

# COATi: statistical pairwise alignment of protein-coding sequences

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## Abstract

Sequence alignment is an essential method in bioinformatics and the basis of many analyses, including phylogenetic inference, ancestral sequence reconstruction, and gene annotation. Sequence artifacts and errors made in alignment reconstruction can impact downstream analyses leading to erroneous conclusions in comparative and functional genomic studies. For example, abiological frameshifts and early stop codons are common artifacts found in protein coding sequences that have been annotated in reference genomes. While such errors are eventually fixed in the reference genomes of model organisms, many genomes used by researchers contain these artifacts, and researchers often discard large amounts of data in comparative genomic studies to prevent artifacts from impacting results. To address this need, we present COATi, a statistical, codon-aware pairwise aligner that supports complex insertion-deletion models and can handle artifacts present in genomic data. COATi allows users to reduce the amount of discarded data while generating more accurate sequence alignments.

## 1 Introduction

Sequence alignment is a fundamental task in bioinformatics and a cornerstone step in comparative and functional genomic analysis (Rosenberg, 2009). While sophisticated advancements have been made, the challenge of alignment inference has not been fully solved (Morrison, 2015). The alignment of protein-coding DNA sequences is one such challenge, and a common approach to this problem is to perform alignment inference in amino-acid space (e.g. Bininda-Emonds, 2005; Abascal et al., 2010). While this approach is an improvement over DNA models, it discards information, underperforms compared to alignment at the codon level, and fails in the presence of artifacts, such as frameshifts and early stop codons. While some aligners can utilize codon substitution models, they are often not robust against coding-sequence artifacts. Additionally these aligners force gaps to occur between codons, whereas in natural sequences, only about 42% of indels occur between codons (Taylor et al., 2004; Zhu, 2022). This mismatch between aligner assumptions and biology can produce sub-optimal alignments and inflated estimates of sequence divergence (Fig. 1).

Bioinformatic pipelines need to be robust to variation in quality across genomic datasets because uncorrected errors in the alignment stage can lead to erroneous results in comparative and functional genomic studies (Schneider et al., 2009; Fletcher and Yang, 2010; Hubisz et al., 2011). While genomes for model organisms often get refined over many iterations and contain meticulously curated protein-coding sequences, genomes for non-model

**a) Biology**

	Ser	His	Lys	Gly	Arg	Ser	Asp	Ala	
A:	TCC	CAT	AAG	GGG	CGG	T-- -CG	GAC	GCC	---
D:	TCC	CA-	--G	GGG	CGG	TCC CCG	GAC	GCC	ACG
	Ser	Gln		Gly	Arg	Ser	Pro	Asp	Ala
									Thr

**b) PRANK (codon) and MACSE**

	Ser	His	Lys	Gly	Arg	Ser		Asp	Ala
A:	TCC	CAT	AAG	GGG	CGG	TCG	---	GAC	GCC
D:	TCC	CAG	---	GGG	CGG	TCC	CCG	GCC	ACG
	Ser	Gln		Gly	Arg	Ser	Pro	Asp	Ala
									Thr

**c) MAFFT and ClustalΩ**

	Ser	His	Lys	Gly	Arg	Ser	Asp	Ala	
A:	TCC	CAT	AAG	GGG	CGG	TCG	GAC	GCC	---
D:	TCC	CAG	GGG	CGG	TCC	CCG	GAC	GCC	ACG
	Ser	Gln	Gly	Arg	Ser	Pro	Asp	Ala	Thr

**d) COATi**

	Ser	His	Lys	Gly	Arg	Ser	Asp	Ala	
A:	TCC	CAT	AAG	GGG	CGG	T-- -CG	GAC	GCC	---
D:	TCC	CA-	--G	GGG	CGG	TCC CCG	GAC	GCC	ACG
	Ser	Gln		Gly	Arg	Ser	Pro	Asp	Ala
									Thr

**Figure 1:** Standard algorithms produce suboptimal alignments. (a) shows the true alignment of an ancestor sequence (A) and a descendant sequence (D). (b), (c), and (d) are the results of different aligners. Nucleotide mismatches are highlighted in red. Phase 1, phase 2, and phase 3 indels are shown in purple, orange, and gray respectively. Additionally, the orange indel is type II (an amino-acid indel plus an amino-acid change) while the purple indel is type I (an amino-acid indel only). COATi is the only aligner able to retrieve the biological alignment in this example.

organisms might only receive partial curation and typically have lower quality sequences and annotations. These genomes often lack the amount of sequencing data needed to fix artifacts, including missing exons, erroneous mutations, and indels (Jackman et al., 2018). When comparative and functional genomics studies include data from non-model organisms, care must be taken to identify and manage such artifacts; however, current alignment methods are ill-equipped to handle common artifacts in genomic data, requiring costly curation practices that discard significant amounts of information.

