MAGPIE/EGRET Annotation of the 2.9-Mb *Drosophila melanogaster Adh* Region

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Our challenge in annotating the 2.91-Mb *Adh* region of the *Drosophila melanogaster* genome was to identify genetic and genomic features automatically, completely, and precisely within a 6-week period. To do so, we augmented the MAGPIE microbial genome annotation system to handle eukaryotic genomic sequence data. The new configuration required the integration of eukaryotic gene-finding tools and DNA repeat tools into the automatic data collection module. It also required us to define in MAGPIE new strategies to combine data about eukaryotic exon predictions with functional data to refine the exon predictions. At the heart of the resulting new eukaryotic genome annotation system is a reverse comparison of public protein and complementary DNA sequences against the input genome to identify missing exons and to refine exon boundaries. The software modules that add eukaryotic genome annotation capability to MAGPIE are available as EGRET (Eukaryotic Genome Rapid Evaluation Tool).

The microbial MAGPIE genome annotation system (Gaasterland and Sensen 1996; Deckert et al. 1998; Gaasterland and Ragan 1998; Romine et al. 1999) accepts assembled, unannotated contiguous genome sequence data as input. For finished genome sequence data, the system performs three phases of analysis. Phase 1 identifies coding regions, builds DNA-level and protein-level analysis requests for the coding regions, manages the execution of the requests on remote or local machines, and parses the output data into local relational facts, each of which is connected to supporting text extracted from the original output. Included in the phase 1 data collection are comparisons of each protein sequence encoded in the query genome with the proteins from each available complete genome or chromosome.

In phase 2, MAGPIE generates a functional report for each coding region by synthesizing all overlapping functional evidence into a single view according to user-specified preferences (Gaasterland and Lobo 1997). A series of decision rules generate one or more suggested functions for the gene product of the coding region. Alignments with proteins from other genomes are used to determine potential boundaries between protein domains. Currently, the system suggests one function for the whole protein and notes potential domains. The system also suggests one or more functional categories for the protein based on categories of similar functions in *Escherichia coli*, yeast, *Synechocystis* sp., and other complete genomes with assigned function categorization. The system treats enzymes as special cases. It looks for all enzyme numbers in the collected evidence and displays the most frequently occurring enzyme numbers together with their function descriptions. The synthesis of evidence overlays PROSITE (Hofmann et al. 1999), BLOCKS (Henikoff et al. 1999), and PRINTS (Attwood et al. 1999) functional motifs with sequence alignments so that a biologist user can easily see whether motif information is consistent with suggested enzyme functions. In phase 2, biologist users are expected to confirm or edit the annotations of individual gene products through interactive forms. Confirmed annotations are saved for later automatic reformatting into an European Molecular Biology Laboratory (EMBL) or GenBank nucleotide database submission form.

In phase 3, the MAGPIE system generates a series of whole-genome reports. The first is an enzyme report that collects links to coding regions with suggested enzymatic functions into one table. The second is a tRNA report that summarizes which tRNAs have been found and which amino acids have at least one type of codon in an annotated tRNA gene. The third is a pathway report that lists for every pathway in the enzyme and metabolic pathway (EMP) database which enzymes have been confirmed manually or suggested automatically. This phase also generates an executive summary of all predicted genes and their current confirmed or suggested annotation. Finally, this phase generates a summary of the distribution of matching proteins from other genomes in the form of a genomic signature (Gaasterland and Ragan 1998a) [also referred to more recently in the literature as a phylogenetic profile (Marcotte et al. 1999)] for every encoded protein. Genomic signatures of ORFs are included in the enzyme

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reports, the individual protein function reports, the pathway reports, and the executive summary.

Adapting MAGPIE for Eukaryotic Genome Annotation

Adapting MAGPIE for eukaryotic genome annotation required four steps related to exon identification. First, we had to add a preliminary module to request and parse gene-finding tools. We evaluated several tools based on (1) their ability to find *Drosophila* exons, (2) whether they could be installed locally or used remotely via an email server, and (3) the difficulty of parsing the output into a relational form. We selected GENSCAN (Burge and Karlin 1998) as the first gene-finding tool to integrate into the system. Second, we had to adapt the visual display and internal relational tables to store coding regions as a series of exons rather than as one ORF. Third, we had to add an automated reverse-similarity feature that extracted the strongest matching proteins for a coding region from the public databases, load those sequences into a search group, and compare the sequences with BLAST (Altschul et al. 1997) to the input genomic sequence data. Fourth, we had to build an exon editing tool that allowed an expert biologist to "tune" exon boundaries based on alignments with complementary DNA (cDNA) and protein sequences from the query organism. Finally, we built a module that assembled the edited exons, translated them into final protein sequences, and generated a new set of requests for final comparison with nonredundant public proteins and all available genomic proteins.

