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Accounting for Solvent Accessibility and Secondary Structure in Protein Phylogenetics is Clearly Beneficial

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ABSTRACT

Amino-acid substitution models are essential to most methods to infer phylogenies from protein data. These models represent the ways in which proteins evolve and substitutions accumulate along the course of time. It is widely accepted that the substitution processes vary depending on the structural configuration of the protein residues. However, this information is very rarely used in phylogenetic studies, though the three-dimensional structure of dozens of thousands of proteins has been elucidated. Here we reinvestigate the question in order to fill this gap. We use an improved estimation methodology and a very large database comprising 1,471 non-redundant globular protein alignments with structural annotations to estimate new amino-acid substitution models accounting for the secondary structure and solvent accessibility of the residues. These models incorporate a confidence coefficient which is estimated from the data and reflects the reliability and usefulness of structural annotations in the analyzed sequences. Our results with 300 independent test alignments show an impressive likelihood gain, compared to standard models such as JTT or WAG. Moreover, the use of these models induces significant topological changes in the inferred trees, which should be of primary interest to phylogeneticists. Our data, models and software are available for download from http://www.atgc-montpellier.fr/phyml-structure/.

Keywords: structural annotation of proteins; amino-acid substitutions; replacement rate matrices; mixture models; partition models; maximum-likelihood; topological impact.

It is widely recognized that evolutionary divergence of protein structures occurs much less rapidly than divergence of protein sequences (e.g. Chothia and Lesk, 1986). Structural constraints act on the protein sites, which in turn impact amino-acid substitutions. Notably, secondary structure and solvent accessibility have been shown to have a strong influence on amino-acid replacement processes (e.g. Koshi and Goldstein, 1995; Thorne et al., 1996; Goldman et al., 1998). For example, buried sites are mostly hydrophobic and tend to remain hydrophobic along the course of time, meaning that substitutions in the buried parts of the proteins most often occur between hydrophobic amino acids. This type of information is only partly captured by standard models of amino-acid substitution, which are based on the use of substitution rate matrices such as PAM (Dayhoff et al., 1972), JTT (Jones et al., 1992), WAG (Whelan and Goldman, 2001) or LG (Le and Gascuel, 2008). These matrices contain replacement rates between every amino-acid pair. These rates were estimated from very large databases, without differentiating among the various site structural configurations, thus resulting in an average model for average sites and proteins. Site-dependent models were thus estimated for several structural categories based on solvent accessibility and secondary structure, showing clear distinctions among categories, most notably between exposed and buried sites (Goldman et al., 1998; Holmes and Rubin, 2002). For example, our recent results (Le et al., 2008a) indicate that highly exposed sites evolve 3-4 times faster than buried sites, on average.

These studies on the variability of evolutionary processes across structural categories greatly improved our understanding of protein evolution. However, they are rarely used today in protein phylogenetics, despite the fact that substitution models are essential in most approaches to phylogenetic inference (e.g. in the estimation of evolutionary distances or the calculation of tree likelihoods; see textbooks: Felsenstein, 2003; Bryant et al., 2005; Yang, 2006). There are several reasons that these structure-based models have not been widely used. First, only a few three-dimensional (3D) structures were available in the 90's. Thus, these models do not use any information on the actual structure of the studied proteins. Each site is assumed to belong to each possible structural category, and the total site likelihood is the weighted average of all possibilities using a

mixture approach (e.g. Le et al., 2008a) or a more sophisticated Hidden Markov Model representing the dependence of structural states along the sequences (Thorne et al., 1996; Goldman et al., 1998). Second, when the 3D structure(s) is known for one (or a few) of the studied proteins, the structural annotations for the whole protein set may not be reliable. These annotations are inferred by homology from known structures, but structure is not fully conserved along evolution. Furthermore, structural annotation procedures rely on numerical thresholds (e.g. to classify buried/exposed sites) and definitions (e.g. using psi and phi angles for the secondary structures) which are somewhat arbitrary and induce uncertainties in the assignments of sites to structural categories. Third, previously implemented structure-based models did not incorporate any gamma distribution of rates across sites (Yang, 1993), which is now an important component in most sequence evolution models. All of these likely explain the low impact of these approaches in phylogenetics. Another factor might be the complexity of the calculations using software and computers of that time.

Today, a number of 3D protein structures are available. The protein data bank (PDB; Berman et al., 2000) contains more than 50,000 proteins. Moreover, using sequence homology we are able to infer likely structures for a number of homologous proteins. The HSSP data bank (Homology-derived StructureS of Proteins; Schneider et al., 1997) contains more than 2 million sequences, that is ~35% of UniProt. In this paper we claim that this information should be used in phylogenetics to build more accurate trees. We use a very large database (extracted from HSSP) of globular protein alignments to estimate new amino-acid replacement matrices for various site structural configurations based on solvent accessibility and secondary structure. We use these matrices in simple substitution models that account for (1) site structural configurations, (2) the fact that structural annotations may not be fully reliable, and (3) the variability of rates across sites. We introduce fast methods and programs to estimate our models and infer phylogenetic trees using these models. The performance of these models and programs is assessed using 300 independent test alignments. We show that using our structural models greatly augments the likelihood values of inferred trees. Moreover, the tree topology is often different in comparison to standard models (e.g. JTT, WAG, or LG). In the

following, we describe our data sets, then the models and their estimation and implementation, and finally the experiments and results. Supplementary material is provided in an online Appendix (http://www.sysbio.oxfordjournals.org). Our data, replacement matrices, detailed results for all test alignments, and software are downloadable from http://atgc.lirmm.fr/phyml-structure/.

DATA SETS

To estimate our models we used a large database of multiple globular protein alignments. It was extracted from HSSP, which comprises ~50,000 alignments of protein families. Each alignment is obtained by aligning a (seed) protein with known 3D structure in the protein data bank (PDB), to all its sequence homologues in Uniprot. The secondary structure and solvent accessibility of the seed protein are calculated using DSSP (Kabsch and Sander, 1983) and are likely to be representative of the structure of all homologues in the alignment.

