

Hidden Markov Models in Bioinformatics

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1 Introduction

The stochastic models known as Hidden Markov models (HMMs) are popular tools in the field of Bioinformatics. They are versatile models that can be used for many different purposes. Some of the problems that can be solved with different variants of HMMs are pairwise sequence alignment (PSA), multiple sequence alignment (MSA), protein structure prediction, protein function prediction, segmentation and protein homology detection. Note that this is by no means an exhaustive list, but only a few examples of problems where HMMs have been successfully applied.

2 Objectives

The main objective of this report is to explain why HMMs are widely used in Bioinformatics. It will explain and highlight why they are popular alternative tools for solving a wide range of problems in the field. In order to understand why HMMs are useful, and why they can be used effectively, some background on stochastic processes and Markov Chains is needed. Moreover, for completeness, a brief theoretical construct of some different HMMs will be done, alongside the most important algorithms for working with the models in practice. The experienced reader may skip these sections. In the remaining part of the report, a range of problems in Bioinformatics will be presented, first solved without the use of HMMs, then contrasted with the HMM based solutions. This will hopefully highlight why HMMs are effective alternatives. Finally, some practical examples in the open-source programming language R will be developed. Note that this report is by no means exhaustive; there exists a plethora of theory and problems where the HMMs can be used.

3 Theoretical Background

Basic knowledge in statistics and probability theory is assumed. Any introductory book on probability and statistics will do, but a quick refresher can be found in the two first chapters of [7].

3.1 Markov Chains

A Markov Chain (MC) $\mathbf{X} = \{X_t\}$ is a stochastic process which is characterized by one important property; given the value of X_t , the values of X_b , $b > t$ are not influenced by the values of X_a , $a < t$, where $a, b \in \mathbb{Z}$ decide what *order* of MC we are working with. Choosing $b = t + 1$ and $a = t - 1$ yields the type of *Markov property* we will be concentrating on in this case, which is referred to as a *first-order* Markov property. More precisely, we have that

$$P(X_{t+1} = j | X_0 = i_0, X_1 = i_1, \dots, X_{t-1} = i_{t-1}, X_t = i) = P(X_{t+1} = j | X_t = i) =: P_{ij}^{t,t+1},$$

where the superscripts highlight an important point; the *one-step transition probability* does not only depend on the state, but also on the time when the transition occurs. In our case, we will concentrate on *time-homogeneous* MCs, which means that the transition probabilities are stationary in time. Thus, we drop the superscripts in the notation and denote the transition probabilities as

$$P_{ij} = P(X_{t+1} = j | X_t = i).$$

In a MC, the random variable X can only take values from a pre-specified set called *states*. We will concentrate on the simplest type of state space, which yields a so-called *discrete-time* MC. This is the case when the set of states is finite or countable set and whose time index is $T = (0, 1, 2, \dots)$. Thus, we have described the assumptions made when working with the simplest form of MC, which we call a *discrete-state time-homogeneous first-order MC* [7]. These lay the foundation of the Hidden Markov models that we will study in the remainder of the report.

Let us define the notation that will be used in the remainder. First of all, let the random vector $\mathbf{X} = (X_0, X_1, \dots, X_L)^T$ represent a MC of length $L + 1$. In the following we will always assume that \mathbf{X} is of finite length $L + 1$. A realization of the random vector will be denoted in lower case, i.e. $\mathbf{x} = (x_0, x_1, \dots, x_L)^T$, following standard notation in most probability or statistics courses. What exactly this means will become clear later. A discrete-time MC consists of a finite set of states, which will be denoted by $\mathcal{H} = \{h_0, \dots, h_m\}$, where $m + 1 = \text{card}(\mathcal{H})$ are the amount of states in the model. Why the letter \mathcal{H} is chosen will hopefully become clear when studying the Hidden Markov models. The MC transitions between each of the states in \mathcal{H} , based on the set of *transition probabilities*, which were denoted by P_{ij} above. In the following, the notation \mathcal{T}_{ij} will be adopted instead, for ease of recalling that they are transition probabilities. These are summarized in a $m \times m$ -matrix \mathcal{T} , where each position \mathcal{T}_{ij} represents the probability of a transition from state i to state j , where $i, j \in \mathcal{H}$. Written in mathematical notation

$$\mathcal{T}_{ij} = P(X_{t+1} = j | X_t = i), \quad i, j \in \mathcal{H}.$$

As noted, the term *time-homogeneous* refers to the fact that \mathcal{T} does not change as time goes on, i.e. as the MC is generated, but stays fixed during the entire simulation of the chain. In mathematical notation, this assumption means that

$$\mathcal{T}_{ij} = P(X_{t+1} = j | X_t = i), \quad i, j \in \mathcal{H}, \forall t \in [0, L].$$

Moreover, the term *first-order* refers to an important assumption in this model, which makes it possible to define \mathcal{T} as we did, which is that the transition probability from the previous state to the current state only depends on the previous state. For example, a time-homogeneous second-order MC would depend on the two previous states when transitioning to the current state, which makes the model more general, but also more complicated. Note that the initial state of a MC is determined based on the initial distribution of the states, commonly denoted by $\pi = (\pi(h_0), \dots, \pi(h_m))$. The assumptions make calculations of probabilities in the MCs very simple, since they are strong independence assumptions.

A MC is completely specified by π and \mathcal{T} (where \mathcal{H} is indirectly given in both these two quantities). Once these quantities are specified, one can make calculations concerning the MC. The probability of a realization $\mathbf{x} = (x_0, x_1, \dots, x_L)^T$ taking place can be calculated recursively as

$$\begin{aligned} P(X_0 = x_0, X_1 = x_1, \dots, X_L = x_L) &= P(X_0 = x_0, X_1 = x_1, \dots, X_{L-1} = x_{L-1}) \\ &\quad \cdot P(X_L = x_L | X_0 = x_0, X_1 = x_1, \dots, X_{L-1} = x_{L-1}) \\ &= P(X_0 = x_0, X_1 = x_1, \dots, X_{L-1} = x_{L-1}) \\ &\quad \cdot P(X_L = x_L | X_{L-1} = x_{L-1}) \\ &= P(X_0 = x_0, X_1 = x_1, \dots, X_{L-1} = x_{L-1}) \cdot \mathcal{T}_{x_{L-1}, x_L} \\ &= P(X_0 = x_0, X_1 = x_1, \dots, X_{L-2} = x_{L-2}) \cdot \mathcal{T}_{x_{L-2}, x_{L-1}} \cdot \mathcal{T}_{x_{L-1}, x_L} \\ &\quad \vdots \\ &= \pi(x_0) \cdot \mathcal{T}_{x_0, x_1} \cdot \dots \cdot \mathcal{T}_{x_{L-2}, x_{L-1}} \cdot \mathcal{T}_{x_{L-1}, x_L} \end{aligned}$$

where the second inequality holds because of the Markov property. Also, note that $\pi(x_0)$ refers to the probability that the initial state is x_0 . Hence, it is apparent that calculating the probability of a specific realization of the MC is very simple.

For a more rigorous treatment of stochastic processes and MCs, the reader is referred [7].

3.1.1 Markov Chain Topologies

The topology of a MC refers to which state transitions are permitted and prohibited, i.e. what the values in the transition probability matrix are. There exists many different ways of constructing MCs.