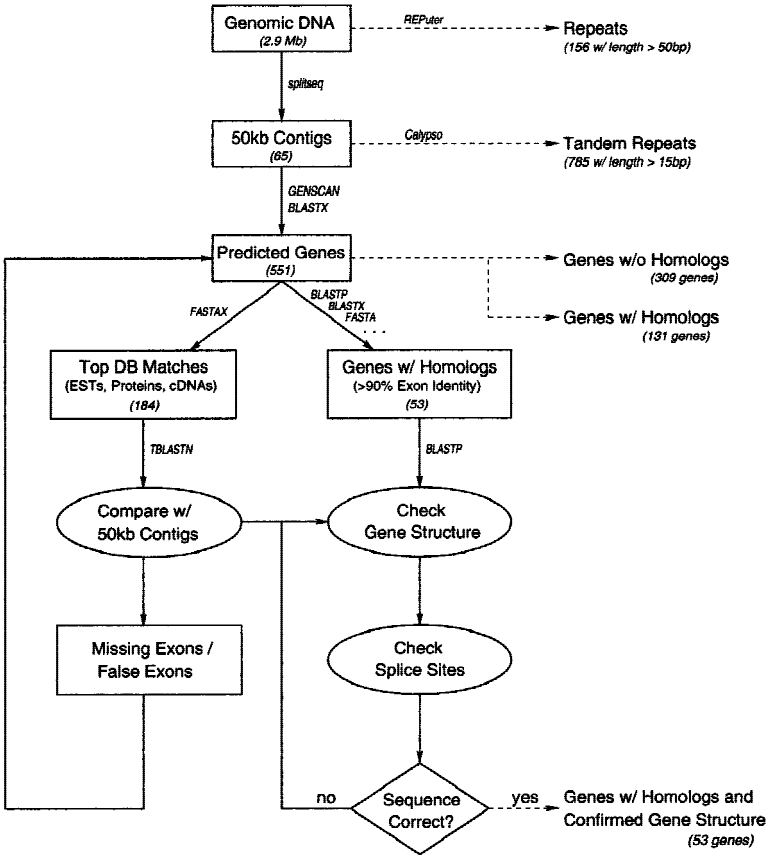


Figure 1 Eukaryotic genome analysis strategy.

Eukaryotic Annotation Strategy

The steps listed above added the general functionality necessary for MAGPIE to be used as a eukaryotic genome annotation system. To execute the annotation of the 2.91-Mb *Adh* region of *Drosophila*, we created a specific configuration of MAGPIE to run the following tools:

REPuter: (Kurtz and Schleiermacher 1999) (input = full genome) to find all forward, reverse, complement, and reverse complement repeats with length >50 bp.

Splitseq: (Gaasterland and Sensen 1996) (input = full genome) to split the input sequence into 65 50,000-base contiguous sequences (contigs) each overlapping with the next by 10,000 bases.

Calypso: (Fields 1999) (input = 65 50-kb subsequences) to identify tandem repeats.

GENSCAN: (Burge and Karlin 1998) (input = 65 50-kb subsequences) to identify exons and assemble them into translatable DNA.

BLASTX: (Altschul et al. 1997) sequence comparison against nonredundant protein databases (input = 65 50-kb subsequences), to identify protein matches both inside and outside predicted exon regions.

BLASTP: against nonredundant protein databases (input = 551 predicted proteins), to find pairwise matching proteins.

BLASTN: against nonredundant GenBank sequences (input = DNA sequences for 551 predicted proteins), to find pairwise matching genes.

BLASTN: against *Drosophila* EST and cDNA sequences (input = DNA sequences for 551 predicted proteins), to confirm exons.

FASTA: (Pearson 2000) against proteins from each complete genome (input = 551 predicted proteins), to find genomic distribution of matching proteins.

