VALIDATION OF A NOVEL EXPRESSED SEQUENCE TAG (EST) CLUSTERING METHOD AND DEVELOPMENT OF A PHYLOGENETIC ANNOTATION PIPELINE FOR LIVESTOCK GENE FAMILIES

A Dissertation

by

ANAND VENKATRAMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Biochemistry

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December 2008

Major Subject: Biochemistry

ABSTRACT

Validation of a Novel Expressed Sequence Tag (EST) Clustering Method and Development of a Phylogenetic Annotation Pipeline for Livestock Gene Families.

(December 2008)

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Dr. Christine G. Elsik

Prediction of functions of genes in a genome is a key step in all genome sequencing projects. Sequences that carry out important functions are likely to be conserved between evolutionarily distant species and can be identified using cross-species comparisons. In the absence of completed genomes and the accompanying high-quality annotations, expressed sequence tags (ESTs) from random cDNA clones are the primary tools for functional genomics. EST datasets are fragmented and redundant, necessitating clustering of ESTs into groups that are likely to have been derived from the same genes. EST clustering helps reduce the search space for sequence homology searching and improves the accuracy of function predictions using EST datasets. This dissertation is a case study that describes clustering of *Bos taurus* and *Sus scrofa* EST datasets, and utilizes the EST clusters to make computational function predictions using a comparative genomics approach.

We used a novel EST clustering method, TAMUClust, to cluster bovine ESTs and compare its performance to the bovine EST clusters from TIGR Gene Indices (TGI) by using bovine ESTs aligned to the bovine genome assembly as a gold standard. This comparison study reveals that TAMUClust and TGI are similar in performance. Comparisons of TAMUClust and TGI with predicted bovine gene models reveal that both datasets are similar in transcript coverage.

We describe here the design and implementation of an annotation pipeline for predicting functions of the *Bos taurus* (cattle) and *Sus scrofa* (pig) transcriptomes. EST datasets were clustered into gene families using Ensembl protein family clusters as a framework. Following clustering, the EST consensus sequences were assigned predicted function by transferring annotations of the Ensembl vertebrate protein(s) they are grouped to after sequence homology searches and phylogenetic analysis. The annotations benefit the livestock community by helping narrow down the gamut of direct experiments needed to verify function.

DEDICATION

To my family members and friends who have been a constant source of encouragement throughout my graduate student life. Without their unstinting support, this roller-coaster of a graduate student journey would not have been possible.

ACKNOWLEDGEMENTS

I would like to thank my co-chairs, Dr. Elsik and Dr. Hu, and my committee members, Dr. Krutovsky and Dr. Park, for their guidance and support throughout the course of this research.

I am greatly indebted to members of the Elsik lab – Dr. Justin Reese, Dr. Juan Anzola, and Dr. Michael Dickens – for the innumerable brain-storming and code-storming sessions we have had, without which many loose ends on this dissertation would have been left untied.

I would also like to thank Dr. Deborah A. Siegele and Dr. Rodolfo Aramayo for critiquing my manuscript and helping me improve the overall quality of work in it. I would like to thank members of the Hu lab – Dr. Brenley McIntosh, Adrienne E. Zweifel, and Daniel Renfro – for all their help and support.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

NOMENCLATURE

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
EST	Expressed Sequence Tag
cDNA	Complementary DNA
OTU	Operational Taxonomic Unit
COG	Clusters of Orthologous Groups
NCBI	National Center for Biotechnology Information
dbEST	Database of Expressed Sequence Tags
TIGR	The Institute of Genomic Research
TGI	TIGR Gene Indices
DFCI	Dana-Farber Cancer Institute
DGI	DFCI Gene Indices
STACK	Sequence Tag Alignment Consensus Knowledgebase
CDS	Coding Sequence
EGAD	Expressed Gene Anatomy Database
BTGI	*Bos taurus* Gene Indices
GO	Gene Ontology
MAFFT	Multiple Alignment Using Fast Fourier Transform

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CHAPTER I

INTRODUCTION

BACKGROUND

Identifying the genes in a genome and predicting their functions are key steps in all genomic sequencing projects. These processes are very important in interpreting genome sequence data and guiding future experimental work [1]. Functional annotations of genes from genome-wide experimental characterization are not yet scaled to match the rapid pace at which genomes are sequenced. This holds true despite the development of new advances in experimental techniques such as DNA microarrays [2, 3], yeast two-hybrid system [4], RNA interference (RNAi) [5, 6] or large-scale systematic deletions [7] and therefore, the annotation of newly sequenced genomes relies mostly on computational methods [8, 9]. In the absence of availability of completed genomes and the accompanying high-quality annotations, expressed sequence tags (ESTs) [8] from random cDNA clones serve as an invaluable and cost-efficient resource for functional genomics in a variety of organisms [9-11]. EST datasets are fragmented and redundant; hence ESTs need to be clustered into groups that are likely to have been derived from the same genes. This dissertation is a case study that describes clustering of *Bos taurus* and *Sus scrofa* EST datasets, and utilizes the EST clusters to make computational function predictions using a comparative genomics approach.

This dissertation follows the style of *BMC Genomics*.

Overview of work in this dissertation

The work in this dissertation uses ESTs in order to determine what genes are there in an organism and what roles they play. To achieve this task, EST datasets from *Bos taurus* (cattle) and *Sus scrofa* (pig) were grouped into gene families using the protein family clusters generated by a clustering algorithm developed in our lab to cluster the different vertebrate proteomes in the Ensembl [9] database. These protein families served as a framework to cluster and assemble the ESTs, and resulted in EST consensus sequences (contigs/singletons) representing putative genes. Anonymous ESTs are of limited value unless connected to function; hence, these EST consensus sequences were annotated by transferring annotations of the Ensembl vertebrate protein(s) following sequence homology searches and phylogenetic analysis. The annotation pipeline developed provides function predictions for *Bos taurus* (cattle) and *Sus scrofa* (pig) using EST datasets. These annotations would benefit the livestock community by serving as a guide that helps narrow down the gamut of direct experiments needed to verify function.

Bos taurus (cattle) and *Sus scrofa* (pig) are good candidate animal models for biomedical research due to parallels with humans [10-12] and represent evolutionary clades distinct from primates, rodents, and fishes (**Figure 1.1**). The genome sequences for these species are in different stages of completion. The *Bos taurus* genome is in the final draft assembly (7.1 fold coverage) [10, 11]; a 6 fold coverage of the *Sus scrofa* has been proposed [12], with the current Pre-Ensembl [13] release of the *Sus scrofa* genome featuring the preliminary assemblies for 15 of the 19 pig chromosomes.

The completed genome and the accompanying high-quality annotations for *Bos taurus* and *Sus scrofa* were unavailable when this study started; hence, EST datasets from these species were used to obtain insights about the functions encoded in the organism.

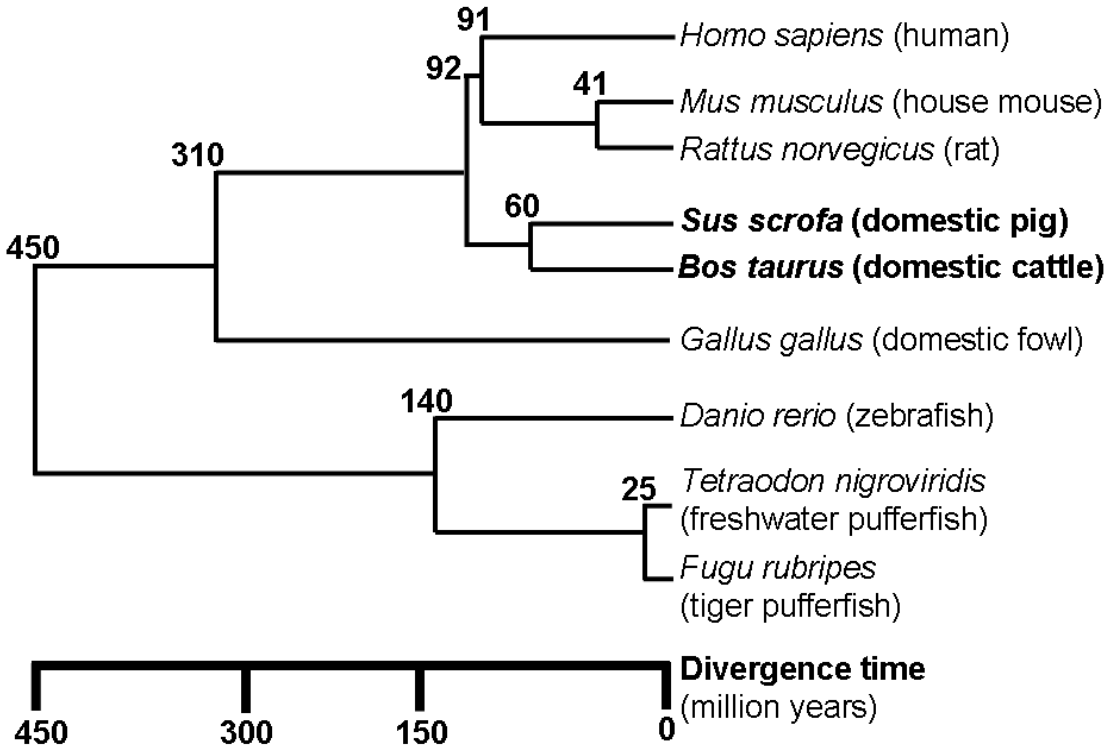


Figure 1.1: Evolutionary distances of the different species being compared in this study.
A phylogenetic tree depicting the evolutionary relationships and divergence times (in million year timescale) for *Sus scrofa* (pig), *Bos taurus* (cattle), and the seven species whose proteomes were used to generate protein family clusters. *Sus scrofa* (pig) and *Bos taurus* (cattle) are shown in bold to convey the fact that EST datasets from these species are utilized to obtain functional insights using a comparative genomics approach involving the other seven proteomes. Figure adapted from [14].

Chapter I

The remaining portion of Chapter I is tailored to provide the relevant background information about the different topics that are discussed in Chapters II, III and IV limited, but not restricted to, concepts in comparative genomics, homology, orthology, paralogy, functional annotations, Expressed Sequence Tags (ESTs), and EST clustering.

Chapter II

Chapter II of this dissertation evaluates the performance of a novel EST clustering method, TAMUClust, developed in our lab; this method clusters ESTs using a protein framework. Using bovine ESTs as an example, evaluation of this clustering method was performed by comparing it with existing EST clustering methods and bovine genome aligned EST clusters.

Chapter III

Chapter III describes the design and implementation of a "Livestock EST Gene Family Database" which houses the annotations for *Bos taurus* (bovine) and *Sus scrofa* (porcine) ESTs. Bovine and porcine ESTs were clustered and assembled using the Ensembl vertebrate protein families as a framework, and resulted in EST consensus sequences which were assigned the function of the 'best match' Ensembl vertebrate protein following FASTX [15] searches.

Chapter IV

Chapter IV describes the design and implementation of a phylogenomic annotation pipeline for *Bos taurus* (bovine) and *Sus scrofa* (porcine) ESTs. The ESTs were grouped into gene families using the Ensembl vertebrate protein families as a

framework, subject to a phylogenetic analysis, and the uncharacterized bovine/porcine EST gene family members were assigned predicted functions based on their positions in the phylogenetic tree involving other protein(s) of the subgroup/subfamily.

Chapter V

Chapter V of this dissertation summarizes the findings from this study and puts into perspective future avenues that can be explored utilizing these findings.

Intersection of evolution and comparative genomics

"Nothing in biology makes sense, except in the light of evolution" [16] is a famous quote of Theodosius Dobzhansky. Comparative genomics is a relatively new field that complements a long history of comparison-based disciplines in biology [17]. Comparative genomics is the analysis and comparison of genomes with the purpose of gaining a better understanding of how species have evolved, and helps obtaining insights into the biology of the organisms being compared [14]. According to Ureta-Vidal et. al [14], "genome sequence comparison is a good example of the application of modern evolutionary theory advocated by Kimura and others [18-20]".

Evolutionary analysis is a powerful tool that aids in the studies of genome sequences and helps to place comparative genomics studies in perspective [21]. Conserved genetic information in the form of DNA sequence forms the foundation of the evolutionary relationships and the underlying functional and anatomical similarities between species [17, 22]. Cross-species comparisons in comparative genomics studies help identify biologically active regions; this is based on the premise that sequences

which carry out important functions are likely to be conserved between evolutionarily distant species [22, 23]. The assumption that underlies all comparative genomics studies is that whenever significant sequence conservation is detected in species separated by a long span of evolution, one can be sure that this conservation is driven by constraints associated with function [24].

Cross-species sequence analyses requires flexibility as no single pairwise comparison can capture all biologically functional sequences based on conservation [17]. A very important decision in the process of designing a comparative genomics based study is to identify which two (or more) species are the most appropriate for comparison in order to address the question under investigation. Given below are the different kinds of questions that can be addressed by comparing genomes at different phylogenetic distances:

1. Comparing DNA sequences between evolutionary distantly related species, such as humans and pufferfish, which diverged approximately 450 million years ago, reveals that the coding sequences are conserved [25]. This is due to the fact that protein coding sequences are tightly constrained to retain function and thus evolve slowly, resulting in readily detectable sequence homology over large evolutionary distances [26]. Therefore, the addition of distantly related organisms (450 million years) to a multi-species sequence comparison improves the ability to classify conserved elements into coding sequences and non-coding sequences.
2. By comparing multiple species that diverged approximately 40–80 million years ago, such as humans with mice and humans with cows, one can determine the

conserved noncoding sequences in several species which is more likely due to active conservation rather than shared ancestry [26].

3. Comparison of DNA sequences between pairs of species that diverged 40–80 million years ago from a common ancestor, such as two species of nematodes (*Caenorhabditis elegans* with *Caenorhabditis briggsae*) [27] or *Escherichia coli* with *Salmonella* species [28], reveals conservation in both coding sequences and a significant number of noncoding sequences.
4. The comparative analysis of very closely related species like human-chimpanzee (separated by 5 million years of evolution) is apt for finding the key sequence differences that may account for the differences in the organisms [23, 29].

Figure 1.2 summarizes the different types of questions that can be addressed by comparing genomes at different evolutionary distances.

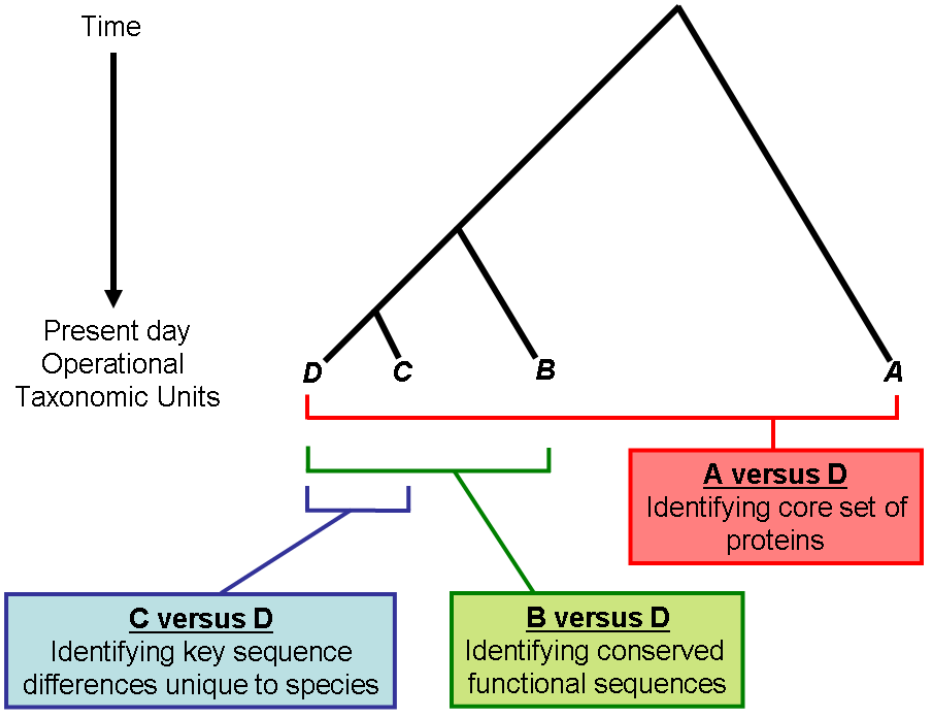


Figure 1.2: Different kinds of comparative genomics questions that can be addressed at different evolutionary distances.
A generalized phylogenetic tree is shown, leading to four present day operational taxonomic units (OTUs), with A and D being the most distantly related pairs. Examples of the different questions that can be addressed are shown in the boxes. Figure adapted from [22].

Developments in the field of comparative genomics

Comparative genomics was born as soon as there were two genomes to compare. The first genome to be sequenced was that of RNA bacteriophage MS2 in 1976 [29], and this was followed by the genome sequence of the bacteriophage phi X174 in 1977 [30]. The complete genome sequence of the bacterium *Haemophilus influenza* [31] signaled the beginning of a new era in biological research [32] as it became clear that genome sequences provide a wealth of information not only about the organism but also the genes encoded.

However, comparative genomics of cellular life forms is a "by-product" of the human genome project [33, 34] and took off [24] after the publication of the human genome in 2001. The increasing awareness of the immense benefits of genome scale sequence comparisons have resulted in a rapid increase in the number of genomes being sequenced post 2001. **Figure 1.3** gives a year-by-year distribution of the number of completely sequenced eukaryotic and prokaryotic genomes from the Entrez Genome Project Database [35] as of September 2008.

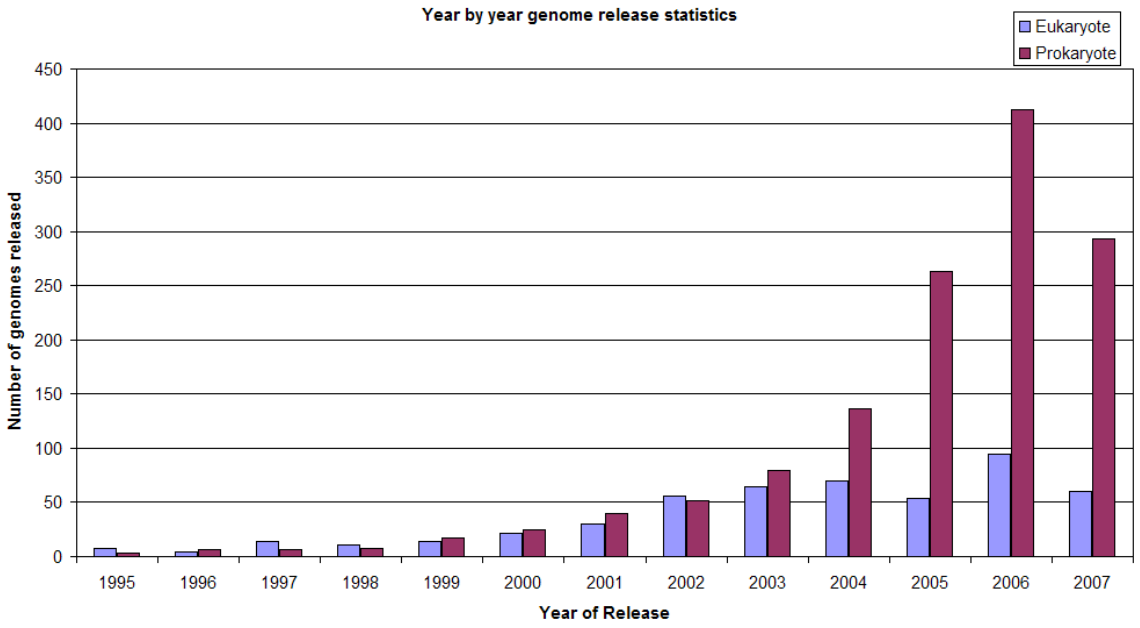


Figure 1.3: Annual genome release statistics from 1995 to 2007.
Year wise distribution of the number of completely sequenced eukaryotic and prokaryotic genomes in the Entrez Genome Database [35] as of September 2008.

Sequence comparisons of human-mouse [36] , human-chicken [37], human-fish [25, 38] have led to the identification of new genes and gene regulatory sequences, and highlighting the benefits of comparative genomics studies. Comparative genomics analyses reveal important information about the functions and evolutionary relationships of great majority of genes in any genome [24]. The number of cross-species sequence comparisons has increased as additional genomes got sequenced. Initial comparative genomics analyses focused on pairwise comparisons to annotate and explore a single species of interest (such as humans), but as genomic data accumulates, simultaneous

analysis from numerous species is needed [17] to catalogue the evolutionary extent of sequence conservation and divergence.

Need for functional annotations

Biologists need tools and annotated databases to deal with the volume of genomic and proteome data. The ability to accurately predict function based on sequence is an important tool in biological research as these predictions are useful in gaining a first-order approximation of the molecular function encoded and help in prioritizing experimental investigation [39] .

Function assignment for proteins identified in genome sequencing projects is a major problem in post-genomic biology as there is no clear function prediction for at least 30-40% of the sequences, and for many of the rest, only general predictions can be made [40, 41]. Until functions are assigned to the unknown genes, the organism's capabilities cannot be completely described as there might be sequences that are species-specific and determine the unique characteristics of the organism; thus, the promise of post-genomic biology will remain elusive until function assignments are complete [41].

Different classes of computational function prediction methods

Many computational methods have been developed to predict function from sequences. These methods can be broadly grouped into two main classes [1] – the homology methods and the non-homology methods.

1. Homology methods: Sequences that share a common ancestor are called homologs. Homology methods rely on the identification, characterization and quantification of sequence similarity. This sequence similarity can exist at

different levels [1]: motifs, domains, entire genes/proteins. The function of the unknown sequence is inferred based on the known (or presumed) function of a statistically significant database hit. Database search programs like BLAST [42, 43], FASTA [44], BLOCKS [45] have revolutionized the role of biological sequence comparisons. Sequence comparison methods are becoming more sensitive (increased number of true positives) and more selective (fewer false positives); improvements in these programs have made the identification of putative homologs much faster, easier and more reliable.

2. Non-homology methods: Here, properties of a gene other than its similarity to other genes are used to aid in function predictions [1]. These include distance from origins of replication, analysis of neighboring genes [46-48], domain patterns [49], and codon usage or nucleotide composition [50-52]. In the non-homology methods, genes in a genome or genes across genomes are grouped by these properties and the function of the unknowns can be predicted if they get grouped with genes of known function [1].

In the subsequent sections, I will be examining homology methods in more detail and discussing the pros and cons of using different approaches for predicting function.

Function predictions using homology based approach

As sequence is the prime determining factor of function, sequence homology based function prediction methods operate on the premise that homology implies

function similarity. In these methods, inference of homology is usually based on finding levels of sequence similarity that are thought to be statistically significant and too high to be arising because of chance or convergence [53]. This similarity can be detected at any or all of these levels [1]: primary structure of DNA or protein, secondary or three-dimensional structure [54, 55]. If statistically significant similarity is detected to a sequence of known function, the sequence with unknown function is tentatively assigned the function of the known sequence.

Popular similarity based function prediction methods

The available homology based function prediction methods differ in the way they choose the homolog whose function is most relevant to an uncharacterized sequence. The different methods available are:

1. Best Hit Method: The uncharacterized sequence is tentatively assigned the function of the database sequence which was identified as the highest hit by a similarity search program [56].
2. Top Hits: The top 10+ hits for the uncharacterized sequence are identified, and depending on the consensus of the functions of the top hits, the query sequence is assigned a specific function or a general activity with unknown specificity or no function [57].
3. Clusters of Orthologous Groups (COG) [58]: In this method, sequences are divided into groups of orthologs based on a cluster analysis of pairwise similarity scores between sequences from different species and the uncharacterized sequences are assigned the functions of characterized orthologs. Although this

method is a major advancement in identifying orthologous groups, it relies on similarity scores and not phylogeny [59] to cluster orthologous groups.

The "best hit" and the "top hits" methods are very fast, can be easily automated, and are accurate in most instances. If no homology is detected and/or if homology is detected to sequences with no known function, no function prediction can be made. However, they do not take advantage of information about how sequences and their functions evolve [39, 53].

Errors associated with homology based function predictions

The errors associated with homology based function prediction methods can be broadly classified into:

1. Gene duplication and neofunctionalization: When gene duplication occurs, one copy retains the original function and the other is free to evolve new functions. Gene duplication and subsequent divergence of function of the duplicates is the single greatest contributor to errors in function prediction by homology [39, 53]. Very often the top database hit may have a different function to the query due to neofunctionalization arising from gene duplication [60].
2. Changes in function due to speciation: Function changes due to speciation are also a contributing factor to errors in function prediction. The proteins can share a common ancestor and be orthologous but can have different function specificities [61, 62].

3. Differences in protein domain architecture between query and database protein: Domain shuffling [63, 64] is also one of the major contributors to errors in function prediction by homology. Standard methods of homology detection by similarity methods ignore whether two proteins align globally or locally, and this leads to errors in function prediction as presence/absence of a domain can have a dramatic impact on the molecular function of the protein. This problem is compounded by the fact that roughly 65% of the eukaryotic proteins and 40% of prokaryotic proteins are composed of multiple domains [65, 66] . Domain fusion and domain fission events produce protein families that may only share a single common domain, and it is also known that some domains are promiscuous [67, 68] in that they are present in combination with other domains, thereby leading to many different domain combinations. **Figure 1.4** depicts a scenario where the query sequences have a different domain architecture compared to the top database hit following a database homology search. In automated function inference approaches, these individual domains of a multi-domain protein are the "local" partial homologs often identified as top hits by the database search program. BLAST [42] and PSI-BLAST [43] are commonly used methods for clustering homologous proteins. As these methods are optimized for homolog detection based on local similarity, the clusters are not screened to remove proteins with different domain architectures. Since the function of a multi-domain protein is a composite of all the constituent domains, annotation transfer based on local homology can be misleading. Function prediction is most reliable

using "homeomorphic" proteins [69] – proteins sharing similar domain architecture.

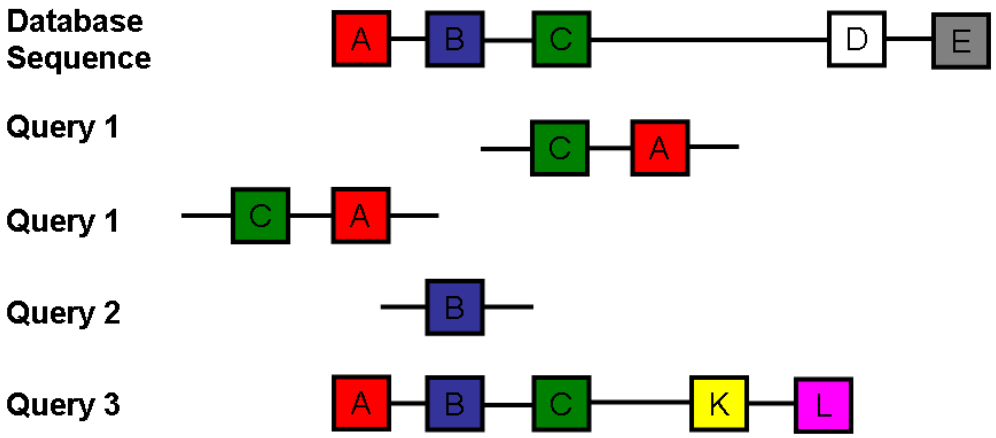


Figure 1.4: Different domain architectures for query and database sequences. Cartoon depicting a scenario where the three query sequences (1, 2, 3) have different architectures compared to the top database hit following a database similarity search.

In my next section, I am going to introduce the two types of homologs: orthologs and paralogs, and going to talk about:

1. the different evolutionary pressures that orthologs and paralogs are subject to when it comes to preserving function;
2. the need to discriminate between orthologs and paralogs; and
3. the need for identifying and using orthologs for function predictions and annotation transfer.

Homologs: orthologs and paralogs

Homologs are of two types: orthologs and paralogs [70, 71]. Orthologs are homologs that have arisen because of speciation events whereas paralogs are homologs hat have arisen because of gene duplication events. **Figure 1.5** depicts a simplified diagram of homolog subtypes showing orthologous and paralogous genes in two species '1' and '2'. These definitions were first introduced by Walter Fitch in 1970. Orthologs are evolutionary counterparts derived from a single ancestral gene in the last common ancestor of the species being compared; paralogs are homologous genes evolved through duplication within the same (perhaps ancestral too) genome.

Function constraints for orthologs and paralogs

Orthologs are under strict evolutionary constraints and this makes them perform the same function as long as the function remains essential for survival or at least confers a substantial selective advantage to its bearers [24, 72]. On the other hand, once paralogs emerge as a result of gene duplication, the pressure of purifying selection decreases for the paralog(s), and they acquire new functions [73]. In some sequenced genomes, substantial fractions (25 to 80%) of genes belong to families of paralogs [74-76] which reflects diversification of function via duplications at different stages of evolution.

The greater likelihood of orthologs retaining the same ancestral function makes a strong case for identifying and using orthologs for function predictions and annotation transfer. Doing so increases the reliability of the transferred functional annotations. The advent of genomics has reinforced the fact that distinction between the two types of

homologs is crucial for understanding evolutionary relationships between genomes and gene functions [24].

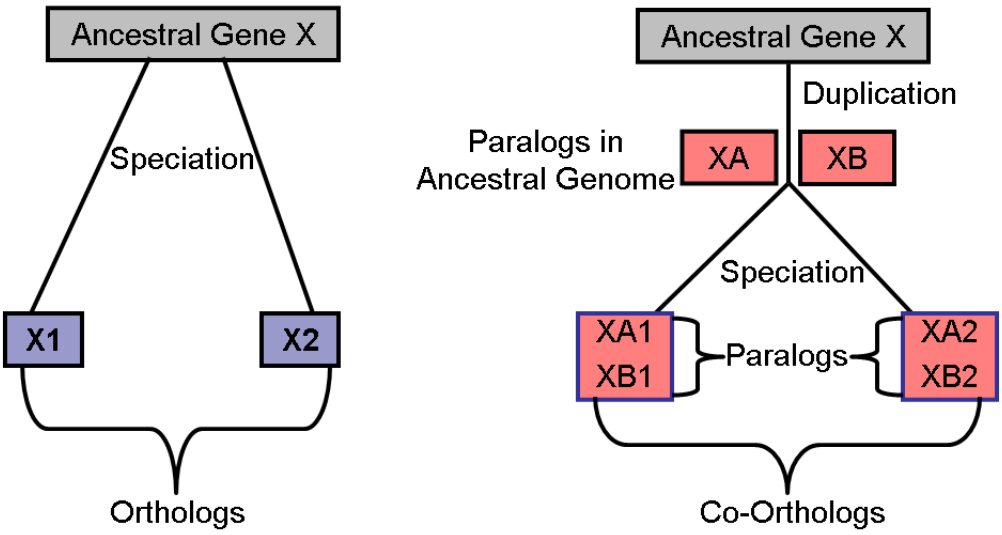


Figure 1.5: Different homolog subtypes - orthologs and paralogs.
Simplified diagram of homolog subtypes showing orthologous and paralogous genes in two species '1' and '2'. The panel of the left shows the direct vertical descent of the ancestral gene 'X' is species '1' and '2' following speciation. The panel on the right depicts the duplication of the ancestral gene 'X' into 'XA' and 'XB' in the ancestral genome followed by subsequent speciation leading to the genes 'XA' and 'XB' in species '1' and '2'.

Identifying orthologs – complex evolutionary scenarios

Since orthology is defined based on phylogeny, it would make more sense to use phylogenetic tools to identify orthologs [1]. However, identification of orthologs is not a simple task because of these complex evolutionary scenarios [72]:

1. when duplication precedes speciation, each of the paralogs gives rise to a distinct line of orthologous descent;
2. when duplication occurs in one or both lineages independently after speciation, this is referred to as lineage-specific gene expansion, and leads to a situation where one-to-one orthologous relationship cannot be delineated;
3. when genes in certain lineages are lost during evolution, this phenomenon is referred to as lineage-specific gene loss. In such cases, genes which appear to be orthologs might actually be paralogs.

Complete phylogenetic analysis of all groups of homologous genes helps decipher true orthologous relationships [24]; however, phylogenetic analysis is extremely labor-intensive and time-consuming.

It becomes imperative that methods need to be developed to make the distinction between orthologs and paralogs. The problem gets confounded with large gene families that have many paralogous members within a species. In such a scenario, the difficulty lies in identifying the orthologs which are more likely to have conserved function.

In the next section, I describe how phylogenetic analysis can be used to predict function, and discuss the pros and cons of this approach.

Function prediction using phylogenetic methods

Incorporating an evolutionary perspective to comparative biology involves going beyond cataloguing similarities and differences between sequences and trying to

understand why those similarities and differences came to be [1]. Phylogenomics combines evolutionary and genomic analysis into a single composite approach [1]. Phylogenomic inference of protein function involves inferring the function of a protein in the larger context of a protein family [1, 39, 53, 77].

Margaret Dayhoff [78], defines protein family as a group of proteins that perform similar biochemical functions; the pairwise identity between any two proteins in a protein family is >50%. However, it is now accepted that all detectable homologs are members of the same protein family. Coding portions of the genome are organized hierarchically as gene/protein families [79, 80]; these families are further subdivided into groups representing distinct subfamilies that have similar functions [32, 53, 60, 77, 81, 82]. A protein family comprises proteins with the same function in different organisms but may also include proteins derived from gene duplications and rearrangements [83] in the same organism. Function prediction methods that utilize the protein family approach include the PANTHER system [84, 85], TIGRfams [86], and some models in PFAM [87].

To most reliably infer the function of a protein in the larger context of protein family, protein families should be divided into groups containing orthologs and paralogs representing distinct subfamilies. In the phylogenomic approach, a phylogenetic tree involving the different members of the protein family is obtained; the tree topology is then analyzed and the phylogenetic information encoded in the subfamily (or subtree) structure is used to infer likely functions for the uncharacterized members of the protein family. Uncharacterized genes can be assigned predicted function based on the

subfamily in which they are placed as function is conserved within orthologous subfamilies [32, 39, 53, 77, 82]. It is only recently that methods have been developed to allow high throughput placement of query genes into phylogenetic groups [88-90].

With the growing recognition that homology-based methods of function classification are prone to systematic error [54, 60, 61], it has been shown that phylogenomic analysis addresses the deficiencies of function prediction by homology and improves the accuracy of prediction [39, 53]. Phylogenomic inference of protein molecular function has been applied to the detailed analysis of individual protein families [81, 91, 92], in comparative genomics [93, 94], and in reconstructing the evolutionary history of a segment of the human genome [95].

Pros and cons of phylogenomic analysis

Phylogenomic inference has its own share of pros and cons. The problem of annotation transfer from paralogous sequences is prevented by identifying orthologous sequences through phylogenetic tree analysis [60]. The disadvantages with this method lie in the fact that inherent technical and computational complexities make it laborious and difficult to automate [60]; in addition, the method cannot be applied to predict function for every sequence [53].

Utility of Expressed Sequence Tag (EST) datasets

Although it is well-known that whole genome sequencing projects are advancing rapidly, it is unlikely [96] that complete genome sequencing will be finished in the foreseeable future for many organisms of scientific, economic or agricultural interest.

For most eukaryotic organisms, the complete genome sequence is not available [97], and sequencing of Expressed Sequence Tags (ESTs) remains the primary tool for functional genomics approaches.

An EST is a tiny portion of an entire gene [8, 98]; usually about 200 to 500 nucleotides long and is representative of the genes expressed in the tissue at the point of cDNA library construction. ESTs are obtained by random sequencing of cDNA copies of mRNA sequences available (**Figure 1.6)**; the utility of EST projects comes by repeatedly sequencing clones from a given library to generate many sequence tags as they provide an opportunity to understand the diversity of genes and the roles they play [99, 100]. The utility of ESTs is illustrated by the phylogenetic diversity of organisms represented in dbEST [101, 102], the NCBI's EST database. As of Sep 2008, there are 55, 796, 748 ESTs deposited in dbEST from thousands of organisms.

EST analysis is a highly cost effective gene discovery method [8, 97, 98, 103] and EST datasets represent an important resource for comparative and functional genomics studies [104-106]. In the absence of completed genomes, ESTs help address these questions:

1. What genes are there in the organism? and
2. What roles do they play?

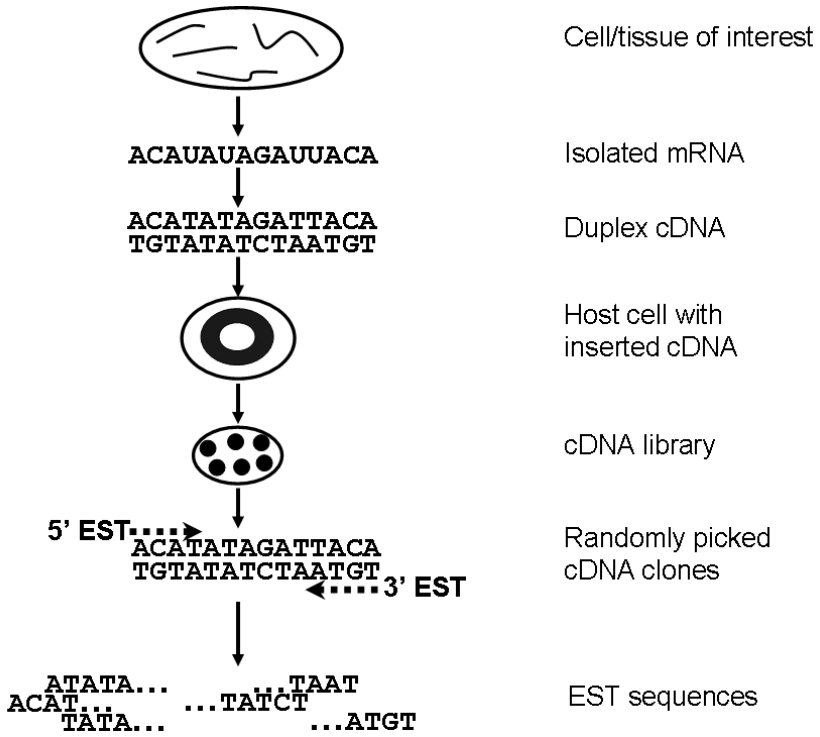


Figure 1.6: Steps involved in obtaining ESTs.
Cartoon depicting how expressed sequence tags (ESTs) are generated.

