Detecting species-site dependencies in large multiple sequence alignments

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ABSTRACT

Multiple sequence alignments (MSAs) are one of the most important sources of information in sequence analysis. Many methods have been proposed to detect, extract and visualize their most significant properties. To the same extent that site-specific methods like sequence logos successfully visualize site conservations and sequence-based methods like clustering approaches detect relationships between sequences, both types of methods fail at revealing informational elements of MSAs at the level of sequence–site interactions, i.e. finding clusters of sequences and sites responsible for their clustering, which together account for a high fraction of the overall information of the MSA. To fill this gap, we present here a method that combines the Fisher score-based embedding of sequences from a profile hidden Markov model (pHMM) with correspondence analysis. This method is capable of detecting and visualizing group-specific or conflicting signals in an MSA and allows for a detailed explorative investigation of alignments of any size tractable by pHMMs. Applications of our methods are exemplified on an alignment of the Neisseria surface antigen LP2086, where it is used to detect sites of recombinatory horizontal gene transfer and on the vitamin K epoxide reductase family to distinguish between evolutionary and functional signals.

INTRODUCTION

Multiple sequence alignments (MSAs) are high dimensional discrete datasets, which play a prominent role in bioinformatics. They are typically involved in the functional classification of proteins and phylogenetic reconstruction of evolutionary trees, for example. In general, there are two aspects of MSAs; analyses are mostly either species- or site focused. Species-driven approaches usually aim at the relationship between sequences, averaging over the alignment columns. Methods for phylogenetic reconstruction as well as general sequence clustering methods are examples, and make (amongst other things) use of distance measures to impose an hierarchy on the species in an alignment. This allows for the detection of closely related species, functional clusters and the reconstruction of gene trees or species trees. Site-driven analyses in contrast put more emphasis on sequence content, looking for specific sequence motifs, conservation profiles, areas with characteristic biochemical properties like hydrophobicity or transmembrane regions, thereby averaging over the sequences or focusing on their conserved regions.

A combination of both types of analyses of an (correctly aligned) MSA helps to distinguish functionally conserved from variable sites, detect clusters of sequences and find sites responsible for a certain splitting of sequence groups. This integration can finally lead to an understanding of the functional evolution of sequences, as tree splits or cluster breaks can be annotated with the associated autapomorphies [an autapomorphy is a trait characteristic for a terminal group in a phylogenetic tree]...
MATERIALS AND METHODS

Embedding

Molecular sequences are typically represented by strings over an alphabet of either 4 or 20 characters.
Post-processing. After SVD, the row $U$ and column $V$ scores are usually rescaled via $X_i = U_i \sqrt{f_i}$ and $Y_j = V_j \sqrt{f_j}$, to obtain the optimal or canonical row ($X$) and column ($Y$) scores. Depending on the implementation of the CA algorithm, these are afterwards further scaled by their corresponding singular values (17).

Interpretation. The selected component axes are then plotted in usually 2D scatterplots. The Euclidean projection of both site and species points in the new space approximates their $\chi^2$ distances as closely as possible. Proximity of points in the CA biplot therefore corresponds to dependencies between items. Furthermore, points are projected such that the further away a point is from the origin, the higher its contribution to the $\chi^2$ statistic. Positive associations lie on the same side of the plot, whereas negative associations lie on the opposite sides. For a more detailed explanation, see ref. (17). Please note that the addition of a positive constant to the original data matrix does not change the proportions of the new coordinate system but implies a rescaling of the result.

Sequence analysis

_Neisseria meningitidis factor H binding protein_. Sequences for the LP2086 and VKOR studies were aligned using Muscle (22) (Supplementary Data). The distance matrix for the LP2086 alignment was calculated using ProlDist (23,24) applying the VT substitution matrix (25). The distance matrix was further analyzed and visualized by SplitsTree’s split decomposition method (26).

