Marked Sequences**. “Selecting Sequences” is when you highlight a portion of the sequence(s) in the sequence viewer using the mouse. This can be either the entire sequence or a portion. However “Marking Sequences” allows you to more easily select an entire sequence by simply checking the box next to the protein ID.

## 5 Edit Menu

Along with the copy/cut/paste options that you expect to see in an edit menu, this menu also provides a power sequence editor.

If you want to edit a sequence, **Enable Editing**. If you are just wanting to align sequences, you can probably choose to just enable gap editing. Once you have enabled editing, you can then use the mouse to choose a residue (or residues). Hit the space bar to insert a gap, or, if you have enabled full editing, you can insert a residue by typing the desired character.

If you want to truly edit the sequences manually, you can choose to **Edit In Text Editor**. VMD’s text editor will be loaded, and you can change the sequence

**Edit Sequence Information**

Sequence Name: 1hrc

Source Organism: Equus caballus

Common Name: equine

Sequence Type: Protein

EC Number:

EC Description:

Description:

Data Sources: pdb=1hrc,A  
sp=\*,CYC\_HORSE

Lineage: Eukaryota  
Metazoa  
Chordata  
Craniata  
Vertebrata  
Euteleostomi

Notes

Secondary Structure

Predict

CCCHHHHHHHHHHHHTTTTBCCTTT  
CTTTTTBTTTTTTETTTTTCCCHH  
HHHHCCEECHHHHHHHHHCHHHH  
TTTCCCCCCCCCHHHHHHHHHH

OK Cancel

Figure 5: Edit Sequence Information

data. Dashes are gaps and the sequence characters can be changed as you see fit.

## 6 Search Menu

**Find, Find Next, Find Previous** Via Search, you can find and highlight residues in sequences. When you use Find, all of the residues will be highlighted, and you can then cycle through them by using Find Next and Find Previous.

**Select Contact Shells** See Figure 6.

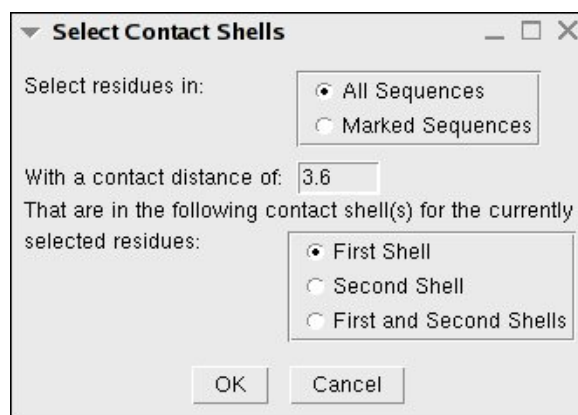


Figure 6: Select Contact Shell Window

**Select residues in:** Lets you choose whether to look through all sequences, or just the ones you have marked.

**With a contact distance of:** defaults to 3.6.

**That are in the following contact shell(s) for the currently selected residues**  
Choose from First, Second, or First and Second shells

**Select Non-Redundant Set** You can use structure QR or sequence QR to select a non-redundant set (See Fig. 7).

**Select from:** Lets you choose whether to look through all sequences, or just the ones you have marked.

**Using Structure QR QH Cutoff:** Can vary from 0 to 1.

**Using Sequence QR Identity Cutoff:**

**Gap Scale Factor:**

**Seed with selected sequences** If you have selected certain sequences, you can seed the algorithm with these sequences to select a non-redundant set based on them.

**Select Residues** The Residue Selection feature (See Fig. 8) lets you analyze conservation, using different measures, and highlight residues in the Sequence Display and Structure Display simultaneously. Residue Selection allows you to examine the conservation on a per residue basis.

There are two options: either Where Sequence Identity is or Where Qres is. Where Sequence Identity is is a sequence identity measure, whereas Where Qres is is a structure measure.