EST sequence analysis

In the case of sequencing of full-length cDNAs, multiple sequencing runs are made for purposes of verification, thereby producing cDNA sequences of high quality. In contrast, ESTs are sequenced only once, and without any verification. Errors arise from substitutions, insertions and deletions in the original mRNA, and from the experimental procedure. This presents a special set of problems for bioinformatics analysis [107]; ESTs are partial and error-prone, resulting in large fragmented datasets

with significant internal redundancy (**Figure 1.7**). Moreover, they are not curated in a highly annotated form and do not have a defined protein product.

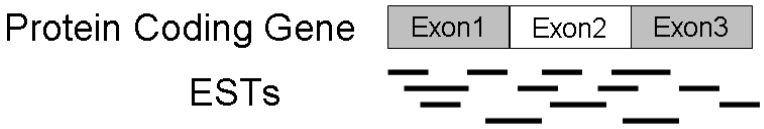


Figure 1.7: Fragmented and redundant nature of ESTs.
Cartoon illustrating the fragmented and redundant nature of EST datasets. A protein coding gene with 13 ESTs spanning 3 exons is shown.

Need for clustering ESTs

Regardless of whether ESTs are used for gene identification or genomic annotation, maximum utilization of the information they encode can be obtained only by reconstructing a high-fidelity set of non-redundant transcripts. EST collections can be organized in the following different ways:

1. Clustering – group ESTs that are derived from the same genes
2. Assembling – derive consensus sequence from clustered ESTs
3. Mapping – associate ESTs with exons in genomic sequences
4. Translating – finding coding regions and identify reading frame

In the clustering step, ESTs are grouped on the basis of sequence similarity into clusters; the similarity threshold is set very high (>95%) and the comparisons are made over a specified window size. The clustered ESTs are then assembled where the assembly program aligns the EST sequences within a cluster and generates a contiguous overlapping sequence (or EST contig), or a singleton might arise when the EST(s) could not be grouped owing to their low similarity to other ESTs. The singletons may represent genes from rare mRNAs where only a single mRNA is available for the expressed gene, or may be a result of contamination or poor quality sequence. The contig is a consensus sequence of several ESTs and is more reliable than the individual ESTs, thereby reducing the effects of errors. The contigs are consensus sequences having improved sequence quality [100]. Clustering and assembly of ESTs has numerous advantages, including, but not limited to, the following:

1. function predictions are significantly improved [97] using the EST consensus sequences coming from the clustering and assembly pipeline; and
2. clustered EST consensus sequences reduce the search space for similarity searches and help obtain an accurate estimate of the number of genes.

What is an EST cluster?

An EST cluster contains fragmented EST and (if known) gene sequence data that has been consolidated and indexed by gene(s) or transcript isoform. In doing so, all expressed data pertaining to a single gene or isoform exists within a single index class [100]. Accurate clustering of ESTs requires a strategy that clusters members based on verifiable information – sequence similarity often being the method of choice.

Different steps involved in EST clustering

EST clustering and assembly is illustrated in **Figure 1.8**, and involves the following steps:

1. Pre-processing: The sequences are screened for low-quality regions, bacterial DNA and other contaminants. The sequences are then masked for vectors, repeats and low-complexity sequences. The 'pre-processed' sequence is referred to as 'high-quality' sequence.
2. Initial Clustering: ESTs by their nature have erroneous sequence data caused by base-calling problems. The goal of this 'initial clustering' step is to incorporate overlapping ESTs into the same cluster and is usually achieved by using a sequence similarity metric over a specified window size (e.g. >95% identity over 100 bp window). This 'initial clustering' can be loose or stringent [108], and either can be either supervised or unsupervised. A loose clustering schema results in larger clusters and a greater inclusion of alternate expressed forms within each cluster. Loose clustering provides for greater coverage at a cost of lower cluster reliability. Stringent clustering provides greater initial fidelity at a cost of lower coverage of expressed gene data and a lower inclusion rate of alternate expressed gene forms; the stringent clustering method results in fewer, shorter consensus sequences. Stringent clustering and assembly results in more singletons while also generating more high-quality contigs [100]. In the supervised clustering methodology, ESTs are grouped with respect to known reference sequences or templates such as full-length mRNAs, protein sequences, and genomic

sequences. In unsupervised clustering, ESTs are grouped without using any reference sequences.

3. EST Assembly: Once the 'initial clustering' is done, a multiple sequence alignment for each cluster is generated to obtain a 'consensus sequence' and/or singletons.

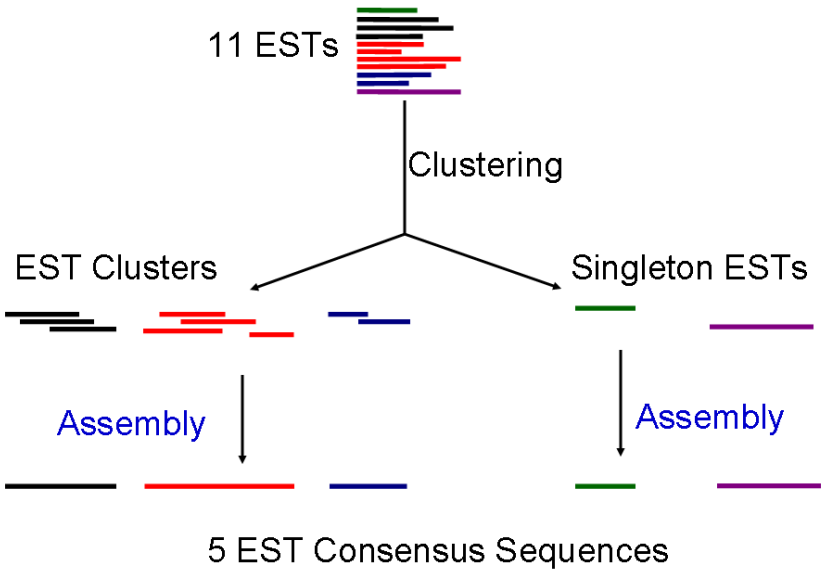


Figure 1.8: Cartoon illustrating EST clustering and assembly.
In the figure, clustering of 11 'High quality' EST sequences results in 11 ESTs getting grouped into 3 clusters and the remaining 2 ESTs are singletons. The assembly process results in 5 EST consensus sequences - 3 EST contigs and 2 singletons.

Comparison of the existing EST clustering systems

The aim of EST clustering is to generate a cluster of ESTs that share a transcript or the gene parent. Systems that are commonly used and have broad acceptance include

TGI (TIGR Gene Indices) [109], NCBI's UniGene [110, 111] and Sequence Tag Alignment Consensus Knowledgebase (STACK) [112] from South African National Bioinformatics Institute (SANBI). (***Note:*** TGI is now known as DFCI Gene Indices or DGI; DFCI denotes the Dana-Farber Cancer Institute). These three systems share an overall approach, but differ in the choice of algorithms used, reconstruction aims and coverage of transcript diversity. The three systems perform a similar pre-processing step that screens out vector, repeat, and low-complexity sequences. Each system differs in the way the 'initial clustering' and 'EST Assembly' is done. **Figure 1.9** characterizes the similarities and differences in EST clustering and assembly between the three methods (TGI, UniGene and STACK).

TIGR Gene Indices (TGI)

ESTs from dbEST are processed to remove vector, poly A/T tails, adaptor sequences and contaminating bacterial sequences. Gene sequences are parsed from the Coding Sequences (CDSs) and CDS-join features in GenBank protein records pertaining to the species in question [96]; additional Expressed Transcript (ET) sequences are obtained from the TIGR EGAD database [113]. For construction of the gene indices, TGI uses Expressed Transcripts (ETs), Tentative Consensus Sequences (TCs) from previous build (if available) and CDSs as templates. Using these templates, pairwise comparisons of previously unclustered sequences (singletons) and new ESTs are performed to identify overlaps and generate initial clusters using NCBI's Megablast [114].

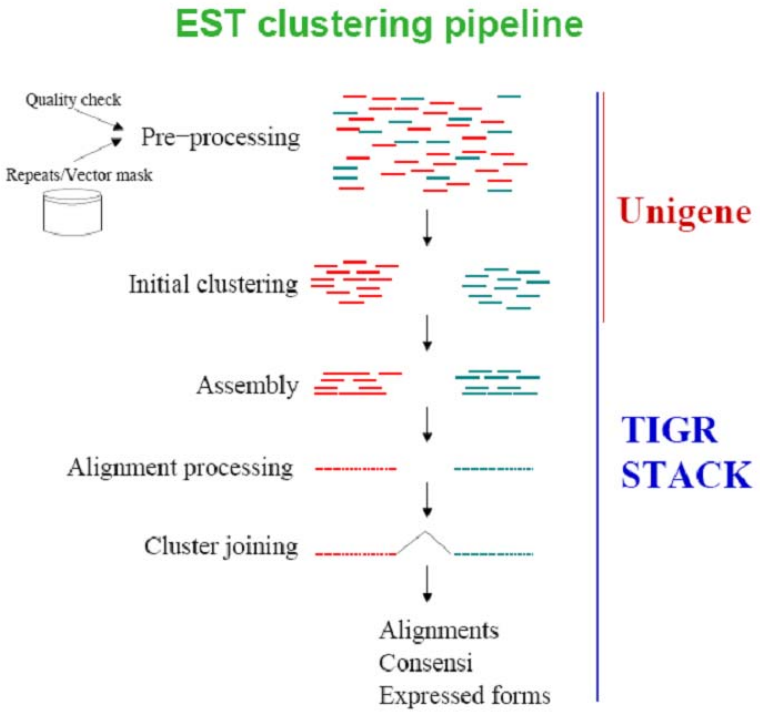


Figure 1.9: Comparison of the EST clustering steps of UniGene, TIGR and STACK.
Cartoon depicting the different steps in the EST clustering pipeline used by existing EST clustering systems like UniGene, TIGR and STACK. Used with permission from Dr. Lorenzo Cerutti [115], Swiss Institute of Bioinformatics. UniGene does not perform an assembly of the initial 'EST clusters'; instead each cluster is represented by the longest sequence. STACK concentrates on human sequences and differs from TIGR and UniGene by not using sequence similarity to cluster ESTs. Both STACK and TIGR assemble the initial EST clusters to generate consensus sequences and singletons.

Sequences are grouped into the same cluster if they have ≥ 95% identity over lengths greater than 40 bases and have < 20 bases mismatch at either end [116]. The resulting clusters are assembled by Paracel Transcript Assembler [117] to obtain Tentative Consensus Sequences, which represent the underlying mRNA transcripts.

These TCs are then annotated using information in GenBank and/or protein homology [116].

The TGI clustering methodology results in shorter consensus sequences [115] compared to UniGene and STACK. TGI tightly groups highly related sequences, separates splice variants into different clusters and discards under-represented or divergent sequences.

UniGene

Clusters are built from genes and mRNAs in GenBank. ESTs are initially clustered using GenBank CDSs and mRNAs as 'seed' sequences. ESTs that join two clusters of mRNA/genes are discarded. To minimize the frequency of multiple clusters being identified for a single gene, UniGene clusters are required to contain at least one sequence carrying readily identifiable evidence of having reached the 3′ terminus. In other words, UniGene clusters must be anchored at the 3′ end of a transcription unit. Hence, any resulting cluster without a polyadenylation signal or not having at least two 3' ESTs is discarded [111].

UniGene clusters ESTs similar to the 'seed' sequence and categorizes the clusters into "highly similar" to the seed (defined as >90% identity in the aligned region), "moderately similar" (70-90% identity), or "weakly similar" (<70% identity) [118]. UniGene uses pairwise comparisons at varying levels of stringency to group sequences, placing closely related and alternatively spliced transcripts into one cluster [115]. UniGene *per se* does not perform an 'EST cluster assembly' of the generated clusters and consensus sequences are not made; instead each cluster is represented by the longest

sequence. In summary, UniGene does not actually reconstruct transcripts but instead attempts to define their cluster membership based on NCBI data.

A common error in UniGene is incorrect clone joining, where irrespective of sequence overlap, 5' and 3' ESTs are placed in the same cluster if they share a parent clone. Another drawback of the sequence comparison method in UniGene results in unrelated clusters being joined together by chimerism and other artifacts.

STACK

STACK concentrates on human data and an initial sub-partitioning step is performed where human ESTs from GenBank are grouped in tissue-based categories. After masking for repeats, vectors and other contaminants, the resulting 'high quality' ESTs are subject to a 'loose' clustering approach using d2_cluster [119]. The 'd2_cluster' approach is not based on alignments; instead it performs comparisons via non-contextual assessment of the compositions and multiplicity of words within each sequence. The 'd2_cluster' looks for the co-occurrence of n-length words (n=6) within window sizes of 150 bases having at least 96% identity.

In a stringent assembly process, the clusters from 'd2_cluster' are initially assembled by PHRAP [120]. CRAW [121] is used to generate consensus sequences with maximized length and CRAW partitions a cluster into sub-ensembles if ≥50% of a 100 base window differs significantly from the remaining sequences in the cluster. The different sub-ensembles are ranked according to the number of assigned sequences and number of called bases for each sub-ensemble.

STACK places highly related sequences into the same cluster, as well as sequences related by rearrangements or alternate splicing. STACK generates longer consensus sequences and integrates 30% more ESTs than UniGene and produces longer consensus sequences compared to TGI [115].

Figure 1.10 is a cartoon that depicts the differences in stringency levels in the EST clustering pipeline used by the existing EST clustering schemes like UniGene, TIGR and STACK.

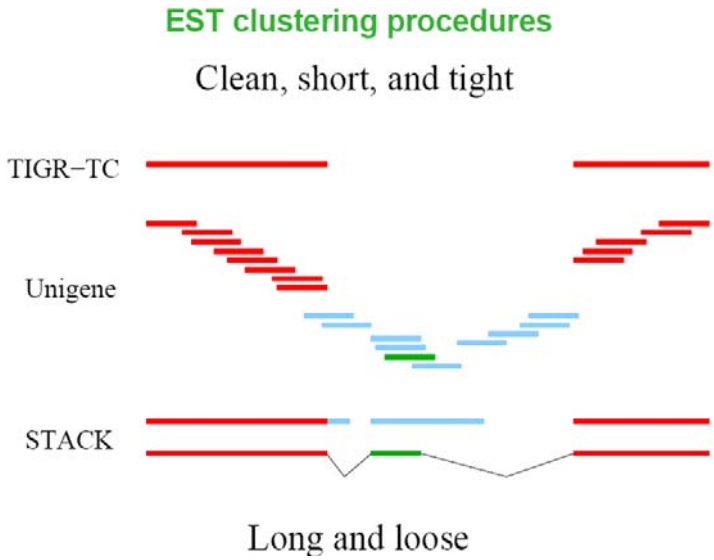


Figure 1.10: Comparison of the EST clustering stringency levels of TIGR, UniGene and STACK.
Cartoon depicting the stringency differences between the three EST clustering methods - TIGR, UniGene and STACK. Used with permission from Dr. Lorenzo Cerutti [115], Swiss Institute of Bioinformatics. TIGR, the most stringent amongst all the methods [115], tightly groups highly related sequences and separates splice variants into different clusters. Both UniGene and STACK place highly related and alternatively spliced transcripts in the same cluster. However, STACK [115] generates longer consensus sequences and integrates 30% more ESTs than UniGene and produces longer consensus sequences compared to TIGR Gene Indices.

Function predictions using EST datasets

In the absence of completely sequenced genomes and the accompanying high-quality annotations, one can use Expressed Sequence Tag (EST) datasets to gain insights about the functions encoded in the genome by first determining what genes are there in the organism and then finding out what roles they play. Annotated EST datasets address the need of connecting ESTs with the functions they encode.

Need for protein sequence comparisons using ESTs

DNA sequences produced by single-pass EST sequencing are of lower quality than traditional "finished" GenBank sequences and the EST sequences are more likely to contain frameshifts when translated to protein. These frameshift errors are troublesome in searches with single-pass EST sequences as these regions are likely to be protein-coding, which are much more effectively identified by protein sequence comparison than by DNA sequence comparison [15]. In addition, the evolutionary look-back time is more than doubled by protein sequence comparisons as against DNA sequence comparisons [122, 123]; hence it makes sense to compare protein sequences if the sequences encode proteins. One of the approaches that has been proved invaluable in comparative genomics and computational biology in general is that whenever distant relationships are involved and sensitivity is an issue, protein sequences rather than nucleotide sequences should be compared directly [24].

Function predictions with EST datasets using sequence homology based approach

A common strategy for obtaining function annotations for EST datasets is to search well-annotated proteomes using BLASTX/FASTX (translated DNA query against

a protein database), obtain information about the homolog with the "best hit" and to transfer the annotations of the "best hit" match to the EST consensus sequence [104-106].

There are limitations of subjecting EST libraries to whole proteome searches due to the short length of the translated EST sequence (the equivalent of 30 to 150 amino acids) which would match only part of the protein; for example, a protein domain or part of it [97]. The accuracy of the computational function predictions is significantly improved [97] when EST consensus sequences are used instead of the individual ESTs.

Function predictions with EST datasets using a phylogeny based approach

ESTs can be coarsely grouped based on their matches to proteins, and subject to clustering and assembly to obtain EST gene families. The EST consensus sequences in these EST gene families can be grouped with other proteins in the family and subject to a phylogenomic inference pipeline. The topologies of the phylogenetic trees can be analyzed to infer likely functions for the EST consensus sequences based on orthologous proteins.

Work in this dissertation

In this dissertation, *Bos taurus* and *Sus scrofa* ESTs were clustered and assembled using vertebrate proteins from Ensembl as the framework. This clustering was performed as part of the TAMUClust pipeline, a new EST clustering method developed by Dr. Elsik. The performance of TAMUClust was compared to currently used EST clustering schemes (TGI/UniGene) by using *Bos taurus* EST clusters from the

respective data sources and designing cluster equivalence comparison studies (Chapter II). The *Bos taurus* and *Sus scrofa* EST consensus sequences obtained were annotated by transferring annotations of the Ensembl vertebrate protein(s) following sequence homology searches and phylogenetic analysis (Chapter III and Chapter IV).

CHAPTER II

VALIDATION OF TAMUClust - A NOVEL EST CLUSTERING METHODOLOGY

SYNOPSIS

Expressed Sequence Tags (ESTs) are single pass sequence reads from randomly selected cDNA clone. They serve as a viable alternative to the genome sequencing of many organisms as they provide a high-throughput method to sample an organism's transcriptome and identify expressed genes. ESTs contain high error rates, and the information encoded is often fragmented and redundant. Therefore, EST sequences are clustered into groups likely to have been derived from the same genes and this process improves the quality of meaningful information that can be derived from ESTs. Chimerism, the single largest contributor to EST misassemblies, can be avoided if ESTs are initially grouped into clusters based on their matches to proteins, and then subject to clustering and assembly. TAMUClust is a new EST clustering method that uses the protein framework to cluster ESTs. The TAMUClust *Bos taurus* EST clusters are compared with the *Bos taurus* EST clusters from TIGR Gene Indices (TGI), the current method of choice in EST clustering. Two types of comparisons are made: (i) determining cluster equivalence for TAMUClust/TGI using bovine genome aligned EST clusters as the reference and (ii) determining how many genes are represented in TAMUClust/TGI using predicted bovine transcripts as the reference. Results indicate that the TAMUClust method compares well with TGI.

BACKGROUND

The utility of Expressed Sequence Tags (ESTs)

Expressed Sequence Tags (ESTs) are single pass sequence reads from randomly selected cDNA clones and provide a high-throughput cost-effective method to sample an organism's transcriptome [8, 98]. ESTs are short (usually 200-500 bases in length), unedited sequence reads and represent a tiny portion of a protein coding gene. ESTs offer a rapid and inexpensive route to gene discovery [8], reveal expression and regulation data [124], and highlight gene sequence diversity and alternative splicing [125]. EST datasets have been utilized as an alternative to the genome sequencing of many organisms, earning the label, the 'poor man's genome' [126].

Need for EST clustering

ESTs are sequenced only once, and without any verification. The high-volume and the high-throughput nature of the EST datasets have a lot of downsides – ESTs contain high error rates with the errors arising from substitutions, insertions and deletions in the original mRNA, and from the experimental procedure. Maximum utilization of the information enoded in ESTs can be obtained by clustering ESTs into groups that have been derived from the same genes, resulting in a high-fidelity set of non-redundant transcripts. Sequence identity between the cluster members is the method of choice in clustering ESTs.

Need of EST clustering using a protein family framework and associated benefits

Chimeric EST clusters are encountered when ESTs representing different genes get grouped together in a single cluster. Chimerism is the single largest contributor to generation of 'incorrect EST assemblies' (misassemblies). Chimeric EST clusters can be eliminated by performing an initial coarse level grouping of ESTs based on their matches to proteins. In a subsequent step, clustering and assembly of ESTs can be done to obtain EST gene families. By incorporating a protein framework to cluster ESTs, the cluster quality can be improved and misassemblies can be minimized.

The need for clustering ESTs using a protein framework lead to the development of a new EST clustering method called TAMUClust. This method was developed in our lab by Dr. Elsik.

Description of TAMUClust - a novel EST clustering system

TAMUClust uses a protein framework to cluster and assemble ESTs in a two-step clustering process.

In the first step, vertebrate proteins are clustered into protein families using a combination of single-linkage and average-linkage clustering. The protein families generated are non-redundant, comprehensive set of full-length protein clusters with homogeneous domain architecture within clusters and the pairwise sequence identity between any two proteins within each cluster is at least 55%. A total of 219,433 proteins from the proteomes for *Homo sapiens, Mus musculus, Rattus norvegicus, Gallus gallus,*

Danio rerio, Takifugu rubripes and *Tetraodon nigroviridis* were obtained from Ensembl [9] in April 2005; these proteins were distributed across 10,992 protein families.

In the second step, TAMUClust uses the protein families obtained in the previous step as a framework to group ESTs into gene families using a translated-DNA:protein search. For ESTs within each family, a DNA:DNA sequence identity metric is used to build consensus sequences. The protein comparison step incorporated before assembling ESTs helps identify coding regions and produce reliable protein translations.

Other commonly used EST Clustering systems such as TGI (TIGR Gene Indices), UniGene, and STACK do not use a protein framework to cluster and assemble ESTs.

Importance of generating the correct EST clusters

DNA microarrays (DNA chips) allow for simultaneous measurement of transcription levels for every gene. A DNA microarray is a slide on which an array of spots has been deposited. Each spot contains many copies of a specified DNA sequence that has been anchored chemically to the slide surface, with a different DNA sequence for each spot.

In the long oligonucleotide array, the oligos are usually 60-70 bases long and are selected for their uniqueness. These are used to profile the gene expression in a genome, and the oligos may be designed such that there is only one oligo per gene or transcript.

In the absence of completed genomes, ESTs are used to determine what genes are there in the organism and to design the oligonucleotide probes. In this scenario, it is

important to ensure that the transcript reconstruction from ESTs is accurate and non-redundant. Inaccuracies with EST clustering would result in problems in selecting sequences for oligonucleotide design. Usually one EST per cluster is selected for oligo design. Overclustering (i.e. grouping unrelated ESTs) would result in lack of representation of some genes; underclustering (i.e. splitting same-gene ESTs into different clusters) would cause multiple oligos to be designed for one gene. Designing oligonucleotide probes by utilizing ESTs clustered using a protein framework could minimize probe redundancy and maximize representation of different genes.

Overview of the work in this chapter

TAMUClust is a novel EST Clustering system developed in our lab by Dr. Chris Elsik. In this method, ESTs are clustered and assembled using a protein framework.

Pilot study

A pilot study was carried out in January 2004 using TAMUClust *Bos taurus* EST clusters generated using Ensembl human protein families as a framework. Using the Q_{single} metric, the results of TAMUClust were compared to those obtained with other commonly used methods like TGI [127] and UniGene [110] for the same set of *Bos taurus* ESTs.

For the purposes of our pilot study comparison analyses, the reference classification was either the *Bos taurus* Gene Indices (BTGI) obtained from TGI or the UniGene *Bos taurus* EST clusters whereas the query classification refers to the TAMUClust *Bos taurus* EST clusters. However, this is not to say that the TGI or

UniGene is “correct”. They were used as “references” in the pilot study as they are the existing methods of choice in EST clustering.

Results from the pilot study indicated that TAMUClust performance compares well with TGI and UniGene. We chose not to include further comparisons of TAMUClust with UniGene as the clustering methodology of TAMUClust is similar to TGI than UniGene.

Comparisons with assembled genome

In August 2006, the third version of the bovine genome assembly, Btau_3.1 was released with a 7.15X coverage [10, 11]. The availability of this high quality bovine assembly came in handy to compare the performance of TAMUClust *Bos taurus* EST clusters against the *Bos taurus* Gene Indices (BTGI) obtained from TGI/DGI [128] by using bovine ESTs aligned to the bovine genome assembly 3.1 as the reference (gold standard). Results from this analysis indicated that the TAMUClust performance was comparable to TGI, and marginally better than TGI.

Comparisons using predicted gene models

In July 2008, the fourth version of the bovine genome assembly, Btau_4.0 was released. Two kinds of ‘megablast’ analyses were carried out by using transcripts from the *Bos taurus* Ensembl Release 50 [129] as the reference (Gold standard).

In the first analysis, TAMUClust/BTGI bovine EST consensus sequences were used as the query and searched against the bovine transcripts in Ensembl Release 50 (July 2008). The performance of TAMUClust and BTGI was compared by obtaining

statistics on the Ensembl transcripts identified or missed by the respective EST clustering methods. More specifically, this analysis was designed to address the question "Does TAMUClust gain anything by including singletons in its gene indices". Since TAMUClust used a protein framework to cluster and assemble ESTs, TAMUClust also included singletons in the gene indices (consensus sequences) it generated where these singletons had a statistically significant sequence similarity to a homologous protein. BTGI performs a nucleotide-nucleotide comparison to cluster and assemble ESTs, and discards ESTs that do not match the clustering criteria. As a result, the gene indices generated by BTGI were devoid of singletons. Based on the bovine transcripts identified by TAMUClust singletons and missed by BTGI consensus sequences, one can conclude that TAMUClust singletons were not clustering artifacts and TAMUClust singletons should be included in the final gene indices.

In the second analysis, the bovine transcripts in Ensembl Release 50 were used as the query and searched against TAMUClust/BTGI bovine EST consensus sequences. This analysis was designed to obtain an estimate of the transcript coverage in TAMUClust and BTGI by obtaining a count of the number of predicted transcripts that match TAMUClust/BTGI consensus sequences. Results from this study indicated that the number of predicted transcripts that match TAMUClust/BTGI consensus sequences were almost the same.

MATERIALS AND METHODS

Q_{single} - cluster equivalence comparison metric

Gracy and Argos [130] have developed a method that helps evaluate the quality of a new classification scheme B in terms of a reference classification A for the same dataset X. Here, the number of True Positives (TP) is given by $(|a \cap b|-1)$, that of False Positives (FP) is given by $|b\backslash a|$, and that of False Negative (FN) is given by $|a\backslash b|$. In this method, each class $a \in A$ is associated with the group $b \in B$ which maximizes the quantity TP-FP-FN. Quality is defined by the percentage of the true positives (100*TP/TP+FP+FN). The underlying meanings of the conventions used in the comparison metric described above are as follows:

1. $a \cap b$ refers to the intersection of 'a' and 'b' – in other words, it refers to the number of members common to 'a' and 'b'.
2. $b\backslash a$ refers to members present in 'b' and absent from 'a'.
3. $a\backslash b$ refers to members present in 'a' and absent from 'b'.

This method of comparing the quality of a new classification in terms of a reference classification as indicated by the mutual agreement of the two classification schemes has been used in the ProtoMap [131] classification evaluation. The ProtoMap authors have termed this 'Quality Index' as 'Q_{single}' and we also use the same term in our cluster equivalence comparison analyses.

Q_{single} *calculation*

The different statistical parameters (*tp*, *fp*, *fn*) and the Q_{single} metric were obtained using the following steps:

1. identify the common ESTs in both datasets – the new classification scheme 'B' and the reference classification scheme 'A'.
2. for every EST 'E' in 'B' do;
3. obtain the cluster_id in A and B for the cluster that has 'E'. Lets call them cluster_id_A and cluster_id_B
4. obtain all the other ESTs in cluster_id_A and cluster_id_B. Lets denoted the members as cluster_id_A_members and cluster_id_B_members
5. identify the cluster_id_A associated with cluster_id_B that maximizes the quantity TP-FP-FN
6. Q_{single} % TP = (100* TP/TP+FP+FN)
7. Q_{single} % FP = (100* FP/TP+FP+FN)
8. Q_{single} % FN = (100* FN/TP+FP+FN)
9. The different Q_{single} indices (Q_{single} %TP, Q_{single} %FP and Q_{single} %FN) are segregated into different bins, for e.g. (0 to 10%, 10 to 20%, and so on).

Methods used in the pilot study

Pilot study: TAMUClust Bos taurus *EST clusters*

Bos taurus ESTs were downloaded from dbEST [101] in March 2003, and after quality control (removal of low quality sequence, untrimmed vector, linker, ribosomal,

mitochondrial, poly A/T tails), 313,503 'High Quality' ESTs were obtained. These ESTs were passed through the TAMUClust pipeline which resulted in 37,025 EST consensus sequences (15,157 contigs and 21,868 singletons).

Pilot study: Bos taurus ***EST clusters from TIGR Gene Indices***

In Sep 2003, *Bos taurus* EST clusters were obtained from *Bos taurus* Gene Indices (BTGI) Release 9 at The Institute of Genome Research (TIGR) Gene Indices database [127]. This BTGI release had 268,328 ESTs distributed in 34,976 clusters.

Pilot study: Bos taurus ***EST clusters from UniGene***

In Sep 2003, *Bos taurus* EST clusters (UniGene Build 49) were obtained from UniGene [110]. This UniGene Build had 209,550 ESTs distributed in 18,153 clusters.

Note: The individual ESTs and also the number of ESTs in BTGI/UniGene are different from what it is in TAMUClust because of the different dates on which the respective sources (TAMUClust/BTGI/UniGene) downloaded the ESTs from dbEST.

Pilot study: Q_{single} ***indices***

Q_{single} quality comparisons as described above were performed by means of these two comparisons:

i. TAMUClust *Bos taurus* EST clusters versus BTGI *Bos taurus* EST clusters using 119,047 ESTs common to both datasets.

ii. TAMUClust *Bos taurus* EST clusters versus UniGene *Bos taurus* EST clusters using 97,245 ESTs common to both datasets.

Methods used in comparisons with the assembled genome (Gold standard comparisons)

Gold standard comparisons: TAMUClust Bos taurus ***EST clusters***

Bos taurus ESTs were downloaded from dbEST [101] in April 2005, and after quality control (removal of low quality sequence, untrimmed vector, linker, ribosomal, mitochondrial, poly A/T tails), 308,132 'High Quality' ESTs were obtained. These ESTs were passed through the TAMUClust pipeline which resulted in 46,731 EST consensus sequences (24,665 contigs and 22,066 singletons).

Gold standard comparisons: BTGI (Bos taurus ***Gene Indices) EST clusters***

In March 2007, the file "BTGI.release_12.zip" was obtained from the ftp site [132] of the *Bos taurus* Gene Indices at DGI [133]. The file was decompressed and the resulting files were parsed to obtain information on the BTGI clusters and the constituent ESTs. The BTGI dataset had 955,223 ESTs clustered into 90,392 EST clusters.

Note: The individual ESTs and also the number of ESTs in BTGI would be different from what it is in TAMUClust depending on the dates on which the respective sources (TAMUClust/BTGI) downloaded the ESTs from dbEST.

Gold standard comparisons: bovine genome aligned EST clusters

Bos taurus ESTs (1,165,913 ESTs) were downloaded from the NCBI dbEST database [101] in April 2007. Using the Splign alignment and splice modeling tool [134] and %identity >95%, bovine ESTs were aligned to the third version of the bovine assembly (Assembly 3.1, 7.1X coverage [10, 11]). Alignments were stored as gff3 [135] files and perl scripts were written to parse the gff3 files to obtain ESTs that have only

one alignment location on the genome. Further perl processing was done to identify overlapping ESTs and grouped into clusters. The bovine genome aligned EST clusters constitute the reference classification (or the gold standard dataset); they comprised of 975,728 ESTs in 44,579 clusters.

Q_{single} *indices in the gold standard comparisons*

Q_{single} quality comparisons as described above were performed by means of these comparisons:

1. One to one comparison of TAMUClust *Bos taurus* EST clusters and gold standard dataset using 209,645 ESTs common to both datasets.
2. One to one comparison of BTGI *Bos taurus* EST clusters and gold standard dataset using 702,128 ESTs common to both datasets.
3. One to one comparisons of TAMUClust *Bos taurus* EST clusters vs gold standard dataset and BTGI *Bos taurus* EST clusters vs gold standard dataset using 198,438 ESTs common to all three datasets.

To account for the fact that BTGI *Bos taurus* EST clusters did not include singletons whereas TAMUClust clusters included singletons, the above Q_{single} analyses were repeated (henceforth referred to as 'singleton minus' analyses) by excluding singletons from the TAMUClust dataset.

1. Singleton minus one to one comparison of TAMUClust *Bos taurus* EST clusters and gold standard dataset using 198,525 ESTs common to both datasets.

2. Singleton minus one to one comparisons of TAMUClust *Bos taurus* EST clusters vs gold standard dataset and BTGI *Bos taurus* EST clusters vs gold standard dataset using 192,920 ESTs common to all three datasets.

Methods used in comparisons with the predicted gene models

Predicted bovine transcripts

The file 'Bos_taurus.Btau_4.0.50.cdna.all.fa.gz' was obtained from the Ensembl ftp site for *Bos taurus* Ensembl Release 50 [129]. Perl scripts were written to identify the longest transcript for each gene, and this transcript dataset served as the 'reference' dataset in the predicted transcript comparison study. The reference dataset had 21, 722 transcripts.

Megablast analysis I

This analysis was designed to address the question "Does TAMUClust gain anything by including singletons in its gene indices". To address this, we used Megablast [114], and the TAMUClust/BTGI consensus sequences were used as the query and searched against the 'reference transcript dataset' from the *Bos taurus* Ensembl Release 50 as mentioned above.

The Megablast results were filtered to include only those alignments which were longer than 100 bases in alignment length and had sequence identity ≥ 95%. Perl scripts were written to parse the Megablast results in order to obtain information on the Ensembl transcripts identified or missed by the respective EST clustering methods. We first determined if there were any Ensembl transcripts that aligned to TAMUClust

consensus sequences but were missed by BTGI consensus sequences; from these TAMUClust hits, we then identified the TAMUClust singletons.

Megablast analysis II

This analysis was designed to identify the extent of coverage of the predicted bovine transcripts in the TAMUClust and BTGI datasets. To address this, the 'reference transcript dataset' from the *Bos taurus* Ensembl Release 50 as mentioned above was used as the query and searched against the TAMUClust/BTGI consensus sequences.

The Megablast results were filtered to include only those alignments which were longer than 100 bases in alignment length and had sequence identity ≥ 95%. Perl scripts were written to parse the Megablast results in order to obtain information on the extent of transcript coverage in the TAMUClust and BTGI datasets using the 'reference transcript dataset'.

RESULTS AND DISCUSSION

As an aid to the reader, **Table 2.1** details the datasets involved in the various Q_{single} analyses performed in this study and the Table/Figure in which the corresponding Q_{single} analysis is discussed. The gold standard dataset in the Q_{single} analyses described below refers to the bovine genome aligned EST clusters (bovine ESTs aligned to the *Bos taurus* Assembly 3.1).

Table 2.1: Table detailing the various Q_{single} analyses performed and the Table/Figure in which they appear in this chapter.

Study	Type of Q_{single} analysis	EST datasets used	Table / Figure
Pilot study	TAMUClust as query and BTGI as reference	ESTs common to TAMUClust and BTGI	Table 2.3, Figures 2.1 and 2.2
	TAMUClust as query and UniGene as reference	ESTs common to TAMUClust and UniGene	Table 2.4, Figures 2.1 and 2.2
Gold standard (bovine genome aligned ESTs) study	TAMUClust as query and bovine genome aligned ESTs as reference	ESTs common to TAMUClust and bovine genome aligned EST clusters	Table 2.7, Figures 2.3 and 2.4
	BTGI as query and bovine genome aligned ESTs as reference	ESTs common to BTGI and bovine genome aligned EST clusters	Table 2.8, Figures 2.3 and 2.4
	TAMUClust or BTGI as query and bovine genome aligned ESTs as reference	ESTs common to TAMUClust, BTGI and bovine genome aligned EST clusters	Table 2.9, Figures 2.5 and 2.6
Singleton minus analysis I	TAMUClust as query and bovine genome aligned ESTs as reference	Excluding TAMUClust singletons and using ESTs common to TAMUClust and bovine genome aligned EST clusters	Table 2.10, Figures 2.7 and 2.8
Singleton minus analysis II	TAMUClust or BTGI as query and bovine genome aligned ESTs as reference	Excluding TAMUClust singletons and using ESTs common to TAMUClust, BTGI and bovine genome aligned EST clusters	Table 2.12, Figures 2.10 and 2.11

A pilot study was carried out in 2004 using TAMUClust *Bos taurus* EST clusters generated using Ensembl human protein families as a framework. Using the Q_{single} metric, the TAMUClust EST clusters (query classification) were compared with other commonly used methods (the reference classification) like BTGI and UniGene for the same set of *Bos taurus* ESTs.

