Protein Complex Structure Prediction Powered by Multiple Sequence Alignment of Interologs from Multiple Taxonomic Ranks and AlphaFold2

Yunda Si and Chengfei Yan*

School of Physics, Huazhong University of Science and Technology, China

Correspondence: chengfeiyan@hust.edu.cn

Abstract

AlphaFold2 is expected to be able to predict protein complex structures as long as a multiple sequence alignment (MSA) of the interologs of the target protein-protein interaction (PPI) can be provided. However, preparing the MSA of protein-protein interologs is a non-trivial task. In this study, a simplified phylogeny-based approach was applied to generate the MSA of interologs, which was then used as the input of AlphaFold2 for protein complex structure prediction. Extensively benchmarked this protocol on non-redundant PPI dataset, we show complex structures of 79.5% of the bacterial PPIs and 49.8% of the eukaryotic PPIs can be successfully predicted. Considering PPIs may not be conserved in species with long evolutionary distances, we further restricted interologs in the MSA to different taxonomic ranks of the species of the target PPI in protein complex structure prediction. We found the success rates can be increased to 87.9% for the bacterial PPIs and 56.3% of the eukaryotic PPIs if interologs in the MSA are restricted to a specific taxonomic rank of the species of each target PPI. Finally, we show the optimal taxonomic ranks for protein complex structure prediction can be selected with the application of the predicted TM-scores of the output models.

Introduction

Proteins often facilitate their functions through interacting with other proteins. Therefore, the knowledge of protein complex structures formed by interacting proteins is valuable for mechanistic investigation of life processes and therapeutic development[1–4]. However, by far, only a small fraction of protein-protein interactions (PPIs) have their experimental complex structures available in the Protein Data Bank (PDB)[5], which is mainly because determining the complex structures through experimental techniques like X-ray crystallography, cryogenic electron microscopy (cryo-EM), etc. is time consuming and expensive[6]. Comparing with experimental structural determination, computational prediction is much more efficient and cheaper. Therefore, computational prediction of protein complex structures has become an important scientific problem for decades. The existing computational methods to predict protein complex structures can be grouped into two categories: protein-protein docking approach and template-based approach[7]. Protein-protein docking approach first applies sampling algorithms to generate a large number of possible binding modes between interacting proteins, then the produced binding modes are ranked using scoring functions to identify the most probable binding mode[8,9]. Protein-protein docking approach has two major limitations. First, protein-protein docking generally uses the structure of each monomeric protein as the input to generate the possible binding modes, however, the monomeric structures of the interacting proteins are not always available. Second, proteins when forming complex structures often have conformational changes, which makes the configurational space of PPIs extremely large, therefore, the sampling algorithms in protein-protein docking is not

always able to successfully generate the correct binding modes, or even near-native binding modes can be generated in the sampling stage, the scoring functions have difficulties to successfully identify these modes[10]. The template-based approach is based on the fact that protein-protein interologs often have similar complex structures, which applies the experimental complex structures of interologs of the target PPIs as the templates to build its complex structure[11]. The template-based approach can be very accurate when high quality templates are available. However, at the current stage, we still lack good templates for most of PPIs.

Recently, AlphaFold2 (AF2) has achieved unprecedented breakthrough in protein monomeric structure prediction[12,13]. After that, in the work of RoseTTAFold, a model based a similar deep learning architecture, the authors further pointed out that this deep learning architecture can also be used to predict the complex structure of intra-species PPIs as long as a multiple sequence alignment (MSA) of a certain number of interologs of the target PPIs is provided, the deep learning architecture is able to infer the intra- and inter-protein coevolutionary information to build the protein complex structure[14]. However, it is not trivial to generate the MSA of interologs, since a protein often have multiple homologous proteins including orthologs and paralogs in each species, and the interaction relationships between the two groups of homologous proteins of the interacting proteins are often unknown[15,16]. In the work of RoseTTAFold, the interaction relationships are inferred based on their genomic distances, in which the sequences with similar Uniprot accession codes (i.e. similar genomic positions) are paired[14]. This protocol is based on the theory of operon, which states that in prokaryotes, proteins with related

functions are usually encoded together within the genome[17,18]. However, operons are mainly observed in prokaryotes, therefore, this protocol may not work well for PPIs of eukaryotes. Besides, even prokaryotes also have a large number of PPIs in which the interacting proteins are not co-localized on the genomes, for which this protocol may also does not work well. Apart from the genomic distance-based approach, in the work of ComplexContact, a web server for predicting inter-protein residue-residue contacts, the authors applied a phylogeny-based approach to generate the MSA of interologs, in which the homologous proteins of each target interacting protein in each specific species were first descendingly sorted according to their sequence similarities to the target protein, and then the proteins with the same rankings within the same species were paired to form MSA of interologs[19]. However, whether the interaction relationships between proteins in the two homologous protein groups can be accurately inferred by this phylogeny-based approach has not been extensively validated.

In this study, we employed a simplified version of the phylogeny-based approach to generate the MSA of interologs, in which only the sequences with the highest sequence similarity to the interacting proteins among the homologous proteins in each species were paired, for these proteins are more likely to be orthologues of the target interacting proteins, thus they have higher probability to interact with each other and are more likely to share a similar binding mode with the target interacting proteins. Different from the phylogeny-based approach of ComplexContact, we did not include the protein pairs with lower rankings in the MSA generation for two reasons: First, the lower ranking proteins are more likely to be paralogs of the interacting proteins, the interaction of paralogs is

often not as conserved as orthologs; second, even though the interaction of parologs is conserved, the accuracy of the inferred interaction relationships between the paralogs cannot be guaranteed. The MSA of interologs prepared by this simplified phylogeny approach was then employed as the input to the deep learning model of AF2 to predict the complex structure of interacting proteins, in which no template information was used to assist the protein complex structure prediction. This protocol was applied to predict complex structures for non-redundant intra-species PPIs including 107 bacterial PPIs and 442 eukaryotic PPIs. The result shows that we can successfully predict complex structures (DockQ≥0.23) for 79.5% of the bacterial PPIs and 49.8% of the eukaryotic PPIs with the application of this protocol. For the purpose of comparison, we also employed the MSAs of interologs prepared by the genomic distance-based approach of RoseTTAFold and by the phylogeny-based approach of ComplexContact as the input to AF2 to predict protein complex structures for the same dataset respectively. The result shows with the application of the genomic distance-based MSA in the protein complex structure prediction, we can successfully predict complex structures for only 56.1% of the bacterial PPIs and 17.6% of the eukaryotic PPIs; and with the application of the phylogeny-based MSA prepared by the protocol of ComplexContact, we can successfully predict complex structures for 78.5% of the bacterial PPIs and 47.0% of the eukaryotic PPIs, from which we can conclude that both the simplified phylogeny-based approach and the phylogeny-based approach of ComplexContact are significantly more effective than the genomic distance-based approach in obtaining the MSA of interologs for protein complex structure prediction, but the inclusion of the lower ranking protein pairs in the phylogeny-

based MSA of interologs can even attenuate the protein complex structure prediction performance. Since PPIs are generally not as conserved as monomeric proteins in the process of evolution, a PPI may not be maintained in another species with very long evolutionary distance, or even though the PPI is maintained, the complex structure of the interolog can be different[20,21]. Including the non-conserved interologs in the MSA may attenuate the prediction performance as well. To very this assumption, we further applied MSAs in which the interologs are from species within different taxonomic ranks (domain, kingdom, phylum, class and order) of the species of the target PPI in the complex structure prediction. We found that for both the bacterial and the eukaryotic PPI datasets, higher prediction accuracies for a significant number of PPIs can be achieved when restricting interologs in the MSA to each taxonomic rank. If the optimal taxonomic rank for each target can be chosen in the MSA generation, the success rates for the protein complex structure prediction can be increased to 87.9% for the bacterial PPIs and 56.3% for the eukaryotic PPIs. Finally, we show that the predicted TM-score (pTM) can be used to select the optimal taxonomic rank for protein complex structure prediction. For each target PPI, if we select the model with highest pTM from multiple models generated on different taxonomic ranks as the predicted model, the success rates for protein complex structure prediction can reach 84.2% for the bacterial PPIs and 54.1% for the eukaryotic PPIs.

Results and Discussions

1. Overview of the prediction and evaluation protocols

1.1 The protein complex structure prediction protocols

In this study, we only focus on predicting heterodimeric complex structures of intra-species PPIs, for there is no clear way to obtain the interologs of inter-species PPIs. Besides, the prediction of homo-oligomeric complex structure is also not covered by our study, for the MSA of interologs of the homo-oligomeric complex can be built trivially by repeatedly concatenating the MSA of homologous monomers, but the prediction of the homo-oligomeric complex structure requires the specification of the degree of polymerization and symmetry of the oligomer.

The complex structure prediction protocol is summarized in Figure 1. As it is shown in the figure, given sequences of the interacting proteins, we first applied JackHMMER[22] to search against UniRef100[22] (2021-04-07) to generate the MSA of homologous proteins for each protein. Then each MSA was filtered by removing sequences with more than 50% gaps, with non-standard amino acids, without species information and with "uncertain", "uncharacterized" and "putative" in their cluster names, and the kept sequences are grouped according to their species. After that, a simplified phylogeny-based approach was applied to pair sequences belonging to the same species in the two MSAs. Specifically, the sequence with the highest similarity to the target sequence in each species was considered as the putative ortholog of the target protein, and the two putative orthologs of the two interacting proteins were paired to form the interolog of the target PPI, considering orthologs of the interacting proteins are more likely to keep interaction. Linkers with each formed of 300 GLYs were then applied to connect the paired sequences to form the MSA of the interologs, which was then inputted to the deep learning model (model_5_ptm) of AF2 with the default setting to predict the complex structure of the

interacting proteins. In the following sections, this basic protein complex structure prediction protocol is referred to as "AF2 + phylogeny-MSA". Besides, the genomic distanced-based MSA of interologs produced by the RoseTTAFold protocol and the phylogeny-based MSA of interologs produced by the ComplexContact protocol were also inputted to the same deep learning model of AF2 to predict protein complex structures respectively for references, which are referred to as "AF2 + genome-MSA" and "AF2 + phylogeny-MSA (all pairs)" in the following sections.

Since PPIs are generally not as conserved as monomeric proteins in the evolution process, including sequences of non-conserved protein pairs in the MSA may attenuate the prediction performance. Therefore, we further applied MSAs in which the interologs are from species within different taxonomic ranks (domain, kingdom, phylum, class and order) of the species of the target PPI in the complex structure prediction. Different target PPIs may have different optimal taxonomic ranks in the complex structure prediction. In this study, the protein complex structure prediction with the application of the MSA of interologs from the optimal taxonomic rank is referred to as "AF2 + phylogeny-MSA + rank". Since the pTM calculated by AF2 for each generated model can be used to estimate the accuracy of the model, we further recalculated the pTM of each generated model with AF2 after the removal of the GLY linker, and then applied the recalculated pTM to select the predicted complex structure from the multiple generated models on different taxonomic ranks (i.e. the model with the highest recalculated pTM), which is referred to as "AF2 + phylogeny-MSA + pTM-rank" in this study.

1.2 The evaluation protocols

The protein complex structure prediction protocols described above were extensively evaluated on non-redundant PPIs including 107 PPIs in bacteria and 442 PPIs in eukaryotes with experimentally resolved complex structures (See the Material and Methods section for the database preparation). For each PPI, we employed DockQ, a continuous quality score in the range [0,1], to assess the quality of the complex structure prediction[23]. Specifically, $\mathrm{DockQ} < 0.23$ corresponds to an incorrect prediction; $0.23 \leq \mathrm{DockQ} < 0.49$ corresponds to an acceptable prediction; $0.49 \leq \mathrm{DockQ} < 0.8$ corresponds to a medium quality prediction; and $0.8 \leq \mathrm{DockQ}$ corresponds to a high quality prediction.

