PRALINE: a multiple sequence alignment toolbox that integrates homology-extended and secondary structure information

V. A. Simossis\(^1\) and J. Heringa\(^{1,2,*}\)

\(^1\)Bioinformatics Section, Faculty of Sciences and \(^2\)Centre for Integrative Bioinformatics VU (IBIVU), Faculty of Sciences and Faculty of Earth & Life Sciences, Vrije Universiteit, De Boelelaan 1081A, 1081 HV, Amsterdam, The Netherlands

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ABSTRACT
PRofile ALIgnNEment (PRALINE) is a fully customizable multiple sequence alignment application. In addition to a number of available alignment strategies, PRALINE can integrate information from database homology searches to generate a homology-extended multiple alignment. PRALINE also provides a choice of seven different secondary structure prediction programs that can be used individually or in combination as a consensus for integrating structural information into the alignment process. The program can be used through two separate interfaces: one has been designed to cater to more advanced needs of researchers in the field, and the other for standard construction of high confidence alignments. The web-based output is designed to facilitate the comprehensive visualization of the generated alignments by means of five default colour schemes based on: residue type, position conservation, position reliability, residue hydrophobicity and secondary structure, depending on the options set. A user can also define a custom colour scheme by selecting which colour will represent one or more amino acids in the alignment. All generated alignments are also made available in the PDF format for easy figure generation for publications. The grouping of sequences, on which the alignment is based, can also be visualized as a dendrogram. PRALINE is available at http://ibivu.cs.vu.nl/programs/pralinewww/.

INTRODUCTION
The alignment of two or more sequences has become an essential sequence analysis technique in biological research. State-of-the-art multiple sequence alignment (MSA) methods, such as T-COFFEE (1) and MUSCLE (2), as well as other MSA methods available to date, perform alignments by only using the sequences in the given set. Although they use profile technology to match distant sequence sets, they do not use further homology information for the sequences that are available in current sequence databases. The benefit of using homologous information to align distant sequences has been shown in a number of studies (3), while the use of profiles to represent the additional homologous information has been shown to have many advantages (4,5). For this reason, the PRALINE toolbox (6,7) has been recently re-designed to include homology-extended multiple alignment (8), where as an initial step a profile for each sequence in a given set is built by using PSI-BLAST (9,10) and the progressive alignment then proceeds using the PSI-BLAST profiles instead of the given sequences. This approach has been previously applied with success to local pairwise alignment methods for homology modelling (11–15) and is extended in PRALINE for global MSA. The recently updated MAFFT alignment tool (3,16) also uses homologous sequences to improve the alignment quality of distant sequences. However, in the MAFFT approach, the additional information is not incorporated in profiles for each of the query sequences, but homologous sequences are added to the original set and then aligned together using the various MAFFT alignment strategies. In the end, the homologous sequences are removed, leaving the aligned original sequences to form the final alignment.

In this paper we present the new web server for the PRALINE toolbox (6,7), where we have added two new alignment features: homology-extended multiple alignment (8) and the integration of predicted secondary structure information with iteration capabilities (V. A. Simossis and J. Heringa, submitted for publication). We show results for the cytochrome P450 HOMSTRAD (17) sequence set as an example to demonstrate how the homology-extended strategy and integrating secondary structure information, in combination

*To whom correspondence should be addressed. Tel: +31 20 5987649; Fax: +31 20 5987653; Email: heringa@cs.vu.nl

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with the visualization possibilities of the server output can lead to meaningful interpretations. Details about the PRALINE strategies and optimizations have been described previously (6–8,18).

**HOMOLOGY-EXTENDED MULTIPLE ALIGNMENT**

The homology-extended MSA strategy enriches the information for each of the sequences in a given set by collecting putative homologous sequences. Each sequence is submitted as a query to PSI-BLAST over a database of choice [default: non-redundant (NR)]. The resulting PSI-BLAST alignments are then filtered for redundancy (100% sequence identity). In the event that no hits or only redundant hits are detected, the PSI-BLAST E-value threshold is automatically adjusted to a 10-fold less stringent setting (e.g. from $10^{-36}$ to $10^{-5}$) and the query is re-submitted. Once all the sequences to be aligned have at least one additional putative homologue, each PSI-BLAST alignment is converted into a profile and progressively aligned. A more detailed account of the PRALINE homology-extended multiple alignment algorithm and its performance is available in Ref. (8).

The advantage of this strategy is that it uses a much larger amount of position-specific information in the homology-extended profiles to score the alignment of two or more positions. As a result, the cases that benefit the most are those that evolution has changed so extensively (<30% identity) that the homology (common ancestry) between them is almost undetectable when compared directly (8).