**Select residues in:** You can choose all sequences or just the marked ones.



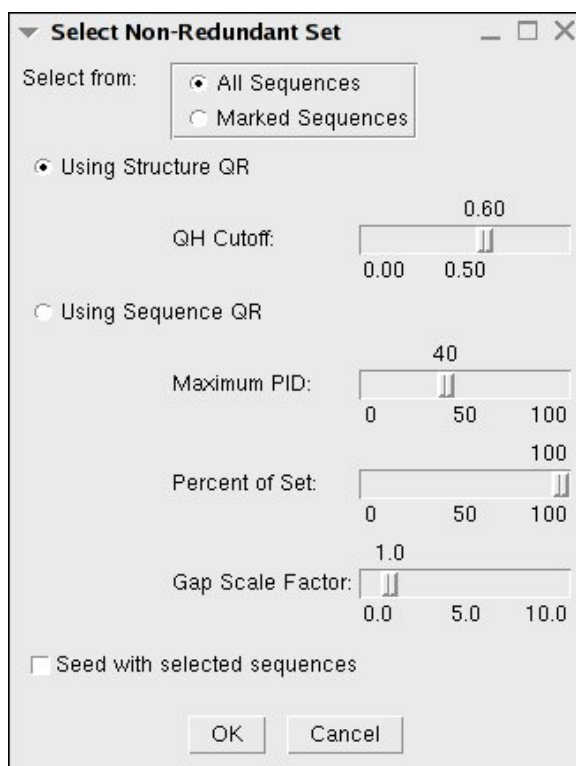


Figure 7: Select Non-Redundant Set Window

**Where Sequence Identity is:** If this option is selected you can select either the less than or equal to option, then a number between 0-99.

**Where Qres is:** If this option is selected you can select either the less than or equal to option, then a number between 0-99.

## 7 Tools Menu

### 7.1 Performing Alignments

MultiSeq can do both structural and sequence alignments. These options are available via the Tools menu in MultiSeq.

#### 7.1.1 Structure Alignments

MultiSeq uses the program STAMP to structurally align protein molecules. The STAMP algorithm minimizes the  $C_{\alpha}$  distance between aligned residues of each molecule by applying globally optimal rigid-body rotations and translations. Also, note that you can perform alignments on molecules that are structurally

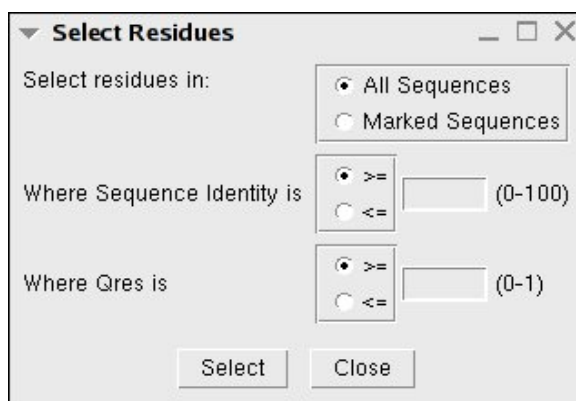


Figure 8: Select Residues Window

etc.

similar. If you try to align proteins that have no common structures, STAMP will have no means to align them. If you would like further information about how the alignment occurs, please refer to the STAMP manual.

**Align the following:** Choose which structures you wish to align

**Number of passes (npass):** Whether one or two fits are to be performed.

The idea is that the initial fit can be used with a conformation biased set of parameters to improve the initial fit prior to fitting using distance and conformation parameters. Default NPASS = 1

**Similarity (scanscore):** Specifies how the Sc value (STAMP algorithm) is to be calculated. This depends on the particular application. As a general rule of thumb, use SCANSORE=6 for large database scans, when you are scanning with a small domain, and wishing to find all examples of this domain - even within large structures. Use SCANSORE=1 when you wish to obtain a set of transformations for a set of domains which you know are similar (and have defined fairly precisely as domains rather than the larger structure that they may be a part of). Default SCANSORE = 6

