Evolutionary Modeling and Prediction of Non-Coding RNAs in *Drosophila*: Text S1

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Comparison of alignment programs

Non-coding RNAs are commonly imperfectly aligned by whole-genome alignment programs [1], limiting the possible sensitivity of alignment-sensitive methods. We tested two alignment programs, MAVID and PECAN, as inputs to our screen. Both use pairwise Hidden Markov Models (Pair HMMs) to iteratively construct a multiple sequence alignment. MAVID [2] performs a progressive alignment, iteratively aligning sibling species while traversing a guide tree, whereas PECAN [3] performs consistency alignment, using posterior probabilities to maximize the expected alignment accuracy. Several studies have suggested that consistency alignment is the more accurate technique [4–6]. We sought empirical evidence for its efficacy for phylo-grammar-based ncRNA detection.

Figure 1 shows ROC curves for the Pfold and ClosingBp grammars on the MAVID and PECAN alignments. While the overall shape of the ROC curves is similar, our method is clearly more effective on the PECAN alignments, suggesting that consistency checking is an effective technique for improving alignments of genomic data. Signals of structural conservation are easily destroyed by small local mis-alignments which consistency checking can help to resolve.

Further integration of alignment and annotation is desirable [7]. As discussed below, mis-alignment can increase false-positive as well as false-negative rates of de novo annotation.
Figure 1. ROC curves for the Pfold and ClosingBp grammars on both the MAVID and PECAN alignments of *Drosophila* genomes. Simulated data was generated with *simgenome* and not realigned. Our ncRNA detection power is greater on the PECAN alignments.
Alignment error and false-positive estimation

Local mis-alignment limits the possible sensitivity of de novo annotation strategies which take fixed alignments as input. Perhaps surprisingly, mis-alignment can increase false-positive rates as well. Alignment programs are generally designed to maximize sensitivity, even at the price of introducing a spurious homology signal [6]. Furthermore, popular whole-genome alignment programs such as MAVID and PECAN are heuristic estimators of insertion and deletion histories and as such are unable to reliably reconstruct overlapping and nested indel events.

In order to test the effect of alignment error on our false-positive estimations, we broke the simulated data into segments of length 50,000 nt, similar to what is produced by orthology mapping programs such as Mercator [8], and re-aligned each segment using PECAN. Figure 3 shows ROC curves for the ClosingBp grammar on the simulated data before and after re-alignment with PECAN.

As Figure 3 suggests, re-alignment with PECAN generally did not significantly increase our estimated false-positive rates (except near the high-specificity discovery threshold which we chose, where the estimated false-positive rate was up to 3-fold higher). This was encouraging but surprising, given that PECAN introduced significant false homology and was unable to reconstruct many of the overlapping indel events present in the original data. PECAN correctly aligned 74% of the nucleotides aligned in the true (original simulated) alignment, but at the price of low positive predictive value (PPV): 46% of the nucleotides aligned by PECAN describe homology relations which are not present in the true alignment.

Our most sophisticated genome simulator allows for many overlapping and nested indel events within intergenic regions, and the indel structure of the re-alignment produced by PECAN was rather different from that present in the original data. We examined the evolutionary gap structure of alignment data by looking at the distribution of overlapping and nested indel events.

Overlapping indel events introduce conditional dependencies between “blocks” of the alignment, where we divide an alignment into blocks by partitioning it into the (adjacent, non-overlapping) sub-alignments defined by all gap boundaries. We say that two adjacent blocks
are conditionally dependent if an insertion or deletion event crosses the boundary between the
two blocks. The number of sequential conditionally-dependent blocks indicates the number of
overlapping or nested indel events; for example, three overlapping indel events give rise to three
sequential conditionally-dependent blocks [9,10].

Figure 2 shows a plot of the distribution of the number of sequential conditionally-dependent
blocks before and after re-alignment. Prior to re-alignment, the many overlapping indels in
the simulated data meant that each segment consisted of a single sequence of conditionally-
dependent blocks; this was no longer the case after re-alignment. PECAN was unable to recover
significant portions of the indel structure of the true alignment.

Although PECAN’s re-alignment introduced significant false homology and imperfectly recon-
structed the true indel history, our estimated false-positive rate actually decreased as a conse-
quence of re-alignment. We conclude that our method’s false-positive rate is robust with respect
to mis-alignment of neutrally-evolving regions of the genome.

**Figure 2.** Distribution of the number of sequential conditionally-dependent blocks, indicating
the number of overlapping or nested indel events, in the simulated data before and after
re-alignment with PECAN. If the indel structure of the true (original) alignment was perfectly
preserved, the two distributions would be identical.
Figure 3. ROC curves for the ClosingBp grammar on simulated data (Indel2Lex3) before and after re-alignment with PECAN. Alignment error introduced by PECAN had surprisingly little effect on our estimated false-positive estimates, except near the high-specificity discovery threshold which we chose, where the estimated false-positive rate was up to 3-fold higher.
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