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SEQUENCE ASSEMBLY AND FINISHING METHODS

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That genome centers are able to produce DNA sequence data at very high speed using robotics and sequencing instruments should not be taken to imply that sequencing is always straightforward or routine. The nature of genomes and the limitations of mapping and sequencing techniques still make such projects challenging and provide scope for improved computing methods. To understand the requirements of sequence assembly software, it is necessary to know a little about genome sequences and sequencing techniques. After a general introduction to the task of sequencing and assembly and an illustration of the reality of routine sequencing operations, we describe how the Staden package can be employed in effectively assembling sequence data. Other packages will include their own, often similar, methods, to deal with the problems.

The large-scale organization and local composition of DNA affects the difficulty and complication of determining its sequence. Genomes of higher organisms vary considerably in their relative GC-to-AT content and in the number and types of repetitive elements they contain. For example, within its components of known utility, the human genome consists of genes for proteins which occur in single or few copies, multigene families scattered throughout the chromosomes, and gene clusters in a variety of arrangements, including multiple copies of genes for proteins and RNAs needed in high abundance. A large proportion of the remainder of the genome consists of various types of repetitive elements including LINEs and SINEs (Hutchinson et al., 1989) of which Alu sequences are the most widely known. Many other simple elements are also repeated, sometimes hundreds of times.

The Sanger dideoxy sequencing technique (Sanger et al., 1977), which is employed by all the major public genome sequencing projects, uses DNA polymerase to synthesize a complementary copy of the target DNA. The inclusion of dideoxy nucleotides (which terminate the growing DNA chains) in the reaction mixture ensures that a proportion of all the fragments so produced stop after each base, hence creating a complete set of nested sequences. The polymerase extends, in the 5′ to 3′ direction, a short segment of DNA (known as a *primer*) that has to be annealed to the 5′ end of the target sequence to initiate the process. Either the primers or the dideoxy ''terminators'' are fluorescently labeled. The fragments are run through a sieving material such as a gel, which separates them according to their length. The fluorescence of the separated fragments is measured, and the resulting traces are analyzed to produce the base calls. The results of individual experiments are known as ''gel readings'' or ''reads.'' If the sequence is 100 bases in length, this requires separating fragments having length differing by only 1%, and this effectively limits the size of sequence that can be obtained by a single experiment to around 1,000 bases. Most sequence runs obtain approximately 500 bases.

There are many potential sources of problems when preparing the DNA ''template'' for sequencing, performing the sequencing reactions, and loading and running the sequencing instrument; all of these factors influence the reliability of the data obtained. The characteristics of individual segments of DNA also influence the difficulty of obtaining reliable sequence, dictating whether special techniques need to be applied.

At the level of the individual gel reading, potential sources of problems include (1) ''compressions,'' in which secondary structure in the DNA fragments causes them to move anomalously in the gel so that more than one size of fragment may migrate to the same position; (2) ''stops,'' where the polymerase has a tendency to dissociate and hence, in dye primer chemistry, produce a large band on the gel; (3) regions of extreme composition, including high GC or GT composition and homopolymer regions; and (4) repetitive DNA. These problems all affect the accuracy and reliability of the data obtained from these regions. At the level of joining the readings together in the correct order, the various types of repeats listed above are the major difficulty (especially if the problem is compounded by poor quality data).

As mentioned above, the primer sequence (of around 20 bases) must be complementary to the sequence at the 5′ end of the target DNA, and the sequence from each experiment is limited to around 1,000 bases. These two factors have led to the widespread use of the so-called ''shotgun'' strategy of DNA sequencing in which the target DNA is randomly broken into overlapping fragments of around 2,000 bases and cloned into a ''sequencing vector.'' This sidesteps the problem of having to know

and synthesize the sequence at the 5′ end of each segment of the target DNA because they now all have a common sequence, namely, that just to the 5′ side of the cloning site in the vector. The use of cloning techniques also provides a way of producing sufficient pure quantities of the DNA. However, this strategy creates the problem of not knowing the order of the fragments obtained from each sequencing experiment. An alternative method of producing random samples from along the target DNA while retaining the ability of using the same primer for each experiment is to use transposons. One further and important experimental strategy is to determine the sequence from both ends of each of the cloned fragments. Together, these ''forward'' and ''reverse'' readings, known as ''read pairs,'' give data from opposite strands of the DNA and provide information about the relative positions and orientations of the pairs of readings from the same fragment or template. The recent introduction of capillary electrophoresis instruments has made the production of forward and reverse readings more useful because this technology removes the problem of gel lanes being confused and, hence, with readings being assigned the wrong clone name (and subsequent incorrect assignment of read pairs).

THE USE OF BASE CALL ACCURACY ESTIMATES OR CONFIDENCE VALUES

When the idea of using numerical estimates of base calling was put forward (Dear and Staden, 1992; Bonfield and Staden, 1995), it was expected that these methods would be supplied as part of the instrument manufacturers' base-calling software. Instead, the first useable numerical values were produced by the program phred, which was devised by an academic group (Ewing, and Green, 1998). This was a very important step forward and has had a major impact on genome projects.

Through the program phrap, phred-derived confidence values have been used to improve the quality of sequence assembly. Through the Staden Package, confidence values are also extensively used during the finishing stages of sequencing projects. Here, much of the tedious and time-consuming trace checking is obviated by the software; the confidence values are used to decide if human expertise is required to adjudicate between conflicting base calls. The result is that the majority of conflicts need never be brought to the user's attention, greatly reducing thetime required to check and edit a contig (Bonfield and Staden, 1995). When the phred-style confidence values became available, the Staden program was adjusted to work on the decibel scale that was then defined. Green's group have written a contig editor known as consed (Gordon et al., 1998), which has some similarities to the one in gap4 (Bonfield et al., 1995) described later in this chapter.

Phred produces a confidence value that defines the probability that the base call is correct. The values were calculated by analyzing the traces from which the readings are derived: peak spacing over seven peaks, peak height ratios over seven and three peaks, and the number of peaks to the nearest uncalled base. The confidence value is given by the formula

$$\text{C-value} = -10 \times \log_{10} (\text{probability of error})$$

A confidence value of 10 corresponds to an error rate of 1/10, 20 to 1/100, 30 to 1/1000, and so on.

Another program that does base calling and produces confidence values is TraceTuner. Also, the program ATQA calculates confidence values and produces probabilities for insertions and deletions but does not recall the bases.

THE REQUIREMENTS FOR ASSEMBLY SOFTWARE

The task that sequence assembly software needs to accomplish is to infer the original sequence from the evidence of the readings and ideally to give, for each base, a probability that it is correct. It might be thought that this is simply a matter of comparing and aligning all the readings in a fully automated process, but this is not the case. The combination of limited reading length, reading errors, and repeats means that this ideal of automation is still some way off; therefore, interactive software tools are essential for helping to solve the many problems that confront sequencing staffs worldwide. As should be clear from the section above, there is ample opportunity for joining readings in the wrong order or wrong orientation, for missing out or duplicating regions, as well as for making minor base assignment errors, insertions, or deletions. Tools are required to check for these contingencies, sometimes in concert with extra experiments such as restriction enzyme digests. At present, the sequencing process is often talked of as consisting of two parts, namely, assembly and finishing, but in practice there is considerable overlap between the two. Assembly is the process of attempting to order and align the readings, and finishing is the task of checking and editing the assembled data. This includes performing new sequencing experiments to fill gaps or to cover segments where the data is poor and adjudicating between conflicting readings when editing.

GLOBAL ASSEMBLY

The global sequence assembly problem can be divided into three steps:

1. Find all possible overlaps between readings by comparing each one, in both orientations, to all the others;
2. from the list of overlaps, produce the best layout of the readings;
3. from the alignment of the readings in the final layout, derive a censensus sequence.

Step 1 is usually performed in two stages. First, a rapid comparison is performed to find all pairs of reading that share an exact match of, for example, 14 consecutive bases. Second, those that contain these matches are aligned using dynamic programming methods. The alignments that satisfy some preset criteria are ‘‘stored’’ in a graph, in which the vertices represent the readings and the edges represent the overlaps. Several different algorithms have been published that can analyze and prune these graphs to produce a consistent left-to-right ordering, orientation, and positioning for the readings. The resulting layout of the readings usually still requires multiple sequence alignment, as it is based only on individual pairwise alignments, each of which may conflict with others that they overlap. Once this has been done, a consensus can be derived. Elegant descriptions of the assembly problem and partic-

ular algorithmic solutions can be found in Kececioglu and Myers (1995) and Myers (1995).

Working programs usually include a number of important and effective extra methods. All the readings can be prescreened to see if they contain the sequences of known repeats. Those that do can be set aside or treated in other special ways. For example, the segments containing repeat elements can be ignored during the search for an exact initial match but then used during the alignment phase. The layout can be checked and altered to be consistent with known read-pair data. The quality of the alignments can be scored by using the confidence values of the bases, and these scores can be used when the overlap graph is analyzed to produce the layout.

There are several widely used global assembly engines. Those that are currently available free of charge to the academic community include phrap (Green, unpublished), FAKII (Myers et al., 1996), CAP3 (Huang, 1996), the TIGR Assembler (Sutton et al., 1995) and gap4 (Bonfield et al., 1995). Those available commercially include Sequencher and DNASTAR.

After the global assembly engines have done their best with the initial shotgun data, the readings will be arranged into overlapping sets, and it is part of the job of the ''finishing'' process to complete the project by obtaining readings to fill the gaps. At the end of the project, there will be only one overlapping set, and it should cover the whole of the target sequence. Although its usage has now been expanded to include any set of overlapping clones, even those for which the sequence is unknown, the word ''contig'' was originally defined to mean a set of sequence readings that overlap (Staden, 1982). A consensus sequence can be calculated for each contig. New readings obtained for the finishing process can be compared against the consensus sequences. If the reading overlaps the end of one contig, it can be extended; if it overlaps two, these can be joined. This more limited assembly problem can be performed by the global engines or more quickly by simpler algorithms that use the consensus sequence.

FILE FORMATS

Raw sequence assembly data consist of traces, base calls, and confidence values, and most programs store these in SCF-format files (Dear and Staden, 1992). In addition to this, processing programs need data about how the readings were obtained—which sequencing vector they were cloned into, which primer was used, which template they were derived from, and which chemistry was used. A variety of methods are in use to manage this data. Many groups store their readings in FASTA-format files and thus have to use so-called ''naming conventions'' or ''naming schemes'' in which the entry name is used to encode data about readings. Others use relational databases, such as ABI's BioLIMS system. The Staden package uses Experiment files. The format is identical to EMBL/SWISS-PROT entries in that two-character record-type identifiers begin each line, but the record types are extended to include the necessary data about sequence readings. This simple text format is easily parsed. A set of C programs for reading and writing SCF and Experiment file formats is available on the Staden package Web site. Assemblies describe readings, chemistries, contigs, alignments and edits, and, for this, a ''gap4 database'' is used for each sequencing project.

The remainder of this chapter illustrates the use of the Staden package as it is applied to sequence assembly data, but readers should be aware that other packages include similar features. Because the examples here use a specific package, we have not gone into the details of how to perform particular tasks but instead give a flavor of the possible operations by describing some of the components of the individual programs.

PREPARING READINGS FOR ASSEMBLY

Data must be passed through several preassembly steps before being entered into the gap4 database, usually via a program called pregap4 (Bonfield and Staden, unpublished), which can operate on batches of traces. The possible steps in this process are shown in Figure 13.1.

Usually, trace files that are in proprietary format, such as those produced by ABI sequencers, are first converted to SCF files. Confidence values are added to the SCF file, and then its Experiment file is initialized with copies of the base calls and confidence values. The trace file is not needed again until the assembly is edited, and, because these programs can uncompress them on the fly, they are now compressed. The trace files are not altered but are kept as archival data so that it is always possible to check the original base calls and traces. Any changes to the data before assembly are made to the copy of the sequence in the Experiment file. It is recommended that no changes are made to the data until readings can be viewed aligned with others. The Experiment file is augmented to include data about how the readings were obtained—which sequencing vector they were cloned into, which primer was used, which template they were derived from, and which chemistry was used. This information can be obtained from a variety of sources. Next, the readings are analyzed to mark segments that are of low confidence, which can aid some assembly engines. With the use of the information in the Experiment file, the reading is searched for the presence of sequencing vectors at each end. A similar search is then done for cloning vectors (for example, for a BAC or cosmid vector). A final check is then performed for missed vectors or other possible contaminant sequences such as *E. coli* or yeast. All of these searches write their results back into the Experiment file, ready for use by later programs in the pipeline. For the assembly stage, pregap4 can use phrap, CAP3, FAKII, or gap4; at the time of this writing, we believe that phrap is the most effective method. Only gap4 can read and write to gap4 databases; therefore, if an external assembly engine is used, the resulting assembly is copied into the gap4 database in the ''enter assembly'' step. When processing has finished, pregap4 will produce a report containing information from each module and the final list of ''passed'' and ''failed'' sequences. From this stage on, all changes are made to the copies of the data in the gap4 database and the Experiment files are no longer required. Pregap4 provides the interfaces to configure all of the above operations, to save the configuration for future use, and to perform the actual work.

Phrapview

Before other components of the gap4 package are described, a brief introduction to the phrapview program is warranted. Phrapview is distributed along with the phrap assembly engine and is a graphical viewer for phrap assemblies. It is intended to

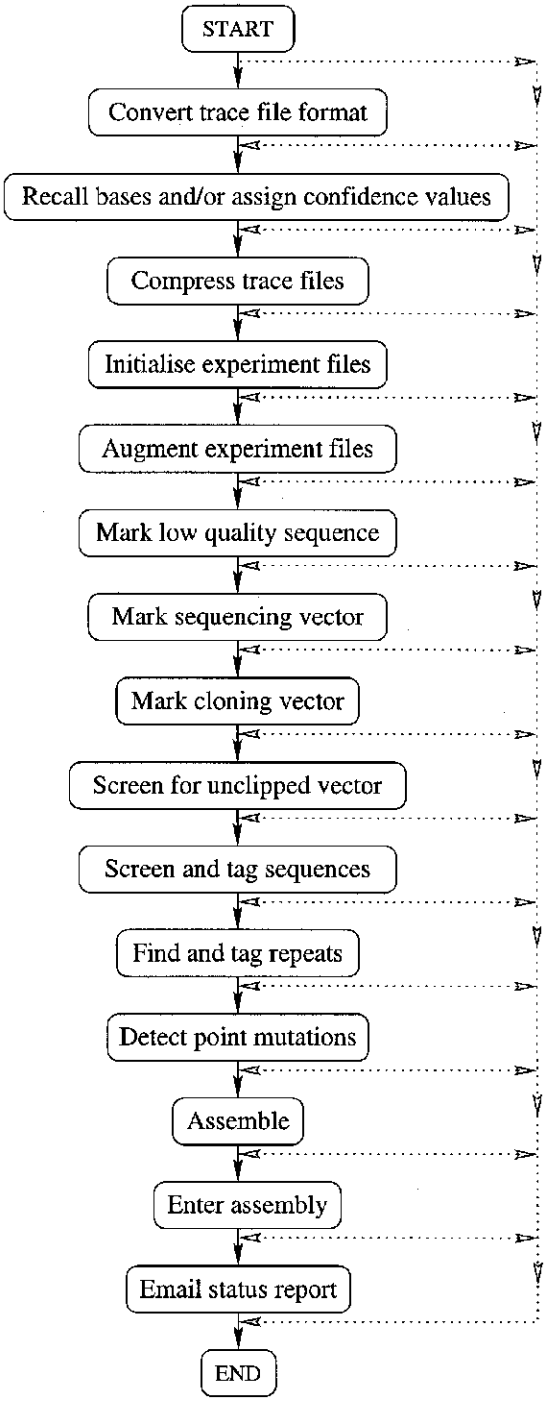

Figure 13.1. A schematic of the tasks performed by the program pregap4 when processing a batch of sequence trace files. See text for details.

provide a ''global' view of the assembly, complementing the individual base and trace view provided by consed. This global view focuses on information pertaining to possible incorrectness, incompleteness, or nonuniqueness of the phrap-generated assembly. Phrapview displays depth of coverage, forward-reverse read pairs, significant pairwise matches involving reads in different locations in the assembly, and chimeric reads.

The input to phrapview is a `.view` file, which is produced by running phrap with the View option. Note that phrapview does not perform any of the analyses itself; rather, it provides a way of displaying a file that contains an already completed analysis of the project. A screen dump for a typical phrapview display of a 40-kb cosmid sequencing project (still in three pieces) is shown in Figure 13.2. In this display, color-coded lines are drawn between read pairs, where red indicates a problem and black indicates ''OK.'' Here, 48 problems and 120 OKs are reported. Read pairs will only be indicated properly if the read-naming convention assumed by phrap is used. The other types of data mentioned above can also be displayed in the same window.

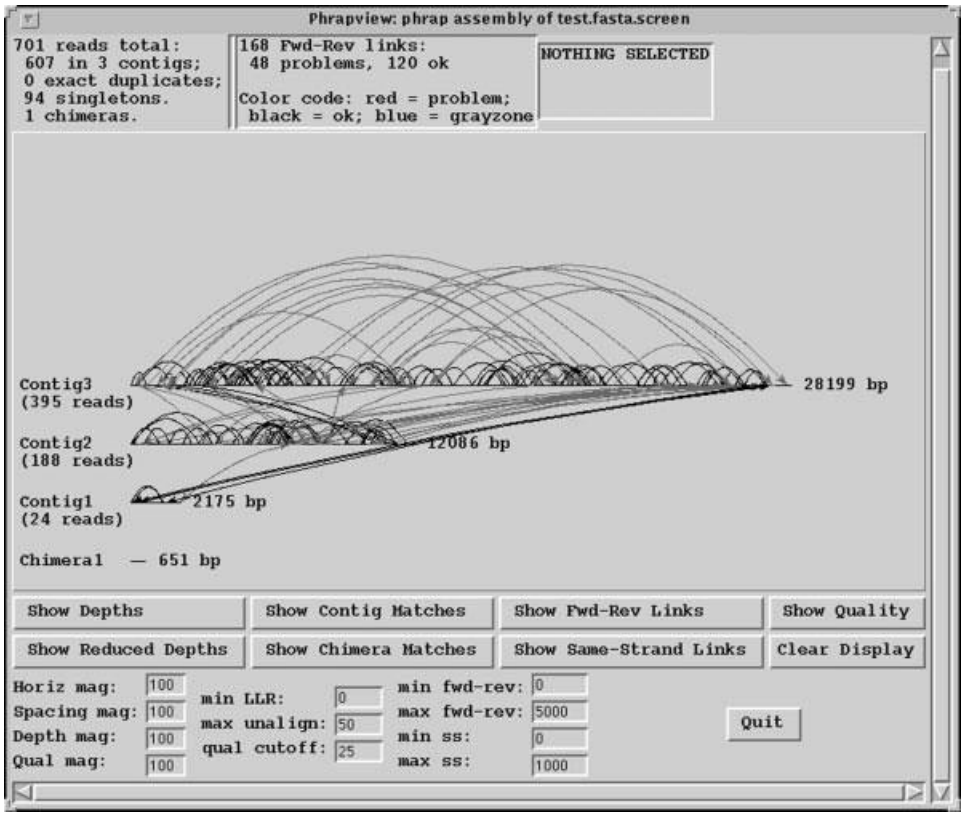

Figure 13.2. A screen dump from the program phrapview, showing a graphical display of the state of the data immediately after a run of the phrap assembly engine. See text for details. (See color plate.)