To address current limitations of alignment software to accurately align protein-coding sequences, we present COATi, short for COdon-aware Alignment Transducer, a pairwise statistical aligner that incorporates evolutionary models for protein-coding sequences and is robust to artifacts present in modern genomic data sets.

## Materials and Methods

### Statistical Alignment via Finite State Transducers

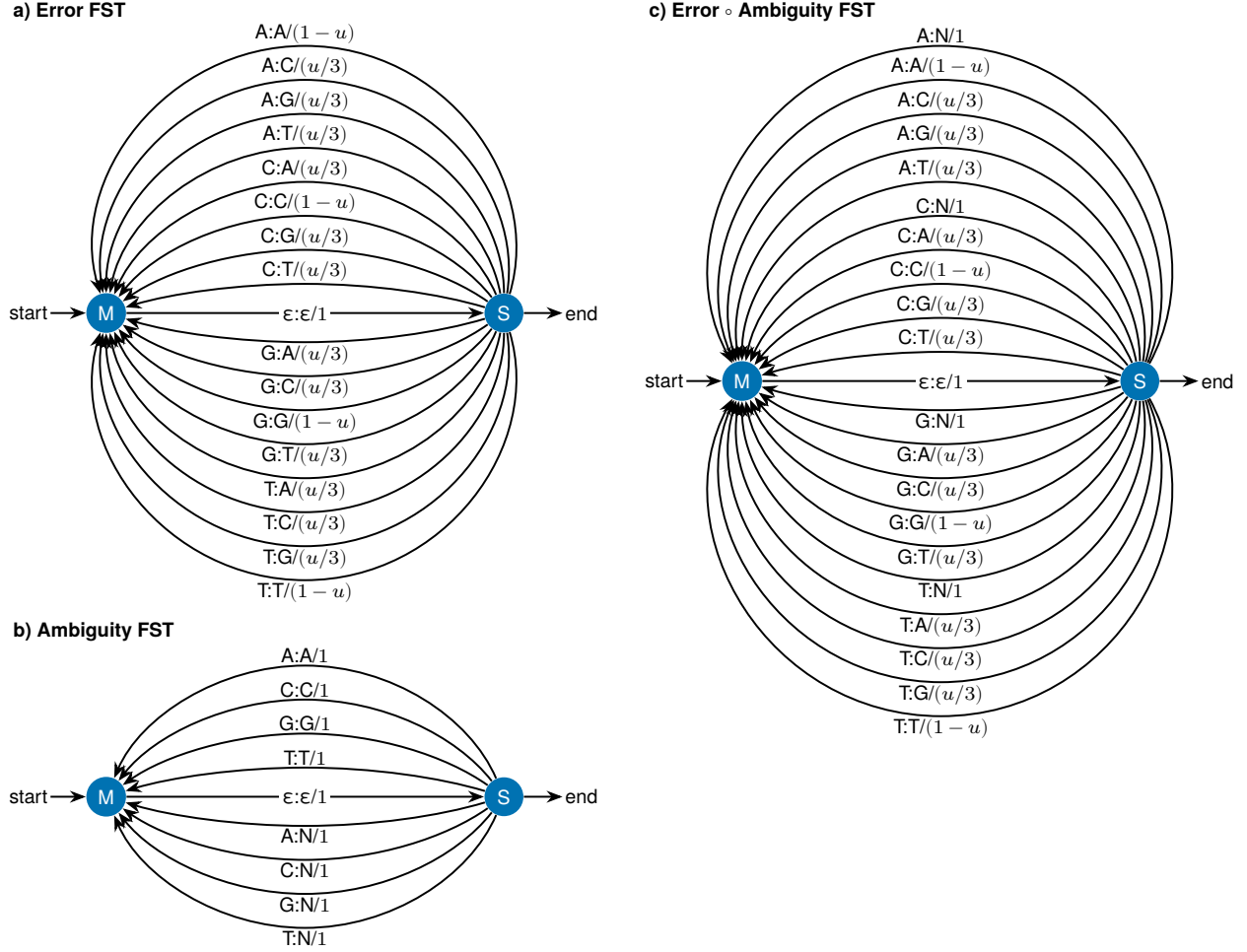
In statistical alignment, sequence alignments are scored based on a stochastic model, typically derived from molecular evolutionary processes (Lunter et al., 2005). An advantage of statistical alignment is that its parameters are derived from biological processes, allowing them to be estimated directly from data or extracted from previous studies. While approaches vary, a statistical aligner for a pair of sequences,  $X$  and  $Y$ , typically finds an alignment,  $Aln$ , that maximizes the joint probability  $P(Aln, X, Y)$  or samples alignments from the posterior  $P(Aln|X, Y)$ . This is typically performed using pairwise hidden Markov models (pair-HMMs; Bradley and Holmes, 2007). Pair-HMMs are computational machines with two output tapes. Each tape represents one sequence, and a path through the pair-HMM represents an alignment of the two sequences. Conceptually, pair-HMMs generate two sequences from an unknown ancestor and can calculate the joint probability  $P(Aln, X, Y)$  (Yoon, 2009).