Table 2.2 compares the number of clusters and cluster size for the *Bos taurus* datasets being compared (TAMUClust vs BTGI and TAMUClust vs UniGene) in the pilot study carried out in Jan 2004 using ESTs common to the datasets being compared. TAMUClust has increased overall number of clusters by 60% compared to BTGI (27,183 vs 16,941) and by 156% compared to UniGene (19,943 vs 7,777). TAMUClust has far more singletons compared to both BTGI and UniGene (12,801 for TAMUClust vs 2640 for BTGI and 10,774 for TAMUClust vs 1203 for UniGene). TAMUClust has a ~2 fold more clusters compared to UniGene for clusters with two to five members, while the numbers were comparable between TAMUClust and BTGI. The cluster sizes for clusters having >5 members were more or less comparable between the datasets being compared (TAMUClust vs BTGI, TAMUClust vs UniGene). The high number of singletons and the relatively higher number of clusters with two to five members for TAMUClust clusters reflects the stringent nature of the TAMUClust clustering process.

Table 2.2: Cluster size distribution for the *Bos taurus ESTs* from the pilot study using ESTs common to TAMUClust, BTGI and ESTs common to TAMUClust, UniGene.

Size of cluster (number of sequences)	**TAMUClust vs BTGI**		**TAMUClust vs UniGene**	
	TAMUClust	**BTGI**	**TAMUClust**	**UniGene**
1	12,801	2,640	10,774	1,203
2 – 5	9,579	9,085	5,114	2,541
6 – 10	2,527	2.621	1,949	1,542
11 – 20	1,505	1,654	1,357	1,350
21 – 50	611	740	592	909
51 – 100	102	134	101	144
> 100	58	67	56	88
Total	**27,183 clusters from 119,047 ESTs**	**16,941 clusters from 119,047 ESTs**	**19,943 clusters from 97,245 ESTs**	**7,777 clusters from 97,245 ESTs**

It is important to understand that the cluster size versus abundance does not necessarily give information about the equivalence of clusters, i.e. whether or not the same cluster members exist in the datasets being compared. To elaborate, Classification Scheme A could have ESTs J and K in a cluster of size two, whereas Classification

Scheme B could have ESTs K and L in a cluster of size two or have ESTs M and N in a cluster of size two, with ESTs J and K existing in some other cluster of a different size. Hence, it is important to first identify the cluster in the different classification schemes housing the EST under comparison and then compare the members of the clusters between the two schemes to determine the degree of agreement. In this scenario, we use the Q_{single} metric which evaluates the quality of a new classification scheme B by comparing it to a reference classification scheme A for the same dataset X.

Interpreting the Q_{single} metric

The Q_{single} index determines quality based on the percentage of True Positives (Q_{single} %TP), percentage of False Positives (Q_{single} %FP) and percentage of False Negatives (Q_{single} %FN) obtained in the analysis. In this method which compares the performance of a new classification scheme B to a reference classification A, each class $a \in A$ is associated with the group $b \in B$ which maximizes the quantity TP - FP - FN. The Q_{single} %TP, Q_{single} %FP and Q_{single} %FN values are determined as described in Materials and Methods.

If the new classification scheme performs just as well as the reference classification scheme, this would result in 100% TP, 0% FP and 0% FN. However, such an idealistic situation is rarely encountered. Instead, what is seen is that the different Q_{single} indices have values ranging from 0% to 100%. These indices are then segregated

into different bins, for e.g. 0 to 10%, 10 to 20%, and so on, where the cluster abundance across the different bins is an indicator of the performance of the new classification scheme when compared to the reference. In such a scenario, interpretation of the different Q_{single} indices give insights about how 'close' or 'similar' the new classification scheme is in comparison to the reference classification schemes. Given below are some of the indicators that signify a "very similar' performance between the two schemes:

1. A high percentage of clusters in the upper bins (>90%) for the Q_{single} %TP parameter.
2. A high percentage of clusters in the lower bins (<10%) for Q_{single} %FP and Q_{single} %FN parameters.

The distribution of the Q_{single} indices for the pilot study comparison between TAMUClust vs BTGI and TAMUClust vs UniGene are detailed in **Tables 2.3** and **2.4** respectively.

Table 2.3: Pilot study Q_{single} analysis with TAMUClust as query and BTGI as reference for the same set of 119,047 *Bos taurus* ESTs.

% Q_{single} bin	TAMUClust vs BTGI pilot study		
	Clusters in Q_{single} True Positive Bins	Clusters in Q_{single} False Positive Bins	Clusters in Q_{single} False Negative Bins
= 0	0	14,147	10,882
≥ 0 and ≤ 10	185	14,533	11,707
> 10 and ≤ 20	346	398	1,108
> 20 and ≤ 30	388	307	733
> 30 and ≤ 40	793	387	973
> 40 and ≤ 50	1,929	368	1,546
> 50 and ≤ 60	582	167	232
> 60 and ≤ 70	1,102	213	364
> 70 and ≤ 80	1,090	228	198
> 80 and ≤ 90	961	193	68
> 90 and ≤ 100	9,565	147	12
= 100	8,973	0	0

Table 2.4: Pilot study Q_{single} analysis with TAMUClust as query and UniGene as reference for the same set of 97,245 *Bos taurus* ESTs.

% Q_{single} bin	TAMUClust vs UniGene pilot study		
	Clusters in Q_{single} True Positive Bins	Clusters in Q_{single} False Positive Bins	Clusters in Q_{single} False Negative Bins
= 0	0	7,612	3,762
≥ 0 and ≤ 10	17	7,648	4,350
> 10 and ≤ 20	37	24	834
> 20 and ≤ 30	101	10	568
> 30 and ≤ 40	264	22	679
> 40 and ≤ 50	715	26	746
> 50 and ≤ 60	423	5	280
> 60 and ≤ 70	612	10	203
> 70 and ≤ 80	712	10	94
> 80 and ≤ 90	734	12	18
> 90 and ≤ 100	4,162	10	5
= 100	3,659	0	0

Pilot study

In the one to one comparisons of TAMUClust with BTGI and TAMUClust with UniGene, there were 119, 047 ESTs common to TAMUClust and BTGI, and 97, 245 ESTs common to TAMUClust and UniGene. **Figure 2.1** depicts the distribution of the different clusters in the upper bins (>90%) for the Q_{single} %TP parameter (from **Tables 2.3** and **2.4** above) in the pilot study comparison between TAMUClust vs BTGI and TAMUClust vs UniGene.

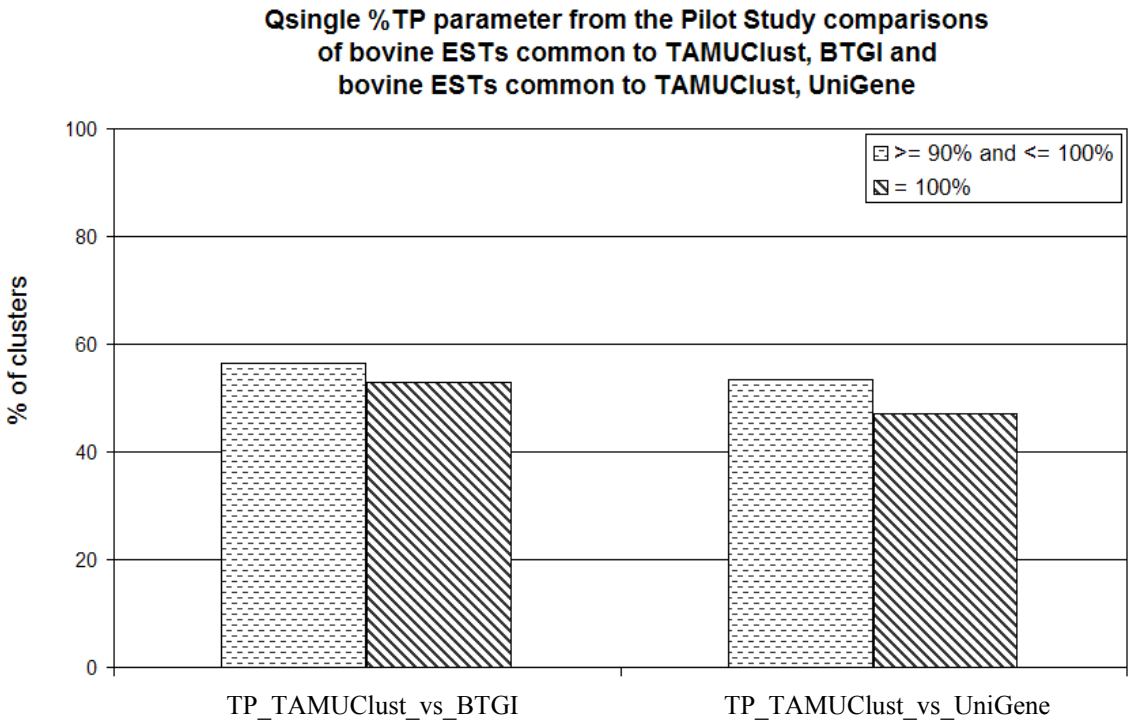


Figure 2.1: Q_{single} %TP for TAMUClust vs BTGI and TAMUClust vs UniGene in the pilot study using ESTs common to datasets being compared.
Cluster abundance of the Q_{single} %TP parameter in the upper bins (>90%) from the pilot study comparisons of bovine ESTs common to TAMUClust, BTGI and bovine ESTs common to TAMUClust, UniGene.

In the TAMUClust vs BTGI comparison, TAMUClust has 56.46% clusters in the 90-100% TP bin and 52.97% clusters in the 100% TP bin. In terms of cluster equivalence comparisons, this means that 56.46% of the TAMUClust clusters have >90% of ESTs in the same cluster as it exists in BTGI. The comparable cluster abundance numbers in the 90-100% TP bin and the 100% TP bin from the TAMUClust vs UniGene pilot study are 53.52% and 47.05% respectively.

Figure 2.2 depicts the distribution of the different clusters in the lower bins (<10%) for the Q_{single} %FP and Q_{single} %FN parameters in the pilot study comparison between BTGI vs TAMUClust and UniGene vs TAMUClust. The Q_{single} %FP metric for the TAMUClust vs BTGI comparison reveals that TAMUClust has 85.79% clusters in the 0-10% FP bin. This indicates that <10% of the ESTs in ~86% of the TAMUClust clusters have been grouped with unrelated members when compared with the corresponding BTGI clusters. The Q_{single} %FN metric for the TAMUClust vs BTGI comparison reveals that TAMUClust has 69.1% clusters in the 0-10% FN bin. This metric indicates that <10% of the relatives (cluster members) for a particular EST are missing in 69.1% of the TAMUClust clusters when compared with the corresponding BTGI clusters. The comparable cluster abundance numbers in the 0-10% FP and the 0-10% FN bins from the TAMUClust vs UniGene pilot study are 98.34% and 55.93% respectively.

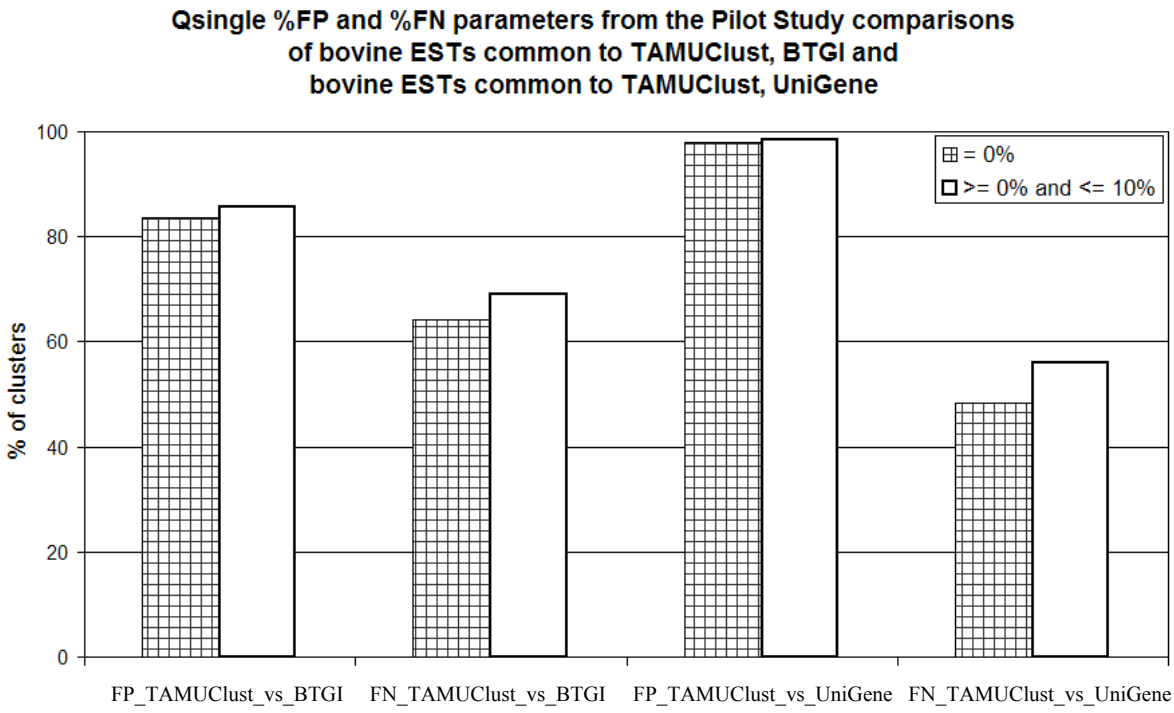


Figure 2.2: Q_{single} %FP, %FN for TAMUClust vs BTGI and TAMUClust vs UniGene in the pilot study using ESTs common to datasets being compared.
Cluster abundance of the Q_{single} %FP and %FN parameters in the lower bins (<10%) from the pilot study comparisons of bovine ESTs common to TAMUClust, BTGI and bovine ESTs common to TAMUClust, UniGene.

For our pilot study, BTGI and UniGene were chosen as the reference sets because they are the most commonly used EST clustering methods. The cluster abundance (**Table 2.2**) from the pilot study highlights the stringency of TAMUClust (singletons, number of clusters with two to five members) compared to BTGI or UniGene. The Q_{single} results (**Tables 2.3** and **2.4**, **Figures 2.1** and **2.2**) indicate that TAMUClust compares well with BTGI and UniGene, and is similar in performance. The

Q_{single} metric was used as an indicator to determine how close or how similar the query classification (TAMUClust) was to the reference classification (BTGI or UniGene). However, disagreement between the query classification and reference classification in Q_{single} analyses does not imply that the reference set is correct and the study set is incorrect.

The clustering methodology of TAMUClust is more similar to BTGI than to UniGene with reference to the way splice variants are handled and consensus sequences are generated. TAMUClust and BTGI separate splice variants into different clusters [115] whereas UniGene places closely related and alternatively spliced transcripts in the same cluster [115]. UniGene clusters often contain 5' and 3' reads from the same cDNA clone even if they do not overlap [115], and it does not perform an 'EST cluster assembly' of the generated clusters. In UniGene, consensus sequences are not made; instead each cluster is represented by the longest sequence [115]. Both TAMUClust and BTGI perform an 'EST cluster assembly' of the generated clusters to obtain consensus sequences. Based on this and in conjunction with the results from the pilot study, we chose to perform further comparisons of TAMUClust only with BTGI. We performed further studies to investigate the reasons for the high number of singletons and clusters having two to five members resulting from TAMUClust. To do so, we obtained the ESTs discarded by TAMUClust in the protein comparison step. The TAMUClust EST clustering statistics for *Bos taurus* ESTs obtained from dbEST in March 2003 are detailed in **Table 2.5**.

Table 2.5: TAMUClust EST clustering statistics in the pilot study using *Bos taurus* ESTs.

Clustering Parameter	Number of ESTs
ESTs downloaded	317,390
High Quality ESTs	313,503
ESTs with protein match	142,741
ESTs without protein match	170,762

Of the 170, 762 ESTs discarded by TAMUClust in the protein comparison step, we found that 62, 832 ESTs were present in BTGI. We analyzed the reasons for rejection of ESTs by TAMUClust and the results for the same are detailed in **Table 2.6**. Our investigations reveal that that ~77% of those ESTs did not have a protein match, whereas ~12% of the ESTs were removed in the sequence trimming step and ~11% of the ESTs were chimeric in nature. These further corroborated the highly stringent clustering nature of TAMUClust compared to BTGI.

Table 2.6: Analysis of the *Bos taurus* ESTs in BTGI discarded by TAMUClust in the pilot study.

Clustering Parameter	Number of ESTs
ESTs in BTGI ignored by TAMUClust	62,832
ESTs without protein match	48,536
Low quality ESTs	7,302
Chimeric ESTs	6,994

Comparison with assembled genome (Gold standard comparisons)

In August 2006, the third version of the bovine genome assembly, Btau_3.1 was released, which is a 7.1X coverage [10, 11]. This came in handy to compare the performance of the TAMUClust *Bos taurus* EST clusters against the *Bos taurus* Gene Indices (BTGI) clusters.

For the purposes of these analyses, the 'query' classification is either TAMUClust or BTGI. The bovine genome aligned EST clusters constitute the 'reference' classification. These bovine genome aligned ESTs can be thought of as the gold standard dataset, and hence, we refer to the analyses using bovine genome aligned ESTs as the Gold standard comparisons.

In the one to one comparisons of TAMUClust vs bovine genome aligned ESTs and BTGI vs bovine genome aligned ESTs using bovine ESTs common to the datasets being compared, 209,645 ESTs common to TAMUClust and the gold standard were identified, and 702,128 ESTs common to BTGI and gold standard were identified. Q_{single} analyses as described in Materials and Methods were performed with these datasets. The distribution of the Q_{single} indices for the comparisons between TAMUClust vs bovine genome aligned ESTs, and BTGI vs bovine genome aligned ESTs are detailed in **Tables 2.7** and **2.8** respectively.

Table 2.7: Q_{single} analysis with TAMUClust as query and bovine genome aligned ESTs as reference for 209,645 *Bos taurus* ESTs common to both datasets.

% Q_{single} bin	TAMUClust ESTs vs bovine genome aligned ESTs		
	Clusters in Q_{single} True Positive Bins	Clusters in Q_{single} False Positive Bins	Clusters in Q_{single} False Negative Bins
= 0	0	13,680	7,426
≥ 0 and ≤ 10	72	13,786	8,240
> 10 and ≤ 20	89	65	1,073
> 20 and ≤ 30	215	43	1,001
> 30 and ≤ 40	655	65	1,208
> 40 and ≤ 50	1,466	94	1,472
> 50 and ≤ 60	830	29	632
> 60 and ≤ 70	1,097	57	444
> 70 and ≤ 80	1,201	50	180
> 80 and ≤ 90	966	35	32
> 90 and ≤ 100	7,692	59	1
= 100	6,975	0	0

Table 2.8: Q_{single} analysis with BTGI as query and bovine genome aligned ESTs as reference for 702,128 *Bos taurus* ESTs common to both datasets.

% Q_{single} bin	BTGI vs bovine genome aligned ESTs		
	Clusters in Q_{single} True Positive Bins	Clusters in Q_{single} False Positive Bins	Clusters in Q_{single} False Negative Bins
= 0	0	23,519	13,877
≥ 0 and ≤ 10	188	23,784	14,552
> 10 and ≤ 20	387	200	1,387
> 20 and ≤ 30	899	151	1,354
> 30 and ≤ 40	1,810	208	2,055
> 40 and ≤ 50	2,891	503	2,323
> 50 and ≤ 60	2,006	100	1,665
> 60 and ≤ 70	1,857	175	1,423
> 70 and ≤ 80	1,537	161	750
> 80 and ≤ 90	1,236	194	138
> 90 and ≤ 100	12,838	173	2
= 100	12,221	0	0

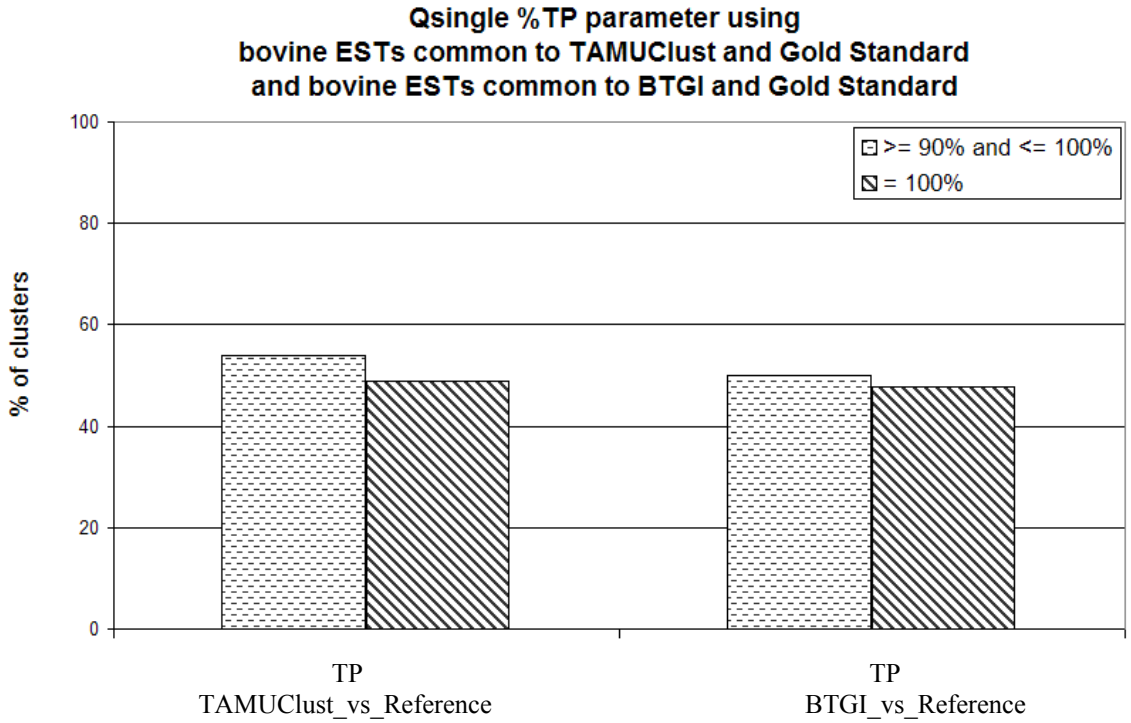


Figure 2.3: Q_{single} %TP for TAMUClust/BTGI vs bovine genome aligned ESTs using ESTs common to datasets being compared.
Cluster abundance of the Q_{single} %TP parameter in the upper bins (>90%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard. Study performed using bovine ESTs common to TAMUClust, gold standard dataset and bovine ESTs common to BTGI, gold standard dataset.

Figure 2.3 depicts the distribution of the different clusters in the upper bins (>90%) for the Q_{single} %TP parameter (from **Tables 2.7** and **2.8**) for the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons. In the TAMUClust vs bovine genome aligned ESTs comparison, TAMUClust has 53.85% clusters in the 90-100% TP bin and 48.83% clusters in the 100% TP bin. In terms of cluster equivalence comparisons, this means that 53.85% of

the TAMUClust clusters have >90% of ESTs in the same cluster as it exists in the gold standard dataset. The comparable cluster abundance numbers in the 90-100% TP and the 100% TP bins from the BTGI vs gold standard comparisons are 50.05% and 47.65% respectively.

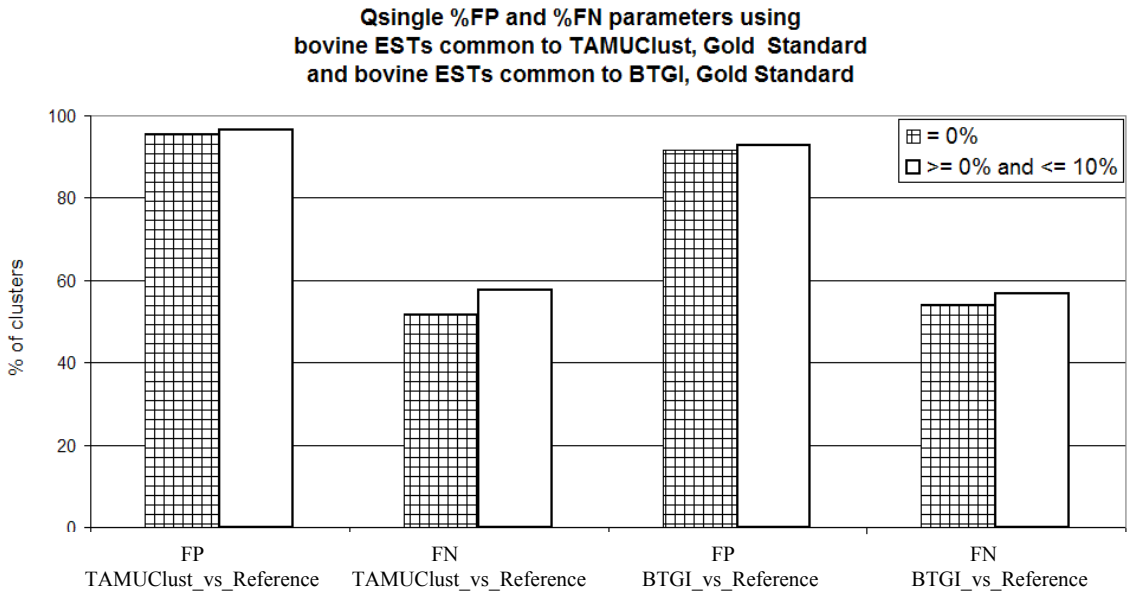


Figure 2.4: Q_{single} %FP, %FN for TAMUClust/BTGI vs bovine genome aligned ESTs using ESTs common to datasets being compared.
Cluster abundance of the Q_{single} %FP and %FN parameters in the lower bins (<10%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard. Study performed using bovine ESTs common to TAMUClust, gold standard dataset and bovine ESTs common to BTGI, gold standard dataset.

Figure 2.4 depicts the distribution of the different clusters in the lower bins (<10%) for the Q_{single} %FP and %FN parameters for the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons. The Q_{single} %FP metric for the TAMUClust vs bovine genome aligned ESTs comparison reveals that TAMUClust has 96.52% clusters in the 0-10% FP bin. This indicates that <10% of the ESTs in ~97% of the TAMUClust clusters have been grouped with unrelated members when compared with the corresponding gold standard clusters. The Q_{single} %FN metric for the TAMUClust vs bovine genome aligned ESTs comparison reveals that TAMUClust has 57.69% clusters in the 0-10% FN bin. This metric indicates that <10% of the relatives (cluster members) for a particular EST are missing in ~58% of the TAMUClust clusters when compared with the corresponding gold standard clusters. The comparable cluster abundance numbers in the 0-10% FP and the 0-10% FN bins from the BTGI vs bovine genome aligned ESTs comparison are 92.73% and 56.73% respectively.

One also needs to take into account the fact that the dataset in the BTGI-gold standard analysis is ~3.3 folds larger compared to the dataset in the TAMUClust-gold standard analysis. The major reason for the ~3.3 folds larger dataset is due to the large difference in the number of ESTs used to generate the clusters. 308, 132 *Bos taurus* ESTs were downloaded from dbEST in March 2005 to generate the TAMUClust EST clusters, whereas 955, 233 ESTs were present in the BTGI release of March 2007. This led us to ask the question "Does this difference in datasets being compared affect the cluster equivalence comparison analysis". To address this, the above Q_{single} analyses were repeated by using only those ESTs which are common to all 3 datasets. 198,438 bovine ESTs common to all three datasets were identified in the one to one comparisons of TAMUClust vs bovine genome aligned ESTs and BTGI vs bovine genome aligned ESTs using bovine ESTs common to all datasets. Q_{single} analyses were performed for the TAMUClust vs bovine genome aligned ESTs and BTGI vs bovine genome aligned ESTs. The distributions of the Q_{single} indices in the different bins and the number of ESTs in each of the Q_{single} %TP, Q_{single} %FP and Q_{single} %FN bins are detailed in **Table 2.9**.

Table 2.9: Q_{single} analysis with TAMUClust/BTGI as query and bovine genome aligned ESTs as reference for 198,438 *Bos taurus* ESTs common to all datasets.

% Q_{single} bin	TAMUClust/BTGI vs bovine genome aligned ESTs											
	Clusters and ESTs in Q_{single} True Positive Bins				Clusters and ESTs in Q_{single} False Positive Bins				Clusters and ESTs in Q_{single} False Negative Bins			
	TAMU Clust	# ESTs	BTGI	# ESTs	TAMU Clust	# ESTs	BTGI	# ESTs	TAMU Clust	# ESTs	BTGI	# ESTs
= 0	0	0	0	0	11,861	0	11,729	0	6,589	0	5,667	0
≥ 0 and ≤ 10	55	60	35	37	11,937	90	11,784	113	7,247	1458	6,119	1018
> 10 and ≤ 20	79	303	122	680	62	117	83	141	984	3288	938	3389
> 20 and ≤ 30	159	1884	330	5194	35	138	46	167	869	4883	905	4858
> 30 and ≤ 40	487	7064	813	9242	58	169	71	209	1,087	6668	1,327	7955
> 40 and ≤ 50	1,171	11116	1,517	12426	94	232	165	292	1,209	10273	1,453	12449
> 50 and ≤ 60	784	11043	1,009	13374	27	176	31	217	508	11041	742	12824
> 60 and ≤ 70	979	11832	1,163	14119	47	230	62	216	340	12483	589	14899
> 70 and ≤ 80	1,014	15458	1,030	14778	49	350	57	364	122	5166	288	15299
> 80 and ≤ 90	902	18434	856	18353	34	335	65	512	25	1077	31	2720
> 90 and ≤ 100	6,762	64891	5,517	34824	49	3978	28	804	1	16	0	0
= 100	6,165	35585	5,136	19133	0	0	0	0	0	0	0	0

Results from **Table 2.9** indicate that TAMUClust has ~50% of the ESTs (98,783 ESTs out of 198,438 ESTs) in clusters with Q_{single} %TP values >70%. In comparison, BTGI has ~34% of the ESTs (67955 ESTs out of 198,438 ESTs) in clusters with Q_{single} %TP values >70%.

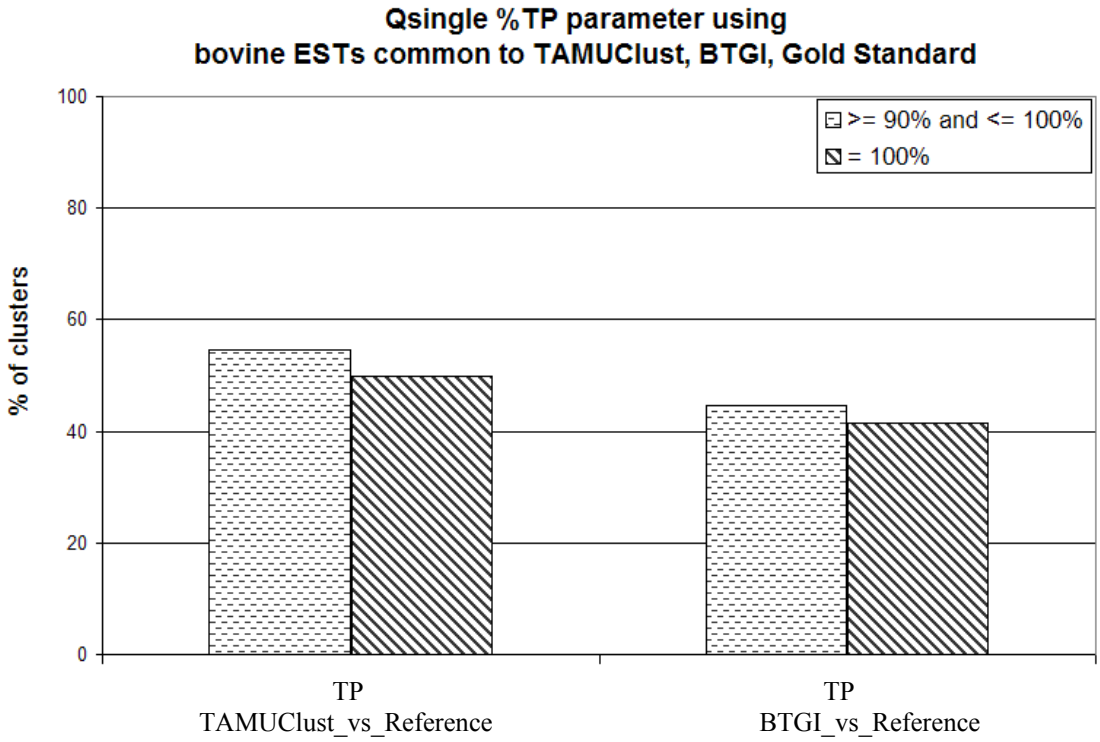


Figure 2.5: Q_{single} %TP for TAMUClust/BTGI vs bovine genome aligned ESTs using ESTs common to all three datasets.
Cluster abundance of the Q_{single} %TP parameter in the upper bins (>90%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard. Study performed using bovine ESTs common to TAMUClust, BTGI and gold standard dataset.

Figure 2.5 depicts the distribution of the different clusters in the upper bins (>90%) for the Q_{single} %TP parameter (from **Table 2.9**) for the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons using ESTs common to all datasets. In the TAMUClust vs bovine genome aligned ESTs comparison, TAMUClust has 54.57% clusters in the 90-100% TP bin and 49.75% clusters in the 100% TP bin. The comparable cluster abundance numbers in the 90-100% true-positives and the 100% true-positives bins from the BTGI vs bovine genome aligned ESTs comparisons are 44.52% and 41.45% respectively.

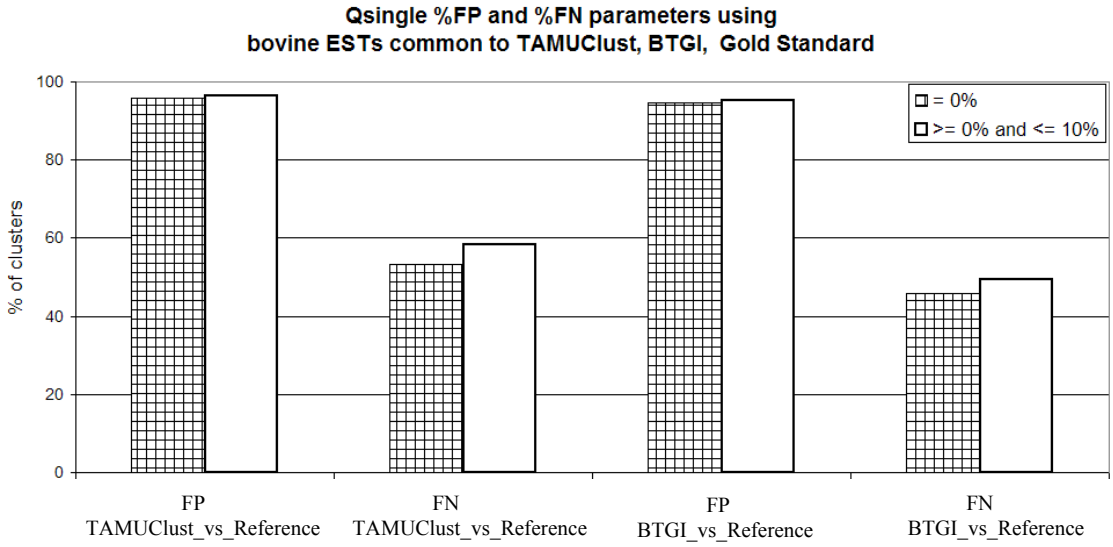


Figure 2.6: Q_{single} %FP, %FN for TAMUClust/BTGI vs bovine genome aligned ESTs using ESTs common to all three datasets.
Cluster abundance of the Q_{single} %FP and %FN parameters in the lower bins (<10%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard. Study performed using bovine ESTs common to TAMUClust, BTGI and gold standard dataset.

Figure 2.6 depicts the distribution of the different clusters in the lower bins (<10%) for the Q_{single} %FP and %FN parameters for the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons using ESTs common to all datasets. The Q_{single} %FP metric for the TAMUClust vs bovine genome aligned ESTs comparison reveals that TAMUClust has 96.32% clusters in the 0-10% FP bin. The Q_{single} %FN metric for the TAMUClust vs bovine genome aligned ESTs comparison reveals that TAMUClust has 58.48% clusters in the 0-10% FN bin. The comparable cluster abundance numbers in the 0-10% FP and the 0-10% FN bins from the BTGI vs bovine genome aligned ESTs comparison are 95.09% and 49.38% respectively.

Figure 2.6 reveals that the cluster abundance in the lower bins (<10%) for the Q_{single} %FP and %FN parameters is almost the same for both the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons.

Based on the analysis so far, one can interpret that TAMUClust is comparable in performance to BTGI. From **Figure 2.5**, one can interpret that TAMUClust marginally outperforms BTGI on the basis of cluster abundance in the >90% Q_{single} %TP bin (~55% clusters for TAMUClust vs ~45% clusters for BTGI). Results from **Table 2.9** (TAMUClust having ~50% ESTs in clusters with Q_{single} %TP values >70% as against BTGI having ~34% ESTs) lend further support to the fact that TAMUClust performs better than BTGI.

Need for 'singleton minus' analyses

TAMUClust uses a protein framework to cluster and assemble ESTs. FASTX is used to compare the ESTs to the vertebrate protein clusters and identify the most closely related vertebrate protein family for a given EST. For ESTs within each protein family, the Megablast [114] tool of the TGICL [136] package groups ESTs into clusters by including ESTs that are at least 95% identical over at least 40 nucleotides and having an overhang of less than 30 nucleotides. The CAP3 [137] tool assembles the clustered ESTs into Contigs/Singletons. In this process, TAMUClust also includes singletons in the gene indices (consensus sequences) it generates where these singletons have a significant sequence similarity to a homologous protein. BTGI performs a nucleotide-nucleotide comparison to cluster and assemble ESTs, and discards ESTs that do not match the clustering criteria. As a result, the gene indices generated by BTGI are devoid of singletons.

'Singleton minus' Q_{single} analyses were performed by excluding singletons from the TAMUClust dataset and then comparing TAMUClust/BTGI clusters with the gold standard (bovine genome aligned ESTs).