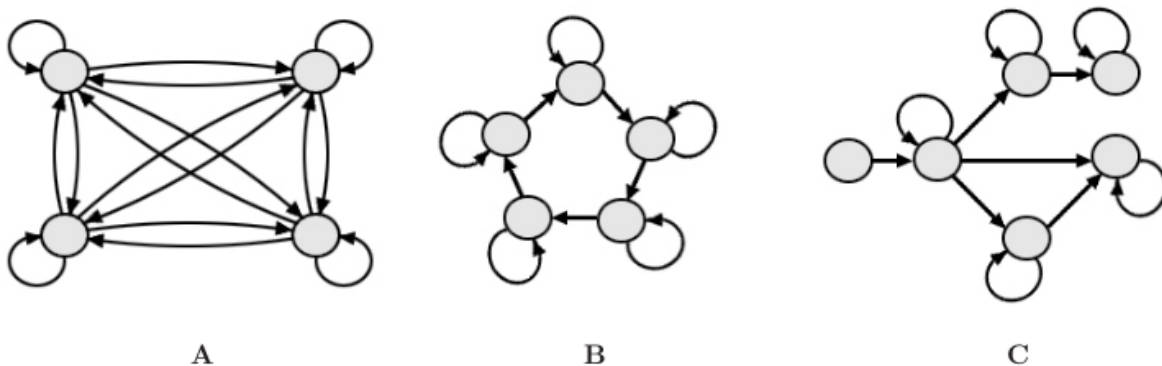


Figure 1: Some existing HMM topologies. **A.** a fully connected HMM; **B.** a circular HMM; **C.** a left-right HMM.

Figure 1: Examples of different MC topologies. Credit to Choo et al. for the figure [2].

Figure 1 gives some simple graphical explanations of these models. The figure is lent from [2], where more details about this fact can be found.

3.2 Hidden Markov Models

Yoon gives a very nice first introduction to Hidden Markov models (HMMs): "A hidden Markov model (HMM) is a statistical model that can be used to describe the evolution of observable events that depend on internal factors, which are not directly observable." [3]. A HMM, simply put, is a model that comprises of two different parts. The first part is the sequence or the result that can be observed. A possible observed sequence could be a sequence of nucleotides or it could be a chain of events, like 20 dice throws in a row. The second part is a MC, which is *hidden*, i.e. it cannot be observed. This very simple idea gives a very flexible model, which can be used for many different types of problems. The main goal is often to infer the characteristics of the hidden MC from the observed sequence.

In a HMM, a hidden sequence of states is generated, according to the MC. This sequence will not be observable and will in (almost) all practical cases be considered as unknown. Each of the states creates, or *emits*, an observed value. All these observed values are what make up the observed sequence. The emission follows a multinomial distribution, where each state follows such a distribution with different parameters. An important assumption for the HMM we will deal with is that the parameters of each multinomial only depends on the state to which it belongs [2]. This means that the emitted value only depends on its respective hidden state and not on other hidden states. A HMM thus represents a doubly stochastic process: the current underlying state in the MC is stochastic, with an added layer of stochasticity in the observed value. Note that each of the hidden states should be able to produce the same symbols, i.e. the same observable values, but in different frequencies [4].

Following the same notation as earlier, let \mathbf{X} be the random vector that represents the underlying MC of length $L + 1$, let \mathcal{T} be the transition probability matrix, let \mathcal{H} denote the hidden states, where $m = \text{card}(\mathcal{H})$, and let π be the initial distribution of states. In addition to this, we need to define some notation for the observable sequence in the HMM. First of all, let the random vector $\mathbf{Y} = (Y_0, Y_1, \dots, Y_L)^T$ represent an observable sequence of length $L + 1$. As earlier, $\mathbf{y} = (y_0, y_1, \dots, y_L)^T$ will represent a realization of \mathbf{Y} . Note that both the hidden and the observed sequence are of length $L + 1$, since it is assumed that each state emits exactly one symbol. The *symbol alphabet*, i.e. the set of symbols that can be emitted from each state, will be denoted by $\mathcal{S} = \{s_1, \dots, s_M\}$, where $M = \text{card}(\mathcal{S})$. The *emission probabilities*, i.e. the probabilities used in each multinomial distribution in each state, are usually arranged in a matrix as well. Denote by \mathcal{E} the emission probability matrix.

\mathcal{E} is of dimension $m \times M$, where each row in the matrix contains the emission probabilities (of each of the symbols) for a given state. Written in mathematical terms

$$\mathcal{E}_{ij} = P(Y_t = j | X_t = i), \quad i \in \mathcal{H}, j \in \mathcal{S}.$$

Time-homogeneity is also assumed in the stochastic process of emission, which means that these emission probabilities are the same $\forall t \in [0, L]$.

A HMM is completely specified by π , \mathcal{T} and \mathcal{E} , where the state space \mathcal{H} and the symbol space \mathcal{S} are indirectly determined by the other quantities [1]. Once these quantities are specified, one can make calculations concerning the HMM. As already noted, calculating the probability of a given realization \mathbf{x} is

$$P(\mathbf{X} = \mathbf{x}) = \pi(x_0) \prod_{t=2}^L \mathcal{T}_{x_{t-1}, x_t}. \quad (1)$$

In a similar fashion, the assumption of independence between states when generating symbols makes it possible to find the probability of the observed sequence simply by taking the product of all appropriate emission probabilities. In mathematical terms, the probability of seeing the sequence \mathbf{y} , given the state sequence \mathbf{x} , is

$$P(\mathbf{Y} = \mathbf{y} | \mathbf{X} = \mathbf{x}) = \prod_{t=1}^L \mathcal{E}_{x_t, y_t}. \quad (2)$$

Finally, combining these results, the joint probability of the sequences \mathbf{x} and \mathbf{y} is

$$P(\mathbf{X} = \mathbf{x}, \mathbf{Y} = \mathbf{y}) = P(\mathbf{X} = \mathbf{x})P(\mathbf{Y} = \mathbf{y} | \mathbf{X} = \mathbf{x}) = \pi(x_0) \mathcal{E}_{x_0, y_0} \prod_{t=2}^L \mathcal{E}_{x_t, y_t} \mathcal{T}_{x_{t-1}, x_t}. \quad (3)$$

Keep in mind that the products in equations (1), (2) and (3) usually become very small in practice. This can give numerical instability when computing the probabilities. A common trick to solve this is to work with the logarithm of the probabilities. In this way, the multiplicative properties become additive, which mitigates some problems that may occur in the calculations. This is especially important when implementing the algorithms that will be discussed later [4].

Note that we here assume that the hidden state sequence \mathbf{x} is known, which is usually not the case in practice. The state sequence needs to be inferred from the observed sequence, which is one of the main problems of a HMM, which will be explained in the following. For a more rigorous treatment of this topic, although in the topic of speech recognition, the reader is referred to [10].

3.3 Basic Problems for HMMs

As Yoon [3] and Rabiner [10] point out in their respective articles, there are three issues that need to be resolved in order to be able to use HMMs in practical applications. In the following, these issues will be presented, together with algorithms that are used to address the problems.

A given HMM will in the following be denoted by $\theta = (\pi, \mathcal{T}, \mathcal{E})$. This means that when we refer to a HMM θ , this means that the HMM is fully specified by the three parameters and that the parameters are known. Moreover, for ease of notation, $P(\mathbf{Y} = \mathbf{y})$ will be denoted as simply $P(\mathbf{y})$ in the following.

