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by

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Abstract

Pupillid land snails (Family Pupillidae) are small (<6mm) snails with a broad geographic distribution and often found in very high density in their habitat. The sequencing of the mitochondrial genome (mt genome) of three pupillid taxa has been undertaken to understand their genome evolution more fully. The *Gastrocopta cristata* mitochondrial genome is 14,060 bp in length and contains 13 protein coding genes, 2 rRNA genes and 22 tRNA genes. The *Pupilla muscorum,* and *Vertigo pusilla* genomes contain all of the same genes but are of differing total sizes, 14,149 bp and 14,078 bp respectively. The AT content of the three genomes is similar at ~71% A+T which is comparable to their closest sequenced relatives. There are no major gene rearrangements among the mt genomes of the three pupillids, but the positions of many tRNA genes differ from those of *Albinaria caerulea*. There is a genomic sequence region of high concentration of thymine on the leading strand of the mt genome that is shared among all three genomes as well as their relatives.

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Introduction

The mitochondrial genomes (mt genomes) of land snails are double-stranded circular DNA molecules and encompass 13 protein coding genes, 22 transfer RNA genes and two ribosomal RNA genes. The sizes of these mitochondrial genomes are presumed to be ~14kb, as *Albinaria caerulea* (Hatzoglou *et al.* 1995)*, Cepaea nemoralis* (Yamazaki *et al.* 1997)*, Succinea putris* (White *et al.* 2011) and *Cylindrus obtusus* (Groenenberg *et al.* 2012) are all approximately 14kb. The taxonomically informative changes in gene order between the mt genomes of different lineages make gastropods superb candidates for inferring evolutionary relationships (Grande *et al.* 2008). Mt genomes have many features which make them ideal for extrapolating taxonomic relationships: they are abundant in animal cells and are easy to work with, they have a higher mutation rate than the nuclear genome, and they lack recombination due to their uni-parental inheritance (Avise *et al.* 1987, Avise 1991, Moriyama *et al.* 1997). All these characteristics make the mitochondria adequate to capture recent taxonomic history despite the issues with introgression and linkage between the nuclear and mitochondrial genomes.

Sequencing of mt genomes is informative for taxonomic identification as well as analysis of evolutionary changes. Pupillid land snails fall under the Pupilloidea super family. They are a wide-ranging geographic group for which completely assembled mitochondrial genome sequences are not available. Among the Stylommatophora, an infra-order of pulmonates (Gastropoda, Mollusca) that the pupillid land snails belong to, only four mitochondrial genomes have been sequenced: *Albinaria caerulea* (Hatzoglou *et al.* 1995)*, Cepaea nemoralis* (Yamazaki *et al.* 1997), *Succinea putris* (White *et al.* 2011) and *Cylindrus obtusus* (Groenenberg *et al.* 2012*)*; all representatives of other super families (White *et al.* 2011, Wade *et al.* 2006). The complete characterization and annotation of additional mitochondrial genomes will provide data for interpretation of evolution among snails in Pupilloidea as well as outside this super family, adding to previous research by Von Proschwitz *et al.* 2009 and Hatzoglou *et al.* 1995 among others.

Three genomes from the Pupilloidea super family were sequenced. Specifically, the entire mitochondrial genomes of *Gastrocopta cristata, Pupilla muscorum,* and *Vertigo pusilla* were sequenced with Sanger sequencing. These particular species were chosen because each are type species within their genus, or as close to type species as easily available. These three genomes were compared with the 21 complete and annotated genomes from Panpulmonata (Gastropoda, Mollusca) from the National Center for Biotechnology Information (NCBI) database. I elucidate in this thesis the genome features, taxonomy and codon bias, G/C skew, D_n/D_s, gene order changes, and nucleotide composition. Some comparative analysis was also undertaken to look at possible evolutionary factors: D_n/D_s comparisons, relative rate of evolution comparisons, nucleotide density comparisons, codon bias comparisons and gene order comparisons.

Materials and Methods

Sample collection:

The *Gastrocopta cristata* (35.0727 N., 106.6160 W.) sample was collected by Jeff Nekola in Albuquerque, NM, while the *Pupilla muscorum* and *Vertigo pusilla* were acquired by Michal Horsak from (49.2509 N; 16.5738 E.) and (48.8586 N., 15.8960 E.) in Moravia, Czech Republic. All samples were collected using standard protocols (Nekola *et al*. 2010) and were single whole snails in estivation that were cleaned with molecular water prior to DNA extraction.

DNA extraction:

Total DNA was extracted from whole body tissue of individual snails, including shells, with the E.Z.N.A. mollusk DNA extraction kit (D3373, Omega Bio-Tek, Inc., Norcross, GA, USA) with the following modifications to manufacturer's protocol. The initial incubation, in lysis buffer, was increased from 4 to 7 hours and vortexing was replaced with inversion mixing. When bonding the DNA to the column, after protein precipitation, instead of filtering once with 750 µl of lysate, filter twice with 350 µl of lysate each time ending with ~100 µl of product.

Sequencing and genome assembly:

Universal primers for *cox1* based on (Gittenberger *et al*. 2004)*, 12S* (Appendix C, Sec 1)*, cox2* (Hugall *et al*. 2002)*, cob* (von Proschwitz *et al*. 2009)*, 16S*F (Tongkerd *et al.* 2004) and 16SR (Jørgensen *et al.* 2004) were used to obtain initial amplicons which were then sequenced and used to design exact primers for long distance PCR and DNA sequencing. Universal primers for *12S* were designed in collaboration with Coenraad Adema by inspecting alignments, BLAST N, of all available alignable *12S, nd1, cox3* and *nd3* gene sequences under Panpulmonata (clade) and identifying the most conserved regions to place primers in. No conserved regions of sufficient length were identified in *nd1, cox3* or *nd3* leaving 12S with the only viable locations for primers. The 5 prime PCR extender system, high fidelity enzyme system (2200510, 5 PRIME, Inc., Gaithersburg, MD, USA), long distance PCR, was used per the following protocol to amplify the mt genome with initially; 5 within gene fragments then later in 2-3 larger fragments each being 6-8 kb covering the entire mt genome (Appendix C, Sec 3). The thermo cycler program was carried out according to LDPCR manufacturer protocol with individual modifications for *G. cristata* (Appendix D, sec. 1), *P. muscorum* (Appendix D, sec. 1) and *V. pusilla* (Appendix D, sec. 1). Each LDPCR reaction was 25 µl in volume containing: 2.5 µl buffer, 1.25 µl of 8 µM DNTP's, .2 µl of TAQ (5U/µl), 16.55 µl of molecular water, 1.5 µl of genomic DNA and 1.5 µl of the forward and reverse 10 µM primers. *Vertigo pusilla* used .5 µl of primer for both the forward and reverse primer and water was used to add up to the final volume. A 1% agarose gel with the stain ethidium bromide was used to ascertain that the LDPCR was producing an amplicon as well as amplifying fragments of the correct size. All reactions were treated with ExoSAP-IT (78201, Affymetrix, Inc., Santa Clara, CA, USA) prior to sequencing to eliminate unincorporated primers and free DNTPs. The amplicons were then completely sequenced via primer walking using exact primers that were designed using previously obtained sequences. All sequencing reactions used the BigDye Terminator v3.1 Cycle Sequencing Kits (4337455, Life Technologies Corporation, Carlsbad, California, USA). A standard thermo cycler protocol (Platt *et al*. 2007) was used except that the anneal temperature was modified between the different species. All sequencing reactions were 10 µl containing 1-6 µl of ExoSAPed LDPCR product, 1 µl of one 10 µM primer, 2 µl of 5X big dye buffer and 1µl of enzyme with any remaining volume being water. *Gastrocopta* *cristata* functioned optimally using 2 µl of ExoSAPed LDPCR product (Appendix D, sec. 2). *Pupilla muscorum* worked best using 4 µl of ExoSAPed LDPCR product (Appendix D, sec. 2). For *Vertigo pusilla* 2 µl of enzyme combined with 6 µl of ExoSAPed LDPCR product (Appendix D, sec. 2). All ethanol precipitations were according to standard procedure (Appendix D, sec. 3). The UNM Molecular Biology Facility ran all sequences, dissolving the DNA pellet in 10 µl of formamide for sequencing using a ABI 3130xl (Applied Biosystems by Life Technologies Corporation, Carlsbad, California, USA).

Genome annotation:

Chromatogram editing and contig assembly were done with Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Genes were tentatively identified with a combination of BLAST X and BLAST N searches on NCBI, restricted to Panpulmonata (NCBI:BLAST 2012). Exact beginning and end points were identified by manually aligning the newly sequenced genes with genes from related snail species represented in NCBI, and then compared to find the correct start and stop codons based on length of the genes and the location of the start codon in related species (Rambaut 2012). The tRNAs were identified by a combination of the tRNAscan-SE Search Server with exceptionally low cutoff scores (1×10^{-66}) and by identifying the anti-codon of each possible tRNA in likely locations and with surrounding sequence that could attain the appropriate secondary structure (Lowe *et al*. 1997, Schattner *et al*. 2005). The predicted secondary structures for the tRNA were made using mt-tRNA-Draw and FASTA sequence exports (Youngblood *et al*. 2012). The ribosomal RNA genes were identified using both the sequence similarity between genomes plus BLAST N searches, in addition to identifying the abutting genes. Genome maps were made with GenomeVx (Conant *et al*. 2008) and the genomes available from GenBank and the experimentally derived genomes from pupillid snails.

Data analyses:

Initially 21 mt genomes from Panpulmonata were acquired from NCBI GenBank (Table 1). These genomes were aligned with the three newly sequenced genomes on a gene-by-gene basis via an amino acid nucleotide alignment with manual correction afterwards (Abascal *et al*. 2010). The tRNA's and rRNA's for the above genomes were visually compared but not sequence aligned. The full mt genomes were analyzed for nucleotide diversity and nucleotide content with a custom Matlab script (Appendix A, sec. 3, 4). The gene-by-gene alignments were concatenated to create protein-only genomes, preserving non-aligning sites. The putative origin of replication was identified by three factors: the region was 40 nucleotides or longer, the region's A+T % was elevated compared to the surrounding sequence, and the region was not overlapping with any genes (Grande *et al.* 2008). The D_n/D_s between these genomes was calculated via a custom Matlab script (Appendix A, sec. 2), which used a window equal to each genes' size, and then graphed in Excel for comparison. The script counts the raw number of synonymous and non-synonymous differences per pairwise comparison for a coding sequence aligned data set, with regions of no alignment, for all possible comparisons while treating gaps as missing data. The codon usage was calculated using a custom Matlab script (Appendix A, sec. 1) and graphed as pie charts per amino acid for comparison. The GC skew was calculated using the concatenated set of aligned protein encoding codons, the non-aligning sites removed, with a fixed 210 bp window and a 3 bp step in DAMBE (Xia *et al*. 2001). The non-synonymous and synonymous divergences between various protein coding sequences of the genomes were calculated using DnaSP set for haploid, mitochondrial, and entire sequence coding DNA (Librado *et al*. 2009). The raw number of non-synonymous and synonymous changes were divided by the number of sites and multiplied by the percentage of sites considered from the total number of coding sites. The relative rate was calculated using Tajima's relative rate test in Mega 5.0 with substitution model A and the coding-only data set with areas of non-alignment eliminated (Tamura *et al*. 2011).

Phylogenetic analyses:

For phylogenetic analysis the gene-by-gene alignments were re-edited and joined to eliminate areas of non-alignment inside of the genes. Four different reconstruction methods were used: maximum parsimony, nearest neighbor joining, maximum likelihood and Bayesian. The maximum parsimony, nearest neighbor joining, and maximum likelihood analyses were all performed using Mega 5.0 (Tamura *et al*. 2011). The maximum parsimony and nearest neighbor joining analyses both incorporated amino acid sequence with support values based on 500 bootstrap replicates. The maximum parsimony search method was to use close neighbor interchange on random trees with 15 initial trees and a search level of one. The nearest neighbor joining analysis was based on the number of differences.