Singleton minus analysis I

In the one to one comparisons of TAMUClust vs bovine genome aligned ESTs and BTGI vs bovine genome aligned ESTs using bovine ESTs common to the datasets being compared, TAMUClust singletons were excluded and ESTs common to TAMUClust and the gold standard were identified. This resulted in 198,525 ESTs common to both datasets.

This TAMUClust versus gold standard dataset Q_{single} analysis (**Table 2.10**) was compared to the BTGI versus gold standard dataset Q_{single} analysis (**Table 2.8**) using ESTs common to BTGI and gold standard (702,128 ESTs).

Table 2.10: Q_{single} analysis excluding TAMUClust singletons and using TAMUClust as query with bovine genome aligned ESTs as reference for 198,525 *Bos taurus* ESTs common to both datasets.

% Q_{single} bin	TAMUClust vs bovine genome aligned ESTs (excluding TAMUClust singletons)		
	Clusters in Q_{single} True Positive Bins	Clusters in Q_{single} False Positive Bins	Clusters in Q_{single} False Negative Bins
= 0	0	10,405	6,454
≥ 0 and ≤ 10	70	10,507	6,903
> 10 and ≤ 20	67	67	733
> 20 and ≤ 30	142	42	732
> 30 and ≤ 40	371	66	938
> 40 and ≤ 50	897	105	934
> 50 and ≤ 60	801	31	433
> 60 and ≤ 70	812	63	273
> 70 and ≤ 80	837	64	92
> 80 and ≤ 90	685	39	8
> 90 and ≤ 100	6,364	62	0
= 100	5,932	0	0

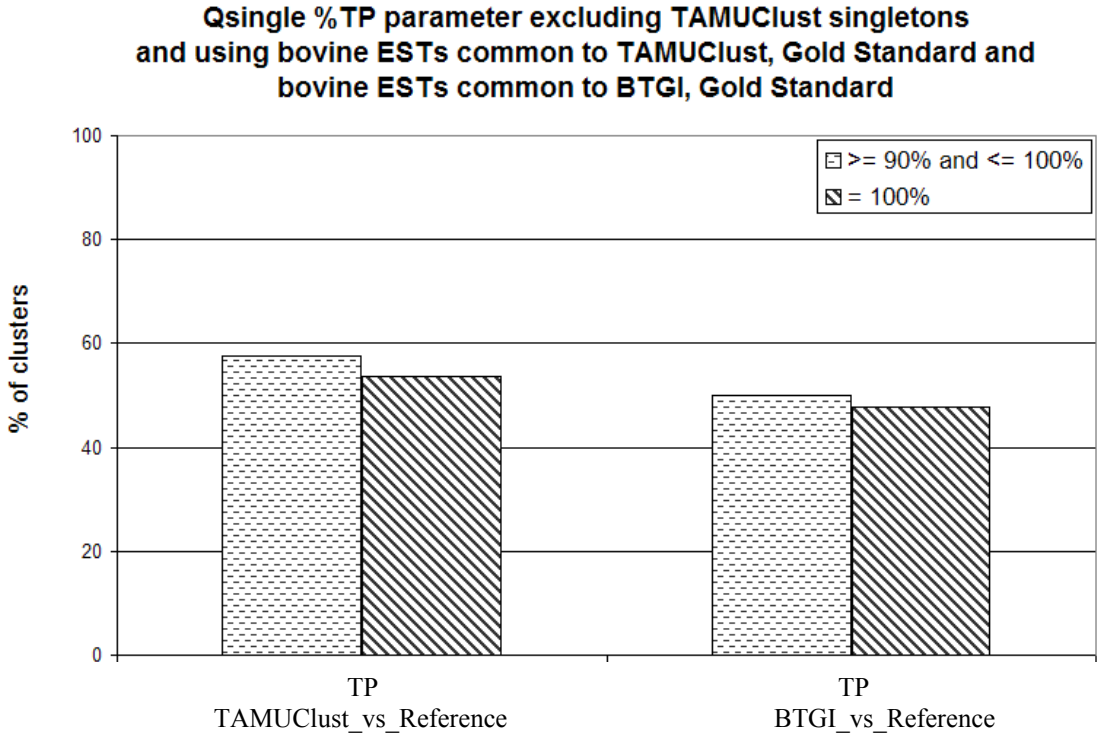


Figure 2.7: Q_{single} %TP for TAMUClust/BTGI vs bovine genome aligned ESTs after excluding TAMUClust singletons and using ESTs common to datasets being compared.
Cluster abundance of the Q_{single} %TP parameter in the upper bins (>90%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard after excluding TAMUClust singletons. Study performed using ESTs common to TAMUClust, gold standard dataset and ESTs common to BTGI and gold standard dataset.

Figure 2.7 depicts the distribution of the different clusters in the upper bins (>90%) for the Q_{single} %TP parameter for the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons after excluding singletons from TAMUClust and using ESTs common to the datasets being compared. In the TAMUClust vs bovine genome aligned ESTs comparison, TAMUClust has

57.61% clusters in the 90-100% TP bin and 53.7% clusters in the 100% TP bin. The comparable cluster abundance numbers in the 90-100% TP and the 100% TP bins from the BTGI vs bovine genome aligned ESTs are 50.05% and 47.65% respectively.

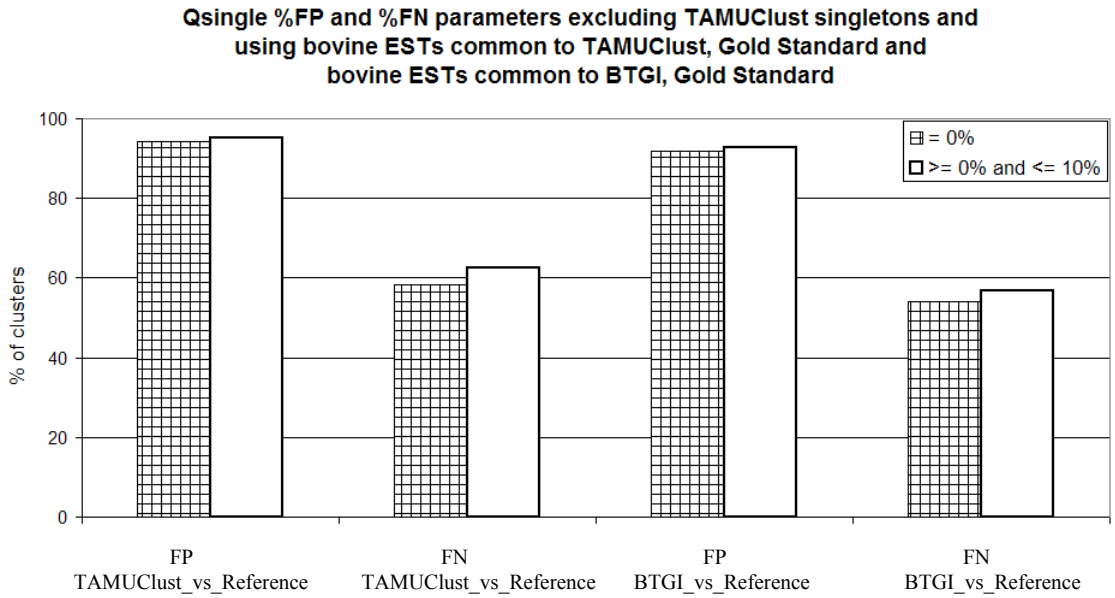


Figure 2.8: Q_{single} %FP, %FN for TAMUClust/BTGI vs bovine genome aligned ESTs after excluding TAMUClust singletons and using ESTs common to datasets being compared.

Cluster abundance of the Q_{single} %FP and %FN parameters in the lower bins (<10%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard after excluding TAMUClust singletons. Study performed using ESTs common to TAMUClust, gold standard dataset and ESTs common to BTGI and gold standard dataset.

Figure 2.8 depicts the distribution of the different clusters in the lower bins (<10%) for the Q_{single} %FP and %FN parameters for the TAMUClust vs bovine genome aligned ESTs and the BTGI vs bovine genome aligned ESTs comparisons after excluding TAMUClust singletons and using ESTs common to the datasets being compared. The Q_{single} %FP metric for the TAMUClust vs bovine genome aligned ESTs comparison reveals that TAMUClust has 95.12% clusters in the 0-10% FP bin. The Q_{single} %FN metric for the TAMUClust vs bovine genome aligned ESTs comparison reveals that TAMUClust has 62.49% clusters in the 0-10% FN bin. The comparable cluster abundance numbers in the 0-10% FP and the 0-10% FN bins from the BTGI vs bovine genome aligned ESTs comparison are 92.73% and 56.73% respectively.

Interpretation of results will be difficult when there is a large difference in the size of datasets being compared. The 'Singleton minus analysis I' described above has a ~3.5 fold larger dataset in the BTGI-gold standard analysis compared to TAMUClust-gold standard dataset. As mentioned before, the ~3.5 fold larger dataset is due to the large difference in the number of ESTs used in the starting material, which arose because of the fact that ESTs for TAMUClust were obtained from dbEST in March 2005 whereas the BTGI build was obtained in March 2007.

Singleton minus analysis II

This analysis was performed where the Q_{single} indices were obtained by using only those ESTs which were common to all 3 datasets after excluding TAMUClust singletons. 192,920 bovine ESTs common to all three datasets were identified after excluding TAMUClust singletons. **Table 2.11** compares the number of clusters and the

cluster sizes for the three *Bos taurus* EST datasets (bovine ESTs aligned to bovine genome, bovine ESTs clustered by TAMUClust, bovine EST clusters from BTGI) using 192,920 ESTs common to all datasets after excluding TAMUClust singletons.

Table 2.11: Cluster size versus number of clusters for the three datasets – bovine genome aligned ESTs, TAMUClust and BTGI – using 192,920 ESTs common to all datasets.

Cluster Size	bovine genome aligned ESTs	TAMUClust	BTGI
1	655	1,254	5,989
2	1,788	5,791	7,904
3	910	2,570	3,550
4	752	1,786	2,286
5	573	1,218	1,547
6 – 10	1,957	3,253	3,834
11 – 20	1,871	2,402	2,518
21 – 30	887	1,027	907
31 – 40	488	477	393
41 – 50	294	229	157
51 – 100	559	308	248
101 - 500	206	101	78
> 500	8	8	4
	Total = 14,283	**Total = 20,424**	**Total = 29,415**

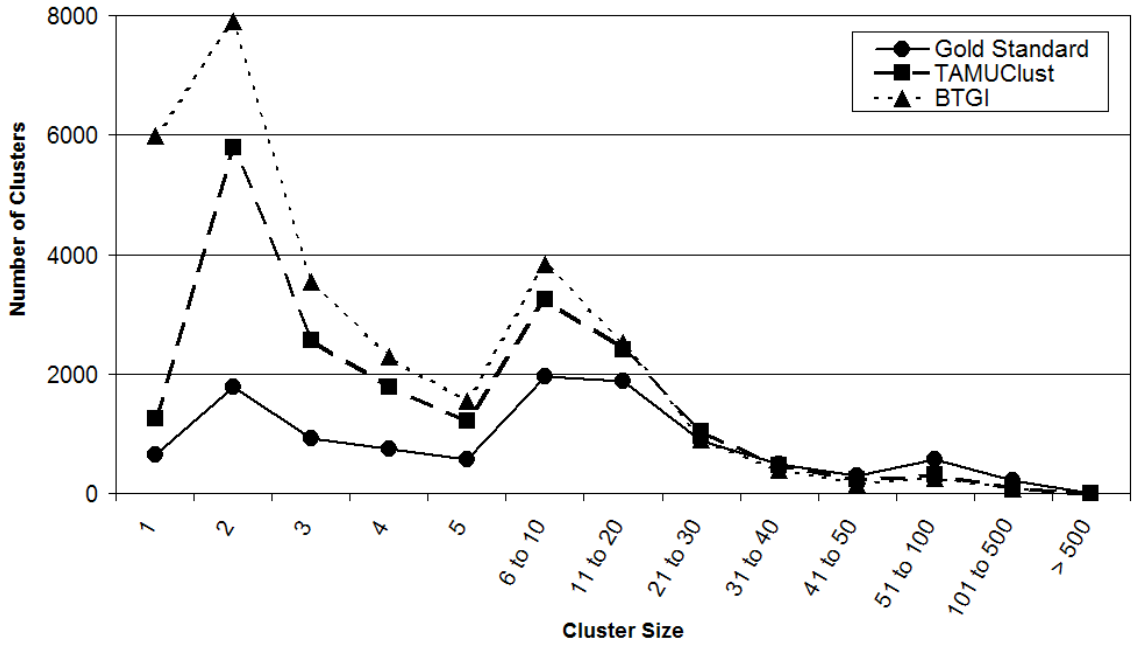


Figure 2.9: Cluster numbers as a function of cluster size for the bovine genome aligned ESTs, TAMUClust and BTGI datasets using ESTs common to all datasets. Number of clusters versus cluster size for the three *Bos taurus* EST cluster datasets – bovine genome aligned ESTs (gold standard), TAMUClust and BTGI – using 192, 920 ESTs common to all datasets.

A plot of number of clusters vs cluster size (**Figure 2.9**) reveals that TAMUClust cluster size is closer to gold standard than the comparable BTGI versus gold standard statistic. As discussed before, it is important to realize that the cluster size metric does not give information about equivalence of clusters, i.e. the same cluster members being grouped together in all datasets. Hence, we use the Q_{single} metric to compare the cluster equivalence of TAMUClust with gold standard and BTGI with gold standard. **Table 2.12** details the distributions of the Q_{single} indices in the different bins and the number of

ESTs in each of the Q_{single} %TP, Q_{single} %FP and Q_{single} %FN bins for the TAMUClust vs gold standard and the BTGI vs gold standard comparisons.

Table 2.12: Q_{single} analysis excluding TAMUClust singletons and using TAMUClust/BTGI as query and bovine genome aligned ESTs as reference for 192,920 *Bos taurus* ESTs common to all datasets.

% Q_{single} bin	TAMUClust/BTGI vs bovine genome aligned ESTs											
	Clusters and ESTs in Q_{single} True Positive Bins				Clusters and ESTs in Q_{single} False Positive Bins				Clusters and ESTs in Q_{single} False Negative Bins			
	TAMU Clust	# ESTs	BTGI	# ESTs	TAMU Clust	# ESTs	BTGI	# ESTs	TAMU Clust	# ESTs	BTGI	# ESTs
= 0	0	0	0	0	10400	0	10444	0	6407	0	4805	0
≥ 0 and ≤ 10	54	59	26	29	10476	90	10493	109	6843	1220	5236	995
> 10 and ≤ 20	63	276	86	601	60	115	65	117	742	3012	849	3249
> 20 and ≤ 30	128	1679	281	4880	35	138	38	152	729	4442	850	4777
> 30 and ≤ 40	368	6573	722	8372	57	161	54	179	926	6713	1201	7264
> 40 and ≤ 50	882	10241	1325	12340	101	241	116	243	917	9164	1307	12063
> 50 and ≤ 60	780	10569	981	13139	28	182	30	213	416	10296	684	12812
> 60 and ≤ 70	786	11420	1013	13326	50	242	45	170	278	11893	550	13833
> 70 and ≤ 80	829	14242	962	14511	56	384	45	316	88	4538	242	14379
> 80 and ≤ 90	687	16745	769	17387	36	359	38	350	9	933	29	2552
> 90 and ≤ 100	6,371	68845	4783	36051	49	3978	24	589	0	0	0	0
= 100	5,952	44263	4413	20151	0	0	0	0	0	0	0	0

Results from **Table 2.12** indicate that TAMUClust has ~52% of the ESTs (99832 ESTs out of 192920 ESTs) in clusters with Q_{single} %TP values >70%. In comparison, BTGI has ~35% of the ESTs (67949 ESTs out of 192920 ESTs) in clusters with Q_{single} %TP values >70%.

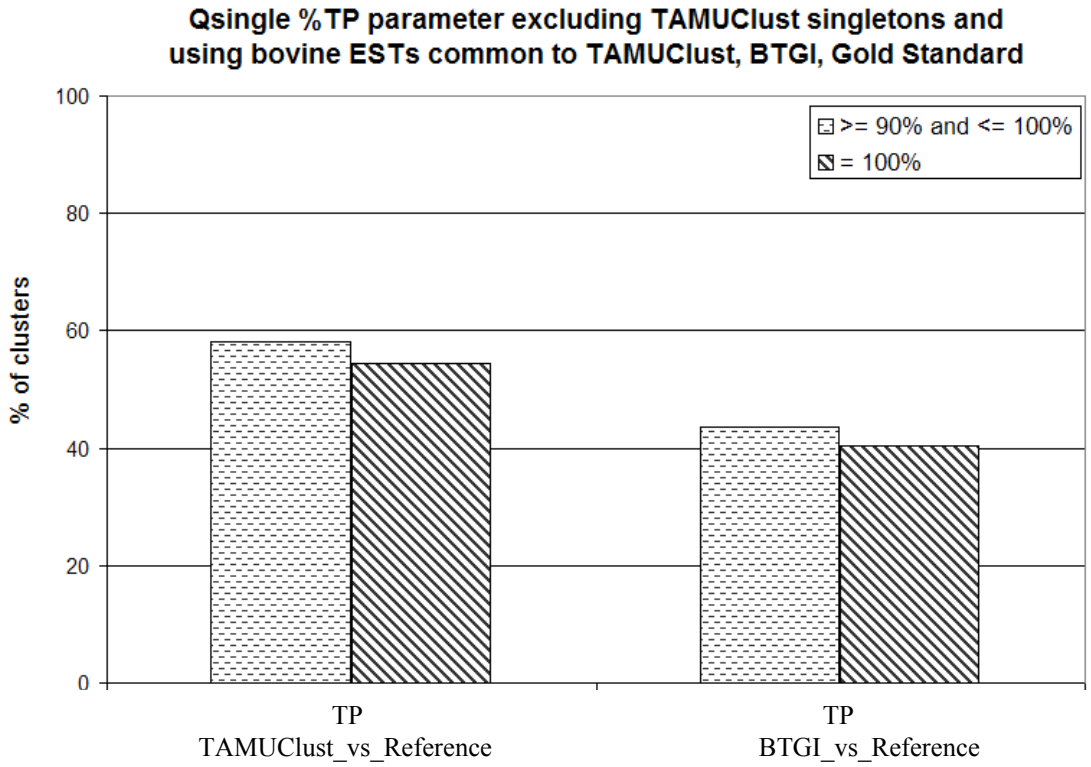


Figure 2.10: Q_{single} %TP for TAMUClust/BTGI vs bovine genome aligned ESTs after excluding TAMUClust singletons and using ESTs common to all three datasets.
Cluster abundance of the Q_{single} %TP parameter in the upper bins (>90%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard. Study performed after excluding TAMUClust singletons and using bovine ESTs common to TAMUClust, BTGI and gold standard dataset.

Figure 2.10 depicts the distribution of clusters in the 90-100% TP bin and the 100% TP bin using the Q_{single} parameter for the TAMUClust vs gold standard and the BTGI vs gold standard comparisons after excluding TAMUClust singletons and using ESTs common to all datasets. **Figure 2.11** depicts the distribution of the different clusters in the lower bins (<10%) for the Q_{single} %FP and %FN parameters for the TAMUClust vs gold standard and the BTGI vs gold standard comparisons after excluding TAMUClust singletons and using ESTs common to all datasets.

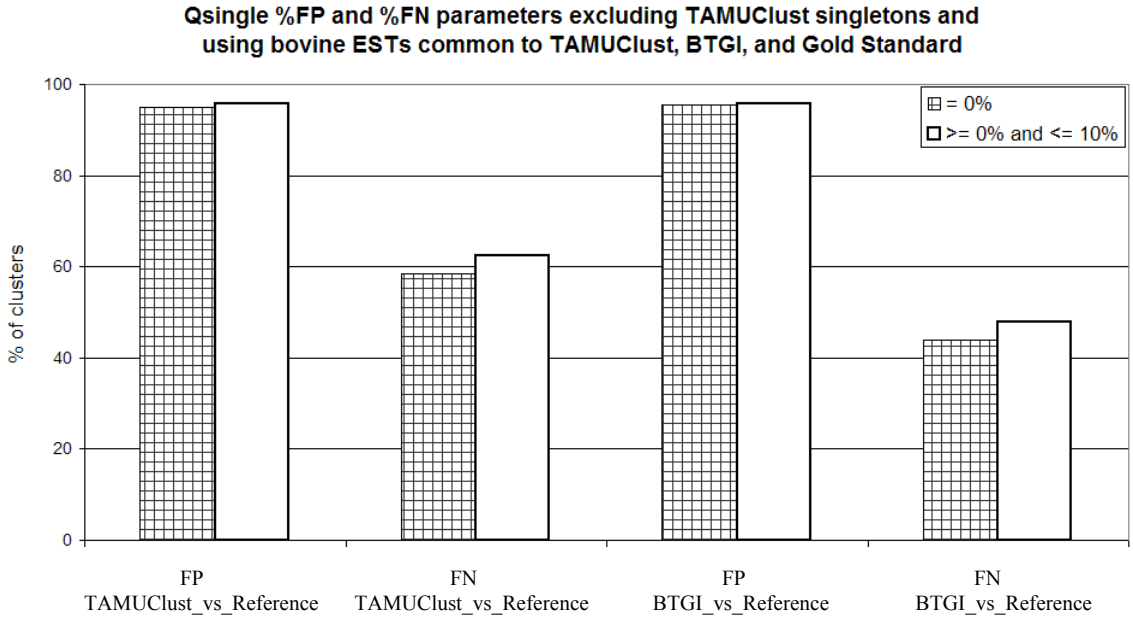


Figure 2.11: Q_{single} %FP, %FN for TAMUClust/BTGI vs bovine genome aligned ESTs after excluding TAMUClust singletons and using ESTs common to all three datasets.
Cluster abundance of the Q_{single} %FP and %FN parameters in the lower bins (<10%) for TAMUClust and BTGI using bovine ESTs aligned to the bovine genome as the gold standard. Study performed after excluding TAMUClust singletons and using bovine ESTs common to TAMUClust, BTGI and gold standard dataset.

Findings from the Q_{single} %FP and %FN indices indicate that TAMUClust and BTGI perform very similarly. Hence, one can compare the cluster abundance of the >90% Q_{single} percentage true-positives parameter for TAMUClust and BTGI using bovine genome aligned ESTs as the gold standard to determine which clustering method performs better.

TAMUClust has 58.19% clusters in the 90-100% true-positives bin compared to 43.69% for BTGI. Comparisons of the 100% true-positives bins reflect a value of 54.37% for TAMUClust and 40.31% for BTGI. In other words, when compared to BTGI, TAMUClust has ~14-15% additional clusters that have the very same members as is seen in the gold standard dataset.

The findings from **Figure 2.10** taken in conjunction with the findings from **Figure 2.7** and **Table 2.12** (TAMUClust having ~52% ESTs in clusters with Q_{single} %TP values >70% as against BTGI having ~35% ESTs) as discussed above illustrate that TAMUClust performs better than BTGI; removal of singletons from the analysis further improves the performance of TAMUClust compared to BTGI.

Summary of findings from the Q_{single} analyses in the Gold standard comparisons

This study was performed to ask the question "Is TAMUClust comparable in performance to TIGR Gene Indices (TGI)". Findings from the Q_{single} analyses from the Gold standard comparisons as discussed above suggest that TAMUClust compares well to TGI and marginally outperforms it.

Comparison with predicted gene models

Megablast analysis I

The Q_{single} metric from singleton minus analyses (I and II) using the gold standard dataset indicates that TAMUClust performs marginally better than BTGI. This led us to ask the question "Does TAMUClust gain anything by including singletons in its gene indices". To address this, we used Megablast [114] to align the consensus sequences of TAMUClust and BTGI to the bovine transcripts in the *Bos taurus* Ensembl Release 50 (July 2008) [129]. For this analysis, we used only the longest transcript for each gene in the Ensembl dataset. The Megablast results were filtered to include only those alignments which were longer than 100 bases in alignment length and had sequence identity greater than 95%. Results from the Megablast analysis I are listed in **Table 2.13**.

Table 2.13: Megablast analysis I - TAMUClust/BTGI EST consensus sequences vs Ensembl Btau predicted transcripts.

Criteria	TAMUClust	BTGI
Number of ESTs	308,132	955,233
Date on which ESTs were obtained	March 2005	March 2007
Number of EST consensus sequences	46,731 (24, 665 contigs + 22, 066 singletons)	90, 392
Ensembl transcript hits	34,199 (22, 041 contigs + 12, 158 singletons)	51, 425
Unique Ensembl transcript hits	14, 842	15, 451
Common Ensembl transcript hits	14, 144	14, 144
Ensembl Transcripts that align to TAMUClust consensus sequences but **not to any** BTGI consensus sequences	698 (57 contigs + **641 singletons**)	-
Ensembl Transcripts that align to BTGI consensus sequences but **not to any** TAMUClust consensus sequences	-	1, 307

We first determined if there were any Ensembl transcripts that aligned to TAMUClust consensus sequences but were missed by BTGI consensus sequences; from these TAMUClust hits, we then identified the TAMUClust singletons. From **Table 2.13**,

we find that there are 698 Ensembl transcripts that are identified by TAMUClust but are missed by BTGI. Of these 698 Ensembl transcript hits from TAMUClust consensus sequences, 641 hits are from singletons.

These results from **Table 2.13** indicate that TAMUClust stands to gain by including singletons. Here are some factors that further reinforce this statement:

1. **Date difference in the TAMUClust and BTGI builds**: ESTs in TAMUClust were obtained from GenBank in Mar 2005, whereas the ESTs in BTGI are from Mar 2007. Despite the fact that there is a ~2 year difference in the dates in favor of BTGI, Megablast analysis reveals that there are 762 Ensembl transcripts which align to TAMUClust consensus sequences but not to BTGI consensus sequences using the alignment filtering criteria (≥95% identity and ≥100 in alignment length). 641 of these 698 hits were from TAMUClust singletons. By the same criteria, there are 1,307 Ensembl transcripts that align to BTGI consensus sequences but not to TAMUClust consensus sequences. We believe that this is largely due to the difference in the dates of obtaining the ESTs to build the EST clusters. The only way to prove this beyond doubt would be to take all the ESTs from the BTGI build and pass it through the TAMUClust pipeline (vertebrate protein family clustering, ESTs vs protein family FASTX and EST assembly) – something that is beyond the scope of this project keeping in mind that the aim of this study is to study how well TAMUClust performs compared to existing EST clustering schemes and not to prove that TAMUClust can outdo those schemes.

2. **Differences in the EST clustering methods**: TIGR Gene Indices (TGI) starts off with gene sequences are parsed from the CDS and CDS-join features in GenBank protein records pertaining to the species in question [96]; additional Expressed Transcript (ET) sequences are obtained from the TIGR EGAD database. Coding Sequences (CDSs), Expressed Transcripts (ETs), and Tentative Consensus Sequences (TCs) from previous build (if available) are used as templates for the clustering process. Given the fact that TGI uses TCs from previous build and CDSs, the average length of the "tentative consensus (TC)" sequence from TGI is bound to be longer compared to *de novo* EST clustering methods like TAMUClust where clusters are built without using information from previous builds and without using CDSs. By including TCs from previous build and CDSs, TGI has "longer" starting sequences. **Despite these differences** in the EST cluster build with TAMUClust and TGI methodologies, 698 TAMUClust consensus sequences (of which 641 are singletons) align to Ensembl transcripts that are missed by BTGI.
3. The TAMUClust singletons have statistically significant similarity to a homologous protein as determined by the coarse protein grouping performed prior to EST clustering and assembly. Therefore, the TAMUClust singletons are not trivial and not some artifacts of the EST clustering method.

Summary of findings from Megablast analysis I

This study was performed to ask the question "Does TAMUClust gain anything by including singletons in its gene indices". Results from the Megablast analysis in conjunction with the singleton minus analyses lend further support to the fact that TAMUClust definitely stands to gain by including singletons in the gene indices (consensus sequences) it generates.

Comparison with predicted gene models

Megablast analysis II

This analysis was designed to determine how many of the predicted transcripts from the *Bos taurus* Ensembl Release 50 (July 2008) [129] have a match to a EST consensus sequence from the TAMUClust and BTGI *Bos* taurus EST clusters. Megablast [114] was used to address this question. The query file being the longest bovine transcript for each gene from the *Bos taurus* Ensembl Release 50 and the database was a file containing either TAMUClust or BTGI EST consensus sequences. There were 21, 722 transcripts in the query file. The Megablast results were filtered to include only those alignments which were longer than 100 bases in alignment length and had sequence identity greater than 95%. Results from the Megablast analysis II are listed in **Table 2.14**.

Table 2.14: Megablast analysis II - Ensembl Btau predicted transcripts vs TAMUClust/BTGI EST consensus sequences.

Criteria	**Ensembl Transcripts vs TAMUClust**	**Ensembl Transcripts vs BTGI**
Number of ESTs	308,132	955,233
Number of EST consensus sequences	46,731 (24, 665 contigs + 22, 066 singletons)	90, 392
Hits	16, 194 (13, 282 contigs + 2, 912 singletons)	16, 853
Unique hits	14, 815 (12, 143 contigs + 2, 672 singletons)	15, 459
Number of Ensembl predicted transcripts in both analyses	15, 559	
Ensembl transcripts that align to TAMUClust consensus sequences but **not to any** BTGI consensus sequences	635 (22 contigs + **613 singletons**)	-
Ensembl transcripts that align to BTGI consensus sequences but **not to any** TAMUClust consensus sequences	-	1, 294

Results from **Table 2.14** indicate that 16194 (out of a total of 21722) predicted bovine transcripts have a hit to a TAMUClust EST consensus sequence, whereas 16853 predicted bovine transcripts have a hit to a BTGI EST consensus sequence. Of the 16194 predicted transcripts that had a hit to a TAMUClust EST consensus sequence, 2912 were to singletons. This further reinforces the point that we had made earlier - TAMUClust does gain by including singletons in the gene indices it generates.

There are 635 predicted transcripts that align to a TAMUClust EST consensus sequence but do not align to a BTGI EST consensus sequence. Likewise, there are 1294 predicted transcripts that align to a BTGI EST consensus sequence but do not align to a TAMUClust EST consensus sequence.

Summary of findings from Megablast analysis II

The overall findings from this analysis suggest that the transcript coverage in TAMUClust and BTGI is more or less same with the TAMUClust dataset reconstructing some transcripts that are missed by BTGI and the BTGI dataset reconstructing some transcripts that are missed by TAMUClust.

CONCLUSIONS

Expressed Sequence Tags (ESTs) are single pass sequence reads from randomly selected cDNA clone. ESTs contain high error rates, and the information encoded is often fragmented and redundant. Therefore, ESTs need to be clustered into groups likely to have been derived from the same genes and this process improves the quality of

meaningful information that can be derived from ESTs. Chimeric EST clusters arise when ESTs representing unrelated genes are grouped together and chimerism is the single largest contributor to EST misassemblies. This can be avoided if ESTs are initially grouped into clusters based on their matches to proteins, and then subject to clustering and assembly. TAMUClust is a new clustering method developed in our lab which clusters ESTs using a protein framework.

Accurate clustering of ESTs derived from the same genes is of utmost importance as ESTs are used to identify genes in an organism and in the design of oligonucleotide probes. Any inaccuracies in the EST clustering step would result in problems associated with improper oligonucleotide design. EST clustering using a protein framework is very handy in selecting the representative sets for oligonucleotide design and helps avoid many of the frequently encountered problems in oligo design - generating oligos with redundant information, and designing oligos based on chimeric ESTs.

We conducted a pilot study using *Bos taurus* ESTs in Jan 2004 to compare the clustering performance of TAMUClust with existing EST clustering methods, *Bos taurus* Gene Indices (BTGI) clusters obtained from DGI/TIGR and UniGene from NCBI. The study was performed by determining cluster equivalence (Q_{single} indices) for the datasets being compared after identifying common ESTs. The results from the pilot study indicate that the performance of TAMUClust is comparable to that of BTGI and UniGene. As the clustering methodology of TAMUClust and BTGI is very much similar

with reference to the stringency and the level of supervision, we chose to perform further comparisons of TAMUClust only with BTGI.

In August 2006, the third version of the bovine genome assembly, Btau_3.1, was released where the genome assembly has a 7.15X coverage [10, 11]. This came in handy to compare the performance of TAMUClust *Bos taurus* EST clusters against the *Bos taurus* Gene Indices (BTGI) clusters by using bovine ESTs aligned to the bovine genome assembly as a gold standard. Findings from the gold standard comparisons reveal that TAMUClust and BTGI are similar in performance.

An ideal result/situation would have been TAMUClust performing as well as the gold standard; this not being the case means that there is scope for improvement in the TAMUClust clustering algorithm. However, one needs to keep in mind that while clustering ESTs aligned to the genome, it is not required to set a minimum overlap length due to the fact that two or more overlapping ESTs, irrespective of the overlap length should belong to the same transcript. This is the reason for the false negatives when TAMUClust/BTGI is compared to the gold standard dataset. The gold standard dataset did not include predicted transcripts. If the known coordinates of these predicted transcripts had been used in the gold standard datasets, the number of false negatives would have reduced significantly.

When TAMUClust singletons are excluded to be on an even keel with BTGI, results from the Q_{single} analyses suggest that TAMUClust performance is improved compared to BTGI. However, TAMUClust singletons are not clustering artifacts; these singletons are included in the gene indices because they have a statistically significant

match to a homologous protein used in the protein framework to cluster the ESTs. Findings from a Megablast analysis using predicted transcripts from the *Bos taurus* Ensembl Release 50 (July 2008) [129] suggest that TAMUClust stands to gain by including the singletons in the gene indices. A second Megablast analysis was designed to determine how many of the predicted transcripts from the Bos taurus Ensembl Release 50 (July 2008) [129] have a match to a EST consensus sequence from the TAMUClust and BTGI *Bos taurus* EST clusters. Findings from this analysis suggest that the transcript coverage in TAMUClust and BTGI is more or less same with the TAMUClust dataset reconstructing some transcripts that are missed by BTGI and the BTGI dataset reconstructing some transcripts that are missed by TAMUClust.

EST clustering methods not based on genome alignments need a framework to cluster and assemble the ESTs. The framework has to ensure that the clustering method is neither too rigid to generate very short consensus sequences nor too relaxed to generate long consensus sequences. Also, in the tradeoff between fewer clusters with longer consensus and larger clusters with short consensus sequences, the EST clustering schema has to decide on certain criteria like minimum pairwise sequence identity, overlap length and overhangs to generate the initial clusters to accurately model the biology behind the sequences being clustered while incorporating splice variants and avoiding chimeras.

Advantages of TAMUClust

Clustering ESTs using a protein framework helps identify distantly related (rapidly diverging) protein homologs that might be missed out using DNA:DNA comparisons. The biggest advantage with the TAMUClust method is that the ESTs are grouped into gene families as a result of the protein framework being used. This comes in handy to predict function for these ESTs on the basis of their similarity to the proteins in the protein family. Moreover, the ESTs in a family can be grouped with the proteins, subject to phylogenetic analysis and prediction of function by analyzing the tree topology. Function predictions made using a phylogenetic approach are expected to be more accurate.

Removal of chimeric ESTs

TAMUClust uses a protein framework to cluster and assemble the ESTs. The protein comparison step introduces a high level of stringency by removing chimeric ESTs and reduces the formation of chimeras in the EST assembly step, thereby preventing misassemblies.

Utility with EST projects from next generation sequencing tools

Sequencing projects are becoming much cheaper nowadays thanks to the use of the massively parallel 454 pyrosequencing technology [138], which is capable of sequencing 25 million bases in a four-hour period and is about 100 times faster than the current Sanger sequencing and capillary-based electrophoresis platform [139]. These next-generation sequencing tools produce large numbers of short DNA reads (80-120 bases) [138], tend to have low error rates (<1%) arising because of homopolymer runs,

and these errors tend to be resolved [140-142] when there is sufficient coverage depth to allow assembly of overlapping reads. However, the short sequence reads make assembly of overlapping sequences problematic [143].

EST projects are likely to become much cheaper in the very near future [144], thanks to next-generation sequencing tools that can sequence large numbers of cDNAs quickly and cheaply. Traditional EST sequencing projects produce sequences of 200–500 bases, where each sequence is anchored at the 3' or 5' end of the mRNA transcript. Using next-generation technology, a form of EST sequencing sometimes referred to as transcriptome sequencing [145] (where each mRNA is sequenced in its entirety in random fragments and assembled computationally) is becoming a more feasible strategy [144] compared to traditional EST sequencing. As of date, published reports of 454 pyrosequencing of transcriptomes are restricted to model species where genomic data or extensive Sanger EST data served as a reference for EST assembly [146-149]. A transcriptome study in wasp using 454 ESTs [150] accomplished annotation by comparisons with the honeybee genome, but did not report EST assembly. Two studies [147, 149] that used the genome or Sanger EST sequences for mapping and annotation of 454 ESTs could not accomplish *de novo* assembly of the 454 EST reads due to the short sequence reads.

The short sequence reads coming from next generation sequencing tools require shorter overlap criteria to be used in the assembly, and this increases the chance of chimeric assemblies. Assembling smaller groups is computationally tractable whereas lack of computational resources can be a problem with huge short read datasets. In this

scenario, the TAMUClust clustering method of using a protein framework is going to be even more beneficial in assembling short reads and reducing formation of chimeric assemblies.

A brief on the work in Chapter III and Chapter IV

Chapter III discusses the design and implementation of the Livestock Gene Family Database – this database has function predictions for bovine and porcine ESTs that were clustered and assembled using the TAMUClust pipeline using Ensembl vertebrate protein families as a framework. The resulting EST consensus sequences are assigned the function of the 'best match' Ensembl vertebrate protein following FASTX [15] searches.

Chapter IV discusses the design and implementation of a phylogenetic annotation pipeline to predict function for the bovine and porcine ESTs.