HSSP is highly redundant and contains a number of gaps. We thus performed an intensive cleaning of HSSP to extract independent alignments and, within each of the alignments, to select sequences and sites corresponding to well aligned, non-gapped regions. Moreover, we discarded membrane proteins (based on their presence in the Membrane Protein Data Bank, Raman et al., 2006), as they show quite specific patterns of amino-acid replacement (Jones et al., 1994). We obtained this way a database comprising 1,771 non-redundant alignments, with an average of ~56 sequences and ~254 sites per alignment, ~27 million amino-acids in total and very few gaps (<0.1%). Moreover, each of these alignments contains the seed protein of the original HSSP alignment and thus (relatively) reliable annotations on the solvent accessibility and secondary structure of each of the sites. Using these annotations we classified each site as extended (E), alpha-helix (H) or other (S, T, B, G, I, "." or "?"). Based on their relative solvent accessibility (Shrake and Rupley, 1973), we also classified sites as either buried or exposed; we used a 10% threshold of relative accessibility, as in Goldman et al. (1998) and several other studies. This threshold induces nearly equally weighted

buried and exposed categories. Finally, we randomly selected 300 alignments for model comparison, while we used the remaining 1,471 to train our models.

We already used this cleaned alignment database to estimate mixture models using amino-acid replacement matrices (Le et al., 2008a) and profiles (Le et al., 2008b). Readers are referred to these papers for details on the cleaning procedure. Note that the purpose of these studies was different of this one. Indeed, in mixture approaches the protein structure is supposed to be unknown when inferring trees, while here we shall use the available structural annotations to improve the accuracy of substitution models and consequently the reliability of tree reconstructions.

MODELS

Our models involve amino-acid replacement matrices that differ depending on the structural properties of the sites (e.g. exposed/buried). We combine these matrices, accounting for both the structural annotations and the fact that this information may not be fully reliable. We first describe the replacement matrices and the way we estimated them from the training data, and then various models to combine these matrices.

Amino-acid Replacement Matrices

We estimated replacement rate matrices for several site partitions based on structural annotations. All of these matrices comply with the general time-reversible (GTR) model, which was first proposed for DNA sequences (Lanave et al., 1984; Tavaré, 1986), and then applied to proteins through a number of empirical (as opposed to mechanistic) models such as PAM, JTT, WAG or LG (see textbooks, e.g. Yang, 2006; see also online Appendix 1). One matrix was estimated per site category, and three site partitions were analyzed:

- EX is a two-category partition corresponding to exposed/buried sites.
- EHO is a three-category partition corresponding to extended/alpha-helix/other sites.

- EX_EHO is a six-category partition that combines both previous criteria; sites are classified as: exposed & extended, buried & extended, exposed & alpha-helix, buried & alpha-helix, exposed & other, or buried & other.

To estimate the corresponding (2+3+6=11) replacement matrices, we applied the procedure detailed in Le and Gascuel (2008) and Le et al. (2008a) to each site category. This procedure is based on the maximum-likelihood (ML) principle and uses XRate (Klosterman et al., 2006) and a new version of PhyML (Guindon and Gascuel, 2003) which implements our multiple-matrix models (see below). A gamma-distribution of rates across sites (Yang, 1993) is incorporated, in both the tree inference and the matrix estimation. PhyML is used to estimate the phylogenies of each of the 1,471 training alignments using all the sites. In the next step we treat the phylogenies as fixed. We preprocess the data to account for rate heterogeneity, and then use XRate to estimate the replacement matrix of each structural category separately. Preprocessing involves assigning each site to the most likely rate category and rescaling the phylogeny associated to this site accordingly (Le and Gascuel, 2008). This procedure is iterated: when a first set of matrices corresponding to the site partition has been estimated, the phylogenies are re-inferred using this new model, the replacement matrices are re-estimated based on the new phylogenies, and so on until convergence. All together, this estimation procedure improves considerably over standard counting approaches that were used to estimate PAM and JTT matrices, as well as all matrices studied by Thorne et al. (1996) and Goldman et al. (1998). It also outperforms Whelan and Goldman's (2001) ML procedure, thanks to the use of rates across sites in the matrix estimation (Le and Gascuel, 2008).

All these matrices are available from our web site and are compared to the (average) LG matrix. EX and EHO matrices have already been analyzed in a mixture context (Le et al., 2008a). One of the main observations (see also Goldman et al., 1998) is that the global average substitution rate is much higher for exposed than for buried sites (ratio=2.45). This is an expected result as buried sites are subject to strong structural constraints, while exposed sites are often in the flexible and variable parts of the proteins. On the other hand, the global average rate does not change much among

secondary-structure categories, some sites within each category being with strong structural constraints and the others not so. Moreover, each matrix defines an equilibrium distribution of amino-acids. As expected, amino-acid distributions are quite different among site categories. For example, the buried category mostly contains hydrophobic amino-acids, while the helix category contains a large proportion of alanines but very few prolines (commonly called "helix-breakers"). Finally, the rates in the matrices (once normalized, i.e. divided by the global average rate) also differ among categories, but to a lesser extent than amino-acid compositions. Again, the exposed/buried partition induces higher contrasts among site categories than the extended/helix/other partition, and thus is expected to bring more information on the substitution processes.

The secondary structure does not impose homogeneous structural constraints within each category. For example, the "other" category contains some deeply buried residues situated in the core of the proteins, as well as residues in the flexible regions. In the same way, it is well known (e.g. Branden and Tooze, 1999) that a number of alpha-helices are amphiphilic, with a side on the protein surface and the other side in the protein core with strong structural constraints. The 6-category partition EX_EHO makes the secondary-structure categorization effective. Indeed, there is a high contrast among EX_EHO categories. The slowest category is "buried & other" (global rate = 0.535), the fastest "exposed & helix" (global rate=2.064), and the two buried/exposed helix categories clearly differ (rate ratio ~2.8). Matrix entries are also more distant from average LG matrix, than are the entries in uncombined EX and EHO matrices. We thus expect good performance of this crossed site partition in modeling amino-acid substitutions.

Models to Account for Site Structural Annotations

Amino-acid replacement matrices are used to compute the likelihood of the data, given a tree with branch lengths, and the ML principle involves maximizing this likelihood to search for the optimal tree (in most cases one must be satisfied with near optimal trees, for computing time reasons). Let D be the analyzed alignment and D_i the data at site i. When all sites are assumed to belong to a

single average category with replacement matrix **Q** (e.g. JTT or WAG), the tree likelihood is expressed using the independence assumption as

$$L\left(T,\mathbf{Q},\Theta\middle|D\right)=\prod_i L\left(T,\mathbf{Q},\Theta\middle|D_i\right), \tag{1}$$

where the product runs over all the sites; T is the tree (including branch lengths), Θ the set of additional model parameters (typically the gamma distribution parameter), and $L\left(T,\mathbf{Q},\Theta\middle|D_i\right)$ the likelihood of the data at site i.