In Table 1, the performance of the homology-extended alignment strategy on 254 HOMSTRAD (17) multiple alignment cases has been compared with the state-of-the-art methods T-COFFEEv2.03 and MUSCLEv3.51. The results show that for the strictest quality measure, column scoring, the overall improvement of the PRALINE strategy is >3.5% relative to T-COFFEE and MUSCLE. Moreover, the improvement is >5% for the most distant and difficult test cases with sequences <30% identity. In addition, PRALINE has also been compared with the PRALINE standard global progressive alignment strategy (PRALINEBASIC) (6) and the PRALINEBASIC and PRALINEPSI strategies with integrated predicted [PSIPRED (19) and YASPIN (20)] secondary structure information, respectively, named as PRALINEBASIC-PSIPRED and PRALINEPSI-YASPIN. The latter secondary structure-guided alignment strategies of PRALINE are discussed in the next section. As shown in Table 1, the improvement in alignment quality achieved by homology-extended alignment (PRALINEPSI) as compared with other methods is significant in the more difficult alignment cases with average sequence identity percentages <60%. As would be expected, in the easier alignment cases that share >60% sequence identity, all the alignments are of comparable high quality.

When used as an option on the server, the homology-extended alignment strategy can further be customized by manually entering the desired iteration count, starting E-value cut-off and database to be searched by PSI-BLAST for the building of the homology-extended profiles (default: 3 iterations, starting with a cut-off of $10 \times 10^{-5}$ on the NR database). The default parameters have been optimized by testing different settings on the HOMSTRAD database of structural alignments (8).

**INTEGRATION OF SECONDARY STRUCTURE**

The rule-of-thumb that structure is more conserved than sequence is a well-documented fact (21–24). As a result, many studies have shown that its use to guide sequence alignment improves alignment quality, especially between distant sequences (6–8,11–15,25). To this end, we have devised a secondary structure scoring scheme for the alignment algorithm that combines exchange weights from four types of matrices: sequence or profile positions that have not been assigned the same secondary structure class are scored using a generic matrix (default: BLOSUM62), otherwise the positions that have matching helix, strand or coil assignments use the Lüthy (26) helix-, strand- and coil-specific matrices, respectively. The use of the secondary structure information significantly improves the PRALINEBASIC alignment quality and also boosts the PRALINEPSI alignments in the very difficult alignment cases <20% sequence identity (V. A. Simossis and J. Heringa, submitted for publication). In Table 1, it is clearly shown that the use of the secondary structure is beneficial for PRALINEBASIC (>4% improvement in cases with <60% identity), albeit not as significant as the improvements seen with PRALINEPSI.

The secondary structure integration options of PRALINE involve the use of any one of the seven prediction methods that are listed [PHDpsi (27), PROFsec (B. Rost, unpublished data), SSPRO 2.01 (28), YASPIN (20), PSIPRED (19), JNET (29) and PREDATOR (30,31)] to predict the secondary structure of the input sequences. In addition, the user can optionally select

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**Table 1.** The quality assessment of 254 HOMSTRAD multiple alignment cases generated by different alignment strategies

| Alignment method              | Overall (%) | 0–30 (%) | 30–60 (%) | 60–100 (%) | P (0–100) |
|-------------------------------|-------------|----------|-----------|------------|-----------|
| Column score                  |             |          |           |            |           |
| PRALINEBASIC                  | 63.8        | 38.7     | 68.5      | 95.5       | –         |
| PRALINEBASIC-YASPIN           | 68.0        | 45.3     | 72.2      | 96.3       | 0.106     |
| PRALINEBASIC-PSIPRED          | 67.4        | 43.5     | 72.1      | 95.9       | 0.337     |
| PRALINEPSI                    | 70.2        | 50.2     | 73.6      | 96.7       | 0.025     |
| PRALINEPSI-YASPIN             | 70.0        | 49.7     | 73.6      | 96.5       | 0.042     |
| PRALINEPSI-PSIPRED            | 70.1        | 50.2     | 73.5      | 96.7       | 0.014     |
| T-COFFEEv2.03                 | 67.6        | 44.0     | 72.2      | 95.8       | 0.237     |
| MUSCLEv3.51                   | 67.5        | 45.0     | 71.6      | 96.3       | 0.461     |

The significance of the results ($P$-value from Kolmogorov–Smirnov test) is calculated with regard to the PRALINEBASIC method. The column scores are the percentage correctly aligned columns with regard to the HOMSTRAD structure alignment.
to also search the Protein Data Bank (PDB) to find 3D structure information for the input sequences and use the DSSP-derived secondary structure for the alignment. If both DSSP and a prediction method are selected, the predictions will only be integrated into the alignment for those sequences that do not have a PDB entry. Finally, in the same list as the seven prediction methods, an optimally segmented (24) or majority voting consensus can be alternatively used that currently combines the predictions of PROFsec, YASPIN and PSIPRED.

PROFILE PRE-PROCESSING AND ITERATION

PRALINE provides a number of alignment strategies, such as profile pre-processing and iterative alignment optimization (6,7). The secondary structure-guided strategies using PHD, PROFsec, JNET and SSPRO, and the profile pre-processing strategies can be set to use consistency information to drive subsequent alignment rounds (iterations), each time drawing upon the theoretically higher quality information from the previous cycle. A detailed account of these strategies can be found in previously published work (6,7,18,25,32,33).

