COMPARATIVE GENOMICS: IDENTIFYING SIMILARITIES AND DIFFERENCES ACROSS THREE LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*

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ABSTRACT
Leafhoppers are considered the second most important vector of agricultural diseases. We examined the gene expression across three leafhopper species, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis),* the blue-green sharpshooter (BGSS; *Graphocephala atropunctata)*, and the black-winged sharpshooter (BWSS; *Oncometopia nigricans),* which are vectors of the plant-infecting bacterium, *Xylella fastidiosa*, which causes Pierce's disease (PD) of grapes. The use of genomic data is providing new information on the biology and relatedness of these and other leafhoppers. Using a genomics approach has also advanced the understanding of leafhopper immunity, pathology, and development. As new developments in genomics and RNAi methodologies emerge, researchers will be able to use this genetic information to design highly specific and effective management tools to reduce either leafhopper populations, and/or leafhopper-transmitted diseases. The importance of these leafhoppers as the vectors of PD, the abundance of Expressed Sequence Tags (ESTs) produced for each, and their differences in host plant preferences, provide an excellent opportunity to conduct comparative examination of these leafhoppers. Several cDNA libraries which had been made from adult GWSS, BGSS, and BWSS, plus nymphs, and tissues, provided a resource totaling almost 50,000 ESTs. When assembled, we obtained ~5,000 specific transcripts for each species for comparison. This is approximately one-third of all the predicted active genes available, as other insect genomes have demonstrated ~15,000 total genes. These were used for analyses between these species as well as for larger analysis to known genomes. Further analyses were conducted *in silico* using software programs available online Internet Resources, NCBI, EXPASY, and others to compare assembled data, predict proteins and compare them to the broader scope of insect genomes.

Many other genes of interest which have various functions in leafhopper biology and physiology have also been identified but are not reported herein. The EST sequences reported in this study have been deposited in GenBank's dbEST (see references: Hunter 2005, 2006, 2007).

INTRODUCTION
Sharpshooter leafhoppers are vectors of a number of economically important destructive plant diseases caused by the plant infecting bacterium, *Xylella fastidiosa (Xf)*. Understanding how these leafhoppers interact with their host plants and the pathogens they transmit is key to developing new management strategies against Pierce's disease (PD). Advances in genomic sequencing now permits researchers to examine thousands of genes which leafhoppers depend during feeding, development, and which are associated with disease acquisition and transmission. We compared the available genetic data for three leafhopper species, *Homalodisca vitripennis* (glassy-winged sharpshooter; GWSS)*, Graphocephala atropunctata*, and *Oncometopia nigricans,* (Hunter 2003, Hunter et al., 2005, 2006, 2007) which are vectors of the plant-infecting bacterium, *Xf,* which causes PD of grapes, and other 'scorch-like' diseases in other woody crops. (Hopkins and Purcell, 2002).

Sharpshooter leafhoppers, belong to the insect order Hemiptera, and feed primarily from the plant xylem, with minor amounts of feeding from the mesophyll and phloem (Backus and Hunter 1989, Hunter and Backus 1989). Xylem unlike plant phloem does not contain large amounts of sucrose and amino acids. Amino acids and soluble proteins are the primary nitrogen nutrients in xylem fluid (Andersen et al., 1989,1992). The dietary nitrogen impacts survival, growth, and reproduction of phytophagous insects (Bi et al., 2005). Consequently, the nutritionally dilute chemistry of the xylem fluid is a probable cause of the extremely high rate of feeding by leafhoppers (Brodbeck et al., 2004). The reported ability of leafhoppers to physiologically assimilate at least 99% of the amino acids, organic acids, and sugars is an evolutionary adaptation in response to their unique food source (Andersen et al., 1989; Brodbeck et al., 1999, 2004, Redak et al., 2004). This adaptation has a genetic basis and using genomics we can start to identify many of the genetic components which are key to leafhopper feeding, digestion, and growth.