**Comparison residues (scanslide):** This is the number of residues that a query sequence is 'slid' along a database sequence to derive each initial superimposition. Initially, the N-terminus of the query is aligned to the 1st residue of the database, once this fit has been performed and refined, and tested for good structural similarity, the N-terminus is aligned with the 1+th position, and the process repeated until the end of the database sequence has been reached. Default SCANSLIDE = 5

**Slow scan:** If set to TRUE, then the SLOW method of getting the initial fits for scanning will be used (See chapter 1). Default SLOWSCAN = FALSE

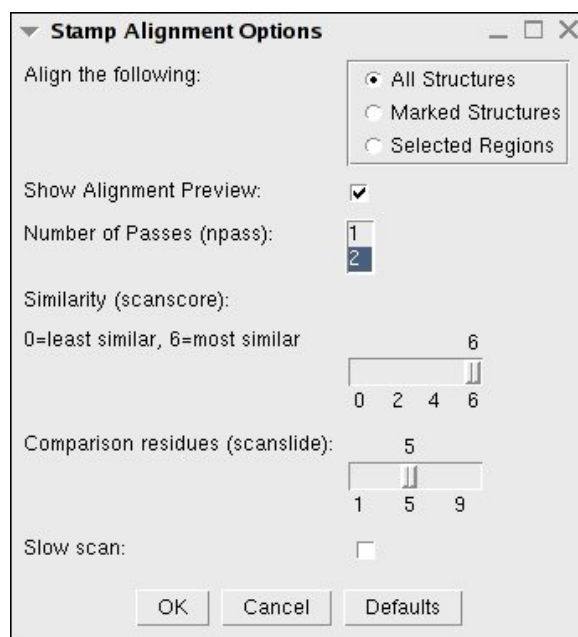


Figure 9: STAMP Structural Alignment Window

**Defaults:** resets the STAMP parameters to their original values

### 7.1.2 Sequence Alignments

Sequence alignment in MultiSeq is done via ClustalW.

You can choose from Multiple Alignment, Profile/Sequence Alignment, or Profile/Profile Alignment. Once you have chosen the desired type of alignment, you can set the proper option.

**Multiple Alignment** Choose which sequences or regions you wish to align.

**Profile/Sequence Alignment** This requires certain sequences to be marked, and they will then be aligned to the group that you specify.

**Profile/Profile Alignment** To align one entire group with another entire group, select this option.

## 7.2 Phylogenetic Tree

The Phylogenetic Tree feature helps in determining the structure and sequence-based relationships between the aligned domains of proteins.

To do this, it uses a modification of Q that accounts for both gapped and aligned regions. This new metric,  $Q_H$ , creates a structure-based phylogeny that