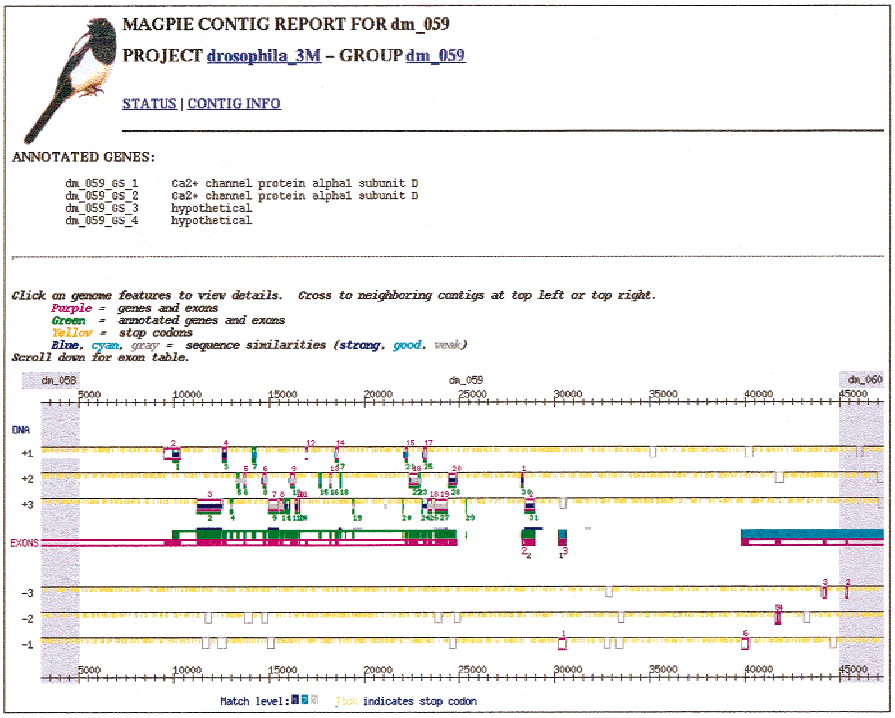


Figure 2 Annotated features in contig 59 of 50-kb subsequences. The 31 exon coding region labeled dm 059 2 encodes a calcium-ion channel protein.

TBLASTN: against the input genome (input = top protein matches for the 184 of 551 proteins that had a match in the nonredundant databases), to find missing exons and extra, overpredicted exons.

BLASTP: against the predicted proteins (input = top protein matches for the 184 of 551 proteins that had a match in the nonredundant databases), to find portions of the known proteins that were not covered by predicted protein sequence.

TBLASTN: against the *Drosophila* cDNA and EST sequences (input = top protein matches for the 184 of 551 proteins that had a match in the nonredundant databases), to identify whether cDNA and EST sequences matched protein boundaries or internal regions.

BLASTP: against the nonredundant sequence databases (input = 53 predicted proteins whose DNA sequences had exact cDNA matches), to confirm that entire protein domains were matched by the predicted proteins and that intron–exon splice sites were correct.

Figure 1 shows the flowchart for executing this analysis strategy. At each stage of the analysis, output at the right of the flowchart was stored in MAGPIE relational tables for further report generation. The REPuter output identified all repeats >50 bp (a threshold that we selected) throughout the 2.91-Mb contig. The Calypso output identified tandem repeats, which we mapped to gene locations together with REPuter repeats in a new repeat report. GENSCAN generated 550 sets of predicted exons, with promoters, terminators, protein sequence translations, and a score indicating confidence that the genes were real. The subsequent BLAST and FASTA analysis of each encoded protein sequence divided the GENSCAN predictions into the following sets: 309 with no evidence beyond GENSCAN prediction; 131 confirmed by protein sequence matches or partial cDNA or EST matches; and 53 confirmed by full-length cDNA sequence matches. These last

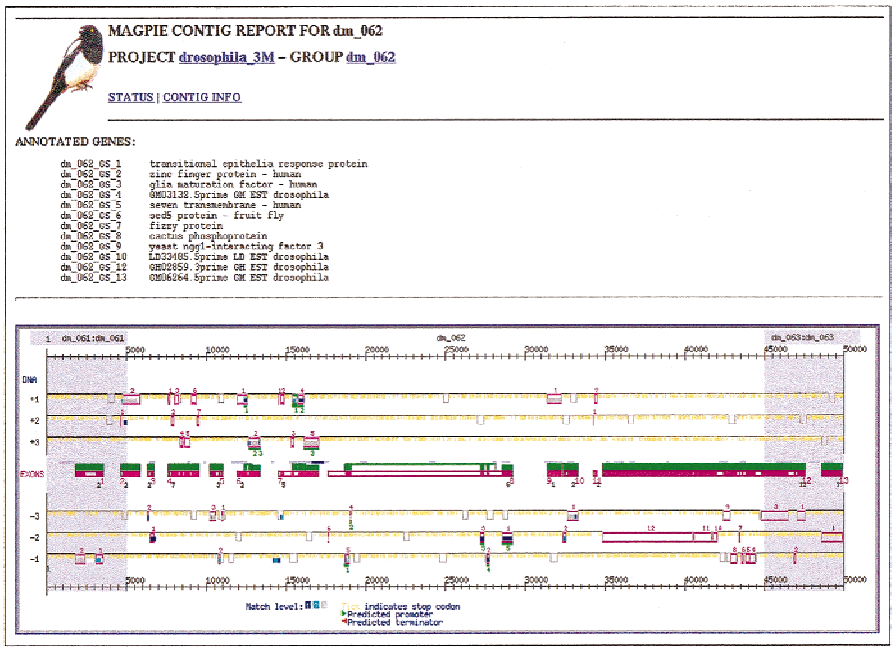


Figure 3 Annotated features in contig 63 of 50-kb subsequences. Of 13 proteins are encoded, 8 are functionally annotated and 4 are confirmed by ESTs. Individual exons are shown in series in the *middle* of the graphic and in frame *top* and *bottom*.

53 predicted proteins all matched a full-length *Drosophila* protein sequence as well, because full-length cDNAs are translated into the public nonredundant protein sequence databases. The best protein matches for the 184 (131 + 53) predicted proteins with evidence were extracted and configured as a group to be compared as queries against the *Drosophila* genome. This step led to intron–exon boundary adjustments for every encoded protein with evidence.