CHAPTER III

COMPUTATIONAL FUNCTION PREDICTIONS OF THE *Bos taurus* AND *Sus scrofa* TRANSCRIPTOMES USING THE BEST MATCH APPROACH

SYNOPSIS

Bos taurus (cattle) and *Sus scrofa* (pig) are important livestock species that occupy a large proportion of the meat industry and also serve as good candidate animal models for biomedical research due to parallels with humans. The genome sequences for these species are in far from being complete and hence, Expressed Sequence Tag (EST) datasets were used to determine what genes are there and what roles they play. Cattle and pig ESTs were first clustered into gene families using the vertebrate proteomes in the Ensembl database as a framework, and the resulting EST consensus sequences were electronically annotated with the function of the 'best match' Ensembl vertebrate protein. These annotations are housed in the 'Livestock EST Gene Family Database' (http://genomes.arc.georgetown.edu/cgi-bin/search_livestock_est_gene_family.cgi). The database currently hosts annotations for 46,731 bovine EST consensus sequences and 39,641 porcine EST consensus sequences. The database has a user-friendly web interface and can be searched in a number of ways: Cattle/Pig EST GenBank identifiers (GI or Accession), Gene Ontology Accession to obtain information about the vertebrate protein family, related EST members and other annotations. These annotations would guide the livestock community in helping narrow down the gamut of direct experiments needed to verify function.

BACKGROUND

Utility of cattle and pigs in biomedical research

Bos taurus (cattle) and *Sus scrofa* (pig) are important livestock species that not only occupy a large proportion of the meat industry, but also serve as candidate animal models for biomedical research. *Bos taurus* and *Sus scrofa* represent evolutionary clades distinct from the primates and the rodents, and serve as important model organisms for health research due to parallels with humans [151, 152]. The cattle genome is useful in comparative genomics for studies that benefit human health [151] in the areas of obesity, reproductive biology, lactation and infectious diseases. It is also useful in studies of endocrinology, physiology and reproductive techniques [151]. Similarities between humans and pigs exist in digestive physiology, renal function, vascular structure, and respiratory rates [152]. Pigs are also used as model organism in many areas of medical research including obesity, cardiovascular disease, endocrinology, alcoholism, diabetes, nephropathy, and organ transplantation [152]. The pig also serves as a possible candidate animal model for biomedical research addressing regenerative medicine or preclinical investigations in pharmacology [153]. The importance of cattle and pigs in agriculture and medicine demands that we have a sound knowledge of the molecular biology of the species and the functions they encode as represented by their genome sequences and gene expression data.

Connecting sequence to biology – function predictions using EST datasets

Genome research requires developing resources like linkage maps, physical maps, and bacterial artificial chromosome (BAC) libraries to organize vast amounts of genetic information that can be easily accessed and characterized [154]. The availability of a high-quality annotated genome sequence is an invaluable tool for the biologist as it bridges the gap from the sequence to the biology of the organism [155], where the aim of high-quality annotation is to identify the key features of the genome – in particular, the genes and their products. The scientific community is becoming increasingly reliant on the tools and resources of annotation to derive information for most aspects of biological research [155].

Most eukaryotic organisms do not have a complete genome sequence available; instead, what is available is a large collection of expressed sequence tags (ESTs) obtained by random sequencing of cDNA copies of cell mRNA sequences. ESTs are short (200 to 500 bases), unedited single-pass sequence reads of cDNA clones, and provide a high-throughput means to sample an organism's transcriptome [8, 98] and identify expressed genes. EST projects provide a wealth of genetic information for a species as they often shorten the laborious process of gene isolation and also provide the raw material for expression profiling utilizing microarrays based on the transcript sequences [154]. One can use EST datasets to identify genes and obtain insights about the functions they encode by comparing the sequence against other sequences in a database, identifying a sequence whose similarity is statistically significant, and transferring the annotation of known (or presumed) function of the database hit to the

query sequence. This strategy of annotation transfer helps connect the ESTs to the 'putative' functions they encode. A common strategy for detecting homologs and obtaining function annotations for EST datasets is to search well-annotated proteomes using BLASTX/FASTX (translated DNA query against a protein database), obtain information about the homolog with the "best hit" and to transfer the annotations of the "best hit" match to the EST sequence [104-106].

Describing biological knowledge using structured annotation vocabularies

Extracting relevant biological information about genes and gene products is often confounded by the lack or incompleteness of annotations. In such a scenario, the controlled (or structured) vocabularies describing domains of molecular biology developed by the Gene Ontology (GO) Consortium [156, 157] can be used to describe gene products in any organism. These ontologies are expert-curated and describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner. Each term can have one or more relationships with other terms, reflecting the complexity of the underlying biology. Formally, the structure and the format [158] of GO is a directed acyclic graph (DAG) [159] wherein the terms are equivalent to nodes of the graph and the relationships are equivalent to edges.

As part of a controlled vocabulary, the different terms in GO have precise definitions and precise relationships to other terms. GO represents the different vocabulary terms in a hierarchically structured format and the vocabulary terms are

linked to each other by "is a" and "part of" relationships, thus ensuring that very general terms as well as very precise terms are both represented. Doing so results in a set of parent-child relationships between different terms, where a child (more specialized term) is a subset of a parent's (less specialized term) elements. Given this hierarchical nature of the GO, all child terms inherit the properties of their parents. This feature comes in handy to profile function at a coarser (parent) level and incorporating the function annotations at the finer (children) level by navigating the GO hierarchy.

Overview of the work in this chapter

This chapter is a case study that utilizes EST datasets and addresses the issue of obtaining insights of the function encoded in the *Bos taurus* and the *Sus scrofa* transcriptomes using computational predictions. The pipeline developed adopts a comparative genomics approach involving database similarity searches of the *Bos taurus* and the *Sus scrofa* EST datasets against well-annotated vertebrate proteomes, and the transfer of function annotations of the database sequence whose similarity was statistically significant. The *Bos taurus* genome is in the final draft assembly (7.1X coverage) [10, 11]; a 6X coverage of the *Sus scrofa* has been proposed [12] with the current assembly (as of Jun 2008) being available with a 3 fold coverage.

Part I of this chapter discusses the design and implementation of the annotation pipeline of "The Livestock Gene Family Database" consisting of cattle and pig ESTs. These ESTs are grouped into gene families using the protein families obtained from an in-house clustering algorithm (described in Chapter II) following the clustering of the

different vertebrate proteomes in the Ensembl [9] database. The resulting EST gene families were electronically annotated using Gene Ontology terms (GO) of the 'best match Ensembl vertebrate protein' following FASTX [15] searches using a BLOSUM62 [160] matrix and a E-value cutoff ≤ 1E-3. Methods described in Chapter II were used to select EST consensus sequences leading to the design of 70-mer oligonucleotide probes. These probes were used to profile the expression of the cattle and pig genomes as part of the Bovine Oligo Microarray Consortium [161] and the Swine Protein-Annotated Oligonucleotide Microarray [162] projects, respectively. Methods described in this chapter ('Best Hit' approach) were used to annotate the oligos.

Part II of this chapter discusses the findings of a Gene Ontology function categorization on the cattle and pig transcriptomes performed using the generic GO Slim [163]. In this analysis, the distribution of the genes in each of the main ontology categories was examined and the percentages of the unique sequences in each of the assigned GO terms from the GO Slim were computed. This GO Slim analysis takes into consideration the directed acyclic graph (DAG) model of the GO schema and in doing so, it accounts for the fact that all child terms of a particular GO accession inherit the property of the parent.

MATERIALS AND METHODS

Clustering of cattle and pig ESTs

ESTs were clustered into gene families using the TAMUClust algorithm as described in Chapter II. This clustering algorithm is a two-step process and uses the

vertebrate proteomes in the Ensembl database as a framework to assemble and translate the ESTs. ESTs were downloaded from dbEST [101] in March 2005 and were subject to cleaning and trimming as described in Chapter II to obtain 'high quality' ESTs. This resulted in 308,132 high quality ESTs from *Bos taurus* and 238,691 high quality ESTs from *Sus scrofa*. Ensembl vertebrate proteins were downloaded from the Ensembl site in April 2005. The different Ensembl vertebrate proteome datasets used were: *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*, *Takifugu rubripes* and *Tetraodon nigroviridis*.

In the first step of the clustering process, vertebrate proteomes are grouped into protein families using a combination of single and average linkage clustering. This resulted in 10,092 clusters of protein families from a total of 219,433 proteins. In the second step of the clustering process, The Gene Indices Clustering tools or TGICL [136] was used to cluster and assemble the ESTs using the vertebrate protein families served as a framework. This resulted in EST consensus sequence (EST gene families) as described in Chapter II. In this process, 308,132 bovine ESTs were grouped into 46,731 EST consensus sequences (24,665 contigs and 22,066 singletons); and the 238,691 porcine ESTs were grouped into 39,641 EST consensus sequences (20,951 contigs and 18,690 singletons).

The EST contigs/singletons generated follow this naming convention: 'Integer1_CLInteger2ContigInteger3', where Integer1 is the Protein Family ID assigned by the protein clustering algorithm, CLInteger2 refers to a particular Cluster ID (assigned by Megablast from the TGICL package) and ContigInteger3 refers to the

Contig ID (assigned by CAP3 from the TGICL package) for that cluster. As an example, for the EST Contig 6144_CL1Contig2, the integer 6144 denotes the Protein Family ID, CL1 represents the first EST Megablast cluster matching the Protein Family 6144 and Contig2 represents the second Contig generated by CAP3 assembly of the different ESTs belonging to ProteinFamily6144_Cluster1. Similarly, the singletons have this naming convention: 'Integer1_GI' where Integer1 is the Protein Family ID assigned by the protein clustering algorithm and GI is the GenBank GenInfo Identifier for the particular EST.

Design of the 'GBrowse EST Assembly Viewers'

EST clustering results in contigs and singletons. The contig is a consensus sequence of several ESTs. The contig files for bovine and porcine ESTs generated by TAMUClust were parsed using Perl to obtain the different EST Contig alignment files. Each file consists of the EST Contig and the alignment of the constituent ESTs to the Contig. Further Perl processing was done to extract information underlying in these EST Contig alignment files to generate gff3 [135] and fasta files required for the design of the GBrowse [164] interface. The information pertaining to the EST Contig alignments for the *Bos taurus* and the *Sus scrofa* ESTs used in this study are accessible over the web:

- "The Cattle EST Assembly Viewer" is available from http://genomes.arc.georgetown.edu/cgi-bin/gbrowse/cattle_ests_test/
- The Pig EST Assembly Viewer" is available from http://genomes.arc.georgetown.edu/cgi-bin/gbrowse/pig_ests/

Annotation pipeline for the Livestock EST gene families

The steps outlined below describe the annotation pipeline developed for annotating the cattle and porcine EST gene families obtained by clustering ESTs using the TAMUClust clustering pipeline (described in more detail in Chapter II).

1. Determining the "best match" Ensembl vertebrate protein for an EST Contig/Singleton: The Ensembl vertebrate proteins were filtered for low-complexity regions using the PSEG [165, 166] tool. The "best match" Ensembl vertebrate protein for a livestock EST Contig/Singleton was determined within a single species following a FASTX [15] search against the different Ensembl vertebrate proteomes using the BLOSUM62 scoring matrix and E-value cutoff $\leq 10^{-3}$. Following the FASTX searches, the "best match" Ensembl vertebrate protein for an EST Contig/Singleton within a proteome was determined from amongst the various proteomes using this order of precedence: *Homo sapiens, Mus musculus, Rattus norvegicus, Gallus gallus, Danio rerio, Takifugu rubripes* and *Tetraodon nigroviridis*. For example, if a livestock EST Contig/Singleton does not have a match in *Homo sapiens*, the best match is searched in the next species using the precedence order mentioned above till a best match in a species is found. This order of precedence used here is based on the evolutionary distance relative to bovine/porcine.
2. Determining the "best match" Ensembl vertebrate protein having a Gene Ontology (GO) Annotation: The Gene Ontology cross-references and other database (NCBI, UniProt) protein cross-references for the Ensembl vertebrate

proteins were obtained from BioMart [167]. Information pertaining to the Gene Ontologies was obtained from the GO CVS repository at the Gene Ontology Consortium [156]. The above mentioned pipeline is used to determine the "best match" Ensembl vertebrate protein for a particular EST Contig/Singleton. If the "best match" Ensembl vertebrate protein does not have a GO Annotation, then the best match Ensembl protein (with GO annotation) is determined from the next species using the order of precedence mentioned above.

Design and implementation of the 'Livestock EST Gene Family Database'

The electronic annotations for the cattle and pig EST gene families can be accessed over the web from "The Livestock EST Gene Family Database" available at: http://genomes.arc.georgetown.edu/cgi-bin/search_livestock_est_gene_family.cgi. The database web server was setup using a LAMPerl (Linux, Apache, MySQL, Perl CGI) environment. The Livestock EST Gene Family Database has two underlying components representing the species-specific webpages:

- the Cattle EST Gene Family Database available at http://genomes.arc.georgetown.edu/cgi-bin/search_cattle_est_gene_family.cgi
- the Pig EST Gene Family Database available at http://genomes.arc.georgetown.edu/cgi-bin/search_porcine_est_gene_family.cgi

'GO Slim' analysis

GO Slim [163] is a list of high level GO terms covering all three GO categories and the GO Slim terms provide biological meaning at a coarser level of resolution. The hierarchical classification of GO was utilized to obtain a broader function analysis of GO mapped annotations. To incorporate and encompass the fine-level (child level) gene annotations to arbitrary coarser levels (parent) found in the GO Slims, we first determined recursively all the descendants (parent-child relationships) for a given GO Slim term. In the next step, the complete descendant information of a given GO Slim term was used to map all the descendants associated with the GO Slim term to their respective gene products from the Livestock EST Gene Families. The steps detailed below outline the pipeline used in the GO Slim analysis of the Livestock EST Gene Families:

1. The "go-term-db" tables were obtained from the Gene Ontology Archive [168]; these tables contain information on the GO terms and relationships. The "go-term-db" tables were loaded into a local MySQL database (say DB1). A different MySQL database (say DB2) housed the information pertaining to the cattle/porcine EST contigs and singletons mapped to their respective "best match" Ensembl vertebrate proteins and GO Annotations associated with the Ensembl protein.
2. A function analysis using Gene Ontology (GO) categories was performed on the cattle and porcine EST gene families using the GO Slim [163] developed and maintained by the Gene Ontology Consortium [156]. The generic GO Slim [169]

was obtained and was parsed using Perl to obtain a tab-delimited file containing the GO ID and GO terms. For every term in the GO Slim, Perl-DBI scripts were used to query the "go-term-db" tables in DB1 and obtain the complete descendant information.

3. Once the complete descendant information for a particular GO Slim term is obtained, Perl-DBI scripts were used to query DB2 for each of the descendants and to obtain the cattle/porcine EST contigs and singletons mapped to it.

RESULTS AND DISCUSSION

The first part of this section describes the utility of the "Livestock EST Gene Family Database". The different search tools allow the user to search by any attribute of a sequence (cattle/pig EST GI or GenBank accession), sequence cluster (cattle/pig EST Contig or Protein Family ID), and Gene Ontology (GO) accessions. The Livestock EST Gene Family Database (**Figure 3.1**) has two underlying components representing the species-specific webpages:

1. The Cattle EST Gene Family Database (**Figure 3.2**) available at: http://genomes.arc.georgetown.edu/cgi-bin/search_cattle_est_gene_family.cgi
2. Pig EST Gene Family Database (**Figure 3.3**) available at: http://genomes.arc.georgetown.edu/cgi-bin/search_porcine_est_gene_family.cgi

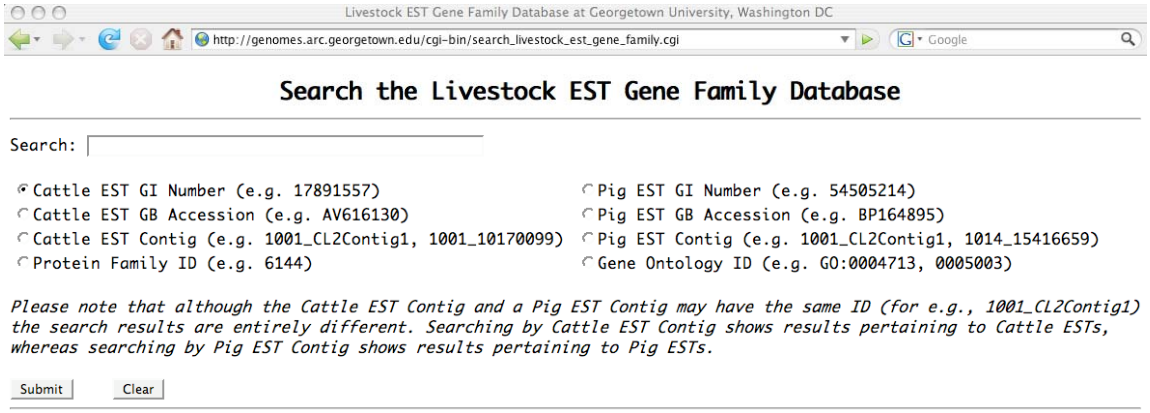


Figure 3.1: Home page of the Livestock EST Gene Family Database.

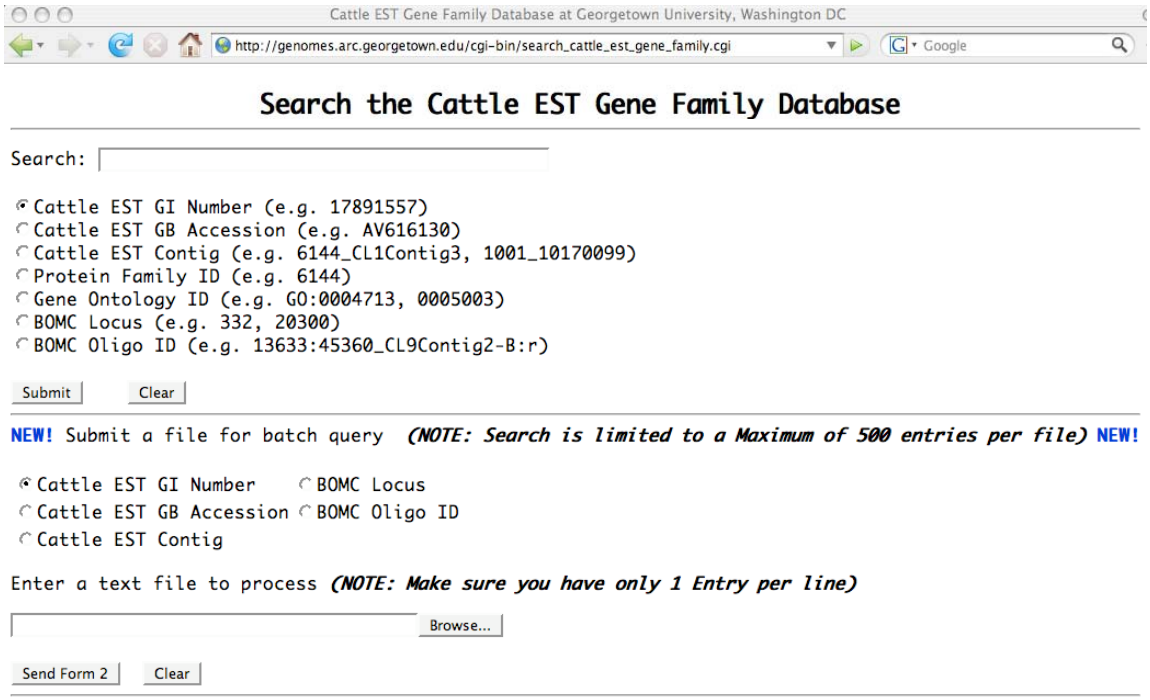


Figure 3.2: Home page of the Cattle EST Gene Family Database.

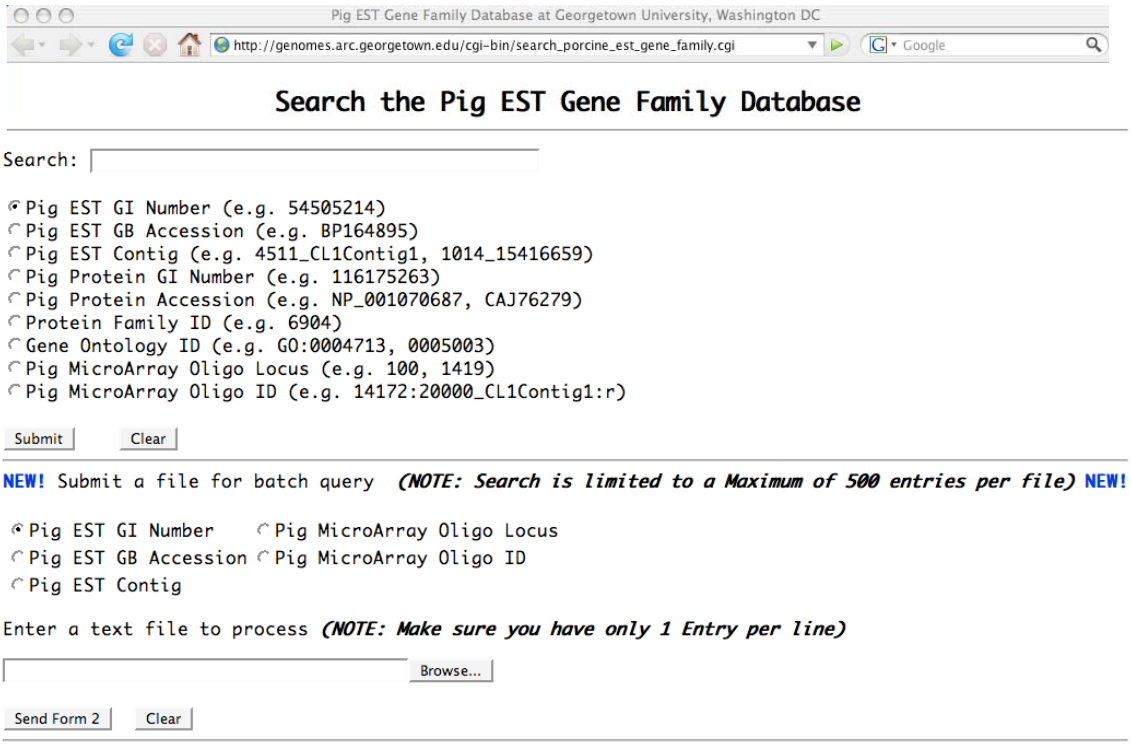


Figure 3.3: Home page of the Pig EST Gene Family Database.

The Livestock EST Gene Family Database provides a user-friendly interface for searching, browsing and retrieval of information. One can search the database in different ways as detailed below.

Search by cattle/pig GenBank identifiers (GI or Accession)

The search results page for this search would indicate if the EST is part of a EST Contig or a Singleton, and for EST Contigs, a GBrowse [164] link depicting the alignment of the different ESTs to the EST Contig is provided. The search results page also has information regarding the 'best match Ensembl vertebrate protein' with/without GO Annotations for the EST Contig/Singleton, and the GO and the NCBI and UniProt protein cross-references for the Ensembl protein. The results page also provides information about other EST contigs and singletons constituting the protein family associated with the EST searched. **Figure 3.4** is a screenshot showing the search results from the "Livestock EST Gene Family Database" upon searching for the *Bos taurus* EST GenBank GI 12123209.

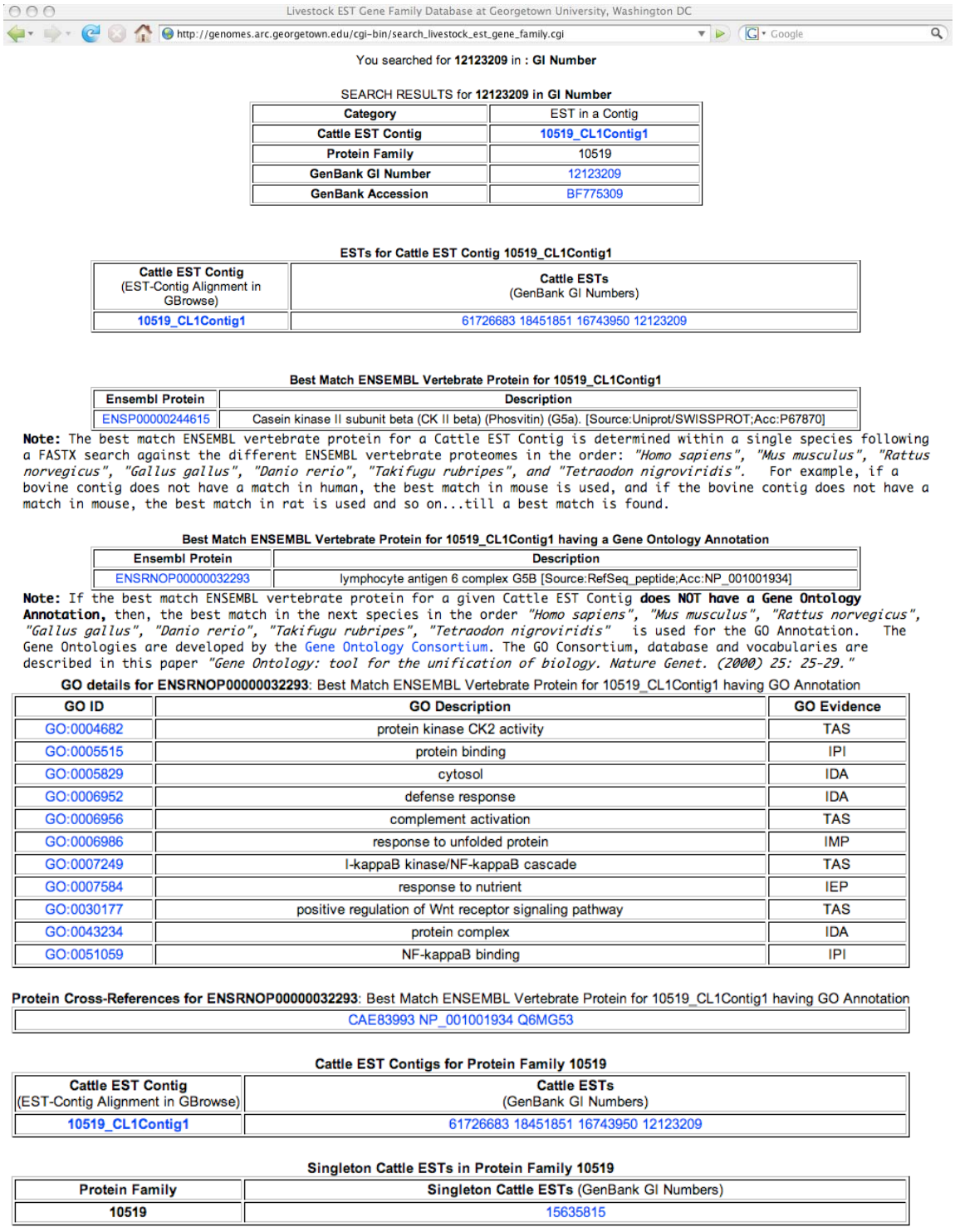


Figure 3.4: Search results for the Cattle EST GenBank GI 12123209 from the Livestock EST Gene Family Database.

Search by cattle/pig 'EST Contig or Singleton'

If a user has prior information about a specific EST Contig or Singleton and is interested in getting further details about the same, one can search the "Livestock EST Gene Family Database" for the same. The search results would detail the annotations, database cross-references, protein family details, and in case of EST contigs a GBrowse [164] link is provided. The EST contigs follow this naming convention: 'Integer1_CLInteger2ContigInteger3', where Integer1 is the Protein Family ID assigned by the protein clustering algorithm, CLInteger2 refers to a particular Cluster ID (assigned by Megablast from the TGICL package) and ContigInteger3 refers to the Contig ID (assigned by CAP3 from the TGICL package) for that cluster. In case of singletons, the names are of the type 'Integer1_GINumber', where Integer1 is the Protein Family ID assigned by the protein clustering algorithm, and GI Number is the GenBank GeneInfo Number for that EST. **Figure 3.5** is a screenshot showing the search results from the "Livestock EST Gene Family Database" upon searching for the *Sus scrofa* EST Contig 10_CL4Contig1.

You searched for **10_CL4CONTIG1** in : **Pig EST Contig**

ESTs for Pig EST Contig 10_CL4Contig1

Pig EST Contig (EST-Contig Alignment in GBrowse)	Pig ESTs (GenBank GI Numbers)
10_CL4Contig1	37798027 37796664

Best Match ENSEMBL Vertebrate Protein for 10_CL4Contig1

Ensembl Protein	Description
ENSP00000297267	Fibronectin type III domain-containing protein 1 (Expressed in synovial lining protein) (Activation-associated cDNA protein). [Source:Uniprot/SWISSPROT;Acc:Q4ZHG4]

Note: The best match ENSEMBL vertebrate protein for a Pig EST Contig is determined within a single species following a FASTX search against the different ENSEMBL vertebrate proteomes in the order: *"Homo sapiens", "Mus musculus", "Rattus norvegicus", "Gallus gallus", "Danio rerio", "Takifugu rubripes", and "Tetraodon nigroviridis".* For example, if a Pig EST Contig does not have a match in human, the best match in mouse is used, and if the Pig EST Contig does not have a match in mouse, the best match in rat is used and so on...till a best match is found.

Best Match ENSEMBL Vertebrate Protein for 10_CL4Contig1 having a Gene Ontology Annotation

Ensembl Protein	Description
ENSMUSP00000063656	neogenin [Source:MGI;Acc:MGI:1097159]

Note: If the best match ENSEMBL vertebrate protein for a given Pig EST Contig **does NOT have a Gene Ontology Annotation,** then, the best match in the next species in the order *"Homo sapiens", "Mus musculus", "Rattus norvegicus", "Gallus gallus", "Danio rerio", "Takifugu rubripes", "Tetraodon nigroviridis"* is used for the GO Annotation. The Gene Ontologies are developed by the Gene Ontology Consortium. The GO Consortium, database and vocabularies are described in this paper *"Gene Ontology: tool for the unification of biology. Nature Genet. (2000) 25: 25-29."*

GO details for ENSMUSP00000063656: Best Match ENSEMBL Vertebrate Protein for 10_CL4Contig1 having GO Annotation

GO ID	GO Description	GO Evidence
GO:0004872	receptor activity	IPI
GO:0005021	vascular endothelial growth factor receptor activity	IEA
GO:0005515	protein binding	IPI
GO:0005524	ATP binding	IEA
GO:0006468	protein amino acid phosphorylation	IEA
GO:0007155	cell adhesion	IEA
GO:0007520	myoblast fusion	IMP
GO:0016020	membrane	IEA
GO:0016021	integral to membrane	IEA
GO:0030528	transcription regulator activity	IDA
GO:0045296	cadherin binding	IDA
GO:0045449	regulation of transcription	IDA

Protein Cross-References for ENSMUSP00000063656: Best Match ENSEMBL Vertebrate Protein for 10_CL4Contig1 having GO Annotation

BAC34991 CAA70727 NP_032710 P97798 Q8C766

Pig EST Contigs for Protein Family 10

Pig EST Contig (EST-Contig Alignment in GBrowse)	Pig ESTs (GenBank GI Numbers)
10_CL1Contig1	18535436 49416043 15031520 41140985 34166707 49412491 41138351
10_CL2Contig1	15414813 15415410 72285009
10_CL3Contig1	74373326 8326165 12010778
10_CL4Contig1	37798027 37796664

Singleton Pig ESTs in Protein Family 10

Protein Family	Singleton Pig ESTs (GenBank GI Numbers)
10	15414730 46176656 59779619 8328290

Figure 3.5: Search results for the Pig EST Contig 10_CL4Contig1 from the Livestock EST Gene Family Database.

Search by 'Protein Family ID'

Searching by Protein Family ID would detail the different members of the *Bos taurus* and *Sus scrofa* EST gene families, and the different Ensembl vertebrate proteins belonging to that protein family. Links for the individual annotations for the different members of the *Bos taurus* and *Sus scrofa* EST gene families, and information pertaining to the different vertebrate proteins constituting the protein family are also provided. It is to be noted that the 'Protein Family ID' is a number internal to this database and denotes the ID assigned by the protein clustering algorithm.

The salient feature of the "Protein Family" search is that one can get a listing of ESTs belonging to the same gene/protein family and represented in two different species (cattle and pig). This information is particularly useful in situations where a researcher, say from the pig community, is interested in the putative functions of a specific pig EST and would like to know the ESTs in cattle which perform a similar function. To obtain this information, the researcher would first search the "Livestock EST Gene Family Database" using the pig EST GenBank accession as a query, obtain the putative function annotations for that pig EST and note the "Protein Family ID" to which the pig EST belongs. In the second step, the researcher would search the "Livestock EST Gene Family Database" using the "Protein Family ID" as the query and obtain information about different cattle and pig ESTs in that protein family. **Figure 3.6** is a screenshot of the search results from the "Livestock EST Gene Family Database" listing the *Bos taurus* and *Sus scrofa* EST gene families, and the different Ensembl vertebrate proteins upon searching for the Protein Family 15939.

You searched for **15939** in : **Protein Family ID**

Cattle EST Contigs for Protein Family 15939 (Note: The **'Bt'** suffix on the Cattle EST Contig stands for *Bos taurus*).

Cattle EST Contig (Click to view Annotation details)	Cattle ESTs (GenBank GI Numbers)
15939_CL1Contig1_Bt	49145592 6988161 17894429 45064528
15939_CL2Contig1_Bt	7425169 6989448

Singleton Cattle ESTs in Protein Family 15939 (Note: The **'Bt'** suffix on the Cattle EST Singleton stands for *Bos taurus*).

Cattle EST Singleton (Click to view Annotation Details)	Singleton Cattle ESTs (GenBank GI Numbers)
15939_10022991_Bt	10022991
15939_49427687_Bt	49427687

Pig EST Contigs for Protein Family 15939 (Note: The **'Ss'** suffix on the Pig EST Contig stands for *Sus scrofa*).

Pig EST Contig (Click to view Annotation details)	Pig ESTs (GenBank GI Numbers)
15939_CL1Contig1_Ss	15039394 48721259 15036614

Singleton Pig ESTs in Protein Family 15939 (Note: The **'Ss'** suffix on the Pig EST Singleton stands for *Sus scrofa*).

Pig EST Singleton (Click to view Annotation Details)	Singleton Pig ESTs (GenBank GI Numbers)
15939_11073023_Ss	11073023
15939_40429937_Ss	40429937
15939_49353420_Ss	49353420

Protein Family Members in Protein Family 15939

Vertebrate ENSEMBL Protein	ENSEMBL Protein Description	Gene Ontology Details	Other Database Cross-References
ENSGALP00000004899	NO Description Found	NO Gene Ontology Details Found	NO Cross-References Found
ENSMUSP00000043555	Telomerase-binding protein EST1A (Ever shorter telomeres 1A) (Telomerase subunit EST1A) (EST1-like protein A). [Source:Uniprot/SWISSPROT;Acc:P61406]	GO:0000781 'chromosome, telomeric region' IEA; GO:0003677 'DNA binding' IEA; GO:0005634 'nucleus' IEA; GO:0007004 'telomerase-dependent telomere maintenance' IEA;	AAH66040.1 BAC98013.1 P61406
ENSMUSP00000052795	Telomerase-binding protein EST1A (Ever shorter telomeres 1A) (Telomerase subunit EST1A) (EST1-like protein A). [Source:Uniprot/SWISSPROT;Acc:P61406]	NO Gene Ontology Details Found	AAH06644.1 Q923C2
ENSP00000263073	Telomerase-binding protein EST1A (Ever shorter telomeres 1A) (Telomerase subunit EST1A) (EST1-like protein A). [Source:Uniprot/SWISSPROT;Acc:Q86US8]	GO:0000781 'chromosome, telomeric region' IEA; GO:0003677 'DNA binding' IEA; GO:0005634 'nucleus' IEA; GO:0007004 'telomerase-dependent telomere maintenance' IEA;	AAH64916.1 AAN46114.1 AAO17581.1 BAA34452.2 BAB14835.1 CAB63733.1 NP_060045.3 NP_079299.1 Q86US8 Q9H7Y8
ENSRNOP00000004058	Rattus norvegicus similar to telomerase subunit EST1A (LOC287522), mRNA. [Source:RefSeq_dna;Acc:XM_220702]	NO Gene Ontology Details Found	NO Cross-References Found
ENSRNOP00000042551	Rattus norvegicus similar to telomerase subunit EST1A (LOC287522), mRNA. [Source:RefSeq_dna;Acc:XM_220702]	NO Gene Ontology Details Found	AAQ96253.1 Q6TXF9
SINFRUP00000130266	NO Description Found	NO Gene Ontology Details Found	NO Cross-References Found

Figure 3.6: Search results for Protein Family ID 15939 from the Livestock EST Gene Family Database.

Search by 'Gene Ontology Accession'

This search will find all gene products (EST Contig/Singletons in this case) directly annotated to the GO accession as well as other gene products transitively annotated. To elaborate, this search functionality above takes into consideration the directed acyclic graph (DAG) model and the hierarchical classification of ontologies in the GO schema where a child (more specialized term) is a subset of a parent's (less specialized term) elements. For example, if gene product GP is annotated to GO:X, and GO:X is beneath GO:Y, then GP is transitively annotated to GO:Y. Therefore, all child terms of a particular GO accession inherit the properties of the parent. Searching by GO accession on this database will retrieve its complete descendant information and the mapping of the different EST contigs/singletons associated with the complete GO descendant tree starting from that GO Accession. This search feature will come in handy for users who want to get a complete profile of gene products associated with a particular GO Accession. As an example, lets say a researcher was interested in finding cattle and pig EST gene products annotated to the GO accession "GO:0006110 – regulation of glycolysis". GO:0006110 has two child terms; GO:0045821 – positive regulation of glycolysis and GO:0045820 – negative regulation of glycolysis. Given the hierarchical organization of GO, the child terms GO:0045820 and GO:0045821 inherit the properties of the parent GO:0006110. A search for GO:0006110 at the "Livestock EST Gene Family Database" would first identify its complete descendant tree and then map the different cattle and pig EST gene products to all of the GO accessions in the descendant tree. Doing it this way, the researcher can get a snapshot of all the cattle and

pig gene products associated with that property (in this case, regulation of glycolysis). Instead, if one were to do a one-to-one mapping of all the EST gene products associated only with the search term GO:0006110 and not its descendants, one would end up obtaining only the coarse level annotation (parent level) and therefore, miss out on the more specific fine-level (child level) annotations. The AmiGO [170] database incorporates a search strategy similar to the one described here. AmiGO however has gene product annotations from model organisms whose complete genome sequences are known and AmiGO does not house GO annotations for EST gene products. To the best of our knowledge, the "Livestock EST Gene Family Database" is the only database of its kind to have an AmiGO like GO term descendant mapping information catered for EST gene products – cattle and pig ESTs in this case. **Figure 3.7** is a screenshot of the search results from the "Livestock EST Gene Family Database" upon searching for the Gene Ontology accession GO:0030295 – protein kinase activator activity.