THE NEW PRALINE SERVER

The PRALINE program is designed to use two or more input protein sequences in the FASTA format (34). The proposed maximum number of sequences that should be submitted to the server is set to 500 with length 2000, but this is mainly to limit the server load and is not the limit of the PRALINE program. In addition, owing to the long running time needed for strategies, such as PRALINEPSI, an optional email notification can be requested that is delivered upon a completion of the job and contains the link to the results and some statistics on the resulting alignment.

Similar to the previous version of the server (18), the gap opening and gap extension penalties and the amino acid substitution matrix can be manually set if needed [default: 12, 1 with BLOSUM62 (35)] for any of the PRALINE alignment strategies. The results page is automatically displayed once the job is complete and contains various sections depending on the options selected (Figure 1). In order to provide all generated files for the user, there is a link to download a compressed file with all the results in the job directory [Figure 1, (D)] and also individual links that allow the user to download specific

Figure 1. The PRALINE results page headers. A: The subtitle indicating which iteration results are presented on this page (only available if iteration >0 is selected). B: The time taken to run the job and statistics related to the visible alignment. C: The links to all other available iteration cycle results (only available if iteration >0 is selected). D: The link to download all job files as a compressed file. E: Links to tabulated specific file types. F: Links to iteration-specific output files (only available if iteration >0 is selected). G: The button that hides/reveals the profile pre-processing scores of the sequence set (only available if profile pre-processing is selected). H: The buttons that switch between colour schemes. I: The button that generates and opens a PDF version of the alignment in the visible colour scheme.
Figure 2. The PRALINEps3 P450 alignment using both PROFsec and DSSP secondary structure integration settings. The alignment has been sectioned to focus on the regions containing the conserved motifs of the cytochrome P450 enzymes (signified by the black bars above the rulers). (A) The oxygen-binding motif, (B) the ExxR motif and (C) the haem-binding motif. For each section, the top colour scheme shows conservation levels according to the colour key and the bottom one shows the secondary structure each residue belongs to (red: helix; green: strand; and clear: coil). The ruler on top of each alignment block shows which parts of the alignment are visible.
files related to each sequence in the set (e.g. a PSI-BLAST profile or a secondary structure file) [Figure 1, (E)].

If the iteration number selected is >0, a subtitle informs the user which iteration cycle results are presented on the page [Figure 1, (A)]. The alignment from each iteration cycle is presented on a different page and is accessible by the corresponding links [Figure 1, (C)]. In addition, it informs the user of the total time taken for the process to complete, provides some statistics related to the visible alignment [Figure 1, (B)] and if the iterations were halted due to alignment convergence or limit cycle convergence and which iteration was the last (not applicable in the Figure 1 example). In the case of iteration-specific output, such as alignment of the iteration or secondary structure prediction, additional links are displayed [Figure 1, (F)].

If profile pre-processing is selected the user has the option of viewing the profile pre-processing scores for all pairwise alignments for deriving an optimum cut-off value [Figure 1, (G)].

Finally, depending on the selected parameters of the job, a series of buttons allows switching between the available colour-coded views [Figure 1, (H)] [details about the colour schemes are described in (18)]. At any point, the visible alignment can be converted into a PDF for printing or further manipulation [Figure 1, (I)]. The remaining of the results page consists of a short description of the visible colour scheme with a key to the colours, after which the colour-coded alignment follows (an example of the conservation and the secondary structure colour-coding is shown in Figure 2).

**SAMPLE OUTPUTS**

Owing to the large number of possible outputs, we have provided a set of nine representative sample outputs for the P450 alignment on the server, each one representing a different combination of PRALINE strategies and settings. These examples are intended as supplementary material to this article and can be accessed through a dedicated link on the server pages or directly at http://ibivu.cs.vu.nl/programs/pralinewww/example/. They can also be used as an indication of CPU times needed by each of the PRALINE strategies.

In Figure 2, we illustrate sections of the PRALINE\textsubscript{psl} alignment of the ‘p450’ HOMSTRAD sequence set (21% average sequence identity) using both DSSP (36) and PROFsec secondary structure integration settings. The colour schemes in the figure are for positional conservation and secondary structure. The secondary structure information for each sequence in this alignment has been derived by using DSSP, since all the corresponding PDB structures of this alignment have been derived by using DSSP. For the secondary structure, only the N-terminal part is more \(\beta\)-sheet rich. The signature motif of the haem-binding site is the only completely conserved amino acids in P450s. These well-documented details are straightforwardly visualized in the PRALINE output conservation colour scheme, while the secondary structure view allows us to relate them in a structural context. As stated in the literature (37), the oxygen binding and ExxR motifs are each part of two distinct C-terminal helices, while the haem-binding motif flanks the N-terminal end of the last helix. Owing to space limitations the alignment has been sectioned to concentrate on these regions, but the full alignment can be viewed online in example 9 of the supplementary material.

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