Example: The Calcium-Ion Channel Region

Figure 2 illustrates the results of the annotation process. It shows a visual display of predicted exons and refined exons for a gene encoding a calcium-ion channel protein. The initial GENSCAN predictions are shown in pink starting at the left. The display runs off to the previous 50-kb subsequence because the GENSCAN tool included exons from the previous subsequence in the gene. The refined exons are shown in green. Each exon is displayed both in the middle and in its translation frame, with respect to the in-strand beginning of the 50-kb subsequence. Pink numbers indicate GENSCAN exons in order; green numbers apply to refined exons. Note that GENSCAN predicted an additional gene with two exons downstream of the predicted calcium-ion channel exons, numbered in pink as 2 2. The reverse similarity step of the analysis indicated that these two exons should be joined with the previous set as encoding the carboxy-terminal end of a single amino acid sequence. Two additional coding regions with no evidence beyond GENSCAN prediction are shown on the reverse strand in dark pink, labeled as 1 3 and 6 4 (label not in Fig. 2). The calcium-ion channel coding region was the most complicated in the entire genome in terms of number and size of exons.

Figure 3 shows the evidence collected when the best matching protein sequence for the calcium-ion channel protein was used as a query against the 2.91-Mb genomic contig, the assembled exons, and the EST and cDNA sequence databases. Portions of the query protein sequence, represented as a ruler across the top of the diagram, failed to match the translated predicted proteins. Matches with the translated proteins are labeled on the left as dm 059 1 and dm 059 2. Notice that this pair of matches indicates that these two GENSCAN predictions should be merged into a single coding region. Comparing the query sequence against the cDNA sequences revealed a full-length match and confirmed that the two sets of exons should be merged. Comparison against the genomic DNA, shown in green, indicates that the full query protein sequence maps completely to the genomic sequence. This match meant that without an automated exon-boundary refinement tool, such as that provided in GeneWise (Birney 1999), the exon boundaries would need to be refined manually, which we did, using the form shown in Figure 5, below. The resulting combined gene corresponds to BG:DS02795.1 (*Ca-α1D*) (Ashburner et al. 1999).

Example: A Well-Annotated Region

Figure 4 shows a well-annotated region of the *Drosophila* genome. The 50-kb subsequence contains 13 predicted coding regions, some on the negative strand, others on the positive strand. The exons for three proteins in the region, labeled as dm 062 6 (6 2), dm 062 7 (7 5), and dm 062 8 (8 6), were confirmed with full-length cDNA sequences and refined via reverse-BLAST against the protein sequences. Four of the remaining proteins were confirmed and functionally annotated through protein sequence similarities. The genes for the remaining four proteins were validated with matches against *Drosophila* EST sequences. These genes

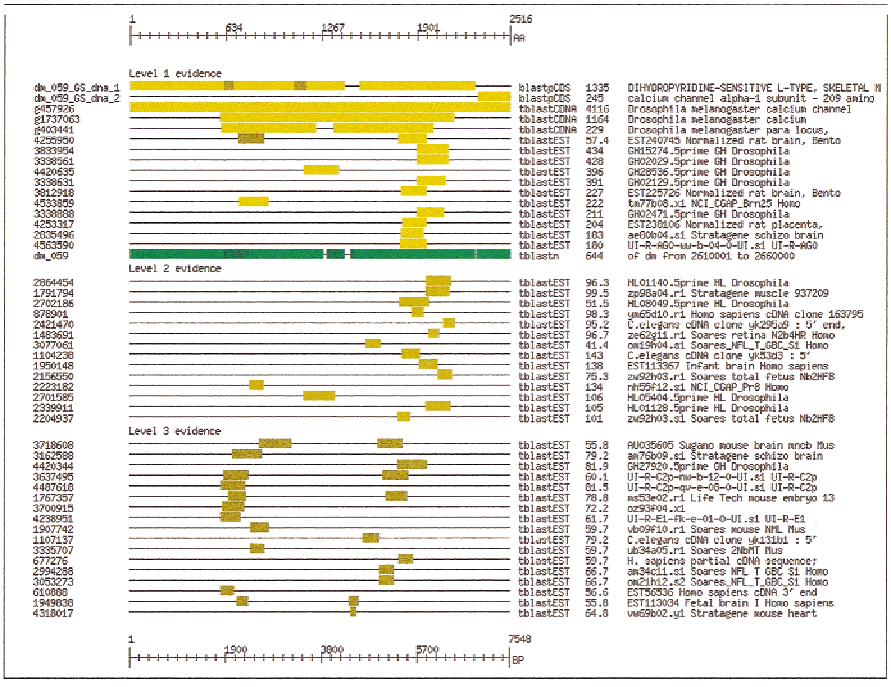


Figure 4 Evidence shows missing and mispredicted exons for the calcium ion channel protein. The first gap in the first row indicates that exons from the next predicted gene should be merged with the calcium-ion channel gene.

correspond to genes *BG:DS02740.12* (*Sed5*), *BG:DS02740.14* (*fzy*), and *BG:DS02740.15* (*cact*) (Ashburner et al. 1999).