While the use of pair-HMMs is ubiquitous in bioinformatics, they are limited to modeling the evolution of two related sequences from an unknown ancestor. As an alternative, finite-state transducers (FSTs, Fig. 2) allow researchers to model the evolution of a descendant sequence from an ancestral sequence. FSTs are computational machines with one input tape and one output tape and provide similar benefits to pair-HMMs, while being more suitable for evolutionary models (Bradley and Holmes, 2007). FSTs consume symbols from an input tape and emit symbols to an output tape based on the symbols consumed and the structure of the FST. Conceptually, FSTs generate a descendant sequence,  $D$ , from a known ancestor,  $A$ , and can calculate the conditional probability  $P(Aln, D|A)$ .

There are well-established algorithms for combining FSTs in different ways allowing the design of complex models by combining simpler FSTs, including concatenation, composition, intersection, union, and reversal (Bradley and Holmes, 2007; Silvestre-Ryan et al., 2021). Specifically, composition is an algorithm to combine two FSTs by sending the output of one FST into the input of another, creating a new, more complex transducer. Figure 2 illustrates how FSTs modeling sequencing errors (Fig. 2a) and ambiguity (Fig. 2b) can be combined via composition to produce an FST that does both (Fig. 2c). Conceptually, composition creates an FST that generates a descendant sequence from a known ancestor via an unknown intermediate,  $J$ , and can calculate the conditional probability  $P(D|A) = \sum_J P(D|J)P(J|A)$ .

### The COATi FST

COATi aligns pairs of sequences using a statistical alignment model, which is implemented as a finite-state transducer derived from the composition of multiple FSTs, each representing a specific biological or technical process: (1) the codon substitution FST, (2) the indel FST, (3) the error FST, and (4) the ambiguity FST (Figs. 2–3; c.f. Holmes and Bruno, 2001). We call this transducer the COATi FST. Codon substitution models are uncommon in sequence aligners, despite their extensive use in phylogenetics. COATi implements the Muse and Gaut (1994) codon model (codon-triplet-mg) and the Empirical Codon Model (codon-triplet-ecm; Kosiol et al., 2007). It also lets the user provide a codon substitution matrix. A key innovation of COATi is that it combines a codon substitution model with a