When the sites are classified into several known categories, the standard approach, called "partition model", uses this knowledge in a natural way. The partition model is commonly used to account for different site categories depending on codon positions or genes in concatenated alignments (e.g. Rannala and Yang, 2008). Here, we consider structural categories, but the mathematical formulation remains the same. Let C denote any given category and $\mathcal{Q}=\left\{\mathbf{Q}_C\right\}$ be the set of replacement matrices corresponding to the studied categories; for example, $\mathcal{Q}$ may contain the exposed and buried matrices for the EX model. Moreover, let C_i be the category of site i (e.g. "buried" or "exposed"), and $\left\{C_i\right\}$ the set of assignments for each site. Then, the tree likelihood is equal to

$$L\left(T,\mathcal{Q},\left\{C_i\right\},\Theta\middle|D\right)=\prod_i L\left(T,\mathbf{Q}_{C_i},\Theta\middle|D_i\right). \tag{2}$$

In other words, for each site we simply use its associated replacement matrix. No free parameter is added in comparison with single-matrix-based Equation (1), and the computing time remains basically unchanged (at least when no extra parameters are added in Θ, see below). Even though this approach is simple and natural, we have not been able to find any paper relating its performance with structural categories. Part of the explanation derives from the fact that structural annotations may be poor or non-informative for some alignments. In these cases, some sites are analyzed with inappropriate replacement matrices and the results may be worse than using a single average matrix as in Equation (1).

We refined the partition model to account for this uncertainty in structural category assignment. A confidence coefficient, denoted as χ, measures the reliability of structural annotations. χ represents the fraction of sites with reliable annotations in the alignment. When χ is equal to 1, all structural annotations are used, just as in Equation (2). When χ is less than 1, some annotations are not reliable or not useful in a phylogenetic context. Given an alignment, all the sites are analyzed using a single χ value which is a free parameter estimated by maximum likelihood from the data. Moreover, we assume that no additional knowledge is available to detect the sites with poor annotations (this hypothesis will be further discussed). For each site we thus envisage two possibilities (reliable/not-reliable) and the site likelihood is the weighted average of both corresponding likelihoods, following the law of total probability. We studied two approaches to deal with non-reliable annotations. In the simplest one, we use a single standard average replacement matrix denoted as **Q** (here LG), and the tree likelihood is equal to:

$$L\left(T,\mathcal{Q},\{C_i\},\Theta|D\right)=\prod_i\left[\chi L\left(T,\mathbf{Q}_{C_i},\Theta|D_i\right)+\left(1-\chi\right)L\left(T,\mathbf{Q},\Theta|D_i\right)\right]. \quad \textbf{(3)}$$

In other words, we make a weighted sum of Equation (1) and Equation (2), assuming that **Q** is well suited to model the substitutions of sites with poor structural annotations. When χ is zero, structural annotations are not used and the sites are analyzed with **Q** only.

In the second approach we replace the single **Q** matrix in Equation (3), by a mixture of matrices (models) corresponding to all categories in the site partition. Mixtures are the standard approach when we assume that the heterogeneity of substitution processes derives from various site categories corresponding to different evolutionary constraints, but ignore the classification of the sites into these categories. Mixtures have been proposed as part of several substitution models (e.g. Pagel and Meade, 2005), including with structural site categories where they show a clear improvement over single-matrix models (Le et al., 2008a). Let P_C be the probability of a site belonging to category *C*. Using (again) the law of total probability the tree likelihood is given by

$$L\left(T,\mathcal{Q},\Theta|D\right)=\prod_i\left[\sum_C P_C L\left(T,\mathbf{Q}_C,\Theta|D_i\right)\right], \quad \textbf{(4)}$$

where the total site likelihood is the weighted sum of the site likelihoods for all possible categories. This approach performs well when the site partition is relevant for the analyzed data set. In this case, the likelihood of each site with the proper (unknown) category is much larger than the likelihood with the average model, and the weighted sum in Equation (4) tends to be larger than the single term in Equation (1). As such a case is encountered with most data sets (Le et al., 2008a), we combine Equation (2) with Equation (4) to obtain

$$L\left(T,\mathcal{Q},\{C_i\},\Theta|D\right)=\prod_i\left[\chi L\left(T,\mathbf{Q}_{C_i},\Theta|D_i\right)+\left(1-\chi\right)\sum_C P_C L\left(T,\mathbf{Q}_C,\Theta|D_i\right)\right]. \quad \textbf{(5)}$$

When χ is 1 (annotations are fully reliable) this becomes identical to the partition model (2), while when χ is zero (annotations are just random) we use the mixture (4). With mixtures, P_C represents the probability of category *C*. Structural category proportions are not the same for all proteins, some only have alpha helices, some beta sheets only, while some have both (e.g. Branden and Tooze 1999). P_C proportions thus are free parameters to be estimated for each analyzed data set. With our compound model (5), the interpretation of P_C proportions is slightly different, as they represent the category proportions among sites with non-reliable annotations. Again, these proportions are free parameters estimated from the data for each alignment separately.

As mentioned above, Θ contains additional model parameters. In our case, Θ contains the shape parameter of the gamma distribution which defines the variability of rates across sites (Yang, 1993). This is used within every structural category, including in Equation (1) where all sites are analyzed with a unique replacement matrix. As usual, the gamma distribution parameter is free and estimated from the analyzed data. More sophisticated models are conceivable, with different gamma distributions among structural categories, but preliminary experiments (not shown) did not reveal any significant improvement, and a single gamma distribution of rates is used in the following.

When using a single gamma distribution, the partition model (2) does not add any free parameter to standard model (1). Our first model (3) to account for uncertainty adds a single free parameter (χ) to be estimated from the analyzed data. Let c be the number of site categories; the mixture approach (4) adds $c-1$ free parameters (category proportions) compared to the standard model, while our second approach to accommodate for uncertainty (5) adds c free parameters (χ and category proportions). Because of the nested relationships of these models, the likelihood value using the confidence-based model (3) is guaranteed to be higher than (or equal to) the likelihood values of the standard (1) and partition (2) models. In the same way, the confidence-based model (5) is guaranteed to obtain better (or equal) likelihood values than (to) the partition (2) and mixture (4) models, at least when the same tree topology is analyzed. However, these gains in likelihood could be counterbalanced by the larger number of free parameters, necessitating a penalized likelihood criterion such as AIC (Akaike, 1974).

These models involve variable computing times, which are basically proportional to the number of categories used to analyze each of the sites (Bryant et al., 2005). The partition model (2) thus requires nearly the same time as the standard model (1), while the confidence-based model (3) is two times slower than the standard model, and the mixture (4) and confidence-based (5) models are c times slower. The same ratios apply to memory consumption. However, this analysis is based on tree likelihood calculation only, while model parameter estimation also induces significant computational cost increase compared to the standard model (see illustrative examples below).