2. Protein complex structure prediction with "AF2 + phylogeny-MSA"

We first applied "AF2 + phylogeny-MSA" to predict complex structures for non-redundant intra-species PPIs including 107 bacterial PPIs and 442 eukaryotic PPIs, and "AF2 + genome-MSA" and "AF2 + phylogeny-MSA (all pairs)" were also applied on the same datasets for reference. In Figure 2, we show the performance comparison between these "AF2 + phylogeny-MSA" and the two reference protocols on protein complex structure prediction. As we can see from Figure 2 that for both the bacterial and the eukaryotic PPIs, "AF2 +phylogeny-MSA" achieved much better performance than "AF2 + genome-MSA", and slightly better performance than "AF2 + phylogeny-MSA (all pairs)". Specifically, "AF2 + phylogeny-MSA" was able to successfully predict complex structures for 79.5% of the bacterial PPIs (acceptable quality: 10.3%, medium quality: 39.3%, high quality: 29.9%, mean DockQ: 0.574) and 49.8% of the eukaryotic PPIs (acceptable quality: 10.2%, medium quality: 24.0%, high quality: 15.6%, mean DockQ: 0.355); "AF2 + genome-

MSA" only successfully predicted complex structures for 56.1% of the bacterial PPIs (acceptable quality: 5.61%, medium quality: 28.0%, high quality: 22.4%, mean DockQ: 0.426) and 17.6% of the eukaryotic PPIs (acceptable quality: 5.43%, medium quality: 8.59%, high quality: 3.62%, mean DockQ: 0.131); and "AF2 + phylogeny-MSA (all pairs)" successfully predicted complex structures for 78.5% of the bacterial PPIs (acceptable quality: 6.54%, medium quality: 39.3%, high quality: 32.7%; mean DockQ: 0.592) and 47.0% of the eukaryotic PPIs (acceptable quality: 10.2%, medium quality: 22.9%, high quality: 14.0%, mean DockQ: 0.332) Therefore, we can conclude from the result that for both the bacterial PPIs and the eukaryotic PPIs, the simplified phylogeny-based approach is more effectively than the genomic distance-based approach in preparing the MSA of interologs to assist protein complex structure prediction, and the inclusion of the lower ranking proteins pairs can even attenuate the protein complex structure prediction performance. It is worth noting that in the work of ComplexContact, a web server for predicting inter-protein residue-residue contacts, the authors claimed that genome-distance approach is more effectively in preparing MSA of interologs for PPIs in bacteria, and the phylogeny-based approach is more effectively in preparing MSA of interologs for PPIs in eukaryotes[19]. However, it should be noted the bacterial PPI dataset used to evaluate ComplexContact is from Ovchinnikov *et al.*[17], which is constituted by Ecoli PPIs formed by proteins with short genomic distances, but our bacterial PPIs do not have genomic distance restrictions.

3. Restriction of interologs in the MSA to different taxonomic ranks of the species of the target PPI in protein complex structure prediction

A PPI may not be maintained in another species with very long evolutionary distance, or even though the PPI is maintained, the complex structure of the interolog can be different, for PPIs are often not as conserved as monomeric proteins in the evolutionary process[20,21]. Including sequences of non-conserved protein pairs may attenuate the prediction performance. Therefore, we further applied MSAs in which the interologs are from species within different taxonomic ranks (domain, kingdom, phylum, class and order) of the species of the target PPI in the complex structure prediction. In Figure 3A-E, we show the performance comparison between the protein complex structure prediction with application of MSA of interologs from each taxonomic rank ("AF2 + phylogeny-MSA (domain/kingdom/phylum/class/order)") and from the whole UniRef100 database ("AF2 + phylogeny-MSA"). As we can see from Figure 3A-E that higher prediction accuracies for a significant number of PPIs can be achieved when restricting the MSA of interologs at each taxonomic rank. Different target PPIs may have different optimal taxonomic ranks for protein complex structure prediction. Of course, for some PPIs, the best prediction performance was achieved when there was no taxonomic rank restriction. In our study, "life" was used to represent all species in the UniRef100 database, which can be considered as the highest taxonomic rank. If the optimal taxonomic rank can be chosen for each target PPI in protein complex structure prediction, higher quality predictions can be obtained for a large number of PPIs (see Figure 3E). Specifically, with the application of the MSA of interologs from the optimal taxonomic rank for each target in protein complex structure prediction ("AF2 + phylogeny-MSA + rank"), the success rates for protein complex structure prediction can be increased to 87.9% for the bacterial PPIs

(acceptable quality: 10.3%, medium quality: 38.3%, high quality: 39.3%, mean DockQ:0.652) and 56.3% for the eukaryotic PPIs (acceptable quality: 10.4%, medium quality: 25.8%, high quality: 20.1%, mean DockQ: 0.406) (see Figure 4A-B). An example of the improvement is shown in Figure 4C-D. As we can see from Figure 4C-D, with the application of MSA of interologs from the whole UniRef100 database as the input to AF2, only a model with acceptable quality (DockQ=0.03) can be predicted for the PPI in PDB 3NK8. However, if we restrict the MSA of interologs to the class of the target PPI, a high quality prediction (DockQ=0.89) can be achieved.

4. Application of pTM to select taxonomic ranks for protein complex structure prediction

Since the pTM calculated by AF2 can be used to evaluate the quality of the predicted models, it was applied to select the taxonomic ranks (including the taxonomic rank of "life") for protein complex structure prediction in this study. Specifically, we predicted protein complex structure for each PPI with the application of MSAs of interologs from the six different taxonomic ranks (life/domain/kingdom/phylum/class/order) of the target PPI respectively, thus six different predicted models can be generated for each PPI. Then we recalculated the pTM for each predicted model after the removal of GLY linker. In Figure 5, we show the relationship between the recalculated pTM and the DockQ of the predicted models. As we can see from the figure, the model with the higher pTM tends to have higher DockQ (i.e. higher quality)), especially when the pTM is high (e.g. pTM>0.7). Therefore, for each target PPI, the model with the highest recalculated pTM was considered as the final predicted model ("AF2 + phylogeny-MSA + pTM-rank"). In Figure

6, we show the performance of “AF2 + phylogeny-MSA +pTM-rank” in protein complex structure prediction, which is also compared with “AF2 + phylogeny-MSA” and “AF2 + phylogeny-MSA + rank”. As we can see from the figure, for only a relative small number of PPIs, the predicted models by “AF2 + phylogeny-MSA + pTM-rank” show significantly lower qualities than those by “AF2 + phylogeny-MSA +rank”, but for a quite a significant number of PPIs, the predicted models by “AF2 + phylogeny-MSA + pTM-rank” show significantly higher qualities (see Figure 5A-B). Specifically, with the application of the pTM-selected taxonomic ranks in the MSA generation, we can successfully predict protein complex structures for 84.2% of the bacterial PPIs (acceptable quality: 11.2%, medium quality: 38.3%, high quality: 34.6%, mean DockQ: 0.615) and for 54.1% of the eukaryotic PPIs (acceptable quality: 10.4%, medium quality: 26.7%, high quality: 17.0%, mean DockQ: 0.382) (see Figure 5C-D).

5. Evaluation of protein complex structure prediction protocols on PPIs deposited after the AF2 training set

Since AF2 was trained on structures of protein monomers deposited in Protein Data Bank (PDB)[5] before April 30, 2018, only structures of protein monomers in each PPI have the possibility to be included in the training of AF2, but none of the complex structures would be included in the model training[13]. Besides, all the predictions were made by inputting the MSA of interologs to the deep learning model of AF2 without using any template information. Therefore, it is unlikely that the protein complex structure prediction was made by trivially recognizing the existing templates in PDB or due to the bias of the deep learning model to the target PPIs. However, the inclusion of the

interacting protein monomers in the training of AF2 can also cause the overestimation of the model performance in protein complex structure prediction. To provide a more objective evaluation of these protein complex structure prediction protocols, we further evaluated their performances only on the 31 PPIs in our dataset (28 eukaryotic PPIs and 3 bacterial PPIs) with their complex structures deposited in PDB after April 30, 2018. In Figure 7, we show the performance comparison between different protein complex structure prediction protocols on the 31 PPIs. As we can see from the figure that the performance comparison between these protocols on the 31 PPIs is consistent with that on the full dataset. Specifically, the mean DockQ of the 31 predicted models by "AF2 + phylogeny-MSA" is 0.295 (acceptable quality: 16.1%, medium quality: 19.4%, high quality: 9.68%), which is significant higher than the performance of "AF2 + genome-MSA" (mean DockQ: 0.05, acceptable quality: 3.22%, medium quality: 3.22%, high quality: 0%) and the performance of "AF2 + phylogeny-MSA (all pairs)" (mean DockQ: 0.239, acceptable quality: 16.1%, medium quality: 3.22%, high quality: 16.1%) ; by further restricting the MSA of interologs to the optimal taxonomic ranks ("AF2 + phylogeny-MSA + rank"), the mean DockQ of the predicted models can be increased to 0.345 (acceptable quality: 19.4%, medium quality: 12.9%, high quality: 19.4%); with the application the pTM-selected taxonomic ranks for protein complex structure prediction ("AF2 + phylogeny-MSA + pTM-rank"), the mean DockQ of the predicted models is 0.324 (acceptable quality: 22.6%, medium quality: 12.9%, high quality: 16.1%), which is only slightly lower than the ideal case (i.e. "AF2 + phylogeny-MSA + rank"). The performances of these protein complex structure prediction protocols on the more recent 31 PPIs (28 of which are eukaryotic

PPIs) are similar to the performances on the full eukaryotic PPI dataset. Therefore, arguably we can say that it has very little overestimation of the performances of these protein complex structure prediction protocols when they were evaluated on the full PPI dataset.

6. The successful cases versus the failed cases

As it was shown above, even when the optimal taxonomic rank was chosen in the MSA generation, "AF2 + phylogeny-MSA + pTM-rank" still failed to predict protein complex structures for 12.1% of the bacterial PPIs and 43.7% of the eukaryotic PPIs. To explore the possible reasons accounting for the failures in the protein complex structure prediction, both the bacterial PPIs and the eukaryotic PPIs were grouped into two subsets respectively based on whether the complex structure of the PPI can be successfully predicted ($\mathrm{DockQ} \geq 0.23$). We found that the failed cases of both the bacterial PPIs and eukaryotic PPIs tend to have fewer effective sequences in their MSAs. As it is shown in Figure 8A, for the bacterial PPIs, the median value of the normalized effective sequence number of the MSA (N_{eff}^{norm}) of the failed cases is only 1.08, which is dramatically lower than the median value of N_{eff}^{norm} of the successful cases (median $N_{eff}^{norm} = 12.85$). For the eukaryotic PPIs, although the median value of N_{eff}^{norm} of the failed cases is not as low as that of the bacterial PPIs (median $N_{eff}^{norm} = 6.48$), which is still significantly lower than that of the successful cases (median N_{eff}^{norm}=10.87). Besides, we also found that the failed cases of the eukaryotic PPIs tend to have significantly smaller inter-protein interfaces, but the interfaces of the failed cases of the bacterial PPIs are with similar sizes to the interfaces of the successful cases. As it shown in Figure 8B, the median number of inter-protein

contacts (a contact is defined if the heavy atom distance of two residues is within 5Å) of the failed cases of the eukaryotic PPIs is 34, which is significantly lower than that of the successful cases (median number is 74), and for the bacterial PPIs, the median numbers of inter-protein contacts of the successful cases and failed cases are 89 and 99 respectively. Obviously, for the failed cases of bacterial PPIs, the lack of effective sequences in the MSA is the main reason for the failures in the protein complex structure prediction. For the failed cases of the eukaryotic PPIs, apart from the lack of effective sequences in their MSAs, we doubt the relatively smaller PPI interfaces are also responsible for the failures. Since the deep learning model of AF2 was trained on protein monomers, which may have difficulty to model transient PPIs with small interfaces. When this manuscript is under preparation, DeepMind also released their deep learning models trained on protein complex structures, it would be interesting to see whether our failed cases can be correctly predicted by inputting our MSAs to the new deep learning models. Besides, since eukaryotic proteins generally contain much more paralogs than prokaryotic proteins, our phylogeny-based sequence pairing protocol may not work well for some eukaryotic PPIs for it is more challenging to select orthologs from homologous sequences. Therefore, we doubt that mis-paired proteins with no interactions may be included in forming the MSA of interologs for some eukaryotic PPI, which can also be the reason that the protein complex structure prediction was failed for some PPIs in which the MSAs contain a large number of paired sequences. It would also interesting to see whether the prediction performance can be further improved if more restrict and complex protocols (e.g. Reciprocal Best Hits) are used for ortholog prediction. Besides, in our study, only the

sequence with the highest similarity to each target interacting protein in each specific species (i.e. the putative ortholog) was included in forming the MSA of protein-protein interologs, appropriately including the lower ranking sequences in the MSA generation may also improve the protein complex structure prediction. However, in our study, we found that including the lower ranking sequences (the putative paralogs) with the protocol of ComplexContact does not help the protein complex structure prediction. Therefore, developing more effectively protocols to pair the putative paralogs of interacting proteins can also be a future direction to improve the protein complex structure prediction performance.