Combining Evidence for Individual Decisions

Figure 5 shows an overview of the evidence gathered for the DNA mismatch repair protein predicted in subsequence dm 029 of the input genome, labeled as BG:DS02740.15 and "Spellchecker" by Ashburner et al. (1999). The protein matched a nonredundant protein sequence from beginning to end with both BLASTP (yellow) and FASTA (pink). In addition, it matched proteins from 11 bacterial genomes (*Aquifex*, *Borrellia*, *Bacillus*, *Camphylobacter*, two *Chlamydia*, *E. coli*, *Haemophilus*, *Treponema*, *Rickettsia*, and *Synechocystis* sp.) and two eukaryotic genomes (*Caenorhabditis elegans* and *Saccaromyces cerevisiae*). The query *Drosophila* sequence matched the yeast sequence nearly entirely. The other genomic matches missed 200 or more of the amino-terminal amino acids. Figure 6 shows the complete display of the high-quality matches for the query sequence, represented at the top of the display with an amino acid sequence ruler.

Genomic Signatures for 53 Confirmed Proteins

We incorporated genomic signatures into the MAGPIE report for the 53 proteins whose exon boundaries were refined using full-length cDNA matches. Figure 8 shows the signatures together with the final protein functional annotations. Only 2 of the 52 proteins had matches only in archaeal and eukaryotic genomes: One was DNA-directed RNA polymerase II, and the other was RNA polymerase I elongation factor, consistent with observations in the yeast (Ragan and Gaasterland 1998) and archaeal genomes (Gaasterland and Ragan 1998) that proteins shared exclusively between archaea and eukaryotes tend to be involved in translation and transcription. Four proteins were conserved across all three phylogenetic domains: glutamic acid decarboxylase, alcohol dehydrogenase, "shuttle craft transcription factor," and acyl-phosphatase. Note that the latter protein had no eukaryotic match outside *Drosophila* in *C. elegans* or yeast. An additional five proteins were shared with bacterial genomes and other eukaryotes. Twenty-six proteins matched a combination of yeast and *C. elegans* but no archaeal or bacterial genome. Finally, 16 cDNA-confirmed *Drosophila* proteins matched nothing in any of the 23 target genomes.

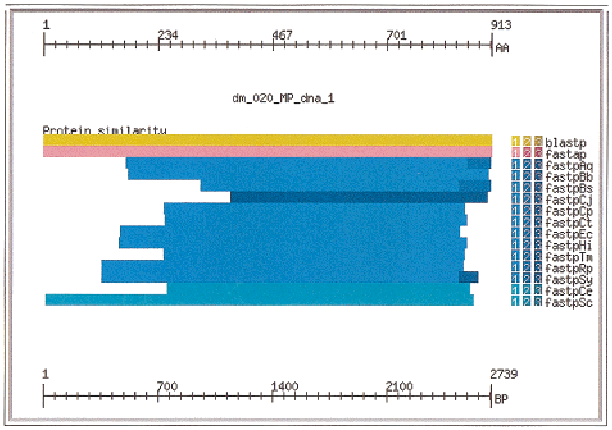


Figure 6 Evidence summary for DNA mismatch repair protein showing matches in 11 bacterial genomes (blue) and 2 eukaryotic genomes (cyan).