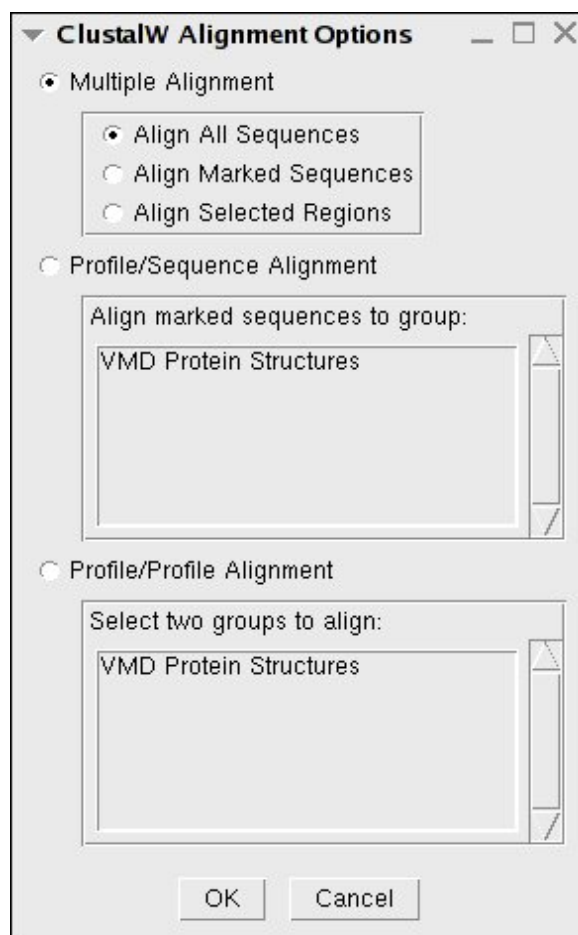


Figure 10: ClustalW Sequence Alignment

is congruent to the sequence-based phylogenies. You can create a Phylogenetic Tree from the **Tools** menu in MultiSeq (See Fig. 11). Once you choose the sequences or regions you wish to create a tree for, you can choose which trees you want to create. The tree viewer can also create a tree from a data file that you provide (if you have created tree data from an external program, for instance).

Once you have chosen which trees to create, the Tree Viewer will be shown in simple black and white. But, you can easily use color and Tree View commands to make the data more useful (see Fig. 12).

The Tree Viewer window is very powerful. In the main window, you can right click on any small black box (in front of an individual sequence, or at any joint in the tree) and remove the element/subtree or look at its properties. Additionally, if you have selected a subtree, you can change the shape of the tree. You can collapse/expand a subtree, as shown in Fig. 12 as well.

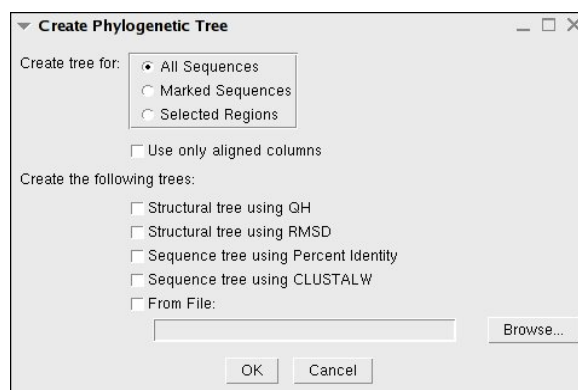


Figure 11: Create Phylogenetic Tree Window

Menu options include:

**File** Trees can be loaded and saved in common formats. Additionally, postscript renderings can be created for use in publications.

**View** If a distance matrix has been created from the data, you can view it. You can also modify the way the tree looks. You can zoom in and out, change the scale (which pushes tree leaves left or right for viewability). Orientation will move the labels from the left side of the tree to the right, and you can even choose whether or not you wish the tree to display the labels and nodes.

The **Leaf Text** option lets you choose the labels that you wish to have displayed, and you can color the labels as well as the tree backgrounds by a variety of different metrics.

You can easily collapse large parts of the tree by choosing a criteria, and, if you have selected a point in the tree, you can make that point the new root node of the tree.

**Trees** If you have chosen to create multiple trees, you can use this menu to rotate through the trees, or you can jump to one directly.

### 7.3 Plot Data

**Plot Data** create graphs of internal MultiSeq data. You can **Plot Data** from the Tools menu in MultiSeq. Once you choose the sequences or regions you wish to plot, you can choose the data (such as **Qres**, **RMSD**, etc) for each residue that you want to display. You can also plot custom data. The data graph will then be displayed (see Fig. 13). If you wish, you can create a postscript file for publication.