3.3.1 The Evaluation Problem

The first issue that needs to be addressed is: how can the probability $P(\mathbf{y} | \theta)$ be calculated? That is, how can one find the probability of observing the realization \mathbf{y} from a given HMM θ ? If the exact underlying state sequence was known, this would have been easily calculated using equation (3). However, the underlying state sequence cannot be observed, which means that there may exist

many different underlying state sequences that can yield the same observed sequence \mathbf{y} . Thus, the first solution that comes to mind would be to use the law of total probability to calculate

$$P(\mathbf{y}|\theta) = \sum_{\forall \mathbf{x}_i \in \mathcal{H}^n} P(\mathbf{y}, \mathbf{x}_i|\theta) = \sum_{\forall \mathbf{x}_i \in \mathcal{H}^n} P(\mathbf{x}_i|\theta)P(\mathbf{y}|\mathbf{x}_i, \theta), \quad (4)$$

where \mathcal{H}^n is the space of all possible state sequences that can yield \mathbf{y} . Of course, \mathcal{H}^n can be very large, which makes this calculation computationally infeasible. In fact, \mathcal{H}^n may consist of m^L sequences, since these are all the possible state sequences. Luckily, the *forward algorithm* exists, which uses dynamic programming to solve this issue in a computationally feasible manner. This algorithm has time complexity $\mathcal{O}(Lm^2)$, which is a significant improvement over the exponential solution by straightforward use of equation (4). The forward algorithm is given below.

Algorithm 1 Forward Algorithm

```

 $V \leftarrow \text{array}((m+1) \times (L+1))$ 
for  $i = 0, 1, \dots, m$  do
     $V(i, 0) \leftarrow \pi(h_i)\mathcal{E}_{h_i, y_0}$ 
end for
for  $i = 1, 2, \dots, L$  do
    for  $j = 0, 1, \dots, m$  do
         $V(j, i) \leftarrow \mathcal{E}_{h_j, y_i} \sum_{k=0}^m V(k, i-1)\mathcal{T}_{h_k, h_j}$ 
    end for
end for
return  $P(\mathbf{y}|\theta) \leftarrow \sum_{k=0}^m V(k, L)$ 

```

Note that the forward algorithm gives a *confidence measure* on the observed sequence - how confident are we that the observed sequence could have been generated by the model θ . This problem is also sometimes referred to as the *scoring problem*, since $P(\mathbf{y}|\theta)$ is a way to score a new observed sequence based on the given HMM [3]. If the calculated probability is lower than some "background distribution" of similar sequences, then the probability of the sequence being emitted by the HMM is low and one would conclude that the HMM is not a good model for the observed sequence. However, if the probability is high, then we can conclude that the HMM can be used in the situation. When this is verified, we tackle the *decoding problem*.

3.3.2 The Decoding Problem

The second issue that needs to be addressed is: what state path \mathbf{x} maximizes the probability of emitting the observed sequence \mathbf{y} , i.e. how can the *optimal state path* be found? This follows the principle of maximum likelihood, which says that we should choose the hidden state path that maximizes the likelihood of yielding the observed sequence. Our best bet when trying to infer the hidden state path from the observed sequence is exactly the one that gives the maximum probability of obtaining the observed sequence. In other words, the hidden state sequence that best explains the observed sequence is the optimal state path

$$\mathbf{x}^* = \arg \max_{\mathbf{x}} P(\mathbf{X}|\mathbf{y}, \theta). \quad (5)$$

Notice that this is equivalent to finding the state sequence that maximizes $P(\mathbf{X}, \mathbf{y}|\theta)$, because of the definition of conditional probability

$$P(\mathbf{X}|\mathbf{y}, \theta) = \frac{P(\mathbf{X}, \mathbf{y}|\theta)}{P(\mathbf{y}|\theta)}.$$

Again, the first solution that comes to mind would be to compare all possible state sequences m^L , and use equation (3), but this is still computationally infeasible in most cases. Therefore, the *Viterbi algorithm*, which also is based on dynamic programming, exists. The time complexity of the Viterbi algorithm is $\mathcal{O}(Lm^2)$, which is the same as for the forward algorithm. This is a large improvement over the exponential time-complexity of the straightforward solution. The Viterbi algorithm is given below.

Algorithm 2 Viterbi Algorithm

```

 $V \leftarrow \text{array}((m+1) \times (L+1))$ 
 $P \leftarrow \text{array}((m+1) \times (L+1))$ 
for  $i = 0, 1, \dots, m$  do
     $V(i, 0) \leftarrow \pi(h_i) \mathcal{E}_{h_i, y_0}$ 
     $P(i, 0) \leftarrow -1$ 
end for
for  $i = 1, 2, \dots, L$  do
    for  $j = 0, 1, \dots, m$  do
         $V(j, i) \leftarrow \mathcal{E}_{h_j, y_i} \max_{k=0,1,\dots,m} \{V(k, i-1) \mathcal{T}_{h_k, h_j}\}$ 
         $P(j, i) \leftarrow \arg \max_{k=0,1,\dots,m} \{V(k, i-1) \mathcal{T}_{h_k, h_j} \mathcal{E}_{h_j, y_i}\}$ 
    end for
end for
return  $P(\mathbf{X}|\mathbf{y}, \theta) \leftarrow \max_{k=0,1,\dots,m} V(k, L); x_L^* = \arg \max_{k=0,1,\dots,m} V(k, L)$ 

```

Note that \mathbf{x}^* is simple to find based on x_L^* ; the optimal path \mathbf{x}^* starts at x_L^* and backtracks all the way to x_0^* by using $P(\cdot, \cdot)$. The backtracking ends when the value -1 is found in P , i.e. when the beginning of the sequence has been reached.

Also notice that both the forward and the Viterbi algorithms can easily be log-transformed in order to gain greater numerical stability, by taking logarithms of all values and switching multiplications with additions.

An important point to make before moving forward is that both the evaluation problem and the decoding problem are related to whether or not an observed sequence can be reasonably modeled by means of a given HMM θ . But how can this model be found in the first place? This is what is often referred to as the *training problem*, which will be covered next.

3.3.3 The Training Problem

The third issue that needs to be addressed is: how can the HMM parameters be reasonably chosen based on a set of observed sequences? For example, we have a set of sequences $\chi = \{\mathbf{y}_1, \dots, \mathbf{y}_G\}$, where each \mathbf{y}_i , $i \in [1, G]$ is an observed sequence of symbols, that we want to represent with a HMM. The difficulty that needs to be solved is how the parameters of the HMM can be estimated based on the set χ . This is what is typically called the *training* or *learning* problem, analogously to the need for training any other machine learning model. There exists no optimal way to train the HMM from a limited number of finite observation sequences, but there exists algorithms that can find local maximums in the observation probability. Some examples are the Baum-Welch algorithm, standard gradient based methods from optimization or simulation with Monte Carlo expectation maximization (MCEM) [3]. For detailed treatments of how the training problem can be solved, the reader is referred to [10], [11] or [12].

3.4 HMM Variants

There exists many variants of what we will call the standard HMM. For completeness, the standard HMM will be specified first, before the most widely used types of HMMs in bioinformatics will be presented. Note that this list is by no means exhaustive.

3.4.1 Standard HMM

The *standard HMM* is the simplest form of a HMM, which has the most restricting assumptions. In fact, the assumptions described in section 3.2 all belong to what we will call the standard HMM. Concisely restated, the hidden MC is a discrete-state time-homogeneous first-order MC. Also, often one assumes that the MC is *ergodic*, which means that every state can be reached from any other state in a finite amount of states [10]. Moreover, each state in \mathcal{H} generates exactly one output and can emit every symbol in \mathcal{S} . Importantly, the emitted value from each states depends only on that respective state.