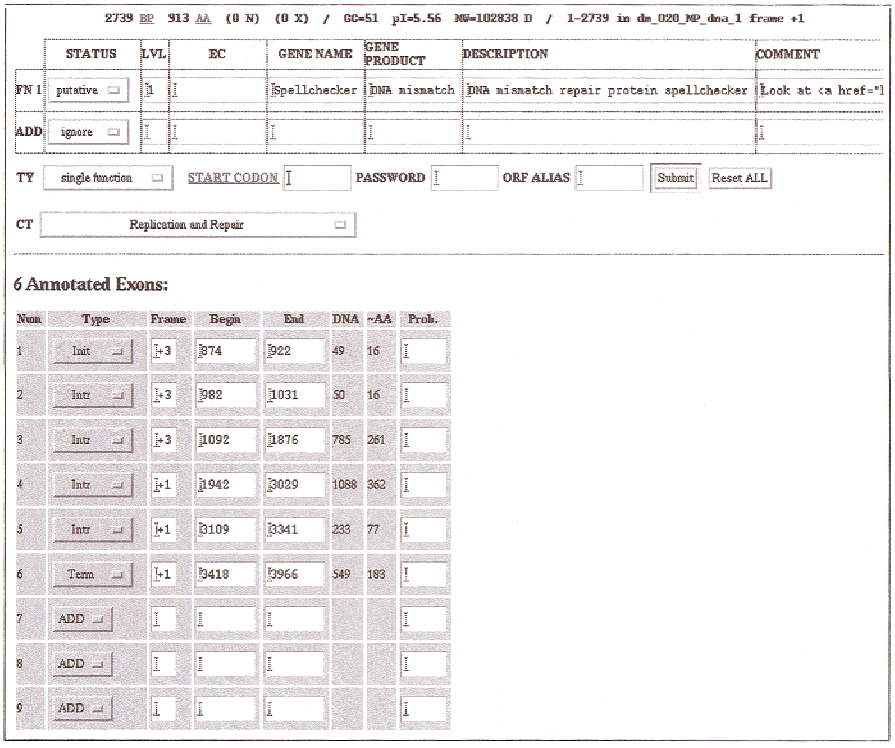
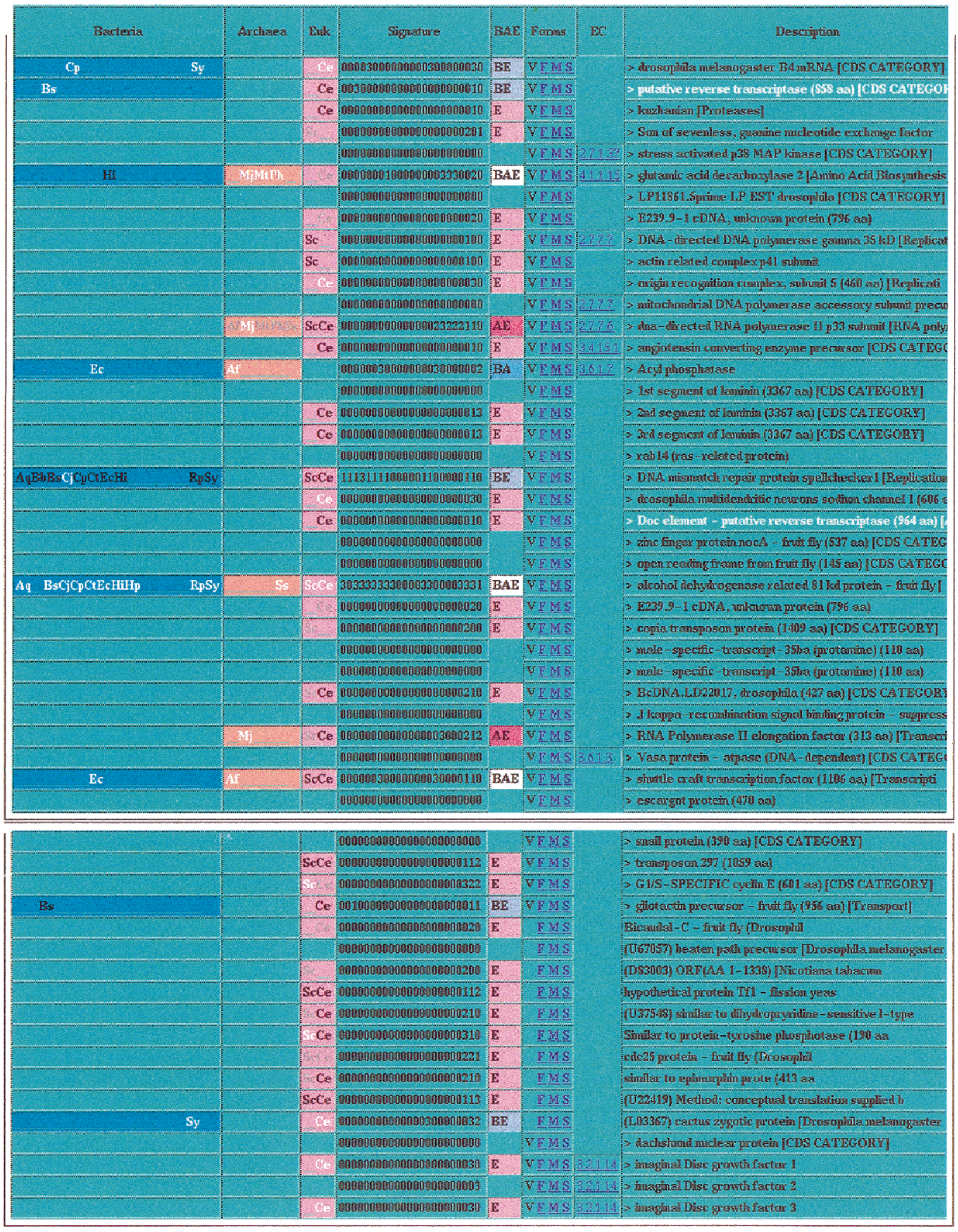


Figure 5 Screen shot of annotation form. Exon boundaries and multiple functions can be edited and saved on the annotation database for further querying.

Data Collection and Manual Annotation Time

Table 1 shows the numbers of seconds spent running each tool in the analysis configuration. A total of 1,515,756 CPU seconds (1515756 sec = 25263 min = 421 hr = 17.5 days) were spent col-

Figure 7 Full evidence view for DNA mismatch repair protein.