Conclusion

The deep learning architecture of AF2 is expected to be able to predict complex structures of PPIs, as long as the MSA of interologs of the target PPI can be provided as the input. However, preparing the MSA of interologs is not trivial due to the existence of paralogs. In this study, a simplified phylogeny approach is employed to generate the MSA of interologs for intra-species PPIs, in which the sequences with the highest similarity to the interacting proteins in each specific species are paired to form the MSA of interologs. Benchmarked on large dataset of bacterial and eukaryotic intra-species PPIs with experimentally resolved complex structures, we show that the complex structures of most of the bacterial PPIs and more than half of the eukaryotic PPIs can be successfully predicted by inputting the multiple sequence alignment (MSA) of interologs prepared by this simplified phylogeny-based approach into the deep learning model of AF2. We further show that the prediction performance can be significantly improved if we only

include species belonging to a specific taxonomic rank of the species of the target PPI in the MSA generation, and the pTM of the predicted models can be used to select the optimal taxonomic ranks for protein complex structure prediction. Furture works on improving the protein complex structure prediction performance include developing more effective protocols to generate the MSA of interologs and employing more accurate deep learning models trained specifically from protein complex structures for protein complex structure prediction. Besides, it is also worth trying weather the current protein complex structure prediction framework can be extended to predict complex structures of inter-species PPIs, for which the preparation of the MSA of interologs will be the biggest challenge.

Materials and Methods

1. Preparing the non-redundant PPI dataset

1.1 Collecting PDB entries of protein complexes from PDB

To prepare the PPI dataset used in this study, PDB entries of protein complexes from PDB were first collected. Specifically, the criteria including 1) Experimental Method equals to X-RAY DIFFRACTION; 2) Data Collection Resolution $\leq$2.5Å; 3) Entry Polymer Types equals to Protein (only); 4) Entry Polymer Composition equals to heteromeric protein; 5) Total Number of Polymer Instances ≥ 2; 6) SCOP classification belongs to (1) All beta proteins, (2) All alpha proteins, (3) Alpha and beta proteins (a+b), (4) Alpha and beta proteins (a/b), (5) Membrane and cell surface proteins and peptides were used to filter the experimental structures deposited in PDB before July 7, 2021. In total, 9509 PDB entries of protein complexes were obtained from the initial filter.

1.2 Extracting PPI complex structures from PDB entries of protein complexes

Then PPI complex structures were then extracted from the obtained PDB entries of protein complexes. Specifically, the PDB entries of protein complexes with more than one assembly were removed in our study, for the complex structure of two interacting chains can not be uniquely determined from the corresponding PDB entry. For PDB entries with only two protein chains, the structure in each PDB entry directly corresponds to the complex structure of the two interacting chains. For PDB entries with more than two chains, the whole complex structure in each PDB entry was split into multiple structures in accordance with different combinations of the chains. Then PPIs which satisfied any of the following conditions: 1) the two interacting proteins belong to different species; 2) either of the interacting protein chains is shorter than 50 or longer than 700; 3) the sequence identity of the two interacting proteins are higher than 40%; 4) either of the two proteins have more than 20% missing residues were removed from the PPI dataset;5) There are more than 10 contact sites in the two chains (a contact is defined if the heavy atom distance of two residues is within 6Å). After the removal, 2895 complex structures of PPIs can be obtained from the previous PDB entries.

1.3 Removing redundant PPIs

Finally, redundant PPIs in the 2895 PPIs obtained above were removed. Specifically, 5790 proteins in the 2895 PPIs were clustered with CD-HIT using 40% as the sequence identity threshold (-c 0.4 -n 0.2 -g 1), from which 640 clusters were obtained. The obtained clusters were then used to label each PPI. The PPIs belonging to the same cluster pair (note: cluster pair AB and cluster pair BA were considered as the same pair) were considered as

redundant PPIs, for which only the PPI with largest number of inter-protein contacts (a contact is defined if the heavy atom distance of two residues is within 6Å) was kept. In total 563 non-redundant PPIs were kept. Among them, 9 are archaea PPIs; 5 are viruses PPIs; 107 are bacterial PPIs; and 442 are eukaryotic PPIs. The PPIs belonging to the two major domains including bacteria and eukaryotes were used to evaluate our protein complex structure prediction protocols in this study.

2. Preparing the MSA of interologs

2.1 Generating the MSA of homologous proteins for each interacting protein

To prepare the MSA of interologs for a given PPI, the MSA of homologous proteins for each interacting protein needs to be generated first. Specifically, JackHMMER[22] was applied to search against the UniRef100 database[24] (released on 2021-04-07) with (--noali --incT L/2), where L is the protein length. Then the MSAs for the two interacting proteins were paired to form the MSA of interologs for the PPI with a simplified phylogeny-based approach.

2.2 Pairing the MSAs with the simplified phylogeny-based approach

Before paring the MSAs, we first removed sequences with more than 50% gaps, with non-standard amino acids, without species information and with "uncertain", "uncharacterized" and "putative" in their cluster names in each MSA. After the filtering, sequences in each MSA were grouped according to their species, and the sequences of each specific species were sorted descendingly according to their sequence similarities ($\frac{\#Idnetical\ positions}{\#Aligned\ positions}$) to the target sequence. The top ranked sequences within each specific species in the two MSAs were considered as the putative orthologs of the target

interacting proteins, which were paired to form the interolog of the target PPI. Finally, Linkers with each formed of 300 GLYs were applied to connect the paired sequences to form the MSA of protein-protein interologs, which can be directly inputted to AF2 to predict the complex structure of the target PPI. Apart from the simplified phylogeny-based approach, the phylogeny-based approach of ComplexContact and the genomic distance-based approach of RoseTTAFold were also used as the reference approaches to prepare the MSA of protein-protein interologs.

2.3 Pairing the MSAs with the phylogeny-based approach of ComplexContact

The only difference between the phylogeny-based approach of ComplexContact and our simplified phylogeny-based approach is that in our simplified phylogeny-based approach, only the top ranked sequences within each specific species were paired, however, in the phylogeny-based approach of ComplexContact, the sequences with the same rankings within each specific species were paired (i.e. the lower ranking protein pairs were included in generating the MSA of protein-protein interologs). Since the tool for preparing the MSA of protein-protein interologs has not been released by ComplexContact, we implemented this approach by ourselves. Specifically, we employed the same protocols in the stages of the MSAs generation for interacting proteins and the sequence filtering, but in the sequence pairing stage, we included the lower ranking proteins pairs in the MSA of protein-protein interologs. Still, linkers with each formed by 300 GLYs were used to connect the paired sequences to form the MSA of protein-protein interologs.

2.4 Pairing the MSAs with the genomic distance-based approach of RoseTTAFold

The protocol provided by the RoseTTAFold website (https://github.com/RosettaCommons/RoseTTAFold/tree/main/example/complex_modeling) was exactly used to pair the MSAs with the genomic distance-based approach. Specifically, the "make_joint_MSA_bacterial.py" script provided by RoseTTAFold was first applied to pair sequences in the two MSA according to their UniProt IDs, and then hhfilter was applied to filter the paired alignment (-id 90 -cov 75). To make the paired MSA compatible with AF2 for protein complex structure prediction, linkers with each formed by 300 GLYS were inserted between the paired sequences to form the MSA of interologs.

3. Restricting interologs in the MSA to different taxonomic ranks of the species of the target PPI

The species and taxonomic ranks information of the target PPI and the searched homologous proteins of the interacting proteins were obtained from the NCBI taxonomy database (https://ftp.ncbi.nlm.nih.gov/pub/taxonomy/new_taxdump/)[25]. To restrict the MSA of interologs to a specific taxonomic rank (domain, kingdom, phylum, class and order) of the target PPI, only the sequences belonging to the corresponding taxonomic rank of the species of the target PPI were included in the MSA generation.

4. Predicting protein complex structures

For a given PPI, the MSA of interologs for the PPI was inputted the fine-tuned model 5 (model_5_ptm) of AF2 to predict the complex structure of the PPI without using any template information. The fine-tuned model was chosen for we would like to use the pTM to evaluate the predicted models. Five fine-tuned models were released in total in AF2. According to a test on a small dataset, the five models achieved very similar

performances on protein complex structure prediction, but among them, the model_5_ptm achieved a slightly better performance, thus which was chosen for protein complex structure prediction in our study. To evaluate the quality of the predicted complex structure, the pTM of predicted complex structure was recalculated with AF2 after removing the GLY linker in the predicted complex structure.

5. Calculating DockQ for the predicted complex structure

The DockQ of the predicted complex structures were calculated with the DockQ software (https://github.com/bjornwallner/DockQ).

6. Calculating N_{eff}^{norm} for the MSA of interologs

For a given MSA of interologs, the GLY linkers in the MSA were first removed. Then the N_{eff}^{norm} for the MSA of interologs was calculated according to:

$$N_{eff}^{norm} = \frac{1}{\sqrt{L}} \sum_{n=1}^{N} \frac{1}{m_n} \quad (1).$$

Where L is the number of columns of the MSA after the removal of the GLY linkers, $\frac{1}{m_n}$ is the weight the n-th interolog with m_n being the number of interologs in the MSA which have a sequence identity higher than 80% to the n-th inerologs; and N is the total number of interologs in the MSA.

Availability

The code for generating the MSA of interologs for a given PPI can be obtained from https://github.com/ChengfeiYan/PPI_MSA-taxonomy_rank.

Funding

This work is supported by the National Science Foundation of China (32101001) and new faculty startup grant (3004012167) of Huazhong University of Science and Technology.

Bibliography

1. Nooren, I. M. A. & Thornton, J. M. Diversity of protein-protein interactions. *EMBO Journal* vol. 22 (2003).
2. Mintseris, J. & Weng, Z. Structure, function, and evolution of transient and obligate protein-protein interactions. *Proc. Natl. Acad. Sci. U. S. A.* 102, (2005).
3. Keskin, O., Gursoy, A., Ma, B. & Nussinov, R. Principles of protein-protein interactions: What are the preferred ways for proteins to interact? *Chemical Reviews* vol. 108 (2008).
4. Wilson, A. J. Inhibition of protein–protein interactions using designed molecules. *Chem. Soc. Rev.* 38, (2009).
5. Berman, H. M. *et al.* The Protein Data Bank. *Nucleic Acids Res.* 28, 235–242 (2000).
6. Petrey, D. & Honig, B. Structural bioinformatics of the interactome. *Annu. Rev. Biophys.* (2014) doi:10.1146/annurev-biophys-051013-022726.
7. Vreven, T., Hwang, H., Pierce, B. G. & Weng, Z. Evaluating template-based and template-free protein-protein complex structure prediction. *Brief. Bioinform.* 15, (2014).
8. Huang, S. Y. Search strategies and evaluation in protein-protein docking: Principles, advances and challenges. *Drug Discovery Today* vol. 19 (2014).
9. Porter, K. A., Desta, I., Kozakov, D. & Vajda, S. What method to use for protein–protein docking? *Current Opinion in Structural Biology* vol. 55 (2019).
10. Yan, C. & Zou, X. Modeling Protein Flexibility in Molecular Docking. in *Comprehensive Medicinal Chemistry III* (2017). doi:10.1016/B978-0-12-409547-2.12351-0.
11. Szilagyi, A. & Zhang, Y. Template-based structure modeling of protein-protein interactions. *Current Opinion in Structural Biology* vol. 24 (2014).
12. Tunyasuvunakool, K. *et al.* Highly accurate protein structure prediction for the human proteome. *Nature* 596, (2021).
13. Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* (2021) doi:10.1038/s41586-021-03819-2.
14. Baek, M. *et al.* Accurate prediction of protein structures and interactions using a three-track neural network. *Science (80-.).* 373, (2021).
15. de Juan, D., Pazos, F. & Valencia, A. Emerging methods in protein co-evolution. *Nat. Rev. Genet.* 14, 249–261 (2013).
16. Szurmant, H. & Weigt, M. Inter-residue, inter-protein and inter-family coevolution: bridging the scales. *Current Opinion in Structural Biology* (2018) doi:10.1016/j.sbi.2017.10.014.
17. Ovchinnikov, S., Kamisetty, H. & Baker, D. Robust and accurate prediction of residue–residue interactions across protein interfaces using evolutionary information. *Elife* 3, e02030 (2014).
18. Hopf, T. A. *et al.* Sequence co-evolution gives 3D contacts and structures of protein complexes. *Elife* 3, e03430 (2014).
19. Zeng, H. *et al.* ComplexContact: A web server for inter-protein contact prediction using deep learning. *Nucleic Acids Res.* (2018) doi:10.1093/nar/gky420.
20. Yu, H. *et al.* Annotation transfer between genomes: Protein-protein interrologs and

protein-DNA regulogs. *Genome Res.* **14**, (2004).
21. Kim, P. M., Lu, L. J., Xia, Y. & Gerstein, M. B. Relating three-dimensional structures to protein networks provides evolutionary insights. *Science (80-.).* **314**, (2006).
22. Johnson, L. S., Eddy, S. R. & Portugaly, E. Hidden Markov model speed heuristic and iterative HMM search procedure. *BMC Bioinformatics* **11**, (2010).
23. Basu, S. & Wallner, B. DockQ: A quality measure for protein-protein docking models. *PLoS One* **11**, (2016).
24. Suzek, B. E., Huang, H., McGarvey, P., Mazumder, R. & Wu, C. H. UniRef: comprehensive and non-redundant UniProt reference clusters. *Bioinformatics* **23**, 1282–1288 (2007).
25. Federhen, S. The NCBI Taxonomy database. *Nucleic Acids Res.* **40**, (2012).