_Vitamin K epoxide reductase family_. Vertebrate sequences were extracted from ENSEMBL using the human VKORC1 and VKORC1L1 proteins as query in a blastp search (27). The ENSEMBL identifiers are:

| Species   | ENSEMBL ID       | Species   | ENSEMBL ID       |
|-----------|------------------|-----------|------------------|
| L1_Human  | ENSP00000353988  | L1_Pan    | ENSPTRP00000047967 |
| L1_Mouse  | ENSMUSP00000027960 | L1_Rat    | ENSNRDOP00000024691 |
| L1_Mouse  | ENSMUSP00000073601 | L1_Monodelphis | ENSMODP00000008083 |
| L1_Xenopus| ENSTXETP00000022173 | L1_Danio  | ENSDARP00000064087 |
| L1_Oryzias| ENSORLP00000014137 | L1_Fugu   | ENSTRUP0000017074 |
| C1_Human  | ENSP00000378426  | C1_Pongo  | ENSPPY00000008254 |
| C1_Cat    | ENSCAP00000002998 | C1_Horse  | ENSECAP0000021915 |
| C1_Dog    | ENSCAP000000024701 | C1_Cow    | ENSBTAP0000000519 |
| C1_Rat    | ENSRNP00000026347 | C1_Mouse  | ENSMUSP00000013074 |
| C1_Fugu   | ENSTRUP00000027115 | C1_Tetraodon | ENSTNIP0000018260 |

The _Ciona savignyi_ homologue was identified only in genomic sequences. The protein sequence was predicted using gene-wise (28) and the human VKORC1 protein as template. The alignment was calculated using Muscle (22) and manually optimized (Supplementary Data). The phylogenetic tree for the VKOR example was calculated with _proml_ of the PHYLIP package (29) and 100 bootstrap replicates. Ancient sequences were reconstructed by _codeml_ of the PAML package (30).

RESULTS

The method we propose here is a novel approach to an explorative analysis and visualization of MSAs. The goal of our method is the detection and depiction of major signals in alignments, ordered by their importance, co-clustering of sequences and sites and resolution of contradictory signals, i.e. different parts of the alignments vote for a different clustering.

The approach comprises three separate steps: (i) the embedding of sequence data into a real valued very high dimensional vector space, (ii) the simultaneous dimension reduction and ordination of both rows and columns of the data matrix (the alignment) and (iii) a biplot visualization of the canonical row and columns scores.

The result is a lower dimensional representation of sequences and sites, which can be analyzed by (two-, or three dimensional) scatterplots, comparable but not identical with the result of classical dimension reduction techniques like PCA, applied to both sequences and sites. In contrast to traditional dimension reduction methods, the sequences are co-clustered to their defining sites and vice versa. In this representation, the sites responsible for a cluster of sequences come to lie close to the sequences.

Embedding (i) is achieved via the Fisher score representation of HMM parameters (14,15,18). Therefore we start by training a profile HMM (pHMM), (9,10) on the previously aligned sequences. The Fisher scores are then computed as the vector of derivatives of the log-likelihood of each training sequence with respect to the emission probabilities of the HMM (see ‘Material and Methods’ section). The sequence is thus transformed in a meaningful way into a vector of real-valued numerical values for the following ordination step.

Steps (ii) and (iii) are done via direct application of CA to the derived data matrix of Fisher scores. CA is a method originating from ecology and designed for the analysis of two-way contingency tables (17). It is capable of performing simultaneous ordination on both rows and columns of a data matrix (often referred to as species and sites in ecology, a nomenclature which also fits well in sequence analysis) and has also been shown to be of use for continuous datasets in the context of microarray analysis (31). In principle, it can be thought of as an oriented MDS on $\chi^2$ distance matrices computed from both sides of a data matrix, which is jointly plotted. In the CA, each axis is a weighted linear combination of the Fisher scores of the data vectors, i.e. of the existent (and due to the way the Fisher scores are generated also non-existent) nucleotides/residues in the alignment. CA is a co-clustering of sequences and sites, where conditionally independent signals are projected onto the component axes.

