Multiple Sequence Alignment System for Pyrosequencing Reads

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Abstract. Pyrosequencing is among the emerging sequencing techniques, capable of generating upto 100,000 overlapping reads in a single run. This technique is much faster and cheaper than the existing state of the art sequencing technique such as Sanger. However, the reads generated by pyrosequencing are short in size and contain numerous errors. Furthermore, each read has a specific position in the reference genome. In order to use these reads for any subsequent analysis, the reads must be aligned . Existing multiple sequence alignment methods cannot be used as they do not take into account the specific positions of the sequences with respect to the genome, and are highly inefficient for large number of sequences. Therefore, the common practice has been to use either simple pairwise alignment despite its poor accuracy for error prone pyroreads, or use computationally expensive techniques based on sequential gap propagation. In this paper, we develop a computationally efficient method based on domain decomposition, referred to as pyro-align, to align such large number of reads. The proposed alignment algorithm accurately aligns the erroneous reads in a short period of time, which is orders of magnitude faster than any existing method. The accuracy of the alignment is confirmed from the consensus obtained from the multiple alignments.

1 Introduction

Pyrosequencing is among the emerging sequencing techniques developed for determining the sequences of DNA bases from a genome. It is capable of generating up to 100,000 overlapping reads in a single run. However, multitude of factors, such as relatively short read lengths (i.e., as of 2008 an average of 100 - 250 nt compared to 800 - 1000 nt for Sanger sequencing), lack of a paired end protocol, and limited accuracy of individual reads for repetitive DNA, particularly in the case of monopolymer repeats, present many computational challenges [14] to make pyrosequencing useful for biology and bioinformatics applications.

For over more than a decade, Sanger sequencing has been the cornerstone of genome sequencing including that of microbial genomes. Improvements in DNA sequencing techniques and the advances in data storage and analysis, as well as developments in bioinformatics have reduced the cost to a mere 8000\$ - 10000\$ per megabase of high quality genome draft sequence. However, the need of more efficient and cost effective

approaches has led to development of new sequencing technologies such as the 454 GS20 sequencing platform. It is a non-cloning pyrosequencing based platform that is several orders of magnitude faster than the Sanger machines. However, the new technology despite its enormous advantage in terms of time and money will not be able to replace the current Sanger technology, unless the reads generated are properly aligned with respect to the reference genome.

The key issues associated with the use of pyrosequencing technique are as under:

Read Length: The read length is expected to be of the order of 100 - 250bp on average. This is much shorter than the other state of the art Sanger machines which give out consistent read lengths of the order of > 800 - 900bp.

Orientation: This is generally the case for most of the sequencing technologies. Each DNA helix will be broken into the original and its Watson-Crick complement. These would be further broken up into pieces, and there is generally no way to reveal which of the two is it. The problem is more severe and usually encountered for genome reconstruction.

Errors: Each individual DNA sequence or read is likely to have errors in the form of insertions and deletions. It may also have mutations and the pyrosequencer may itself make errors. These errors correspond to homopolymer effects, including extension (insertions), incomplete extensions (deletions), and carry forward errors (insertions and substitutions). Insertions are considered the most common type of error (36% of errors) followed by deletions (27%), ambiguous bases, Ns (21%), and substitutions (16%) [28].



Fig. 1. Pairwise alignment of the reads with the reference genome is shown

For most practical purposes, pyroreads without any post processing are of limited use. One of the most widely required tasks as a pre processing step for many applications, including haplotype reconstruction [12] [13], analysis of microbial community analysis [3], analysis of genes for diseases [2], is the alignment of these reads with the

wild type. For important applications such as viral population estimation or haplotype reconstruction of various viruses e.g., HIV in a population, scientists usually have the information about the wild type genome of the virus. While for other sequencing technologies, such as Sanger, simple pair-wise alignment with the wild type may produce reasonable multiple alignment, in the case of pyrosequencing, the variation in the haplotype population compounded with the errors introduced in the reads does not allow feasible multiple alignment by simple pair-wise alignment. Fig. 1 depicts simple pairwise alignment of pyrosequence reads with a reference genome. We assert that accurate and workable multiple alignment is often necessary for a variety of applications and statistical packages to work with these pyroreads, as demonstrated in [12] [13] [3] [2].