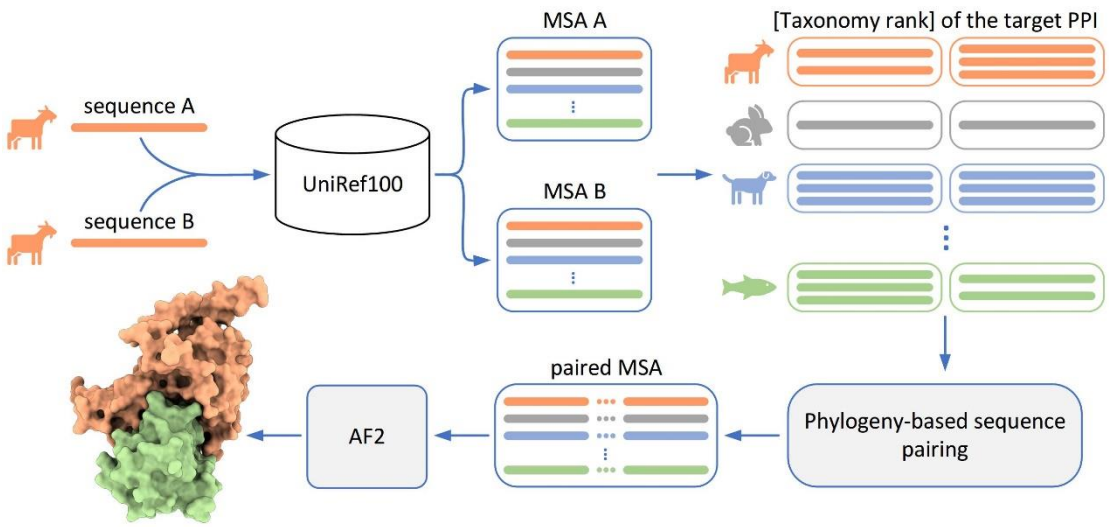


Figure 1. The flow chart of protein complex structure with the simplified phylogeny-based MSA of interologs and AF2.

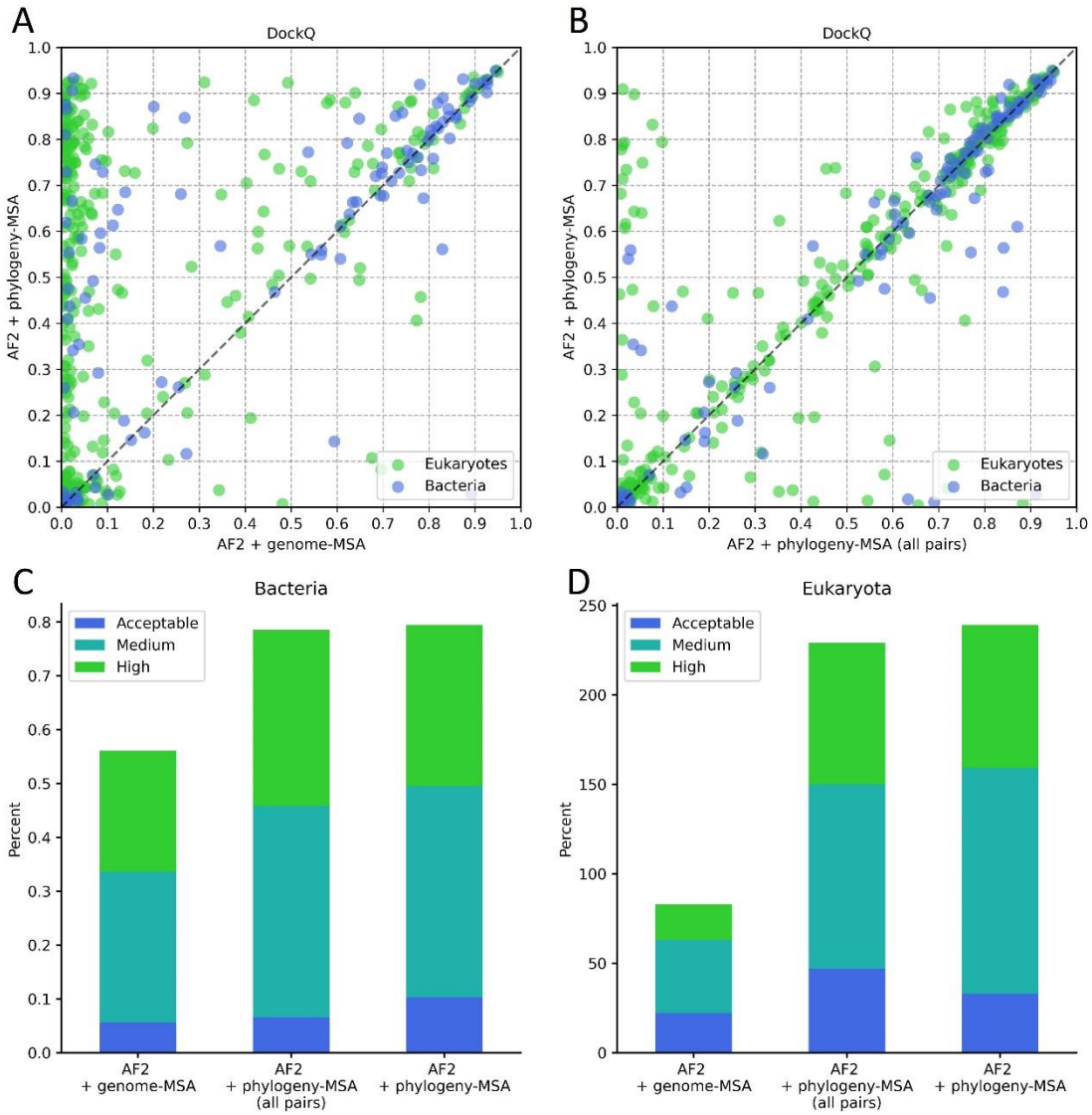


Figure 2. Protein complex structure prediction with the application of the MSA of interologs prepared by different approaches. (A) "AF2 + phylogeny-MSA" versus "AF2 + genome-MSA" for each target PPI. (B) "AF2 + phylogeny-MSA" versus "AF2 + phylogeny-MSA (all pairs)" for each target PPI. (C) Performance comparison between "AF2 + genome-MSA", "AF2 + phylogeny-MSA (all pairs)" and "AF2 + phylogeny-MSA" on the bacterial PPI dataset. (D) Performance comparison between "AF2 + genome-MSA", "AF2 + phylogeny-MSA (all pairs)" and "AF2 + phylogeny-MSA" on the eukaryotic PPI dataset.

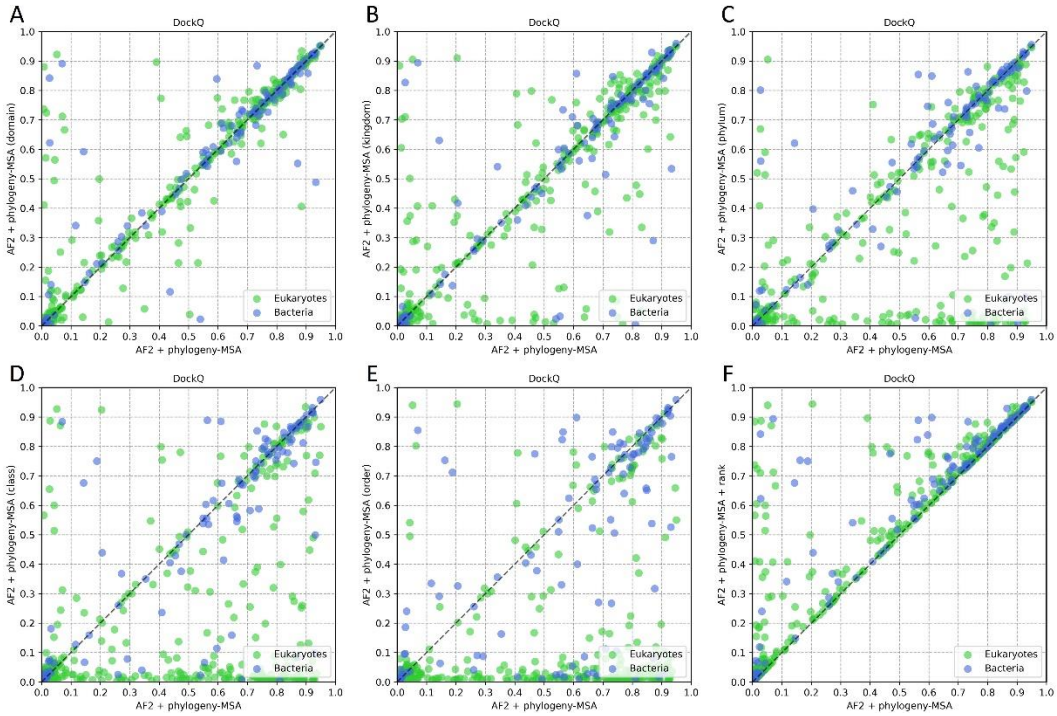


Figure 3. Protein complex structure prediction with the restriction of MSA of interologs to different taxonomic ranks of species of the target PPI. (A) "AF2 + phylogeny-MSA (domain)" versus "AF2 + phylogeny-MSA" for each target PPI. (B) "AF2 + phylogeny-MSA (kingdom)" versus "AF2 + phylogeny-MSA" for each target PPI. (C) "AF2 + phylogeny-MSA (phylum)" versus "AF2 + phylogeny-MSA" for each target PPI. (D) "AF2 + phylogeny-MSA (class)" versus "AF2 + phylogeny-MSA" for each target PPI. (E) "AF2 + phylogeny-MSA (order)" versus "AF2 + phylogeny-MSA" for each target PPI. (F) "AF2 + phylogeny-MSA + rank" versus "AF2 + phylogeny-MSA" for each target PPI.