Therefore, in a phylogenetic context, one would expect that the first component axis corresponds to the branch of a phylogenetic tree which discriminates most between the most different sequence groups. Typically this refers to the longest branch of the tree. This means that the two major phylogenetic sequence groups are expected to lie consistently on one side of the first component axis, or the other, respectively. Other long-branched subgroups of the tree are then likely to be found in higher order component axes. The co-clustered sites are major candidates for the autapomorphies defining
the split. In the same manner, alignments can be decomposed, even when a well-supported phylogenetic tree cannot be constructed, either due to contradictory signals within the alignment or different evolutionary histories. In summary, our proposed method yields a complete decomposition of the considered MSA. In particular, it visualizes information content and species-site dependencies with respect to a given sequence family, modeled by the underlying pHMM.

Example on an artificial dataset

To illustrate the concept of our proposed method, we created an artificial DNA MSA (Figure 1a) of four sequences. The main split of the associated cluster tree (Figure 1b) distinguishes the sequences 1 and 2 from 3 and 4. Given the first split, split II distinguishes between sequences 1 and 2 and split III distinguishes between sequences 3 and 4.

Application of our method illustrates how it is able to recover the sequence groups and the nucleotide replacements responsible for the grouping. The procedure decomposed the alignment into a 3D space, without loss of information. Figure 1c and d are CA plots of the MSA showing the first three component axes (1 versus 2, and 3 versus itself). In Figure 1c, the first component axis corresponds to the main split of the cluster tree and separates sequences 1 and 2 from sequences 3 and 4, thereby indicating the sites responsible for this split, i.e. G and C versus both Ts at position 3 and 4. The second component axis explains the (conditional) split between sequences 1 and 2 identified by an A or T at position 10. The last conditional split to be explained is the one separating sequences 3 and 4. This is shown in Figure 1d, the third component axis, which identifies the differences at position 16 (G versus C) as being responsible for the split.

Neisseria meningitidis factor H binding protein

To validate our method on a biological example we chose the *N. meningitidis* factor H binding protein (fHBP), also termed lipoprotein 2086 and GNA1870, which has become a prominent target in the development of a novel vaccine against serogroup B meningococci (32–34). Alignment of the factor H binding protein (fHBP), Neisseria meningitidis

To compare our results with classical methods, we computed a matrix of evolutionary distances between all 47 unique sequences, which was then visualized as an evolutionary network using split decomposition (26). The main cluster, as found by Fletcher et al. (33) and our analysis of component axis 1, was also recovered in the evolutionary network (Figure 2a). These findings strongly suggest that the evolutionary split which lead to development of subfamilies A and B must have happened early in the history of this protein.

Remarkably, axis 3 divided both subfamilies A and B into two sub-clusters (A1, A2 and B1, B2, respectively). When we investigated the most prominent representatives of these groups (i.e. the ones closest to the borders of the plot), the co-clustered sites showed that these groups contain identical sequence elements (positions 37–69), including a three-residue long lys-asp-asn insertion between alignment position 67 and 69.

This indicates that if the development of subfamilies A and B was prior to the emergence of the second split, clusters 1 and 2 have developed within subfamily A (Figure 2a), and parts of the sequence has afterwards been transferred to members of subfamily B by means of an horizontal gene transfer (HGT)/recombination event. This uncertainty in the evolutionary hierarchy between the sequences is also reflected in the large rectangles contained in the split decomposition visualization of the distance matrix (Figure 2a). This finding was further supported by a PHI test for recombination, which was carried out on the complete alignment (P-value <1.07 × 10⁻¹¹) (35).
Vitamin K epoxide reductase family