INTRODUCTION TO GAP4

Gap4 is an interactive program used for working on data from sequencing projects. It contains a comprehensive set of functions, many of which present their results graphically. Others, such as the Experiment Suggestion functions, produce textual output ready for parsing by external programs. One of its important components, used by many of the other functions, is the consensus algorithm. The gap4 database does not store the consensus sequence; rather, it is calculated whenever it is needed. When appropriate, it can be calculated separately for each strand, and, in the Contig Editor and Contig Joining Editor, it is instantly updated for each edit made. When phred-style confidence values are available, the algorithm uses them with strand and chemistry data to calculate a confidence value for each base in the consensus. At the end of a project, the algorithm can produce a FASTA-format file or an Experiment file containing the consensus and its confidence values. Preprocessing programs used by pregap4 and routines within gap4 can add annotations to readings (for example, the position of an Alu segment or a custom primer). Throughout the text, these annotations are referred to as ''tags.''

The gap4 top-level window is divided into an Output Window for showing textual results, an Error Window for displaying error messages, and, at the top, an array of menus for selecting the main functions. The contents of the two text windows can be searched, edited, and saved. Each set of results is preceded by a header containing the time and date when it was generated. Most of gap4's tools are used within other windows, which are invoked when required or when an analytic function producing graphical output is selected. The most important windows are the Contig Selector, Contig Comparator, Template Display, Consistency Plot, Restriction Map, Contig Editor, Contig Joining Editor, and Trace Display. All of the graphical displays and the Contig Editors can be scrolled in register. The base of the graphical display windows usually contains an Information Line that shows short textual data about results or items touched by the mouse cursor. The next sections give short descriptions of the windows in order of their resolution.

THE CONTIG SELECTOR

When an assembly database is opened, gap4 will bring up the Contig Selector, which is used to display, select and reorder contigs. In the Contig Selector, all contigs are shown as colinear horizontal lines separated by short vertical lines. The length of the horizontal lines is proportional to the length of the contigs, and their left-to-right order represents the current ordering of the contigs. The contig order can be changed by using the mouse to drag the lines representing the contigs. The Contig Selector can also be used to select contigs for processing. For example, clicking with the right mouse button on the line representing a contig will invoke a menu containing the commands that can be performed on that contig. As the mouse is moved over a contig, it is highlighted and the contig name and length are displayed in the Information Line.

A sample Contig Selector is shown in Figure 13.3. Along the top are three menus: File, View, and Results. Below this are buttons for zooming, activating the crosshair, and showing its position. The leftmost button, labeled Next, is used to sequentially step through sets of results. For example, if the user employs the Find

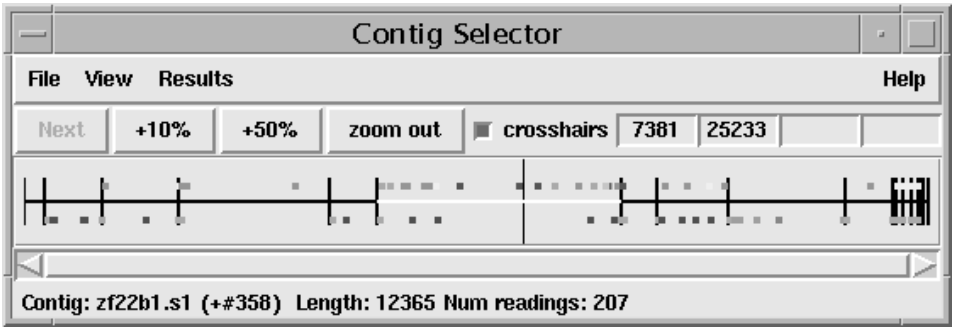

Figure 13.3. A screen dump of the gap4 Contig Selector, which gives an overview of the state of a sequencing project and provides a method for users to select contigs for processing. See text for details. (See color plate.)

Internal Joins option (see below), which finds possible overlaps between contigs, the results are automatically sorted into descending order of overlap quality, and the Next button can be used to process them in that order. Each time the user clicks on the Next button, the Contig Joining Editor will be invoked to show the next overlap, enabling the user to examine the match and edit the two contigs and make the join. Below this row of buttons is the schematic of the contigs. The small boxes around the contig lines show the positions of tags on the readings and the consensus sequence.

THE CONTIG COMPARATOR

Gap4 commands such as Find Internal Joins, Find Repeats, Check Assembly, and Find Read Pairs automatically transform the Contig Selector to produce the Contig Comparator. For this transformation, a copy of the Contig Selector is added at right angles to the original window to create a two-dimensional rectangular surface on which to display the results of comparing or checking contigs. It is therefore equivalent to the ''dot plot'' display commonly used for comparing pairs of sequences (cf. Chapter 8).

As mentioned above, Find Internal Joins compares the ends of contigs to see if there are possible overlaps. Find Repeats is similar, but, unlike Find Internal Joins, it does not require the found matches to continue to the ends of contigs. Check Assembly compares every reading with the segment of the consensus it overlaps to see how well they align. Those that align poorly are plotted along the main diagonal of the Contig Comparator. Find Read Pairs plots the positions of consistent read pairs that may indicate the order of contigs. Each of the functions plots its results as diagonal lines of different colors. Lines parallel to the main diagonal represent contigs that are in the correct orientation relative to one another. Those perpendicular to the main diagonal show results for which one contig would need to be reversed before the pair could be joined. The manual contig-dragging procedure mentioned above can be used to change the relative positions of contigs. As the contigs are dragged, the plotted results will automatically be moved to their corresponding new positions. Because this plot can simultaneously show the results of independent types

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of search, users are able to determine whether different analyses produce corroborating or conflicting evidence for the ordering of readings or contigs. The plotted results can be used to invoke a subset of commands by the use of pop-up menus. For example, if the user clicks the right mouse button over a result from Find Internal Joins, a menu containing Invoke Join Editor and Invoke Contig Editors will pop up. If the user selects Invoke Join Editor, the Join Editor will be started with the two contigs aligned at the match position contained in the result. If required, one of the contigs will be complemented to allow their alignment.

A typical display from the Contig Comparator is shown in Figure 13.4. Although they cannot be distinguished without their usual color coding, this screen dump includes results for Find Internal Joins (black), Check Assembly (green), and Find Read Pairs (blue). Superimposed on the bottom left corner are the View menu and the Results Manager menu. The crosshairs show the positions for a pair of contigs. The vertical line continues into the Contig Selector part of the display, and the position represented by the horizontal line is also duplicated there.

THE TEMPLATE DISPLAY

The next level of resolution is the Template Display, which can show schematic plots of readings, templates, tags, restriction enzyme sites, and the consensus quality. It can show one or several contigs and uses color coding to distinguish reading, primer, and template types. It is often used to view the results of the Order Contigs option, which uses read-pair data to find the most likely left-to-right order of the contigs. These data may contain errors due to misnaming of readings (and hence which templates they were obtained from); thus, it is useful to view the results and, if required, modify the order. Again, this can be done by dragging the lines representing the contigs (but this time in the Template Display rather than in the Contig Selector). If the contigs are dragged, the plot is immediately redrawn to reflect the new ordering. Figure 13.5 shows a Template Display containing the data for several contigs after they have been ordered by Order Contigs.

Under the menus and buttons, the largest section of the display contains stacks of lines and arrows representing the readings and templates for the contigs (shown by single nonoverlapping lines surrounded by tags directly below). Beneath this is a representation of the consensus quality for one contig and below that its restriction map. Note that the high depth of coverage seen here is template coverage. An alternative plot in which only the readings (and not their templates) are drawn would show much lower coverage. The template and reading sections of the display are in two parts. The top part contains the templates that have been sequenced from both ends but that are in some way inconsistent—for example, given the current relative positions of their readings, they may have a length that is larger or greater than that expected or the two readings may point in opposite directions. In this screen dump, there are four inconsistent templates, three within contigs and one spanning a pair of contigs. Color coding is used to distinguish between different types of inconsistencies and whether the inconsistencies involve readings within or between contigs. The rest of the data (mostly for templates sequenced from only one end) is plotted below the data for the inconsistent templates. Templates with only one reading are shown in dark blue. Superimposed on the lines representing the templates are forward readings, shown as light blue arrows, and reverse readings, shown as orange arrows.

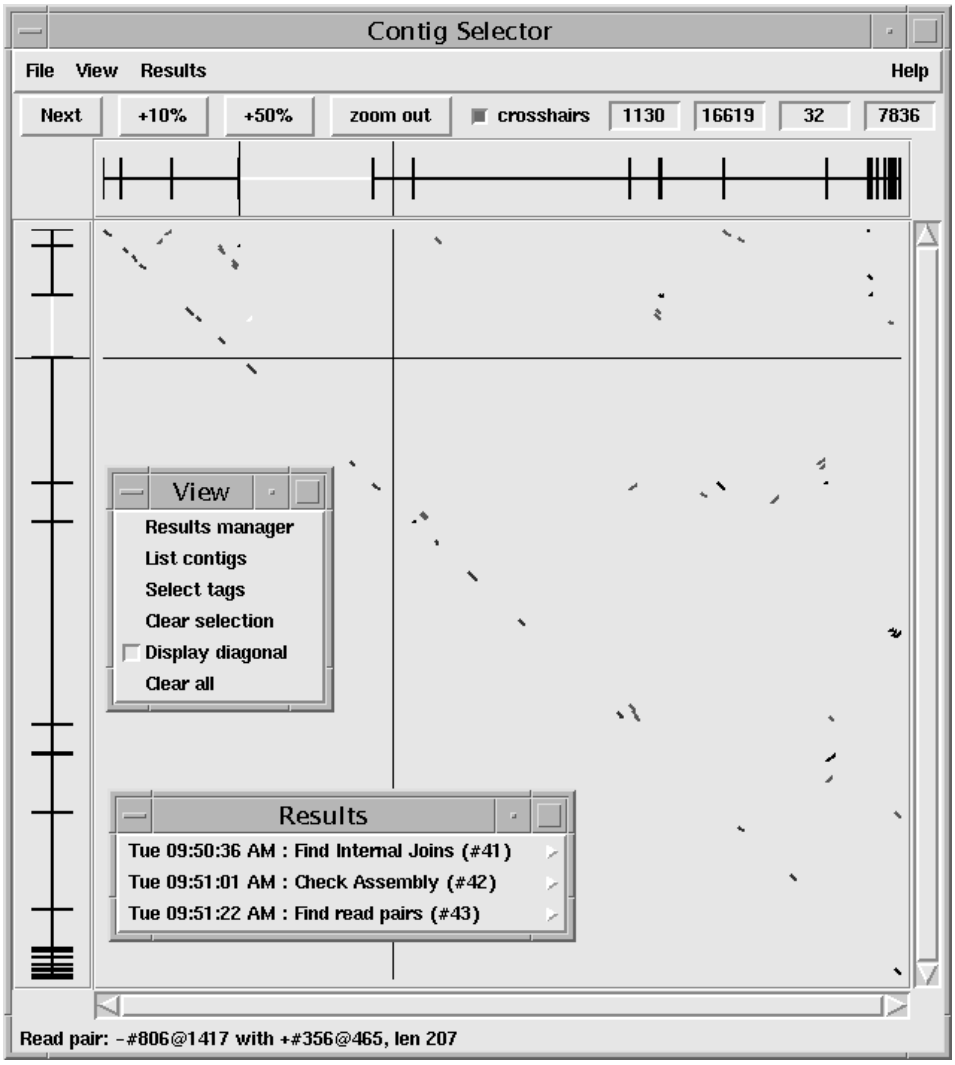

Figure 13.4. A screen dump of the gap4 Contig Comparator. This transformed version of the Contig Selector is used to display the results of analytical methods that give information about the relationships between contigs. For example, it can show sequence matches between contigs and the positions of read pairs that span contigs. See text for details. (See color plate.)

Templates in bright yellow have been sequenced from both ends, are consistent, and span a pair of contigs (and thus show the relative orientation and separation of the contigs). All data are scaled: the templates with one reading are given their expected length (here, 2,000 bases), templates within single contigs and with readings from both ends are shown with their actual length, and templates spanning contigs are shown with a length calculated from the positions of their readings. The screen dump contains two vertical lines that extend the full height of the plot. One is the crosshair, and the other shows the position of the Contig Editor editing cursor. Not only does

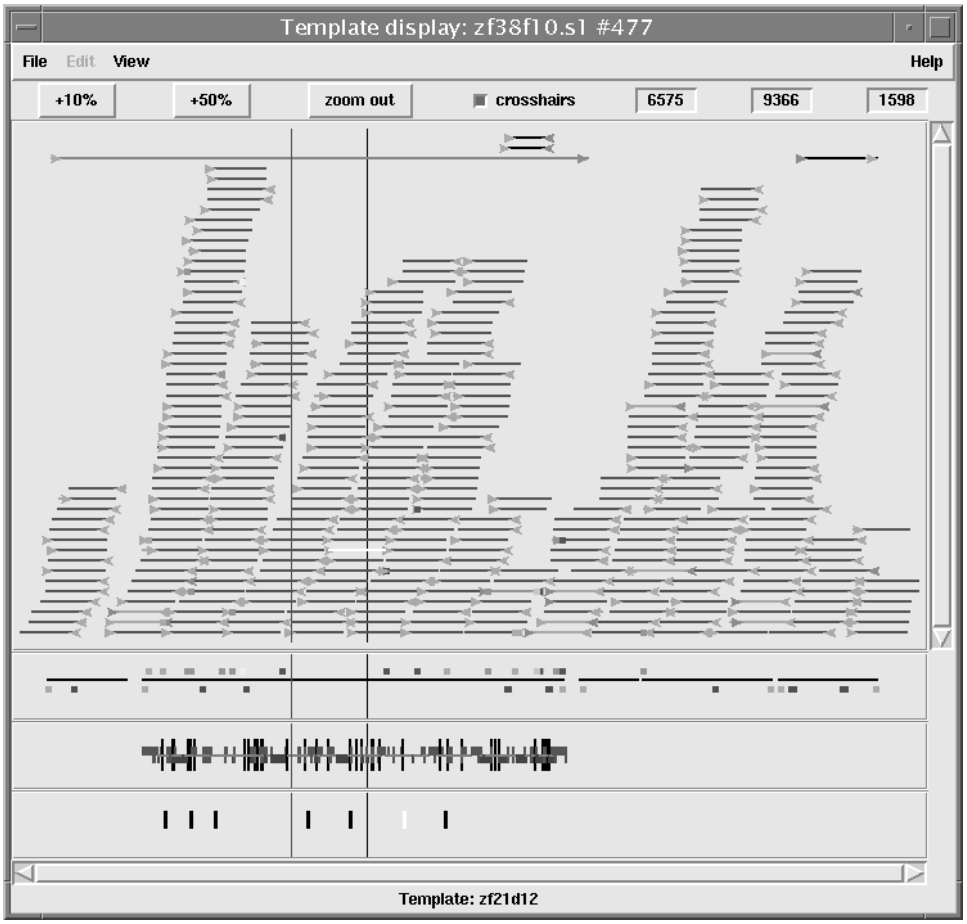

Figure 13.5. A screen dump of the gap4 Template Display, which shows the positions of DNA templates and the extent of readings derived from them. Color coding is used to distinguish between forward and reverse readings and to show consistent and inconsistent read pairs. See text for details. (See color plate.)

this vertical line show the editing cursor position but, by using the mouse, it can be used to scroll the editor.

In the plot, below the line representing the longest contig, the quality of its consensus is illustrated by color and line height. Here, the ''quality'' of the consensus is calculated using a simple algorithm and is mostly useful for data that has no confidence values. If a consensus base in a contig has good data from both strands and the consensus, when calculated separately for each of the two strands, is in agreement, then the plot for that position will show nothing. However, if the two strands disagree, or there are good data for only one strand, lines will be plotted of a color and height that signifies the problem. An alternative plot for data with confidence values, which shows the confidence for the consensus, is also available.

The position of a crosshair is shown in the two leftmost boxes in the top right-hand corner. The leftmost shows the distance in bases between the crosshair and the start of the contig underneath the crosshair. The middle box shows the distance

between the crosshair and the start of the first contig. The bottom plot is of the positions of restriction enzyme cut sites, and the rightmost box at the top of the display shows the distance between two selected cut sites. Comparing the predicted restriction pattern and that obtained from the original physical map clone can be an important final check on the overall correctness of the assembly.

THE CONSISTENCY DISPLAY

An alternative way of viewing the data at a scale similar to that of the Template Display is provided by the Consistency Display, which can plot histograms of the reading coverage and the read-pair coverage. An example is shown in Figure 13.6. The blocky histogram shows two lines (one red, one black) depicting the number of readings covering each position in a contig. The coverage for each strand is summarized below by the two sets of horizontal lines; where a line appears, there is no data for the strand. In the example, there are no data for the minus strand at the left end of the contig and none for the plus strand just left of the right-hand cursor or crosshair.

