MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability

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Abstract

We report a major update of the MAFFT multiple sequence alignment program. This version has several new features, including options for adding unaligned sequences into an existing alignment, adjustment of direction in nucleotide alignment, constrained alignment and parallel processing, which were implemented after the previous major update. This report shows actual examples to explain how these features work, alone and in combination. Some examples incorrectly aligned by MAFFT are also shown to clarify its limitations. We discuss how to avoid misalignments, and our ongoing efforts to overcome such limitations.

Key words: multiple sequence alignment, metagenome, protein structure, progressive alignment, parallel processing.

Introduction

Multiple sequence alignment (MSA) plays an important role in evolutionary analyses of biological sequences. MAFFT is an MSA program, first released in 2002 (Kato et al. 2002). Because of its high performance (Nui et al. 2006; Golubchik et al. 2007; Dessimoz and Gil 2010; Letsch et al. 2010; Sahraeian and Yoon 2011; Sievers et al. 2011), MAFFT is becoming popular in recent years. After reviewing the previous version (version 6) in Katoh and Toh (2008b), we have been continuously improving its accuracy, speed, and utility in practical situations. These improvements and techniques were mostly reported in individual papers (Kato et al. 2009; Kato and Toh 2010; Kato and Frith 2012; Katoh and Standley 2013). In this report, we demonstrate the different kinds of analyses that can be achieved with the new features, alone and in combination, using realistic examples. We also discuss limitations of current version by giving examples of sequences incorrectly aligned by MAFFT, and describe our ongoing efforts to overcome these limitations.

Basic Concepts and Usage

As listed in table 1, MAFFT version 7 has options for various alignment strategies, including progressive methods (PartTree, FFT-NS-1, and L-INS-1) (Feng and Doolittle 1987; Higgins and Sharp 1988; Kato and Toh 2007), iterative refinement methods (FFT-NS-i, L-INS-i, E-INS-i, and G-INS-i) (Barton and Sternberg 1987; Berger and Munson 1991; Gotoh 1993; Kato et al. 2005), and structural alignment methods for RNAs (Q-INS-i and X-INS-i; Kato and Toh 2008a). This method separately converts alignment1 and alignment2 to profiles and then aligns the two profiles. It means that the two input alignments are assumed to be significantly different classes of MSA methods, similarity-based methods and evolution-based methods. MAFFT is classified as a similarity-based method. However, evolutionary information is useful even for similarity-based methods, because the sequences to be aligned are generated from a common ancestor in the course of evolution. In this respect, MAFFT takes evolutionary information into account.

All the options of MAFFT assume that the input sequences are all homologous, that is, descended from a common ancestor. Thus, all the letters in the input data are aligned. Genomic rearrangement or domain shuffling is not assumed, and thus the order of the letters in each sequence is always preserved, although the sequences can be reordered according to similarity. Most options in MAFFT assume that almost all the pairs in the input sequences can be aligned, locally or globally. In such a situation, there is a tradeoff between accuracy and speed. For example, the PartTree option (Kato and Toh 2007) is a fast and rough method, whereas L-INS-i and G-INS-i are slower and more accurate. RNA structural alignment methods are generally more accurate and computationally more expensive because they need additional calculations (Kato and Toh 2008a). However, this tradeoff does not always hold. In particular, the new options to add sequences into an existing alignment (Kato and Frith 2012), requires careful consideration of this tradeoff, as discussed later.

Profile Alignments

MAFFT has a subprogram, mafft-profile, to align two existing alignments.

mafft-profile alignment1 alignment2 > output

This method separately converts alignment1 and alignment2 to profiles and then aligns the two profiles. It means that the two input alignments are assumed to be...
### Table 1. Options of MAFFT Version 7.