The maximum likelihood analysis employed the mitochondrial reversible model in addition to the frequency F+ model, a gamma distribution with invariant sites and 12 categories for the rates among sites and support values based on 500 bootstrap replicates. The heuristic method for the maximum likelihood analysis was nearest neighbor interchange with an automatic initial tree. The Bayesian analysis was generated using Mr.Bayes (Huelsenbeck *et al*. 2001, Ronquist *et al*. 2003). The Bayesian analysis is amino acid general time reversible with variable rates and 1,000,000 generations with a burn-in of 250,000 generations. The tree is a consensus tree with a 25% burn-in on the number of trees (~5000). The out-group is *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006), a non-pulmonate venomous gastropod.

Results

Complete mt-genomes of three Pupillid species

Genome organization and nucleotide composition

The exact sizes of *G. cristata* (Figure 1), *P. muscorum* (Figure 2) and *V. pusilla* (Figure 3) are 14,060 bp, 14,149 bp and 14,078 bp, all which fall into the typical size range for pulmonate mt genomes (Figure 4). All three mt genomes consist of 13 protein-coding genes, 22 tRNA genes and two rRNA genes with no duplications. Sequencing coverage was three-fold for the majority of the genome and two-fold everywhere else. The genomes' A+T richness is similar at: 69.2% *G. cristata*, 71.8% *P. muscorum* and 72.3% *V. pusilla*. *Gastrocopta cristata* (Figure 5), *P. muscorum* (Figure 6) and *V. pusilla* (Figure 7) have parallel regions of A+T and G+C richness across their genomes.

Protein coding genes

Pupillid mitochondrial genomes have 13 protein coding genes. Of those genes, *cox1, nd6, nd5, nd1, nd4L, cytb, cox2, nd4* and *nd2* are on the L-strand while *atp8, atp6, nd3* and *cox3* are on the H-strand. The protein-coding genes make up ~77% of their mt genomes. Non-standard starting codons are also moderately common and which start codon is used for which gene sometimes varies between different species (Serb *et al.* 2003, Boore *et al.* 2004). The genes *cox1* and *cytb* have consistently begun with alternative start codons among the Pupillids. *Cox1* starts with the alternative start codon for leucine but for *cytb* and the rest of the genes the particular start codon utilized varies (Table 2). In *G. cristata* the common genes, *cox1* and *cytb*, as well as *nd3* all commence with non-standard start codons. In *P. muscorum* the common genes plus *nd6, atp6, nd3* and *nd2* begin with alternative start codons. In *V. pusilla* the common genes, in addition to *nd1, nd4L, nd3* and *nd4*, start with alternative start codons. I hypothesize that one gene, *nd5*, has a stop codon completed via polyadenylation in *G. cristata.* Furthermore, some protein coding genes also appear to overlap. *Gastrocopta cristata* has an overlap between *nd5* and *nd1* of 10 bp. The same overlap occurs in *P. muscorum,* except it is 16 bp, and additionally there is another overlap between *nd6* and *nd5* that is 8 bp. *Vertigo pusilla* has the same overlap as *G. cristata*, except it is 34 bp in length and there is also an overlap between *nd4L* and *cytb* of 15 bp.

Transfer and ribosomal RNA genes

There are 22 tRNA genes and 2 rRNA genes in pupillid land snail mitochondrial genomes and tRNA genes make up ~10% of the mt genomes of the three species. Rather than on the H-strand (~ 8) most of the tRNA's (~14) for all three species are on the L-strand with the majority of the genes. There are frequent overlaps between different tRNA's and between tRNA's and protein coding genes in all three species. For *G. cristata* the tRNA sizes range from 73 bp in tRNA$^{\mathrm{Lys}}$ and tRNA$^{\mathrm{Gln}}$ to 61 bp in tRNA$^{\mathrm{Thr}}$ and tRNA$^{\mathrm{Ser\ (AGN)}}$. In *P. muscorum* the size range is between 68 bp for tRNA$^{\mathrm{Glu}}$ and tRNA$^{\mathrm{Ala}}$ to 61 bp for tRNA$^{\mathrm{His}}$. *Vertigo pusilla* is different from the others in that the range is from 69 bp in tRNA$^{\mathrm{Ile}}$ to 52 bp for tRNA$^{\mathrm{Arg}}$. Most, but not all, of the tRNAs for all three species fit the "cloverleaf" secondary structure model. In *G. cristata* there are six tRNAs which do not fit the model (Figure 8), for *P. muscorum* there are three tRNAs (Figure 9), and for *V. pusilla* there are five tRNAs (Figure 10). These tRNAs are referred to as non-standard tRNAs and are identified by the following criteria: missing leaves, the number of missing bonds, number of mispaired bases, and non-standard secondary structure.

Pulmonate snail mitochondria have two rRNA genes, *12S* and *16S*, which is the same with the new genomic sequences. The rRNAs are arranged in the typical manner, *16S* between *cox1* and nd6 with the tRNAs on either side and *12S* between *atp6* and *nd3* with it's own tRNAs on either side (Boore 1999). rRNA makes up ~12.5 % of the three pupillid mt genomes. The rRNA in *G. cristata* is mostly composed of A/T with a G/C content of only ~27%. The same is true of the other two Pupillidae except their GC content is ~25%.

Non-coding regions

Most of the mitochondria genome sequences in pupillid snails is taken up by protein coding genes, but there are still gaps between the coding sequence and differences in the distribution of those regions. While the percentages of tRNA genes, rRNA genes and protein coding genes are approximately the same across genomes, the percentage of non-coding DNA varies more. *Gastrocopta cristata* has .01% intergenic DNA, *P. muscorum* has .7% and *V. pusilla* has .06%. This difference is probably not significant however it is interesting to note that the difference in mt genome size between *V. pusilla and G. cristata* versus *P. muscorum* can be accounted for almost entirely by an increase in the amount of intergenic space in *P. muscorum.* All three mt genomes have many non-coding regions with most of them being in the 1-14 bp range, the size of the putative origin of replication (POR) being an exception. In *G. cristata* there are two regions that are longer: 26 bp between tRNA$^{\mathrm{Ala}}$ and *ND6* as well as 14 bp between tRNA$^{\mathrm{Gln}}$ and tRNA$^{\mathrm{Leu\ (UUR)}}$. In *P. muscorum* the most significant non-coding regions are between tRNA$^{\mathrm{Val}}$ and *16S* (18 bp) and between tRNA$^{\mathrm{Ala}}$ and *ND6* which is 30 bp. *Vertigo pusilla* has one large non-coding region between tRNA$^{\mathrm{Ala}}$ and *ND6,* which is 23 bp. All 3 species have their POR between *COX3* and tRNA$^{\mathrm{Ile}}$ and they are all in the 40 bp range. Specifically *G. cristata's* POR is 46 bp long with 87% A/T, *P. muscorum's* POR is 42 bp with 81% A/T and *V. pusilla's* POR is 45 bp long with 89% A/T.