Bacteria	Archaea	Euk	Signature	BAE	Forms	EC	Description
Cp Sy		Ce	00000300000000300000030	BE	V F M S		> drosophila melanogaster B4 mRNA [CDS CATEGORY]
Bs		Ce	00300000000000000000010	BE	V F M S		> putative reverse transcriptase (858 aa) [CDS CATEGO
		Ce	00000000000000000000010	E	V F M S		> kazbanian [Proteases]
			00000000000000000000201	E	V F M S		> Son of sevenless, guanine nucleotide exchange factor
			00000000000000000000000		V F M S	2.7.1.37	> stress activated p38 MAP kinase [CDS CATEGORY]
Hi	MjMtPh		00000001000000003300020	BAE	V F M S	4.1.1.15	> glutamic acid decarboxylase 2 [Amino Acid Biosynthesis
			00000000000000000000000		V F M S		> LP11861.5prime LP EST drosophila [CDS CATEGORY]
			00000000000000000000020	E	V F M S		> E239.9-1 cDNA, unknown protein (796 aa)
		Sc	00000000000000000000100	E	V F M S	2.7.7.-	> DNA-directed DNA polymerase gamma 35 kD [Replicat
		Sc	00000000000000000000100	E	V F M S		> actin related complex p41 subunit
		Ce	00000000000000000000030	E	V F M S		> origin recognition complex, subunit 5 (460 aa) [Replicati
			00000000000000000000000		V F M S	2.7.7.-	> mitochondrial DNA polymerase accessory subunit precu
	Mj	ScCe	00000000000000023222110	AE	V F M S	2.7.7.6	> dna-directed RNA polymerase II p33 subunit [RNA poly
		Ce	00000000000000000000010	E	V F M S	3.4.15.1	> angiotensin converting enzyme precursor [CDS CATEG
Ec	Af		00000030000000030000002	BA	V F M S	3.6.1.7	> Acyl phosphatase
			00000000000000000000000		V F M S		> 1st segment of laminin (3367 aa) [CDS CATEGORY]
		Ce	00000000000000000000013	E	V F M S		> 2nd segment of laminin (3367 aa) [CDS CATEGORY]
		Ce	00000000000000000000013	E	V F M S		> 3rd segment of laminin (3367 aa) [CDS CATEGORY]
			00000000000000000000000		V F M S		> rab14 (ras-related protein)
AqBbBsCjCpCtEcHi RpSy		ScCe	11131111000001100000110	BE	V F M S		> DNA mismatch repair protein spellchecker1 [Replicatio
		Ce	00000000000000000000030	E	V F M S		> drosophila multidendritic neurons sodium channel 1 (606 a
		Ce	00000000000000000000010	E	V F M S		> Doc element - putative reverse transcriptase (964 aa) [
			00000000000000000000000		V F M S		> zinc finger protein nocA - fruit fly (537 aa) [CDS CATEG
			00000000000000000000000		V F M S		> open reading frame from fruit fly (145 aa) [CDS CATEGO
Aq BsCjCpCtEcHiHp RpSy	Ss	ScCe	30333333330000330000331	BAE	V F M S		> alcohol dehydrogenase related 81 kd protein - fruit fly [
		Sc	00000000000000000000020	E	V F M S		> E239.9-1 cDNA, unknown protein (796 aa)
		Sc	00000000000000000000200	E	V F M S		> copia transposon protein (1409 aa) [CDS CATEGORY]
			00000000000000000000000		V F M S		> male-specific-transcript-35ha (protamine) (110 aa)
			00000000000000000000000		V F M S		> male-specific-transcript-35ha (protamine) (110 aa)
		Ce	00000000000000000000210	E	V F M S		> BcDNA.LD22017, drosophila (427 aa) [CDS CATEGORY
			00000000000000000000000		V F M S		> J kappa-recombination signal binding protein - suppress
	Mj	Ce	00000000000000003000212	AE	V F M S		> RNA Polymerase II elongation factor (313 aa) [Transcr
			00000000000000000000000		V F M S	3.6.1.3	> Vasa protein - atpase (DNA-dependent) [CDS CATEG
Ec	Af	ScCe	00000030000000030000110	BAE	V F M S		> shuttle craft transcription factor (1106 aa) [Transcripti
			00000000000000000000000		V F M S		> escargot protein (470 aa)
			00000000000000000000000		V F M S		> small protein (390 aa) [CDS CATEGORY]
		ScCe	00000000000000000000112	E	V F M S		> transposon 297 (1059 aa)
		Sc	00000000000000000000322	E	V F M S		> G1/S-SPECIFIC cyclin E (601 aa) [CDS CATEGORY]
Es		Ce	00100000000000000000011	BE	V F M S		> glutactin precursor - fruit fly (956 aa) [Transport]
			00000000000000000000020	E	F M S		Bicaudal-C - fruit fly (Drosophil
			00000000000000000000000		F M S		(U67057) beaten path precursor [Drosophila melanogaster
			00000000000000000000200	E	F M S		(DS3003) ORF(AA 1-1338) [Nicotiana tabacum
		ScCe	00000000000000000000112	E	F M S		hypothetical protein Tf1 - fission yeas
		Ce	00000000000000000000210	E	F M S		(U37548) similar to dihydropyridine-sensitive L-type
		ScCe	00000000000000000000310	E	F M S		Similar to protein-tyrosine phosphotase (190 aa
			00000000000000000000221	E	F M S		cdc25 protein - fruit fly (Drosophil
		Ce	00000000000000000000210	E	F M S		similar to ephrin protein (413 aa
		ScCe	00000000000000000000113	E	F M S		(U22419) Method: conceptual translation supplied b
Sy		Ce	00000000000000300000032	BE	F M S		(L03367) cactus zygotic protein [Drosophila melanogaster
			00000000000000000000000		V F M S		> dachshund nuclear protein [CDS CATEGORY]
		Ce	00000000000000000000030	E	V F M S	3.2.1.14	> imaginal Disc growth factor 1
			00000000000000000000003		V F M S	3.2.1.14	> imaginal Disc growth factor 2
		Ce	00000000000000000000030	E	V F M S	3.2.1.14	> imaginal Disc growth factor 3