Vitamin K is an essential cofactor for the post-translational $\gamma$-glutamyl carboxylation of the vitamin K-dependent proteins such as several coagulation factors, bone proteins, cell growth regulating proteins and others of unknown function (36,37). During the carboxylation vitamin K hydrochinone is converted into vitamin K 2,3-epoxide (38). The recycling reaction of vitamin K epoxide back to the hydrochinone form is catalyzed by the vitamin K epoxide reductase (VKORC1) in the so-called vitamin K cycle (39). VKORC1 is the key protein in this redox reaction and the molecular target of coumarin derivatives, such as warfarin, which act as vitamin K antagonists (40). They reduce coagulation activity by interfering with the vitamin K epoxide reductase. Worldwide, coumarins are used in therapy and prevention of thromboembolic events and also in higher doses for rodent pest control. Mutations in the VKORC1 gene cause one form of combined deficiency of vitamin K-dependent coagulation factors (VKCFD type 2) as well as resistance or hypersensitivity to warfarin (41,42). The human VKORC1 gene is localized on chromosome 16 (43) and consists of three exons encoding a 163-amino acid endoplasmic reticulum membrane protein with three or four predicted transmembrane $\alpha$-helices (44). With the identification of the VKORC1 gene in 2004 (45,46) a paralog gene was discovered, which is called vitamin K epoxide reductase complex 1-like 1 (VKORC1L1) and which is highly conserved over the species. Its physiological function is completely unknown.

Extensive database searches in a wide variety of metazoan genomes and subsequent phylogenetic reconstruction allowed us to time the duplication event to the base of the vertebrates. To identify candidate positions for functional analyses, we built a MSA including both variants over different vertebrates, namely a group of fish species (danio, tetraodon, fugu and oryzias), a group of mammals (macaca, pan, human, pongo, mouse, rat, cow, cat, dog and horse), Monodelphis and Xenopus. Furthermore, the alignment contained the VKOR ortholog of Ciona savigny, pre-dating the duplication event. As expected, a first phylogenetic tree revealed two groups, VKORC1 and VKORC1L1, and placed the C. savigny sequence as outgroup. It further clearly separated the fish species from the rest in both groups and correctly clustered the subgroups of mammals in contrast to the singletons Chicken, Monodelphis and Xenopus (Figure 3a).

Application of our method to this alignment revealed the following: the first (and most informative) axis separated all species, i.e. all duplicated genes, from the C. savigny sequence (data not shown). This corresponds to the longest branch and rootsplit in the phylogenetic tree, but as we were more interested in variation between species with both paralogs present, we did not investigate this further. We expected axis 2 to either separate the C1 from the L1 sequences or the fish from the land animals, in analogy to the phylogenetic tree. Axis 3 in general separated C1 from L1, for all but the C1 fish sequences, which came to lie near the origin. Analysis of

Figure 1. Artificial example: the MSA (a) and its cluster tree (b) as used in our toy example. The subparts (c) and (d) are scatterplots of the first three component axes, which together account for 100% of the inertia in the data. The CA plots present sequences (black circles) and sites (red crosses) in an integrated manner. For better interpretation, the most important sites are explicitly shown in the plots with their nucleotide letters and alignment positions. Roman numbers indicate the splits in the cluster tree and the component axes resolving them.
the subsequent axes of the ordination results separated the L1 fish sequences from its main group (axis 5) and showed that the Danio sequence within the C1 fish group was evolutionarily more distant to the other C1 fish (axis 4), as reflected in the phylogenetic tree.

The co-clustered sites showed us that positions which are otherwise completely conserved within the C1 or L1 family were different in the C1 fish sequences. For example, alignment positions 73–77 (marked with yellow dots in Figure 3b) showed a typical EHVL motif for the C1 family and a GSIF sequence for the L1 cluster. The C1 fish sequences in contrast had a QYFV motif (QIFT for Danio) instead. The missing information was caught by axis 2 which separated the C1 fish sequences from all others (for a combined scatterplot of axes 2 and 3, see Figure 3b). In addition, different positions in the alignment were identified, where the fish C1 sequences harbored the same amino acids as the L1 land animal group but differed from the rest of the C1 group. A prominent example is the Warfarin binding motif which is found as a TYA in the C1 non-fish and L1 fish sequences, but as a TYV/TYI/TYL in the C1 fish and L1 non-fish sequences. Reconstruction of ancient sites revealed that this motif evolved in the C1 group only after the split of fishes from the other vertebrates (Figure 3a). Following this observation, we extracted all positions specific for the L1 group and the L1/C1 fish groups, respectively.