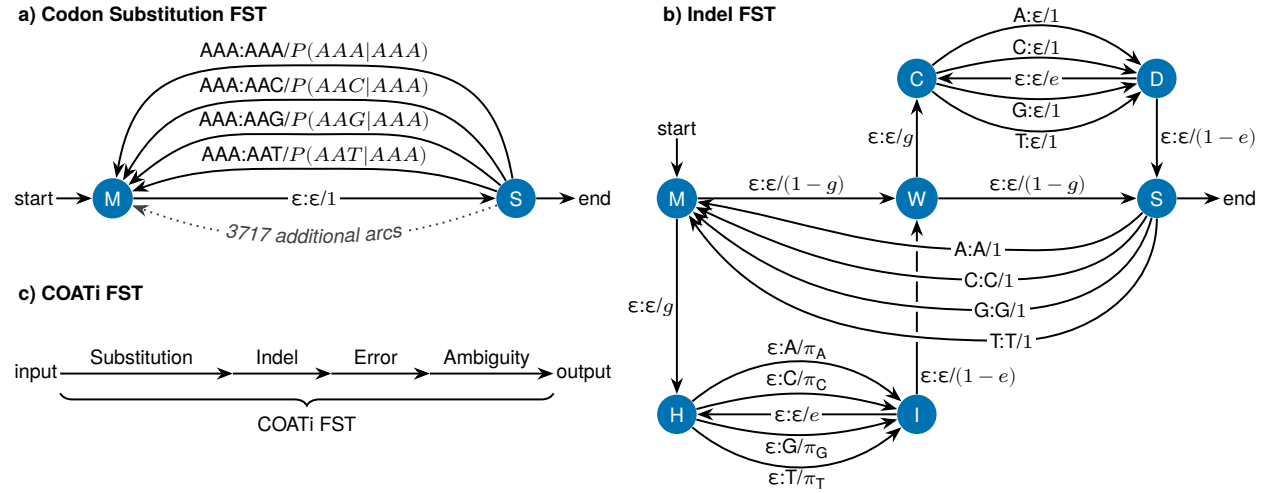


**Figure 2:** Finite state transducers (FSTs) model the generation of an output sequences based on an input sequence. (a) A graph of a probabilistic FST (Cotterell et al., 2014) for base-calling errors using a Mealy-machine architecture, where parameter  $u$  is the error rate. This graph contains two states (S and M) connected by arcs, with labels “input symbols : output symbols / weight”. Arcs consume symbols from the input sequence and emit symbols to the output sequence. Weights describe the probability that an arc is taken given the input symbols. Epsilon ( $\epsilon$ ) is a special symbol denoting that no symbols were either consumed or emitted. (b) An FST for matching sequences against ambiguous nucleotides (N). This FST is not a true probabilistic FST and cannot be used to simulate output sequences since it is missing a parameter to control how often Ns are added to the output sequence. (c) An FST that results from the composition ( $\circ$  operation) of the Error FST with the Ambiguity FST. As with (b), this composed FST cannot be used to simulate output sequences; however, it does properly weight the ambiguous nucleotide N as representing any other symbol.

nucleotide-based indel model, allowing gaps to occur both between and within codons (c.f. Ranwez et al., 2011, 2018). This also allows the aligner to be robust against sequencing artifacts that produce sequences with disrupted reading frames.

Since codon substitution is the first process in COATi’s model, the input sequence to the COATi FST must be compatible with a codon-substitution model, i.e. be a multiple of three nucleotides and not contain any ambiguous symbols or stop codons. The phases, reading

frames, and amino-acid contexts of alignment columns is determined by the input sequence, and better alignments will be generated if the input is of high quality and free of artifacts. Depending on context, we may refer to the input sequence as the “ancestral” or “reference” sequence. In contrast, the output sequence must be compatible with the ambiguity FST, can be of any length, and can contain any nucleotides or “N”. This allows COATi to align lower-quality sequences that may contain artifacts against a high-quality reference sequence. We may refer to the output sequence as the “descendant”, “non-reference”, or “query” sequence. The choice of which sequence is the input sequence and which sequence is the output sequence is left up to the user.



**Figure 3:** The COATi FST is built from simpler FSTs via composition. (a) The substitution FST encodes a  $61 \times 61$  codon substitution model with 3721 arcs from S to M. These arcs consume three nucleotides from the input tape and emit three nucleotides to the output tape. The weight of each arc is a conditional probability derived from a codon substitution model. See Fig. 2 for more details about reading this graph. (b) The indel FST allows for insertions (H to I) and deletions (C to D). Here  $g$  is the “gap opening parameter” and  $e$  is the “gap extension” parameter. Insertion arcs are weighted according to the codon model’s stationary distribution of nucleotides, and deletion arcs have a weight of 1. This FST is structured such that if insertions and deletions are contiguous, insertions will precede deletions. (c) The COATi FST is derived via composition from the codon substitution, indel, error, and ambiguity FSTs.