| Option name | Command | Description |
|-------------|---------|-------------|
| **For a large-scale alignment: progressive methods with the PartTree algorithm** |
| NW-NS-PartTree1 | `mafft --parttree --retree 1 input` | Distance is by the 6mer method. |
| NW-NS-PartTree2 | `mafft --parttree --retree 2 input` | Distance is by the 6mer method. Guide tree is re-built. |
| NW-NS-DPPartTree1 | `mafft --dpparttree --retree 1 input` | Distance is estimated based on DP. |
| NW-NS-DPPartTree2 | `mafft --dpparttree --retree 2 input` | Distance is estimated based on DP. Guide tree is re-built. |
| **For a medium-scale alignment: progressive methods** |
| FFT-NS-1 | `mafft --retree 1 input` | Approximately two times faster than the default. |
| FFT-NS-2 | `mafft input` | Default. |
| **For a small-scale alignment: iterative refinement methods** |
| FFT-NS-i | `mafft --maxiterate 16 input` | Fastest of the four in this category. Uses WSP score (Gotoh 1995) only. |
| G-INS-i | `mafft --maxiterate 16 --globalpair input` | Uses WSP score and consistency (Notredame et al. 1998) score from global alignments. |
| L-INS-i | `mafft --maxiterate 16 --localpair input` | Uses WSP score and consistency score from local alignments. |
| E-INS-i | `mafft --maxiterate 16 --genafpair input` | Uses WSP score and consistency score from local alignments with a generalized affine gap cost (Altschul 1998). |
| **If not sure which option to use** |
| Automatic | `mafft --auto` | Selects an appropriate option from FFT-NS-2, FFT-NS-i and L-INS-i, according to the size of input data. |
| **For a small-scale RNA alignment: structural alignment methods** |
| Q-INS-i | `mafft-qinsi input` | Structure information is included in iterative refinement step. |
| X-INS-i-scarnapair | `mafft-xinsi --scarnapair input` | Uses pairwise structural alignment by MXSCARNA (Tabei et al. 2008). |
| **To add new sequences into an existing MSA** |
| Add | `mafft --add new msa` | The simplest option for alignment extension. |
| Addprofile | `mafft --addprofile msa1 msa2` | msa1 must form a monophyletic cluster. |
| Addfragments | `mafft --addfragments new msa` | Suitable for short new sequences. |
| Addfragments, LAST | `mafft --addfragments new --lastmultipair msa` | Faster option, LAST (Kielbase et al. 2011) is required. |
| Addfragments, 6mer | `mafft --addfragments new --6merpair msa` | Faster option for conserved data. |
| **Parameters** |
| `--bl #, --jtt #, --tm #` | Score matrices for protein alignment. |
| `--kimura #` | Score matrix for nucleotide alignment. |
| **Utility options** |
| `--anysymbol` | See main text. |
| `--reorder` | |
| `--clustalout` | |
| `--phylipout` | |
| `--namelength #` | |
| `--adjustdirection` | |
| `--adjustdirectionaccurately` | |
| `--seed msa1 --seed msa2 ...` | |
| `--treein treefile` | |
| `--treeout` | |
| `--thread #` | |

**Note:** N, the number of sequences; L, the sequence length; input, new, unaligned sequences in the multi-fasta format; msa, msa1, msa2, aligned sequences in the multi-fasta format; treefile, input guide tree file.
phylogenetically isolated from each other, like figure 1A. Careless application of this method results in serious misalignments, as discussed in later section.

MAFFT version 7 has an alternative option, --addprofile, which is safer against misuses.

```
mafft --addprofile alignment1 alignment2 > output
```

This option accepts two existing alignments, alignment1 and alignment2, and assumes a phylogenetic relationship shown in figure 1B. That is, alignment1 is assumed to form a monophyletic cluster, but alignment2 is not assumed to form a monophyletic cluster. The cluster of alignment1 can be placed in any phylogenetic position in the tree of alignment2. Moreover, this option checks whether alignment1 forms a monophyletic cluster. If not, it returns an error message and asks user to use the --add option (see the following section).

### Adding Unaligned Sequences into an MSA

As a result of advances in sequencing technologies, we increasingly need MSAs consisting of a larger number of sequences. There are several different approaches to enable construction of large MSAs, such as rapid algorithms and parallelization. Here, we describe an alternate approach: use of an existing alignment. There already exist databases of carefully aligned and annotated sequences (Cole et al. 2009; Sigrist et al. 2010; Punta et al. 2012), in which each MSA consists of a small number (typically up to ~1,000) of sequences. We can use such MSAs as a backbone to build a larger MSA containing newly sequenced data. This is more efficient than rebuilding the entire MSA from a set of ungapped sequences. Moreover, this approach is relatively robust to low-quality sequences resulting from sequencing errors, misassemblies, and other factors. Such noise usually has a negative effect on the quality of an MSA, but there are situations where biologically important information is contained in low-quality sequences. In such a case, we first select highly reliable sequences to build a backbone MSA, and then add the other sequences, including low-quality ones, into the MSA. As a result, the quality of the final MSA is less affected by the low-quality sequences.