All the assumptions made in the simple model restricts the usefulness of the HMM. For example, standard HMMs do not deal well with correlations between residues, since they assume that each residue depends only on one underlying state [1]. Since the Markov property is assumed, an HMM has no way of "remembering" any distant correlation between the symbols in \mathcal{S} . Moreover, Choo et. al writes that "the linear nature of HMM also makes it difficult to capture higher-level information or correlations among amino acids" [2]. These are only some examples of how restricted the standard HMMs are.

Despite the fact that the strong assumptions of the standard HMM restrict the flexibility of the model, one can only imagine how the assumptions can be tweaked to create a new type of HMM. Some examples of how changes in the assumptions can mitigate some of the problems of the standard HMM are shown next.

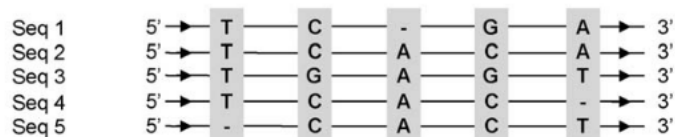
3.4.2 Profile-HMM

Profile-HMMs (pro-HMMs) have specific architectures, which make them suitable for modeling sequence profiles [3]. Because of this, in the context of bioinformatics, the topology of pro-HMMs are best explained with multiple sequence alignment in mind. Two simple ways to think of a pro-HMM is as an abstract description of a protein family or a statistical summary of multiple sequence alignment [4]. A pro-HMM is constructed to not contain any cycles. Moreover, a pro-HMM consists of three different types of hidden states: match states (M_k), insert states (I_k) and delete states (D_k). These are used to describe symbol frequencies, insertions and deletions, respectively, at each specific position in a sequence. A great example on how to build a pro-HMM, which helps to clarify the ideas, can be found in [3]. **Can I just steel this example? Yes, that is fine, not everything has to be original!** The illustration from the example is shown in 2.

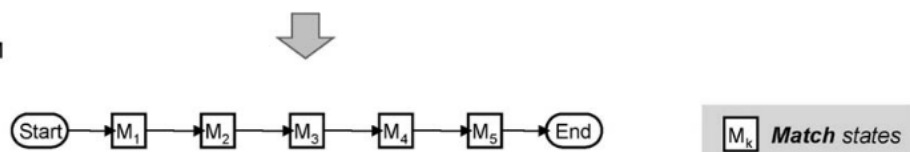
The example shows how a pro-HMM can be built from a multiple sequence alignment. The idea is that the pro-HMM can be used as a profile to classify new observations; either the new observation fits into the family of sequences that have been aligned, or it does not fit and should be aligned with some other profile. The M_k 's are used to indicate match states, i.e. they represent the case where a symbol in a new observation matches with the state of the pro-HMM. The emission probabilities from each match state are easily estimated by using the frequencies of each symbol in the match state. The ungapped HMM can represent ungapped sequences. The states I_k and D_k are added so that the HMM can represent sequences that contain gaps, which makes the pro-HMM a much more powerful model compared to the simpler models that describe a multiple sequence alignment, like regular expressions and PSSM, which we will come back to in section 4.3. It is important to note that the delete states D_k are silent states; they are placeholders for missing symbols in the consensus sequence of the alignment when compared to a shorter new observed sequence.

Note that the pro-HMM parameters can be set in two different ways [13]; The first option is to set the parameters from a pre-aligned set of sequences, simply by counting state transitions and emissions, before converting the counts to probabilities. This is how the parameters in the previous example would be set. The second option is to use a set of sequences that is not aligned before training the pro-HMM. This problem is thus analogous to running a multiple sequence alignment before building the model. In this case, algorithms mentioned in section 3.3.3 are used. Since these algorithms are only local optimizers it is always advisable to build HMMs on pre-aligned data when this is possible

(a) Sequence Alignment



(b) Ungapped HMM



(c) Profile-HMM

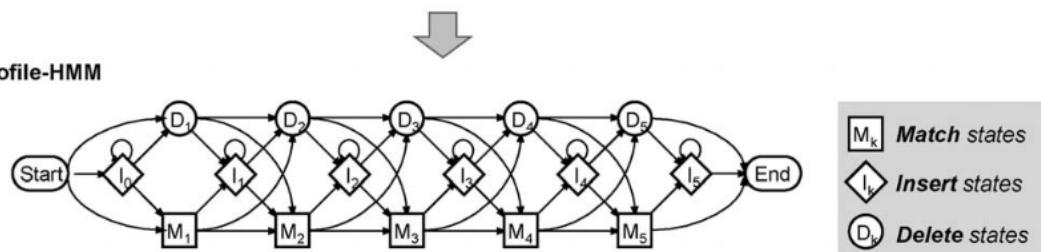


Fig. (2). Profile hidden Markov model. (a) Multiple sequence alignment for constructing the profile-HMM. (b) The ungapped HMM that represents the consensus sequence of the alignment. (c) The final profile-HMM that allows insertions and deletions.

Figure 2: Example from [3]

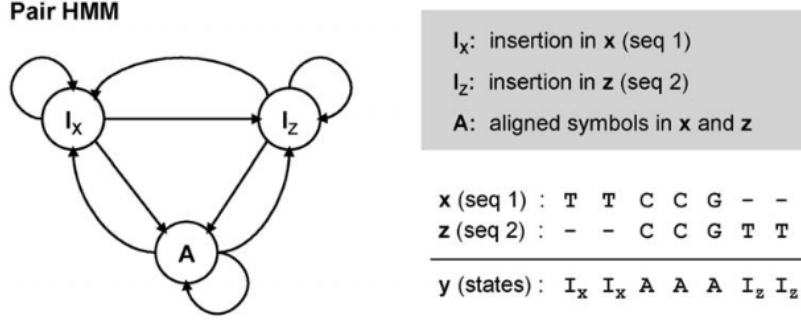


Fig. (3). Example of a pair hidden Markov model. A pair-HMM generates an aligned pair of sequences. In this example, two DNA sequences \mathbf{x} and \mathbf{z} are simultaneously generated by the pair-HMM, where the underlying state sequence is \mathbf{y} . Note that the state sequence \mathbf{y} uniquely determines the pairwise alignment between \mathbf{x} and \mathbf{z} .

Figure 3: Example from [3]

[13].

3.4.3 Pair-HMM

In the context of bioinformatics, a *pair-HMM* (p-HMM) is especially useful for finding sequence alignments and evaluating the significance of the alignments [3]. To contrast the standard HMM, a p-HMM generates an aligned pair of sequences, instead of only one sequence. An example is shown in [3] Just steel this example as well!? Yes, that's fine The illustration from the example is shown in 3. The example presents a very simple p-HMM, with hidden states I_x , I_y and A . This hidden MC produces the two observed sequences denoted by \mathbf{x} and \mathbf{z} , where I_x emits an unaligned symbol in sequence \mathbf{x} and I_z emits an unaligned symbol in \mathbf{z} . Additionally, state A generates an aligned pair of two symbols, i.e. it inserts two identical symbols, each inserted into each of the observed sequences.

Since there is a one-to-one relationship between a hidden state sequence \mathbf{x} of a p-HMM and the alignment between two observed sequences, the alignment problem reduces to finding the optimal state path in the hidden MC. This can be found by a simple variation of the Viterbi algorithm and has time complexity $\mathcal{O}(L_a L_b)$, where \mathbf{a}, \mathbf{b} are the two sequences that are aligned and L_a, L_b are their respective lengths. The p-HMM model is an improvement over the classical sequence alignment methods, since it can be used to compute the alignment probability of the pair of sequences indepently of a specifif alignment. A problem in the classical methods is how one should choose a punctuation scheme in order to find a biologically meaningful optimal alignment of the sequences. In cases where this is difficult to choose, it is more meaningful to compute the probability that the sequences are related, which a p-HMM makes possible [3]. This probability can be calculated by a slight modification of the forward algorithm. The improvement that a p-HMM gives with respect to pairwise sequence alignment will be further discussed in section 4.1.