Thus an important part of our project involves gaining a better understanding of the digestive physiology of leafhoppers vectors of PD. Genomics is providing the molecular tools needed to investigate the role proteins and peptides play in leafhopper nutrition. The increased comprehension of leafhopper digestive physiology also provides a more thorough understanding of the nutritional requirements, effects of host plants, and will provide the information needed to produce more effective mass rearing methods for application in the production of leafhoppers for parasitoid production.

Although the full extent to which leafhoppers, like GWSS, utilize host plant proteins is not understood, the ability to utilize xylem proteins as a nutrient source depends heavily on the presence and activity of the kinds of proteases within the digestive tract. Therefore, identifying these proteases and other enzymes will influence current tenets and advance our understanding of the underlying mechanisms of leafhopper digestive physiology. The use of expression libraries is a timely approach to understanding the genetic basis of proteolytic activity as it relates to insect development (Hunter et al., 2003; Sabater-Munoz et al., 2006), feeding and digestion (Colebatch et al., 2002; Coudron et al., 2007).

OBJECTIVES

Apply comparative genomics to advance the understanding of leafhopper biology, digestion, and development. These data support development and application of emerging management strategies which rely on an understanding of leafhopper genetics.

RESULTS

The datasets were produced in the Hunter lab (2005-2007), with sequencing performed at the Genomic lab, ARS, U.S. Horticultural Research Lab, Ft. Pierce, FL.

Sequence Analysis: Base calling was performed using TraceTuner™ (Paracel, Pasadena, CA) and low-quality bases (quality score <20) were stripped from both ends of each Expressed Sequence Tag (EST). Quality trimming, vector trimming, and sequence fragment alignments were executed using Sequencher™ software (Gene Codes, Ann Arbor, MI). Sequencher contig assembly parameters were set using a minimum overlap of 50 base pairs (bp) and 90% identity. Contigs joined by vector sequence were flagged for possible misassembly and manually edited. Putative sequence identity was determined based on BLAST similarity searches using the NCBI BLAST server (www.ncbi.nlm.nih.gov) with comparisons made to both non-redundant nucleic acid and protein databases using BLASTN and BLASTX, respectively (Altschul et al., 1997). Matches with an E-value ≤-10 were considered significant and were classified according to the Gene Ontology (GO) classification system (Schäffer et al., 2001). A partial list of ~29 transcripts (**Table 1**) show homologous matches between leafhoppers, and the E-values showing relative homology. As the value approaches zero, the more significant the homology match (yellow), as sequences diverge, having less homology the values become farther away from zero, approaching a positive number (*note:* all values in Table 1 under E-value are negative or zero).

Digestive Enzymes: Aminopeptidases, several cathepsin L–like cysteine proteases, and other proteases have been identified in these leafhoppers which are also in other piercing-sucking feeding insects (Foissac et al., 2002, Wright et al., 2006, Zhu et al., 2003). In aphids, a cathepsin B protease has been shown to be constitutively expressed in all aphid individuals, suggesting gene duplication and evolution of a novel biological function of cathepsin B in the aphid lineage (Houseman and Downe 1983). Cathepsin B proteases were also identified in these leafhoppers and may show similar duplication.

Cleavage of food proteins into peptides and amino acids is an important process for which an array of proteases of different substrate specificity and enzymatic activities are produced in the alimentary tract and are involved in protein digestion (Terra et al., 1996, Sajid and McKerrow 2002). Gene duplications relevant to biological requirements such as those which encode digestive proteases, have been documented in: lepidopteran insects (Chougule et al. 2005), coleopteran insects (Zhu-Salzman et al. 2003; Brown et al. 2004), parasitic helminths (Dvora´k et al. 2005), and will most likely be found to have occurred within leafhoppers. The number of genes associated with leafhopper biology continues to expand as more genetic information is produced and compared from different species of leafhoppers (**Figure 1**). Annotation of these data advances current understanding of leafhopper biological pathways while providing clues to the genetic basis of such processes in insect-pathogen, and insect-plant interactions (**Figures 2 and 3**). The availability of genomic data for these leafhoppers continues to increase, thus uses of the current data provides a solid foundation for future studies in leafhopper functional genomics.