All these models are implemented in a new version of PhyML (available from http://atgc.lirmm.fr/phyml-structure/). An initial ML tree is first inferred with LG in the usual manner, and then the chosen model is used to refine this first tree, in terms of both topology and branch lengths, with the model parameters (χ value, category proportions and gamma shape parameter) being adjusted along the way.

RESULTS AND DISCUSSION

In this section we present the results obtained with the 300 HSSP test alignments: (1) we show the likelihood gains provided by our models compared to standard approaches; (2) we analyze the values of our confidence coefficient (χ) regarding the various structural categories; (3) we discuss the impact on the topologies of inferred trees.

Likelihood Gains

We used the 300 HSSP test alignments to compare standard single-matrix models (JTT, WAG and LG) to our new models based on EX (solvent accessibility), EHO (secondary structure) and EX_EHO (both solvent accessibility and secondary structure) site partitions. For each of the multi-matrix models, we used the four combinations detailed in the previous section:

- MIX is the mixture approach, expressed by Equation (4), where the site categories are supposed to be unknown.
- PART is the partition approach, expressed by Equation (2), where one uses the known HSSP site categories.
- CONF/LG is our first confidence-based approach, expressed by Equation (3), where sites with unreliable annotations are analyzed using LG (HSSP version, see below).
- CONF/MIX is our second confidence-based approach, expressed by Equation (5), where sites with unreliable annotations are analyzed using a mixture.

These abbreviations are combined with the name of the studied site partition; for example, EX_PART is the partition model with exposed and buried categories. All models were run using the new version of PhyML (http://atgc.lirmm.fr/phyml-structure/) with 4 gamma rate categories ($\Gamma 4$), BioNJ (Gascuel, 1997) starting tree, and SPR-based tree topology search (Hordijk and Gascuel, 2005; Guindon et al., 2009).

For all models, we measured the AIC criterion (Akaike, 1974) on each of the test alignments:

$$AIC(M,D) = 2\#\,parameters(M) - 2\,ln\,L(M,T|D),$$

where: $\#\,parameters(M)$ is the number of free parameters of model *M*; $ln\,L(M,T|D)$ is the log-likelihood value of alignment *D* given model *M* and inferred tree *T*. This criterion has to be minimized; best scores are given to models with low number of free parameters and high likelihood value. We also computed the average AIC per site of model *M* for all test alignments, which is simply

$$AIC/site(M) = \sum_D AIC(M,D) \Big/ \sum_D s, \qquad \textbf{(6)}$$

where *s* is the number of sites in *D*. All models were compared to LG using criterion (6) by computing the difference $AIC/site(\mathrm{LG}) - AIC/site(M)$, which is positive when *M* has a better fit than LG. To complete this global average result, we also compared all models to LG by counting the number of alignments where $AIC(M,D)$ is better/worse than $AIC(\mathrm{LG},D)$. Moreover, to assess the statistical significance of the observed difference between *M* and LG for any given alignment, we used a Kishino-Hasegawa (KH; 1989) test with $p<0.01$. As the number of free parameters may differ between *M* and LG, we used AIC-corrected log-likelihood values instead of simple log-likelihood values (see Shimodaira, 1997, for explanations and justifications of this procedure).

Average AIC results using criterion (6) are displayed in Figure 1, while Figure 2 provides the number of alignments where each model is (significantly) better/worse than LG. Main observations and conclusions are as follows.

The improvement from JTT to WAG and then LG is quite significant. The AIC gain per site of LG compared to JTT is 0.71, meaning that with 300 sites (standard length of protein alignments) the gain is as high as ~200 AIC points, that is ~100 log-likelihood points. Moreover, LG is often significantly better than JTT and WAG, but rarely worse.

We also tested a new version of LG, obtained from HSSP instead of Pfam (Bateman et al., 2002), but using the same estimation procedure as for original LG (Le and Gascuel, 2008). The aim

was to check that the improvements provided by our new models do not derive from HSSP training alignments, but from the models themselves. Indeed, this HSSP version is only slightly better than original LG with the 300 HSSP test alignments (AIC gain per site ~0.02). Both LG versions are actually very close (the correlation of log-entries is equal to 0.98), which shows that LG matrices are relatively stable when estimated from different general databases.

All our multiple-matrix models improve a lot compared to single-matrix models, including LG. For example, the gain between our best model (EX_EHO_CONF/MIX; both solvent-accessibility and secondary-structure site partitions, non-reliable sites are analyzed using a mixture) and JTT is 1.96 AIC points per site, meaning that with 300 sites the gain will be close to 600 AIC points. This gain is of the same magnitude as that provided by the use of a gamma distribution of rates across sites. For example, the gain between LG with and without gamma distribution equals 2.90 with HSSP test alignments, and similar values are found for JTT and WAG. We thus believe that multiple-matrix models should become standard in the near future, just as the gamma distribution is standard today and used in most phylogenetic analyses.

However, using a gamma distribution of rates across sites is still required with multiple-matrix models, though they involve site categories with variable global rates. For example, EX_EHO_ CONF/MIX without gamma distribution is not any better than LG+Γ4 (AIC gain per site < 0.01). Another example is PASSML (Lio et al., 1998), which is clearly worse than JTT+Γ4 due to its lack of gamma distribution (AIC difference per site ~0.5; see online Appendix 2 for comparisons of PASSML with our models). These results show that sites within structural categories are not subject to the same constraints and do not evolve at the same rate. However, part of the rate heterogeneity is accounted for by the structural categories and their variable global rates; the estimated value of the gamma distribution parameter tends to be higher (i.e. the rate variability is lower) with our multi-matrix models (~0.9 on average) than with single-matrix models (~0.65 on average).

Overall, the site partition based on solvent accessibility (EX) has higher gains than the secondary structure (EHO) partition, while the combination of both (EX_EHO) is clearly best. As discussed earlier, this illustrates that secondary structures are not homogeneous, with highly conserved, hydrophobic sites, and other sites situated in the exposed and variable regions. However, EHO models clearly improve over single matrices, as they still account for a part of site heterogeneity.

The partition approach (PART) performs better than mixtures (MIX), except with EHO where both obtain similar average performance (see online Appendix 3 for significance results). This is explained by the fact that secondary structure annotation is somewhat arbitrary and not fully reliable; for example, the extremities of α helices are typically difficult to define. A quantitative measurement of this uncertainty is provided below. With reliable annotations PART benefits this information and outperforms MIX.