3.4.4 Context-Sensitive HMM

As noted in section 3.4.1, the standard HMM cannot properly model correlations between residues, which makes the model unsuitable for several applications in biology, for example when dealing with RNA sequences [3]. In order to apply the HMM-methodology successfully to such situations, one needs to extend the standard HMM to allow for pairwise correlations between non-adjacent symbols in the observed sequence. This is where the *context-sensitive HMMs* (cs-HMMs) are appropriately applied.

The main difference between cs-HMMs and the standard HMMs is that the former can use information about earlier emissions to adjust the emission probabilities at future states. This information is referred to as the "context". Thus, it is possible to model correlation between non-adjacent states in a cs-HMM. These HMMs use three different types of states: single-emission states (ξ_i), pairwise-emission states (ϕ_i) and context-sensitive states (γ_i). ξ_i are very similar to the normal states in ordinary HMMs, since they have usual emission probabilities and do not use any additional information for emitting symbols. ϕ_i have fixed emission probabilities, and, in addition, they store the symbols that are admitted in memory. γ_i first retrieves the emitted symbol from ϕ_i , before adjusting its emission probabilities based on this retrieved context. Thus, we can state the emission probability for this context-sensitive state as

$$P(y_j|y_k, x_k, x_j) = P(y_j \text{ emitted at } x_j \text{ given that } y_k \text{ was emitted at } x_k), \quad j > k$$

where y_j is the emitted symbol at a context-sensitive state x_j , y_k is the emitted symbol at a pairwise-emission x_k and $j > k$, i.e. the MC transitions to the context-sensitive state some time after the pairwise-emission state. Using the fact that y_k is independent of x_j , the joint emission probability of y_k and y_j can be calculated

$$P(y_k, y_j|x_k, x_j) = P(y_k|x_k)P(y_j|y_k, x_k, x_j) = \mathcal{E}_{x_k, y_k}P(y_j|y_k, x_k, x_j).$$

Thus, using pairs of states (ϕ_i, γ_i) allow modeling of pairwise symbol correlation by specifying the emission probabilities that appear in equation (3.4.4). Notice that these two states always exist in pairs, since ϕ_i are needed to describe the emission probabilities at γ_i . Moreover, the model is built such that it is never possible to transition to a context-sensitive state before transitioning to the corresponding pairwise-emission state [3].

A simple example of a cs-HMM, which is a modified version of an example appearing in [3], is given in figure 4. The model has three different states: **inconsistent notation!** S_1 is the only single-emission state, P_1 is the only pairwise-emission state and C_1 is the only context-sensitive state. The MC first transitions to P_1 and emits one or more symbols which are stored in a queue. When the MC eventually transitions to C_1 , a symbol is retrieved from the queue and the emission probabilities in C_1 are adjusted in such a way that the same symbol is emitted. Moreover, in this model, the transition probabilities are adjusted such that the MC transitions only to C_1 as long as the queue is not empty. When the queue is empty, the MC transitions to the end state and the simulation is over. Thus, this simple example can be used to create repeating sequences of two different formats. Firstly, the sequence

$$\mathbf{y} = (y_1, y_2, \dots, y_{(L+1)/2}, y_1, y_2, \dots, y_{(L+1)/2})^T,$$

of even length $L + 1$ can be generated by the model. In this case, the first $(L + 1)/2$ underlying states are P_1 and the next $(L + 1)/2$ are C_1 . Secondly, the sequence

$$\mathbf{y} = (y_1, y_2, \dots, y_{L/2}, y_0, y_1, y_2, \dots, y_{L/2})^T,$$

of odd length $L + 1$ can be generated by the model. In this case, the first $L/2$ underlying states are P_1 , the underlying state for the $L/2 + 1$ emitted symbol y_0 is S_1 and the underlying states for the last $y_{L/2}$ are C_1 . Notice that changing the FIFO queue for a LIFO enables generation of palindromes instead of repeating sequences from the model [3].

Recall that pro-HMMs were used to make profiles that represent multiple alignments of sequences, which in the context of biology can be DNA or proteins (sequences of aminoacids). Imagine that we want to make profiles that take column-wise correlations in the multiple alignment into account. This can be done relatively easily by combining the ideas of the pro-HMMs and the cs-HMMs, into what is called *Profile Context-Sensitive HMMs* (pro-cs-HMMs). Like for the pro-HMMs studied earlier, the pro-cs-HMMs contain match states (M_k), insert states (I_k) and delete states (D_k). The new addition is that the match states can be of three different types ($\xi_i^m, \phi_i^m, \gamma_i^m$), to account for symbols

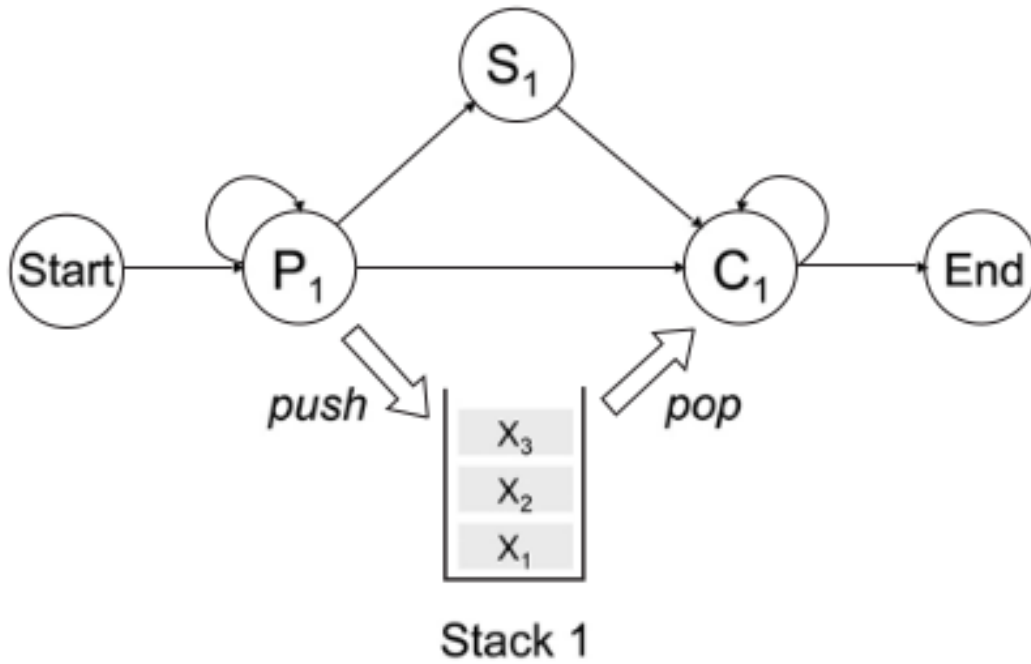


Fig. (4). A context-sensitive HMM that generates only symmetric sequences, or palindromes.

Figure 4: Example from [3]. I changed the example a bit, generating repeating sequences instead of for palindromes, as Yoon did. Changing the stack (LIFO) for a queue (FIFO) gives repeating patterns in the sequences, instead of palindromes. .