### Inappropriate Applications of Profile Alignment

The mafft-profile program is not useful for this purpose. There are two types of misapplications. One is as follows: 1) convert an existing alignment to a profile, 2) align new sequences and convert them to a profile, and 3) align the two profiles. This procedure is inappropriate for adding new sequences because it assumes a phylogenetic relationship as illustrated in figure 1A.

Another misapplication is as follows: 1) convert the existing alignment to a profile, 2) separately align each new sequence to the profile of the existing alignment, and 3) construct a full alignment from the individual alignments computed in the previous step. This approach is more reasonable than the first one but still problematic, because the phylogenetic positions of new sequences are assumed at the root of the tree, as illustrated in figure 1C. Results of this procedure for two cases are shown in table 2 and figure 2.

### The --add and --addfragments Options

To overcome this limitation of profile alignment, in 2010, we implemented an option, --add, to add unaligned sequences to an existing MSA. This option assumes that each new sequence was derived from a branch in the tree of an existing alignment, as illustrated in figure 1D. This option works almost identically to the standard progressive method, except that the alignment calculation is skipped at the nodes whose children are all in the existing alignment.

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**Fig. 1.** Assumptions on the phylogenetic relationship in different options of MAFFT. (A) mafft-profile, (B) --addprofile, (C) misuse of mafft-profile, and (D) --add or --addprofile.
Along with popularization of second-generation sequencers, we sometimes need to align short reads to an existing alignment. Several tools (Berger and Stamatakis 2011; Löytynoja et al. 2012; Sun and Buhler 2012) for this purpose were developed between 2011 and 2012. A limitation of the --add option in MAFFT for this purpose was pointed out in Löytynoja et al. (2012). Thus, we implemented a new option, --addfragments, which does not consider the relationship among the sequences to be added. Details of the --add and --addfragments options are described in Katoh and Frith (2012).

**Test Case 1: Fungal Internal Transcribed Spacers Sequences**

Here, we discuss how the --addfragments option works, using an actual case. Internal transcribed spacers (ITSs) are spacer regions located between structural ribosomal RNAs. The structure of the rDNA region in a eukaryotic genome is 18S – ITS1 – 5.8S – ITS2 – 28S. Here, we use a data set consisting of ITS1 and ITS2 sequences obtained from environmental samples (Chen W, personal communication). Each sequence has either ITS1 or ITS2 region only, extracted from 454 pyrosequencing data using FungalITSextractor (Nilsson et al. 2010). In addition, several fungal genomic sequences that fully cover ITS1 + 5.8S rRNA + ITS2 are available from public databases.

Suppose a situation where we need an MSA of approximately 300 full-length sequences and approximately 5,000 ITS1 or ITS2 sequences. One possible solution is to build an entire MSA at once. The result of the default option (FFT-NS-2) of MAFFT is obviously incorrect, as shown in figure 2A. ITS1 and ITS2 regions are forced to be aligned correctly.

**Fig. 2.** ITS alignments by different options of MAFFT, displayed on Jalview (Waterhouse et al. 2009). (A, B) Incorrect alignments by the FFT-NS-2 and L-INS-i algorithms, respectively. (C) An incorrect alignment by mafft-profile. The full-length sequences were aligned with the L-INS-i algorithm and then each new sequence was separately added to the full-length alignment, using mafft-profile. (D) Reasonable alignment by a two-step strategy. The --6merpair --addfragments option was used at the second step. (E) Reordered version of D; sequences are ordered such that similar sequences are placed closely. All calculations were performed using 16 cores on a Linux PC with 2.67 GHz Intel Xeon E7-8837/256 GB RAM.
aligned to build a backbone MSA, and then the new ITS1 and a set of full-length sequences taken from databases are first consumed method, L-INS-i, is not always the most accurate appropriate to the problem of interest. The most time-consuming method, L-INS-i, is applied (CPU time = 98 h). The former option --addfragments option in E. In this alignment, ITS1 and ITS2 are identical that in fig. 2B). These misalignments are due to an incorrect assumption on phylogenetic placement of new sequences shown in figure 1C.