In order to use the COATi FST to align an output sequence against an input sequence, we first convert each sequence into an acceptor, represented as a linear transducer where the input and output symbols of each transition are identical and each transition represents one nucleotide of a sequence (Allauzen et al., 2007). By composing the input and output acceptors with the COATi FST, we generate a transducer of all possible alignments of the two sequences. Any path through this FST represents a pairwise alignment, while the shortest path (by weight) corresponds to the best alignment. If more than one optimal alignment exists, ties are broken according to the implementation of the shortest-path algorithm. All FST operations in COATi, including model development, composition, search for the shortest path, and other optimization algorithms, are performed using the C++ openFST library (Allauzen et al., 2007). An example of an FST-based alignment can

be found in Supplementary Materials Figure 2.

## The Marginal Model

The COATi FST has a large state space to keep track of codon substitution rates when codons can be interspersed with indel events. This additional state space increases the computational complexity of the alignment algorithm. To reduce the runtime complexity of COATi, we have also developed an approximation of the COATi FST that can be implemented with standard dynamic programming techniques. This approximation uses a marginal substitution model where the output nucleotides are independent of one another and only depend on the input codon and position. This produces a  $(61 \times 3) \times 4$  substitution model and eliminates the need to track dependencies between output nucleotides.

A marginal substitution model is calculated from a standard substitution model by calculating the marginal probabilities that each ancestral codon produces specific descendant nucleotides at each reading frame position. Specifically, let

$$P_{\text{cod}}(Y_1 = y_1, Y_2 = y_2, Y_3 = y_3 | X_1 = x_1, X_2 = x_2, X_3 = x_3)$$

represent transition probabilities from a codon model, and

$$P_{\text{mar}}(Y_p = y | X_1 = x_1, X_2 = x_2, X_3 = x_3) = \sum_{y_1, y_2, y_3} I(y_p = y) P_{\text{cod}}(y_1, y_2, y_3 | x_1, x_2, x_3)$$

represent the marginal transition probabilities, where  $p \in \{1, 2, 3\}$  is the position of the descendant nucleotide relative to the ancestral reading frame and  $I()$  is an indicator function. COATi contains marginal models for both Muse and Gaut (1994) or the Empirical Codon Model, resulting in the marginal models codon-marginal-mg and codon-marginal-ecm. These models emphasize the position in a codon where the substitution occurs, help restrict the effects of low-quality data in the descendant sequence, and allow more than one substitution per codon. In combination with the indel model, alignment using the marginal model is implemented using dynamic programming.

## Empirical Simulation Algorithm

In order to compare COATi against other aligners on realistic datasets, we developed a procedure to introduce realistic gap patterns into human-gorilla orthologous gene pairs that did not previously contain indels. Briefly, we downloaded 16000 human genes and their gorilla orthologs from the ENSEMBL database (Hubbard et al., 2002). After downloading, we removed 2232 sequence-pairs longer than 6000 nucleotides and aligned the remaining pairs with five alignment methods: (1) COATi's FST model (i.e. codon-triplet-mg), (2) the amino-acid model of ClustalΩ v1.2.4 via translation and reverse translation, (3) the amino-acid aware nucleotide model of MACSE v2.06, (4) the DNA model of MAFFT v7.505, and (5) the codon model of PRANK v.150803 (Sievers et al., 2011; Ranwez et al., 2018; Katoh and Standley, 2013; Löytynoja, 2014). Next we separated sequence pairs into two sets: (1) 6048 sequence pairs for which at least one aligner added gaps, and (2) 7761 sequence pairs for which all five aligners did not introduce any gaps.

We identified gap patterns from the sequence pairs that were aligned with gaps and randomly introduced them into the ungapped sequence pairs. We used an equal number

of randomly sampled gap patterns from each aligner to produce a benchmark set of known alignments. The phases of gaps were preserved as were the spacings of any clusters of gaps. Segments of matches that were 99 nucleotides or longer were allowed to change length to accommodate the length of the ungapped sequence pair. (This criteria was recursively lowered if gap pattern did not fit the ungapped sequence pair.) We deleted nucleotides from the gorilla sequences of ungapped pairs to create deletions, and we inserted random nucleotides according to their stationary frequency to the gorilla sequences to create insertions. The human sequences were unchanged.

## Alignment Accuracy Measures

We used multiple statistics to quantify the similarity between each alignment in the benchmark and the corresponding output obtained by the different tools.