Comparative analyses

Mt genome comparisons

The mt genome lengths compared across Panpulmonata are fundamentally the same with one exception, *Pedipes pedipes* (Figure 4). The patterns of nucleotide usage demonstrated by the Pupillids are preserved across genera (Figure 5,6,7,11,12). T is the most favored nucleotide followed by A, C, and G on the leading strand. A/T content is between 55% and 75% for all of the genera reviewed (Figure 13), and the G/C content is between 25% and 45%. All these gastropod mt genomes have the majority of genes on the L-strand rather than the H-strand.

The gene order across species is largely consistent sans the frequent changes in the organization of tRNA (Figure 14). The D_s is greater than one for most comparisons regardless of which species are being compared (Table 4). The D_n was fairly consistent (~.2) across all gene and species comparisons (Table 4). Correspondingly, the D_n/D_s ratios for those comparisons are unreliable due to silent site saturation. While the newly sequenced genomes are not unique in having non-standard tRNA's, which tRNAs are non-standard is inconsistent across genera except for $tRNA^{Lys}$ which was non-standard in every species.

Protein coding genes

The patterns of nucleotide usage in the protein coding genes differ slightly from the whole genome although the ratios of nucleotides remain the same. The universally favored nucleotide in protein coding genes is A, and T never overtakes it in overall prevalence. The A/T richness of the protein coding genes is not significantly different from that of the overall genome. When comparing the protein coding portion of the mitochondrial genomes against each other, the non-synonymous divergence between them is 18% while the synonymous divergence is 14%. The G/C skew indicates what nucleotide is mutationally preferred on the L-strand, in this case it is G most of the time with C being usually preferred when genes switch strands (Figure 15). All three genomes show some form of codon bias although slight in some cases. When all three are compared to each other the differences between them are minor. On the whole, codons ending in A or T in addition to codons that are more A/T rich are preferred (Figure 16, 17, 18). More distantly related species are only slightly A/T biased as demonstrated by their more equal codon usage (Figure 19, 20), seeming to indicate that A/T selection is stronger, or at least more apparent, in *G. cristata, P. muscorum*, and *V. pusilla*.

When examining the relative evolutionary rates between the three species as compared to *A. caerulea,* with *S. putris* as the out-group, the results are non-significant and confirm the null hypothesis (Table 3). The null hypothesis states that all species in the comparison are evolving at the same rate.

Comparative phylogenetics

The phylogenetic relationships of the pupillid land snails were inferred using the three newly sequenced genomes and 22 existing genomes. All of the phylogenetic analyses were somewhat similar in results but they did contain significant differences. As such the Bayesian (Figure 21), maximum likelihood (Figure 22), maximum parsimony (Figure 23), and nearest neighbor joining (Figure 24) consensus trees were all included for comparison. Overall the outer clades, clades close to the taxa, are more supported than the inner clades, clades closer to the root.

Despite the fluctuating support values for various clades the groupings themselves are reasonably consistent across analyses. One of the consistent clades was that all three of the genomes recently sequenced clustered together. *Cepaea nemoralis* also persistently groups as a sister with *C. obtusus. Ascobulla fragilis* and *E. chlorotica* are sister species and consistently clade with the two *Siphonaria* sister species. The *A. bidentata, O. vulcani* and *T. reticulatus* clade consistently comes out as a sister to the *P. mortoni, P. peronii,* and *O. celtica* clade. On average all the species belonging to a particular super family group together.

Succinea putris, *M. myosotis*, *P. pedipes*, *P. dolabrata* and the *Biomphalaria* sister species, however, all move depending on the method of analysis. For example, in the Bayseian and maximum likelihood phylogenies *S. putris* is in a clade with *C. nemoralis* and *C. obtusus*. This is unlike the nearest neighbor joining phylogeny where *S. putris* is in a clade with *A. caerulea*, *G. cristata, P. muscorum,* and *V. pusilla* or like in the maximum parsimony phylogeny where *S. putris* is sister to *A. caerulea.* The other species move between clades in an analogous manner to *S. putris,* depending on the method analysis.

Discussion

When comparing the mt genomes of *G. cristata, P. muscorum,* and *V. pusilla* to previously sequenced mt genomes it is apparent that there is a great deal of similarity between them and that there are also some minor differences. The gene orientation, despite re-arrangements, usually remains consistent; the genes start and end in approximately the same locations across all genomes referenced. The protein and rRNA gene order remains constant while the tRNA's frequently move around across genera. Despite all of the movement, the tRNA's are mainly located in the same regions across the genome rather than scattered.

Seventy percent of the genome is protein coding. Of the changes that occur in the protein coding genes most are synonymous. This makes sense based on the expectation that most mutations are synonymous in order to preserve fitness by reducing the number of deleterious changes. An explanation for the amount of non-standard tRNA may be that the higher mutation rates in the mitochondrion compared to the nucleus, in combination with the absence of recombination, results in the accumulation of deleterious mutations by Muller's ratchet (Lynch *et al*. 1996). The number of changes, synonymous or non-synonymous between species, also means that designing primers for LDPCR is difficult because species-specific primers are necessary for large amplicons.