Our confidence-based models (CONF/LG, which uses LG for unreliable sites, and CONF/MIX using a mixture instead of LG) clearly improve over both PART and MIX. Due to nested relationships (see above), CONF/MIX always has higher likelihood values than MIX and PART. Figure 3 shows that the AIC differences are significant in a large number of cases: CONF/MIX is significantly better than MIX with >200 alignments, and significantly better than PART with 55 to 136 alignments. The AIC differences between CONF/LG, MIX and PART are positive in most cases, but less often significant (see online Appendix 3). All of these indicate that some alignments (some sites within some alignments) are poorly annotated from a phylogenetic perspective, and that our simple confidence-based models offer efficient solutions to deal with this uncertainty. CONF/MIX has better AIC values than CONF/LG since unreliable sites are analyzed with a mixture that outperforms LG in most cases. However CONF/LG is clearly of interest with EX_EHO site partition where it is about 3 times faster than CONF/MIX with similar likelihood performance. Actually, EX_EHO_CONF/LG is even faster than EX_CONF/MIX and EHO_CONF/MIX, while providing much better likelihood values. Its interest would be even higher with a larger number of categories,

for example combining 3 solvent accessibility categories (Le et al., 2008a) or 4 secondary structure ones (Goldman et al., 1998), with a computing time still nearly equals to twice that required by standard single-matrix models.

The computing time required by our best (and slowest) model, EX_EHO_CONF/MIX, is ~9 times the computing time required by LG with the same program options (Γ4, SPR, etc). The average running time with the test alignments (~56 sequences and ~254 sites on average) equals 40 minutes and 4.5 minutes per alignment using a standard PC (Intel(R) Xeon(R) 1.86GHz) for EX_EHO_CONF/MIX and LG, respectively. The slowest alignment (1o98, 92 taxa and 352 sites) requires 270 and 30 minutes for EX_EHO_CONF/MIX and LG, respectively, but a number of alignments are dealt with much faster. Other models require running times in between these two extreme models. The observed ratio of ~9 between EX_EHO_CONF/MIX and LG is significantly higher than 6, as expected based on tree-likelihood calculation (see above). The difference is basically explained by the estimation of model parameters (i.e. χ, 5 category proportions, and the gamma distribution parameter) which are highly correlated.

All together, these results strongly support the use in phylogenetics of solvent accessibility and secondary structure annotations. Using this information in the most appropriate way (EX_EHO_CONF/LG and EX_EHO_CONF/MIX models) provides very high AIC gains compared to the best known single matrices (LG, WAG or JTT), and these gains are significant for most alignments (~290/300 in our experiments). Moreover, computing times are still acceptable for most data sets using standard computers.

Reliability and Usefulness of Structural Annotations

Our confidence coefficient χ in Equations (3) and (5) is estimated separately for each of the alignments and reflects the reliability of the annotations in the given alignment. Actually, χ combines several factors: (1) precision of the annotation which may be somewhat arbitrary, for example at both α helix extremities; (2) reliability of the annotation, as some errors may be induced by the complex

computations to infer them from crystallographic or NMR data (3D structure elucidation, multiple alignment, DSSP, etc); (3) conservation of the annotation, as the structures of proteins in the multiple alignment may have evolved and become slightly different to the structure of the seed protein; (4) usefulness of the annotation, which can be more or less appropriate to analyze site evolution in a phylogenetic context.

Figure 4 plots the distribution of χ depending on the site partition with CONF/MIX model. The solvent accessibility-based partition (EX) is associated with the highest χ values (i.e. the most reliable annotations), while EHO corresponds to the lowest ones. This is an expected result, as solvent accessibility is well defined and associated with high likelihood gains (Fig. 1), while secondary structure annotations are not fully precise and useful, as seen from Figure 1 where the partition approach (PART) is not any better than the mixture (MIX). When combining these two site partitions to obtain EX_EHO, the χ values are intermediary. This reflects both the uncertainty in EX_EHO annotations, which cannot be more reliable and precise than EHO annotations, and the fact that the 6 EX_EHO categories are specially relevant and useful in a phylogenetic context. Overall, we thus see that χ combines several different aspects. Moreover, Figure 4 shows that the χ values are highly variable, ranging from ~0 to ~1. Further investigation would be needed to characterize the sites and alignments with poor annotation, and build new models, for example with χ values modulated along the sequences (typically close to 0 at α helix extremities) and among proteins (e.g. disregarding secondary structure with small proteins dominated by disulfide bridges).

Topological Impact

The true tree is usually unknown with real data (as opposed to simulated data), and thus it is hard to assess the topological accuracy induced by any tree building approach in a realistic setting. Here, we studied the topological impact of our models, that is, whether using these models we frequently infer trees that differ from those inferred with standard models.

Using the 300 HSSP test alignments and previous experimental conditions (Γ4, SPR tree search, etc), we compared the topologies obtained with our multiple-matrix models to the LG topologies. We counted the number of alignments where the tree built using any given model *M* is not the same as the tree inferred with LG. Both *M* and LG trees were also compared using the Robinson and Foulds (1981) distance, which is the number of clades that belong to one tree but not to the other. When different topologies are found, one should prefer the one with best likelihood value, or best AIC (or similar criterion) value, when evolutionary models used for tree inference involve different numbers of parameters. However, the difference may be slight and non-significant, so one cannot reject the topology with a lower fit to data. We thus counted the number of alignments where *M* and LG topologies differ, and where *M* is significantly better than LG, using a KH test on AIC values with $p<0.01$.

Finally, we checked that the observed topological differences comprised some clades with significant support. Indeed, the topological impact would be low if all differences corresponded to poorly supported clades. To this end we performed bootstrap analyses and compared the topologies and clade supports of EX_EHO_CONF/MIX (our best model), LG and JTT. We counted the number of clades with notable bootstrap support (BP1 ≥ 50%) in one tree, which were not recovered in the other tree, or had a much lower support in this tree (BP2 + 50% ≤ BP1). For example, one clade with (BP1 = 40%) was not counted, even when it was not recovered in the other tree; on the opposite, one clade with (BP1 = 80%) in one tree was counted when it was found in the other tree with (BP2 = 20%). This measure thus summarizes the topological and clade support differences. We used only 50 bootstrap replicates for computing time reasons, but the differences in clade supports corresponding to our gap of 50% between BP1 and BP2 are highly significant (p-value ~ 0.0 using a Z-test for two proportions). Moreover, 50% of bootstrap support was shown to be optimal in terms of topological error (Berry and Gascuel, 1996; see also Holder et al., 2008).