THE CONTIG EDITOR

The most detailed level of resolution provided by gap4 is the Contig Editor and its associated Trace Display, which are used for the final checking and editing of the aligned readings. It makes this an efficient and rapid procedure by providing a variety

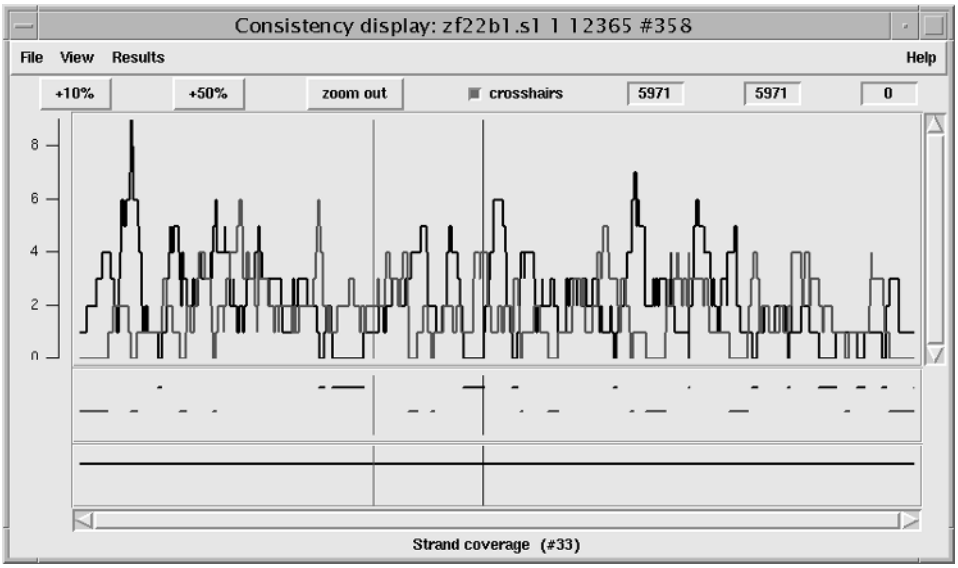

Figure 13.6. A screen dump of the gap4 Consistency Display. Here, it is being used to plot a histogram of the number of readings from each strand covering each position along a contig. Below that it is showing the segments with no data from one strand or the other. See text for details. (See color plate.)

of search methods and by automatically displaying the trace data for any problems found. Users can alter bases or their confidence values to resolve errors, and the consensus sequence is instantly updated. Editing should be performed only to obtain the correct consensus at the required level of confidence. The less editing done, the quicker the project will be completed, and the more original base calls visible in the Contig Editor, the easier it will be to check if any doubts arise in the future. The consensus calculation is the same as that used to produce the final consensus to be sent to the sequence library, and it will remove all padding characters that appear in the Contig Editor consensus. Note that the numbering shown along the top of the Contig Editor window can be set in two ways: positions can include or exclude consensus padding characters.

Figure 13.7 shows a screen dump from the Contig Editor that contains segments of aligned readings, their consensus, and a three-phase translation. Below this is the Trace Display. The Search dialogue is obscuring the right-hand side of the Editor window. The main components of the editor are the controls at the top, reading names on the left, sequences to their right, and status lines at the bottom. To the left of each reading name is the reading number, which is negative for readings that have been reversed and complemented. The reliability of the individual base calls is shown using gray scale—the lighter the background, the higher the confidence. Below the reading data is their consensus sequence, with its reliability again shown using gray

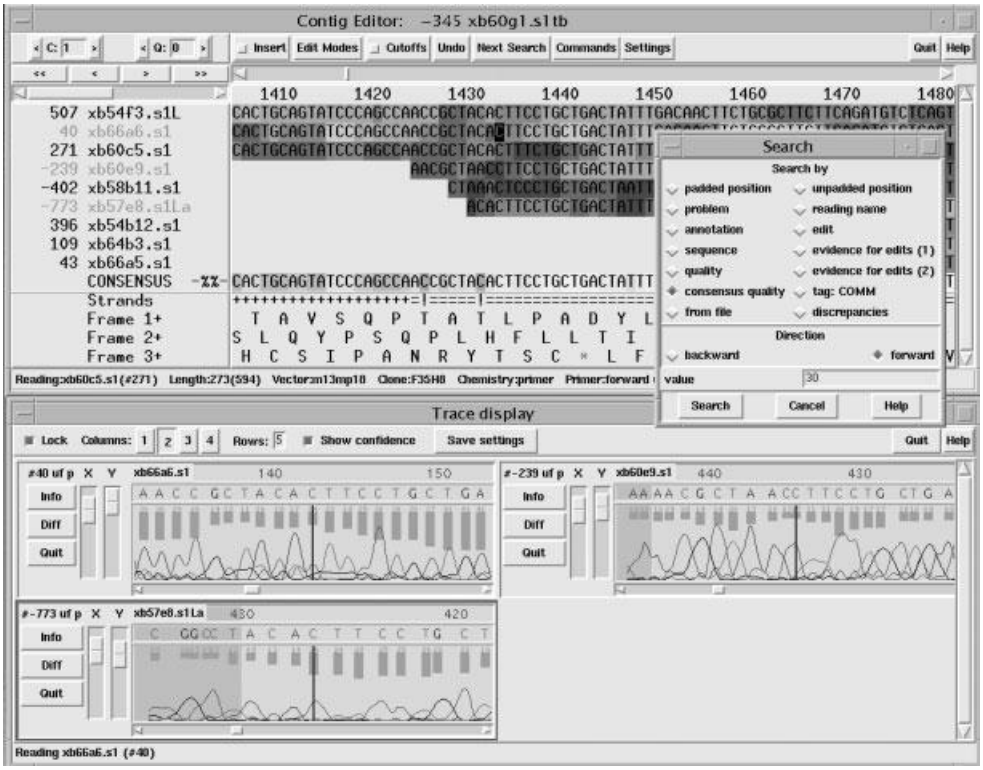

Figure 13.7. A screen dump of the gap4 Contig Editor and Trace display. See text for details. (See color plate.)

scale. The first of the status lines, labeled ''Strands,'' shows a summary of strand coverage. The left end of the segment of sequence being displayed is covered only by readings from one strand of the DNA (+), but the rest contains data from both strands (=). Two positions are marked with an exclamation mark (!), indicating that, when the consensus is calculated separately for each strand, they disagree.

Along the top of the editor window is a row of command buttons and menus. The rightmost pair of buttons provide help and exit. To their left are two menus, Settings and Commands. To the left of this is a button that initially displays the search dialogue (shown); by pressing it again, the selected search will be performed. Further left is the Undo button; each time the user clicks on this box, the program reverses the previous edit command. The next button, labeled Cutoffs, is used to toggle between showing or hiding the reading data of poor quality or are vector sequences. The next button to the left is the Edit Modes menu, which allows users to select which editing commands are enabled. The next command toggles between insert and replace and thus governs the effect of typing in the edit window. The Information Line at the bottom of the window can show information about readings, annotations, and base calls, depending on what is under the mouse cursor.

With the use of the Settings menu, the Contig Editor can be configured to display disagreements and edits. Disagreements between the consensus and individual base calls are shown in dark green. Edits are shown with a light green background, replacements/insertions are shown in pink, deletions are shown in red, and confidence value changes are shown in purple. As can be seen from the contents of the Search dialogue superimposed on the Contig Editor, there are many types of search that can be performed. For example, the cursor can explicitly be moved to any position in the contig, either ignoring or counting padding characters. Other operations include finding the next non-ACGT character in the consensus; the next edit, the next place where the confidence of the consensus falls below some user-defined value, and the next place where an edit has been performed, but there is no evidence for the change in the original data (i.e., the current base does not appear in any of the original readings covering the position). The type of search used varies depending on the user's role. For example, a user finishing a sequence project may search mainly for low-confidence consensus sequences, whereas someone in a supervisory role may go through and check every edit made.

The Contig Editor can display the traces for any reading or set of readings. The number of rows and columns of traces displayed can be set by the user. The traces scroll in register with one another, as well as with the cursor in the Contig Editor. Conversely, the Contig Editor cursor can be scrolled by the trace cursor. A typical view containing three traces is shown in the screen dump of the Contig Editor (Figure 13.7). These are the best two traces from each strand plus a trace from a reading that contains a disagreement with the consensus. With the use of the Settings menu, the program can be configured to automatically bring up this combination of traces for each problem located by the Next Search option. The histogram or vertical bars plotted top-down show the original confidence values for each base call. The reading number, together with the direction of the reading (+ or −) and the chemistry by which it was determined, is given at the top left of each subwindow. There are three buttons (Info, Diff, and Quit) arranged vertically, with X and Y scale bars to their right. The Info button produces a window containing notes about the trace. The Diff button is mostly used for mutation detection and causes a pair of traces to be subtracted from one another and the result plotted, hence revealing their differences.

THE CONTIG JOINING EDITOR

The Join Editor looks like a pair of Contig Editors stacked one above the other with a strip in between, labeled `Diffs` for displaying the disagreements between the two consensus sequences. The other two main differences are the Align and Lock buttons. The former performs an alignment between the two consensus sequences and inserts padding characters accordingly, and the latter toggles between scrolling the contigs separately or in unison. Each contig can be edited separately, and it is important that, before a join is confirmed, the alignment is checked over its full extent. The Contig Join Editor is usually invoked by clicking on a Find Internal Joins or Find Repeats result in the Contig Comparator, in which case the two contigs will appear aligned at the match found by these searches. The few differences between the Join Editor and the Contig Editor can be seen in Figure 13.8. This figure shows the right-hand end of one contig in the lower editor and the left-hand end of another in the top editor. The Cutoff or Hidden data are displayed for the right-hand contig but not for the left-hand contig.

DISASSEMBLING READINGS

Pregap4 and gap4 use lists of readings and contigs for processing batches of data. Obviously, the input to pregap4 is usually a list of trace files. Within gap4, users can create these lists in a number of ways. For example, when using the Contig Editor, users can create a list of readings to be disassembled. These are automatically sent to the gap4 Disassemble Readings function when the editor is exited. Readings can either be removed from the database or from the contigs they were in. If they are removed only from the contigs, they each start new contigs of their own; therefore, if the Find Internal Joins function is applied to all the contigs in the database, it will reveal any overlaps they may have elsewhere. If they are removed from the database, the list can be used by the assembly function to reassemble them. During its disassembly, if a reading is the only one covering part of a contig, the contig will be automatically broken in two. Gap4 also has a specific function for this purpose that will break a contig at the start of a given reading.

EXPERIMENT SUGGESTION AND AUTOMATION

Much of the foregoing has been to illustrate the types of tools that are available for solving difficult problems interactively, but ideally one would prefer to automate as much of the work as possible. Genome sequencing center staff have put many hours into developing their own in-house procedures for automating their work. The current release of gap4 enables this approach in two ways. The first is by use of its Experiment Suggestion functions, and the second is by use of the gap4 scripting language.

Using its consensus algorithms, gap4 can analyze assembly databases to find segments of sequence that require further readings, either to resolve disagreements, to fill the requirement for data from both strands of the DNA, or to extend contigs to try join them to others. It can then suggest the experiments to perform and the templates to use because it has all the necessary information in its assembly database. However, in the current version of gap4, the types of experiments are limited, and

Figure 13.8. A screen dump of the gap4 Join Editor, which is used to align, edit, display traces, and join contigs. See text for details. (See color plate.)

it has no knowledge of the very latest techniques or the ones that may be available in any particular laboratory. For large laboratories using gap4, at present, it is better to use its algorithms to analyze the database and to use the results of that analysis with a set of external routines customized to local methods. As outlined below, this is enabled by the design of gap4.

An entirely new set of gap4 finishing functions is currently being tested at the Sanger Center. In addition to the consensus algorithm, these new functions employ an analysis of reading and template depthas well as a knowledge of sequencing chemistry to design sets of potential problem-solving experiments. These experiments are geared towards satisfying the finishing criteria specified by the user.

Tcl (Ousterhout, 1994) is a portable and extensible scripting language written in the C language. The programs in the Staden package are written in the form of additional commands understood by the Tcl interpreter. Each of gap4's algorithms can therefore be used in Tcl scripts. This means that users can write scripts to perform analysis of gap4 databases (e.g., apply a consensus calculation or read-pair analysis algorithm), and these scripts could also be linked to local laboratory information management systems and robotics. A further type of automation is furnished by the fact that the gap4 Contig Editor can be driven by an external file of commands.

CONCLUDING REMARKS

We have tried to give insight into the types of tasks performed during routine sequence assembly projects and used our own software as an illustration. We recommend interested readers to look at the Web sites of some of the main sequencing centers to find out more about their finishing criteria and the software they have developed to aid their projects. At the time of this writing, much of the effort of publicly funded genome centers was devoted to producing a low-coverage sequence of the human genome; therefore, the emphasis was on assembly. When that stage is completed, the low-coverage data will need to be finished, which will require more detailed attention of the types we have attempted to describe here.

INTERNET RESOURCES FOR TOPICS PRESENTED IN CHAPTER 13

ATQA	*http://www.wagner.com*
CAP3	*http://www.cs.mtu.edu.faculty/Huang.html*
Consed	*http://bozeman.genome.washington.edu/consed/consed.html*
DNASTAR	*http://www.dnastar.com*
FAKII	*http://www.cs.arizona.edu/faktory*
Phrap	*http://bozeman.genome.washington.edu/phrap.docs/phrap.html*
Phred	*http://bozeman.genome.washington.edu/phrap.docs/phred.html*
Gap4	*http://mrc-lmb.cam.ac.uk/pubseq/index.html*
Sequencher	*http://www.genecodes.com*
Staden package	*http://www.mrc-lmb.cam.ac.uk/pubseq/index.html*
TIGR Assembler	*http://www.tigr.org/softlab*
TraceTuner	*http://www.paracel.com/html/tracetuner.html*

PROBLEM SET

Data and lecture notes for a short course in the use of pregap4 and gap4 are available on the book's Web site (http://www.wiley.com/bioinformatics). This information is derived from a two-day practical course, but the notes are written such that users can perform the exercises in an autotutorial fashion. The exercises revolve around a series of ABI trace files and expose the user to format conversion, experiment file creation, vector clipping, quality clipping, contaminant screening, assembly, join finding, read-pair analysis, finishing experiments, use of confidence values, use of the contig editor and trace display, and consensus calculation. The notes are available in both PostScript and Microsoft Word format, and the data can be used with either the UNIX or Microsoft Windows versions of the programs.

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14

PHYLOGENETIC ANALYSIS

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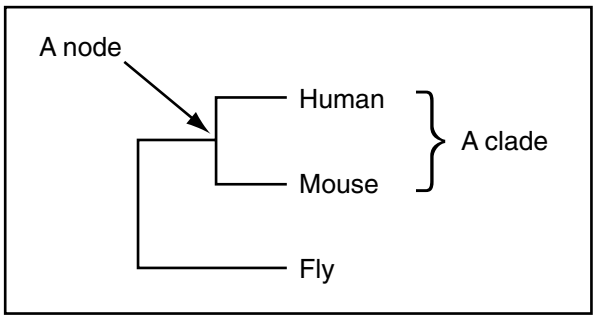
Phylogenetics is the study of evolutionary relationships. Phylogenetic analysis is the means of *inferring* or estimating these relationships. The evolutionary history inferred from phylogenetic analysis is usually depicted as branching, treelike diagrams that represent an estimated pedigree of the inherited relationships among molecules (''gene trees''), organisms, or both. Phylogenetics is sometimes called cladistics because the word ''clade,'' a set of descendants from a single ancestor, is derived from the Greek word for branch. However, cladistics is a particular method of hypothesizing about evolutionary relationships.

The basic tenet behind cladistics is that members of a group or clade share a common evolutionary history and are more related to each other than to members of another group. A given group is recognized by sharing unique features that were not present in distant ancestors. These shared, *derived* characteristics can be anything that can be observed and described—from two organisms having developed a spine to two sequences having developed a mutation at a certain base pair of a gene. Usually, cladistic analysis is performed by comparing multiple characteristics or ''characters'' at once, either multiple phenotypic characters or multiple base pairs or amino acids in a sequence.

- There are three basic assumptions in cladistics: Any group of organisms is related by descent from a common ancestor (fundamental tenet of evolutionary theory).
- There is a bifurcating pattern of cladogenesis. This assumption is controversial.
- Change in characteristics occurs in lineages over time. This is a necessary condition for cladistics to work.

The resulting relationships from cladistic analysis are most commonly represented by a phylogenetic tree:



Even with this simple tree, a number of terms that are used frequently in phylogenetic analysis can be introduced:

- A **clade** is a monophyletic taxon. Clades are groups of organisms or genes that include the most recent common ancestor of all of its members and all of the descendants of that most recent common ancestor. Clade is derived from the Greek word ''klados,'' meaning branch or twig.
- A **taxon** is any named group of organisms but not necessarily a clade.
- In some analyses, **branch** lengths correspond to divergence (e.g., in the above example, mouse is slightly more related to fly than human is to fly).
- A **node** is a bifurcating branch point.

Macromolecules, especially sequences, have surpassed morphological and other organismal characters as the most popular form of data for phylogenetic or cladistic analysis. It is this molecular phylogenetic analysis that we will introduce here.

It is unrealistic to believe that an all-purpose phylogenetic analysis recipe can be delineated (Hillis et al., 1993). Although numerous phylogenetic algorithms, procedures, and computer programs have been devised, their reliability and practicality are, in all cases, dependent on the structure and size of the data. The merits and pitfalls of various methods are the subject of often acrimonious debates in taxonomic and phylogenetic journals. Some of these debates are summarized in a series of useful reviews of phylogenetics (Saitou, 1996; Li, 1997; Swofford et al., 1996). An especially concise introduction to molecular phylogenetics is provided by Hillis et al. (1993).

The danger of generating incorrect results is inherently greater in computational phylogenetics than in many other fields of science. The events yielding a phylogeny happened in the past and can only be inferred or estimated (with a few exceptions,

see Hillis et al., 1994). Despite the well-documented limitations of available phylogenetic procedures, current biological literature is replete with examples of conclusions derived from the results of analyses in which data had been simply run through one or another phylogeny program. Occasionally, the limiting factor in phylogenetic analysis is not so much the computational method used; more often than not, the limiting factor is the users' understanding of what the method is actually doing with the data.

This brief guide to phylogenetic analysis has several objectives. First, a conceptual approach that describes some of the most important principles underlying the most widely and easily applied methods of phylogenetic analyses of biological sequences and their interpretation will be introduced. The aim is to show that practical phylogenetic analysis should be conceived as a search for a correct model, as much as a search for the correct tree. In this context, some of the particular models assumed by various popular methods and how these models might affect analysis of particular data sets will be discussed. Finally, some examples of the application of particular methods to the inferences of evolutionary history are provided.

Note that the principles for DNA analysis will be initially discussed, although most also apply to protein sequences (except where further description of protein sequences is indicated). As there is a growing interest in the analysis of protein sequences, the reader is directed to further descriptions of protein-specific problems, as reviewed by Felsenstein (1996).

FUNDAMENTAL ELEMENTS OF PHYLOGENETIC MODELS

Phylogenetic tree-building methods presume particular evolutionary models. For a given data set, these models can be violated because of occurrences such as the transfer of genetic material between organisms. Thus, when interpreting a given analysis, one should always consider the model used and its assumptions and entertain other possible explanations for the observed results. As an example, consider the tree in Figure 14.1. An investigation of organismal relationships in the tree suggests the eukaryote 1 is more related to the bacteria than to the other eukaryotes. Because the vast majority of other cladistic analyses, including those based on morphological features, suggest that eukaryote 1 is more related to the other eukaryotes than to bacteria, we suspect that for this analysis the assumptions of a bifurcating pattern of evolution are incorrect. We suspect that horizontal gene transfer from an ancestor of the bacteria 1, 2, and 3 to the ancestor of eukaryote 1 occurred because this would most simply explain the results.