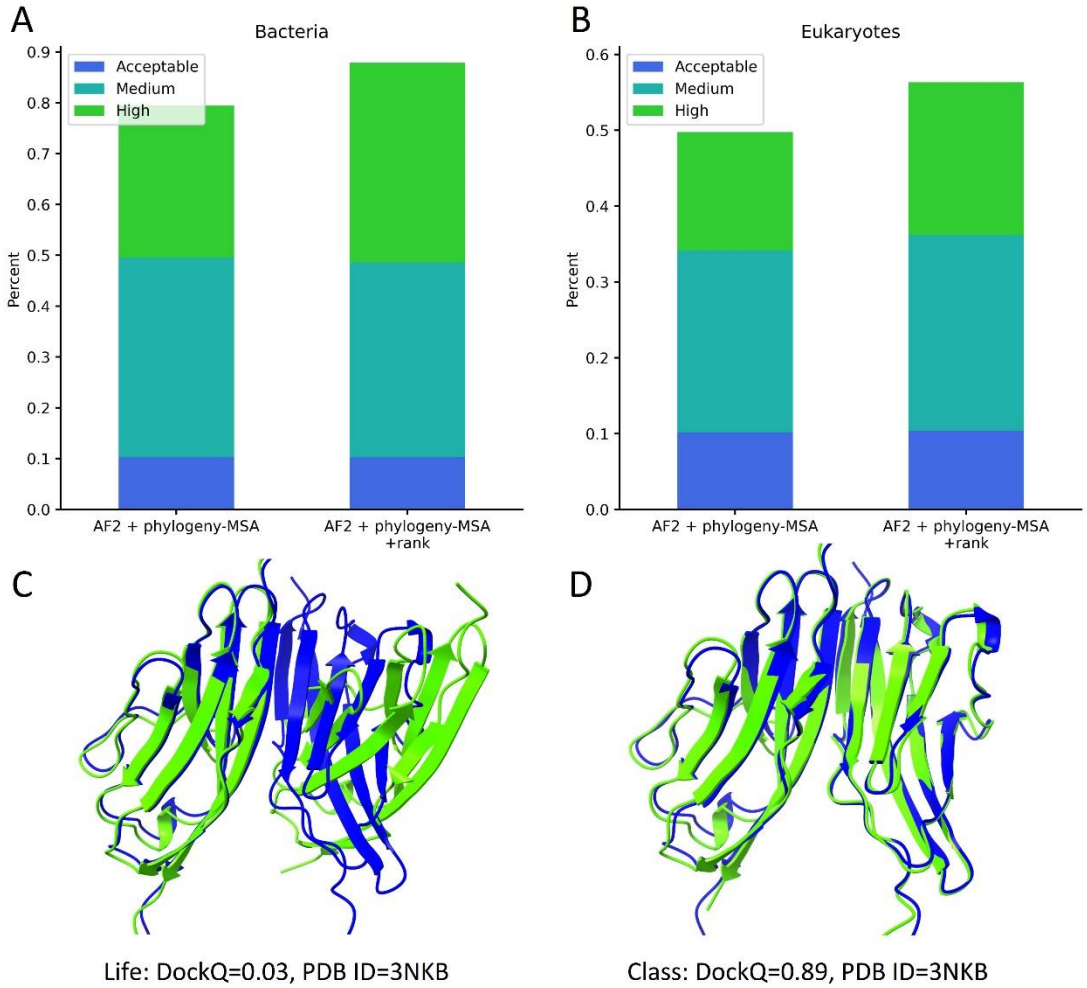


Figure 4. Protein complex structure prediction with the restriction of MSA of interologs to the optimal taxonomic rank of the species of each target PPI. (A) Performance comparison between "AF2 + phylogeny-MSA" and "AF2 + phylogeny-MSA +rank" on the bacterial PPIs. (B) Performance comparison between "AF2 + phylogeny-MSA" and "AF2 + phylogeny-MSA +rank" on the eukaryotic PPIs. (C) The quality of the predicted model for PDB 3KNB when the MSA of interologs was not restricted to a specific taxonomic rank of the species of the target PPI. (D) The quality of the predicted model for PDB 3KNB when the MSA of interologs was restricted to the order of the target PPI.

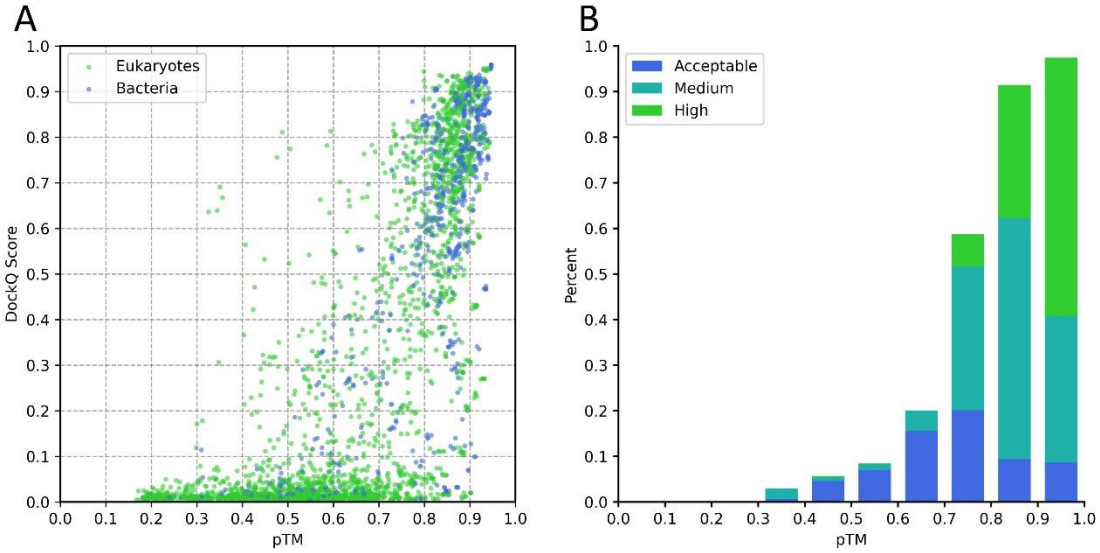


Figure 5. The relationship between the pTM and the qualities of the predicted models. (A) DockQ versus pTM for each predicted model, all the generated models using the MSAs of interologs within different taxonomic ranks of the species of the target PPIs are included in the figure. (B) The proportions of the models with acceptable quality, medium quality and high quality when the pTM values are within different intervals.

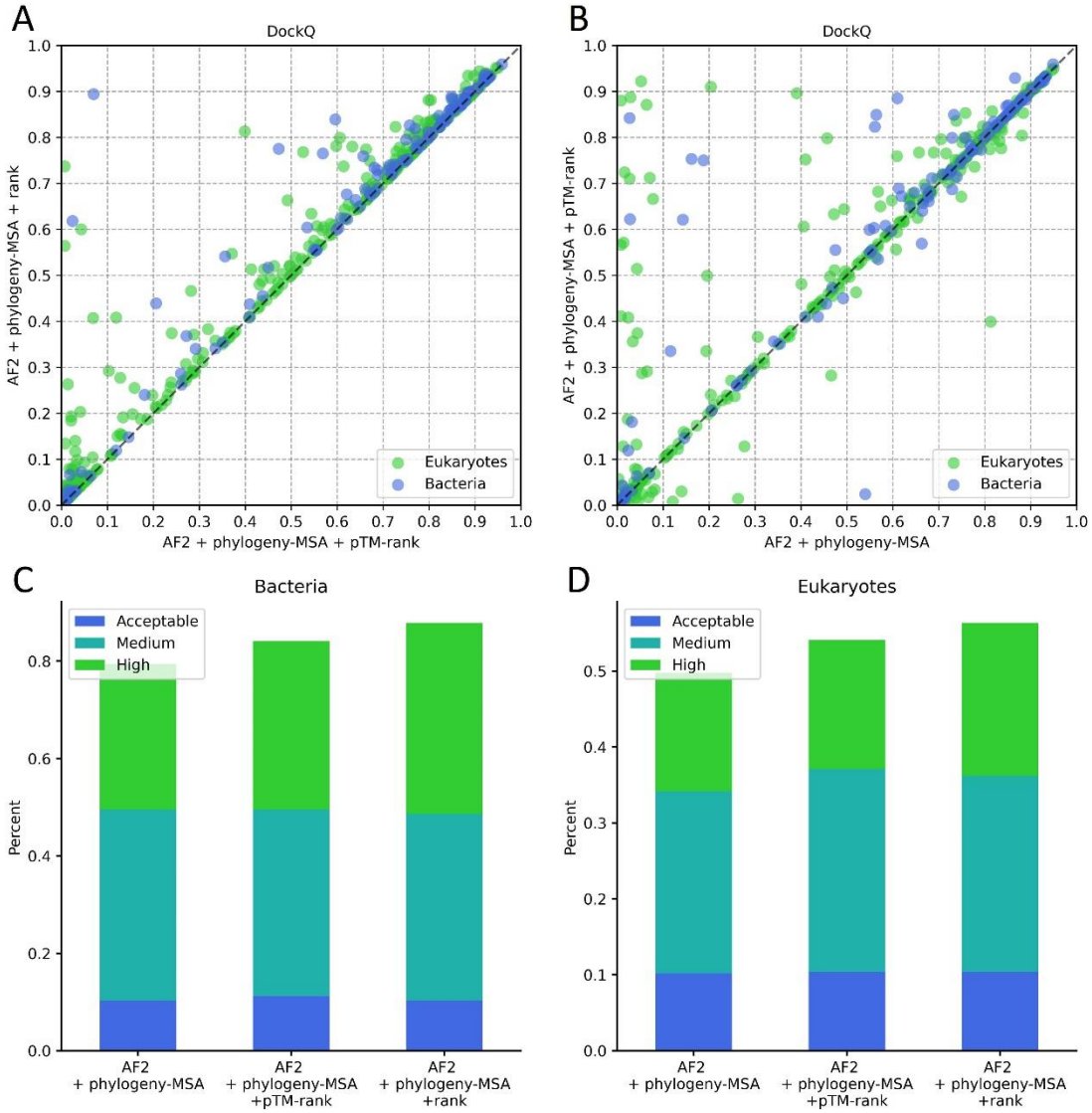


Figure 6. Protein complex structure prediction with the restriction of MSA of interologs to the pTM-selected taxonomic rank of the species of each target PPI. (A) "AF2 + phylogeny-MSA + pTM-rank" versus "AF2 + phylogeny-MSA + rank" for each target PPI. (B) "AF2 + phylogeny-MSA + pTM-rank" versus "AF2 + phylogeny-MSA" for each target PPI. (C) Performance comparison between "AF2 + phylogeny-MSA", "AF2 + phylogeny-MSA + pTM-rank" and "AF2 + phylogeny-MSA + rank" on the bacterial PPI dataset. (D) Performance comparison between "AF2 + phylogeny-MSA", "AF2 + phylogeny-MSA + pTM-rank" and "AF2 + phylogeny-MSA + rank" on the eukaryotic PPI dataset.