**Test Case 2: Bacterial SSU rRNA**

Another case is the 16S.B.ALL data set by Mirarab et al. (2012). It consists of an MSA of 13,822 bacterial SSU rRNA sequences, taken from the Gutell Comparative RNA Website (CRW) (Cannone et al. 2002) and 138,210 fragmentary sequences, which are originally included in the CRW alignment but ungapped and artificially truncated. In Katoh and Standley (2013), we used a subset (13,821 fragmentary sequences) prepared by Mirarab et al. (2012). In addition to this subset, here we use the full data set (138,210 fragmentary sequences), to examine the scalability. Suppose a situation where we already have a manually curated (or backbone) MSA and a newly determined set of many fragmentary sequences in a metagenomics project, and we need an entire MSA of them.

The first four lines in table 2 (case 1) show the performances of various options for such an analysis, with a relatively small data set (13,822 sequences in the existing one. The difficulty of this problem for standard approaches comes from the fact that ITS1 sequences and ITS2 sequences are not homologous to each other and most pairwise alignments are impossible. Because of these nonhomologous pairs, the distance matrix used for the guide tree calculation is not additive; the distances between ITS1 and full-length sequences and those between ITS2 and full-length sequences are close to zero, whereas the distances between ITS1 and ITS2 are quite large. In this situation, it is difficult for normal distance-based tree-building methods to give a reasonable tree. Moreover, in the alignment step, the objective function of the L-INS-i is affected by inappropriate pairwise alignment scores between ITS1 and ITS2. Such problems can be avoided by just ignoring the relationship between ITS1 and ITS2, as done in the --addfragments option.

In addition, a result of the second type of misuse of mafft-profile (discussed earlier) is shown in figure 2C. Some new sequences are correctly aligned but others are obviously incorrectly aligned (note that the order of sequences in fig. 2C is identical that in fig. 2D). These misalignments are due to an incorrect assumption on phylogenetic placement of new sequences shown in figure 1C.

### Table 2. Comparison of Different Options Using the 16S.B.ALL Data Set (Mirarab et al. 2012).

| Data Method                                         | Accuracy | CPU Time | Actual Time |
|-----------------------------------------------------|----------|----------|-------------|
| Case 1 mafft --multipair --addfragments frags existingmsa | 0.9969   | 6.67 days| 18.3 h      |
| mafft --6merpair --addfragments frags existingmsa     | 0.9949   | 3.76 h   | 36.2 min    |
| mafft --localpair --add frags existingmsa             | 0.9707   | 23.4 days| 2.43 min    |
| mafft --6merpair --add frags existingmsa              | 0.9604   | 1.32 h   | 1.44 h      |
| profile alignment                                    | 0.2779   | 15.5 h   | 1.60 h      |
| Case 2 mafft --6merpair --addfragments frags existingmsa | 0.9969   | 4.54 h   | 33.8 min    |
| mafft --6merpair --add frags existingmsa              | 0.9949   | 1.79 days| 5.91 h      |

Note.—The estimated alignments were compared with the CRW alignment to measure the accuracy (the number of correctly aligned letters/the number of aligned letters in the CRW alignment). Calculations were performed on a Linux PC with 2.67 GHz Intel Xeon E7-8837/256 GB RAM (for the other cases).

Case 1: 13,822 sequences in the existing alignment Case 2: 1,000 sequences in the existing alignment Case 3: 13,822 sequences in the existing alignment

138,210 fragments. In this alignment, ITS1 and ITS2 are quite large. In this situation, it is difficult for normal distance-based tree-building methods to give a reasonable tree. Moreover, in the alignment step, the objective function of the L-INS-i is affected by inappropriate pairwise alignment scores between ITS1 and ITS2. Such problems can be avoided by just ignoring the relationship between ITS1 and ITS2, as done in the --addfragments option.