Figure 8 Bacterial, archaeal, eukaryotic genomes matched by each gene product with cDNA or protein sequence verified exon boundaries.

Table 1. Automated Data Collection Run Times for Each Tool and Total

Tool	Sec/tool	Responses	Total sec
GENSCAN	1800	65	117000
BLASTX nr	8880	65	577200
BLASTN nt	960	65	62400
BLASTN EST	360	65	23400
FASTA dmEST	120	65	7800
FASTA dmcDNA	8	65	520
REPORTS	120	65	7800
Subtotal		455	822120
BLASTX nr	400	841	336400
Subtotal		4205	336400
BLASTN *Adh*	8	551	4408
TBLASTN cDNA	4	551	2204
BLASTP CDS	1	551	551
FASTAX nr	480	551	264480
REPORTS	60	551	33060
Subtotal		2755	304703
TBLASTN *Adh*	2	184	368
BLASTP CDS	1	184	184
FASTAP CDS	1	184	184
REPORTS	60	184	11040
Subtotal		736	11776
TBLASTN cDNA	4	53	106
TBLASTX cDNA	12	53	636
BLASTN cDNA	4	53	106
BLASTN *Adh*	8	53	212
BLASTP CDS	1	53	53
FASTAP nr	480	53	25440
FASTA 26 genomes	8	1378	11024
REPORTS	60	53	3180
Subtotal		1537	40757
Total		5483	1515756

Run times and totals were determined using Ultrasparc 336-mHz CPUs writing to disk via an UltraSCSI fast-and-wide connection.

lecting and parsing 5483 individual analysis outputs automatically.

Table 2 shows the total amount of time spent manually annotating the functions and exon boundaries for proteins predicted by GENSCAN based on the evidence collected above. The full annotation required a total of 1966 person min (1966 min = 33 hours = 5.5 workdays) in addition to the CPU seconds listed above. In practice, the work was distributed over a cluster of 21 Sun Ultrasparc 336 megahertz CPUs with UltraSCSI fast-and-wide connections to local disk arrays, interconnected via gigabit and 100 BaseT ethernet links.

DISCUSSION

In the 6 weeks from the opening of the genome annotation competition to the submission deadline, we designed and implemented a new set of modules, called EGRET (for Eukaryotic Genome Rapid Evaluation Tool), to enable the MAGPIE genome annotation system to handle eukaryotic genome sequence data. Once the software modules were in place, we configured the system to collect gene predictions, EST, cDNA and protein sequence similarities, and functional protein sequence patterns. The system executed an automated functional annotation, and our annotation team per-

Table 2. Total Manual Confirmation and Editing Times via MAGPIE Forms

Activity	Min/CDS	CDSs	Total min
db matches → exon regions	5	184	920
Exon regions → splice site	15	53	795
db matches → function	1	551	551
Total		788	1966

(db) Database; (CDS) coding sequence.

formed manual exon refinement and a final manual confirmation of function annotations. The new software modules, EGRET, were implemented and run as separate new programs compatible with the original microbial MAGPIE. It remains to integrate the new modules into the entire system so that a full eukaryotic genome can be accepted as input and processed from beginning to end without human intervention. The EGRET modules are available through the Rockefeller University, and the MAGPIE modules are available through Argonne National Laboratory and the National Research Council of Canada.

The integrated EGRET system provides biologists with a useful tool to perform annotation of functional and genomic features of megabases of eukaryotic sequence data. We are currently in the process of designing and implementing a hardware architecture for supporting the timely application of the new eukaryotic genome annotation system, built for this competition, to full eukaryotic genomes.

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