**Alignment Error.** To quantify the error between estimated alignments and the benchmark alignments, we used the alignment error metric  $d_{seq}$  (Blackburne and Whelan, 2011). Intuitively,  $d_{seq}$  ranges between zero and one and can be interpreted as the fraction of nucleotides in the sequence pair that are aligned differently between estimated and benchmark alignments. This metric summarizes each alignment by building homology sets for each nucleotide in the sequence pair. Briefly, if an alignment column contains position  $i$  from the first sequence and position  $j$  from the second sequence, then  $\{j\}$  is the homology set for position  $i$  in the first sequence and  $\{i\}$  is the homology set for position  $j$  in the second sequence. If a position is aligned against a gap, then its homology set is simply  $\{\text{gap}\}$ . This treats all gaps equally as the location of the gap is not recorded. Finally,  $d_{seq}$  is calculated between an estimated and a benchmark alignment as the average, normalized Hamming distance between the homology sets for each nucleotide in the sequence pair. See Blackburne and Whelan (2011) for further details.

**Perfect, Imperfect, and Best Alignments.** We quantified the number of perfect, imperfect and best alignments each aligner produced. We define perfect alignments as alignments with a distance of zero to their benchmark alignment ( $d_{seq} = 0$ ) or any alignment that is evolutionary equivalent to an alignment with a distance of zero. Equivalency was included in our definition of perfect alignments to prevent the manner by which aligners break ties between multiple optimal alignments from impacting our results. Evolutionary equivalence was determined by scoring alignments using COATi's marginal model. Any alignment that had a score which matched the score of its benchmark alignment was considered perfect even if its distance to the benchmark was greater than zero. Imperfect alignments are defined as alignments that are not perfect when another method successfully produces a perfect alignment for the same pair of sequences. Best alignments are the alignments that the lowest distance  $d_{seq}$  to the true alignment, including ties. Taken together, these three statistics not only allow a direct comparison of aligners but also expose instances where all aligners fall short of achieving a perfect result.

**Selection.** Alignment error negatively impacts the identification of genes that have experienced positive and negative selection. To evaluate the impact of aligners on selection identification, we estimated  $k_s$  and  $k_a$  statistics (also known as  $d_s$  and  $d_n$ ) for each estimated alignment and compared them to the benchmark alignment. Briefly,  $k_s$  is the number of substitutions per synonymous site and  $k_a$  is the number of substitutions per

non-synonymous site between two protein-coding sequences. We used the method of Li (1993) to estimate these statistics as implemented in the R package *seqinr* (Charif and Lobry, 2007). We considered any alignment to be showing evidence of positive selection if  $k_a/k_s > 1$  and negative selection if  $k_a/k_s < 1$ . We estimated  $F_1$  scores for both positive and negative selection by comparing estimated alignments to benchmark alignments.  $F_1$  is the harmonic mean of precision and recall and allows us to measure how well aligners produce alignments that correctly identify or exclude positive selection or negative selection.

**Evolutionary Distance.** Alignment error also negatively impacts the estimation of evolutionary distances and phylogenetic trees. To quantify the impact of aligners on the estimation of evolutionary distances, we compared Kimura’s 2-parameter distance (K2P; Kimura, 1980) calculated for the estimated and benchmark alignments. This distance corrects for multiple mutations at a site and takes into account differences in transition and transversion rates. It also assumes equal nucleotide frequencies and no variations in the rate of substitution across sites. While Kimura’s 2-parameter distance is more suitable for non-coding sequences, it is straight forward to calculate and provides a quantitative measure of the evolutionary divergence between sequences. We used the R software package *ape* (Paradis and Schliep, 2019) to calculate K2P distances.

## Results and Discussion

COATi obtained better results compared to a wide variety of alignment strategies. It was significantly more accurate (lower  $d_{seq}$ ) at inferring the empirically simulated alignments compared to other methods; all p-values were less than  $1.3 \cdot 10^{-76}$  according to the one-tailed, paired Wilcoxon signed-rank tests (Supplementary Materials Figure 1). Notably, the average alignment error of the second best protocol was six times larger than COATi’s. In addition, COATi produced more perfect alignments, less imperfect alignments, and more accurately inferred events of positive and negative selection (Table 1). Furthermore, the estimated evolutionary divergence from the alignments retrieved by COATi was substantially less overestimated than other methods (Table 1, Supplemental Materials Figure 8).