CONCLUSIONS

The information gained from this study provides the first investigation using comparative genomics of the transcriptomes from three leafhopper vectors of PD of grapes: *H. vitripennis, G. atropunctata*, and *O. nigricans*. Amino acid sequence comparisons BLASTX, BLASTP with other known proteins relies on conserved motifs of specific domain(s), NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blast). *In silico* analysis based on protein domains is a widely accepted method which continues to increase in quality and demonstrates the application of Bioinformatics to address many biological questions. Many of the discoveries made in other insects, such as *Drosophila*, Honey Bee, or Lepidopteran species, can be applied within the Hemiptera when the same genetic transcripts can be identified. For example, we increased our

understanding of the roles and pathways of heat shock proteins in leafhoppers by examining the data completed in Locusts, Flies, and Nematodes. The same is true for digestive enzymes.

The increasing application of transcriptional data is leading the way in the development of new strategies to reduce plant diseases and their insect vectors. Application of RNAi against a wide range of insect species from spruce budworm to whiteflies are viewed as the future in insect pest control, and many new methods which incorporate the use of native endophytic bacteria and/or viruses as the mechanism for delivery or expression of dsRNA within plants are being widely evaluated. The main advantages of applying genomic data in this manner to solve agricultural problems is that the plants are not 'transformed', thus the quality of the crop is not altered, saving time, money, and reducing the effort needed to find solutions to many emerging devastating agricultural problems. Collectively, these genetic sequences provide the foundation needed for further functional genomic studies which will enable the development of more biorational management strategies to reduce losses from the diseases spread by these and other leafhopper pests.

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Table 1. **Partial Comparison of cDNA's in three leafhopper species**, *Homalodisca vitripennis, Graphocephala atropunctata*, and *Oncometopia nigricans*. Analysis using BlastX, Values approaching zero are more significant in sequence identities (**Yellow**). Genes which have more variability **(Blue)**. Sequence homology was greater between *Homalodisca* and *Oncometopia* than to *Graphocephala*, which supports current taxonomy separating these leafhoppers. Only a partial list is shown for sequences within Molecular Function.