In theory, alignment of multiple sequences can be achieved using pair-wise alignment, each pair getting alignment score. But for optimal alignment the sum of all the pair-wise alignment scores need to be maximized, which is an NP complete problem [15]. Towards this end, dynamic programming based solutions of $O(L^N)$ complexity have been pursued, where N is the number of sequences and L is the average length of a sequence. Such accurate optimizations are not practical for large number of sequences -as is the case in pyrosequencing-, thus making heuristic algorithms as the only feasible option. The literature on these heuristics is vast and includes widely used works, such as Notredame et. al. [16], Edgar [18], Thompson et. al. [17], Do et. al. [22], and Morgenstern et.al. [20]. These heuristics are complex combination of ad-hoc procedures with some flavor of dynamic programming. Despite the usefulness of these widely used heuristics, they scale very poorly with increasing number of sequences.

For multiple alignment of pyroreads, 'out of the box' use of these heuristics is not feasible because of two main reasons: 1) the pyrosequencing reads can be very large in number (up to 100,000 usable reads in a single run (with a Roche GS20 platform), and 2) the heuristics do not take into account the positions of the reads with respect to the reference genome. Additional factors such as short lengths and errors, and the fact that these reads have preceding or trailing 'gaps' pose further alignment challenges. In [12], an alignment technique based on sequential gap propagation has been used. This technique is computationally expensive and its alignment quality decreases with the increase in the mutation value.

In this paper, we present a computationally efficient algorithm *pyro-align*, specifically designed for multiple alignment of DNA reads obtained from pyrosequencing. The proposed algorithm is based on a novel domain decomposition concept, therefore it is capable of aligning very large number of pyrosequences. It takes into account the position of the reads with respect to the reference genome, and assigns weight to the leading and trailing gaps for the reads.

The objective of our work is to develop a multiple alignment system for small error prone reads, such that the errors in the alignment are 'highlighted' and the system is able to handle large number of reads, as may be expected from pyrosequencing reads.

We assume that the reads may be generated from one or many genomes, with 'forward' orientation. We also assume that the reference genome (or its wild type) from which the reads are generated is available, as is generally the case for haplotype reconstruction. In our experiments, we have used HIV-pol gene virus as the reference genome (with length of 1970bp) and simulator Readsim [11] to generate these reads.

The algorithm uses concepts from domain decomposition and parallel multiple alignment techniques [1,21].

For the sake of completeness, let's first formally define the Multiple Sequences Alignment problem in its generic form, without indulging with the issues such as scoring functions. Let N sequences be presented as a set $S = \{S_1, S_2, S_3, \dots, S_N\}$ and let $S' = \{S'_1, S'_2, S'_3, \dots, S'_N\}$ be the aligned sequence set, such that all the sequences in S' are of equal length, have maximum overlap, and the score of the global map is maximum according to some scoring mechanism suitable for the application.

A perfect multiple alignment for pyroreads would be, that the reads are aligned with each other such that the position of the reads with respect to the reference genome is conserved; the reads have maximum overlap and are of equal lengths after the alignment, including leading and trailing gaps.

The intuitive idea behind the proposed *pyro-align* algorithm is to first place the reads in correct orientation with respect to the reference genome and then use progressive alignment to achieve the final alignment. For efficient progressive alignment, the correctly placed reads are reordered according to the starting position, and a computationally low complexity similarity metric is extracted from this ordering position. The similarity metric is then used to align pairs of aligned reads using a hierarchical decomposition strategy. The proposed multiple alignment algorithm takes advantage of the pyroreads characteristics and brings in techniques from data structures and parallel computing to realize a low complexity solution in terms of time and memory.

The proposed alignment algorithm, *pyro-align*, consists of the following two main components:

- 1. Semi-Global alignment
- 2. Hierarchical progressive alignment
 - (a) Reordering of reads to generate guidance tree
 - (b) Pairwise and profile-profile alignment

Each component is designed considering the characteristics of pyroreads and it is described in the following sections along with its justification.