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MAFFT Version 7 has an option for parallel processing, --thread (Katoh and Toh 2010). This feature is currently supported on Mac OS X in addition to Linux, but not yet supported on Windows for technical reasons. With the --thread n option, it runs in parallel with n threads. The number of threads can be automatically determined by --thread -1. This option sets the number of threads as the number of physical cores, not the number of logical cores in Intel’s hyperthreaded CPUs.

For progressive methods, the result with the multithread version is identical to that of the serial processing version. However, for iterative refinement methods, the results are not always identical. We confirmed that the accuracy of the parallel version in this case is comparable with that of the serial version (Katoh and Toh 2010). The efficiency of parallelization depends on the alignment strategy. In the case of the --addfragments option, the efficiency is acceptably high as shown in table 2.

**Utility Options**

MAFFT version 7 also has several enhanced options for peripheral functions.

**Estimating the Direction of DNA Sequences**

In the case of nucleotide alignments, if some of input sequences have an incorrect direction relative to the other sequences, the directions can be automatically adjusted by the --adjustdirection option. MAFFT cannot handle more complicated sequences with genomic rearrangements (translocations, duplications, or inversions). The web version of MAFFT displays dot plots between the first sequence and the remaining sequences, using the LAST local alignment program (Kielbasa et al. 2011), for every nucleotide alignment run. By viewing the dot plots, a user can easily check for genomic rearrangements and the directions of input sequences. See Katoh and Standley (2013) for details and an example.

**Input/Output**

MAFFT version 7 has several enhancements in the flexibility of input/output. The following options related to input/output are available and can be combined with other options.

--anysymbol If the input data include unusual letters, like U, J, etc., (in the case of protein data), MAFFT stops by default. The --anysymbol option allows these letters and nonalphabetical letters.

--preservecase By default, amino acid sequences are converted to upper case and nucleotide sequences are converted to lower case. This behavior can be changed by using the --preservecase option.

--reorder The order of sequences is the same as the input sequences by default, but the sequences can be sorted according to similarity to each other by the --reorder option.

--phylipout and --clustalout The output format is multi-fasta by default, but the phylip (interleaved) format and the clustal format can be selected.

**Guide Tree and Phylogenetic Positions of New Sequences**

Users can check the guide tree by using the --treeout option. In the case of --addfragments, the estimated phylogenetic positions of new sequences are shown together with the estimated tree of the existing alignment. The alignment calculation is performed based on this phylogenetic estimation. It is also possible to compute such phylogenetic information only, without alignment, by the --retree 0 option. An example of output is shown in Figure 3A.
Note that this phylogenetic information is roughly estimated before the MSA calculation, not based on the MSA. Especially, with the fast option, --6merpair, the estimation is very rough. With the --multipair option (default), the estimation is expected to be better, but it needs a relatively long computational time. For more rigorous estimation of phylogenetic positions of new sequences, specially designed tools, such as pplacer (Matsen et al. 2010), PaPaRa (Berger and Stamatakis 2011), PAGAN (Lo¨ytynoja et al. 2012), SEPP (Mirarab et al. 2012), or combinations of them including MAFFT, should be tried.

Parameters

For amino acid alignment, MAFFT uses the BLOSUM62 matrix by default. For nucleotide alignment, a 200PAM log-odds scoring matrix is generated assuming that the transition rate is twice the transversion rate. These matrices are suitable for aligning distantly related sequences. We selected these default parameters based on an expectation that, if the program works well for difficult (distantly related) cases, it should also work well for easy cases.

It is unclear whether this expectation is always correct. For example, in a benchmark using simulated protein sequences (Lo¨ytynoja et al. 2012) generated by INDELiBLE (Fletcher and Yang 2009), when we tested a more stringent scoring matrix, JTT IPAM (Jones et al. 1992) with weaker gap penalties than the default, the benchmark scores were considerably improved. Despite this observation, we consistently used the default parameters in the benchmark in Katoh and Frith (2012), because it does not make sense to arbitrarily adjust parameters to a simulation setting. This observation suggests that the current default parameters of MAFFT might not be very suitable for aligning closely related sequences. However, this idea must be checked using actual biological sequences.