Models inherent in phylogenetics methods make additional ''default'' assumptions:

1. The sequence is correct and originates from the specified source.
2. The sequences are homologous (i.e., are all descended in some way from a shared ancestral sequence).
3. Each position in a sequence alignment is homologous with every other in that alignment.
4. Each of the multiple sequences included in a common analysis has a common phylogenetic history with the others (e.g., there are no mixtures of nuclear and organellar sequences).

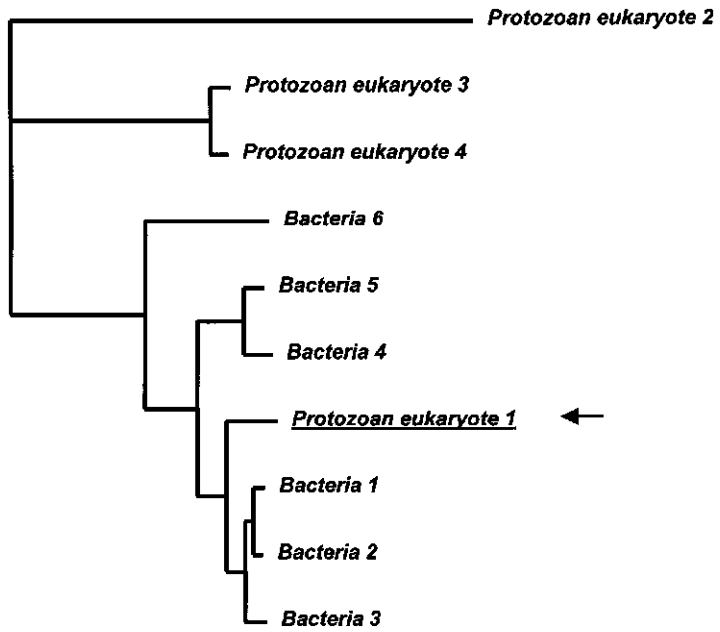


Figure 14.1. Example of a phylogenetic tree based on genes that do not match organismal phylogeny, suggesting horizontal gene transfer has occurred. The ancestor of protozoan eukaryote 1 (underlined and marked with an arrow) appears to have obtained the gene from the ancestor of Bacteria 1, 2, and 3, as this is the simplest explanation for the results. This unexpected result is not without precedent: there have been a number of reported phylogenetic analyses that suggest that protozoa have taken up genes from bacteria, most likely from bacteria that they have ingested.

5. The sampling of taxa is adequate to resolve the problem of interest.
6. Sequence variation among the samples is representative of the broader group of interest.
7. The sequence variability in the sample contains phylogenetic signal adequate to resolve the problem of interest.

There are additional assumptions that are defaults in some methods but can be at least partially corrected for in others:

1. The sequences in the sample evolved according to a single stochastic process.
2. All positions in the sequence evolved according to the same stochastic process.
3. Each position in the sequence evolved independently.

Errors in published phylogenetic analyses can often be attributed to violations of one or more of the foregoing assumptions. Every sequence data set must be

evaluated against these assumptions, with other possible explanations for the observed results considered.

TREE INTERPRETATION—THE IMPORTANCE OF IDENTIFYING PARALOGS AND ORTHOLOGS

As more genomes are sequenced, we are becoming more interested in learning about protein or gene evolution (i.e., investigating gene phylogeny, rather than organismal phylogeny). This can aid our understanding of the function of proteins and genes.

Studies of protein and gene evolution involve the comparison of *homologs*—sequences that have common origins but may or may not have common activity. Sequences that share an arbitrary, threshold level of similarity determined by alignment of matching bases are termed *homologous*. They are inherited from a common ancestor that possessed similar structure, although the structure of the ancestor may be difficult to determine because it has been modified through descent.

Homologs are most commonly either orthologs, paralogs, or xenologs.

- *Orthologs* are homologs produced by speciation. They represent genes derived from a common ancestor that diverged due to divergence of the organisms they are associated with. *They tend to have similar function.*
- *Paralogs* are homologs produced by gene duplication. They represent genes derived from a common ancestral gene that duplicated within an organism and then subsequently diverged. *They tend to have different functions.*
- *Xenologs* are homologs resulting from horizontal gene transfer between two organisms. The determination of whether a gene of interest was recently transferred into the current host by horizontal gene transfer is often difficult. Occasionally, the %(G + C) content may be so vastly different from the average gene in the current host that a conclusion of external origin is nearly inescapable, however often it is unclear whether a gene has horizontal origins. Function of xenologs can be variable depending on how significant the change in context was for the horizontally moving gene; however, in general, the function tends to be similar.

An example of how the identification of orthologs and paralogs can be used to aid prediction of protein function is illustrated in Figure 14.2.

PHYLOGENETIC DATA ANALYSIS: THE FOUR STEPS

A straightforward phylogenetic analysis consists of four steps:

1. Alignment (both building the data model and extracting a phylogenetic dataset)
2. Determining the substitution model
3. Tree building
4. Tree evaluation

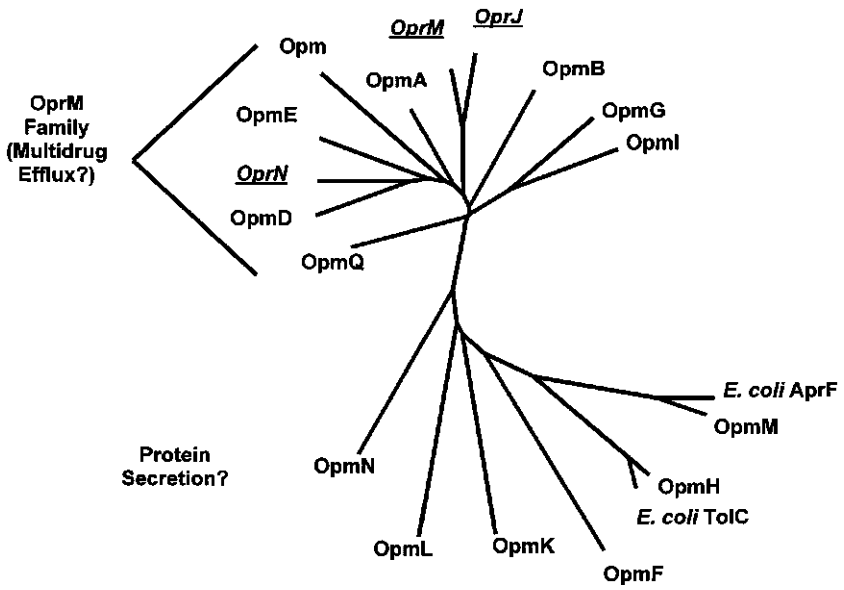


Figure 14.2. Insight into protein function from an investigation of paralogs and orthologs —an example. *Pseudomonas aeruginosa*, a bacteria that is one of the top three causes of opportunistic infections, is noted for its antimicrobial resistance and resistance to detergents. Three homologous outer membrane proteins, OprJ, OpM, and OprN, have been identified as playing a role in this antimicrobial resistance, by pumping different antimicrobials out of the cell as they entered. When the genome of this bacterium was sequenced, it was found that there were no less than 14 homologs of the genes encoding these three proteins (given names starting with "Opm"). Phylogenetic analysis of these protein sequences, using the neighbor joining distance method within the PHYLIP 5.3 package, showed that this 17-member family was divided into two clades, one containing all three genes with roles in antimicrobial efflux pumps (underlined italics). Two members of the other clade were found to share highest similarity with proteins AprF and TolC from another organism, *E. coli*. AprF and TolC are both involved in secreting proteins. This analysis allowed us to hypothesize that the clade containing OprM, OprJ, and OprN, nicknamed the OprM family, comprises a series of paralogous genes involved in efflux of different antimicrobials or antimicrobial-like compounds. The other cluster with homologs to AprF and TolC may be a functionally rated group of paralogs involved in secretion of proteins (of which OpmM appears to be the ortholog of AprF and OpmH is likely the ortholog of TolC). Currently, efforts are expanding to characterize *P. aeruginosa* with mutations in these genes to evaluate their ability to efflux antimicrobials. This phylogenetic analysis allows us to prioritize the analysis of genes in this extended family, analyzing the OprM family genes first as they are more likely to have the functions of interest. This tree was drawn using Treeview.

Each step is critical for the analysis and should be handled accordingly. For example, trees are only as good as the alignment they are based on. When performing a phylogenetic analysis, it often insightful to build trees based on different modifications of the alignment to see how the alignment proposed influences the resulting tree.

ALIGNMENT: BUILDING THE DATA MODEL

Phylogenetic sequence data usually consist of multiple sequence alignments; the individual, aligned-base positions are commonly referred to as ''sites.'' These sites are equivalent to ''characters'' in theoretical phylogenetic discussions, and the actual base (or gap) occupying a site is the ''character state.''

Multiple alignment methods are reviewed in Chapter 9. This chapter reviews similar alignment methods in the context of phylogenetic analysis. Aligned sequence positions subjected to phylogenetic analysis represent a priori phylogenetic conclusions because the sites themselves (not the actual bases) are effectively assumed to be genealogically related, or homologous. Sites at which one is confident of homology and that contain changes in character states useful for the given phylogenetic analysis are often referred to as ''informative sites.''

Steps in building the alignment include selection of the alignment procedure(s) and extraction of a phylogenetic data set from the alignment. The latter procedure requires determination of how ambiguously aligned regions and insertion/deletions (referred to as *indels*, or gaps) will be treated in the tree-building procedure.

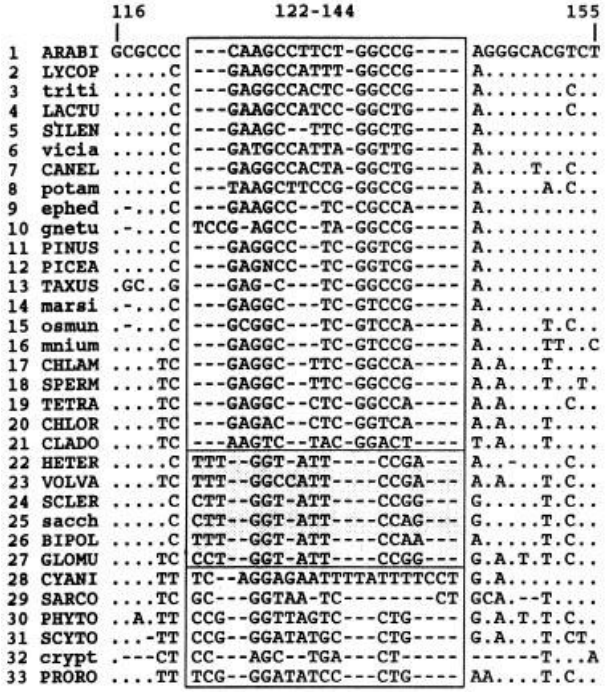
A typical alignment procedure involves the application of a program such as CLUSTAL W, followed by manual alignment editing and submission to a tree-building program. This procedure should be performed with the following questions and considerations in mind:

How much computer dependence? Fully computational multiple alignment is sometimes advocated on the grounds that manual editing is inexplicit and/or unobjective (Gatesy et al., 1993). Usually, however, manual alignment editing is advocated (e.g., Thompson et al., 1994) because alignment algorithms and programs are not optimally adapted for phylogenetic alignment (see Fig. 14.3).

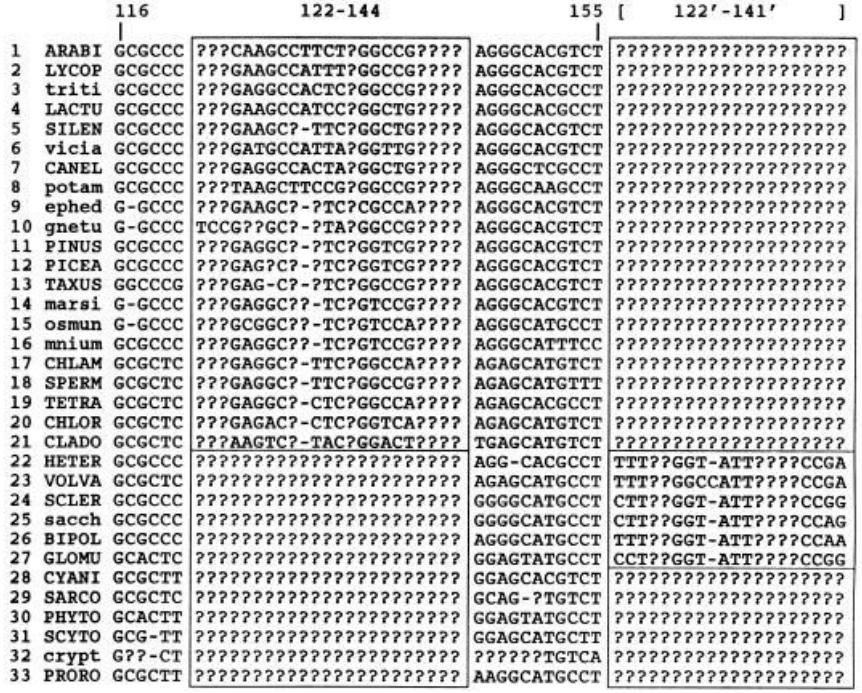
Phylogenetic criteria preferred. Some computational multiple alignment methods align sequences strictly based on the order they receive them (the input order) without any consideration of their relationship. However, many current methods (e.g., CLUSTAL W, PileUp, ALIGN in ProPack) align according to an explicitly phylogenetic criterion (a ''guide tree''). These guide trees are generated on the basis of initial pairwise sequence alignments. SAM (Hughey et al., 1996) and MACAW (Lawrence et al., 1993) are examples of multiple alignment programs that do not explicitly invoke phylogenetic criteria, although it is possible to manipulate parameters in these programs to mimic phylogenetic processes. Theory holds that more closely related sequences should be aligned first and then the resulting groups of sequences, which may be less related to one another but still have a common ancestor, should share the same ancestral indels. This means that they could then be more accurately aligned.

The guide tree from CLUSTAL W (Fig. 14.4) is formatted as a PHYLIP tree file and can be imported in various tree-drawing programs. Some programs are designed to simultaneously (recursively) optimize an alignment and a phylogenetic tree (e.g., TreeAlign and MALIGN). In theory, an optimal simultaneous solution or set of solutions to an alignment/phylogeny problem exists, but the hazard of the recursive approach lies in the possibility of funneling the analysis toward a wrong or

A


116
122-144
155
1 ARABI GCGCCC ---CAAGCCTTCT-GGCCG---- AGGGCACGTCT
2 LYCOP .....C ---GAAGCCATTT-GGCCG---- A.....
3 triti .....C ---GAGGCCACTC-GGCCG---- A.....C..
4 LACTU .....C ---GAAGCCATCC-GGCTG---- A.....C..
5 SILEN .....C ---GAAGC--TTC-GGCTG---- A.....
6 vicia .....C ---GATGCCATTA-GGTTG---- A.....
7 CANEL .....C ---GAGGCCACTA-GGCTG---- A....T..C..
8 potam .....C ---TAAGCTTCCG-GGCCG---- A.....A.C..
9 ephed .-...C ---GAAGCC--TC-CGCCA---- A.....
10 gnetu .-...C TCCG-AGCC--TA-GGCCG---- A.....
11 PINUS .....C ---GAGGCC--TC-GGTCG---- A.....
12 PICEA .....C ---GAGNCC--TC-GGTCG---- A.....
13 TAXUS .GC..G ---GAG-C---TC-GGCCG---- A.....
14 marsi .-...C ---GAGGC---TC-GTCCG---- A.....
15 osmun .-...C ---GCGGC---TC-GTCCA---- A.....T.C..
16 mnium .....C ---GAGGC---TC-GTCCG---- A.....TT..C
17 CHLAM ....TC ---GAGGC--TTC-GGCCA---- A.A...T....
18 SPERM ....TC ---GAGGC--TTC-GGCCG---- A.A...T..T.
19 TETRA ....TC ---GAGGC--CTC-GGCCA---- A.A.....C..
20 CHLOR ....TC ---GAGAC--CTC-GGTCA---- A.A...T....
21 CLADO ....TC ---AAGTC--TAC-GGACT---- T.A...T....
22 HETER .....C TTT--GGT-ATT----CCGA--- A..-....C..
23 VOLVA ....TC TTT--GGCCATT----CCGA--- A.A...T.C..
24 SCLER .....C CTT--GGT-ATT----CCGG--- G.....T.C..
25 sacch .....C CTT--GGT-ATT----CCAG--- G.....T.C..
26 BIPOL .....C TTT--GGT-ATT----CCAA--- A.....T.C..
27 GLOMU ....TC CCT--GGT-ATT----CCGG--- G.A.T.T.C..
28 CYANI ....TT TC--AGGAGAATTTTATTTTCCT G.A.....
29 SARCO ....TC GC---GGTAA-TC--------CT GCA.--T....
30 PHYTO ..A.TT CCG--GGTTAGTC---CTG---- G.A.T.T.C..
31 SCYTO ...-TT CCG--GGATATGC---CTG---- G.A...T.CT.
32 crypt .---CT CC---AGC--TGA---CT----- ------T...A
33 PRORO ....TT TCG--GGATATCC---CTG---- AA....T.C..