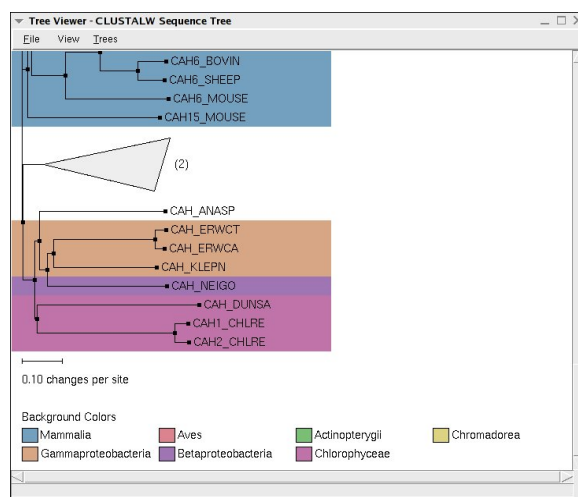


Figure 12: Phylogenetic Tree Viewer - CLUSTALW Sequence Tree

## 8 Options Menu

**Atom Picking** Normally disabled, but can be turned on.

**Grouping** MultiSeq can automatically create groups and show the sequences in the MultiSeq window sorted accordingly. Just choose the grouping that you want to use.

## 9 View Menu

The View menu provides several useful options for coding and looking at large amounts of data.

**Zoom** To change the amount of data seen in the MultiSeq window, you can zoom in and out. As you zoom farther out (choosing a percentage that is smaller) MultiSeq will make the sequence letters smaller and smaller until you will only see the background colors and then, not even that. If you need to see the entire sequence, the **Zoom Window**, discussed below, might be more useful.

**Coloring** You can choose to color the sequences by a wide range of attributes. First, you can choose *what* you want to color by choosing **Apply to All**, **Group**, or **Marked**. Then, you can choose the coloring method that you wish to apply.

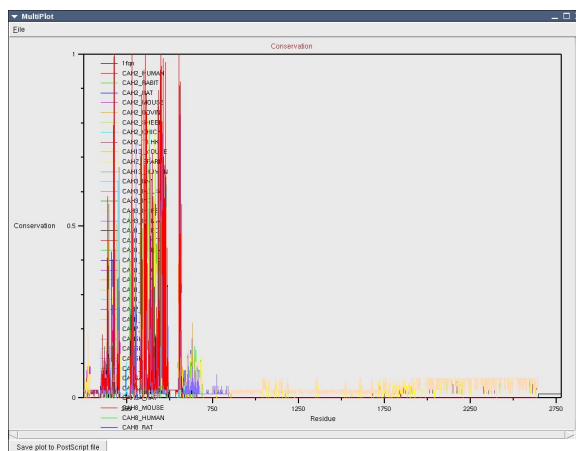


Figure 13: Plot Data - Sequence Conservation

For **Qres**, traditionally,  $Q$  has meant “the fraction of similar native contacts” between the aligned residues in two proteins<sup>6</sup>, or in two different conformational states of the same protein. When  $Q = 1$ , it indicates that the structures are identical. When  $Q$  has a low score ( $0.1$ ), it means the structures do not align well, or, in other words, only a small fraction of the C-alpha atoms superimpose. You will discover that homologs typically have  $Q \geq 0.4$ .  $Q$  per residue is the contribution from each residue to the overall average  $Q$  score. For more information see Appendix A.

For **Sequence Identity**, the aligned domains are colored by how much of the sequence is conserved. The **Sequence Identity** coloring method colors each amino acid according to the degree of conservation within the alignment: blue means highly conserved, whereas red means very low or no conservation.

**Highlight Style** **Highlight Style** is an option for the OpenGL diplay. The style refers to drawing method in VMD<sup>7</sup>. This option allows a user to highlight residues of a structure in the sequence display and see the areas simultaneously highlighted in the OpenGL display.

**Highlight Color** **Highlight Color** is another option for the OpenGL diplay. Alongside **Highlight Style**, **Highlight Color** is the color or coloring method<sup>8</sup> used in the OpenGL display when highlighting residues in the Sequence Display. The default **Highlight Color** is yellow.