1.1 Semi-Global Alignment

The first step is to determine the position of each read with respect to the reference genome. If this step is omitted, there are number of alignments that would be correct, but would be inaccurate if analyzed in the global context. A read that is not constricted in terms of position, may give the same score (SP score) for the multiple alignment but would be incorrect in context of the reference. To accomplish the task of 'placing' the reads in the correct context with respect to the reference genome we employ semi-global alignment procedure.

The semi global alignment is also referred to as overlapping alignment because the sequences are globally aligned ignoring the start and end gaps. For semi-global alignment we use a modified version of Needleman-Wunsch algorithm [5].

The modification in the basic version of Needleman-Wunsch is required to handle the leading and trailing gaps of the reads when aligning to the reference genome. If the leading and trailing gaps are not ignored, considering the short length of the reads, the alignment scores would be dominated by these gaps, hence giving an inaccurate alignment with respect to the genome.

Let the two sequences to be aligned be s and t, and M(i, j) presents the score of the optimal alignment. Since, we do not wish to penalize the starting gaps, we modify the dynamic programming matrix by initializing the first row and first column to be zero. The gaps at the end are also not to be penalized. Let M(i, j) represent the optimal score of s_1, \dots, s_i and t_1, \dots, t_j . Then M(m, j) is the score that represents optimally aligning s with $t_{1,\dots,j}$. The optimal alignment therefore, is now detected as the maximum value on the last row or column. Therefore the best score is $M(i, j) = max_{k,l}(M(k, n), M(m, l))$, and the alignment can be obtained by tracking the path from M(i, j) to M(0, 0). For additional details on semi-global alignment we refer the reader to [8].

Once each read has been semi-globally aligned with the reference genome, we obtain reads with leading and trailing gaps, where the first character after the gaps is the starting position of the read with respect to the reference genome. The information for these alignments are stored in hashtables that are further used for processing in reordering the reads for alignment.

2 Hierarchical Progressive Alignment

Generally multiple sequence alignment (MSA) procedures are either based on iterative methods or employ progressive techniques. Although, progressive techniques relative to iterative techniques are more efficient, they are not suitable when the sequences are relatively diverse or the number of sequences is very large. Considering the fact that the pyroreads are highly similar, we develop a hierarchical progressive alignment procedure that is also computationally efficient for large number of reads.

Progressive alignment techniques develop final MSA by combining pair-wise alignments beginning with the most similar pair and progressing to the most distantly related. All progressive alignment methods require two stages: a first stage in which the relationships between the sequences are represented as a tree, called a guide tree, and a second step in which MSA is built by adding the sequences sequentially to the growing MSA according to the guide tree. In the following, we describe the low complexity components of *pyro-align*.

2.1 Reordering Reads

The method followed by most of the progressive multiple alignment algorithms is that a quick similarity measure is computed that is based on k-mer counting [4] or some other heuristic mechanism. These pair-wise similarity measures (distances) are tabulated in a matrix form and a tree is constructed from this distance matrix using UPGMA or neighboring joining. The progressive alignment is thus built, following the branching order of the tree, giving a multiple alignment. These steps require $O(N^2)$ time each, where N is the number of reads. To reduce this complexity, we exploit the fact that the reads are coming from the same reference or nearly the same reference. This in turn implies

that the reads starting from the same or near same 'starting' point with respect to the reference genome are likely to be similar to each other. Therefore, we already have the ordering information or the 'guide tree' from the first step of the algorithm. Our guide tree, or the order in which sequences will be aligned in the progressive alignment is from the starting position of the reads from the first stage. Of course the decomposition of the reads (the subtree of the profiles that we built) doesn't render the reads in the same order as in traditional progressive alignment, but nevertheless the order is more or less the same when the profiles of these reads are aligned.