To analyze their functional relevance, we mapped these positions onto the transmembrane topology of this protein [Figure 3b, (44)]. Two clusters of these sites reside on the cytoplasmic extensions of the transmembrane helices I and III. Further sites are localized within the transmembrane helix II. Here, the positions were placed regularly on every fourth position (alignment positions 111, 115, 119 and 123, Figure 3b).

With a standard helix turn taking on about every 3.5 amino acids, there seems to be a spatially aligned position, where the sites of this transmembrane helix are specific for the subgroups. Although highly speculative, these findings might suggest the following model of action for
this family of transmembrane proteins. First, a substrate, differing between the C1 and L1 subfamilies, is bound by the cytoplasmic extensions of helices I and III. Possibly, a further region in the first, large cytoplasmic loop (position 73–77 in Figure 3b) assists in substrate recognition. Second, the substrate is channeled into the membrane along one site of transmembrane helix II. Finally, it is presented to the catalytic center built by the CIVC motif residing in helix III (blue dots, Figure 3b).

**DISCUSSION**

Recent advances in genome sequencing technology have lead to a noticeable shift in focus toward methods dealing with contig- or genome-sized sequences, be it for contig assembly or phylogenomics. Nevertheless, accurately reconstructed MSAs on the gene or protein level are still of major importance. Most tools or algorithms introduced in this context are dedicated to a specific task like the reconstruction of phylogenetic trees, transmembrane prediction or conservation profiling.

The method we propose here is different in that it is a method for the explorative unsupervised analysis of MSAs. It decomposes the alignment into its major signals and co-clusters sequences and sites, thereby simultaneously finding sequence groups and the sites responsible for their grouping. The probabilistic model (pHMM) used to describe the alignment is a known and approved method for sequence modeling (10) and due to their nature the Fisher score embedding is advantageous to other embeddings proposed and applied before (3,13). These advantages include the possibility to directly

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**Figure 3.** Analysis of the VKOR sequence family. (a) Phylogenetic tree of the VKOR protein family. (b) Sequence logo of the MSA including the proposed membrane topology of VKORC1 with conserved positions for VKORC1L1 (44). The conserved VKORC1L1-specific amino acids are marked in yellow. Pink-labeled amino acids are specific to VKORC1L1 and to the VKORC1 protein of fish. In the third transmembrane domain, the blue circles symbolize the redox center (CIVC motive) and the supposed warfarin binding site with the TYA motive is highlighted in red. (c) Scatterplot of the second and third principal factors. Sequences are depicted as black circles, sites as red crosses. Closeness of sequences and sites in the plot shows strength of association.
model and encode transition probabilities of the pHMM and thereby insertions and deletions in the alignment. Further, apart from a pure probabilistic representation of the alignment itself, the HMM fitting process allows integration of prior knowledge about amino acid distributions. Biologically meaningful priors can be derived, e.g. via Dirichlet mixtures (47,48) or from log-odds-based substitution matrices (49). These incorporate the desired biological signal into the pHMM, giving, e.g. amino acid positions with similar chemical or physical properties, more similar probabilities than obtained from the alignment alone.

Fisher scores are known to be 'sufficient statistics' for the underlying HMM parameters, i.e. they contain all available information about the parameters (14). In contrast to a direct embedding via the HMM scores or site probabilities, they do not suffer from the effect that highly divergent, but from the HMM’s perspective equally probable sequences receive the same representation. This would project those unrelated sequences close to each other during the ordination step. Additionally, Fisher scores are a fixed-length representation of the original sequences, thus preventing length-driven biases in the analyses. Computational problems due to the high dimensionality of the Fisher score representation itself can be circumvented by application of the economy-sized SVD variant. The computational complexity of the Fisher score calculation is similar to the forward-backward algorithm \( O(N^2T) \) for \( N \) states and sequence length \( T \).