<sup>6</sup>Eastwood, M.P., C. Hardin, Z. Luthey-Schulten, and P.G. Wolynes. "Evaluating protein structure-prediction schemes using energy landscape theory." IBM J. Res. Dev. 45: 475-497. 2001

<sup>7</sup>For more information about drawing methods, please refer to the VMD manual.

<sup>8</sup>For more information, please refer to the VMD manual

**Color Scale** Once you have chosen a coloring, you might wonder what the specific colors mean. The **Color Scale** option will show you the scale of colors according to value.

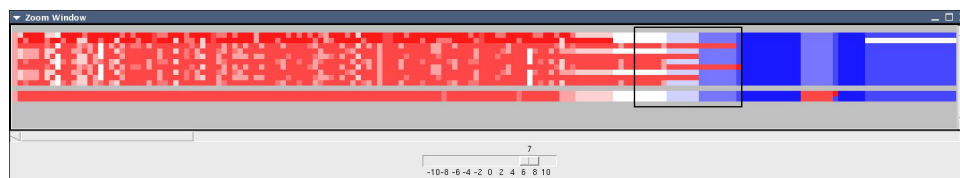


Figure 14: Zoom Window

**Zoom Window** (See Fig. 14) If you need to see the entire collection of sequences and quickly move from area to area, the **Zoom Window** will be useful to you. It shows the entire sequence palette. You can choose the zoom factor using the sliding bar at the bottom of the window, and the black box shows you the area of the sequences that are currently visible in the MultiSeq window. To see other areas, just click the mouse and the black box will be moved to the mouse pointer location.

Note: When you have the **Zoom Window** open, the MultiSeq window will redraw more slowly. If this is a problem for you, just close the **Zoom Window** and reopen as needed.

## 10 Working With Sessions

The **Load** and **Save Session** options from the **File** menu provide a way to save and load all of the files, alignments, and visual representations currently in use within MultiSeq in a convenient package.

### 10.1 Save Session

You can save a session of MultiSeq, with all of the files, alignments, and visual representations, by simply going to the **File Menu** and selecting **Save Session**. You will be prompted to save the session, and will have the opportunity to create a unique name for the session here. Hit the **OK** button. A file will be generated with a `.multiseq` extension along with a directory filled with various files necessary to load the saved session into MultiSeq. Please note that both the generated file and directory have to be in the same directory location in order to load up the session in the future properly.

### 10.2 Load Session

Unlike **Import Data** (also in the **File** menu), **Load Session** opens up a previous session of MultiSeq with all of the sequence and structure files aligned, and using



previous coloring and drawing methods. To load a previously saved MultiSeq Session, simply select the File menu and Load Session. A file browser will appear allowing you to select a file with the extension `.multiseq` and make sure it has a corresponding directory of the same name.

## 11 Other Ways To Export Data

### 11.1 Save to PostScript

From the File menu, if you choose Save Screenshot, you will be able to save a postscript version of the MultiSeq window.

## 12 Appendices

### 12.1 Appendix A: $Q$

The following equation is from the article “Evaluationg protein structure-prediction schemes using energy landscape theory” by Eastwood, et al.

$$Q = \frac{2}{(N-1)(N-2)} \sum_{i < j-1} \exp \left[ -\frac{(r_{ij} - r_{ij}^N)^2}{2\sigma_{ij}^2} \right]$$

$r_{ij}$  is the distance between a pair of  $C^\alpha$  atoms.

$r_{ij}^N$  is the  $C^\alpha$ - $C^\alpha$  distance between residues  $i$  and  $j$  in the native state.

$\sigma_{ij}^2 = |i - j|^{0.15}$  is the standard deviation, determining the width of the Gaussian function.

$N$  is the number of residues of the protein being considered.

## 12.2 Appendix B: $Q_H$

The following text is in the article “On the evolution of structure in aminoacyl-tRNA synthetases.” by O’Donoghue et al.