You searched for **0030295** in : **Gene Ontology ID**

View details for **GO:0030295** at the EBI QuickGO site

Shown here is the complete descendant tree (children, grand-children, and so on) for GO:0030295

GO:0030295 :: protein kinase activator activity
| GO:0043539 :: protein serine/threonine kinase activator activity
| GO:0042557 :: eukaryotic elongation factor-2 kinase activator activity
| GO:0030296 :: protein tyrosine kinase activator activity
| | GO:0030298 :: receptor signaling protein tyrosine kinase activator activity
| | GO:0030297 :: transmembrane receptor protein tyrosine kinase activator activity
| GO:0019912 :: cyclin-dependent protein kinase activating kinase activity
| GO:0016534 :: cyclin-dependent protein kinase 5 activator activity

The different GO IDs from the descendant tree of GO:0030295 shown above were found to be associated with these Cattle EST Contigs. **Click on the Cattle EST Contig** to get more information on the ESTs and other details. The **'Bt'** suffix on the Cattle EST Contig stands for *Bos taurus*.
Note: *If a particular GO (from above) is not found in the Cattle EST Gene Family Database, then the same would be* ***missing*** *from the table below.*

Cattle EST Contig	GO List
15543_CL1Contig1_Bt	**GO:0043539** :: *protein serine/threonine kinase activator activity* **GO:0016534** :: *cyclin-dependent protein kinase 5 activator activity*
23222_CL1Contig1_Bt	**GO:0030296** :: *protein tyrosine kinase activator activity*
45360_51807642_Bt	**GO:0030297** :: *transmembrane receptor protein tyrosine kinase activator activity*
45360_CL766Contig1_Bt	**GO:0030297** :: *transmembrane receptor protein tyrosine kinase activator activity*
4643_CL6Contig1_Bt	**GO:0030296** :: *protein tyrosine kinase activator activity*
851_45489909_Bt	**GO:0030295** :: *protein kinase activator activity*
851_CL2Contig1_Bt	**GO:0030295** :: *protein kinase activator activity*

The different GO IDs from the descendant tree of GO:0030295 shown above were found to be associated with these Pig EST Contigs. **Click on the Pig EST Contig** to get more information on the ESTs and other details. The **'Ss'** suffix on the Pig EST Contig stands for *Sus Scrofa*.
Note: *If a particular GO (from above) is not found in the Pig EST Gene Family Database, then the same would be* ***missing*** *from the table below.*

Pig EST Contig	GO List
15543_37855376_Ss	**GO:0016534** :: *cyclin-dependent protein kinase 5 activator activity*
25189_CL2Contig1_Ss	**GO:0030295** :: *protein kinase activator activity*
6810_54527672_Ss	**GO:0030297** :: *transmembrane receptor protein tyrosine kinase activator activity*
851_54532656_Ss	**GO:0030295** :: *protein kinase activator activity*
851_CL2Contig1_Ss	**GO:0030295** :: *protein kinase activator activity*

Figure 3.7: Search results for the Gene Ontology Accession GO:0030295 from the Livestock EST Gene Family Database.

Search by microarray 'Oligo Locus' or 'Oligo ID'

The EST clustering process resulted in a set of Unique Transcripts (UT) which includes EST Contigs and Singletons. Unique Transcripts overlapping the same vertebrate Ensembl protein were identified and these "groups of Unique Transcripts" are in effect "groups of genes". As part of the Bovine Oligo Microarray Consortium [161] and the Swine Protein-Annotated Oligonucleotide Microarray [162] projects, novel 70-mer oligonucleotide probes were designed for profiling gene expression of the cattle and pig genomes with one oligo being designed per gene. The Cattle EST Gene Family Database has information pertaining to the 16,846 oligonucleotide sequences designed for the Bovine Oligo Microarray Consortium [161]; the Pig EST Gene Family Database has information pertaining to the 18,254 oligonucleotide probes designed for the Swine Protein-Annotated Oligonucleotide Microarray [162] project. On the specific EST Gene Family webpages (**Figure 3.2** and **Figure 3.3**), searching for the Microarray Oligo Locus or ID retrieves information about the consensus oligo sequence it represents, the EST Contig or Singleton associated with the oligo sequence, and the annotations and other cross-references associated with EST Contig or Singleton. **Figure 3.8** is a screenshot of the search results from the "Cattle EST Gene Family Database" upon searching for the Bovine Oligo Microarray Consortium (BOMC) Locus 11695. **Figure 3.9** is a screenshot of the search results from the "Pig EST Gene Family Database" upon searching for the Pig MicroArray Oligo ID 10033:7213_CL4Contig1:F.

You searched for **11695** in : **BOMC Locus**

SEARCH RESULTS for **11695 in BOMC Locus**

Cattle EST Contig	3438_CL1Contig1
Oligo Locus	11695
Oligo ID	11695:3438_CL1Contig1-A:f
Oligo Sequence	GTGCAGCGGGAGCAATCCTTCGAGCCAACTCAAGCCAAGTGCCCCCTAATGAATCAAACACAAACAGCGA

Best Match ENSEMBL Vertebrate Protein for 3438_CL1Contig1

Ensembl Protein	Description
ENSP00000305815	Recombining binding protein suppressor of hairless (J kappa- recombination signal-binding protein) (RBP-J kappa) (RBP-J) (RBP-JK) (CBF-1) (Renal carcinoma antigen NY-REN-30). [Source:Uniprot/SWISSPROT;Acc:Q06330]

Note: The best match ENSEMBL vertebrate protein for a Cattle EST Contig is determined within a single species following a FASTX search against the different ENSEMBL vertebrate proteomes in the order: *"Homo sapiens", "Mus musculus", "Rattus norvegicus", "Gallus gallus", "Danio rerio", "Takifugu rubripes", and "Tetraodon nigroviridis".* For example, if a bovine contig does not have a match in human, the best match in mouse is used, and if the bovine contig does not have a match in mouse, the best match in rat is used and so on...till a best match is found.

Best Match ENSEMBL Vertebrate Protein for 3438_CL1Contig1 having a Gene Ontology Annotation

Ensembl Protein	Description
ENSMUSP00000040694	recombination signal binding protein for immunoglobulin kappa J region [Source:MGI;Acc:MGI:96522]

Note: If the best match ENSEMBL vertebrate protein for a given Cattle EST Contig **does NOT have a Gene Ontology Annotation,** then, the best match in the next species in the order *"Homo sapiens", "Mus musculus", "Rattus norvegicus", "Gallus gallus", "Danio rerio", "Takifugu rubripes", "Tetraodon nigroviridis"* is used for the GO Annotation. The Gene Ontologies are developed by the Gene Ontology Consortium. The GO Consortium, database and vocabularies are described in this paper *"Gene Ontology: tool for the unification of biology. Nature Genet. (2000) 25: 25-29."*

GO details for ENSMUSP00000040694: Best Match ENSEMBL Vertebrate Protein for 3438_CL1Contig1 having GO Annotation

GO ID	GO Description	GO Evidence
GO:0001525	angiogenesis	IMP
GO:0001837	epithelial to mesenchymal transition	IMP
GO:0003677	DNA binding	IEA
GO:0003682	chromatin binding	IDA
GO:0003700	transcription factor activity	IEA
GO:0005515	protein binding	IEA
GO:0005634	nucleus	IEA
GO:0006355	regulation of transcription, DNA-dependent	IEA
GO:0006357	regulation of transcription from RNA polymerase II promoter	IGI
GO:0007219	Notch signaling pathway	IMP
GO:0007507	heart development	IMP
GO:0008134	transcription factor binding	IPI
GO:0008284	positive regulation of cell proliferation	IGI
GO:0021983	pituitary gland development	IMP
GO:0030097	hemopoiesis	IMP
GO:0030183	B cell differentiation	IMP
GO:0042742	defense response to bacterium	IMP
GO:0045165	cell fate commitment	IMP
GO:0045449	regulation of transcription	IEA
GO:0045596	negative regulation of cell differentiation	IMP
GO:0048505	regulation of timing of cell differentiation	IMP

Protein Cross-References for ENSMUSP00000040694: Best Match ENSEMBL Vertebrate Protein for 3438_CL1Contig1 having GO Annotation

A0N920 AAA39018 AAA39019 AAA39020 AAA39021 AAA39022 AAA39023 AAB20195 AAH51387 BAC37889 BAE31773 CAA35501 CAB38078 NP_033061 P31266 Q3U6F1

Figure 3.8: Search results for Bovine Oligo Microarray Consortium Locus 11695 from the Cattle EST Gene Family Database.

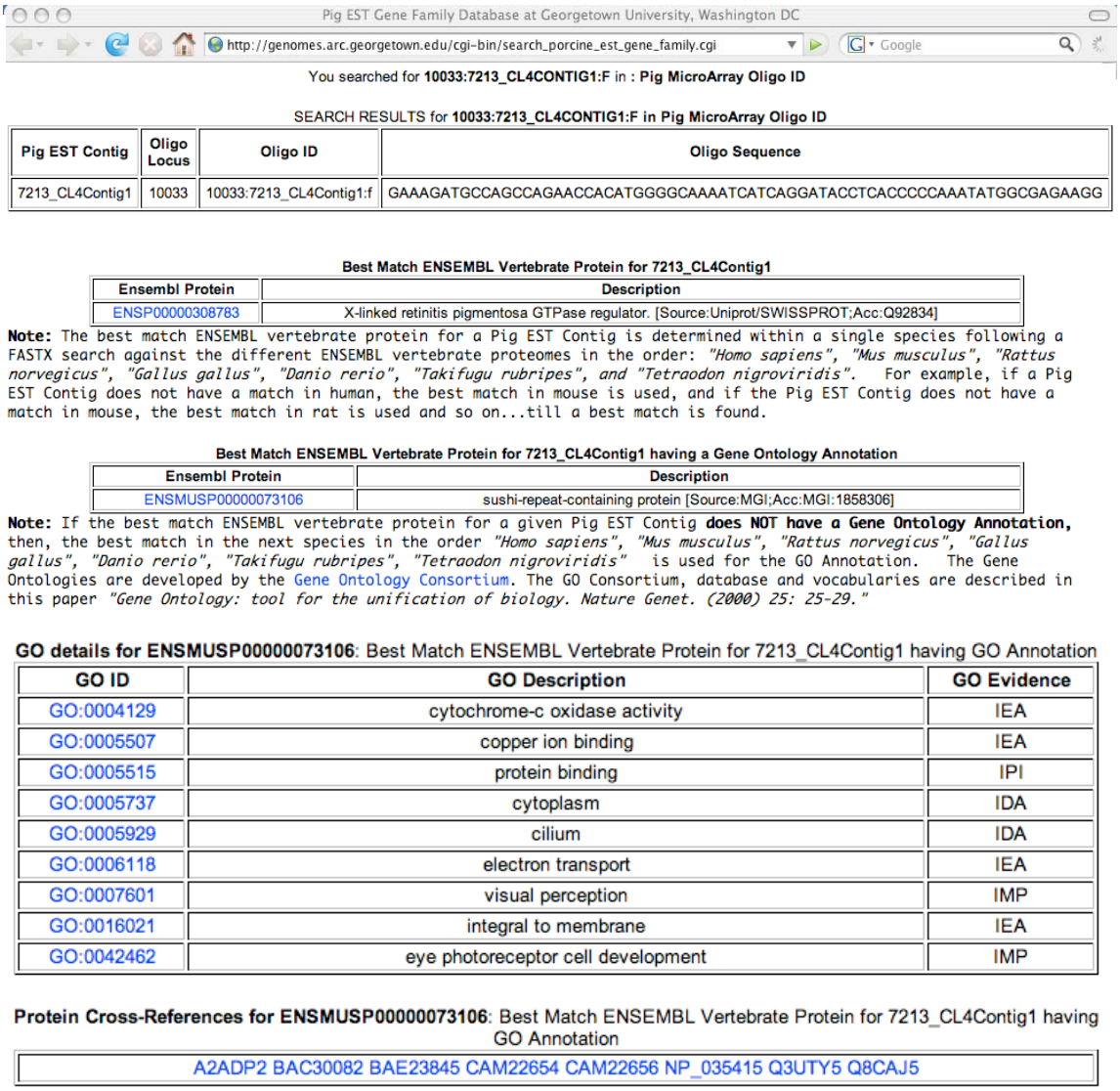


Figure 3.9: Search results for Pig MicroArray Oligo ID 10033:7213_CL4Contig1:F from the Pig EST Gene Family Database.

Cattle/Pig 'EST Assembly Viewers' in GBrowse

Generic Genome Browser (GBrowse) is a web-based application for displaying genomic annotations that uses a combination of database and interactive web pages to manipulate and display sequence annotations. The "Cattle EST Gene Family Database" and the "Pig EST Gene Family Database" have outgoing links to the respective EST Assembly Viewers in GBrowse. The "Cattle EST Assembly Viewer" is available at: http://genomes.arc.georgetown.edu/cgi-bin/gbrowse/cattle_ests_test/ and the "Pig EST Assembly Viewer" is available at: http://genomes.arc.georgetown.edu/cgi-bin/gbrowse/pig_ests/.

The GBrowse back-end comprises a MySQL database housing information about the consensus sequence of EST Contigs, and the alignment coordinates and sequence information of the constituent ESTs. Integration of GBrowse with the main database(s) happens at the level of the web site with link rules to forge outgoing links (e.g., annotation information for the EST contig, GenBank link for the EST). Incoming links to GBrowse from the main database(s) use GBrowse's standard URL calling conventions to specify the region of interest (e.g., EST Contig or coordinates of an EST on a particular EST Contig). **Figure 3.10** is a screenshot showing results from the "GBrowse Cattle EST Assembly Viewer" upon searching for the Cattle EST Contig 4944_CL2Contig2. **Figure 3.11** is a screenshot of the search results from the "GBrowse Pig EST Assembly Viewer" upon searching for the Pig EST having GenBank GI 34172655.

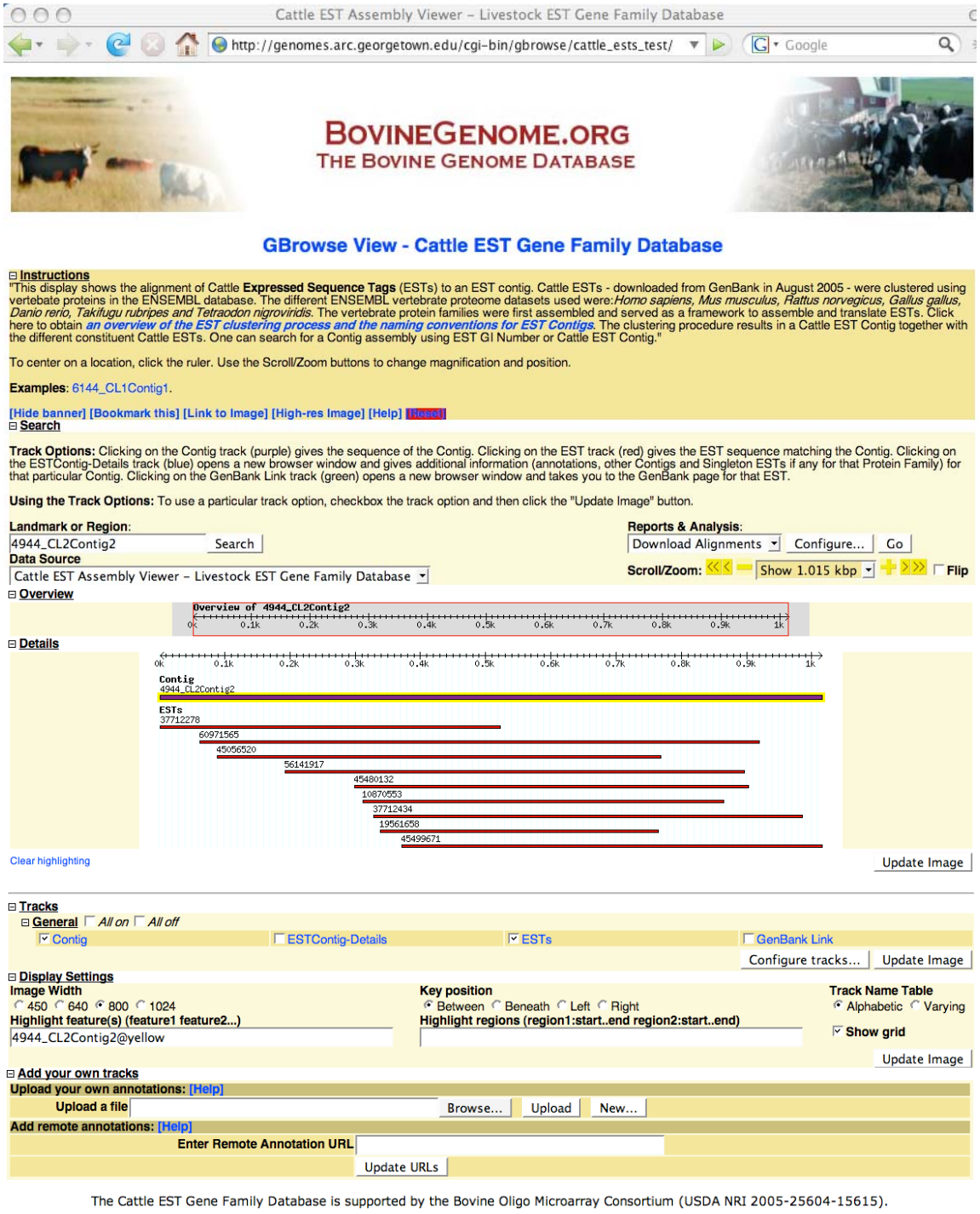


Figure 3.10: Search results for Cattle EST Contig 4944_CL2Contig2 from the Cattle EST Assembly Viewer.

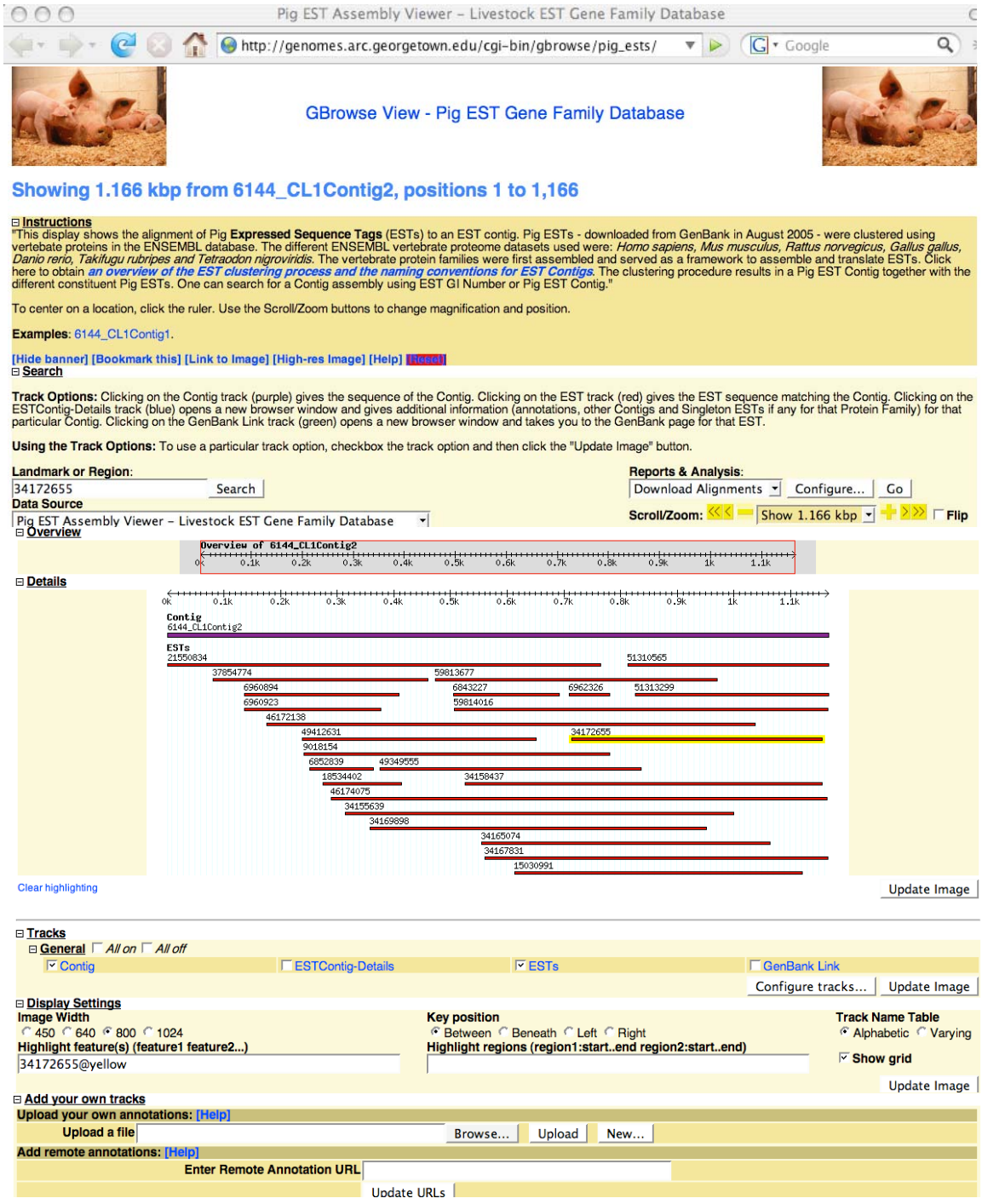


Figure 3.11: Search results for Pig EST GI 34172655 from the Pig EST Assembly Viewer.

‘GO Slim’ analysis: function categorization of the cattle and pig transcriptomes

The significance of this GO Slim analysis lies in the fact that it enables categorization of the function encoded in the cattle and pig transcriptomes within arbitrary coarser (parent) levels of the three independent gene ontologies - biological process, molecular function and cellular component. This categorization helps identify the putative functions of the different EST gene products using the structured vocabularies in the gene ontologies. By conveying biological meaning at a coarser level, it helps overcome any inconsistencies like categorization errors and/or incomplete coverage of certain GO categories.

This analysis was carried out by first determining the ‘best match Ensembl vertebrate protein’ having a Gene Ontology (GO) Annotation for a EST Contig/Singleton using the pipeline described in Materials and Methods; subsequently, these EST Contigs/Singletons via their GO annotations were mapped to the different GO Slim terms in the generic GO Slim [169]. In this analysis, the fine-level (child level) gene annotations are encompassed within arbitrary coarser levels (parent) found in the GO Slims.

By first recursively determining the complete descendant information for a given GO Slim term and then mapping all the descendants associated with that GO Slim term to their respective gene products from the cattle/pig EST Gene Families, the percentages of the unique cattle/pig EST Contig/Singleton sequences for each of the assigned GO terms in the GO Slim were computed. These percentages were normalized based on the total number of gene products having GO annotations; i.e., the number of gene products assigned to a particular GO term was divided by the total number of sequences having GO annotations to obtain percentages of the unique cattle/pig EST Contig/Singleton sequences mapped to every GO Slim term.

Tables 3.1, **3.2** and **3.3** provide a breakdown of the number of *Bos taurus* and *Sus scrofa* consensus sequences associated with the "Biological Process", "Molecular Function" and "Cellular Component" namespaces of Gene Ontology annotations using the terms in the generic GO Slim.

Table 3.1: Biological Process Gene Ontology annotation statistics for cattle and pig EST consensus sequences using the GO Slim terms in the generic GO Slim.

Gene Ontology ID	Gene Ontology Term	Number of *Bos taurus* EST consensus sequences	Number of *Sus scrofa* EST consensus sequences
GO:0007582	Physiological Process	27, 477	23, 315
GO:0008152	Metabolism	19, 374	16, 435
GO:0044238	Primary Metabolism	17, 301	14, 777
GO:0019538	Protein Metabolism	8, 178	7, 014
GO:0006139	Nucleic Acid Metabolism	7, 987	6, 657
GO:0050789	Regulation of Biological Process	8, 015	6, 859
GO:0006810	Transport	7, 075	5, 804
GO:0007154	Cell Communication	6, 089	5, 032
GO:0007165	Signal Transduction	5, 599	4, 659
GO:0006350	Transcription	4, 535	3, 833
GO:0006464	Protein Modification	4, 016	3, 610
GO:0016043	Cell Organization, Biogenesis	3, 985	3, 576
GO:0007275	Development	3, 846	3, 139
GO:0009058	Biosynthesis	3, 593	3, 006
GO:0006950	Stress Response	2, 452	2, 065
GO:0006996	Organelle Organization, Biogenesis	2, 290	2, 051
GO:0009607	Biotic Stimulus Response	2, 289	2, 085
GO:0006412	Protein Biosynthesis	2, 136	1, 667

Table 3.2: Molecular Function Gene Ontology annotation statistics for cattle and pig EST consensus sequences using the GO Slim terms in the generic GO Slim.

Gene Ontology ID	Gene Ontology Term	Number of *Bos taurus* EST consensus sequences	Number of *Sus scrofa* EST consensus sequences
GO:0005488	Binding	25, 749	22, 143
GO:0003824	Catalytic Activity	14, 419	12, 272
GO:0005515	Protein Binding	11, 499	9, 973
GO:0003676	Nucleic Acid Binding	7, 555	6, 536
GO:0000166	Nucleotide Binding	5, 731	4, 915
GO:0016787	Hydrolase Activity	5, 454	4, 609
GO:0004871	Signal Transducer Activity	4, 794	4, 355
GO:0016470	Transferase Activity	4, 604	4, 102
GO:0003677	DNA Binding	4, 181	3, 646
GO:0005215	Transporter Activity	3, 490	2, 778
GO:0004872	Receptor Activity	2, 864	2, 717
GO:0030528	Transcription Regulator Activity	2, 627	2, 266
GO:0005198	Structural Molecule Activity	2, 364	1, 751
GO:0016301	Kinase Activity	2, 233	1, 988
GO:0003723	RNA Binding	2, 094	1, 743
GO:0005509	Calcium Ion Binding	1, 909	1, 642
GO:0030234	Enzyme Regulator Activity	1, 763	1, 506
GO:0003700	Transcription Factor Activity	1, 596	1, 396

Table 3.3: Cellular Component Gene Ontology annotation statistics for cattle and pig EST consensus sequences using the GO Slim terms in the generic GO Slim.

Gene Ontology ID	Gene Ontology Term	Number of *Bos taurus* EST consensus sequences	Number of *Sus scrofa* EST consensus sequences
GO:0005623	Cell	26, 772	22, 754
GO:0005622	Intracellular	19, 802	16, 700
GO:0043226	Organelle	16, 271	13, 788
GO:0005737	Cytoplasm	10, 015	8, 239
GO:0005634	Nucleus	8, 787	7, 581
GO:0043234	Protein Complex	5, 389	4, 252
GO:0005886	Plasma Membrane	3, 175	2, 668
GO:0005576	Extracellular Region	2, 950	2, 461
GO:0005856	Cytoskeleton	2, 192	1, 843
GO:0005739	Mitochondrion	2, 058	1, 741
GO:0005615	Extracellular Space	1, 583	1, 270
GO:0005783	Endoplasmic Reticulum	1, 459	1, 217
GO:0005829	Cytosol	1, 148	823
GO:0005794	Golgi Apparatus	1, 090	1, 036
GO:0005840	Ribosome	1, 038	720
GO:0005654	Nucleoplasm	727	628
GO:0005694	Chromosome	705	667
GO:0005578	Extracellular Matrix	682	601

The findings from **Tables 3.1**, **3.2** and **3.3** after profiling the cattle and pig transcriptomes in the "Biological Process", "Molecular Function" and "Cellular Component" namespaces of Gene Ontology are illustrated in **Figures 3.12, 3.13** and **3.14**. Given below is a summary of the findings from **Tables 3.1**, **3.2** and **3.3**.

Biological Process profile

Figure 3.12 depicts the Biological Process Gene Ontology profile for the cattle and the pig transcriptomes. The largest Biological Process GO category in both the transcriptomes was the 'physiological process' category with approximately 67% of both the cattle and pig EST gene products coming under it (27, 477 Cattle EST Consensus sequences and 23, 315 Pig EST Consensus sequences). The next largest categories in the Biological Process GO were the EST gene products pertaining to the 'metabolism' category with ~47% of cattle and pig EST gene products in it (19,374 Cattle EST Consensus sequences and 16,435 Pig EST Consensus sequences). The 'metabolism' category encompasses 'primary metabolism' (~42% of cattle and pig EST gene products), 'protein metabolism' (~20% of cattle and pig EST gene products), and 'nucleic acid metabolism' (~20% of cattle and pig EST gene products). Among the other GO terms in the Biological Process namespace, the 'transport' category has approximately 17% of cattle and pig EST gene products, and the 'stress response' as well as the 'biotic stimulus response' categories having approximately 6% of cattle and pig EST gene products.

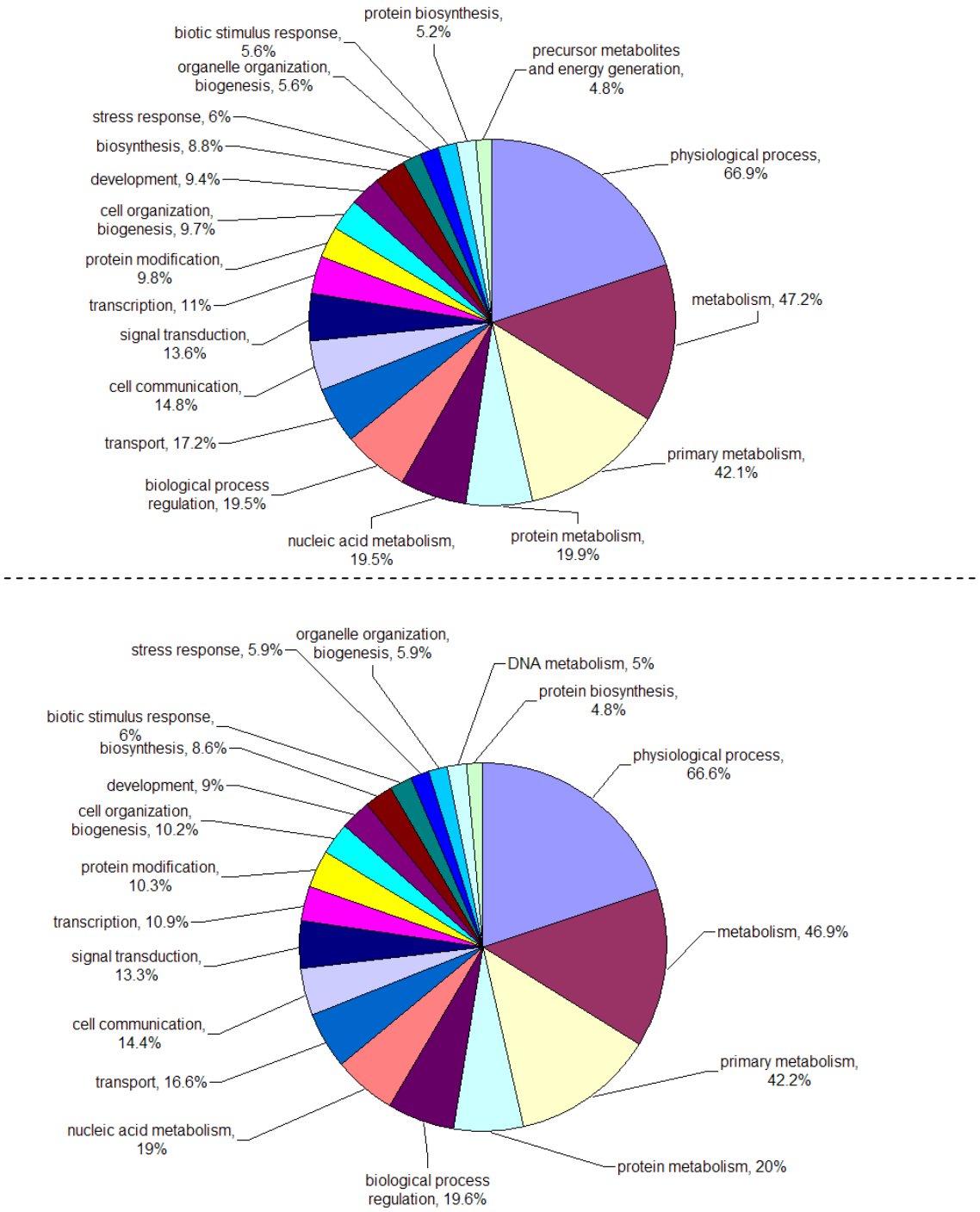
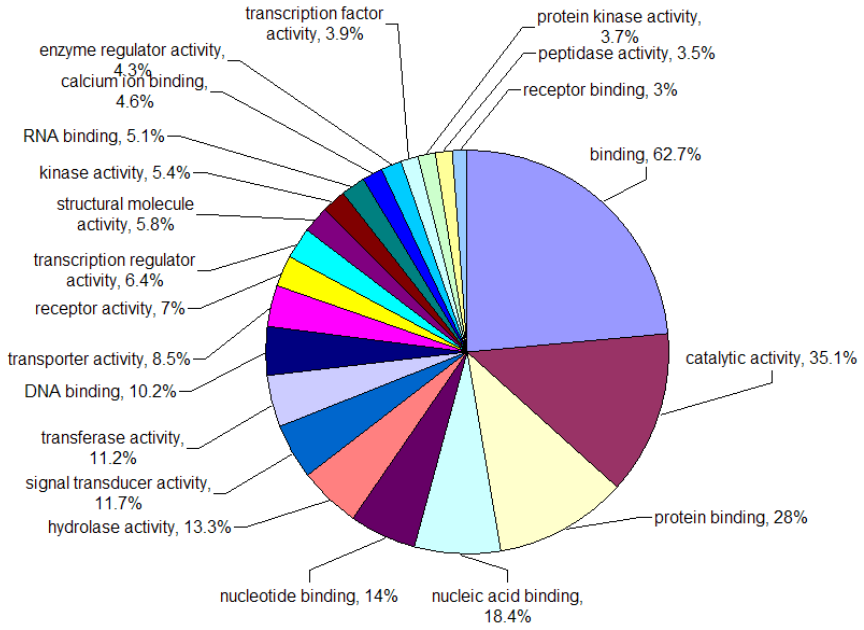


Figure 3.12: Biological Process Gene Ontology (GO) profiles for cattle and pig EST consensus sequences.
Assignment to the different GO Slim terms for cattle (**top panel**) and pig (**bottom panel**) EST consensus sequences normalized on number of sequences having GO annotations.

Molecular Function profile

Figure 3.13 depicts the Molecular Function Gene Ontology profile for the cattle and the pig transcriptomes. The largest Molecular Function GO category in both the transcriptomes was the 'binding' category with approximately 63% of both the cattle and pig EST gene products coming under it (25,749 Cattle EST Consensus sequences and 22,143 Pig EST Consensus sequences). The 'binding' category encompasses the following categories: 'protein binding' (~28% of cattle and pig EST gene products), 'nucleic acid binding' (~19% of cattle and pig EST gene products), 'nucleotide binding' (~14% of cattle and pig EST gene products), 'DNA binding' (~10% of cattle and pig EST gene products) and 'RNA binding' (~5% of cattle and pig EST gene products). The next largest category in the Molecular Function GO was the 'catalytic activity' category with ~35% of cattle and pig EST gene products in it (14,419 Cattle EST Consensus sequences and 12,272 Pig EST Consensus sequences). The 'catalytic activity' GO encompasses the 'hydrolase activity' (~13% of cattle and pig EST gene products) category and the 'transferase activity' (~11% of cattle and pig EST gene products) category. Among the other GO terms in the Molecular Function namespace, the 'signal transducer activity' category has ~12% of cattle and pig EST gene products, the 'transporter activity' category has ~9% of cattle and pig EST gene products, and the 'receptor activity' as well as the 'transcription regulator activity' categories mapping to approximately 6-8% of the cattle and pig EST gene products.



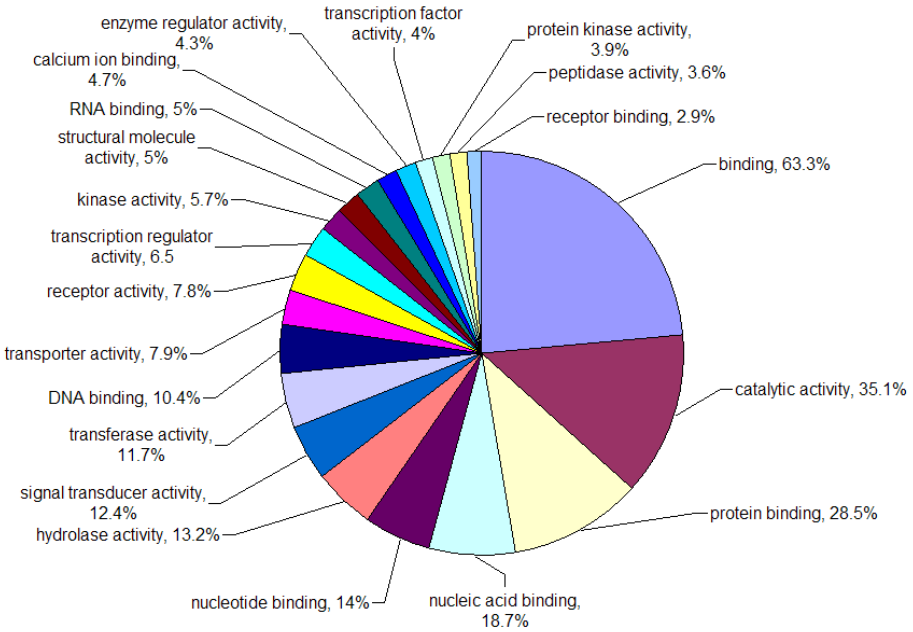


Figure 3.13: Molecular Function Gene Ontology (GO) profiles for cattle and pig EST consensus sequences.
Assignment to the different GO Slim terms for cattle (**top panel**) and pig (**bottom panel**) EST consensus sequences normalized on number of sequences having GO annotations.