B


116
122-144
155
[122'-141']
1 ARABI GCGCCC ???CAAGCCTTCT?GGCCG???? AGGGCACGTCT ??????????????????
2 LYCOP GCGCCC ???GAAGCCATTT?GGCCG???? AGGGCACGTCT ??????????????????
3 triti GCGCCC ???GAGGCCACTC?GGCCG???? AGGGCACGCCT ??????????????????
4 LACTU GCGCCC ???GAAGCCATCC?GGCTG???? AGGGCACGCCT ??????????????????
5 SILEN GCGCCC ???GAAGC?-TTC?GGCTG???? AGGGCACGTCT ??????????????????
6 vicia GCGCCC ???GATGCCATTA?GGTTG???? AGGGCACGTCT ??????????????????
7 CANEL GCGCCC ???GAGGCCACTA?GGCTG???? AGGGCTCGCCT ??????????????????
8 potam GCGCCC ???TAAGCTTCCG?GGCCG???? AGGGCAAGCCT ??????????????????
9 ephed G-GCCC ???GAAGC?-?TC?CGCCA???? AGGGCACGTCT ??????????????????
10 gnetu G-GCCC TCCG??GC?-?TA?GGCCG???? AGGGCACGTCT ??????????????????
11 PINUS GCGCCC ???GAGGC?-?TC?GGTCG???? AGGGCACGTCT ??????????????????
12 PICEA GCGCCC ???GAG?C?-?TC?GGTCG???? AGGGCACGTCT ??????????????????
13 TAXUS GGCCCG ???GAG-C?-?TC?GGCCG???? AGGGCACGTCT ??????????????????
14 marsi G-GCCC ???GAGGC??-TC?GTCCG???? AGGGCACGTCT ??????????????????
15 osmun G-GCCC ???GCGGC??-TC?GTCCA???? AGGGCATGCCT ??????????????????
16 mnium GCGCCC ???GAGGC??-TC?GTCCG???? AGGGCATTTCC ??????????????????
17 CHLAM GCGCTC ???GAGGC?-TTC?GGCCA???? AGAGCATGTCT ??????????????????
18 SPERM GCGCTC ???GAGGC?-TTC?GGCCG???? AGAGCATGTTT ??????????????????
19 TETRA GCGCTC ???GAGGC?-CTC?GGCCA???? AGAGCACGCCT ??????????????????
20 CHLOR GCGCTC ???GAGAC?-CTC?GGTCA???? AGAGCATGTCT ??????????????????
21 CLADO GCGCTC ???AAGTC?-TAC?GGACT???? TGAGCATGTCT ??????????????????
22 HETER GCGCCC ???????????????????????? AGG-CACGCCT TTT??GGT-ATT????CCGA
23 VOLVA GCGCTC ???????????????????????? AGAGCATGCCT TTT??GGCCATT????CCGA
24 SCLER GCGCCC ???????????????????????? GGGGCATGCCT CTT??GGT-ATT????CCGG
25 sacch GCGCCC ???????????????????????? GGGGCATGCCT CTT??GGT-ATT????CCAG
26 BIPOL GCGCCC ???????????????????????? AGGGCATGCCT TTT??GGT-ATT????CCAA
27 GLOMU GCACTC ???????????????????????? GGAGTATGCCT CCT??GGT-ATT????CCGG
28 CYANI GCGCTT ???????????????????????? GGAGCACGTCT ??????????????????
29 SARCO GCGCTC ???????????????????????? GCAG-?TGTCT ??????????????????
30 PHYTO GCACTT ???????????????????????? GGAGTATGCCT ??????????????????
31 SCYTO GCG-TT ???????????????????????? GGAGCATGCTT ??????????????????
32 crypt G??-CT ???????????????????????? ??????TGTCA ??????????????????
33 PRORO GCGCTT ???????????????????????? AAGGCATGCCT ??????????????????

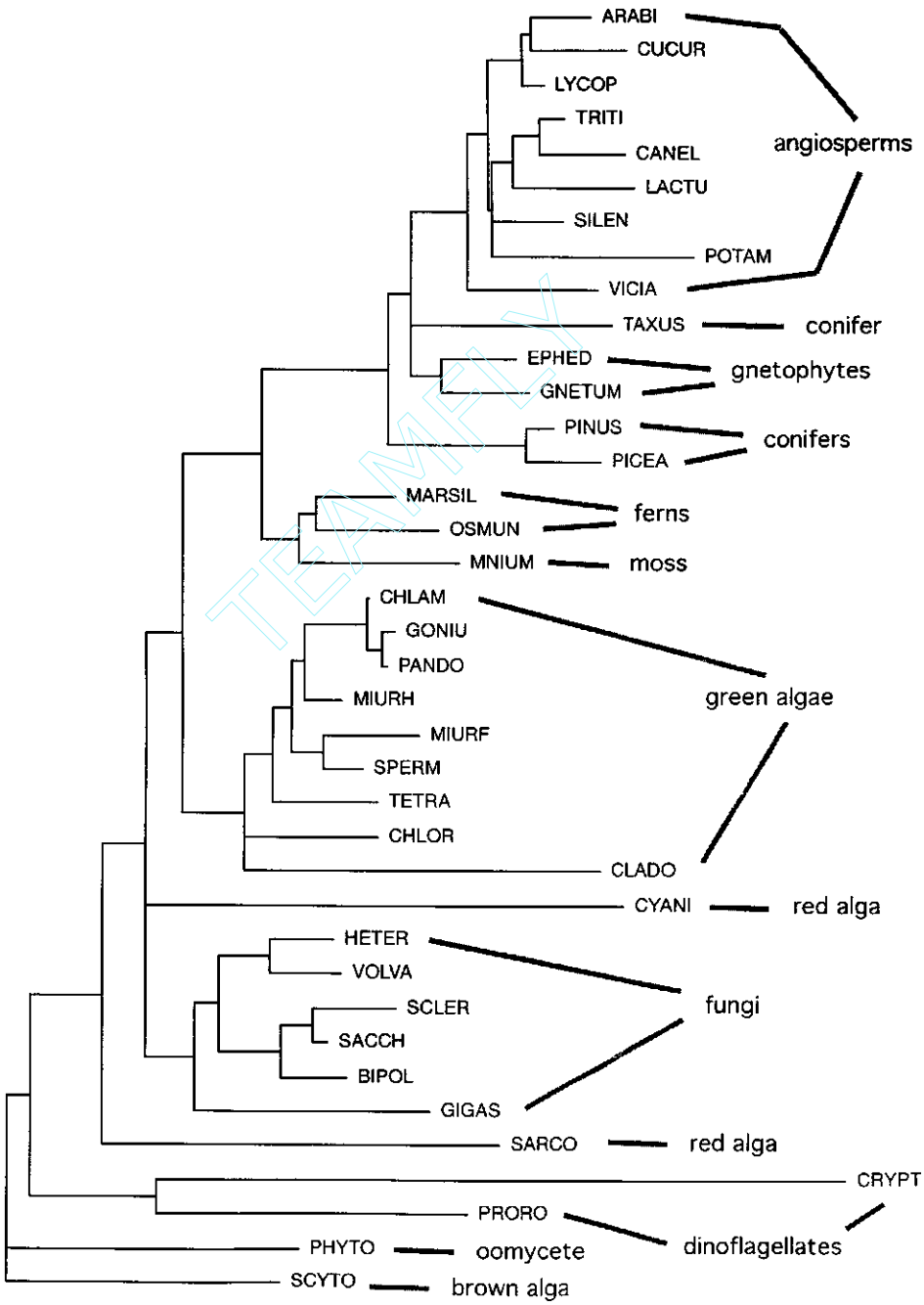
incomplete solution (Thorne and Kishino, 1992). Thus, when the tree-building analysis based on the alignment is followed, one should consider whether other evolutionary relationships might be favored using a slightly modified alignment.

Alignment Parameter Estimation. The most important parameters in an alignment method are those that determine the placement of indels or gaps in an alignment of length-variable sequences. Alignment parameters should vary dynamically with evolutionary divergence (Thompson et al., 1994), such that base mismatches are more likely as the sequences become more divergent. Alignment parameters should also be adjusted to prevent closely related, overrepresented sequences from adversely influencing the alignment of underrepresented sequences (Thompson et al., 1994, Hughey et al., 1996). This is accomplished by downweighting the alignment score contribution of closely related sequences. These dynamic parameter adjustments are both implemented in CLUSTAL W, whereas sequence weighting is implemented in SAM.

Which Alignment Procedure is Best for Phylogenetic Analysis? The short answer is ''the method that is closest to understanding the evolutionary relationships

←

Figure 14.3. Alignment modification for phylogenetic analysis. (A) Alignment showing a length-variable region (boxed) of 5.8S rDNA for the taxa in the guide tree of Figure 14.4 Taxa 1–8 are angiosperms; 9 and 10, gnetophytes; 11–13, conifers; 14 and 15, ferns; 16, moss; 17–21, green algae; 22–27, fungi; and 28–33, protists. The alignment positions correspond to those published elsewhere (Hershkovitz and Lewis, 1996). Each sequence is unique in the shaded region. Taxa represented in the Figure 14.4 tree having the same sequence as any shown here were omitted for brevity. Note that taxa grouped in the guide tree (based on the entire sequence) appear to form alignment groups in the length-variable region. On a pairwise basis, alternative alignments of some of the distantly related taxa seem plausible. For example, if moved two spaces to the left, the TAC in the center of the CLADO sequence might appear to align better with YAY in several angiosperms than the YYC in other green algae. Sufficient sampling, however, shows that YAY is not universal in the angiosperms, and the guide tree supports the present alignment, which allows no length variability in green algae. In the absence of sufficient sampling, a guide tree, or other prior phylogenetic evidence, no such conclusion could be drawn. Note also that the taxa of the green plant lineage (1–21) do not align well with the fungi and protists. The variability in the shaded region and the divergences indicated in the guide tree suggest that there is no true alignment between these distantly related groups, that the alignment indicated is arbitrary, and that the actual bases are not likely homologous. (B) The same alignment, modified as follows for phylogenetic analysis: (1) the fungi and protists are rescored as "missing" for all positions in the shaded region, where alignment with the green plant lineage is ambiguous; (2) the length-variable regions of the fungi were appended to the end of the alignment because these sequences are alignable among fungi and include phylogenetically useful variation; and (3) multiple-position gaps were rescored as one gap position and the rest missing, so that, in MP analysis, multiposition gaps are not counted as several independent deletions. The length-variable region of protists was not appended to the end of the alignment because both the alignment and the guide tree indicate that the original alignment is arbitrary.



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between the sequences being examined.'' Unless the actual phylogenetic relationships are known beforehand, there is no clear way to determine which alignment procedure is best for a given phylogenetic analysis. In general, it is inadvisable to simply subject a computer-generated alignment to a tree-building procedure because the latter is blind to errors in the former. This caution especially applies to tree-building programs included in alignment packages (e.g., CLUSTAL W and TREE in ProPack) because the tree-building methods in these programs are not rigorous (Feng and Doolittle, 1996). However, as long as the entire alignment is scrutinized in view of independent phylogenetic evidence, methods such as CLUSTAL W that utilize some degree of phylogenetic criteria are among the best currently available.

Mathematical optimization and analysis of structures. Some alignment programs (e.g., MACAW, SAM) optimize according to a statistical model, but the relationship of these statistics to phylogenetic models is not yet clear. No methods are yet available for determining whether one multiple alignment is significantly better than another based on a phylogenetic model.

Aligning according to secondary or tertiary sequence structure is considered phylogenetically more reliable than sequence-based alignment because confidence in homology assessment is greater when comparisons are made to complex structures rather than to simple characters (primary sequence). However, there does not appear to be any way to computationally facilitate phylogeny-based structural multiple alignment. Hopefully, new insights into these areas will be developed in the near future.

ALIGNMENT: EXTRACTION OF A PHYLOGENETIC DATA SET

In alignments that include length variation, the phylogenetic data set is usually not identical to the alignment. Even in alignments of length-invariable sequences, the data set can be different—for example, when only first and second codon positions are to be analyzed to avoid the strong G + C bias in the third codon position from affecting the final results.

Figure 14.4. CLUSTAL guide tree for 5.8S rDNA sequences of selected plants, fungi, and protists. The taxa and sequences corresponding to the acronyms are described elsewhere (Hershkovitz and Lewis, 1996). The tree is a neighbor-joining (distance) resolution of pairwise sequence similarities determined by pairwise alignment according to specified (in this case, default) gap penalties in CLUSTAL. Similarity is calculated as the proportion of pairwise shared bases, ignoring gap positions in either sequence. The tree can be generated as an end product or as a preliminary step in a multiple-alignment procedure. Either way, it is saved to a PHYLIP-formatted tree file. For the multiple-alignment procedure, the guide tree topology determines the sequence input order (outermost clusters are aligned first) and the branch lengths determine the sequence weights. This tree includes (see Hershkovitz and Lewis, 1996) several groupings that contradict broader evidence (e.g., polyphyly of conifers and red algae; monophyly of ferns plus moss). Such inaccuracies potentially mislead the multiple alignment. This tree was drawn and printed using the tree-drawing feature in the Macintosh version of PAUP.

In the case of length-variable sequences, the degree of difference between an alignment and the phylogenetic data set is determined mainly by how alignment ambiguities and indels are treated. The most extreme way to treat indels is to remove from the analysis all sites that include gaps (cf. Swofford et al., 1996). This approach has the advantage of permitting all the variation in the sequences to be described in terms of the substitution model, without the need for an ad hoc model to account for indels. The disadvantage of this approach is that phylogenetic signals contained within the indel regions are discarded.

Maximum parsimony (MP; see below) is the only method that permits for the incorporation of alignable gaps as characters. These can be included in either of two ways: as an additional character state (a ''fifth'' nucleotide base or ''twenty-first'' amino acid) or as a set of characters independent of base substitutions. The first approach is not tenable for gaps occupying more than one site, for these will be counted as independent character state changes. The latter approach is useful for analyzing an alignment in which subsets of sequences contain perfectly aligned gaps. A set of gap characters can be appended to the aligned sequence data set, or the gaps can be scored ''in place'' by using the extra base approach but scoring only one of the gap positions in a sequence as a gap and the remainder as missing. These approaches can be implemented using PAUP.

For some alignments, procedures that ignore all gap scores or all sites including gap scores are less than ideal. However, there is not yet any program that allows one to ignore individual sites in individual sequences. When alignment might be unambiguous within groups of sequences but ambiguous among them, alignment ''surgery'' is warranted to ensure that unambiguous information relevant to groups of sequences can be retained and ambiguous information removed.

An example of alignment surgery is given in Figure 14.3. In gapped regions, one should determine whether alternative alignments seem reasonably plausible and, just as important, whether they might bias the tree-building analysis. When alignment ambiguities are resolved manually, phylogenetic relations, substitution processes, and base composition should be considered. It is perfectly reasonable at this stage to resolve ambiguities in favor of phylogenetic evidence and in some cases to delete ambiguous regions in the alignment. The advantage of this latter approach is that unambiguous information relevant to particular sequences can be retained over ambiguous data. The disadvantage is that parsimony and likelihood tree-building methods can interpret the ''missing'' information as zero divergence.

In summary, the following points should be considered when constructing a multiple sequence alignment for a phylogenetic analysis:

- The alignment step in phylogenetic analysis is one of the most important because it produces the data set on which models of evolution are used.
- It is not uncommon to edit the alignment, deleting unambiguously aligned regions and inserting or deleting gaps to more accurately reflect probable evolutionary processes that led to the divergence between sequences.
- It is useful to perform phylogenetic analyses based on a series of slightly modified alignments to determine how ambiguous regions in the alignment affect the results and what aspects of the results one may have more or less confidence in.

DETERMINING THE SUBSTITUTION MODEL

The substitution model should be given the same emphasis as alignment and tree building. As implied in the preceding section, the substitution model influences both alignment and tree building; hence, a recursive approach is warranted. At the present time, two elements of the substitution model can be computationally assessed for nucleotide data but not for amino acid or codon data. One element is the model of substitution between particular bases; the other is the relative rate of overall substitution among different sites in the sequence. Simple computational procedures have not been developed for assessing more complex variables (e.g., site- or lineage-specific substitution models). An overview of substitution models is presented below.

Models of Substitution Rates Between Bases

In general, substitutions are more frequent between bases that are biochemically more similar. In the case of DNA, the four types of transition (A $\rightarrow$ G, G $\rightarrow$ A, C $\rightarrow$ T, T $\rightarrow$ C) are usually more frequent than the eight types of transversion (A $\rightarrow$ C, A $\rightarrow$ T, C $\rightarrow$ G, G $\rightarrow$ T, and the reverse). Such biases will affect the estimated divergence between two sequences.

Specification of the relative rates of substitution among particular residues usually takes the form of a square matrix; the number of rows/columns is four in the case of bases, 20 in the case of amino acids (e.g., in PAM and BLOSUM matrices), and 61 in the case of codons (excluding stop codons). The off-diagonal elements of the matrix correspond to the relative costs of going from one base to another. The diagonal elements represent the cost of having the same base in different sequences.

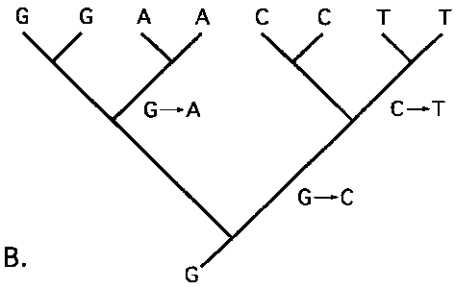
The cost schedule can be fixed a priori to ensure that the tree-building method will tally an exact cost for each substitution incurred. Fixed-cost matrices are character-state weight matrices and are applied in maximum parsimony (MP) tree building (Fig. 14.5). When such weights are applied, the method is referred to as ''weighted parsimony.'' For distance and maximum likelihood (ML) tree building, the costs can be derived from instantaneous rate matrices representing ML estimators of the probability that a particular type of substitution will occur (Fig. 14.6). Although application of the MP weight matrix is just simple arithmetic, application of the distance and ML rate matrices can involve complex algebra. To avoid the blind application of possibly inappropriate methods, practitioners are advised to familiarize themselves with the relevant underlying theory (see Li, 1997; Swofford et al., 1996).

Character-state weight matrices have usually been estimated more or less by eye, but they can also be derived from a rate matrix. For example, if it is presumed that each of the two transitions occurs at double the frequency of each transversion, a weight matrix can simply specify, for example, that the cost of A-G is 1 and the cost of A-T is 2 (Fig. 14.5). (The parsimony method dictates that the diagonal elements of the matrix, or the cost of having the same base in different sequences, be zero. This proves to be a shortcoming of parsimony; this will be discussed further below.) In the subsequent tree-building step, this set of assumptions will minimize the overall number of transversions and tend to cluster sequences differing mainly by transitions.

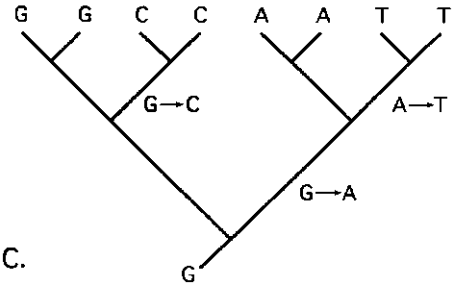
A simplified substitution rate matrix used in ML and distance phylogenetic analysis is presented in Figure 14.6. The matrix is analogous to that presented in Figure

A.

	A	C	G	T
A	-	2	1	2
C	2	-	2	1
G	1	2	-	2
T	2	1	2	-



B.



C.

Figure 14.5. Character weight matrix and application in MP phylogenetic analysis. (A) Matrix indicating that a transversion substitution costs twice that of a transition. Because, according to MP bases shared between two sequences cannot ever have changed, diagonal elements of the matrix are ignored. (B, C) Two phylogenetic resolutions and reconstructions of the evolution of a hypothetical pattern of aligned bases at a particular site in eight sequences. With unweighted MP, both reconstructions (among several others) have the same cost (three steps); hence, they are equally acceptable. With the weight matrix in (A), the reconstruction of (B) requires four steps, and the reconstruction of (C) requires five. Thus, the first reconstruction (B) and others requiring four steps are preferred.

14.5, but the actual computation of divergence involves more complex algebra and cannot be determined by simply counting steps between bases.