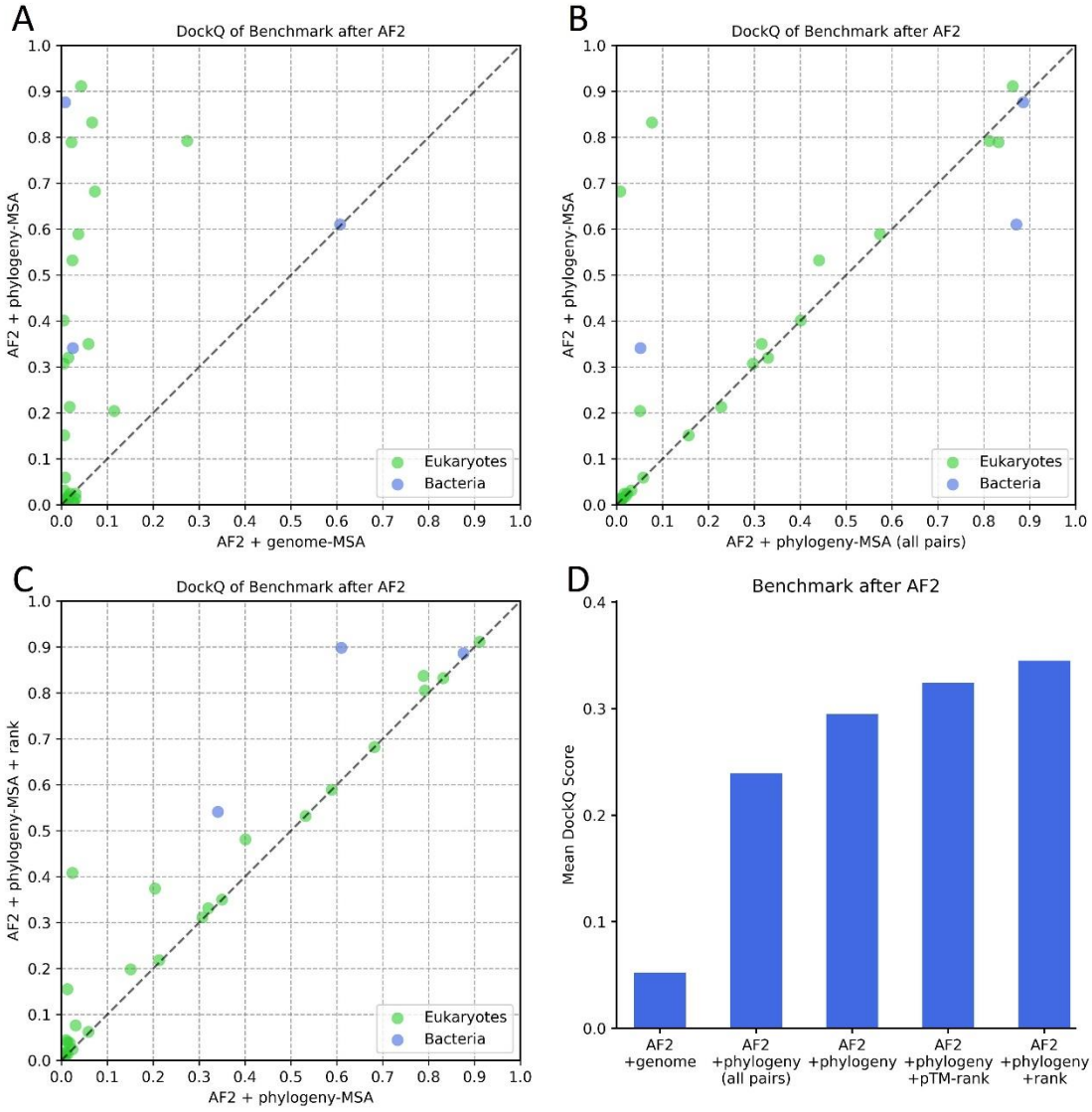


Figure 7. Protein complex structure prediction on PPIs after the training of AF2. (A) "AF2 + genome-MSA" versus "AF2 + phylogeny-MSA" for each target PPI. (B) "AF2 + phylogeny-MSA" versus "AF2 + phylogeny-MSA (all pairs)" for each target PPI. (C) "AF2 + phylogeny-MSA + rank" versus "AF2 + phylogeny-MSA" for each target PPI. (D) The mean DockQ scores of the predicted complex structures with the application of different protein complex structure prediction protocols.

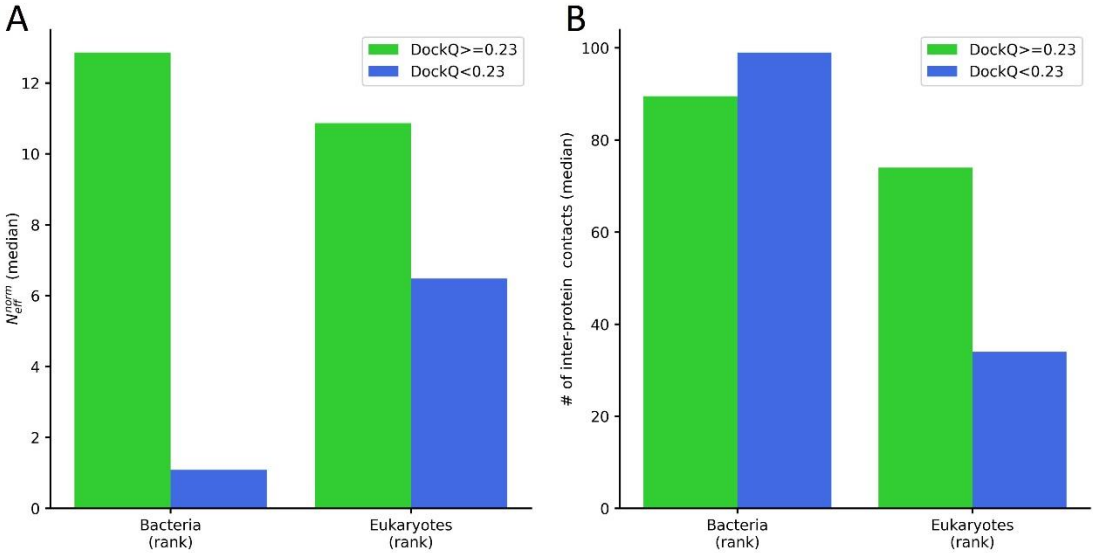
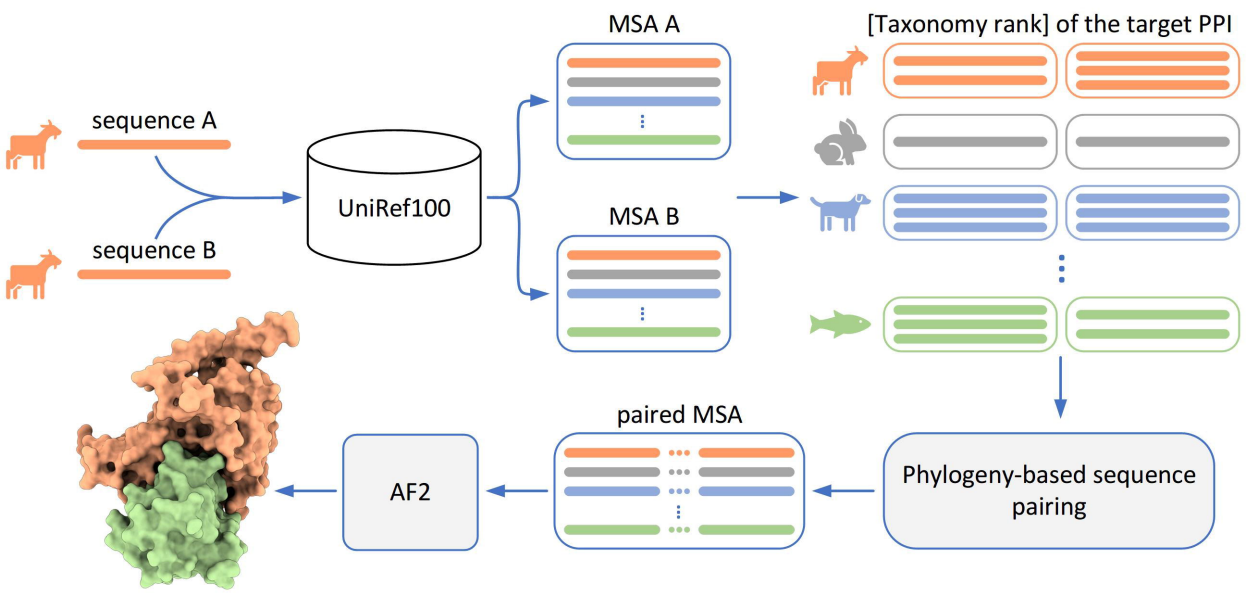
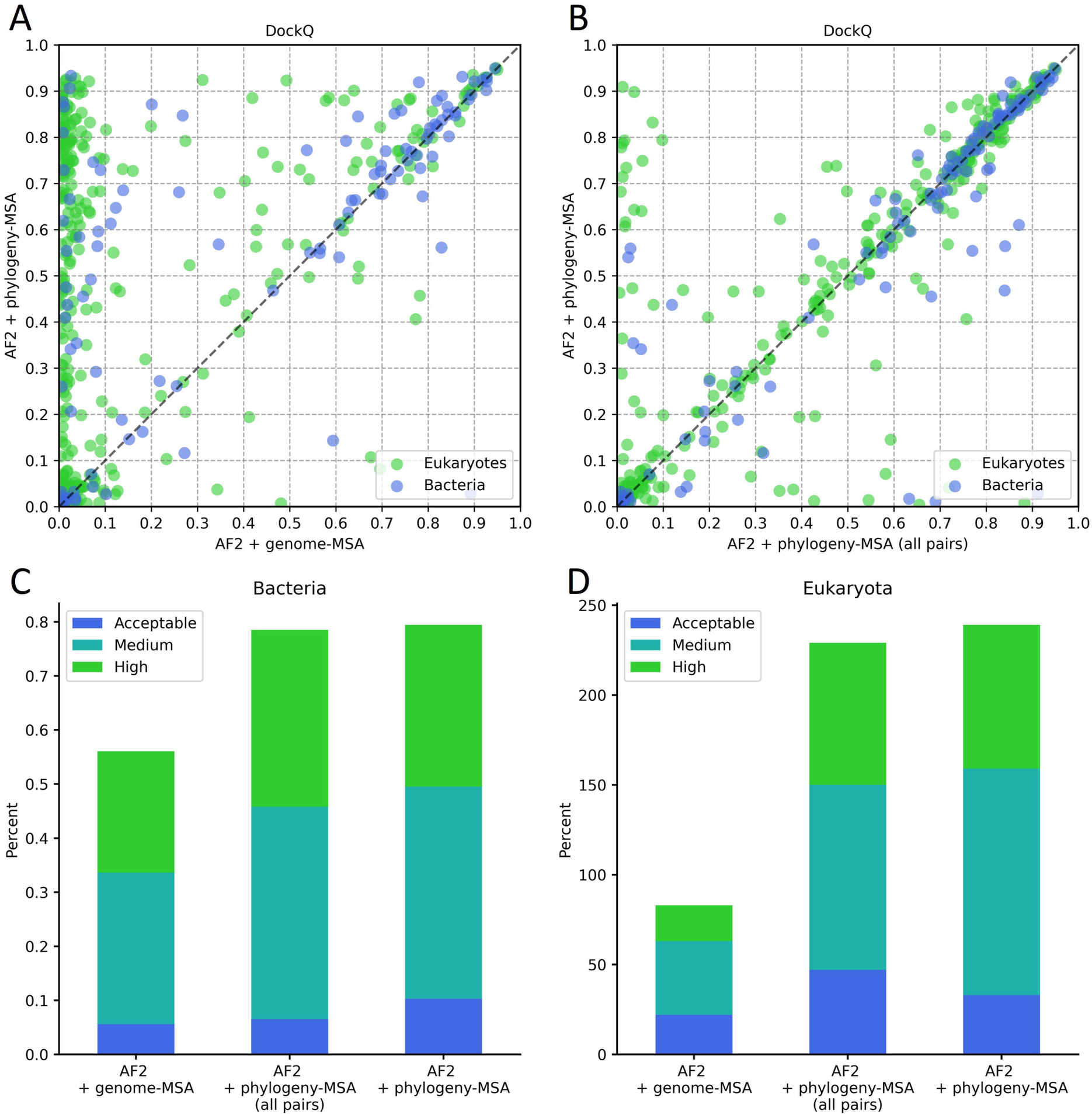
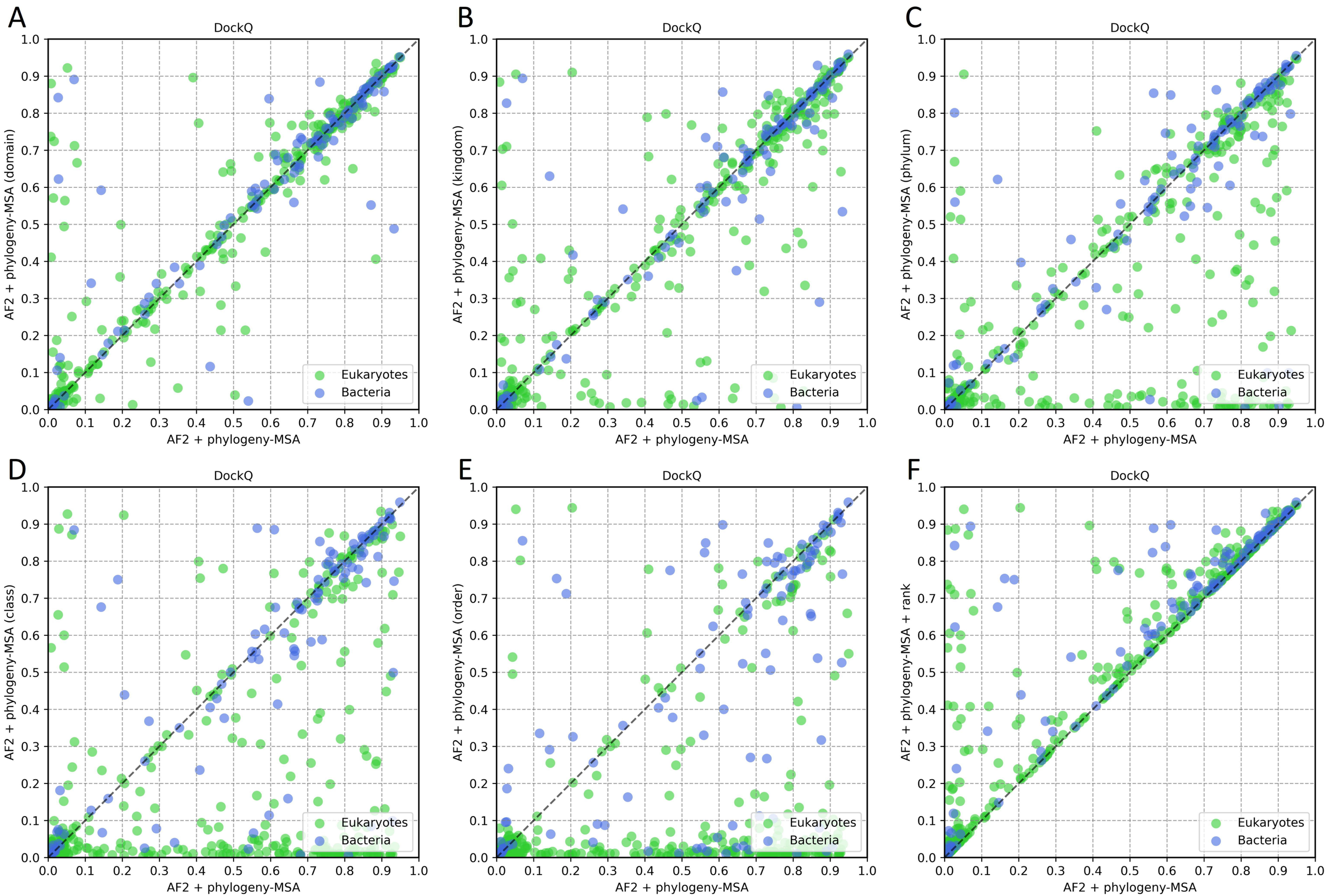
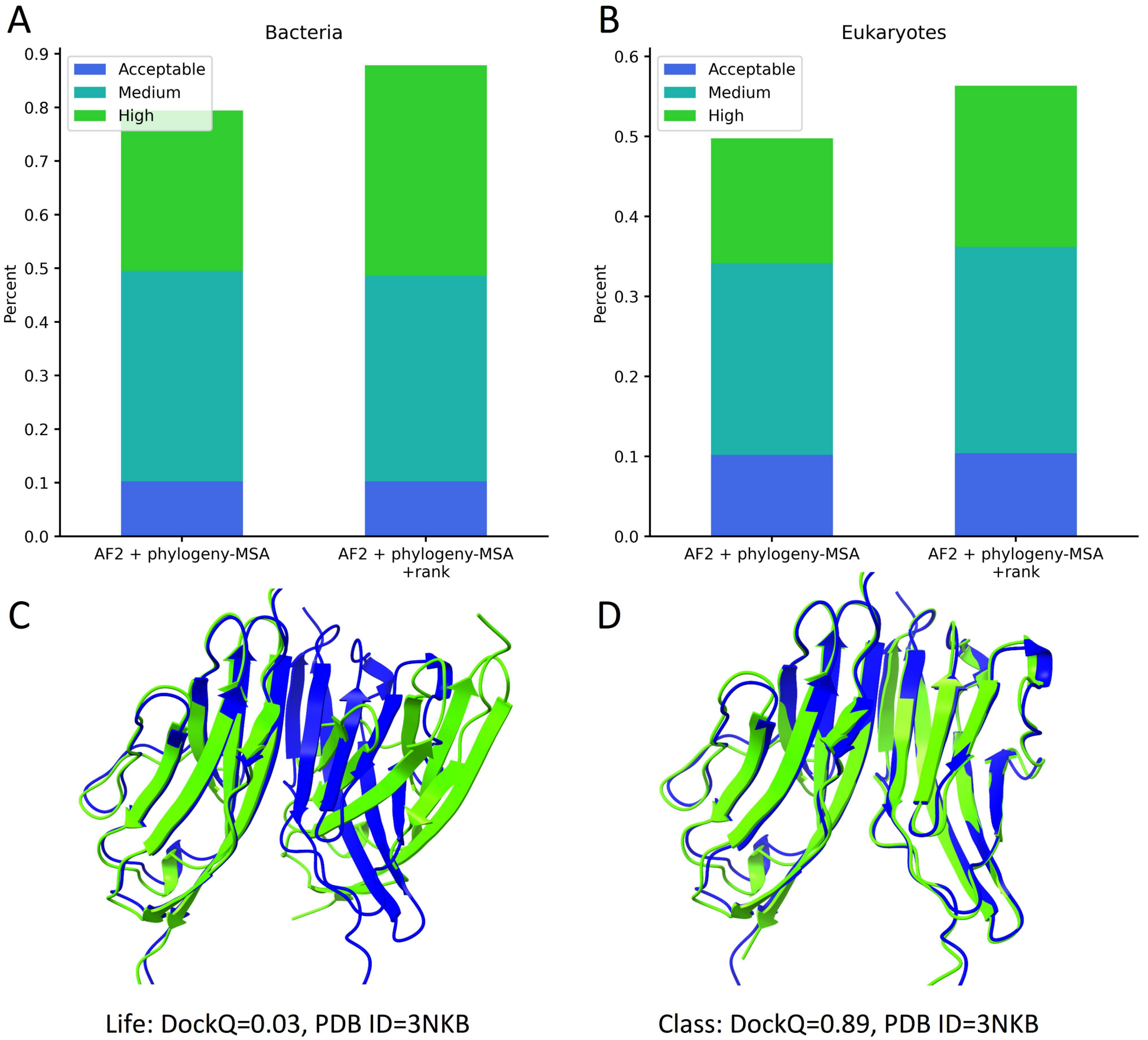