Results are displayed in Table 1. We see that all models frequently infer topologies that differ from LG topologies. Moreover, these topologies are significantly better than LG topologies in most

cases. For example, EX_EHO_CONF/MIX finds 230 topologies that differ from LG ones, and among these 225 correspond to significant AIC gains. In practice, one should thus retain EX_EHO_CONF/MIX topology and discard LG one with 225 alignments among 300. The difference with JTT is even larger, as EX_EHO_CONF/MIX topology should be retained with 248 alignments. The topological distance between these trees is also appreciable. Comparing LG and EX_EHO_CONF/MIX, the average distance equals 0.136, meaning that on average the LG and EX_EHO_CONF/MIX topologies differ with ~11 clades (among ~106 on average). With JTT the average topological distance with EX_EHO_CONF/MIX trees is even larger and corresponds to ~14 clades on average.

Bootstrap analyses result in 171 alignments where LG and EX_EHO_CONF/MIX trees differ by at least one clade with notable support (BP1 ≥ BP2 + 50%, see above); the total number of such clades equals 338 with all 300 HSSP test alignments. Comparing JTT and EX_EHO_CONF/MIX, we found 213 alignments where both trees have notable differences, corresponding to 509 clades in total. These numbers of clades (< 2 per alignment on average) are low compared to the topological differences obtained with the standard Robinson and Foulds topological distance. This effect was expected, as it is commonly observed in phylogenetics that clades with strong support tend to be recovered with all models and inference methods. To scale the differences observed between JTT, LG and EX_EHO_CONF/MIX, we thus used the same protocol to compare LG+Γ4 (as in former experiments) and LG without gamma distribution of rates across sites; we found 209 alignments where trees show notable differences, corresponding to 510 clades in total. These results are very close to those of JTT versus EX_EHO_CONF/MIX. This indicates that the topological impact of our models versus standard models (JTT, WAG, LG) is in the same range as that induced by the use of a gamma distribution of rates, a finding which is consistent with the results above on likelihood gains.

Table 1 (without regard on branch supports) seems to indicate that the topological impact of CONF/LG models is lower than that of CONF/MIX ones. Most notably, the topological distance with LG trees is about twice lower for CONF/LG than for CONF/MIX, thus showing some (expected)

closeness between CONF/LG and LG. However, when only counting clades with notable support difference (BP1 ≥ BP2 + 50%, see above), CONF/LG has similar impact as CONF/MIX. Comparing EX_EHO_CONF/LG and JTT (LG), we found 225 (168) alignments where both inferred trees have notable differences, corresponding to 530 (323) clades in total. With JTT, these results are even a bit higher than those obtained by EX_EHO_CONF/MIX (209 alignments corresponding to 510 clades). Moreover, as expected, there is a few notable differences between EX_EHO_CONF/LG and EX_EHO_CONF/MIX (61 clades in total). This further supports the use of CONF/LG models, which obtain similar likelihood values and topologies as CONF/MIX models, but require much lower computing times.

All together, these results demonstrate that our models have a notable topological impact. Although it is not clear whether the resulting topologies are closer to the true phylogenies, they are, in most cases, different from the topologies inferred using standard models (JTT, WAG, LG …), with significantly higher likelihood values, and new well supported clades. These alternative topologies should thus be of great interest for phylogeneticists. Just as was observed with other substitution model improvements (e.g. transition/transversion for DNA in Kimura (1980), or gamma distributed site rates in ML methods by Yang (1993)), it is most likely that the high likelihood gains obtained here, will also result in more accurate phylogeny reconstructions.

CONCLUSIONS AND PERSPECTIVES

We have described simple phylogenetic models to benefit from the structural annotations available for a large number of proteins (~50,000 entries in PDB, ~35% of Uniprot covered by HSSP). These models continue previous research, mainly by Koshi and Goldstein (1995), Thorne et al. (1996), and Goldman et al. (1998). Our results confirm their observations that evolutionary processes are quite heterogeneous depending on site structural configurations, and that highly significant likelihood gains are obtained when accounting for this heterogeneity. The main difference between these previous studies and our paper is that we explicitly use available structural annotations,

while their approaches were based on mixtures or HMMs, where all structural categories were envisaged for each site, a solution imposed by the low number of 3D structures available in the 1990's. Moreover, our models cope with the uncertainty inherent in structural annotations, and include a gamma distribution of rates across sites, which is an essential component in substitution modeling. All together, our models provide likelihood gains over standard models (JTT, WAG, LG) which are of the same magnitude as the gain obtained by adding a gamma distribution of site rates to standard models. The topological impact of these new models is also notable and (again) in the same range as that induced by the use of a gamma distribution with standard models. We thus believe that accounting for structural annotations in protein phylogenetics should become routine in near future, using our models or their foreseeable refinements.

Several research directions deserve to be explored. First some tools should be implemented to connect the phylogeny domain to structural bioinformatics. For example, extracting and synthesizing the structural annotations associated with multiple alignments is still a difficult task, despite the progress of some recent web servers (e.g. Pollastri et al., 2007). Moreover, our models could be refined in several ways, for example, using different structural categories (e.g. 3 exposure classes, other groupings of the 8 DSSP secondary structure states), or studying solutions with variable confidence coefficients (χ) among site categories or along the sequences. Finally, we have described general models for globular proteins, but it is well known that substitution processes vary among life domains (e.g. apicomplexa or viruses) and protein groups (e.g. mitochondrial or membrane proteins). Specific substitution rate matrices and models should be estimated and implemented to analyze these data and non-globular proteins.

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Table 1: Topological impact

	MIX	PART	CONF/LG	CONF/MIX
EX				
≠ LG top	210 (158)	219 (171)	172 (162)	207 (191)
R&F LG top	0.127	0.140	0.061	0.117
EHO				
≠ LG top	177 (122)	200 (108)	157 (110)	186 (147)
R&F LG top	0.098	0.117	0.053	0.103
EX_EHO				
≠ LG top	214 (184)	232 (208)	195 (190)	230 (225)
R&F LG top	0.132	0.157	0.075	0.136

These results are obtained with the 300 HSSP test alignments. "≠ LG top" is the number of alignments (among 300) where given model and LG topologies differ; the bracketed number is the number of cases where given model topology has significantly better AIC value than LG topology (Kishino-Hasegawa test on AIC values with $p<0.01$; each topology is analyzed with its own model). "R&F LG top" is the average Robinson and Foulds (1981) topological distance between given model and LG topologies for all 300 alignments; this distance is normalized between 0 (both trees are identical) and 1 (they do not share any clade in common). MIX: mixture model; PART: partition model; CONF/LG: confidence-based model using LG for non-reliable sites; CONF/MIX: confidence-based model using mixtures for non-reliable sites; EX: solvent accessibility-based site partition; EHO: secondary structure-based site partition; EX_EHO: combination of EX and EHO site partitions.