Accession	EC #	BLAST DeKegg Ids	GO TYPE	GO1	GO2	GO3	HC_ID	E-value (HC)	GA_ID	E value (GA)	ON_ID	-value (ON)
Q7QEH5		AgCP7442	Molecular Function	motor activity	structural molecu	structural	WHMg 2758	0	WHGA067-6_	5.4086E-102	WHON042_E12	1.1E-134
Q86GF8		Hypothetic Citrate cycle (TCA cycl	Molecular Function	aconitate hydratase	mitochondrion	tricarboxyli	WHMg 2485	0	WHGA2354	7.01792E-63	WHON0097	0
Q6PPI6		Putative cytoplasmic actin A3a1	Molecular Function	motor activity	structural molecu	structural	WHHC51531	0	WHGA2663	4.55082E-67	WHON0017	0
Q6RFY9	[3.6.5.3]	Putative elongation factor 1-alpha	Molecular Function	translation elongatio	GTP binding	cytoplasm	WHHC51398	0	WHGA0038	0	WHON0147	0
Q6PPI5		Putative muscle actin	Molecular Function	motor activity	structural molecu	structural	WHHC51395	0	WHGA2643	1.92228E-73	WHON0021	0
Q7PMH3	1.6.5.3	ENSANGP Ubiquinone biosynthes	Molecular Function	iron ion binding	electron transpor	mitochond	WHHC2058	0	WHGA1900	8.2498E-107	WHON1268	0
Q7PPE7	2.7.1.40	ENSANGP Glycolysis / Gluconeog	Molecular Function	magnesium ion bind	pyruvate kinase	glycolysis	WHHC0110	0	WHGA2158	1.54504E-62	WHON0828	2.1E-138
Q6PPH2		Putative activated protein kinase (	Molecular Function	receptor activity	kinase activity		WHHC2042	0	WHGA006-44_	1.6435E-120	WHON1478	1.98E-48
Q6PPI6		Putative cytoplasmic actin A3a1	Molecular Function	motor activity	structural molecu	structural	WHHC0062	0	WHGA2663	4.55082E-67	WHON0017	0
Q6PPH9	1.14.19.1	Putative delta-9 desaturase 1	Molecular Function	stearoyl-CoA 9-desa	iron ion binding	endoplasm	WHHC0255	0	WHGA0206	0	WHON0283	0
Q6RFY9	3.6.5.3	Putative elongation factor 1-alpha	Molecular Function	translation elongatio	GTP binding	cytoplasm	WHHC0034	0	WHGA0038	0	WHON0147	0
Q6PPI0	4.1.2.13	Putative fru Glycolysis / Gluconeog	Molecular Function	fructose-bisphospha	glycolysis	lyase activ	WHHC0177	0	WHGA1213	0	WHON0128	0
Q6PPI5		Putative muscle actin	Molecular Function	motor activity	structural molecu	structural	WHHC0074	0	WHGA2643	1.92228E-73	WHON0021	0
Q6PPI1		Putative rhodopsin	Molecular Function	rhodopsin-like recep	receptor activity	G-protein	WHHC0120	0	WHGA051-49_	3.1982E-122	WHON0281	0