Aligning sequences using amino-acid translation via ClustalΩ obtained the highest average alignment error and also had difficulties correctly identifying positive selection and estimating evolutionary distances. These results are not surprising because alignments in amino-acid space only permit phase-3 gaps and are sensitive to frameshift artifacts, which inflate divergence estimates and impact selection detection. Conversely, we obtained better results when aligning in nucleotide space using both amino-acid aware MACSE and amino-acid agnostic MAFFT. These results indicate that aligning protein-coding sequences in nucleotide space works reasonably well if the sequences come from closely related species, such as humans and gorillas, as it supports all three gap phases and is robust to frameshift artifacts. If the sequences were further apart, we predict that amino-acid agnostic approaches would begin to produce unreasonable alignments. And finally, our PRANK protocol used a codon substitution model which should have been an improvement over both amino-acid and nucleotide models; however, PRANK’s codon model only permits phase-3 gaps and is very sensitive to frameshift artifacts. In fact, PRANK refuses to align any sequence using a codon model if its length is not a multiple of three.

COATi is not a symmetric pairwise aligner, as reference sequences are more constrained



	COATi	MAFFT	PRANK*	MACSE	ClustalΩ
Method	Trip-MG	DNA	Codon	DNA+AA	AA
Avg alignment error ( $d_{seq}$ )	0.00221	0.01471	0.01828	0.01399	0.02929
Best alignments	5139	4692	4774	3737	2615
Perfect alignments	5793	5292	4725	2861	2893
Imperfect alignments	1048	1549	2116	3980	3948
F <sub>1</sub> score for positive selection	98.1%	84.3%	86.7%	79.5%	68.7%
F <sub>1</sub> score for negative selection	99.8%	98.4%	98.7%	98.2%	96.9%
Overestimated K2P distances	10.9%	26.6%	33.8%	48.7%	61.8%

\*PRANK produced 42 empty alignments, calculations are based on 7719 alignments.

**Table 1:** COATi generates better alignments than other alignment algorithms. Results of COATi, PRANK, MAFFT, ClustalΩ, and MACSE aligning 7761 empirically simulated sequence pairs. Best alignments have the lowest  $d_{seq}$  (including ties), perfect alignments have the same score as the true alignment, and imperfect alignments have a different score than the true alignment when at least one method found a perfect alignment.

than non-reference sequences. To test how well COATi performs when the roles of sequences are reversed, we realigned the 7761 benchmark sequence alignments using gorilla as the reference. Notably, COATi was only able to align 4003 sequence pairs due to the presence of early stop codons or other artifacts in 3758 simulated gorilla sequences. While the simulation algorithm prevents disrupting the reading frame and introducing frameshifts, it does not prevent early stop codons from being formed in the descendant sequence. Despite this limitation, we analyzed the 4003 alignments and compared the results across methods, including COATi using the human sequence as the reference. The results show a decrease, albeit small, in accuracy across all metrics when the low-quality sequence is used as the reference in comparison to the reverse (Supplementary Materials Tab. 6). However, the results for COATi continue to be a significant improvement over other aligners.

COATi uses finite-state transducers to align pairs of sequences using a codon-aware statistical model. While COATi has multiple modes and models, here we have focused on evaluating the utility of COATi FST for estimating pairwise alignments. We have shown that COATi offers a biologically significant improvement over other methods when aligning pairs of sequences separated by short evolutionary distances in the presence of genomic artifacts. In other work, we have begun to explore application of COATi to more divergent sequences and the accuracy of the marginal approximation of the COATi FST (García Mesa, 2023).

COATi is under active development. We plan on extending it to support more complex gap models, e.g. mixtures of single-nucleotide and triple-nucleotide indel models and weighing gap openings to reflect known selection on indel phases (Zhu, 2022). We plan on improving its multiple-sequence alignment and alignment sampling capabilities, as well as implement new models for aligning long-read sequences of genes against reference genomes. Our goal is to develop COATi into a user-friendly suite of tools that will allow researchers to analyze more data with higher accuracy and facilitate the study of important biological processes that shape genomic data.

## Availability

The source code for COATi, along with documentation, is freely available on GitHub: <https://github.com/CartwrightLab/coati> and is implemented in C++. Additional information, code, and workflows to replicate the analysis can be found on GitHub: <https://github.com/jgarciamesa/coati-testing>.

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*Conflict of interest:* none declared.

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