Let there be N number of reads $R = R_1, R_2, \dots, R_N$ generated from pyrosequencing technique, from the reference genome of length L_g . Also, let the length of each read denoted by $L(R)_p$. After executing semi-global alignment using the algorithm discussed in the previous section, let each read be presented by R_{pq} , where the p^{th} read has q leading gaps and $L_g - q - L(R)_p$ trailing gaps. Then the reordering algorithm would reorder the reads such that after the reads are reordered using the information from the leading gaps, the read R_{pq} comes in ordering 'before' $R_{p(q+1)}, \forall p, q \in L_g$.

To execute the reordering in an efficient manner, we employ hashtables that speed up the search process. We create two hashtables: $hashtable_1$ uses fasta sequence tag as the hash key and stores the corresponding starting position of the read; $hashtable_2$ stores the read names (fasta sequence tag) and the dna sequence it is associated with. Using these tables, the reads are reordered in the database in linear time.

2.2 Pair-wise and Profile-Profile Alignments

The ordering of the reads determined in the preceding step is now used to conduct the progressive alignment. Traditional progressive alignment requires that the sequences most similar to each other are aligned first. Thereafter, sequences are added one by one to the multiple alignments determined according to some similarity metric. This sequential addition of sequences for progressive alignment is not suitable for large number of sequences. In order to devise a low complexity system, we design a hierarchical progressive alignment procedure that is based on domain decomposition [1], as described below and depicted in Figure 2.

First of all, pair-wise local alignment using standard Needle-Wunsch is executed on each overlapping pair of reads (the ordering is still the same as discussed in the previous section). After this stage, the reads are aligned in pairs such that we have N/2pairs of aligned reads. These N/2 pairs of reads are then used for profile alignments as discussed below.

Profile-profile alignments are used to re-align two or more existing alignments(in our case the pairs of aligned reads). It is useful for two reasons; one being that the user may want to add sequences gradually, and second being that the user may want to keep one high quality profile fixed and keep on adding sequences aligned to that fixed profile [17].

We take advantage of both of these properties in our domain decomposition.

In this stage of the algorithm, the N/2 pairs of aligned reads have to be combined to get a multiple alignment. We have shown in [21] that the decomposition of the profiles gives a fair amount of time advantages even on a single processor. Therefore a hierarchical model similar to [1] is implemented (see Fig. 2). The model requires that instead



Fig. 2. Hierchical profile-profile alignments for pyro-align is shown

of combining the profiles in a sequential manner (one by one), a binary tree is built such that the profiles to be aligned are the leafs of the tree.



Fig. 3. Two profiles(X and Y) are aligned under the columns constrains, producing profile Z

In order to apply pair-wise alignment functions to profiles, a scoring function must be defined, similar to the substitution methods defined for pair-wise alignments. One of the most commonly used profile functions is the sequence-weighted sum of substitution matrix scores for each pair of amino acid letters. Let *i* and *j* be the amino acid, p_i the background probability of *i*, p_{ij} the joint probability of *i* and *j* aligned to each other, S_{ij} the substitution matrix being used, f_i^x the observed frequency of *i* in column *x* of the first profile, x_G the observed frequency of gaps in that column. The same attributes are assumed for the profile *y*. Profile sum of pairs (PSP) is the function used in Clustalw [17], Mafft [23] and Muscle [19] to maximize Sum of Pairs(SP) score, which in turn maximizes the alignment score such that the columns in the profiles are preserved, as depicted in Fig. 3.The PSP score can be defined as in [24] and [19]:

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$$S_{ij} = \log(p_{ij}/p_i p_j) \tag{1}$$

$$PSP^{xy} = \sum_{i} \sum_{j} f_i^x f_j^y log(p_{ij}/p_i p_j)$$
(2)



Fig. 4. The final Alignment of the reads

For our purposes, we will take advantage of PSP functions based on 200 PAM matrix [25] and the 240 PAM VTML matrix [26]. Some multiple alignment methods implement different scoring functions such as Log expectation (LE) functions, but for our purposes PSP scoring suffices. Profile functions have evolved to be quite complex and good discussion on these can be found at [19] and [27]. We use the profile functions from the clustalw system. The final alignment from the *pyro-align* algorithm can be seen in Fig. 4. Different steps of the proposed *pyro-align* Algorithm are outlined below.