Q6PPI3		Glyceralde Glycolysis / Gluconeog	Molecular Function	glyceraldehyde-3-ph	glucose metabol	glycolysis	WHMg 1552	4.3383E-168	WHGA0261	1.9645E-169	WHON0243	4.9E-173
Q6PPH2		Putative activated protein kinase (	Molecular Function	receptor activity	kinase activity		WHHC52008	4.6847E-166	WHGA2745	9.75547E-72	WHON1478	1.98E-48
Q6PPI0		Putative fr Glycolysis / Gluconeog	Molecular Function	fructose-bisphospha	glycolysis	lyase activ	WHMg 2297	2.3954E-147	WHGA1213	0	WHON013_H01	2.59E-19
Q17083		Vitellogenin precursor	Molecular Function	lipid transporter activ	lipid transport	nutrient re	WHHC0041	3.3648E-143	WHGA019-70_	2.47424E-39	WHON0059	1.1E-105
Q9VFF0		CG3731-PA	Molecular Function	metalloendopeptidas	proteolysis		WHHC52247	5.2528E-142	WHGA085-15_	4.12314E-65	WHON036_H10	5.27E-30
Q7PXA8		AgCP12715	Molecular Function	catalytic activity	GTP binding	tricarboxyli	WHHC1385	8.5228E-136	WHGA2684	2.53721E-45	WHON007_F09	2.43E-73
Q7PF83		ENSANGP00000024398	Molecular Function	electron transporter	iron ion binding	tricarboxyli	WHHC2462	1.1902E-129	WHGA1354	1.55212E-69	WHON030_F08	2.06E-64
Q7PPE7	[2.7.1.40]	ENSANGP00000021580	Molecular Function	magnesium ion bind	pyruvate kinase	glycolysis	WHSg044_B07	7.9038E-108	WHGA2158	1.54504E-62	WHON0828	2.1E-138
Q7PPE7		ENSANGP Glycolysis / Gluconeog	Molecular Function	magnesium ion bind	pyruvate kinase	glycolysis	WHMg 2868	2.4039E-107	WHGA2158	1.54504E-62	WHON0828	2.1E-138
Q9VFF0		CG3731-PA	Molecular Function	metalloendopeptidas	proteolysis		WHMg046_G12	6.9007E-106	WHGA085-15_	4.12314E-65	WHON036_H10	5.27E-30
Q9V4E0		CG1970-PA	Molecular Function	electron transporter	mitochondrion	electron tra	WHSg031_H08	6.3715E-104	WHGA2174	1.50812E-85	WHON032_H08	4.29E-96
Q17083		Vitellogenin precursor	Molecular Function	lipid transporter activ	lipid transport	nutrient re	WHHC0115	4.2689E-103	WHGA1453	4.34174E-57	WHON0059	1.1E-105
Q6PPI2		Putative ferritin GF2	Molecular Function	binding	iron ion transport	iron ion ho	Contig 1413	3.50913E-95	WHGA0709	7.98921E-91	WHON0085	2.56E-92
Q7QB64		AgCP2476	Molecular Function	malic enzyme activit	malate metabolis	oxidoreduc	WHSg041_F10	3.9499E-95	WHGA1635	5.71834E-84	WHON034_G07	5.85E-93
Q6PPI2		Putative ferritin GF2	Molecular Function	binding	iron ion transport	iron ion ho	WHMg 1739	4.47344E-95	WHGA0709	7.98921E-91	WHON0085	2.56E-92