Even though our proposed method of ordination (CA) was originally designed for two-way contingency tables (17), it has been shown earlier that the method is very suitable for the analysis of continuous datasets, in which dependencies between rows and columns of a data matrix are of interest (31).

We compared our method to a standard approach of ordination with an Euclidean metric (e.g. PCA). Representatives are, for example, the SeqSpace and Jalview programs (3,13,50), although these tools additionally suffer from the inexpressiveness of the binary embedding employed. For a fair competition, we compared our CA decomposition to classical PCA on the same dataset, in both cases embedded via Fisher scores, and found CA to be more sensitive toward biological signals. For example, PCA analysis of the LP2086 dataset moved sequences ACB38144.1 and AC131835.1 (close to the blue HGT candidate group in Figure 2c) even though they do not share the 30 amino acid region characteristic for sequences of that cluster (Figure 2b). In the original CA ordination, they clearly separate from the other sequences of their cluster on the x-axis (the two points on the far right side of Figure 2c), but show no grouping with the HGT candidates. Similar effects were found in other regions of the sequence and in the VKOR example (data not shown). It seems that CA profits from application of the \( \chi^2 \) distance in that it focuses on sequence-site associations rather than simple one-way Euclidean ordination. We finally also directly loaded our datasets into Jalview, but as the software is missing the ordination of sites in the alignment, no functional annotation of sequence clusters could be made. The SeqSpace software, which is supposed to also cluster the sites, was not available anymore at the time of this writing.

The advantages of detecting associations in terms of the \( \chi^2 \) distance become apparent in the fHBP example. Neither sequence-based nor site-based methods are on their own able to detect any recombination event. Phylogenetic algorithms average over the length of the alignment, rightfully discarding the subtle 30 amino acid transfer region in the beginning of the alignment. The HGT never shows in the tree, it can be suspected from the evolutionary network, but due to the short length and the low number of representatives carrying the motif, the signal is only weakly reflected in the distance matrix and therefore in the split decomposition. Conservation profiles like sequence logos or clustering procedures on sites would not reveal the HGT either, which can only be identified by detection of incompatible sites (35), i.e. sites for which contradicting sequence clusters can be built. Our method was able to resolve the recombinated group and identify the responsible sites. It allows for an explorative analysis of the MSA without focusing on any specific type of signal, e.g. phylogenetic signals or HGT alone. It is important to note that this is by no means a test for recombination nor a method to thoroughly find all possible sites of HGT within an alignment, but it can provide an unbiased and structured view on an MSA from different perspectives.

Studying the VKOR protein family again showed how major phylogenetic signals appear on one of the first axes in the ordination, like separation of the C. savignyi outgroup. But it is also a good indication of how interesting features of the alignment are completely missed by sequence-based methods, like the phylogenetic tree, or site-base methods, like the depicted sequence logo alone. The co-clustering of species and sites, i.e. the identification of associations between the two, bring insight into the dependencies and—maybe—functional relations, between sequences in the alignment, thereby annotating them with the necessary sequence features. It showed us for example, that in contrast to the L1 fish sequences, the C1 fish sequences do not share the typical C1-L1 site differences of the other groups and identified the positions where those sequences differed. Recovering this tiny signal covered by the large phylogenetic trend would not be possible by methods considering complete sequences, as in the calculation of phylogenetic trees.

From these findings we are convinced that the method proposed here provides researchers with a new and unique way to analyze MSAs. Our method provides a structured decomposition of an alignment and depiction of its information content with increasing granularity. The modularity of the approach allows for a variety of statistical methods applicable to high-dimensional datasets to be used. Its explorative nature can give rise to hypotheses which might then be validated by, for example, statistical tests. On the modeling side, future work might extend the algorithm to include combined sequence structure alignments suitable for analysis of RNA sequences. In general, all types of sequential data (DNA, RNA and protein sequences) are in principle suitable for such an
analysis, provided they can be modeled in a probabilistic fashion via, for example, an HMM and from which Fisher scores can be derived.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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The software implementing the above described method is available on request from the authors.

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