Figure 8. The successful cases versus the failed cases in protein complex structure prediction with "AF2 + phylogeny-MSA + rank". (A) The comparison of the median values of N_{eff}^{norm} for the successful cases and the failed cases in the bacterial PPI dataset and eukaryotic PPI dataset respectively. (B) The comparison of the median value of the median value of the number of inter-protein contacts for the successful cases and the failed cases in the bacterial PPI dataset and eukaryotic PPI dataset respectively.


sequence A
sequence B
UniRef100
MSA A
MSA B
[Taxonomy rank] of the target PPI
Phylogeny-based sequence pairing
paired MSA
AF2

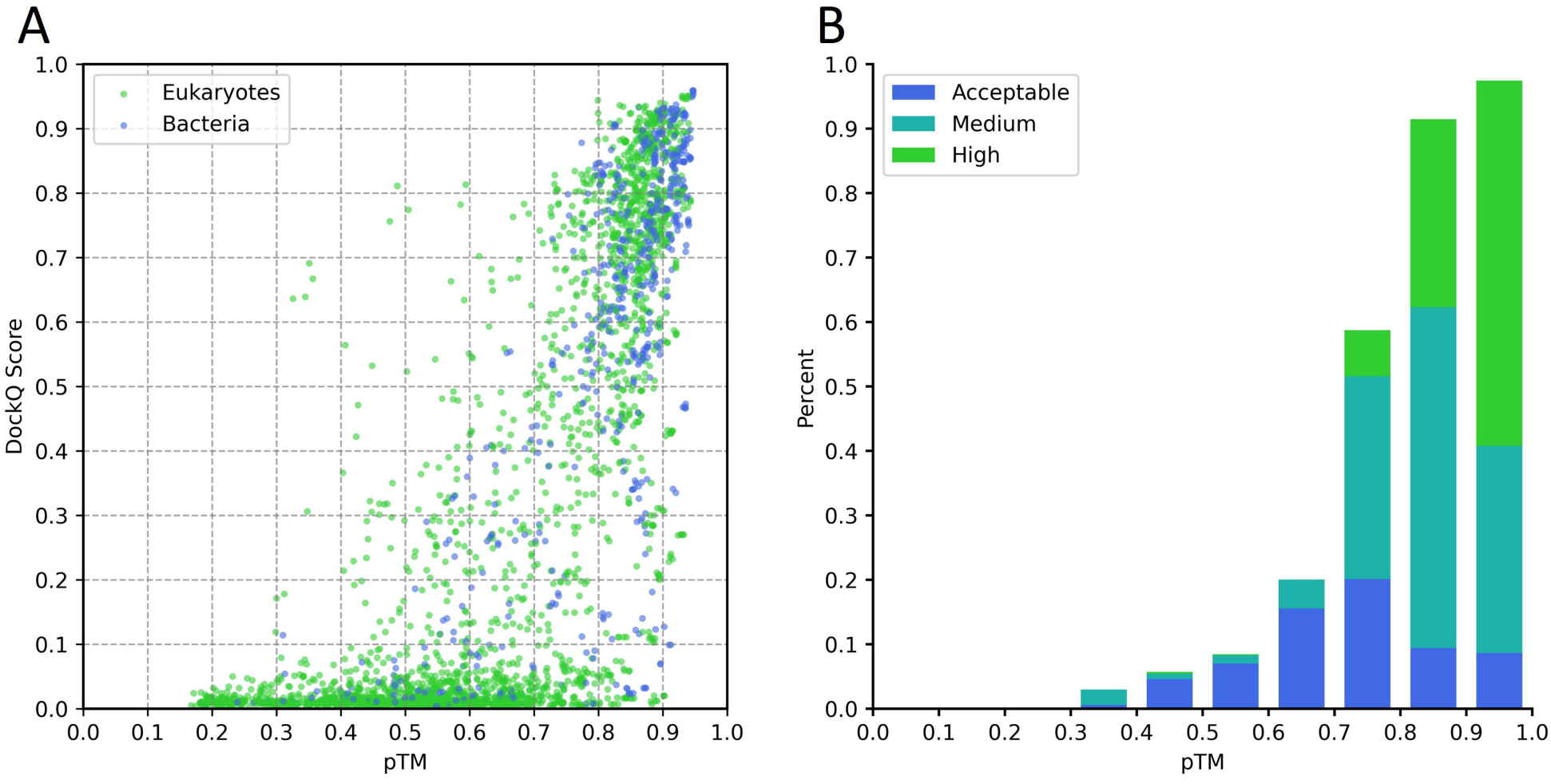

A
DockQ
AF2 + phylogeny-MSA
AF2 + genome-MSA
Eukaryotes
Bacteria
B
DockQ
AF2 + phylogeny-MSA
AF2 + phylogeny-MSA (all pairs)
Eukaryotes
Bacteria
C
Bacteria
Acceptable
Medium
High
Percent
AF2 + genome-MSA
AF2 + phylogeny-MSA (all pairs)
AF2 + phylogeny-MSA
D
Eukaryota
Acceptable
Medium
High
Percent
AF2 + genome-MSA
AF2 + phylogeny-MSA (all pairs)
AF2 + phylogeny-MSA