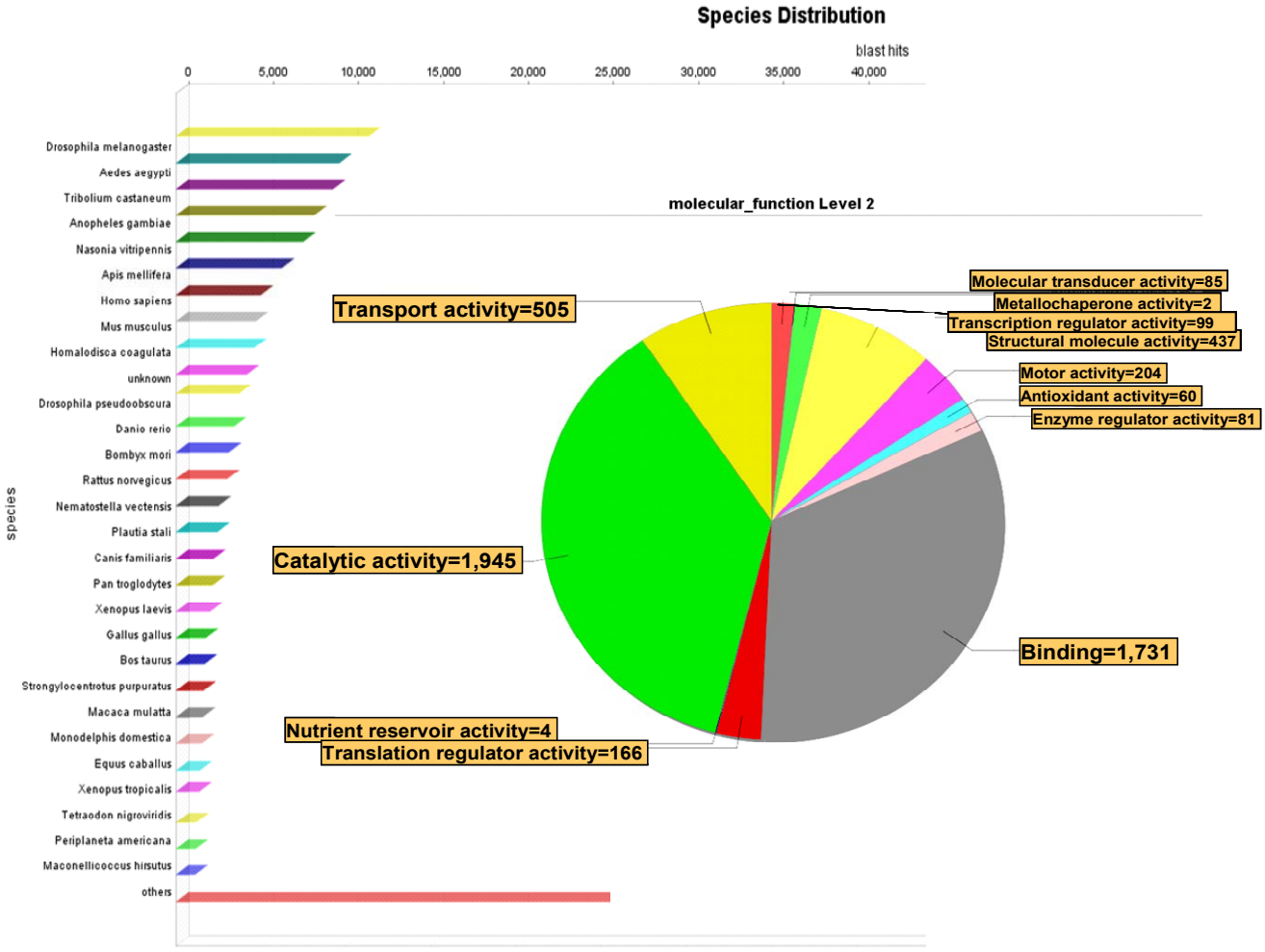

Figure 1. Composite figure showing distribution of *Homalodisca vitripennis* transcripts across other species (along left), with the top 6 species homologies being in these insects whose genomes have been completed: *Drosophila melanogaster*, *Aedes aegyptii*, *Tribolium castaneum, Anopheles gambiae, Nasonia vitripennis, and Apis mellifera.* **Molecular functions** of transcripts gave the greatest number within: Catalytic activity=1,945; Binding=1,731; and then transporter activity=505. Broad Categories. Represents EST's from three cDNA libraries, Adults, 5th instar, and Midgut. *H. vitripennis*, (Blast2GO analysis).