Input: Reads generated from pyrosequencing procedure and Reference Genome **Output**: A Multiple Alignment of Reads is returned //Calculate overlapping of each of the reads with respect to the reference Genome **for** $(i = 1; i \le N; i + +)$ **do** | Overlapped-Reads \leftarrow Semi-Global-Alignment(R_i ,Genome); **end**

 $Reordered-Reads \leftarrow Reordering(Overlapped-Reads);$

//Pairwise alignment using standard Needle-Wunsch is exectued, for pairs of ordered reads ;

 $Pair-wise-aligned \leftarrow Needle-Wunsch(Reordered-Reads);$

//Profile-profile alignment is obtained using Sample-align-D strategy

 $Final-Alignment \leftarrow Profile-Profile-alignment(Pair-wise-aligned);$

return Final-Alignment;

Algorithm 1: Steps of the Proposed Multiple Sequence Alignment *pyro-align* Algorithm

3 Performance Analysis

As discussed earlier in the paper, the exact solution for multiple alignment is not feasible and heuristics are employed. Most of these heuristics perform well in practice but there is generally no theoretical justification possible for these heuristics [9]. For pyro-align it can be shown that the semi-global alignment of the reads with the reference genome is analogous to center star alignment. The center star alignment is shown to give results within 2-approx of the optimal alignment [9] in worst case and same can be expected from the semi-global alignment of reads with reference genome. The accuracy of the later stages is confirmed by rigorous quality assessment procedure described in the section below.

3.1 Experimental Setup and Quality Assessment

The performance evaluation of the algorithm has been carried on a single desktop system 2x QuadCore Intel 5355 2.66 GHz, 2x4 MB Cache and 16GB of RAM. The operating system on the desktop is RedHat Linux with kernel 2.6.18-92.1.13.el5. The software uses libraries from Biojava [7] and is built using java version "1.6.0" Java(TM) SE Runtime Environment,IBM J9 VM.

To investigate the quality of the alignment produced by the algorithm we used Readsim simulator [11] to generate the reads. The quality assessment of multiple alignment is generally carried out using benchmarks such Prefab [18] or BaliBase [6]. However, these benchmarks are not designed to access the quality of the aligned reads produced from pyrosequencing, and there are no benchmarks available specifically for these reads. Therefore, a system has to be developed to access the quality of the aligned reads. The experimental setup for the quality assessment of the alignment procedure is shown in the Fig. 5 and is explained below.

Our quality assessment have two objectives: (1) to assess the quality of the alignment produced by pyro-align with respect to the original genome (2) ensure that the system must be able to handle reads from multiple haplotype for alignment.

To achieve these objectives, we setup the quality assessment system as shown Fig. 5. We used a HIV pol gene virus with length of 1970bp as the wildtype for the experiments. The wildtype is then used to produce 4 sets of genomes, randomly mutated at different rate; The four sets of genomes are Dist-003, Dist-005, Dist-007 and Dist-010, with mutations of 3%, 5%, 7% and 10%, respectively. Now using the mutated genomes, 2000 and 5000 reads from the Readsim were generated using standard ReadSim parameters with forward orientation.

The generated reads from these mutated genomes were then aligned with the wildtype. This procedure is adopted because generally scientists only have a wildtype of the microbial genomes available and therefore it depicts a more practical scenario.

After the alignment, a majority consensus of the reads is obtained. A distance based similarity is then calculated of the consensus obtained from the aligned reads with the original genome from which the reads were generated. The results of the alignment obtained and the accuracy of the consensus thus obtained are shown in Fig. 6 and Fig. 7 for 2000 and 5000 reads respectively.



Fig. 5. The experimental setup for the quality assessment of the multiple alignment program

We compare the accuracy of the algorithm with two different methods. First being the simple pair-wise alignment of the reads with the reference genome. Secondly, we compare it with a sequential gap propagation method, used in recent pyrosequencing systems [12]. Simply put, gap propagation method builds multiple alignment from pairwise alignments by sequentially 'propagating' the gaps from each pairwise alignment to all the reads in the system. Propagation of gaps is accomplished for every position where at least one read has an inserted base. A gap is inserted in the reference genome and, consequently, in all reads that overlap the genome at that position. The complexity of the procedure is of the order of $O(N^2)$.