The paralinear or ''log-det'' transformation corrects for nonstationarity (see Swofford et al., 1996). In this method, which is applicable only to distance tree building, the numbers of raw substitutions of each type and in each direction are tallied for each sequence pair in a four-by-four matrix as shown in Figure 14.7. Each matrix has an algebraic determinant, the log of which becomes a factor in estimating sequence divergence, hence the name ''log-det.'' Pairwise comparisons of sequences having various and assorted patterns of base frequencies will yield a variety of matrix patterns, giving a variety of determinant values. Thus, each estimated pairwise distance will be affected by the determinant particular to each pair, which effectively

$$\begin{array}{c} \\ A \\ C \\ G \\ T \end{array} \begin{array}{c} \begin{array}{cccc} A & C & G & T \end{array} \\ \begin{bmatrix} -(a_1+a_2+a_3) & a_1 & a_2 & a_3 \\ a_4 & -(a_4+a_5+a_6) & a_5 & a_6 \\ a_7 & a_8 & -(a_7+a_8+a_9) & a_9 \\ a_{10} & a_{11} & a_{12} & -(a_{10}+a_{11}+a_{12}) \end{bmatrix} \end{array}$$

Figure 14.6. Simplified substitution rate matrix used in ML and distance phylogenetic analysis. The off-diagonal values a_n represent a product of an instantaneous rate of change, a relative rate between the different substitutions, and the frequency of the target base. In practice, the forward rates (upper triangular values) are presumed to equal the reverse rates (corresponding lower triangular values). The diagonal elements are nonzero, which effectively accounts for the possibility that more divergent sequences are more likely to share the same base by chance. In the simplest model of sequence evolution (the Jukes-Cantor model), all values of a are the same: all substitution types and base frequencies are presumed equal.

allows the substitution model to be different for each, varying along different branches of a phylogenetic tree. Log-det is especially sensitive to among-site rate heterogeneity (see below), since base frequency bias can exist only in sites that are subject to variation.

Models of Among-Site Substitution Rate Heterogeneity

In addition to variation in substitution patterns, variation in substitution rates among different sites in a sequence has been shown to profoundly affect the results of tree building (Swofford et al., 1996). The most obvious example of among-site rate variation, or heterogeneity, is that evident among the three codon positions in a coding

		Sclerotinium sclerotiorum				
		A	C	G	T	total
Spinacia oleracea	A	340	6	13	4	363
	C	10	229	6	36	281
	G	25	8	229	12	372
	T	5	22	6	312	345
	total	380	265	352	364	1361

Figure 14.7. Pairwise sequence comparison. The table compares 1,361 sites of 18S rDNA aligned between spinach (*Spinacia oleracea*) and a rust fungus (*Sclerotinium sclerotiorum*). The rows indicate the distribution of bases in the fungus aligned to particular bases in spinach. The columns indicate the reverse. The diagonal values are the number of site-wise identities between the sequences. Note the AT bias in the fungus: 83 (10 + 36 + 25 + 12) sites that are G or C in spinach are A or T in the fungus. In contrast, only 47 sites (6 + 22 + 13 + 6) that are G or C in the fungus are A or T in spinach. This bias is muted in simple comparison of base frequencies in the two sequences (the totals) because most sites are the same in both sequences and are probably mutationally constrained. Note also the obviously larger number of transition (13 + 36 + 25 + 22 = 96) versus transversion (6 + 4 + 10 + 6 + 8 + 12 + 5 + 6 = 57) substitutions and that C-T transitions account for 58/153 total differences. The data shown can be generated using the PAUP or MEGA programs.

sequence. Due to the degeneracy of the genetic code, changes in the third codon position can more frequently occur without affecting the ultimately encoded protein sequence. Therefore, this third codon position tends to be much more variable than the first two. For this reason, many phylogenetic analyses of coding sequences exclude the third codon position. In some cases, however, rate variation patterns are more subtle (e.g., those corresponding to conserved regions of proteins or rRNA).

Approaches to the estimation of substitution rate heterogeneity are the nonparametric models (Yang et al., 1996), the invariants model, and the gamma distribution models (Swofford et al., 1996). The nonparametric approach derives categories of relative rates for particular sites. This approach can be used with MP tree building simply by weighting particular sites according to relative mutation frequency, although such weighting tends to require prior knowledge of the true tree. The approach is also applicable to ML tree building, but it is considered computationally impractical (Yang et al., 1996). The invariants approach estimates a proportion of sites that are not free to vary. The remaining sites are presumed to vary with equal probability. The gamma approach assigns a substitution probability to sites by assuming that, for a given sequence, the probabilities vary according to a gamma distribution. The shape of the gamma distribution, as described by the shape parameter , describes the distribution of substitution probabilities among sites in a sequence (Swofford et al., 1996, p. 444, Fig. 13; cf. Li, 1997, p. 76, Fig. 3.10; note that the scales differ). In a combined approach, it can be presumed that a proportion of sites are invariant and that the remainder varies according to a gamma distribution.

In practice, gamma correction can be continuous, discrete, or ''autodiscrete'' (Yang et al., 1996). ''Continuous gamma'' means that sites are assigned to a change probability along a continuous curve. At present, this approach is computationally impractical in most cases. The discrete gamma approximation assigns sites to a specified number of categories that approximate the shape of the gamma curve. The autodiscrete model assumes that adjacent sites have correlated rates of change. Groups of sites are assigned to categories, and sites within a category can be assumed to have either constant or heterogeneous rates.

Various rate heterogeneity corrections are implemented in several tree-building programs. For nucleotide data, PAUP 4.0 implements both invariants and discrete gamma models for separate or combined use with time-reversible distance and likelihood tree-building methods and invariants in conjunction with the log-det distance method (see below). For nucleotide, amino acid, and codon data, PAML implements continuous, discrete, and autodiscrete models. For nucleotide and amino acid data, PHYLIP implements a discrete gamma model.

Models of Substitution Rates Between Amino Acids

The most widely used models of amino acid substitution include distance-based methods, which are based on matrixes such as PAM and BLOSUM. Again, such matrices are described further in other chapters in this book. Briefly, Dayhoff's PAM 001 matrix (Dayhoff, 1979) is an empirical model that scales probabilities of change from one amino acid to another in terms of an expected 1% change between two amino acid sequences. This matrix is used to make a transition probability matrix that allows prediction of the probability of changing from one amino acid to another and also predicts equilibrium amino acid composition. Phylogenetic distances are calculated with the assumption that the probabilities in the matrix are correct. The

distance that is computed is scaled in units of expected fraction of amino acids changed. Kimura's distance is another method used in PROTDIST, one of the PHYLIP family of programs (mentioned further below), and is a rough distance formula for approximating PAM distance by simply measuring the fraction of amino acids that differ between two sequences and computing the distance by a set formula (see Kimura, 1983). This is a more rapid method, but it has some obvious limitations. It does not take into account which amino acids differ or what amino acids are changed, so some information is lost. The distance measure is represented as the fraction of amino acids differing; this is also the case with PAM distances. If the fraction of amino acids differing gets larger than 0.8541, the distance becomes infinite.

Although PROTDIST is one of the most widely used programs providing substitution models for calculating protein distances, others that are faster and make use of additional matrices such as BLOSUM are now more widely-used (e.g., PUZZLE).

The model used in parsimony (not a distance-based method) insists that any amino acid changes be consistent with the genetic code so that, for example, lysine is allowed to substitute to methionine but not to proline. However, changes between two amino acids via a third are allowed *and* are counted as two changes if each of the two replacements is individually allowed. This sometimes allows changes that, at first sight, one would think should be outlawed. Thus, phenylalanine can be changed to glutamine via leucine in two steps total. Genetic code translation tables show that there is a leucine codon one step away from a phenylalanine codon; there is also a leucine codon one step away from a glutamine codon. These leucine codons, however, are not identical. It actually takes three base substitutions to get from either of the phenylalanine codons (UUU and UUC) to either of the glutamine codons (CAA or CAG). Why, then, does this program count only two? The answer is that recent DNA sequence comparisons seem to show that synonymous changes (changes in the nucleotide sequence of a codon region that do not change what amino acids are encoded by that region) are considerably faster and easier than ones that change the amino acid outright. We are assuming that, in effect, synonymous changes occur so much more readily that they need not be counted. Thus, in the chain of changes UUU (Phe) $\rightarrow$ CUU (Leu) $\rightarrow$ CUA (Leu) $\rightarrow$ CAA (Glu), the middle one is not counted because it does not actually change the amino acid (leucine).

Which Substitution Model to Use?

Although any of the parameters in a substitution model might prove critical for a given data set, the best model is not always the one with the most parameters. To the contrary, the fewer the parameters, the better. This is because every parameter estimate has an associated variance. As additional parametric dimensions are introduced, the overall variance increases, sometimes prohibitively (see Li, 1997, p. 84, Table 4.1). For a given DNA sequence comparison, a two-parameter model will require that the summed base differences be sorted into two categories and into six for a six-parameter model. Obviously, the number of sites sampled in each of the six categories would be much smaller (and perhaps too small) to give a reliable estimate.

A good strategy for substitution model specification for DNA sequences is the ''describe tree'' feature in PAUP, which uses likelihood to simultaneously estimate the six reversible substitution rates, the -shape parameter of the gamma distribution, and the proportion of invariant sites. These parameters can be estimated by means

of equal or specified base frequencies. Usually, any reasonable phylogenetic tree (e.g., an easily generated neighbor-joining tree) is suitable for this procedure because parameter estimates are apparently influenced predominantly by the character pattern rather than by the tree topology (Swofford et al., 1996). This estimation procedure is not overly time consuming for up to 50 sequences. If there will be more sequences or less time, the test tree can be selectively pruned to reduce the number of taxa while retaining the overall phylogenetic range and structure. From the estimated substitution parameters, one can determine whether a simpler model is justified (e.g., whether the six substitution categories can be reduced to two) by comparing likelihood scores estimated for this tree using more or fewer parameters. Parameters for and the proportion of invariant sites sometimes can substitute for each other, so one should compare likelihoods with each estimated alone versus both together. Note that, unlike MP and ME, the ML scores derived using different parameter values are directly comparable (Swofford et al., 1996).

In the case of protein-coding DNA sequences, it is sometimes obvious that, depending on the divergence of the samples, the useful variation is essentially either in the first and second codon positions, with the third positions randomized across the data set, or in the third position, with the first and second positions invariant. The procedure above will correct for this rate heterogeneity, although removing the ''useless'' sites may permit a more precise estimate of rate heterogeneity in the remaining sites.

For protein sequences, the model used is often dependent on the degree of sequence similarity. For more divergent sequences, the BLOSUM matrices are often better, whereas the PAM matrix is suited for more highly similar sequences. Both parsimony and distance matrix methods (mentioned further below) have benefits and disadvantages, and their use depends on one's philosophy about protein sequence changes: Is it better to retain information about each character when determining a tree (i.e., through parsimony) or to derive distance measures to base the tree (i.e., using a distance matrix)? Is a matrix based on empirical data a more accurate reflection of evolutionary change than a matrix based on generated theories about sequence change? Again, although cladistic analysis can be a powerful method for investigating evolutionary relationships, keep in mind that there is no one clear method that is better than the other. Each has its own benefits and disadvantages that differ depending on the type of analyses performed and the philosophy of the investigator.

TREE-BUILDING METHODS

Tree-building methods implemented in available software are discussed in detail in the literature (Saitou, 1996; Swofford et al., 1996; Li, 1997) and described on the Internet. This section briefly describes some of the most popular methods. Tree-building methods can be sorted into distance-based vs. character-based methods. Much of the discussion in molecular phylogenetics dwells on the utility of distance- and character-based methods (e.g., Saitou, 1996; Li, 1997). Distance methods compute pairwise distances according to some measure and then discard the actual data, using only the fixed distances to derive trees. Character-based methods derive trees that optimize the distribution of the actual data patterns for each character. Pairwise distances are, therefore, not fixed, as they are determined by the tree topology. The

most commonly applied distance-based methods include neighbor-joining and the Fitch-Margoliash method, and the most common character-based methods include maximum parsimony and maximum likelihood.

Distance-Based Methods

Distance-based methods use the amount of dissimilarity (the distance) between two aligned sequences to derive trees. A distance method would reconstruct the true tree if all genetic divergence events were accurately recorded in the sequence (Swofford et al., 1996). However, divergence encounters an upper limit as sequences become mutationally saturated. After one sequence of a diverging pair has mutated at a particular site, subsequent mutations in either sequence cannot render the sites any more ''different.'' In fact, subsequent mutations can make them again equal (for example, if a valine mutates to an isoleucine, which mutates back to a valine). Therefore, most distance-based methods correct for such ''unseen'' substitutions. In practice, application of the rate matrix effectively presumes that some proportion of observed pairwise base identities actually represents multiple mutations and that this proportion increases with increasing overall sequence divergence. Some programs implement, at least optionally, calculation of uncorrected distances, whereas, for example, the MEGA program (Kumar et al., 1994) implements only uncorrected distances for codon and amino acid data. Unless overall divergences are very low, the latter approach is virtually guaranteed to give inaccurate results.

Pairwise distance is calculated using maximum-likelihood estimators of substitution rates. The most popular distance tree-building programs have a limited number of substitution models, but PAUP 4.0 implements a number of models, including the actual model estimated from the data using maximum likelihood, as well as the log-det distance method.

Distance methods are much less computationally intensive than maximum likelihood but can employ the same models of sequence evolution. This is their biggest advantage. The disadvantage is that the actual character data are discarded. The most commonly applied distance-based methods are the unweighted pair group method with arithmetic mean (UPGMA), neighbor joining (NJ), and methods that optimize the additivity of a distance tree, including the minimum evolution (ME) method. Several methods are available in more than one phylogenetics software package but not all implementations allow the same parameter specifications and/or tree optimization features (e.g., branch swapping; see below).

Unweighted Pair Group Method with Arithmetic Mean (UPGMA). UPGMA is a clustering or phenetic algorithm—it joins tree branches based on the criterion of greatest similarity among pairs and averages of joined pairs. It is not strictly an evolutionary distance method (Li, 1997). UPGMA is expected to generate an accurate topology with true branch lengths only when the divergence is according to a molecular clock (ultrametric; Swofford et al., 1996) or approximately equal to raw sequence dissimilarity. As mentioned earlier, these conditions are rarely met in practice.

Neighbor Joining (NJ). The neighbor-joining algorithm is commonly applied with distance tree building, regardless of the optimization criterion. The fully resolved tree is ''decomposed'' from a fully unresolved ''star'' tree by successively

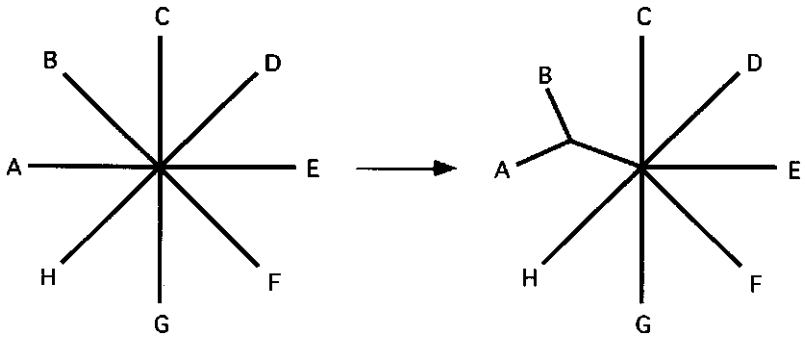


Figure 14.8. Star decomposition. This is how tree-building algorithms such as neighbor-joining work. The most similar terminals are joined, and a branch is inserted between them and the remainder of the star. Subsequently, the new branch is consolidated so that its value is a mean of the two original values, yielding a star tree with n-1 terminals. The process is repeated until only one terminal remains.

inserting branches between a pair of closest (actually, most isolated) neighbors and the remaining terminals in the tree (Fig. 14.8). The closest neighbor pair is then consolidated, effectively reforming a star tree, and the process is repeated. The method is comparatively rapid.

Fitch-Margoliash (FM). The Fitch-Margoliash (FM) method seeks to maximize the fit of the observed pairwise distances to a tree by minimizing the squared deviation of all possible observed distances relative to all possible path lengths on the tree (Felsenstein, 1997). There are several variations that differ in how the error is weighted. The variance estimates are not completely independent because errors in all the internal tree branches are counted at least twice (Rzhetsky and Nei, 1992).

Minimum Evolution (ME). Minimum evolution seeks to find the shortest tree that is consistent with the path lengths measured in a manner similar to FM; that is, ME works by minimizing the squared deviation of observed to tree-based distances (Rzhetsky and Nei, 1992; Swofford et al., 1996; Felsenstein, 1997). Unlike FM, ME does not use all possible pairwise distances and all possible associated tree path lengths. Rather, it fixes the location of internal tree nodes based on the distance to external nodes and then optimizes the internal branch length according to the minimum measured error between these ''observed'' points. It thus purports to eliminate the nonindependence of FM measurements.

Which Distance-Based Tree-Building Procedure Is Best? ME and FM appear to be the best procedures, and they perform nearly identically in simulation studies (Huelsenbeck, 1995). ME is becoming more widely implemented in computer programs, including METREE (Rzhetsky and Nei, 1994) and PAUP. For protein data, the FM procedure in PHYLIP offers the greatest range of substitution models but no correction for among-site rate heterogeneity. The MEGA (Kumar et al., 1994) and METREE packages include a gamma correction for proteins, but only in conjunction with a raw (''*p*-distance'') divergence model (no distance or bias correction), which is unreliable except for small divergences (Rzhetsky and Nei, 1994). MEGA also computes separate distances for synonymous and nonsynonymous sites, but this

method is valid only in the absence of substitution or base frequency bias and when there is no correction for among-site rate heterogeneity. Thus, for most data sets, using the nucleotide data under a more realistic model might be preferable to MEGA's methods.

Simulation studies indicate that UPGMA performs poorly over a broad range of tree shape space (Huelsenbeck, 1995). The use of this method is not recommended; it is mentioned here only because its application seems to persist, as evidenced by UPGMA gene trees appearing in publications (Huelsenbeck, 1995).

NJ is clearly the fastest procedure and generally yields a tree close to the ME tree. (Rzhetsky and Nei, 1992; Li, 1997). However, it yields only one tree. Depending on the structure of the data, numerous different trees might be as good or significantly better than the NJ tree (Swofford et al., 1996).

Character-Based Methods

The character-based methods have little in common with each other, besides the use of the character data at all steps in the analysis. This allows the assessment of the reliability of each base position in an alignment on the basis of all other base positions.

Maximum Parsimony (MP). Maximum parsimony is an optimization criterion that adheres to the principle that the best explanation of the data is the simplest, which in turn is the one requiring the fewest ad hoc assumptions. In practical terms, the MP tree is the shortest—the one with the fewest changes—which, by definition, is also the one with the fewest parallel changes. There are several variants of MP that differ with regard to the permitted directionality of character state change (Swofford et al., 1996).