Cellular Component profile

Figure 3.14 depicts the Cellular Component Gene Ontology profile for the cattle and the pig transcriptomes. The 'cell' category was the largest Cellular Component GO category in both the transcriptomes with approximately 65% of both the cattle and pig EST gene products coming under it (26,772 Cattle EST Consensus sequences and 22,754 Pig EST Consensus sequences). The 'cell' category encompasses the following categories: 'intracellular' (~48% of cattle and pig EST gene products), 'cytoplasm' (~24% of cattle and pig EST gene products), 'nucleus' (~21% to ~22% of cattle and pig EST gene products), 'plasma membrane' (~8% of cattle and pig EST gene products) and 'cytoskeleton' (~5% of cattle and pig EST gene products). The next largest categories in the Cellular Component GO was the 'organelle' category with ~39% to ~40% of cattle and pig EST gene products in it (16,271 Cattle EST Consensus sequences and 13,788 Pig EST Consensus sequences) and the 'protein complex' category with ~12% to ~13% of cattle and pig EST gene products in it (5,389 Cattle EST Consensus sequences and 4,252 Pig EST Consensus sequences).

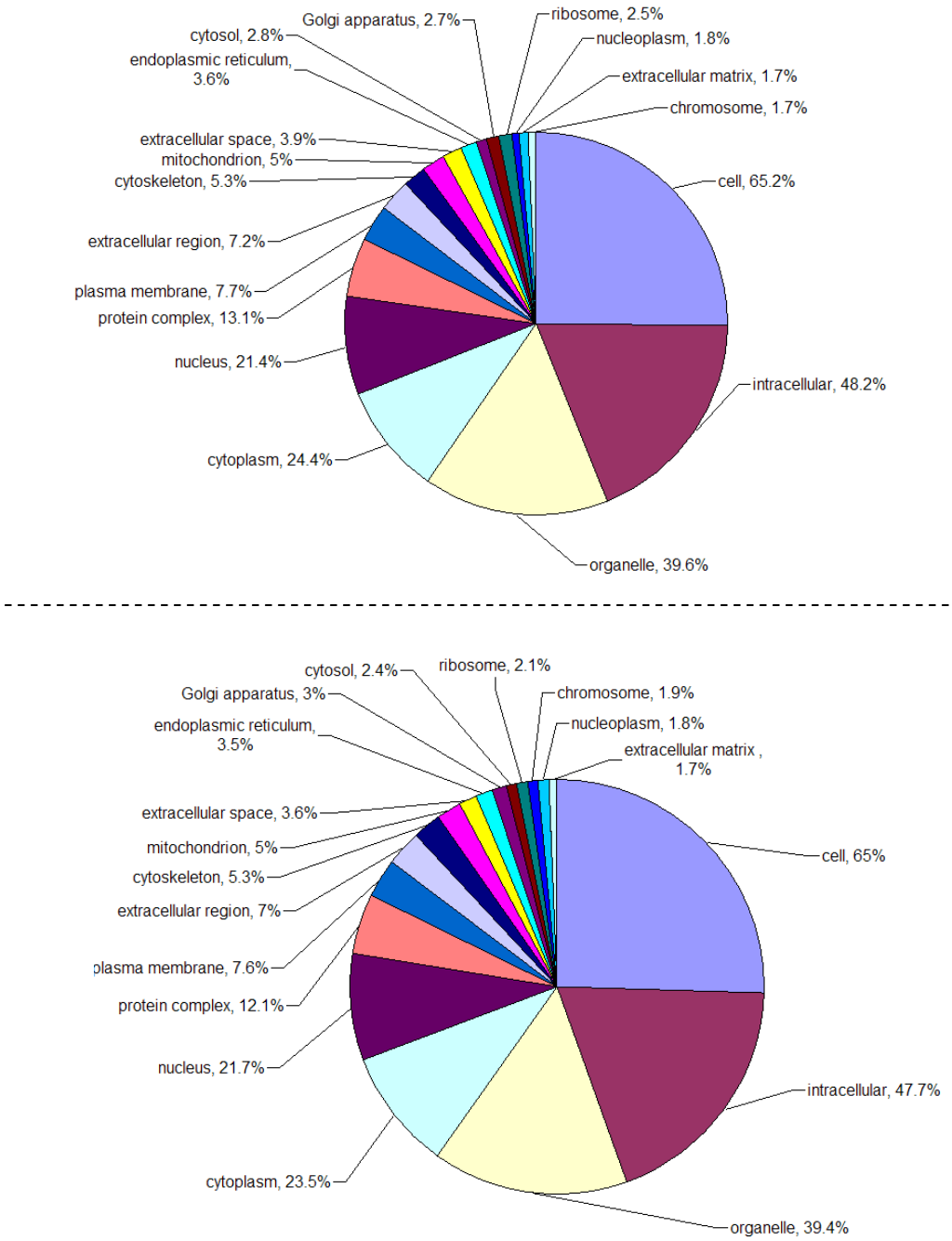


Figure 3.14: Cellular Component Gene Ontology (GO) profiles for cattle and pig EST consensus sequences.
Assignment to the different GO Slim terms for cattle (**top panel**) and pig (**bottom panel**) EST consensus sequences normalized on number of sequences having GO annotations.

Bovine transcriptome GO mappings from the 'best match' approach versus GO mappings for predicted bovine transcripts

We compared the GO mappings identified by our 'best match' approach for the bovine EST consensus sequences with the GO mappings for the predicted bovine transcripts in the *Bos taurus* Ensembl Release 50 (July 2008) [129]. The GO mappings for the predicted bovine transcripts were obtained from BioMart [167]. The 'best match' approach mapped the bovine EST consensus sequences to 6,322 unique GO terms; the Ensembl approach mapped the bovine transcripts to 5,960 unique GO terms. Perl scripts were written to identify the GO terms common to both methods as well as the GO terms unique to the datasets. **Figure 3.15** is a cartoon depicting how the two GO mapping methods compare.

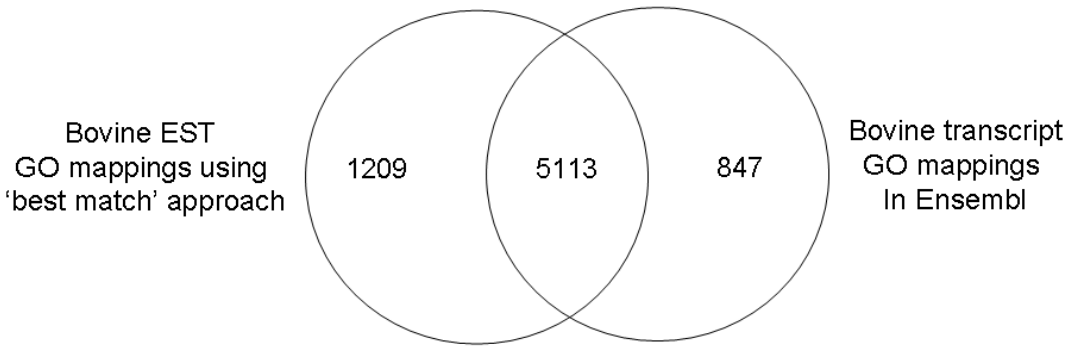


Figure 3.15: Comparison of the GO mappings for bovine EST consensus sequences using 'best match' approach and GO mappings for predicted bovine transcripts in Ensembl.

Figure 3.15 reveals that the two methods have 5113 GO terms in common and ~1000 GO terms unique to either set. We believe that the ~1000 or so GO terms unique to either set could be due to the fact that the GO annotations for the Bovine Genome project are ongoing and not yet complete. The 'best match' approach of GO annotations for the bovine EST consensus sequences does not rely on GO mappings from the bovine genome; instead, GO annotations are transferred to the bovine EST consensus sequence based on the vertebrate protein match found using FASTX and using the species precedence order described earlier. In spite of the differences in the approach taken by the two methods for the GO mappings, the fact that there are 5113 GO terms are in common between the two methods signifies that the procedure of transferring GO annotations using the 'best match' approach can be relied upon for species that do not have complete genome sequences and/or do not have GO mappings derived from a complete genome sequence being available.

CONCLUSIONS

Bos taurus (cattle) and *Sus scrofa* (pig) are important livestock species that serve as candidate animal models for biomedical research. In the absence of availability of completed genomes for the *Bos taurus* and *Sus scrofa* species, very less information exists about the genes and the gene products. In this scenario, EST datasets were utilized to obtain insights of the functions encoded in the cattle and the pig transcriptomes using computational predictions.

Anonymous ESTs are of limited value unless connected to function, thereby necessitating the need for annotated datasets. The Livestock EST Gene Family Database consists of cattle and pig ESTs clustered into gene families using the vertebrate proteomes in the Ensembl database as a framework for assembly and translation. Cattle and Pig ESTs were electronically annotated making use of the rich annotations of the vertebrate proteomes; these annotated datasets would help researchers to deal with the volume of information, and to utilize the information embedded in the gene expression data gathered from assembling the EST datasets. These annotations would immensely aid the livestock community in narrowing down the gamut of direct experiments needed to verify function. The database has a user-friendly web interface and can be searched in a number of ways: Cattle/Pig EST GenBank identifiers (GI or Accession), Gene Ontology Accession to obtain information about the vertebrate protein family, related EST members and other annotations.

The Livestock EST Gene Family Database is the first of its kind where ESTs with similar functions in more than one species are grouped together in the same gene family. This information comes in handy when one wants to get a listing of ESTs from different species performing the same function. To do so, one could search the database using an EST from species 1; obtain the Protein Family Id for that EST. Upon searching the database using the Protein Family Id as the query, one can get a listing of all the ESTs from different species belonging to that Protein Family Id.

The Livestock EST Gene Family Database is also the first of its kind that incorporates an AmiGO [170] like search strategy to transitively annotate EST gene

products with a GO accession and all its descendants. The AmiGO database houses gene product annotations from model organisms whose complete genome sequences are known and AmiGO does not house GO annotations for EST gene products. Searching by GO accession on the Livestock EST Gene Family Database will retrieve the complete descendant information for the GO accession and give a mapping of the different livestock EST contigs/singletons associated with the complete GO descendant tree starting from that GO Accession. This search feature will come in handy for users who want to get a complete profile of the cattle and pig EST gene products associated with a particular GO Accession.

The GO terms mapped to the bovine EST consensus sequences identified by the 'best match' approach were compared to the GO terms mapped to the bovine transcripts by Ensembl. Despite the different strategies used by the two methods, the findings reveal that the 'best match' approach compares well with the Ensembl approach and can be used reliably for species that do not have complete genome sequences and/or do not have GO mappings derived from a complete genome sequence being available.

A GO Slim analysis was performed to functionally categorize the cattle and pig transcriptomes within arbitrary coarser (parent) levels of the three independent ontologies - biological process, molecular function and cellular component - and helped in the identification of the roles of the different EST gene products. The GO Slim analysis helped profile the cattle and pig transcriptomes by conveying biological meaning at a coarser level, thereby making up for inconsistencies like categorization errors and/or incomplete coverage of certain categories.

The work described in this chapter provides an invaluable resource to explore the orthologous relationships and evolutionary analysis of the genes and gene families in cattle and pig, and for functional genomics. The computational function predictions of the cattle and the pig transcriptomes would help in obtaining a first-order approximation of the molecular function of the proteins encoded and come in handy while prioritizing experimental investigations.

CHAPTER IV

DESCRIPTION OF A PHYLOGENOMIC ANNOTATION PIPELINE FOR COMPUTATIONAL FUNCTION PREDICTIONS OF THE *Bos taurus* AND *Sus scrofa* TRANSCRIPTOMES

SYNOPSIS

In this chapter, EST consensus sequences, which had been generated from cattle and pig ESTs after grouping ESTs into gene families, were combined with proteins in their respective protein family, and subject to multiple sequence alignment and phylogenetic analysis. The phylogenetic trees obtained were then analyzed using a subtree neighbors approach to predict function of the cattle ad pig ESTs consensus sequences. This analysis was able to identify function for ~23% of the Livestock EST gene products. The remaining ~77% of the sequences were excluded due to various reasons that include, but are not restricted to: a) belonging to families with 50 or more members, b) ambiguous regions in the multiple sequence alignment, c) failure to match tree resolving criteria in the consensus phylogenetic tree reconstruction after bootstrapping, d) tree ambiguity problems. This work thus outlines the processes required for phylogenomic annotation and identifies the technical/biological pitfalls of the same. This work, to our knowledge, is the first of its kind where phylogenomic inference has been used in predicting functions for EST gene products, and the function predictions of the uncharacterized cattle/pig EST gene products using this approach would help reduce the number of direct experiments required to verify function.

BACKGROUND

Orthologs are evolutionary counterparts derived from a single ancestral gene in the last common ancestor of species being compared [70-72] . Orthologs are likely to have equivalent or similar functions because of their phylogenetically close relationships; however, function is not part of the definition. The high level of function conservation between orthologs makes orthology relevant for protein function prediction.

Phylogenetic analysis helps improve function predictions by incorporating an evolutionary perspective. Phylogenomics combines evolutionary and comparative genomic analysis into a single composite approach [1], and involves inferring function of a unknown sequence (protein) in the larger context of a protein family based on evolutionary relationships [32, 39, 53, 77]. In this approach, a phylogenetic tree is constructed following multiple sequence alignment of the different members of a protein family. As a result, the various members of the family are segregated into subfamilies; each subfamily representing a functionally and evolutionarily distinct group of homologous proteins with similar functions and activities. The tree topology is then analyzed and the phylogenetic information encoded in the subfamily (or subtree) structure is used to infer likely functions for the uncharacterized members of the protein family. Since function is conserved within orthologous subfamilies [82], uncharacterized genes can be assigned predicted function based on the subfamily in which they are placed.

Making accurate function predictions is a key step and these predictions have become increasingly important as numerous sequences are being generated with little or no accompanying experimentally determined information regarding the function they encode. *Bos taurus* (cattle) and *Sus scrofa* (pig) represent evolutionary clades distinct from the primates and rodents, and therefore are good candidate animal models for biomedical research due to parallels with humans. The genome sequences for these species are in different stages of completion [10, 12], and in this scenario Expressed Sequence Tags represent a good alternative to indentify the genes and the functions encoded in the genome.

Overview of the work in this chapter

In this chapter, we use a phylogenomic approach to predict the functions encoded in the *Bos taurus* (cattle) and *Sus scrofa* (pig) transcriptomes. Cattle and pig ESTs were first grouped into gene families using the vertebrate protein family clusters obtained from an in-house clustering algorithm developed in our lab. Phylogenetic analysis of the protein family clusters results in subdivisions of groups containing orthologs and paralogs, each group representing distinct subfamilies.

As part of the TAMUClust EST clustering pipeline, cattle and pig ESTs were grouped into gene/protein families using the Ensembl protein families as the framework. The different proteins and the EST gene products in each protein family are passed through a multiple sequence alignment and phylogenetic analysis pipeline. The resulting phylogenetic trees were analyzed for their tree topology and the functions of the

uncharacterized EST gene family members were predicted by their positions on the phylogenetic tree involving other member(s) of the subgroup/subfamily.

To our knowledge, this is the first time that phylogenomic analysis, and that too on a large scale, has been used in predicting functions for EST gene products. This analysis helps obtain improved function predictions of the uncharacterized EST gene family members in cattle and pig, and would thereby provide the livestock community with an invaluable resource to verify function of the EST gene products by designing experiments based on these predictions.

MATERIALS AND METHODS

Assembling a dataset

One of the preliminary requirements for constructing a phylogenetic tree is building the dataset. Given below are the steps used in assembling a protein family dataset comprising of Ensembl vertebrate proteomes and bovine/porcine translated EST Contig/Singleton sequences:

1) *Bos taurus* and *Sus scrofa* ESTs were clustered into gene families using Dr. Elsik's clustering algorithm as described in Chapter II. This clustering algorithm is a two step process and uses the vertebrate proteomes in the Ensembl database as a framework to assemble and translate the ESTs. A total of 219,433 Ensembl vertebrate proteins from *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*, *Takifugu rubripes* and *Tetraodon nigroviridis* were clustered into 10,092 protein families. The vertebrate protein families served as a framework

to group the *Bos taurus* and *Sus scrofa* ESTs into gene families using Dr. Elsik's algorithm as described in Chapter II. 308,132 bovine ESTs were grouped into 46,731 EST Clusters (24,665 contigs and 22,066 singletons); 238,691 porcine ESTs were grouped into 39,641 EST Clusters (20,951 contigs and 18,690 singletons).

2) The "best match" Ensembl vertebrate protein for every EST Contig/Singleton was determined using the pipeline mentioned in Chapter III. The 'protein2dna' model of the Exonerate [171, 172] program in conjunction with the "ryo" (roll your own) output option was used to translate the EST Contig/Singleton using the "best match" Ensembl vertebrate protein as the framework. The 'protein2dna' model compares a protein sequence to a DNA sequence while incorporating all the appropriate gaps and frameshifts. Perl scripts were written to automate the procedure and to parse the Exonerate outputs to generate a fasta file of the translated EST Contig/Singleton.
3) Perl scripts were written to make directories of protein families generated by Dr. Elsik's single-linkage and average-linkage clustering results of the Ensembl vertebrate proteins. Each directory (protein family) contains fasta files of the different Ensembl vertebrate proteins assigned to that protein family by Dr. Elsik's clustering algorithm.
4) Perl scripts were written to first identify the protein family to which a translated EST Contig/Singleton (from ***Step 2*** above) belongs and then to assign it to its respective protein family directory (from ***Step 3*** above).

The above mentioned steps resulted in different protein family directories (10092 directories), each of which having fasta files of the vertebrate proteins and fasta files of the bovine/porcine translated EST Contig/Singleton. The different sequences in each protein family were subject to multiple sequence alignment (MSA) as described in detail below.

Multiple Sequence Alignment (MSA)

Molecular trees are based on multiple sequence alignments (MSA); these multiple sequence alignments form the heart of the matter when in comes to inferring relationships based on phylogenetic trees [173]. Earlier, these alignments were assembled by hand [173] as the exhaustive alignment of >8 sequences was computationally unfeasible. Nowadays, with advances in computing resources, MSAs can be performed with large number of sequences.

With reference to our study, it was critical to understand the subtleties and nuances of the underlying dataset to determine the ideal MSA program that could answer our questions. In our dataset, each protein family had a mix of full-length vertebrate proteins along with bovine/porcine translated EST Contig/Singleton sequences. Moreover, each protein family was diverse in terms on total number of sequences range from 2 to 1593. Hence, it was of utmost importance to select a MSA program that could work with a mix of full-length as well as fragmented sequences.

Choice of the MSA program

In a recent study [174] evaluating different MSA methods using four sets of reference alignments, three MSA methods – T-Coffee [175], MUSCLE [174, 176], and Multiple Alignment using Fast Fourier Transform (MAFFT) [177] – were found to be highly accurate. T-Coffee has a drawback that the number of sequences cannot exceed 50 [178] and was hence unsuitable for our study. We evaluated the performance of MUSCLE (version 3.6) and MAFFT (version 5.861) with our dataset; we found MUSCLE to be crashing with certain protein families having large sizes whereas MAFFT would work well in those scenarios. Moreover, the MAFFT algorithm has different alignment options like (linsi, ginsi, einsi) [179]; each option being suitable for different types of sequences. The 'linsi' (l' standing for local) option is suitable for aligning a set of sequences containing flanking sequences around one alignable domain. The 'ginsi' ('g' standing for global) options assumes that entire region can be aligned and tries to globally align them. The MAFFT 'einsi' option is suitable for MSAs which have large unalignable regions [179]; it is also the recommended option if the nature of sequences to be aligned is not clear.

We decided to use MAFFT and the 'einsi' option to perform the multiple sequence alignments of the sequences in the different protein family directories as it was tailor-made for the nature of sequences that we had. We used the 'clustalout' option of MAFFT to generate 'clustal-like' MSAs.

Phylogenetic analysis

There are three methods – maximum parsimony, maximum likelihood, and distance – to determine the evolutionary tree or trees that best account for the observed variation in a group of sequences.

1) Maximum parsimony (MP) predicts the evolutionary tree that minimizes the number of steps required to generate the observed variation in the sequences from common ancestral sequences. Analysis is performed on every single column of the MSA and for each aligned position, phylogenetic trees that require smallest number of evolutionary changes are found. This is repeated for every position in the sequence alignment. Finally, only those trees that produce smallest amount of changes overall for all sequence positions are determined. Because the maximum parsimony method tries to fit all possible trees to the data, this method is best suited [97] for analysis involving sequences that are quite similar and is limited to small number (<12) of sequences in a MSA. The MP method is also not suitable for sequences from large evolutionary distances as it does not take into account a model of evolution.
2) Maximum likelihood (ML) methods start with a simple model of rates of evolutionary changes in sequences and tree models that represent a pattern of evolutionary change, and then adjust the model until a best fit for the observed data is found. Similar to the MP method, ML methods also perform analysis on each column of the alignment. The ML method resembles the MP method in that trees with the least number of changes will be the most likely. However, the ML method allows one to evaluate tress with variations in mutation rates in different lineages, or

site-to-site variations within the MSA [97] – conditions that are not well handled by MP methods. Because all possible trees are considered by the ML method in its analysis, this method is feasible only for a small number of sequences.

3) Distance methods are based on evolutionary distances between sequence pairs in a MSA. The goal of distance methods is to identify a tree that positions the neighbors correctly and that has branch lengths which, when added up between each sequence pair, closely reproduce the original distance measurements. Distance matrix programs use a substitution model to generate a table with the distances between all pairs of sequences in a MSA. Distance analysis programs use the information in the distance matrices obtained from above to obtain a tree that best accounts for the observed variation in a group of sequences. The biggest advantage [97] that 'distance' methods have over MP and ML is that the 'distance' methods can handle large numbers of sequences and usually are not significantly affected by variations in rates of mutation over evolutionary times.

Given the large number of sequences in our analysis, we decided to use the 'distance' method for phylogenetic analysis. We chose to use the phylogeny inference package (PHYLIP) [180] to generate phylogenetic trees of the different protein families in our dataset.

Phylogeny inference package (PHYLIP) pipeline

The PHYLIP pipeline requires the different PHYLIP programs in a sequential way; the output from Program 1 is used as input for Program 2, and the output of

Program 2 is used as input for Program 3, and so on. The perl modules Bio::AlignIO and Bio::SimpleAlign from the BioPerl toolkit [181] were used in perl scripts to convert the MSAs from a 'clustal-like' format to a 'phylip infile' format required by PHYLIP. This 'phylip infile' was passed though a PHYLIP pipeline as detailed below:

1. Seqboot: Seqboot is a general bootstrapping and data set translation tool which generates multiple data sets that are resampled versions of the input data set. The input dataset can be resampled in many ways: bootstrapped, jackknifed, or permuted. We used Seqboot with the default settings to generate 100 bootstrapped datasets. Bootstrapping tests whether the entire dataset is supporting the tree or if the tree is a marginal winner among many equally possible alternatives. Bootstrapping is done by taking random subsamples of the dataset. Building trees from these and calculating the frequency with which various parts of the tree are reproduced in each of the random subsamples. Seqboot requires the input file to be called 'infile' and produces an output file called 'outfile'. The file called 'outfile' has to be renamed to 'infile' before it can be used as an input for ProtDist.
2. ProtDist: ProtDist is a 'distance matrix' program which uses amino acid replacement models on protein sequences to compute a distance matrix. The distance for each pair of species is the estimated total branch length between the two species, and this distance can be used by 'distance analysis' programs like Fitch to generate trees. We used the Jones-Taylor-Thornton (JTT) model [182] – the default model in this program. We changed the default settings on ProtDist using the 'M' option to analyze 100 datasets to account for the fact that we had

generated 100 bootstrapped datasets using Seqboot earlier. ProtDist requires the input file to be called ‘infile’ and produces an output file called ‘outfile’. The file called ‘outfile’ has to be renamed to ‘infile’ before it can be used as an input for Fitch.

3. Fitch: Fitch is a ‘distance analysis’ program that estimates phylogenies from distance matrix data under the "additive tree model"; according to this model, the distances are expected to equal the sums of branch lengths between the species. Fitch uses the Fitch-Margoliash criterion [183] and does not assume an evolutionary clock. G is the Global search option. We used the Fitch-Margoliash method – the default method in this program. We changed the default settings on Fitch using the ‘M’ option to analyze 100 datasets to account for the fact that we had generated 100 bootstrapped datasets using Seqboot earlier. We also changed the default settings on Fitch using the ‘G’ option to do global rearrangements on the tree. The ‘G’ option causes, after the last species is added to the tree, each possible group to be removed and re-added. Using the ‘G’ option in Fitch approximately triples the run-time of the program but improves the result, since the position of every species is reconsidered. Fitch requires the input file to be called ‘infile’ and produces two output files called ‘outtree’ and ‘outfile’. The file called ‘outtree’ has to be renamed to ‘intree’ before it can be used as an input for Consense.
4. Consense: Consense is a tree program that reads a file of computer-readable trees and prints out (and may also write out onto a file) a consensus tree. This consensus

tree serves as the consensus from amongst the different phylogenetic trees generated from the bootstrapped datasets (100 datasets in our case). Out of the four consensus methods used by this program, we chose to use the 'majority rule extended' (MRe) method – the default method in this program. The MRe method first includes all sequences that appear in more than 50% of the trees. The program then considers the other sets of sequences in order of the frequency with which they have appeared, adding to the consensus tree any which are compatible with it until the tree is fully resolved. Consense requires the input file to be called 'intree' and produces two output files called 'outtree' and 'outfile'. The 'outtree' is a tree file where the trees are represented in a linear form by a series of nested parentheses, enclosing names and separated by commas. This type of representation is called the 'Newick' format [184] and the originator of this format was the English mathematician Arthur Cayley; the pattern of the parentheses in the 'Newick' format indicates the tree topology.

Making images of phylogenetic trees

The perl module Bio::Tree::Draw::Cladogram from the BioPerl toolkit [181] was used in perl scripts to parse the phylogenetic tree files and obtain a cladogram of the tree in postscript format. A cladogram is a tree that depicts the tree topology by means of ancestor-descendant relationships. The postscript cladogram files were converted to pdf using the unix utility 'ps2pdf'.

RESULTS AND DISCUSSION

Multiple Sequence Alignment (MSA) editing

Most multiple sequence alignments are constructed by the 'progressive sequence alignment' method [185]; this method begins with the construction of a crude 'guide tree' which involves pairwise comparison of all sequences to determine the order in which sequences are progressively added to build the alignment. With this 'guide tree', the MSA program starts with the most similar sequences, builds an alignment and progressively adds more dissimilar sequences. Each alignment position is assumed to include residues that share common ancestry among species; hence it is important to edit the MSA to remove regions of ambiguous alignment from the MSAs before phylogenetic analysis.

We edited the MSAs to exclude any ambiguous regions and include only the densest portions over a given window length, which is the larger of these two indices:

(i) length of 100

(ii) 90% of the length of the longest sequence in the MSA

We then trim the alignment to include this window alone; in this process, the sequences which do not satisfy the above mentioned criteria are excluded from the phylogenetic analysis. The statistics pertaining to number of vertebrate proteins, bovine/porcine contigs/singletons that passed our MSA editing criteria are detailed in **Table 4.1**.

Entire dataset or large enough dataset

Large datasets often bring up an interesting dilemma; whether to include all the data or to include data that is large and representative enough to answer the biological question being asked within a reasonable timeframe. In this tradeoff, we decided on the latter and we chose to run the PHYLIP pipeline only on those families which have ≤50 sequences in the edited MSA. The entire process (MSA, PHYLIP pipeline on protein families having ≤50 sequences) took ~4-5 months with the different jobs running in parallel on a computer cluster having 12 worker nodes.

Table 4.1: Comparative statistics of the number of sequences present in the original and final multiple sequence alignment.

	Present in original MSA	**Not Included in final (edited) MSA**	**Included in final (edited) MSA**
Vertebrate proteins	133,874	11,543	122,331
Translated bovine contigs	24, 665	8,778	15,887
Translated bovine singletons	22, 006	10,636	11,370
Translated porcine contigs	20, 951	7,211	13,740
Translated porcine singletons	18,690	9,568	9,122
Total number of sequences	**220,186**	**47,736**	**172,450**

Phylogenetic tree analysis

Phylogenetic analysis is a powerful tool for interpreting molecular data. Prediction of trees produced by 'distance' methods can be improved by rerooting the trees using an 'outgroup' sequence. The 'outgroup' sequence is one that is more distantly related to the other sequences than they are to each other; the 'outgroup' acts as an external reference and aids in correctly arranging the other sequences.

Choosing an outgroup

In our analysis, each protein family can have sequences from a maximum of nine species (translated EST Contig/Singleton sequences from cattle and pig, in addition to the protein sequences from the seven vertebrate species). The number of species in each tree would vary depending on the species represented in each protein family. In this scenario, each tree had to be first analyzed to determine the 'outgroup' and then rerooted using the 'outgroup'. Keeping in mind that our analysis focused on determining annotations using a phylogenomic pipeline for 'cattle' and 'pig' EST gene products, the 'outgroup' was determined from amongst the various proteomes using this order of precedence: *Takifugu rubripes, Tetraodon nigroviridis, Danio rerio, Gallus gallus, Rattus norvegicus, Mus musculus and Homo sapiens*. For example, if a phylogenetic tree has sequences from *Takifugu rubripes*, then *Takifugu rubripes* was used as the 'outgroup'. If the tree does not have *Takifugu rubripes* sequences, the next species using the order of precedence mentioned above was used as the 'outgroup'.

Perl scripts incorporating the perl module Bio::TreeIO from the BioPerl toolkit [181] were written to navigate the tree and to determine the 'outgroup' using the strategy

mentioned above. Outgroups were determined for every phylogenetic tree; in other words, outgroups were determined for every protein family that got included in the phylogeny analysis. With this knowledge of the 'outgroup', all the phylogenetic trees were rerooted, subject to a 'subtree neighbors' approach to determine orthologs and subsequent function annotation of the bovine/porcine EST gene products.

Livestock EST gene products function classification using subfamily annotation

Subtree neighbors approach for assigning predicted function

In the subtree neighbors approach, multiple seqeunce alignment (MSA) of proteins in a protein family is performed in the lead up to phylogenetic tree construction. Following this, the tree topology is analyzed and sequences without known function can be assigned a predicted function on the basis of other sequences in the subtree containing the sequence with unknown function.

We adopt this 'subtree neighbors' approach to identify the most recent diverging sequence from a different species with reference to the sequence (bovine/porcine) whose function we are predicting. In other words, by using the subtree neighbors approach, we are finding the best ortholog from amongst many possible orthologs. Using this approach, we end up identifying orthologs for a particular bovine/porcine EST gene product based on the subfamily the EST gene product gets grouped with in the phylogenetic tree. The EST gene product is then assigned predicted function from amongst one of the ortholog(s). Here is the pseudocode used in this study to determine the ortholog(s) for a bovine/porcine EST gene product:

1. in a given phylogenetic tree, for each leaf node which is a bovine or a porcine sequence, do;
2. go one node toward root node and analyze the new branch;
3. if all leaf nodes from step 2 are from same species; then repeat step 2;
4. excluding the sequence(s) in question, if all leaf node(s) of the new branch are from a different species and do not include the 'outgroup' species, then report orthology and use the sequence(s) for function annotation and go to step 1;
5. if the new branch contains at least one sequence of the 'outgroup' species OR at least one sequence of the same species as the sequence in question, it denotes ambiguity in the tree topology pertaining to the sequence in question; hence discard the sequence in question and go to step 1.

Table 4.2 gives a breakdown of the Livestock EST Contigs/Singletons for which function could be predicted using the 'subtree' approach described above.

Table 4.2: Breakdown of the Livestock EST gene products for which function could be predicted using the subtree approach.

	Contigs	**Singletons**	**Total**
Bos taurus	8156	2284	**10440**
Sus scrofa	7274	2061	**9335**
Total	**10430**	**4345**	**19775**

Given that orthologs are sequences derived from a single ancestral gene in the last common ancestor of the species being compared, there is no requirement for orthology to be a one-to-one relationship. In case of one or more duplication events happening after speciation, it can result in one-to-one, one-to-many, many-to-one and many-to-many orthologous relationships.

Usage notes for the phrase 'one-to-many orthologous relationship' in this work

In the ensuing discussions in this chapter, we would like to mention that the phrase 'one-to-many orthologous relationship' is not used in the evolutionary sense; instead, it is used in an algorithmic sense with reference to the subtree neighbors method. From an evolutionary standpoint, the 'one-to-many orthologous relationship' would reflect a scenario where gene duplication in a family (say with members A, B, C) occurs after a speciation event in one lineage or in both lineages independently (lineage-specific expansion); in these situations, the functional correspondence between the two orthologous families is less straightforward than it is between one-to-one orthologs [24]. Instead, one can only state the members (say A_1, B_1, C_1) of a family in lineage 1 are co-orthologous to the members (say A_2, B_2, C_2) of the same family in lineage 2.

From the subtree neighbors algorithmic standpoint, 'one-to-many orthologous relationships' encompass these three scenarios:

1. the bovine/porcine EST gene product is orthologous to multiple splice isoforms produced by the same gene in a species as well as duplicated gene products in a species resulting from lineage specific expansions;

2. the bovine/porcine EST gene product is part of multiple one-to-one orthologous relationships with proteins in more than one species; and
3. combination of the above-mentioned scenarios '1' and '2'.

The 'subtree' approach mentioned above was used to determine the best ortholog for the bovine/porcine EST gene product from amongst a set of orthologs. In cases where a one-to-one orthology relationship could not be detected because of one or more duplication events happening after speciation, we term the orthologous relationships as 'one-to-many' from the algorithmic standpoint as specified above. We do not attempt to distinguish between these three scenarios: multiple splice isoforms produced by the same gene in a species, duplicated gene products in a species as a result of lineage specific expansions and multiple one-to-one orthologous relationship with proteins in more than one species. The most recent diverging sequence(s) from a different species with reference to the bovine/porcine EST gene product identified by the 'subtree' method was used for assigning predicted function and for annotation transfer. Using this approach, we were able to predict function for 19775 Livestock EST gene products and this resulted in a mix of gene products which were part of one-to-one orthologous relationships as well as one-to-many orthologous relationships from the 'subtree neighbors' algorithmic standpoint.

From amongst the 19775 Livestock EST gene products for which function could be predicted using the above approach, 13895 of them had a one-to-one orthologous relationship. **Figure 4.1** depicts the statistics pertaining to the different one-to-many

orthologous relationships found for the remaining 5880 Livestock EST gene products for which putative orthologs could be identified.

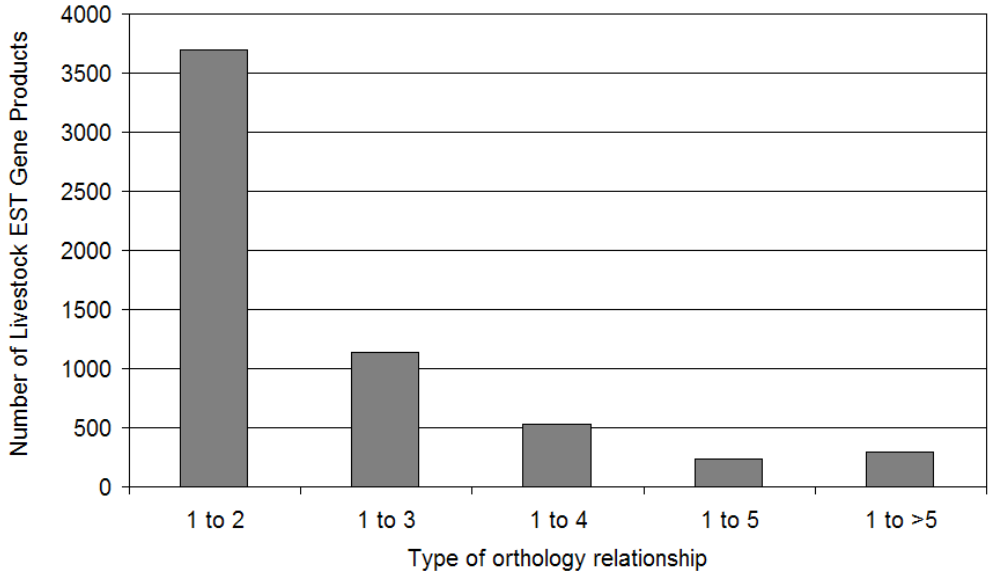


Figure 4.1: One-to-many orthologous relationship statistics for Livestock EST gene products.
One-to-many orthologous relationship statistics for 5880 of the 19775 Livestock EST gene products for which putative orthologs could be identified.

As the algorithm is catered to determine predicted function for Livestock EST gene products using the subtree/orthology approach, it is worth mentioning that a fraction of the one-to-many orthologous relationships from the bovine/porcine perspective could in fact be a part of many-to-many orthologous relationships when the whole protein family is taken into consideration.

Table 4.3 serves as a guide to identify the different species to which the sequences belong for the different phylogenetic tree examples discussed here.