User can select different scoring matrices other than the default. For amino acid alignment, --bl 45, --bl 62, --bl 80, --jtt N, and --tm N are accepted, where N is an expected evolutionary distance among input sequences. The --bl, --jtt, and --tm options mean BLOSUM (Henikoff S and Henikoff JG 1992), JTT (Jones et al. 1992), and a transmembrane model (Jones et al. 1994), respectively. A user-defined scoring matrix can also be accepted, by --aamatrix. For nucleotide alignments, --kimura N is accepted, where N is an expected evolutionary distance among input sequences. Gap penalties can be adjusted by --op, --exp, --lop, and --lexp options.

One possible extension is to use different scoring matrices and gap penalties for different sequence pairs according to the divergence level, like ClustalW (Thompson et al. 1994). More studies using actual sequence data will be necessary before implementing this extension. It will also be necessary to adjust gap penalties, preferably based on a realistic evolutionary model of insertions and deletions.

Use of Structural Information

We have discussed possible improvements in MSAs of closely related sequences in the previous section. MSA of distantly related sequences is still a challenging problem.
Test Case 3: PIN Domain

Figure 4 shows a typical limitation of sequence level alignment for a highly divergent set of three PIN-domain containing proteins: human regnase-1, VPA0982 from Vibrio parahaemolyticus, nuclease domain of taq polymerase from Thermus aquaticus. These three proteins share a magnesium-binding site composed of three conserved aspartic acids. Figure 4A shows a superposition of the three structures (Protein Databank identifiers 3v33, 2qip, and 1taq, respectively). The middle aspartic acid is indicated by sphere-representation, colored red. In Figure 4B, a typical MSA (by MAFFT-L-INS-i) is shown wherein the middle aspartic acid position is misaligned. In Figure 4C, a structure-informed MSA with correctly aligned Ds; Alpha helices and beta sheets are shown in blue and yellow, respectively, in (A–C).

Strategy for Integrating Structural Alignments and MAFFT

It has long been known that structural information can be used to improve MSA calculations. This was the basis of the 3D Coffee program (O’Sullivan et al. 2004), and later the PROMAL3D package (Pei et al. 2008). Here, we address incorporation of protein structural information in MAFFT-based MSA construction. There are both conceptual issues and technical issues that complicate the process. Conceptually, we have to define structural similarity in such a way that it can easily be used in sequence alignments. We discuss our approach to this problem below in the context of integrating MAFFT with the structural alignment program ASH (Standley et al. 2004, 2007). On the technical level, structural information complicates matters simply because protein structures contain more information and more noise than sequence information.

A particular element in the structural similarity matrix takes the form of a Gaussian-shaped function of the inter-residue distance

$$e_{ij} = \exp\left(-(d_{ij}/d_0)^2\right),$$

where $d_{ij}$ is the distance between two alpha carbons $i$ and $j$ in the two input structures and $d_0$ is a parameter that defines tolerance in the score. The default behavior is to set $d_0$ to 4 Å.

The goal of ASH is to maximize the sum of $e_{ij}$ over aligned residues. The residue-level equivalences, which form the basis of all ASH alignments, provide a convenient route for combining MAFFT and ASH. We can, for example, set a threshold value of $e_{ij}$ and incorporate highly confident parts of the alignment into MAFFT to “seed” the MSA calculation. If we consider the case of the three PIN domain-containing structures in Figure 4, we can first compute structural alignments for the three unique pairs using ASH (ash_3v33A-2qipA, ash_3v33A-1taqA, and ash_2qipA-1taqA). If we set a threshold for residue equivalence at $\geq 0.5$, we can then combine the equivalence-filtered alignments into MAFFT using the seed option (Katoh et al. 2009):

```
mafft-linsi --seed ash_3v33A-2qipA
--seed ash_3v33A-1taqA
--seed ash_2qipA-1taqA
sequences > output
```

Because the sequence identities between the aligned structures are low, we see an improvement in the resulting MSA relative to conventional MAFFT (Fig. 4). Based on this approach, we are developing an integrative service for protein structure-informed MSA construction.

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