The accuracy of the consensus obtained using just the pairwise alignment is less than 55% and that obtained from the pyro-align is always greater than 96%. An even better alignment quality is achieved for greater number of reads, because more number of reads provide a better coverage for a genome of given length. The accuracy of the gap propagation procedure, is comparable to pyro-align for small mutations, but as the mutations increase the accuracy of gap propagation based method decreases.

To illustrate that the alignment system also works with a 'mixture' of reads from different haplotype, we use the mutated reads from Dist-003, Dist-005 and Dist-007 to generate a new set of reads. The new set contains equal number of reads from the mutated sets e.g. 2000 reads from each mutated genome for the results shown. The reads are then aligned by the pyro-align algorithm using wildtype as the reference genome. The results of alignment for this mixture set are shown in Fig. 8 for Dist-003/Dist-005 and Dist-005/Dist-007 mixtures. It must be noted here that we don't have a 'ground



Fig. 6. The quality of the alignment using pairwise, pyro-Align and 'propagation' methods for 2000 reads

truth' genome in these cases and hence no genome is available to compare the consensus obtained from the alignment.

However, we do know the mutation rates for the genomes from which the mixture sets were generated. Therefore, if an optimal alignment of these reads is obtained, the 'mutation' in the consensus should not be greater than the combined mutations of the genomes. For example consider the case of Dist-003/Dist-005 mixture. We know the mutation rates for the genomes from which the reads generated were 3% and 5% with respect to the wildtype. Therefore, for accurate alignment, the consensus of the alignment should not vary more than 8%, in the worst case, when compared to the wildtype. Same would be true for the other cases considered according to the mutation rates of the genomes. As can be seen that the results of the alignment compared with the wildtype are well within the expected limits. The accuracy of the pairwise alignment of the reads with the reference genome(in this case the wildtype), and that obtained using propagation method is also shown for comparison.

4 Complexity Analysis

In this section we briefly outline the complexity of the proposed pyro-align algorithm. Recall the pyro-align algorithm consists of these major steps: semi-global alignment, reordering, pair-wise alignment, and profile-profile alignment.

We assume that the number of reads is N with the average length of the read equal to L_R . Let's further assume that the length of the reference genome is equal to L_g . Then, the complexity of the semi-global alignment (overlapping alignment) is equal to $O(NL_RL_g)$. The clustering of the reads can be done in $O(NL_g)$ and the reordering using hashtables can be achieved in O(N), making the total for this stage equal to $O(NL_g+N)$. The pairwise alignment of the reads is shown to be achieved in $O(NL_g^2)$



Fig. 7. The quality of the alignment using pairwise, pyro-Align and 'propagation' methods for 5000 reads

and the profile-profile alignment can be achieved in $O(NlogN \times L_g^2)$. This makes the total complexity equal to $O(NL_RL_g + NL_g + N + NL_R^2 + NlogN \times L_g^2)$. This is asymptotically equal to $O(NlogN \times L_g^2 + NL_R^2)$. The advantage of low complexity of pyro-align was further evident by our experimentation. We were able to align 2000 reads of average length 250bp from a genome of length 1970bp in about 12 minutes compared to 6 hours of computation using more traditional multiple alignment systems such as Clustalw.

5 Conclusion

The short reads from the pyrosequencing method are rendered useless if they are not multiple aligned for magnitude of important applications, such as haplotype reconstruction and error elimination. We have presented an efficient hierarchical procedure to multiple align large number of short reads from the pyrosequencing procedure.

We demonstrated that simple-pair-wise alignment is not feasible in the case of pyroreads. We also showed that the proposed method is much faster than traditional time consuming multiple alignment methods such as Clustalw or Tcoffee. We also presented the quality assessment results and compared those with the results obtained by simple pair-wise alignment procedure and 'propagation' methods.

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Fig. 8. The quality of the alignment using pairwise, pyro-Align and 'propagation' methods for mixed genome reads

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