FIGURE CAPTIONS

Figure 1: AIC gain per site compared to LG (and WAG and JTT)

All models are compared to LG (Le and Gascuel, 2008) using the average AIC/site criterion (Equation (6)), estimated with the 300 HSSP test alignments. Both WAG (Whelan and Goldman, 2001) and JTT (Jones et al., 1992) are worse than LG, with a negative difference of -0.30 and -0.71, respectively. All multiple-matrix models are better than LG with positive AIC/site difference. MIX: mixture model; PART: partition model; CONF/LG: confidence-based model using LG for non-reliable sites; CONF/MIX: confidence-based model using mixtures for non-reliable sites; EX: solvent-accessibility-based site partition; EHO: secondary-structure-based site partition; EX_EHO: combination of EX and EHO site partitions.

Figure 2: number of alignments with better/worse likelihood values than LG

Number of alignments (among the 300 HSSP test alignments) where each model provides a better (positive side) and a worse (negative side) likelihood value than LG. The black bars correspond to the numbers of significant differences using the Kishino-Hasegawa test on AIC values with $p<0.01$. MIX: mixture model; PART: partition model; CONF/LG: confidence-based model using LG for non-reliable sites; CONF/MIX: confidence-based model using mixtures for non-reliable sites; EX: solvent-accessibility-based site partition; EHO: secondary-structure-based site partition; EX_EHO: combination of EX and EHO site partitions.

Figure 3: Comparison of CONF/MIX, PART and MIX

Number of alignments where CONF/MIX (confidence-based model using mixtures for non-reliable sites) is significantly better than MIX (mixture model) and PART (partition model), using the Kishino-Hasegawa test on AIC values with $p<0.01$. EX: solvent-accessibility-based site partition; EHO: secondary-structure-based site partition; EX_EHO: combination of EX and EHO site partitions.

Figure 4: Distribution of the confidence coefficient χ depending on the site partition

This graph was obtained with CONF/MIX (confidence-based model using mixtures for non-reliable sites) and the 300 HSSP test alignments. When χ equals 1 annotations are fully reliable, while $\chi=0$ means that available annotations are not any better than random guessing. EX: solvent-accessibility-based site partition; EHO: secondary-structure-based site partition; EX_EHO: combination of EX and EHO site partitions.

Figure 1: AIC gain per site compared to LG (and WAG and JTT)