All of the mitochondrial genomes examined have high A/T content and low G/C content (Figure 13). The three new genomes are on the higher end of that spectrum, as defined by my dataset at ~77%. The A/T richness is not particular to a certain region; the relative amount of A/T is equal from the protein coding genome to the remainder of the genome. There are several regions were T content is extremely high (Figure 5,6,7). This region is conserved among *G. cristata, P. muscorum,* and *V. pusilla* as well as with other species. *A. caerulea* has a similar region (Figure 11), while *C. nemoralis* has another similar region but on a different scale (Figure 12). The region of high T content potentially varies between the different species because the comparisons are between distinctive super families. Codons with more A/T are usually favored over codons with more G/C (Figure 16,17,18,19,20). While this relationship exists in the other species of the data set, they don’t have the same amount of bias towards particular codons (Hatzoglou *et al*. 1995). Some amino acids which particularly highlight this trend are valine, glutamic acid and tyrosine.

The G/C skew indicates that G is favored by the three genomes over C on the L strand. The fact that the D_n estimates are usually higher than one suggests that the sequences are already saturated at silent sites. The saturation of synonymous sites indicates that D_n and D_n/D_s are unreliable in cases where D_s is greater than one.

The relative rate test which supports the null, that there is no difference in evolutionary rates between the compared species, indicates that any differences in the effective population sizes or phenotypic differences between these snails have little to no effect on the mutation rates.

The phylogeny recovered from this data set is not well conserved across different methods. Any clade containing *S. putris*, *M. myosotis*, *P. pedipes*, *P. dolabrata* or the *Biomphalaria* sister species varies across analyses with low (55% <) support values. This uncertainty about where to place these species might be due to lack of data for intervening taxa, skewing the data based on a few informative sites or perhaps the data set itself does not contain enough informative sites to place these particular species. Another possibility is that the data set is too divergent, despite being an amino acid alignment of protein coding genes with the indels in the alignment concatenated out.

The Bayesian phylogeny is an exception to low support values on the clades containing the above species, however there is reason for the values to be suspect. The maximum likelihood phylogeny doesn’t agree with the support the Bayesian phylogeny assigns to identical nodes by as much as 65%. It has also been suggested that the posterior probabilities from Bayesian phylogenetics are consistently overconfident (Misawa *et al.* 2003). The nearest neighbor joining and maximum parsimony phylogenies similarly do not agree with the Bayesian phylogeny's support values, although the discrepancies are smaller.

When comparing the retrieved phylogenies to previous work by Wade *et al.* 2006 and White *et al.* 2011 some striking similarities and differences are revealed. Both authors recovered the same groupings by super family as is retrieved in the four phylogenies above. Interestingly enough among the list of species with low support values and uncertain positions that White uses he also has low support values for those clades but the positions are fixed across his analyses while they varied in mine. There is also a descrepancy in the location of the *Biomphalaria* clade, White has *Biomphalaria* as a clade sister to but outside of the Stylommatophorans and the Eupulmonates, while my analysies have *Biomphalaria* as a clase nested within the non-Stylommatophoran clade.

All of the phylogenies support a few conclusions. The monophyly of the Stylommatophoran snails is supported by all of the phylogenies. The groupings of species by superfamily are supported across all phylogenies. The relations among the super families when using mt DNA seem to depend on the data set and analyses run. Theses phylogenies support the previous work by Wade and White but they don't provide additional resolution on the relations between the super families and deeper nodes.

Conclusion

Gastrocopta cristata, P. muscorum and *V. pusilla* genomes' are the first fully sequenced mitochondrial genomes in their families. These three genomes, *G. cristata*, *P.*

muscorum and *V. pusilla* have been sequenced to provide for the first time coverage of the Pupilloidea super family. These sequences add genera to improve the accuracy of phylogenetics in areas where there previously has been no data.

In addition to improving future taxonomic efforts these three genomes provide improved insight into the general features and organization of pupillid mt genomes. The *G. cristata* (KC185403), *P. muscorum* (KC185404) and *V. pusilla* (KC185405) genomes are not yet released on NCBI GenBank but they can be found in Appendix B. Interesting features such as the region with an increased prevalence of thymine need more work and characterization of mt genomes of comparable genera to more fully understand. This work has hopefully improved the ease of sequencing pupillid mt genomes and adds to the growing body of knowledge about mitochondrial features as well as snail mitochondrial genomic features. Some future extensions of this work will be to cover all of the families, if not genera, that lack representative sequences using a next generation sequencing approach similar to the one suggested by Jex *et al.* 2010. This will expand the taxonomy and provide additional data on the evolutionary processes shaping pupillid mitochondrial genomes.

Figure 1: Mt genome map of *G. cristata*. Protein coding genes are blue, tRNA are green and rRNA are red.