### Homology Measure

We employ a structural homology measure which is based on the structural similarity measure,  $Q$ , developed by Wolynes, Luthey-Schulten, and coworkers in the field of protein folding. Our adaptation of  $Q$  is referred to as  $Q_H$ , and the measure is designed to include the effects of the gaps on the aligned portion:  $Q_H = \aleph(q_{aln} + q_{gap})$ , where  $\aleph$  is the normalization, specifically given below.  $Q_H$  is composed of two components.  $q_{aln}$  is identical in form to the unnormalized  $Q$  measure of Eastwood et al. and accounts for the structurally aligned regions. The  $q_{gap}$  term accounts for the structural deviations induced by insertions in each protein in an aligned pair:

$$Q_H = \aleph [q_{aln} + q_{gap}]$$

$$q_{aln} = \sum_{i < j-2} \exp \left[ -\frac{(r_{ij} - r_{i'j'})^2}{2\sigma_{ij}^2} \right]$$

$$q_{gap} = \sum_{g_a} \sum_j^{N_{aln}} \max \left\{ \exp \left[ -\frac{(r_{g_a j} - r_{g'_a j'})^2}{2\sigma_{g_a j}^2} \right], \exp \left[ -\frac{(r_{g_a j} - r_{g''_a j'})^2}{2\sigma_{g_a j}^2} \right] \right\}$$

$$+ \sum_{g_b} \sum_j^{N_{aln}} \max \left\{ \exp \left[ -\frac{(r_{g_b j} - r_{g'_b j'})^2}{2\sigma_{g_b j}^2} \right], \exp \left[ -\frac{(r_{g_b j} - r_{g''_b j'})^2}{2\sigma_{g_b j}^2} \right] \right\}$$

The first term,  $q_{aln}$ , computes the unnormalized fraction of  $C^\alpha$ - $C^\alpha$  pair distances that are the same or similar between two aligned structures.  $r_{ij}$  is the spatial  $C^\alpha$ - $C^\alpha$  distance between residues  $i$  and  $j$  in protein a, and  $r_{i'j'}$  is the  $C^\alpha$ - $C^\alpha$  distance between residues  $i'$  and  $j'$  in protein b. This term is restricted to aligned positions, e.g., where  $i$  is aligned to  $i'$  and  $j$  is aligned to  $j'$ . The remaining terms account for the residues in gaps.  $g_a$  and  $g_b$  are the residues in insertions in both proteins, respectively.  $g'_a$  and  $g''_a$  are the aligned residues on either side of the insertion in protein a. The definition is analogous for  $g'_b$  and  $g''_b$ .

The normalization and the  $\sigma_{ij}^2$  terms are computed as:

$$\aleph = \frac{1}{\frac{1}{2}(N_{aln} - 1)(N_{aln} - 2) + N_{aln}N_{gr} - n_{gaps} - 2n_{cgaps}}$$

$$\sigma_{ij}^2 = |i - j|^{0.15}$$

where  $N_{aln}$  is the number of aligned residues.  $N_{gr}$  is the number of residues appearing in gaps, and  $n_{gaps}$  is sum of the number of insertions in protein “a”, the number of insertions in protein “b” and the number of simultaneous insertions (referred to as bulges or c-gaps).  $n_{cgaps}$  is the number of c-gaps. Gap-to-gap contacts and intra-gap contacts do not enter into the computation, and terminal gaps are also ignored.  $\sigma_{ij}^2$  is a slowly growing function of sequence separation of residues  $i$  and  $j$ , and this serves to stretch the spatial tolerance of similar contacts at larger sequence separations.  $Q_H$  ranges from 0 to 1 where  $Q_H = 1$  refers to identical proteins. If there are no gaps in the alignment, then  $Q_H$  becomes  $Q_{aln} = \aleph_{qaln}$ , which is identical to the Q-measure described into the  $Q$  measure described before.