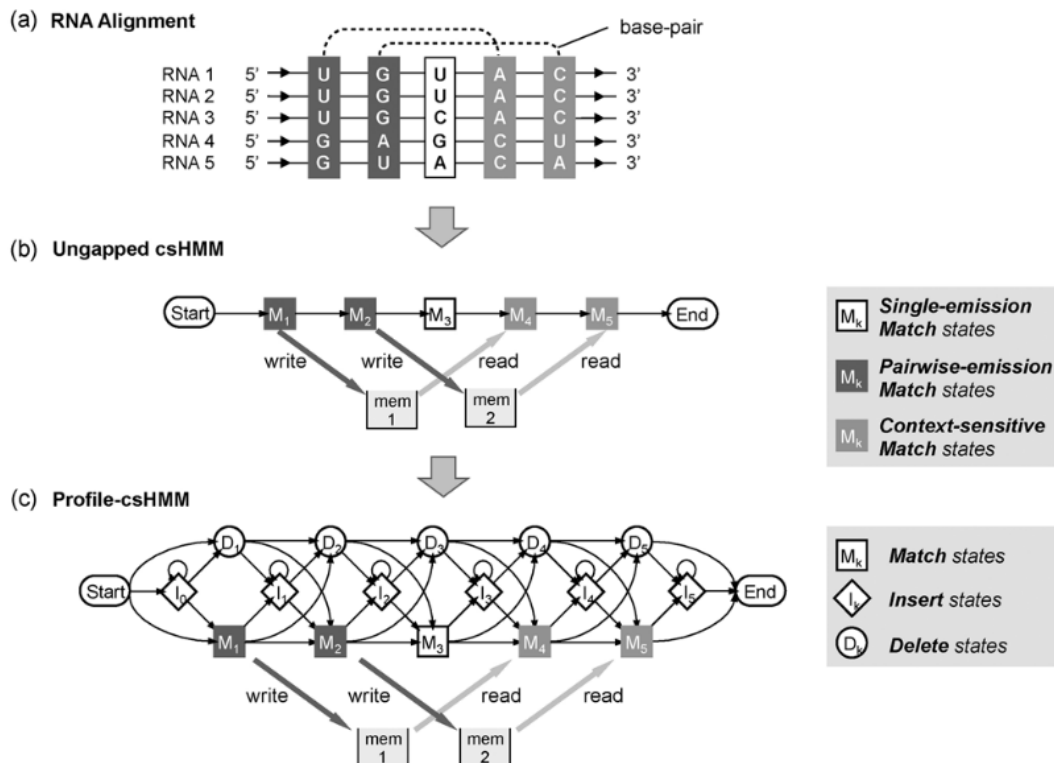


Fig. (5). Constructing a profile-csHMM from a multiple RNA sequence alignment. (a) Example of an RNA sequence alignment. The consensus RNA structure has two base-pairs. (b) An ungapped csHMM constructed from the given alignment. (c) The final profile-csHMM that can handle symbol matches, insertions, and deletions.

Figure 5: Example from [3]. Tenker at jeg stjeler dette eksempelet, da det er veldig godt (eller bare henviser), men lager de to andre litt selv etter hvert!

that are pairwise correlated. The single-emission match states ξ_i^m are used to represent columns in the multiple alignment that are uncorrelated with the others, while the pairing of (ϕ_i^m, γ_i^m) are used to model pairwise correlation between symbols in different columns. An example, taken from [3], is shown in figure 5 **Not described yet**.

For a detailed treatment of cs-HMMs, the reader is referred to [14].

4 Applications in Bioinformatics

Some more applications listed on the Wikipedia page: https://en.wikipedia.org/wiki/Hidden_Markov_model#Applications

4.1 Pairwise Sequence Alignment

Pairwise sequence alignment (PSA) is carried out in order to infer functional, structural and/or evolutionary relationships between two sequences. Such an alignment can be done both locally and globally and there exists a variety of methods of doing it. Optimal algorithms exist, such as the Needleman-Wunsch algorithm for optimal global alignment and the Smith-Waterman algorithm for optimal local alignment. These algorithms are based on dynamic programming and can be time consuming to use. Therefore, algorithms based on heuristics were developed, where the two most popular algorithms are called BLAST and FASTA. All the mentioned algorithms work well for highly similar sequences, but produce mediocre results for highly divergent sequences [2]. Profile based analysis was developed to improve these results, in which HMMs play a crucial role.

A big issue when it comes to finding the best alignment between sequences using the previously mentioned algorithms is that one has to define a reasonable scoring scheme for ranking the possible alignments. It can be difficult to define this scoring scheme, since different scoring schemes perform better or worse in different applications, depending on how similar the sequences are. Thus, the major drawback of these algorithms is the dependence on the scoring scheme, which to a large degree dictates how the alignment is produced, which in turn limits what type of relationship one is able to find between sequences. The p-HMMs introduce an alternative method of finding relationships between a pair of sequences, which distances itself from ad-hoc scoring schemes.

When using p-HMMs the pairwise sequence alignment problem is tackled as a stochastic process, as used by Smith et al [5] and Durbin et al [12]. Because the p-HMM generates pairs of observations, it can be used to calculate the relationship between two sequences independent of a specific alignment, simply by using the forward algorithm [2]. A more detailed treatment on how the p-HMMs are used in practice for local and global PSA can be found in Chapter 4 in [12]. The bottomline is that p-HMMs give a more general method of PSA compared to the previously mentioned algorithms, because the parameters θ of the HMM can be estimated, as discussed in the training problem, instead of having to manually define a scoring scheme that makes biological sense in the specific problem. For a more detailed treatment of PSA, the reader is referred to [12].

This section only discusses PSA in the context of sequences of nucleotides or aminoacids, i.e. PSA of DNA sequences or proteins. Alignment of RNA sequences can become more complex, which will be shortly discussed in section 4.10.

4.2 Multiple Sequence Alignment

Multiple sequence alignment (MSA) is commonly used to find conserved regions in a group of sequences and predicting protein structures. Functional biological sequences typically come in families, which means that one is interested in discovering possible relationships between a sequence and a known sequence family. Despite the fact that sequences will have diverged from each other in evolution, MSA can be used to identify which sequences belong to a family, in order to allow for inferences about their function [12]. This application of MSA will be studied further in sections 4.4, 4.5 and 4.6. Moreover,

MSA is interesting in the case of protein structure prediction, because one can align structural data instead of sequences [13]. This application of MSA will be further discussed in section 4.7.

In contrast to PSA, there does not exist any optimal algorithms to solve the multiple alignment problem. However, there exists a variety of heuristic methods for solving it. One possible solution is to reduce the multiple alignment problem to a set of pairwise comparisons between the sequences, in an orderly fashion, in order to end up with the multiple alignment in the end. Here one can choose to use either the optimal algorithms or the heuristic algorithms when performing the pairwise alignment. One type of methods that employs this paradigm is referred to as *progressive sequence alignment*. More details on a progressive algorithm that uses Needleman-Wunsch for the pairwise alignment can be found in [6]. Some commonly available implementations of this solution include the T-Coffee and the Clustal package [8]. Another type of methods that uses this paradigm is referred to as *iterative alignment*. These methods are slightly different from the progressive methods, since they allow re-alignment of the pairwise sequences during multiple iterations. The progressive methods do not facilitate this, which means that they depend highly on the initial pairwise alignment of the first two sequences [9]. One popular openly available implementation of an iterative method is MUSCLE [8].