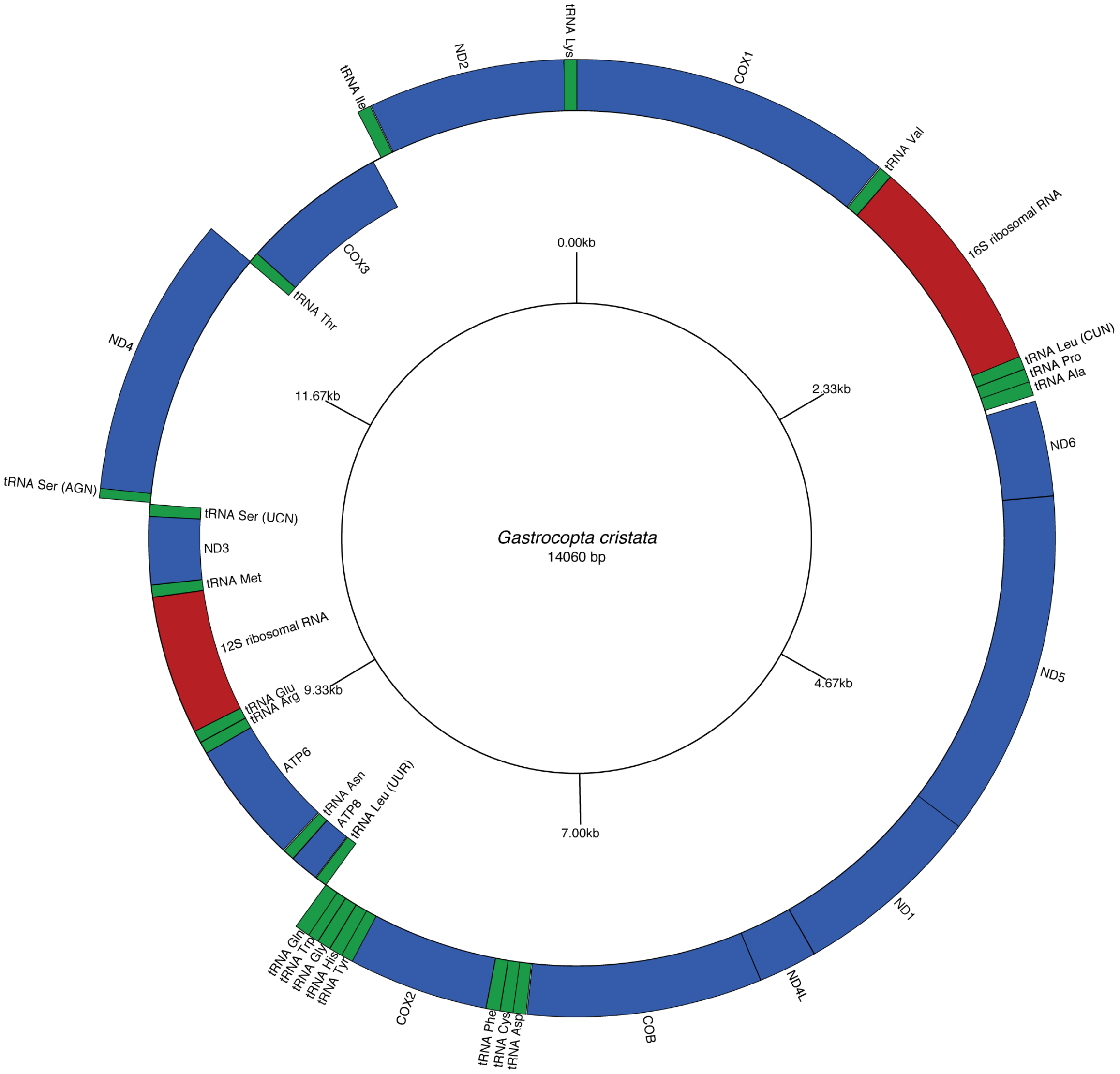

Figure 2: Mt genome map of *P. muscorum*. Protein coding genes are blue, tRNA are green and rRNA are red.

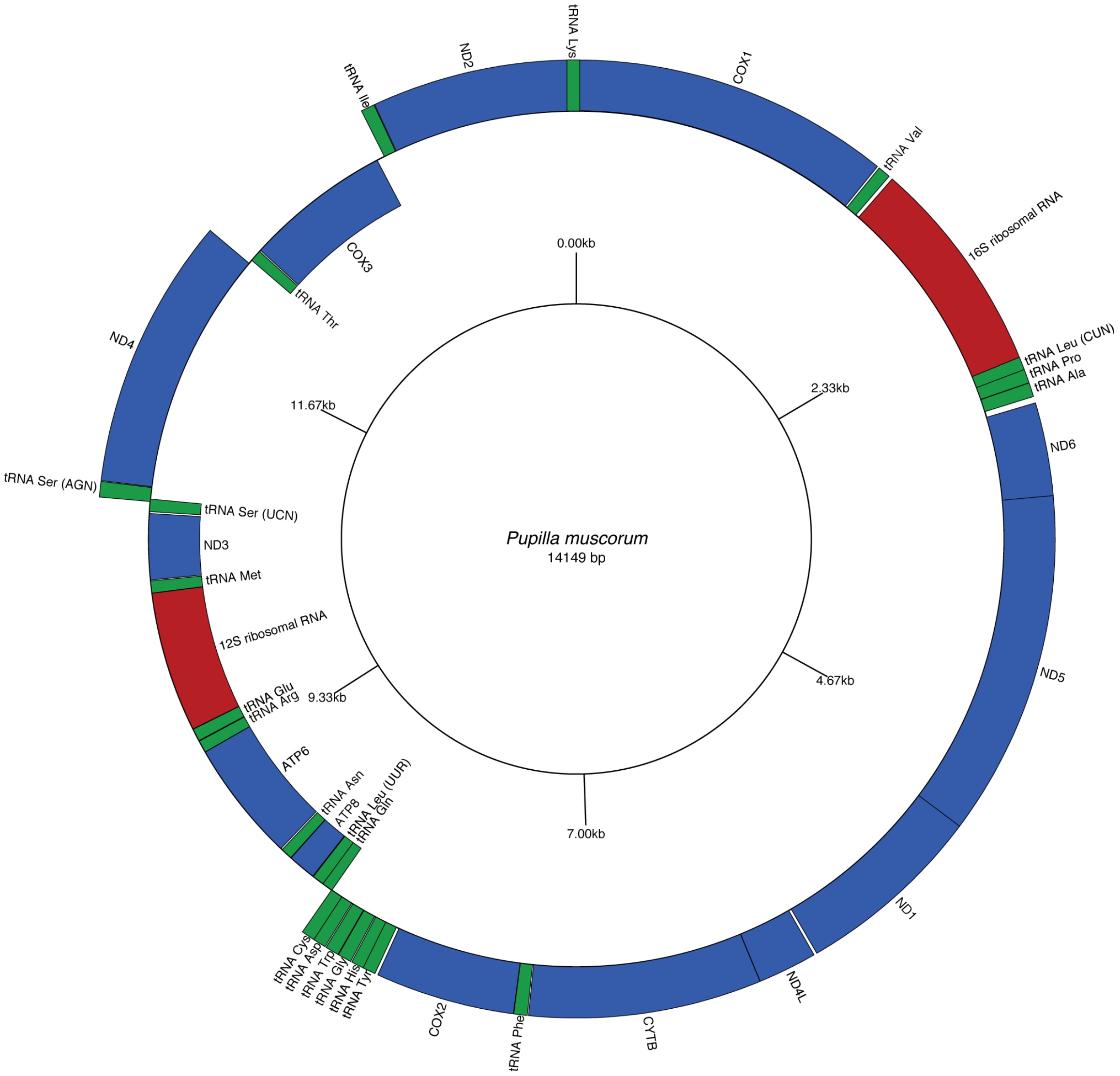

Figure 3: Mt genome map of *V. pusilla*. Protein coding genes are blue, tRNA are green and rRNA are red.