To accommodate substitution bias, MP is amenable to weighting; for example, the transformation of a transversion can be weighted relative to a transition (see above). The easiest way to do this is to create a weighting step matrix in which the weights are the reciprocal of the rates estimated using ML as described above. However, step-matrix weighting can greatly slow MP computation.

The MP method performs poorly when there is substantial among-site rate heterogeneity (Huelsenbeck, 1995). There are few good fixes for this problem. One approach is to modify the data set to include only sites that exhibit little or no heterogeneity as determined by likelihood estimation (see above). Another approach is to recursively reweight positions according to their propensity to change as observed in preliminary trees. This ''successive approximations'' approach is automatically facilitated in PAUP, but it is prone to error to the degree that the preliminary trees are incorrect.

MP analyses tend to yield numerous (and sometimes many thousands of) trees that have the same score. Because each is held to be as optimal as any other, only groupings present in the strict consensus of all trees are considered to be supported by the data. The reason that distance and ML tree methods tend to arrive at a single best tree is that their calculations involve division and decimals, whereas MP merely counts discrete steps. For a given data set, a strict consensus of all ME or ML trees that are not significantly worse than optimal probably would yield resolution more or less comparable to the MP consensus. Unfortunately, whereas MP users conventionally present strict consensus (and sometimes consensus of trees one or two steps worse), ME and ML users typically do not.

Simulation studies have shown that MP performs no better than ME and worse than ML when the amount of sequence evolution since lineages diverged is much greater than the amount of divergence that occurred between lineage splits (i.e., in a tree with very long terminal branches and short internal internodes) (Huelsenbeck, 1995). This condition produces ''long branch attraction''—the long branches become artificially connected because the number of nonhomologous similarities the sequences have accumulated exceeds the number of homologous similarities they have retained with their true closest relatives (Swofford et al., 1996). Character weighting improves the performance of MP under these conditions (Huelsenbeck, 1995).

Maximum Likelihood (ML). ML turns the phylogenetic problem inside out. ML searches for the evolutionary model, including the tree itself, that has the highest likelihood of producing the observed data.

In practice, ML is derived for each base position in an alignment. The likelihood is calculated in terms of the probability that the pattern of variation at a site would be produced by a particular substitution process, given a particular tree and the overall observed base frequencies. The likelihood becomes the sum of the probabilities of each possible reconstruction of substitutions under a particular substitution process. The likelihoods for all the sites are multiplied to give an overall ''likelihood of the tree'' (i.e., the probability of the data given the tree and the substitution process). As one can imagine, for one particular tree, the likelihood of the data is low at some sites and high at others. For a ''good'' tree, many sites will have higher likelihood, so the product of likelihoods is high. For a ''poor'' tree, the reverse will be true.

The substitution model should be optimized to fit the observed data. For example, if there is a transition bias, evident by an inordinate number of sites that include only purines or pyrimidines, the likelihood of the data under a model that assumes no bias will never be as good as one that does. Likewise, if a substantial proportion of the sites are occupied by a single base and another substantial proportion have equal base frequencies, the likelihood of the data under a model that assumes that all sites evolve equally will be less than that of a model that allows rate heterogeneity. Modifying the substitution parameters, however, modifies the likelihood of the data associated with particular trees. Thus, the tree yielding the highest likelihood under one substitution model might yield much lower likelihood under another.

Because ML uses great amounts of computational time, it is usually impractical to perform a complete search that simultaneously optimizes the substitution model and the tree for a given data set. An economical, heuristic approach is recommended (Adachi and Hasegawa, 1996; Swofford et al., 1996). Perhaps the best time saver in this regard is preliminary ML estimation of the substitution model (as can be performed using PAUP). This procedure can be applied iteratively, searching for better ML trees, then reestimating the parameters, and then searching for better trees.

As algorithms, computers, and phylogenetic understanding have improved, the ML criterion has become more popular for molecular phylogenetic analysis. In simulation studies, ML has consistently outperformed ME and MP when the data analysis proceeds according to the same model that generates the data (Huelsenbeck, 1995). ML will always be the most computationally intensive method of all, however, so there will always be situations in which it is not practical.

DISTANCE, PARSIMONY, AND MAXIMUM LIKELIHOOD: WHAT'S THE DIFFERENCE?

Distance matrix methods simply count the number of differences between two sequences. This number is referred to as the evolutionary distance, and its exact size depends on the evolutionary model used. The actual tree is then computed from the matrix of distance values by running a clustering algorithm that starts with the most similar sequences (i.e., those that have the shortest distance between them) or by trying to minimize the total branch length of the tree. The principle of maximum parsimony searches for a tree that requires the smallest number of changes to explain the differences observed among the taxa under study.

A maximum-likelihood approach to phylogenetic inference evaluates the probability that the chosen evolutionary model has generated the observed data. The evolutionary model could simply mean that one assumes that changes between all nucleotides (or amino acids) are equally probable. The program will then assign all possible nucleotides to the internal nodes of the tree in turn and calculate the probability that each such sequence would have generated the data (if two sister taxa have the nucleotide ''A,'' a reconstruction that assumes derivation from a ''C'' would be assigned a low probability compared with a derivation that assumes there already was an ''A''). The probabilities for all possible reconstructions (not just the more probable one) are summed up to yield the likelihood for one particular site. The likelihood for the tree is the product of the likelihoods for all alignment positions in the data set.

Searching for Trees

The number of unique phylogenetic trees increases exponentially with the number of taxa, becoming astronomical even for, say, 50 sequences (Swofford et al., 1996; Li, 1997). In most cases, computational limitations permit exploration of only a small fraction of possible trees. The exact number will depend mainly on the number of taxa, the optimality criterion (e.g., MP is much faster than ML), the parameters (e.g., unweighted MP is much faster than weighted; ML with fewer preset parameters is much faster than with more and/or simultaneously optimized parameters), computer hardware, and computer software (some algorithms are faster than others; some software allows multiprocessing; some software limits the number and kind of trees that can be stored in memory). The search procedure is also affected by data structure: poorly resolvable data produce more ''nearly optimal'' trees that must be evaluated to find the most optimal.

Branch-swapping algorithms successively modify existing trees built by an initial step (Swofford et al., 1996). The algorithms range from those that generate all possible unique trees (exhaustive algorithms) to those that evaluate only minor modifications.

Quartet puzzling is a relatively rapid tree-searching algorithm available for ML tree building (Strimmer and von Haeseler, 1996) and is available in PUZZLE.

One of the best ways to economize the search effort is to prune the data set. For example, it might be apparent from the data alone or from preliminary searching

that a particular cluster of five terminals is unresolvable, that the arrangement of these terminals does not impact the remainder of the topology, and/or that resolution of these terminals is not the objective of the analysis. Removing four of the terminals from the analysis simplifies the search by several orders of magnitude.

Every analysis is unique. The elements that influence the choice of optimal search strategy (amount of data, structure of data, amount of time, hardware, objective of analysis) are too variable to suggest a foolproof recipe. Thus, researchers must be familiar with their data; they must also have specific objectives in mind, understanding the various search procedures as well as the capabilities of their hardware and software.

Rooting Trees

The methods described above produce unrooted trees (i.e., trees having no evolutionary polarity). To evaluate evolutionary hypotheses, it is often necessary to locate the root of the tree. Rooting phylogenetic trees is not a trivial problem (Nixon and Carpenter, 1993).

If one accepts a molecular clock, then the root will always be at the midpoint of the longest span across the tree (Weston, 1994). Whether molecular evolution is indeed clocklike generally remains a contentious issue (Li, 1997), but most gene trees exhibit unclocklike behavior regardless of where the root is placed. Thus, rooting is generally evaluated by extrinsic evidence, that is, by means of determining where the tree would attach to an ''outgroup,'' which can be any organism/sequence not descended from the nearest common ancestor of the organisms/sequences analyzed (for example, a bird sequence could be used to root an analysis of mammals). Outgroup rooting, however, creates a dilemma: an outgroup that is closely related to the ingroup might be simply an erroneously excluded member of the ingroup. A clearly distant outgroup (e.g., a fungus for an analysis of plants) can have a sequence so diverged that its attachment to the ingroup is subject to the long-branch attraction problem mentioned above. It is wise to examine the results obtained for trees both with and without an outgroup.

Another means of rooting involves analysis of a duplicated gene or gene with an internal duplication (Lawson et al., 1996). If all the paralogs from most or all of the organisms are included in the analysis, then one can logically root the tree exactly where the paralog gene trees converge, assuming that there are not long branch problems in all trees.

TREE EVALUATION

Several procedures are available that evaluate the phylogenetic signal in the data and the robustness of trees (Swofford et al., 1996; Li, 1997). The most popular of the former class are tests of data signal versus randomized data (skewness and permutation tests). The latter class includes tests of tree support from resampling of observed data (nonparametric bootstrap). The likelihood ratio test provides a means of evaluating both the substitution model and the tree.

Randomized Trees (Skewness Test)

Simulation studies indicate that the distribution of random MP tree lengths generated using random data sets will be symmetrical, whereas those using data sets with

phylogenetic signal will be skewed. The critical value of the g_1 statistic of skewness will vary with the number of taxa and variable sites in the sequence. The test does not estimate the reliability of a particular topology, and it is sensitive to even very small amounts of signal present in an otherwise random data set. If taxa from groups that are obviously well supported by the data are selectively deleted, the test can be used to determine whether a phylogenetic signal remains, provided at least 10 variable characters and 5 taxa are examined. The procedure is implemented in PAUP.

Randomized Character Data (Permutation Tests)

The randomized data approach determines whether an MP tree or portion of it derived from the actual data could have arisen by chance. The data are not truly randomized but permuted within each aligned column, so that covariation in the initial data is removed. The result is an alignment of sequences that are not random sequences; rather, the base at each site in these sequences is randomly drawn from the population of bases occupying that site in the overall alignment. The permutation tail probability test (PTP) compares the score for the MP tree with trees generated by numerous permutations of the data at each site, determining only whether there is a phylogenetic signal in the original data. A topology-dependent test (T-PTP) compares the scores for specific trees to determine whether the difference can be attributed to chance. This method does not evaluate whether the tree or any portion of it is correct (Swofford et al., 1996). In particular, the T-PTP test will appear to corroborate groups that are in trees close to the MP tree but not in it. This is because the method detects the collective signal that places a taxon even approximately, if not actually, in its correct position. The results can be fine-tuned, however, by additional applications using relevant subsets of the data (Faith and Trueman, 1996). The procedure is implemented in PAUP.

Bootstrap

Bootstrapping is a resampling tree evaluation method that works with distance, parsimony, likelihood, and just about any other tree derivation method. It was invented in 1979 (Efron, 1979) and introduced as a tree evaluation method in phylogenetic analysis by Felsenstein (1985). The result of bootstrap analysis is typically a number associated with a particular branch in the phylogenetic tree that gives the proportion of bootstrap replicates that supports the monophyly of the clade.

How is this done practically? Bootstrapping can be considered a two-step process comprising the generation of (many) new data sets from the original set and the computation of a number that gives the proportion of times that a particular branch (e.g., a taxon) appeared in the tree. That number is commonly referred to as the bootstrap value. New data sets are created from the original data set by sampling columns of characters at random from the original data set with replacement. ''With replacement'' means that each site can be sampled again with the same probability as any of the other sites. As a consequence, each of the newly created data sets has the same number of total positions as the original data set, but some positions are duplicated or triplicated and others are missing. It is therefore possible that some of the newly created data sets are completely identical to the original set—or, on the other extreme, that only one of the sites is replicated, say, 500 times, whereas the remaining 499 positions in the original data set are dropped.

Although it has become common practice to include bootstrapping as part of a thorough phylogenetic analysis, there is some discussion on what exactly is measured by this method. It was originally suggested that the bootstrap value is a measure of repeatability (Felsenstein, 1985). In more recent interpretations, it has been considered to be a measure of accuracy—a biologically more relevant parameter that gives the probability that the true phylogeny has been recovered. On the basis of simulation studies, it has been suggested that, under favorable conditions (roughly equal rates of change, symmetric branches), bootstrap values greater than 70% correspond to a probability of greater than 95% that the true phylogeny has been found (Hillis and Bull, 1993). By the same token, under less favorable conditions, bootstrap values greater than 50% will be overestimates of accuracy (Hillis and Bull, 1993). Simply put, under certain conditions, high bootstrap values can make the wrong phylogeny look good; therefore, the conditions of the analysis must be considered. Bootstrapping can be used in experiments in which trees are recomputed after internal branches are deleted one at a time. The results provide information on branching orders that are ambiguous in the full data set (cf. Leipe et al., 1994).

Parametric Bootstrap

The parametric bootstrap differs from the nonparametric in that it uses simulated (yet actual) replicates rather than pseudoreplicates. In the case of phylogenetic sequence analysis, replicate data sets of the same size as the original data set are generated according to a specified model of sequence evolution, including the optimal tree topology determined according to that model (Huelsenbeck et al., 1996). Each data set is then analyzed according to the method of interest. Support for the branches in the test tree can be determined in much the same way as in the nonparametric bootstrap.

Likelihood Ratio Tests

As the name implies, likelihood ratio tests are applicable to ML analyses. A suboptimal likelihood value is evaluated for significance against a normal distribution of the error in the optimal model. In ideal applications, the error curve is presumed to be a χ^2 distribution. Thus, the test statistic is twice the difference between the optimal and test values, and the degrees of freedom is the number of parameter differences.

Application of the χ^2 test to alternative phylogenetic trees is problematic, especially because of the ''irregularity of [the] parameter space'' (Yang et al., 1995), but its use has been advocated for evaluating optimality of the substitution model when the number of parameters between models is known.

PHYLOGENETICS SOFTWARE

PHYLIP and PAUP compete as the most widely used phylogenetic analysis software, although other newer applications such as PUZZLE are beginning to compete. Here, PHYLIP and PAUP will be described in the most detail, with references made to other available packages that have useful features. However, the number of programs available is now so numerous, many each having their own useful features, that the

reader is referred to the list of Internet resources at the end of this chapter for further information.

PHYLIP

PHYLIP (for **phyl**ogeny **i**nference **p**ackage) is a package now consisting of about 30 programs that cover most aspects of phylogenetic analysis. PHYLIP is free and available for a wide variety of computer platforms (Mac, DOS, UNIX, VAX/VMS, and others). According to its author, PHYLIP is currently the most widely used phylogeny program.

PHYLIP is a command-line program and does not have a point-and-click interface, as programs like PAUP do. The documentation is well written and very comprehensive, and the interface is straightforward. A program within PHYLIP is invoked by typing its name, which automatically causes the data to be read from a file called ''infile'' or a file name you specify if no infile exists. This infile must be in PHYLIP format; this format is clearly described in the documentation, and most sequence analysis programs offer the ability to export sequences in this format. For example, if an alignment is produced using CLUSTAL W or edited using GeneDoc, the alignment may be saved in PHYLIP format and then used in PHYLIP programs directly. Once the user activates a given PHYLIP program and loads the infile, the user can then choose from an option menu or accept the default values. The program will write its output to a file called ''outfile'' (and ''treefile'' where applicable). If the output is to be read by another program, ''outfile'' or ''treefile'' must be renamed before execution of the next program, as all files named outfile/tree file in the current directory are overwritten at the beginning of any program execution. The tree file generated is a widely used format that can be imported into a variety of tree-drawing programs, including DRAWGRAM and DRAWTREE that come with this package. However, these PHYLIP tree-drawing programs produce low-resolution graphics, so a program such as TreeView (described below) is instead recommended. Particulars of some of the PHYLIP tree-inference programs are discussed below.

PROTDIST is a program that computes a distance matrix for an alignment of protein sequences. It allows the user to choose between one of three evolutionary models of amino acid replacements. The simplest, fastest (and least realistic) model assumes that each amino acid has an equal chance of turning into 1 of the other 19 amino acids. The second is a category model in which the amino acids are redistributed among different groups; transitions in this model are evaluated differently depending on whether the change would result in an amino acid in the same or in a different group. The third (default) method, which is recommended, uses a table of empirically observed transitions between amino acids, the Dayhoff PAM 001 matrix (Dayhoff, 1979). More details can be found in the PHYLIP documentation and in a publication (Felsenstein, 1996).

NEIGHBOR is a tree-generating program that utilizes the distance matrix data generated from a program such as PROTDIST and generates a tree using the neighbor-joining method. This is one of the more popular methods, due to its speed of computation.

FITCH is another tree-generating program similar to NEIGHBOR but much more robust. It also uses distance matrix data, such as that described in PROTDIST, and generates a tree using the method of Fitch-Margoliash. This method, while more robust than NEIGHBOR, tends to produce a similar final answer, yet takes longer

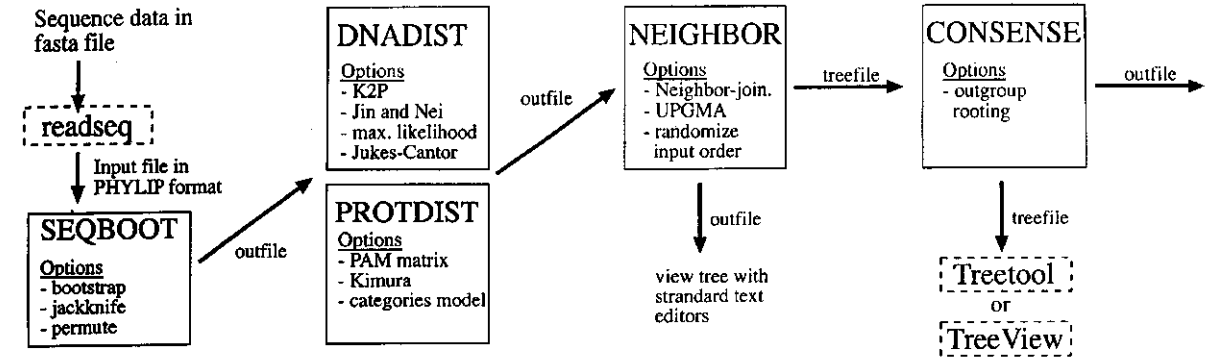
to compute. Although computational times are often significantly longer, the quality of the results produced by the method often makes this method the method of choice in these types of analyses.

PROTPARS is a parsimony program for protein sequences that generates trees without utilizing a distance matrix. The evolutionary model is different from the ones used in the PROTDIST program in that it considers the underlying changes in the nucleotide sequence to evaluate the probabilities of the observed amino acid changes. Specifically, it makes the (biologically meaningful) assumption that synonymous changes [e.g., GCA (alanine) → GCC (alanine)] occur more often than nonsynonymous changes. As a consequence, a transition between two amino acids that would require, for example, three nonsynonymous changes in the underlying nucleotide sequences, is assigned a lower probability than an amino acid change calling for two nonsynonymous changes and one synonymous change. PROTPARS does not have an option that uses empirical values for amino acid changes (e.g., PAM matrices).