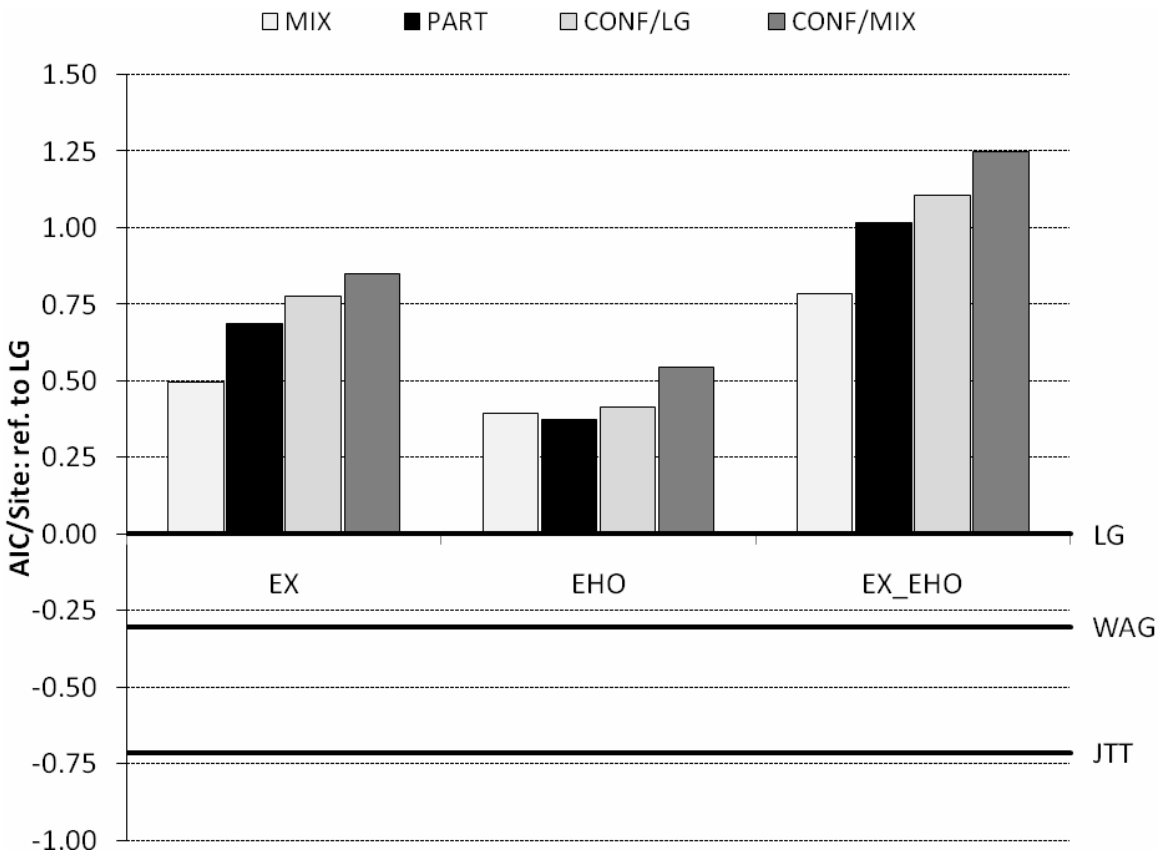


Figure 2: Number of alignments with better/worse likelihood values than LG

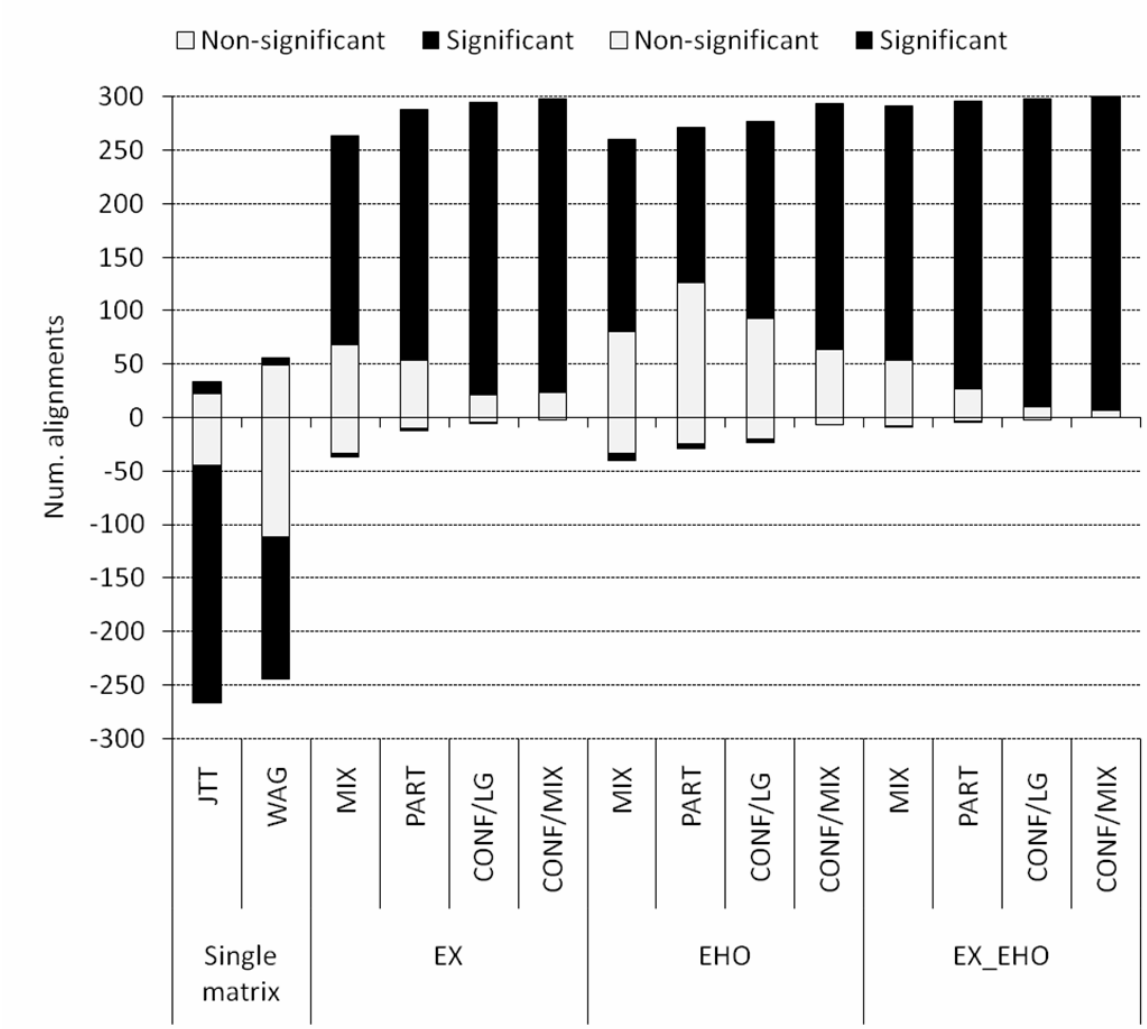
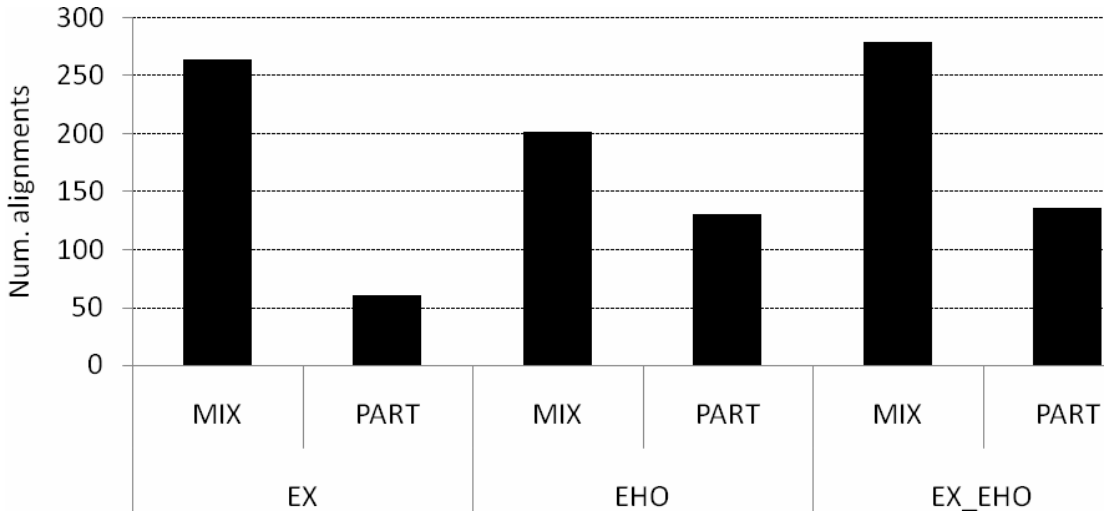
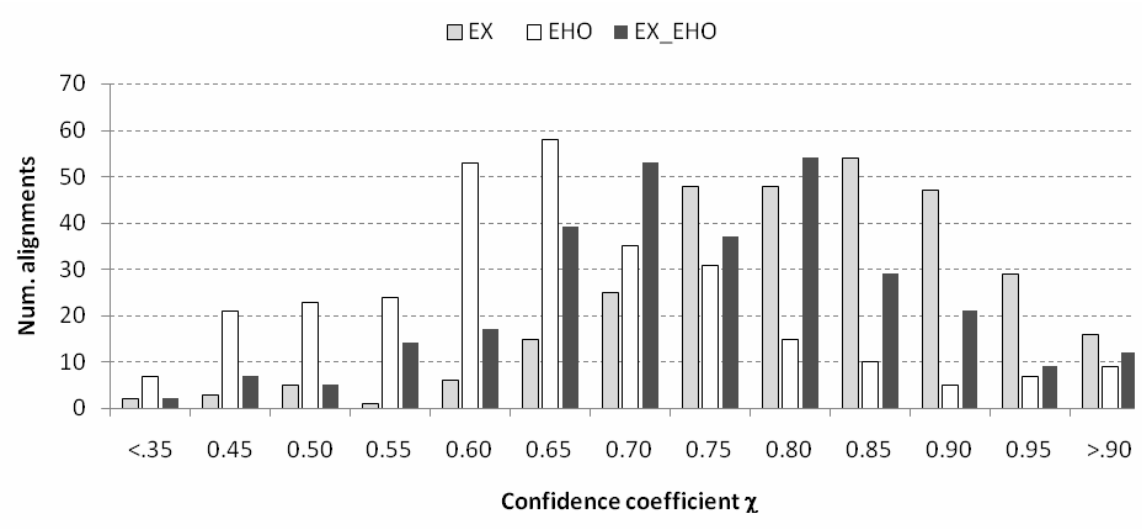


Figure 3: Comparison of CONF/MIX, PART and MIX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