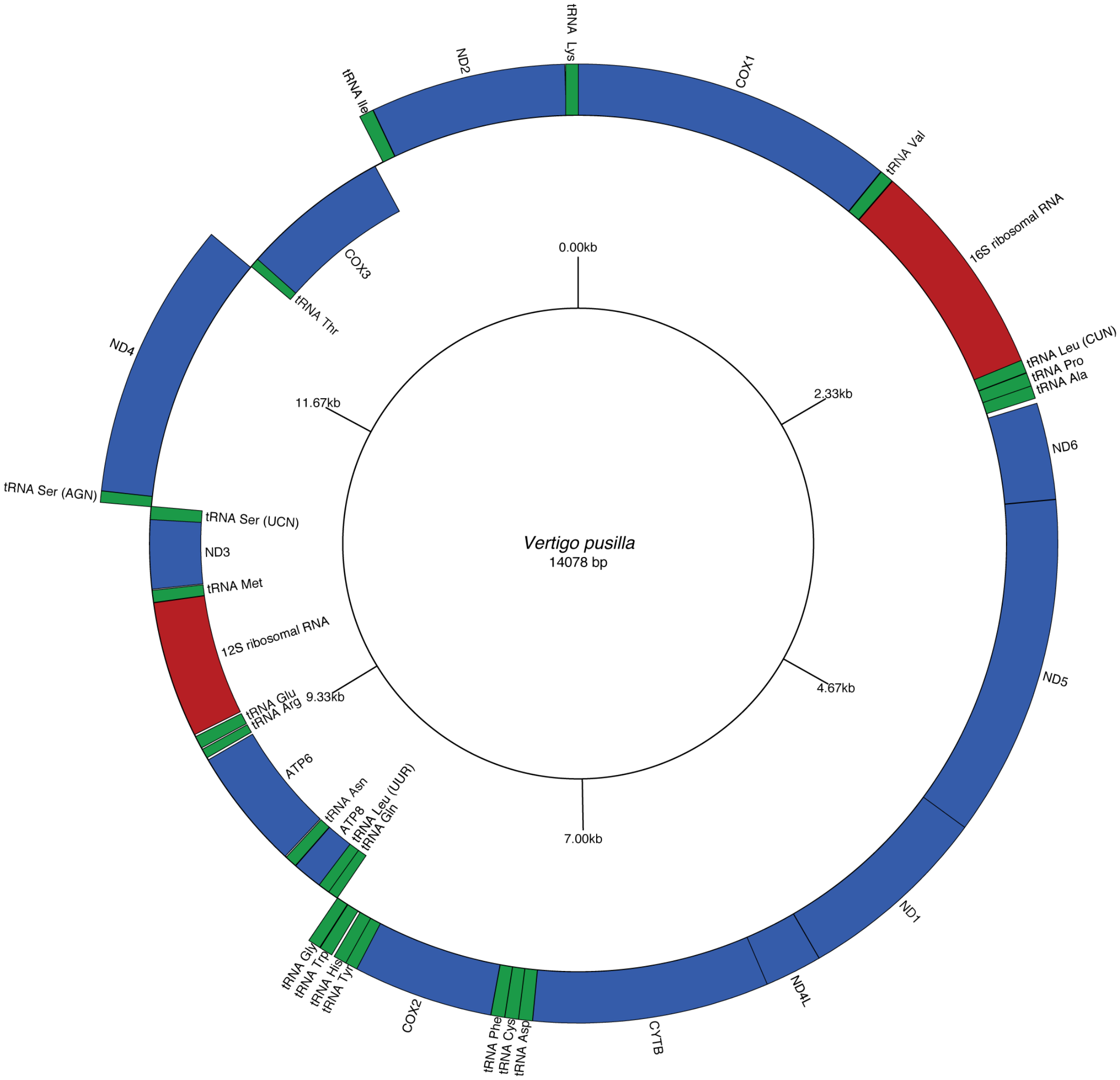

Figure 4: Comparison of genome length across data set.