Table 4.3: Key for identifying the different species using the prefix or suffix on the sequence identifiers of the vertebrate proteins and Livestock EST gene products.

Prefix/Suffix	Example sequence identifier prefix/suffix	Species
Suffix	_Bt	*Bos taurus*
Suffix	_Ss	*Sus scrofa*
Prefix	ENSP	*Homo sapiens*
Prefix	ENSMUSP	*Mus musculus*
Prefix	ENSRNOP	*Rattus norvegicus*
Prefix	ENSGALP	*Gallus gallus*
Prefix	ENSDARP	*Danio rerio*
Prefix	GSTENP	*Tetraodon nigroviridis*
Prefix	SINFRUP	*Takifugu rubripes*

Annotation transfer – one-to-one orthologous relationships

For Livestock EST gene products having a one-to-one putative orthologous relationship, the Livestock EST gene product was annotated with the function of the 'putative' ortholog.

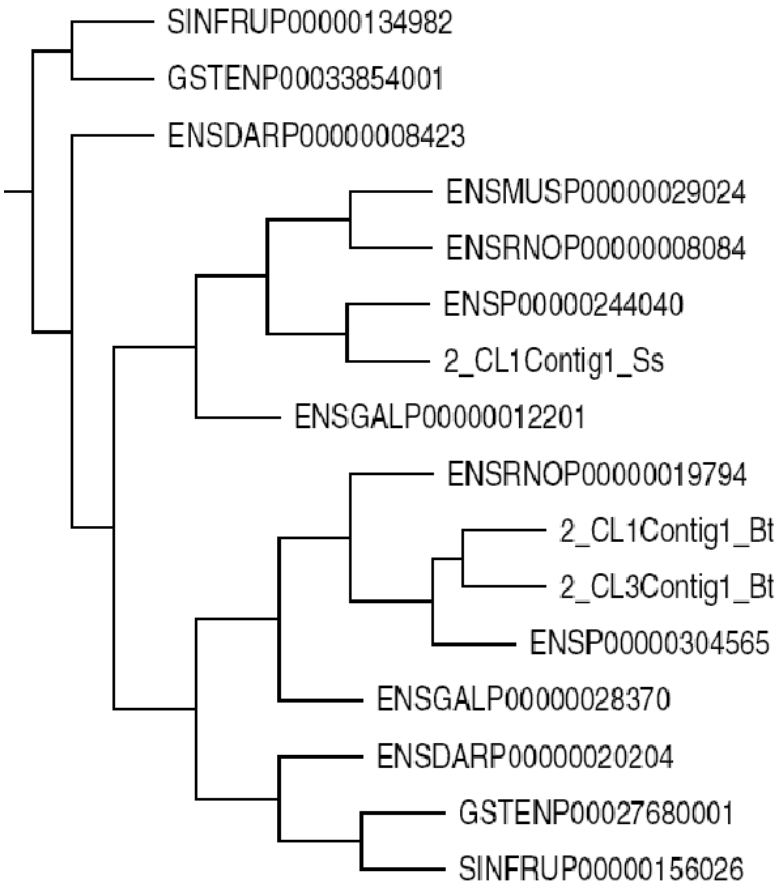


Figure 4.2: Phylogenetic tree for Protein Family 2 – a family comprised of 'Ras related' proteins.
The tree is rooted using the fugu protein (SINFRUP00000134982) as the outgroup. Orthology assignments for the three Livestock EST gene products (2_CL1Contig1_Ss, 2_CL1Contig1_Bt, 2_CL3Contig1_Bt) shown in this tree are made as detailed in the text.

Figure 4.2 depicts the phylogenetic tree for protein family '2' – a family comprised of 'Ras related' proteins. Protein Family 2 has three Livestock EST gene products (2_CL1Contig1_Ss, 2_CL1Contig1_Bt, 2_CL3Contig1_Bt), and proteins from all the other seven vertebrate species used in this study. The tree has been rooted using the fugu protein (SINFRUP00000134982) as the outgroup.

From the phylogenetic tree for protein family '2', the following orthologous relationships for the Livestock EST gene products were identified:

- porcine EST gene product 2_CL1Contig1_Ss is orthologous to the human protein ENSP00000244040 (Ras-related protein Rab-22A), hence annotated as "putative Ras-related protein Rab-22A".
- bovine EST gene product 2_CL1Contig1_Bt is orthologous to the human protein ENSP00000304565 (Ras-related protein Rab-31), hence annotated as "putative Ras-related protein Rab-31".
- bovine EST gene product 2_CL3Contig1_Bt is orthologous to the human protein ENSP00000304565 (Ras-related protein Rab-31), hence annotated as "putative Ras-related protein Rab-31".

The tree topology for Protein Family '2' (**Figure 4.2)** also highlights one of the salient aspects of the orthology assignment algorithm wherein it takes care of the scenario where there is duplication in one species after a speciation event. In such a scenario, the algorithm traverses an additional node toward the root in the phylogenetic tree and assigns orthology if other criteria are also met. This aspect is evident from the

subtree involving the two bovine EST gene products (2_CL1Contig1_Bt and 2_CL3Contig1_Bt) where there is a duplication event in bovine after speciation; hence the two bovine EST gene products have been assigned as orthologs to the human protein ENSP00000304565.

Bovine-porcine one-to-one orthologous combination

Interestingly, 5420 of the 13895 one-to-one orthologous relationships comprised of a bovine-porcine one-to-one orthologous combination. An example illustrating this is evident from two of the three orthologous relationships depicted in the phylogenetic tree for Protein Family '15208' (**Figure 4.3**). This is a protein family comprised of 'nuclear transport factor' like proteins; it is represented by three Livestock EST gene products and proteins from six of the other seven vertebrate species used in this study. The tree has been rooted using the fugu protein (SINFRUP00000135058) as the outgroup. From the bottom segment of phylogenetic tree for protein family '15208', one can deduce the bovine-porcine one-to-one orthologous relationship between the bovine EST gene product 15208_CL1Contig1_Bt and the porcine EST gene product 15208_CL1Contig1_Ss. (**Note**: The third Livestock EST gene product in this tree, 15208_CL2Contig1_Bt, is involved in a one-to-many orthologous relationship as it is orthologous to the human proteins ENSP00000339705 and ENSP00000218004. Annotation transfer in one-to-many orthologous relationships is discussed later in the text).

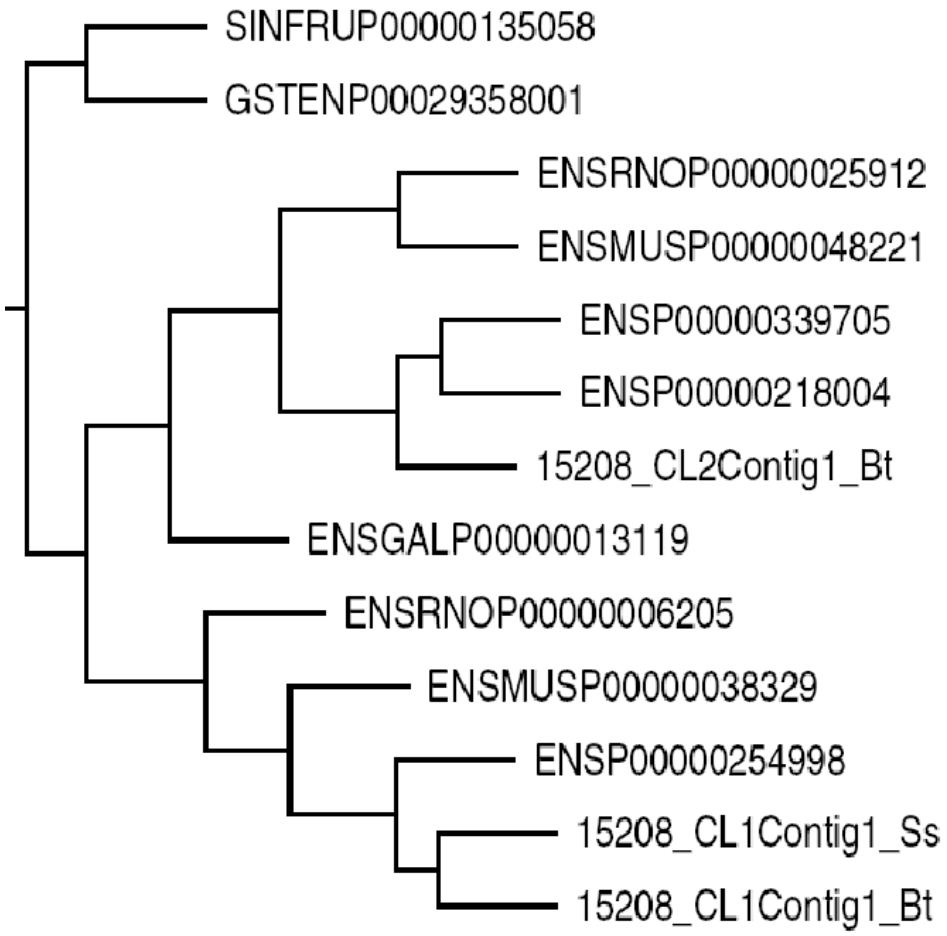


Figure 4.3: Phylogenetic tree for Protein Family 15208 – a family comprised of 'nuclear transport factor' like proteins.
The tree is rooted using the fugu protein (SINFRUP00000135058) as the outgroup. Orthology assignments for the three Livestock EST gene products (15208_CL2Contig1_Bt, 15208_CL1Contig1_Ss, 15208_CL1Contig1_Bt) shown in this tree are made as detailed in the text.

Annotation transfer – one-to-many orthologous relationships

After identifying putative orthologs for a particular bovine/porcine EST gene product using the 'subtree neighbors' approach, 5880 Livestock EST gene products were part of different one-to-many orthologous relationships. We had to come up with a strategy to choose one ortholog, from amongst the many orthologs identified, for annotation transfer. To do so, we devised a strategy which relies on identifying the "source" database identifiers from the "Descriptions" of the different Ensembl proteins identified as putative orthologs. Whenever possible, the 'Description' field for a given Ensembl protein lists the "source" database identifier from which the Ensembl protein was derived. When information about the database identifier was available, we first made a note of the source database and we made a choice of which database identifier to use based on this database order of precedence: UniProtKB [186], RefSeq [187], Ensembl (when the source identifier was not UniProtKB or RefSeq). We give preference to UniProtKB for the following reasons:

1. Whenever possible, all the protein products encoded by one gene in a given species are described within a single UniProtKB entry, including isoforms generated by alternative splicing, alternative promoter usage, and alternative translation initiation. In cases where some alternative splicing isoforms derived from the same gene share only a few exons, the isoforms have to be described in separate entries [188]. In our dataset, the isoform variants of the different Ensembl proteins are in the same protein family; hence, there might be instances

where an EST gene product involved in a one-to-many orthologous relationship might actually have the different orthologs mapped to the same UniProtKB entry.

2. UniProtKB is a secondary database, in that it collects information from primary databases, curates the information if necessary and makes it available. Information in primary databases (GenBank, GenPept) contain information submitted by the experimenter and cannot be changed by anybody other the person who submitted the information. As a result, the primary databases are essentially archival in nature and cannot correct errors, if any, that are found later. In other words, annotations from secondary databases like UniProtKB are more reliable in nature.

For the one-to-many orthologous relationship scenarios, the UniProtKB entry extracted from the 'Source' field of the Ensembl protein is used for annotating the Livestock EST gene product. Whenever the source database identifier for a given Ensembl protein was not UniProtKB, we would check to see if the Ensembl protein had a RefSeq identifier and then we use the Database Mapping tool [189] at UniProtKB to find the corresponding UniProt entry for the RefSeq identifier. If the RefSeq identifier did not map to a UniProt entry, we used the RefSeq entry for annotating the Livestock EST gene product. If we did not find a UniProt or a RefSeq cross-reference for the Ensembl protein, we ended up using the Ensembl protein itself for annotating the Livestock EST gene product.

The examples below illustrate how the above annotation transfer strategy was used for Livestock EST gene products involved in one-to-many orthologous relationships.

Example I: 'Protein Family 15208' – nuclear transport factor like proteins

Figure 4.3 depicts the phylogenetic tree for Protein Family '15208', comprised of 'nuclear transport factor' like proteins, and represented by three Livestock EST gene products and proteins from six of the other seven vertebrate species used in this study. One of the Livestock EST gene products, 15208_CL2Contig1_Bt, is involved in a one-to-many orthologous relationship as it is orthologous to the human proteins ENSP00000339705 and ENSP00000218004, which are isoforms. **Table 4.4** lists the descriptions of these two human proteins.

Table 4.4: Descriptions of Ensembl proteins identified as orthologs for 15208_CL2Contig1_Bt.

Ensembl protein	Description
ENSP00000339705	NTF2-related export protein 2. [Source: UniProtKB Q9NPJ8]
ENSP00000218004	NTF2-related export protein 2. [Source: UniProtKB Q9NPJ8]

As evident from **Table 4.4**, the two isoform variants ENSP00000339705 and ENSP00000218004 map to the same UniProtKB entry, Q9NPJ8. By the annotation transfer strategy described above, 15208_CL2Contig1_Bt is annotated as "putative

NTF2-related export protein 2" – from the "Description" field of the UniProtKB entry, Q9NPJ8.

Example II: 'Protein Family 23614' – metastatic lymph node (MLN) proteins

Figure 4.4 depicts the phylogenetic tree for Protein Family '23614', comprised of 'metastatic lymph node (MLN)' protein homologs. This protein family is represented by four Livestock EST gene products and proteins from six of the other seven vertebrate species used in this study. Two of the Livestock EST gene products, 23614_CL1Contig1_Ss and 23614_29253671_Bt, are involved in a one-to-one bovine-porcine orthologous relationship. The remaining two Livestock EST gene products, 23614_CL1Contig1_Bt and 23614_CL2Contig1_Ss, are part of one-to-many orthologous relationships. 23614_CL2Contig1_Ss is involved in a one-to-five orthologous relationship as it is orthologous to the human protein ENSP00000341409, the mouse proteins ENSMUSP00000042792 and ENSMUSP00000043328, and the rat proteins ENSRNOP00000012812 and ENSRNOP00000012820. 23614_CL1Contig1_Bt is involved in a one-to-six orthologous relationship as it is orthologous to 23614_CL2Contig1_Ss and the five putative orthologs identified for 23614_CL2Contig1_Ss. **Table 4.5** lists the descriptions of the five Ensembl proteins which were identified as orthologs to both 23614_CL1Contig1_Bt and 23614_CL2Contig1_Ss.

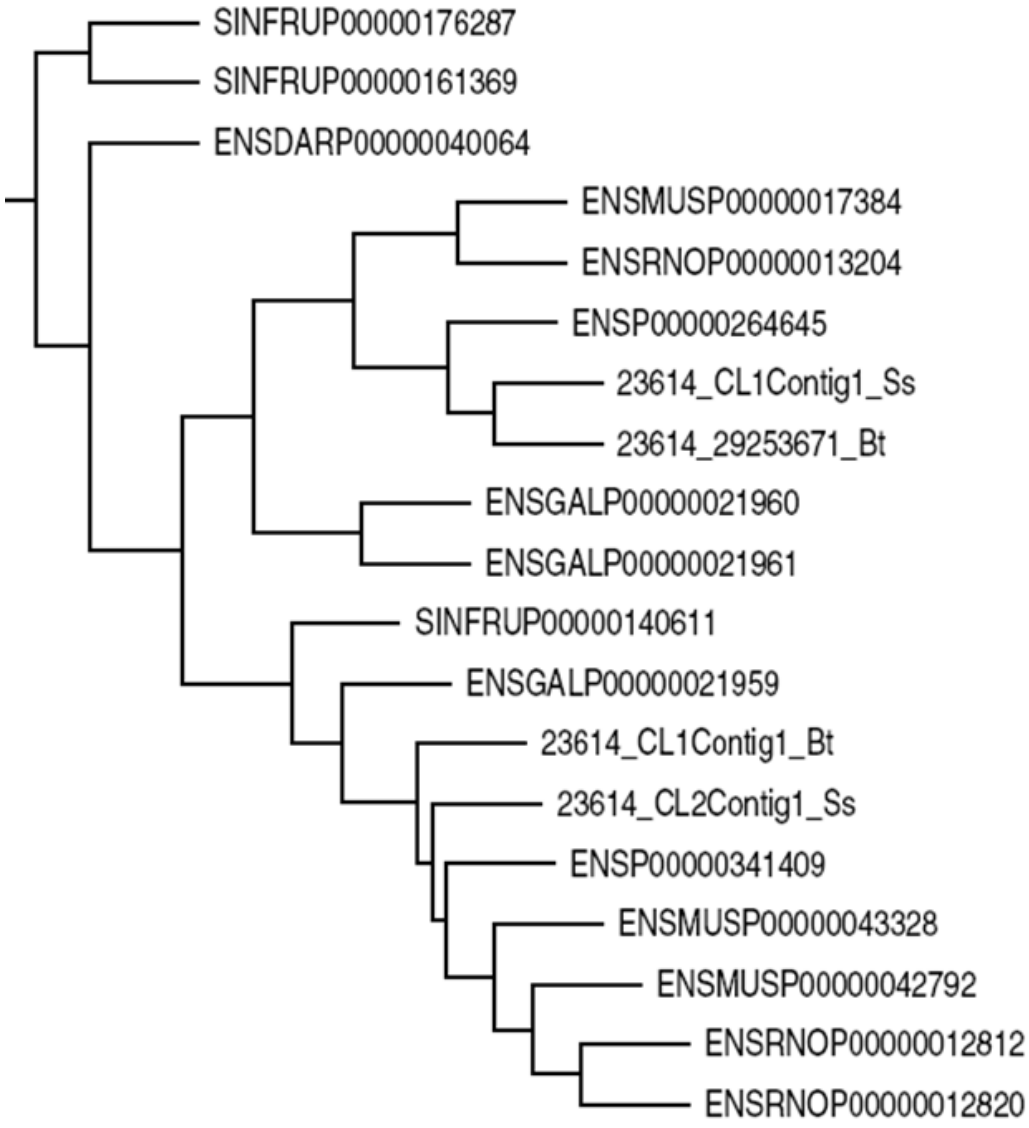


Figure 4.4: Phylogenetic tree for Protein Family 23614 – a family comprised of 'metastatic lymph node' protein homologs.
The tree is rooted using the fugu protein (SINFRUP00000176287) as the outgroup. Orthology assignments for the four Livestock EST gene products (23614_CL1Contig1_Ss, 23614_29253671_Bt, 23614_CL1Contig1_Bt, 23614_CL2Contig1_Ss) shown in this tree are made as detailed in the text.

Table 4.5: Descriptions of Ensembl proteins identified as orthologs for 23614_CL1Contig1_Bt and 23614_CL2Contig1_Ss.

Ensembl protein	Description
ENSP00000341409	PREDICTED: similar to RIKEN cDNA 4121402D02 [Source:RefSeq_peptide;Acc:XP_496217]
ENSMUSP00000042792	Cancer susceptibility candidate gene 3 protein [Source:UniProtKB Q8K3W3]
ENSMUSP00000043328	Cancer susceptibility candidate gene 3 protein [Source:UniProtKB Q8K3W3]
ENSRNOP00000012812	similar to RIKEN cDNA 4121402D02 [Source:RefSeq_dna;NM_001107048]
ENSRNOP00000012820	similar to RIKEN cDNA 4121402D02 [Source:RefSeq_dna;NM_001107048]

From **Table 4.5**, one can make the observation that three of the five putative orthologs for 23614_CL1Contig1_Bt and 23614_CL2Contig1_Ss are annotated as "similar to RIKEN cDNA 4121402D02" with the "source" field database being RefSeq. The remaining two putative orthologs are annotated as "Cancer susceptibility candidate gene 3 protein" with the "source" field database being UniProtKB. By using the annotation rules regarding database precedence mentioned above, we tried to identify UniProtKB mappings for each of the RefSeq identifiers from the above table, but none

of the RefSeq identifiers mapped to any UniProtKB entry. Hence, using the annotation transfer strategy mentioned above, 23614_CL1Contig1_Bt and 23614_CL2Contig1_Ss were annotated as "putative Cancer susceptibility protein" – from the "Description" field of the UniProtKB entry, Q8K3W3.

Example III: 'Protein Family 32278' – zinc finger proteins

Figure 4.5 depicts the phylogenetic tree for Protein Family '32278', comprised of 'zinc finger proteins'. This family is represented by two Livestock EST gene products and proteins from six of the other seven vertebrate species used in this study. One of the two Livestock EST gene products, 32278_CL8Contig1_Bt is involved in a one-to-one orthologous relationship with the human protein ENSP00000262630, which is a "Zinc finger and BTB domain-containing protein 32". ENSP00000262630 mapped to the UniProtKB entry Q9Y2Y4, which had the same description as the Ensembl protein. Since this is one-to-one orthologous relationship, 32278_CL8Contig1_Bt was annotated as "similar to human Zinc finger and BTB domain-containing protein 32" using the "Description" of the UniProtKB entry Q9Y2Y4. The other Livestock EST gene product in protein family 32278, 32278_CL2Contig1_Ss, is involved in a one-to-five orthologous relationship as it is orthologous to three human proteins (ENSP00000346516, ENSP00000352262 and ENSP00000333556), one mouse protein ENSMUSP00000002095, and one rat protein ENSRNOP00000020573. **Table 4.6** lists the descriptions of the five Ensembl proteins which are identified as orthologs for 32278_CL2Contig1_Ss.

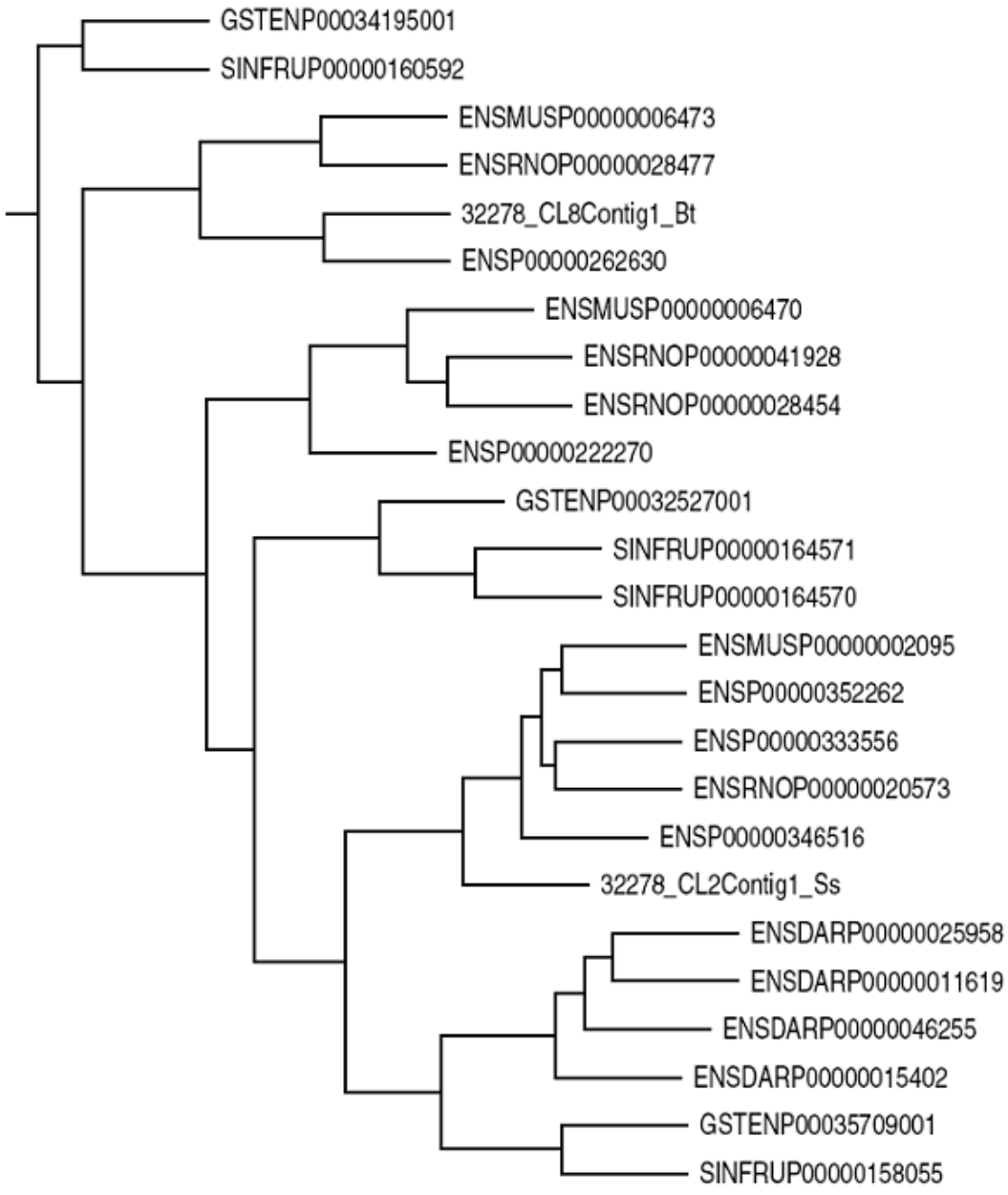


Figure 4.5: Phylogenetic tree for Protein Family 32278 – a family comprised of 'zinc finger' proteins.
The tree is rooted using the fugu protein (SINFRUP00000160592) as the outgroup. Orthology assignments for the two Livestock EST gene products (32278_CL8Contig1_Bt and 32278_CL2Contig1_Ss) shown in this tree are made as detailed in the text.

Table 4.6: Descriptions of Ensembl proteins identified as orthologs for 32278_CL2Contig1_Ss.

Ensembl protein	Description
ENSP00000346516	Zinc finger protein HRX [Source:UniProtKB Q03164]
ENSP00000352262	Zinc finger protein HRX [Source:UniProtKB Q03164]
ENSP00000333556	Zinc finger protein HRX [Source:UniProtKB Q03164]
ENSMUSP00000002095	Zinc finger protein HRX [Source:UniProtKB P55200]
ENSRNOP00000020573	myeloid/lymphoid or mixed-lineage leukemia [Source:RefSeq_peptide; NP_001101609]

From **Table 4.6**, the consensus description for four of the five putative orthologs is "Zinc finger protein HRX"; each of these four orthologs had UniProtKB as the "source" field database. The other putative ortholog (ENSRNOP00000020573) had the description "myeloid/lymphoid or mixed-lineage leukemia" and was sourced from a RefSeq entry NP_001101609. This RefSeq entry could not be mapped to any UniProtKB entry. Hence, using the annotation transfer strategy mentioned above, 32278_CL2Contig1_Ss was annotated as "putative Zinc finger protein HRX" – from the "Description" field of the UniProtKB entry, Q03164.

The annotation transfer strategy for the different one-to-many orthologous relationship examples discussed above gives precedence to UniProtKB cross-references of the putative orthologs, where available, for transferring the annotation of one of the

putative orthologs to the bovine/porcine EST gene product. This UniProtKB preference is given irrespective of the number of putative orthologs that had UniProtKB cross-references. To elaborate, in **Table 4.5** (**Example II**) above, the Livestock EST gene product, 23614_CL2Contig1_Ss, was part of a one-to-five orthologous relationship. Two of the five putative orthologs had UniProtKB cross-references and the rest of the orthologs had RefSeq cross-references. We chose to use the ortholog having a UniProtKB cross-reference for the transfer of annotation to the Livestock EST gene product in question. If the putative ortholog does not have a UniProtKB cross-reference, we give preference to those orthologs that have RefSeq cross-references and if RefSeq cross-references are not available, one of the Ensembl protein(s) identified as putative ortholog is used for transfer of annotation.

For the purposes of annotation transfer in one-to-many orthologous scenarios, we believe that it really should not matter whether on not the putative ortholog had a UniProtKB or RefSeq cross-reference. We say so because the 'subtree' approach used in our orthology algorithm identifies the distinct subfamily to which a Livestock EST gene product belongs; since function is conserved within orthologous subfamilies [82], any of the orthologs in that subfamily can be used for 'reliable' annotation transfer. By first short listing putative orthologs that had UniProtKB cross-references and using them for annotation transfer, we are only enriching the quality of the annotation for the reasons discussed earlier.

Phylogenomic annotations – can it predict functions for all sequences?

It is well-known that the phylogenomic method cannot predict functions for all sequences [53], but the predictions that are obtained tend to be accurate; hence, the annotations can be transferred reliably. In our analysis, we had 86,312 Livestock EST gene products to begin with, of which only 50,119 sequences passed our multiple sequence alignment (MSA) editing criteria. From these 50,119 sequences, we were able to identify predicted functions for only 19,775 Livestock EST gene products at the end of our phylogenomic annotation pipeline. Sequences get excluded from the phylogenomic pipeline at the following different stages:

1. ***Protein families excluded from phylogenetic analysis***: we chose to run the PHYLIP pipeline only on those protein families which have ≤50 sequences in the edited MSA.
2. ***Consense step in PHYLIP pipeline***: The 'majority rule extended' (MRe) method from the 'Consense' step in our PHYLIP pipeline excludes some sequences from the final tree that did not match its tree resolving criteria.
3. ***Tree ambiguity in our subtree orthology assignment***: If any branch of the phylogenetic tree contains at least one sequence of the 'outgroup' species OR at least one sequence of the same species as the sequence in question, it denotes ambiguity in the tree topology pertaining to the sequence in question; hence the sequence in question is discarded.

Currently, there do not exist genomic-scale error-free 'ortholog gold standard' datasets [190] and this makes it difficult to analyze and evaluate the performance of the orthology algorithm. Benchmark datasets like PREFAB [174], BALiBASE [191] and SABMark [192] are available for evaluation of multiple sequence alignment programs. Likewise, in the protein structure prediction field, the availability of challenging benchmark datasets from the Protein Structure Prediction Center [193] in the international biennial CASP event [194] helps in the assessment of the protein structure prediction methods. Analogous to the MSA and protein structure prediction benchmark datasets, the accuracy of phylogenomic methods can be assessed only if rigorously validated biological data is available [77].

Future work

Future work would include making the phylogeny based function annotations for bovine/porcine EST gene products accessible from the "Livestock EST Gene Family Database". Cattle and pig are more closely related to each other than to the other vertebrate species used in this study. It would be interesting to know the different cases in which a protein family has at least one bovine and one porcine EST gene product where these are not orthologous – using a 'best hit' method in this scenario for bovine vs porcine would have resulted in function prediction and assignment that is not based on orthology, which would have been erroneous. Comparing the function predictions made using the 'best hit' method with the predictions from the phylogeny based approach would reveal instances where the 'best hit' was not used for function prediction and

annotation transfer in the phylogeny based approach. Future work on this front could address the issue as to why the 'best hit' sequence was not the closest neighbor in the phylogeny based approach.

CONCLUSIONS

In this chapter, we have discussed the development and implementation of a phylogenomic annotation pipeline to make computational predictions of the functions encoded in *Bos taurus* (bovine) and *Sus scrofa* (porcine) expressed sequence tag (EST) datasets. As part of the TAMUClust EST clustering pipeline, *Bos taurus* and *Sus scrofa* ESTs were grouped into gene families using the Ensembl vertebrate protein family clusters. The different EST consensus sequences and proteins in each protein family were subject to a pipeline involving multiple sequence alignment and phylogenetic tree construction. This resulted in subdivisions of groups containing orthologs and paralogs, each group representing distinct subfamilies. Following this, the tree topology was analyzed and the functions of the uncharacterized EST gene family members were predicted using a subtree neighbors approach. This approach involves identifying the subgroup having the sequence (bovine/porcine) whose function is being predicted, and assigning predicted function based on the most recent diverging sequence from a different species in that subgroup. Our approach requires estimating a new phylogenetic tree for each protein family, and the trees need to be recomputed each time a new sequence is added to the family.

For phylogenomic inference to be accurate, all sequences in the set must share the same domain architecture [68]. In this study, we ensure that this requirement is taken care of by the clustering algorithm when Ensembl vertebrate proteins are clustered into protein families. The protein families generated are non-redundant, comprehensive set of full-length protein clusters with homogeneous domain architecture within clusters and the pairwise sequence identity between any two proteins within each cluster is at least 55%. Phylogenetic tree construction of these full-length protein clusters with homogeneous domain architecture enhances the specificity of function annotation for phylogenomic inference of function [68].

The phylogenomic approach uses phylogenetic trees to determine orthology between homologs; this requires that the phylogenetic tree topology used as a basis for inference of function is correct. We address this by performing bootstrap phylogenetic analysis and obtaining the consensus tree in our PHYLIP pipeline. We use the 'majority rule extended' (MRe) method in the 'Consense' step in PHYLIP to obtain the consensus tree. The MRe method first includes all sequences that appear in more than 50% of the trees and then considers the other sets of sequences in order of the frequency with which they have appeared, adding to the consensus tree any sequence compatible with the tree until the tree is fully resolved. In doing so, the MRe method may exclude some sequences from the final tree as they did not match the criteria.

We have used the phylogenomic inference method to make computational predictions of the functions encoded in *Bos taurus* (bovine) and *Sus scrofa* (porcine) expressed sequence tag (EST) datasets. We were able to identify putative functions for

~23% of the Livestock EST gene products in our dataset. This, to our knowledge, is the first time that phylogenomic inference has been used in predicting functions for EST gene products. The livestock community would largely benefit from the function predictions of the uncharacterized EST gene family members in cattle and pig from this analysis. By designing experiments based on these predictions, the number of direct experiments required to verify function would be drastically reduced.

CHAPTER V

SUMMARY

Prediction of functions of genes in a genome is one of the key steps in all genomic sequencing projects. Sequences that carry out important functions are likely to be conserved between evolutionarily distant species and cross-species comparisons help identify these biologically active and conserved regions.

Obtaining insights about the function encoded in an organism by means of computational function predictions using EST datasets forms the crux of the work in this dissertation. In this dissertation, the EST clusters from *Bos taurus* and *Sus scrofa* EST datasets were utilized to make computational function predictions. We have shown here how some of the tools for comparative genomics like sequence homology searching, annotation transfer, phylogenetic analysis can be used to predict the functions of the sequence with unknown function.

The TAMUClust method described in this dissertation uses a protein framework to cluster the ESTs and in doing so, it helps avoid many of the frequently encountered problems in oligo design - generating oligos with redundant information, and designing oligos based on chimeric ESTs. The performance of the TAMUClust *Bos taurus* EST clusters and the *Bos taurus* Gene Indices (BTGI) clusters were compared by using bovine ESTs aligned to the bovine genome assembly as a gold standard. Findings from the gold standard comparisons reveal that TAMUClust and BTGI are similar in performance. Comparisons of TAMUClust and TGI with predicted bovine gene models reveal that both datasets are similar in transcript coverage.

The Ensembl protein families from seven vertebrate species were used as a framework to cluster and assemble the cattle/pig EST datasets, resulting in 'EST consensus sequences' (contigs/singletons) that represent putative genes. These EST consensus sequences were assigned predicted functions by transferring annotations of the Ensembl vertebrate protein(s) they get grouped with following sequence homology searches. The 'Livestock EST Gene Family Database' available at http://genomes.arc.georgetown.edu/cgi-bin/search_livestock_est_gene_family.cgi houses the annotations. The database has a user-friendly web interface and can be searched in a number of ways: Cattle/Pig EST GenBank identifiers (GI or accession), Gene Ontology accession to obtain information about the vertebrate protein family, related EST members and other annotations. The Livestock EST Gene Family Database is the first of its kind where ESTs from different species (cattle and pig in this case) are grouped together in the same gene family. This information comes in handy when a researcher wants to get a listing of ESTs from different species performing the same function. The Livestock EST Gene Family Database is also the first of its kind that incorporates a search strategy that transitively annotates EST gene products with a GO accession and all its descendants. Searching by GO accession on the Livestock EST Gene Family Database will retrieve the complete descendant information for the GO accession and give a mapping of the different cattle and pig EST gene products associated with the complete GO descendant tree starting from the GO Accession being searched for. This search feature is useful for researchers wanting to obtain a complete profile of the cattle and pig EST gene products associated with a particular GO Accession. The Livestock

EST Gene Family Database and the annotations housed would help researchers to deal with the volume of information, and to utilize the information embedded in the gene expression data gathered from assembling the EST datasets.

Phylogenomic inference of function combines evolutionary and comparative genomic analysis into a single composite approach, and the function predictions using this method are more accurate. This approach relies on inferring the function of an unknown sequence (protein) in the larger context of a protein family based on evolutionary relationships. Using the phylogenomic method, bovine/porcine EST consensus sequences belonging to a protein family were grouped with other proteins in the family, and subject to multiple sequence alignment followed by phylogenetic analysis. This resulted in subfamilies with each subfamily representing a functionally and evolutionarily distinct group of sequences with similar functions. By analyzing the tree topology using a subtree neighbors approach, uncharacterized bovine/porcine EST gene products were assigned predicted function based on the proteins from the subfamily in which they got grouped with. To our knowledge, this is the first time that phylogenomic analysis, and that too on a large scale, has been used in predicting functions for EST gene products. The annotations for the uncharacterized EST gene family members in cattle and pig developed using the phylogenomic annotation pipeline provide the livestock community with a valuable resource to verify function by designing experiments based on the predictions.

Experimental characterization of sequences, function prediction of sequences and genomic sequencing can be thought of a three-horse race with the leader being ‘genomic

sequencing' followed by 'function prediction of sequences' and 'experimental characterization of sequences' lagging far behind the other two. Computational analysis of genomes keeps yielding interesting function predictions, even years after the publication of the sequence; what is most often lacking is systematic experimental testing of these predictions. In addition, there do not exist genomic-scale error-free 'ortholog gold standard' datasets and this makes it difficult to analyze and evaluate the performance of orthology algorithms. The accuracy of the function prediction methods can be assessed only if rigorously validated biological data is available, *a la*, the MSA and protein structure prediction benchmark datasets that help evaluate the performances of the multiple sequence alignment and the protein structure prediction methods, respectively.

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