DNADIST computes a distance matrix from nucleotide sequences. Trees are generated by running the output through NEIGHBOR or other distance matrix programs in the PHYLIP package. DNADIST allows the user to choose between three models of nucleotide substitution. The older Jukes and Cantor model is similar to the simple model in the PROTDIST program in that it assumes equal probabilities for all changes. The more recent Kimura two-parameter model is very similar but allows the user to weigh transversion more heavily than transitions. PHYLIP also comprises DNAML, a maximum-likelihood program for nucleotide data. Because the program is fairly slow, the use of its faster ''sibling,'' the fastDNAml program (Olsen et al., 1994) described below, is recommended.

SEQBOOT and CONSENSE are required for bootstrap analysis. SEQBOOT is used to generate any number of replicates of the data; these replicates are then used in programs within the PHYLIP suite for analysis. The resulting tree file contains as many trees as there are replicates of the data, so this file needs to be run through CONSENSE to generate the consensus tree from the analysis. As an example, the steps involved in building a bootstrapped neighbor-joining tree for protein sequences are outlined in Figure 14.9.

Figure 14.9. Work flow for bootstrap analysis with the PHYLIP program. SEQBOOT accepts a file in PHYLIP format as input and multiplies it a user-specified number of times (e.g., 1,000). The resulting outfile can be used to calculate 1,000 distance matrices for DNA (DNADIST) or protein (PROTDIST) data. In this step, the actual data (nucleotides, amino acids) are discarded and replaced by a figure that is a measure for the amount of divergence between two sequences. The NEIGHBOR program will create 1,000 trees from these matrices. The CONSENSE program reduces the 1,000 trees to a single one and indicates the bootstrap values as numbers on the branches. The topology of the CONSENSE tree can be viewed with any text editor in the "outfile," whereas the "treefile" can be further processed for publication purposes. Treetool and TreeView allow the user to manipulate the tree (rerooting, branch rearrangements, conversions from dendrograms to phylograms, and so forth) and to save the file in commonly used graphic formats. Although these are not part of the PHYLIP package (indicated by boxes with dashed lines), they are freely available (see end-of-chapter list). Different file formats used during date processing through the stages of bootstrap analysis are also shown. Periods to the right and at the bottom of a box indicate files that were truncated to save space.



PHYLIP input file

```
 5 60
human   TKYHSDDYST GGSVASAVNW AG..
mouse   TKYHSDEYST GGSVAGAVNW AG..
frog    TKYHSDDYSA GGSVASSVNW SG..
yeast   CQFHTDEYSG GGSMEGAVNY AG..
bact.   ELVHEPDYVG GTILTAACNT AG..
```

SEQBOOT output

```
    5    60
human   TTTKKYDDDY YYTGGGSASA VV..
mouse   TTTKKYDEEY YYTGGGSAGA VV..
frog    TTTKKYDDDY YYAGGGSASS VV..
yeast   CCCQQFDEEY YYGGGGSEGA VV..
bact.   EEELLVPDDY YYGGGTITAA CC..
    5    60
human   TKKYSSSDYS GGSAAVNWWW AA..
mouse   TKKYSSSEYS GGGAAVNWWW AA..
frog    TKKYSSSDYS GGSSSVNWWW SS..
yeast   CQQFTTTEYS GGGAAVNYYY AA..
bact.   ELLVEEEDYV GGAAACNTTT AA..
    5    60
human   TYYYHHSDDD DYYSTTASAA VV..
mouse   TYYYHHSDDD EYYSTTAGAA VV..
  .               .           .
  .               .           .
```

PROTDIST output

```
    5
human   0.00000  0.00982  0.10084  0.24911  1.01684
mouse   0.00982  0.00000  0.13747  0.21005  1.05809
frog    0.10084  0.13747  0.00000  0.34655  1.19283
yeast   0.24911  0.21005  0.34655  0.00000  0.97720
bact.   1.01684  1.05809  1.19283  0.97720  0.00000
    5
human   0.00000  0.00000  0.08682  0.41532  1.13122
mouse   0.00000  0.00000  0.08682  0.41532  1.13122
  .        .        .        .        .        .
  .        .        .        .        .        .
```

NEIGHBOR/CONSENSE outfile

```
          +---------mouse
     +-93.0
     !    !    +----frog
+-94.0    +-72.0
!    !         +----human
!    !
!    +--------------yeast
!
+-------------------bacterium
```

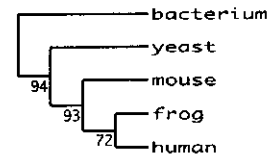
NEIGHBOR treefile

```
(frog:0.09252,((yeast:0.20912,bacterium:1.29
294):0.16052,mouse:-0.00846):0.02131,human:-
0.02075);

(mouse:-0.00225,frog:0.09130,(human:-0.00225,
(yeast:0.16225,bacterium:0.63517):0.11338):0
.00225);

((frog:0.14362,human:-0.01934):0.03099,(yeast:
0.12204,bacterium:0.80782):0.10822,mouse:-
0.00667);
```

Treeview output



PAUP

The objective of the development of PAUP is to provide a phylogenetics program that includes as many functions (including tree graphics) as possible in a single, platform-independent program with a menu interface. PAUP stands for **p**hylogenetic **a**nalysis **u**sing **p**arsimony and still contains one of the most sophisticated parsimony programs available. Version 3 performed only MP-associated tree-building and analytical functions. PAUP version 4 also includes distance and ML functions for nucleotide data and other new features.

Current tree-building functions in PAUP include MP, and, for nucleotide data, distance and ML using the fastDNAml algorithm. In addition, PAUP performs Lake's method of invariants (Swofford et al., 1996; Li, 1997). Each tree-building program permits a variety of options. The MP options include specification of any character-weighting scheme. Distance options include choice of NJ, ME, FM (see PAUP release notes regarding PHYLIP), and UPGMA procedures. The full range of options and their current values can be examined using the menu and/or by typing `pse[ttings] ?`, `dse[ttings] ?`, and `lse[ttings] ?` for parsimony, distance, and likelihood, respectively. Both distance and ML allow detailed specification of the substitution model (values of substitution, gamma, and invariant-sites parameters, assuming equal, specified, or empirical base frequencies), and these can be estimated for any tree by setting the parameter values to `est[imate]` and applying the `des[cribe tree]` command with a desired tree in memory.

According to the release notes accompanying PAUP test version 4, PAUP* *usually* finds trees with likelihoods as high or higher [i.e., better] than PHYLIP (both because PAUP*'s tree rearrangements are more extensive and because its convergence criterion for branch-length iteration is stricter).

With any tree-building method, PAUP allows a variety of tree search options. These include algorithm specification for generating the initial tree (starting tree): NJ, stepwise addition, or input tree(s). The stepwise-addition algorithm allows numerous options, including addition of taxa ''as is'' (taxa added in file order): closest, furthest, or random with any number of replicates. All the stepwise options allow for any maximal number of partial trees to be retained and built on during taxon addition. Increasing this number to, say, 100, is another means of increasing the diversity of starting topologies, although these are not random.

A random addition strategy provides a useful complement to the default search strategy (closest addition, TBR swapping, saving all best trees). In the random search, a large number of replicates can be combined with the faster NNI swapping algorithm. For MP analysis, in which a large number of equal-length trees might exist, the search should specify saving from each replicate only a few trees that match or are better than the score of the slower search. In addition, the number of suboptimal trees (the trees that will be swapped on to find better trees) should be limited by setting MAXTREES to a low number (e.g., 10). By using this strategy to explore areas of ''tree space'' possibly missed in the slow search, one sometimes finds better trees and/or additional unique optimal trees.

PAUP performs the nonparametric bootstrap for distance, MP, and ML, using all options available for tree building with these methods. When a bootstrap or jackknife with MP is under way, MAXTREES should be set between 10 and no more than 100. This is because poorly resolvable portions of an MP tree will usually be even less resolvable with resampled data; hence, a replicate could find astronomical num-

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bers of equal-length trees. Because tree branches weakly supported by the full data set will not have high bootstrap or jackknife values, limiting MAXTREES will have little, if any, bearing on the results, especially if the number of replicates is increased to, say, 1,000.

In addition, PAUP performs the Kishino-Hasegawa test to compare MP or ML trees, computes four types of consensus of multiple trees (usually used for multiple equal-length MP trees), computes stepwise differences between MP trees, and evaluates signal conflicts between specified partitions of sites (e.g., nuclear and organellar sequence data in a combined analysis).

Other Programs

In addition to PAUP and PHYLIP, there are phylogenetics programs that have some unique capabilities but are generally more limited in their procedures and portability. These include FastDNAml, PUZZLE, MACCLADE, and MOLPHY.

FastDNAml. FastDNAml (Olsen et al., 1994) is a freestanding maximum-likelihood, tree-building program. Although it is currently not part of the PHYLIP package, it uses largely the same input and output conventions, and the results of fastDNAml and PHYLIP's DNAML should be very similar or identical. FastDNAml can be run on parallel processors, and it comes with a number of useful scripts (in particular for bootstrapping and jumbling the sequence input order). To take full advantage of the program, knowledge of UNIX is beneficial. The source code for UNIX systems is publicly available from the RDP Web site, and a Power Macintosh version is available by FTP.

PUZZLE or TREE-PUZZLE

PUZZLE or TREE-PUZZLE (Strimmer and von Haeseler, 1996), as it is now called, is a maximum likelihood-based program that implements a fast tree search algorithm (quartet puzzling) that allows analysis of large data sets and automatically assigns estimations of support to each internal branch. PUZZLE also computes pairwise maximum-likelihood distances as well as branch lengths for user-specified trees. PUZZLE also offers a novel method, likelihood mapping, to investigate the support of a hypothesized internal branch without computing an overall tree and to visualize the phylogenetic content of a sequence alignment. It conducts a number of statistical tests (χ^2 test for homogeneity of base composition, likelihood ratio clock test, Kishino-Hasegawa test) and includes a large range of models of substitution. Rate heterogeneity is modeled by a discrete gamma distribution and by allowing invariable sites.

MACCLADE. MACCLADE is an interactive Macintosh program for manipulating trees and data and studying the phylogenetic behavior of characters (Maddison and Maddison, 1992). It uses the NEXUS file format and will read PAUP data and tree files. Some information in PAUP files will be ignored in MACCLADE (e.g., gap mode), but information in a PAUP ''assumptions'' block will be imported, including character weightings and character and taxon sets. Several subtle differences exist between PAUP and MACCLADE files. Thus, PAUP files edited with MACCLADE and vice versa should be saved under new names and the unedited file

maintained separately. PHYLIP, NBRF-PIR, and text files are also readable by MACCLADE. Any method can be used to generate the trees, but MACCLADE's functions are based strictly on parsimony. For example, the program allows one to trace the evolution of each individual character on any tree. The MP and ML reconstruction functions differ, however, and the ML function is considered more realistic (Swofford et al., 1996). Tree topologies can be manipulated by dragging branches, and flipping branches can produce aesthetic modifications in tree symmetry.

MACCLADE includes additional features relevant to sequence analysis, including a chart of character number versus number of changes in a tree, which is useful for visualizing among-site rate heterogeneity, and a chart of the overall numbers of changes from one base to another over an MP tree (''state changes and stasis'' chart: the values are sometimes erroneously reported in the literature as substitution ''rates,'' but there is no correction for branch lengths or among-site rate heterogeneity).

MOLPHY. MOLPHY is a shareware package of programs and utilities for ML analysis and statistics of nucleotide or amino acid sequences (Adachi and Hasegawa, 1996). It has been tested on Sun OS and HP9000/700 systems. Practical application requires some knowledge of UNIX file management. The ML procedures are similar to those in PHYLIP, but there is a wider range of amino acid substitution models and options for faster, heuristic searches, including an option to use ''local bootstrap'' analyses (i.e., a bootstrap on subtrees under the assumption that the remainder of the tree is correct) to search for better ML trees. The output includes branch-length estimates and standard error. Analysis of separate codon positions is possible. MOLPHY uses a subset of the nucleotide substitution models available in PAUP, although it allows user-specified parameter values. The current MOLPHY lacks a bootstrap option and also has no accommodation for among-site rate heterogeneity.

Tree Drawing. There are a number of tree-drawing programs available now, such as TreeTool (X-windows), TreeDraw (Macintosh), PHYLODENDRON (Macintosh), TreeView (Macintosh, Microsoft Windows), or the tree-drawing tool in PAUP, and all handle standard tree files. These programs facilitate not only the generation of trees suitable for publication or other presentation but also facilitate viewing of the data in general. For example, programs such as the freely available TreeView enable the user to manipulate the view of branching order, root the tree, and other graphical manipulations that aid the user.

INTERNET-ACCESSIBLE PHYLOGENETIC ANALYSIS SOFTWARE

Currently, there are few Web-based applications that will permit an investigator to perform phylogenetic analyses over the Web. However, these kinds of resources are appearing in increasing numbers, and, presumably as the Internet bandwidth increases and servers have faster CPUs, this may become even more common. Highlighted here are three Internet-based applications that provide phylogenetic analysis capabilities: WEBPHYLIP, PhyloBLAST, and the ''BLAST2 & Orthologue Search Server.'' These illustrate the variety of applications currently available. Although all use PHYLIP programs, the latter two combine phylogenetic analysis with BLAST to aid the user in retrieving sequences for analysis.

WEBPHYLIP

WEBPHYLIP uses CGI/Perl scripts to produce a Web-based cut-and-paste interface to the PHYLIP programs. Unfortunately, the programs are not linked together; therefore, to generate a neighbor-joining tree, for example, the user must run multiple analyses (PROTDIST and NEIGHBOR in this case). Analyses may time-out if too intensive of an analysis is requested. Also, the trees cannot be easily viewed; for example, if the user has a PC, they must have ghostview or another such PostScript viewer installed to actually view the results. However, the Web site provides excellent flowcharts and other helpful documentation about running the programs, and an extensive collection of the PHYLIP programs is available.

PhyloBLAST

PhyloBLAST is also based on CGI/Perl scripts. PhyloBLAST compares a user's protein sequence to the SWISS-PROT/TREMBL databases using BLAST2 and then allows user-defined phylogenetic analyses to be performed on selected sequences from the BLAST output. Neighbor-joining and parsimony analyses may be performed, either with or without bootstrapping, using PHYLIP programs. Flexible features, such as the ability to input premade multiple sequence alignments and use all options found in the PHYLIP programs, provide additional functionality that goes beyond the simple analysis of a BLAST result. Because PHYLIP programs need to generate trees that are linked, there is less input required by the user than for WEBPHYLIP; however, DNA analysis and analysis using some programs (for example, FITCH) are not currently available. However, PhyloBLAST's ability to generate trees-containing hyperlinks to further protein sequence information or generate JPEG graphics of trees is a considerable advantage. Also, the program is set up to handle Web page time-outs so long analyses are not a problem (and can be E-mailed to the user if preferred).

BLAST2 & Orthologue Search Server

This is a fairly specialized application of phylogenetic analysis of a BLAST output for the identification of orthologs verses paralogs. It is based on the use of CLUSTALW, WU-BLAST2, and the tree-reconciling algorithm of Page (1994). This tool first performs a BLAST analysis and then performs a phylogenetic analysis on user-selected sequences based on a CLUSTAL W alignment and PHYLIP's neighbor-joining methods. This resulting neighbor-joining tree (''gene tree'') is ultimately compared with a predicted species tree and the reconciled tree viewed for analysis. The philosophy here is that, whenever the phylogeny of the species matches the phylogeny of the gene tree, these genes will be deemed orthologous.

Although this is a useful tool, users should be cautioned that this does not represent a comprehensive phylogenetic analysis, due to the automated nature of the application. Its use should be primarily as intended: to gain insight into what homologous sequences are orthologous in an automated fashion. Further analysis should be performed for any particularly in-depth investigation using less automated alignment and phylogenetic analysis that suits the sequences being investigated.

SOME SIMPLE PRACTICAL CONSIDERATIONS

1. Paradoxical as it may sound, by far the most important factor in inferring phylogenies is not the method of phylogenetic inference but the quality of the input data. The importance of data selection and in particular of the alignment process cannot be overestimated. Even the most sophisticated phylogenetic inference methods are not able to correct for erroneous input data.
2. Look at the data from as many angles as possible. Use each of the three main methods (distance, maximum parsimony, maximum likelihood) and compare the resulting trees for consistency. At the same time, be aware that one cannot rely on having arrived at a good estimate for the true phylogeny just because all three methods produce the same tree. Unfortunately, consistency among results obtained by different methods does not necessarily mean that the result is statistically significant (or represents the true phylogeny), since there can be several reasons for such correspondence.
3. The choice of outgroup taxa can have as much influence on the analysis as the choice of ingroup taxa. Complication will occur in particular when the outgroup shares an unusual property (e.g., composition bias or clock rate) with one or several ingroup taxa. It is therefore advisable to compute every analysis with several outgroups and check for congruency of the ingroup topologies.
4. Be aware that programs can give different answers (trees) depending on the order in which the sequences appear in the input file. PHYLIP, PAUP and other phylogenetic software provide a ''jumble'' option that reruns the analysis with different (jumbled) input orders. If for whatever reason the tree must be computed in a single run, sequences that are suspected of being ''problematic'' should be placed toward the end of the input file, to lower the probability that tree rearrangement methods will be negatively influenced by a poor initial topology stemming from any problematic sequences.

INTERNET RESOURCES FOR TOPICS PRESENTED IN CHAPTER 14

Compilation of available phylogeny programs	*http://evolution.genetics.washington.edu/phylip/software.html*
BLAST2 & Orthologue Search	*http://www.Bork.EMBL-Heidelberg.DE/Blast2e/*
CLUSTAL W	*http://www-igbmc.u-strasbg.fr/BioInfo/*
GeneDoc	*http://www.psc.edu/biomed/genedoc/*
GeneTree	*http://taxonomy.zoology.gla.ac.uk/rod/genetree/genetree.html*
PHYLIP	*http://evolution.genetics.washington.edu/phylip.html*
PhyloBLAST	*http://www.pathogenomics.bc.ca/phyloBLAST/*
Phylogenetic Resources	*http://www.ucmp.berkeley.edu/subway/phylogen.html*
PUZZLEBOOT	*http://www.tree-puzzle.de*
ReadSeq	*http://dot.imgen.bcm.tmc.edu:9331/seq-util/Options/readseq.html*
RDP Tree	*http://rdp.life.uiuc.edu/RDP/commands/sgtree.html*

TreeView	*http://taxonomy.zoology.gla.ac.uk/rod/treeview.html*
WebPHYLIP	*http://sdmc.krdl.org.sg:8080/~lxzhang/phylip/*
WHS	*http://www.cladistics.org/education.html*

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