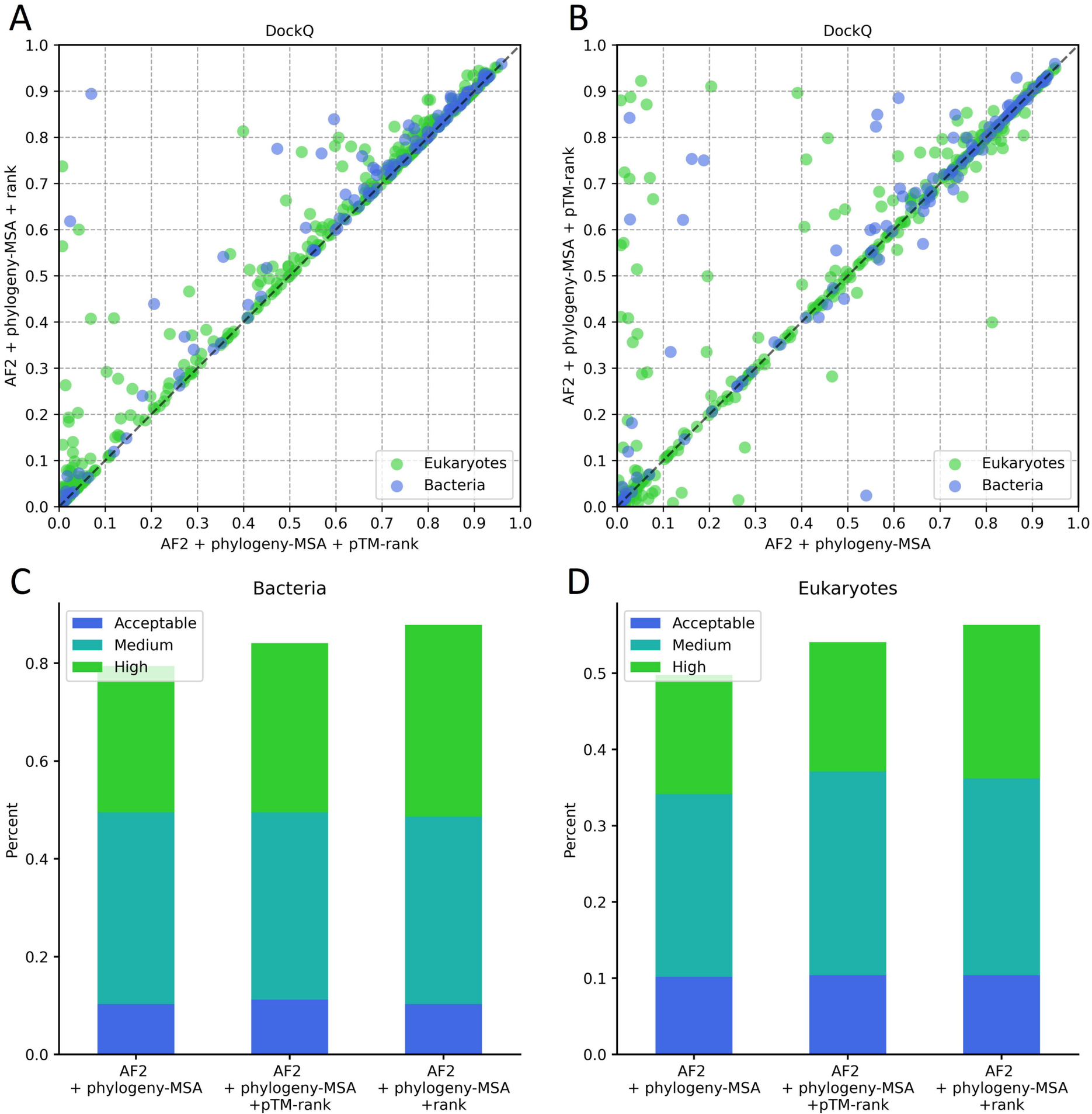

A
DockQ
AF2 + phylogeny-MSA (domain)
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
B
DockQ
AF2 + phylogeny-MSA (kingdom)
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
C
DockQ
AF2 + phylogeny-MSA (phylum)
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
D
DockQ
AF2 + phylogeny-MSA (class)
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
E
DockQ
AF2 + phylogeny-MSA (order)
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
F
DockQ
AF2 + phylogeny-MSA + rank
AF2 + phylogeny-MSA
Eukaryotes
Bacteria

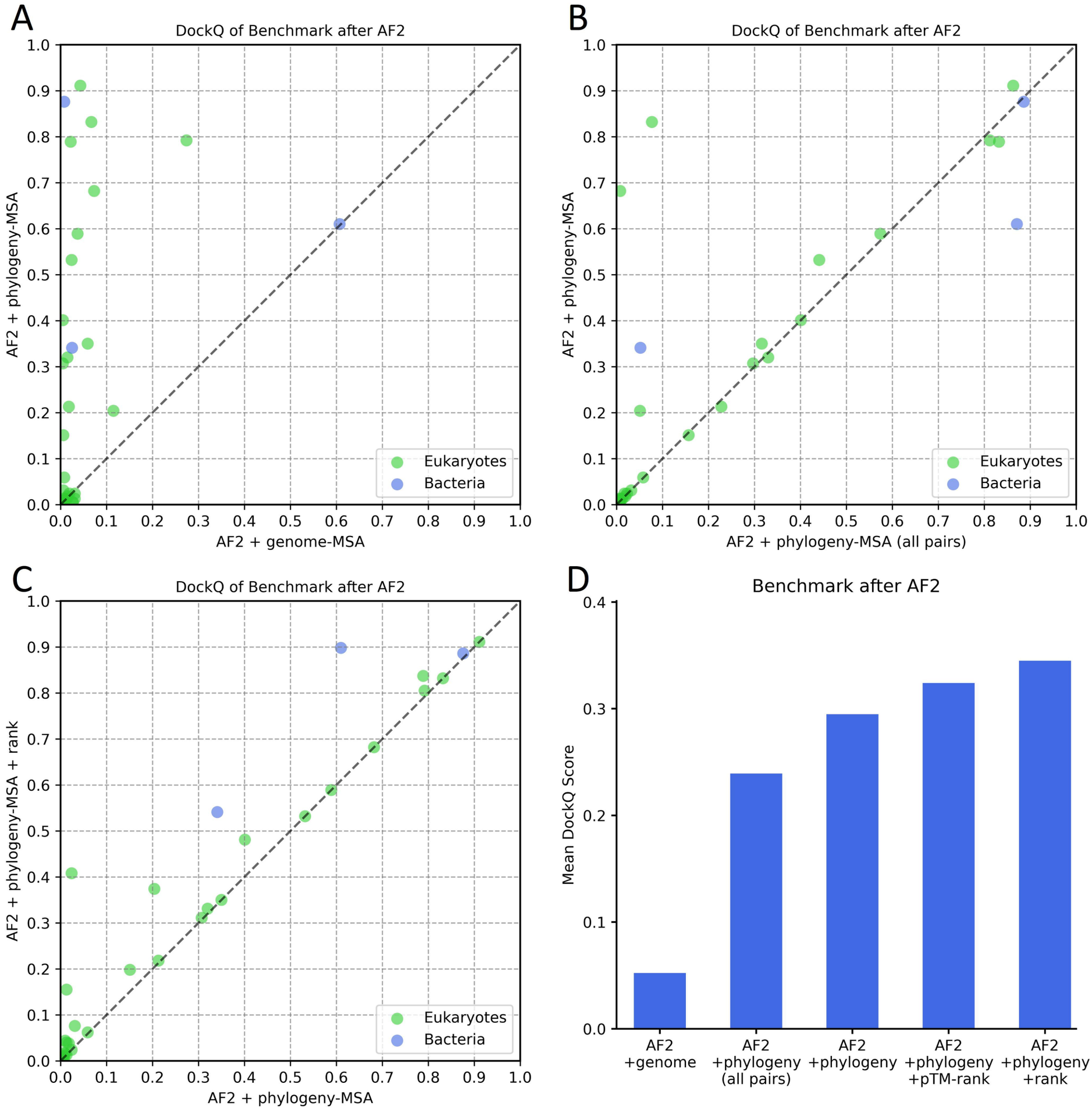


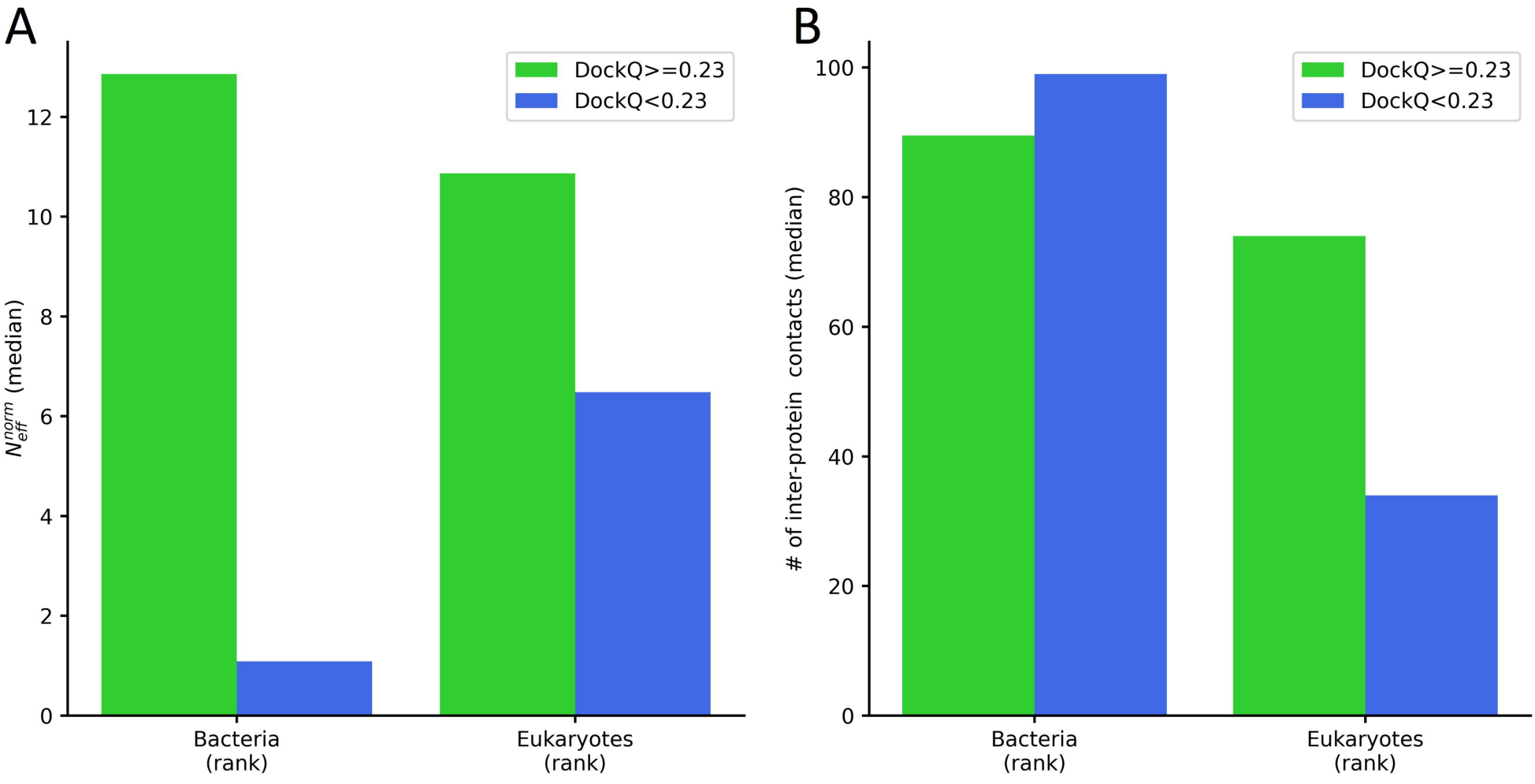
Life: DockQ=0.03, PDB ID=3NKB

Class: DockQ=0.89, PDB ID=3NKB


A
Eukaryotes
Bacteria
DockQ Score
pTM
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
B
Acceptable
Medium
High
Percent
pTM


A
DockQ
AF2 + phylogeny-MSA + rank
AF2 + phylogeny-MSA + pTM-rank
Eukaryotes
Bacteria
B
DockQ
AF2 + phylogeny-MSA + pTM-rank
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
C
Bacteria
Acceptable
Medium
High
Percent
AF2 + phylogeny-MSA
AF2 + phylogeny-MSA +pTM-rank
AF2 + phylogeny-MSA +rank
D
Eukaryotes
Acceptable
Medium
High
Percent
AF2 + phylogeny-MSA
AF2 + phylogeny-MSA +pTM-rank
AF2 + phylogeny-MSA +rank


A
DockQ of Benchmark after AF2
AF2 + phylogeny-MSA
AF2 + genome-MSA
Eukaryotes
Bacteria
B
DockQ of Benchmark after AF2
AF2 + phylogeny-MSA
AF2 + phylogeny-MSA (all pairs)
Eukaryotes
Bacteria
C
DockQ of Benchmark after AF2
AF2 + phylogeny-MSA + rank
AF2 + phylogeny-MSA
Eukaryotes
Bacteria
D
Benchmark after AF2
Mean DockQ Score
AF2 +genome
AF2 +phylogeny (all pairs)
AF2 +phylogeny
AF2 +phylogeny +pTM-rank
AF2 +phylogeny +rank


A
B
DockQ>=0.23
DockQ<0.23
N_{eff}^{norm} (median)
# of inter-protein contacts (median)
0
2
4
6
8
10
12
20
40
60
80
100
Bacteria (rank)
Eukaryotes (rank)