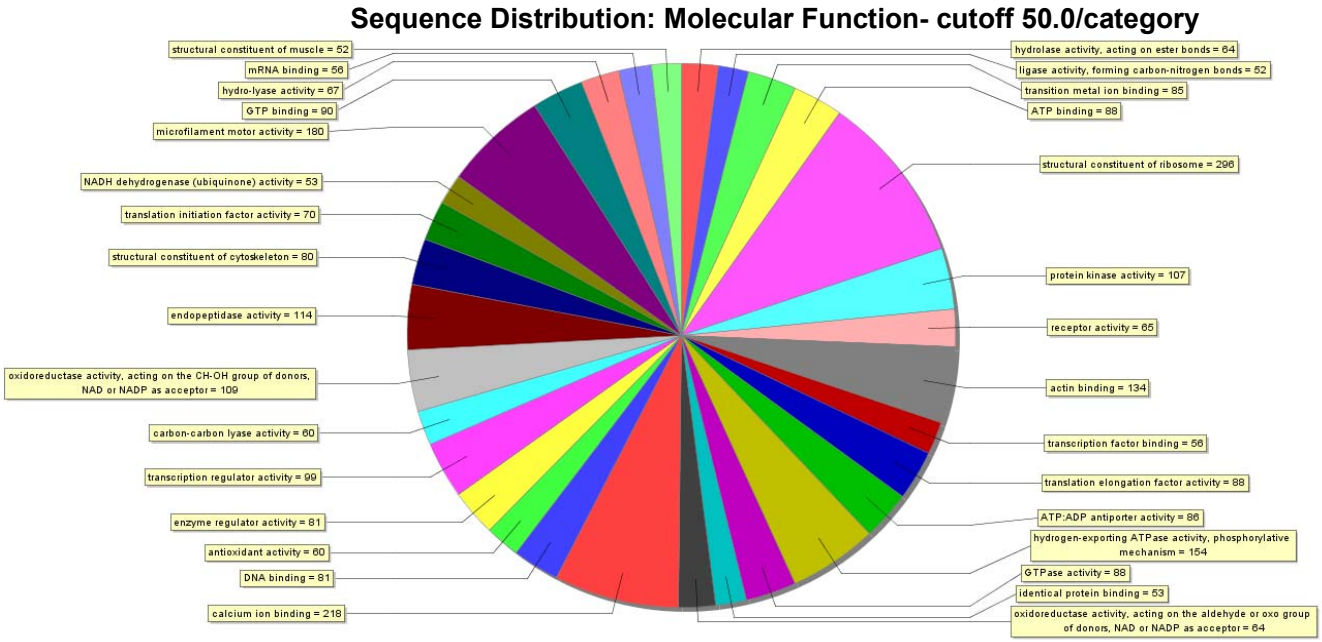

Figure 2. Sequence Distribution: Molecular Functions. Categories had to have at least 50 members. Represents EST's from three cDNA libraries, Adults, 5th instar, and Midgut. *Homalodisca vitripennis*, (Blast2GO analysis). Highest Categories in descending order: Ribosome structure= 296, Calcium ion binding=218, ATPase activity=154, Actin binding= 134, Microfilament motor activity=180, Endopeptidase activity=114, Oxidoreductase activity= 109, Protein Kinase activity= 107.

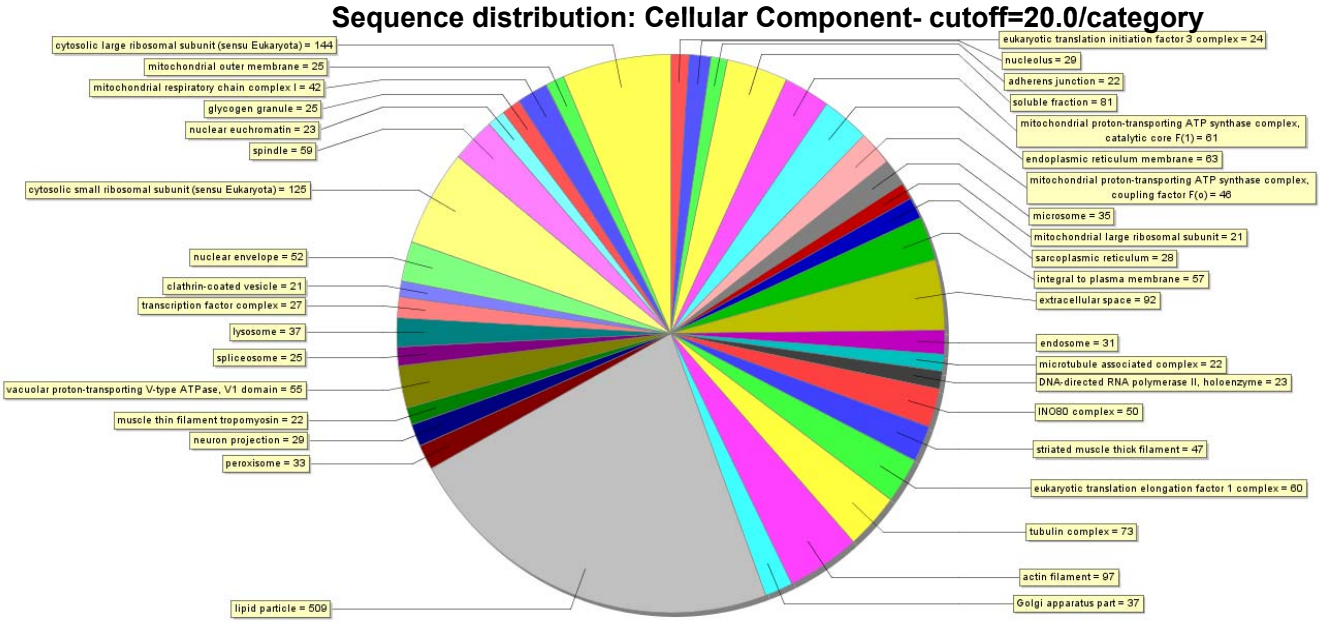

Figure 3. Sequence Distribution: Cellular Component. Categories had to have at least 20 members. Represents EST's from three cDNA libraries, Adults, 5th instar, and Midgut. *Homalodisca vitripennis,* (Blast2GO analysis).