As in the case of PSA, HMMs provide powerful alternatives to the mentioned methods for MSA. In this problem, pro-HMMs have been particularly successful. Choo et al states that "Multiple alignments from a group of unaligned sequences are automatically created using the Viterbi algorithm" [2]. Each match state in the pro-HMM corresponds to a column in the MSA. We refer back to section 3.4.2 for a brief description of how the pro-HMM relates to MSA.

The beauty of a pro-HMM in the case of MSA is that they can be treated as a summary of a multiple alignment of sequences or as a model for a family of such sequences [4]. These pro-HMM representations of MSAs make it possible to use the MSA for many different applications in biology. They facilitate quick assignment of protein function, based on alignment of a new sequence against the model. Moreover, a pro-HMM that represents a family of sequences can be used to search for homologues. The model can be used to search in a database of sequences, in order to find additional homologues to the family. Similarly, given a database of pre-built pro-HMMs, one can look for matching profiles to a symbol sequence. Thus, such a database can be used to classify and annotate the given sequence. Additionally, pro-HMMs can be used to compare two different MSAs. This can be beneficial for detecting remote homologues [3]. Thus, the pro-HMM representation of a MSA is very flexible, as it can be applied to many different problems by following different principles. Once a pro-HMM is constructed one can align new sequences to it, one can use it to query in databases of sequences, one can query a database of such pro-HMMs for matching profiles to one symbol sequence or one can compare two different pro-HMMs in order to discover similarities between two different MSAs.

Finally, we mention two free online repositories that store pre-built pro-HMMs of many known protein families: PFAM and PROSITE.

For a more detailed treatment of MSA, the reader is referred to [12].

4.3 Motif Representation

A motif is a recurring pattern in DNA or proteins, which is assumed to be related to biological function. Thus a motif can be a sequence of nucleotides or of aminoacids. Finding such motifs is interesting to a biologist because *transcription factor binding sites* (TFBS) appear as such motifs in sequences. These are regulatory sequences that control gene transcription, which is important information for a biologist because they repress or promote the expression of many other genes [4].

There are several ways of representing such sequence motifs, where the performance of each method generally depends on what type of motifs one wants to represent. For shorter, ungapped motifs of fixed length, methods like *consensus sequences*, *regular expressions* (RegEx), *sequence logos* and *position specific scoring matrix* (PSSM), in ascending order of complexity, are commonly used. Note that

these can also be seen as different ways of visualizing or representing MSAs, which are useful tools in practice, since the alignments themselves are not very easy in use.

The drawbacks of these methods are that they do not work well for longer motifs with variable length gaps. This is where pro-HMMs shine in comparison. The HMMs work well for all types of motifs, including the short and fixed length motifs, but they are more complicated models. Thus, even though the HMMs always will produce good results, given that they can be built correctly in each specific case, one should not always use these models because of their complexity. Always keep the principle of parsimony in mind; for competing explanations, or models, where all are reasonable, one should always choose the simplest, which makes the least amount of assumptions. Hence, even though the HMMs are great tools also for motif representation, they should be used when appropriate.

But how can the HMMs be used to represent motifs with variable length gaps? pro-HMMs have gained much traction for this problem, since they yield very reliable results. How they work can be seen easily from the example presented about the pro-HMMs in section 3.4.2, since the example shows how a MSA can be represented as a pro-HMM. The same principle explained in the example can be used when working with much larger and more complicated alignments. As you have noted by now, motif representation is very closely related to MSA, which means that the tactics used for querying with pro-HMMs described in section 4.2 also apply here.

Note that the problem of identifying the motifs is not discussed in detail here, as this is a much more complicated problem compared to simply representing the motifs. However, this problem is similar to multiple local alignment. An example of an algorithm to solve this problem is given in chapter 10.3 in [4], which is a variant of the Gibbs sampling algorithm based on PSSMs.

4.4 Protein Homology Detection

Protein homology detection concerns the problem of discovering homologous proteins, i.e. which proteins are derived from a common ancestor. The scientist wants to characterize sets of homologous proteins (gene families) based on common patterns in their sequence. Once such a characterization is found from a known family, one can determine if a new protein belongs to this family or not [4]. The attentive reader will notice that this simply can be seen as an application of MSA.

How is this solved without HMMs? Look at MSA! Or does there exist other methods as well? Google quickly!

When treating this as an application of MSA it is readily seen that this type of homology detection can be done with pro-HMMs. As noted by Christianini and Hahn in *Introduction to Computational Genomics* "profile HMMs encode position-specific information about the frequency of particular amino acids as well as the frequency of insertions and deletions in the alignment. They are constructed from multiple alignments of homologous sequences" [4]. Thus, aligning a sequence with a pro-HMM is equivalent to aligning the sequence to many other sequences, which were used to establish the pro-HMM in the first place. The pro-HMM can be used to query a database of sequences or a database of other pro-HMMs in order to find additional homologues.

Remote protein homology detection can be more complicated, which means that the standard pro-HMMs can be unreliable. To improve the performance of pro-HMMs in such cases, models known as *feature-based profile-HMMs* can be used [3].

Tror jeg sa noe om dette i bio-kladdeboka når jeg øvde til midterm! Sjekk der også!

4.5 Prediction of Function

Probably the most important characteristic of a protein is its function, which is not inherently known, unless it already has been discovered. This means that, given a specific protein with unknown function, a scientist is interested in predicting its function. One way of making this prediction possible is the attempt of inferring functional similarity between a given protein, with unknown function, and a protein with previously discovered function. But how can this similarity be inferred? As noted in section 4.2, functional biological sequences typically come in families. This assumption makes it

possible to infer functional similarity based on similarities in the sequences of proteins. The attentive reader will notice that this can be solved via sequence alignment as well. The simplest possible solution could be to compare the protein with unknown function pairwise, sequentially, to a database of proteins with known functions. This can be time-consuming and inaccurate however **Sjekk at dette stemmer, tror jeg skrev noe i bio-kladdeboka om dette når jeg øvde til midterm!**, since PSA does not guarantee finding similarity between the sequences. The use of MSA is more **"utbredt"** because of the familiarity assumption. Thus, having built a pro-HMM from a family of proteins with known function, this model can be used to infer whether or not a new protein shares the same function. Searching an extensive database of pre-built pro-HMMs one can find what function a specific protein most likely possesses.

How is this solved without HMMs? Look at MSA! Or does there exist other methods as well? Google quickly!

4.6 Protein Sequence Classification

Hva mener jeg egentlig med dette? Classification in function, structure or other characteristics?

How is this solved without HMMs?

Profile HMMs (analogously to multiple sequence alignment).

Another application of MSA.

4.7 Protein Structure Prediction

Another important characteristic of a protein is its structure. The structure of a protein provides "a key to understanding its biological function" [11]. Generally, the structure of a protein comes in four different levels; primary, secondary, tertiary and quaternary **Se kladdebok i bio (øving til midterm), sjekk at det er de samme**. Without going into closer details about what the different levels mean, each of them describe different characteristics of the protein's structure, where the primary structure is the aminoacid sequence itself. The main goal of the structure prediction is to predict the secondary, tertiary and quaternary levels from the primary level of a specific protein. This is interesting because the experimental approaches used to discover all the structural levels of a protein are expensive, difficult and time-consuming. Because of these difficulties, the ideal is to use computational and statistical models to predict unknown structures. However, note that prediction of all the levels from the primary level can be very optimistic. In practice, one can only hope to predict the secondary structure, and sometimes the tertiary structure, as noted by Isaev [11].

How is this solved without HMMs? I think I have written enough about this above, but a quick google search could be smart still.

One set of widely used models for predicting the secondary structure of a protein are pro-HMMs. This problem can, again, be seen as an application of MSA. When searching for a particular structural feature in a protein with unknown structure, one constructs a pro-HMM from a set of proteins with this feature. Then, aligning the query sequence to the pro-HMM can decide whether or not the sequence in question has the structural feature. In practice, one would query a database of pro-HMMs constructed based on different structural features, in order to (hopefully) uncover one of the features in the query protein.

For prediction of the tertiary structure of a protein, one possible methodology is described by Yoon [3]. He notes that pro-HMMs can be used to "model sequences of protein secondary structure symbols: helix (H), strand (E) and coil (C)" [3]. Such models can be used to recognize the three-dimensional fold of new protein sequences based on their secondary protein structure predictions. Thus, this methodology assumes the secondary structure of the protein as known, either from experimental approaches or prediction.

Despite the vast improvements the HMMs have yielded, Eddy notes that "Profile HMMs and HMM-based gene finders have probably been the most successful applications of HMMs in computational biology. On the other hand, protein secondary structure prediction is an area in which the state

of the art is neural net methods that outperform HMM methods by using extensive local correlation information that is not necessarily easy to model in an HMM” [13]. This shows that the HMMs that are used up until now are not the end all be all for statistical methods in bioinformatics, and that today’s most successful method may be surpassed in the future.

More details about this problem, which is also known as the *protein folding problem*, can be found in Isaev’s *Introduction to Mathematical Methods in Bioinformatics* [11]. A more technical note can be found in [2].

4.8 Segmentation

How is this solved without HMMs?

Segmentation is about defining exact boundaries between distinct regions with different chemical properties. Moreover, segmentation is about defining larger sequences of heterogeneous nucleotide in genome and to identify biological features that are responsible for the heterogeneity that is observed [4]. Some classical methods are ...

HMMs can be used effectively for segmentation as well... They can help to define regions of gene and protein sequences with various chemical properties [4].

Example 4.2 in [4] shows an example of segmentation using HMMs.

In the setting of segmentation, the hidden states are interpreted as different types of the sequence and the hidden alphabet is typically very small. The underlying MC is cyclic in this case, allowing returns to the same state, i.e. to the same type of sequence, many times during the simulation [4].

4.9 Gene Prediction / Genomic Annotation (?) / Gene Finding (?)

Is Gene Prediction and Genomic Annotation (and Gene Finding) the same problem? Two different words for the same name or are they slightly different problems, with different goals in mind?

How is this solved without HMMs?

HMMs are employed to find eukaryotic genes and to find pseudogenes, which look like functioning genes except for some misplaced stop codons. They are very useful for these problems because of their flexibility [4].

If the hidden states in the HMM can be inferred, the genome can be better annotated or one can understand the dynamics of the genome better [4].

Genomic annotation: Generalized HMMs [2].

Eukaryotic genes can be modeled using HMMs [3].

Pair HMMs can be used for gene prediction [3].

4.10 RNA Structural Alignment

How is this solved without HMMs?

As explained when talking about context-sensitive HMMs (csHMMs), profile-csHMMs can be used to perform structural alignment of RNA and performing similarity searches, analogously to how the profile-HMMs can be used to perform multiple alignment of DNA or proteins and performing similarity searches in these cases. RNA sequence analysis is often of high computational complexity, because the alignment algorithms have to deal with base-pair correlations in the sequences that may be very complicated. More about this problem in [3].

Pair Hidden Markov models on tree structures (PHHMTS) can be used for aligning trees. Since most RNA secondary structures can be represented as trees, this provides a useful framework for aligning RNA sequences.

4.11 Other Applications

Some more applications of HMMs, all described by Yoon, are *base-calling*, *modeling DNA sequencing errors* and *mcRNA identification* [3]. These are all interesting problems, but will not be described here.

Either way, based on the short list of applications that have been described, it has hopefully become clear to the reader that HMMs are very widely used in Bioinformatics.

5 Examples in R

5.1 Toy Example

Simple HMMs can relatively easily be programmed manually. Imagine that you want to build a simple HMM for the cleavage of a DNA sequence between areas rich in CG or AT. The HMM would take the form as depicted in figure 6. The hidden MC has two states (AT and CG), where AT represents an AT rich state and CG represents a CG rich state. In mathematical terms, we would write that $\mathcal{H} = \{AT, CG\}$. Moreover, since we are working with sequences of DNA, $\mathcal{S} = \{A, C, G, T\}$. The transition probabilities and the emission probabilities are shown in the figure. Note that the initial state probabilities are omitted from the figure, but they are set in the simulated model below. Based on these probabilities, the matrices are

$$\mathcal{T} = \begin{bmatrix} \mathcal{T}_{AT,AT} & \mathcal{T}_{AT,CG} \\ \mathcal{T}_{CG,AT} & \mathcal{T}_{CG,CG} \end{bmatrix} = \mathcal{T} = \begin{bmatrix} 0.8 & 0.2 \\ 0.2 & 0.8 \end{bmatrix},$$

and

$$\mathcal{E} = \begin{bmatrix} \mathcal{E}_{AT,A} & \mathcal{E}_{AT,C} & \mathcal{E}_{AT,G} & \mathcal{E}_{AT,T} \\ \mathcal{E}_{CG,A} & \mathcal{E}_{CG,C} & \mathcal{E}_{CG,G} & \mathcal{E}_{CG,T} \end{bmatrix} = \begin{bmatrix} 0.4 & 0.1 & 0.1 & 0.4 \\ 0.1 & 0.4 & 0.4 & 0.1 \end{bmatrix}.$$

The code block below shows one simple example of how this example HMM can be simulated. The function takes four inputs; the transition matrix, the emission matrix, the length of sequence that one wants to simulate and the initial state probabilities.

INSERT CODE BLOCK HERE!

For example, the code block below shows how a HMM of length $n = 1000$, according to the specifications listed above, can be simulated, using the function ‘generate.sequences’.

INSERT CODE BLOCK HERE!

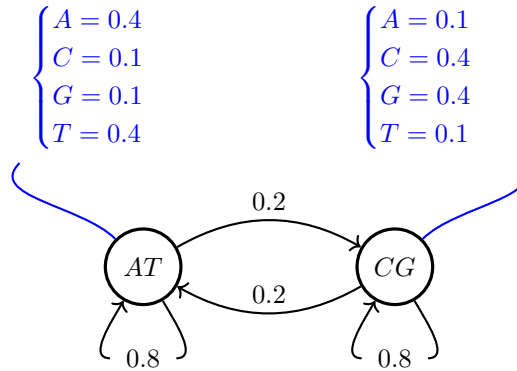


Figure 6: HMM topology for example on modelling the cleavage of a DNA sequence between areas rich in CG or AT. Note that the initial state probabilities are omitted from this figure.

Chapter 10.6 in [15] is a good reference for some more simple manual implementations of MCs and HMMs in R. Although it is always recommended to try to implement methods manually at first, because it helps to understand the theory to a greater degree, the use of libraries always gives more flexibility. Moreover, libraries are very often optimized, such that the performance and reliability of the code is guaranteed. We will be diving deeper into one such library for HMMs in R in the following.

5.2 The ‘aphid’ library

<https://cran.r-project.org/web/packages/aphid/vignettes/aphid-vignette.html>

5.3 CG-islands and the ”Fair Bet Casino”

”An introduction to bioinformatics algorithms” - Jones and Pevzner Page 388.

Hay una tarea sobre esto tambien, descargado en pdf en ”Books/bioinfo”.

The reference list should be alphabetically ordered later!

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