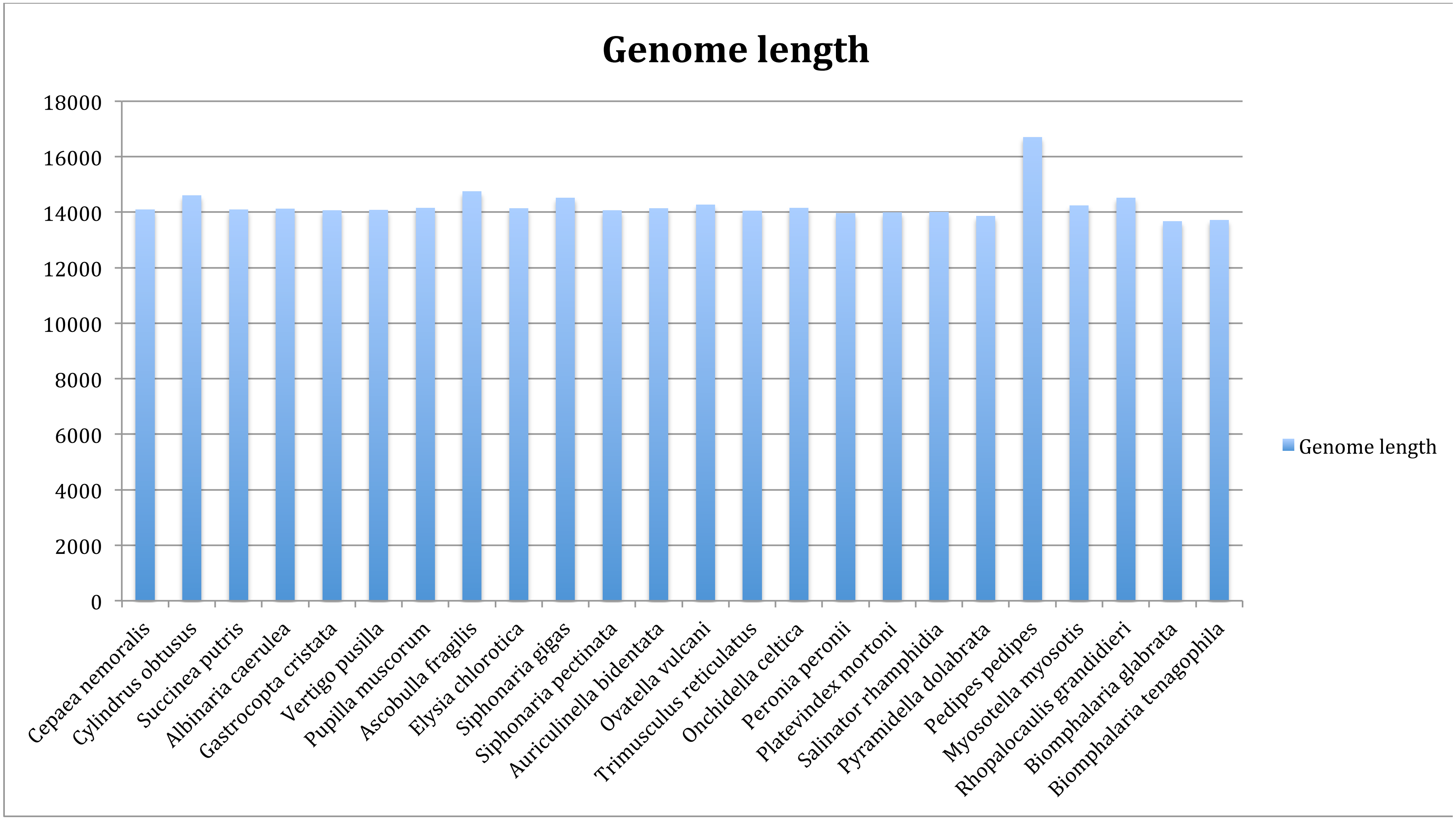

Figure 5: Nucleotide density plot for mt genome of *G. cristata*. Matlab automatically determined the bin size. Position 1 is at the begining of *cox1*.

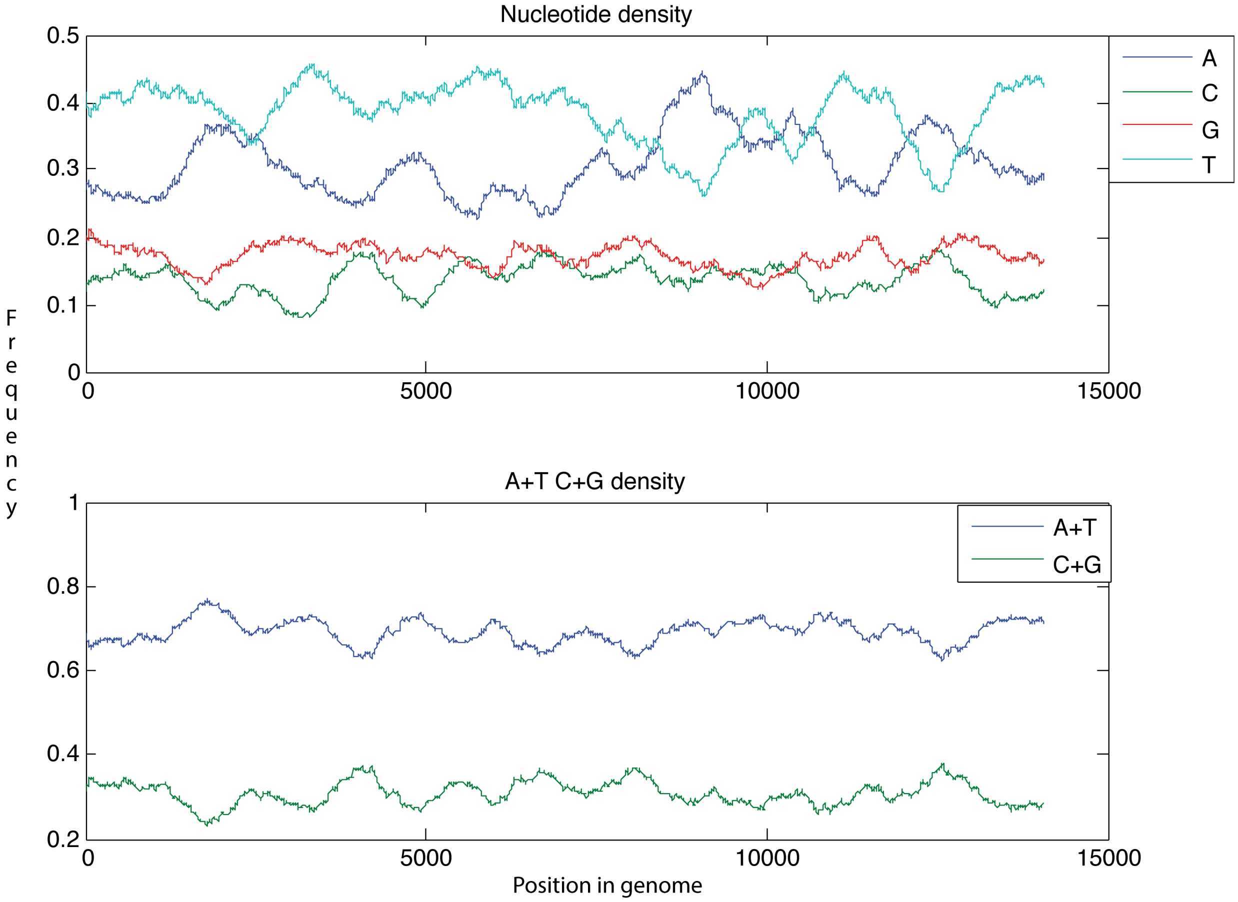

Figure 6: Nucleotide density plot for mt genome of *P. muscorum*. Matlab automatically determined the bin size. Position 1 is at the begining of *cox1*.

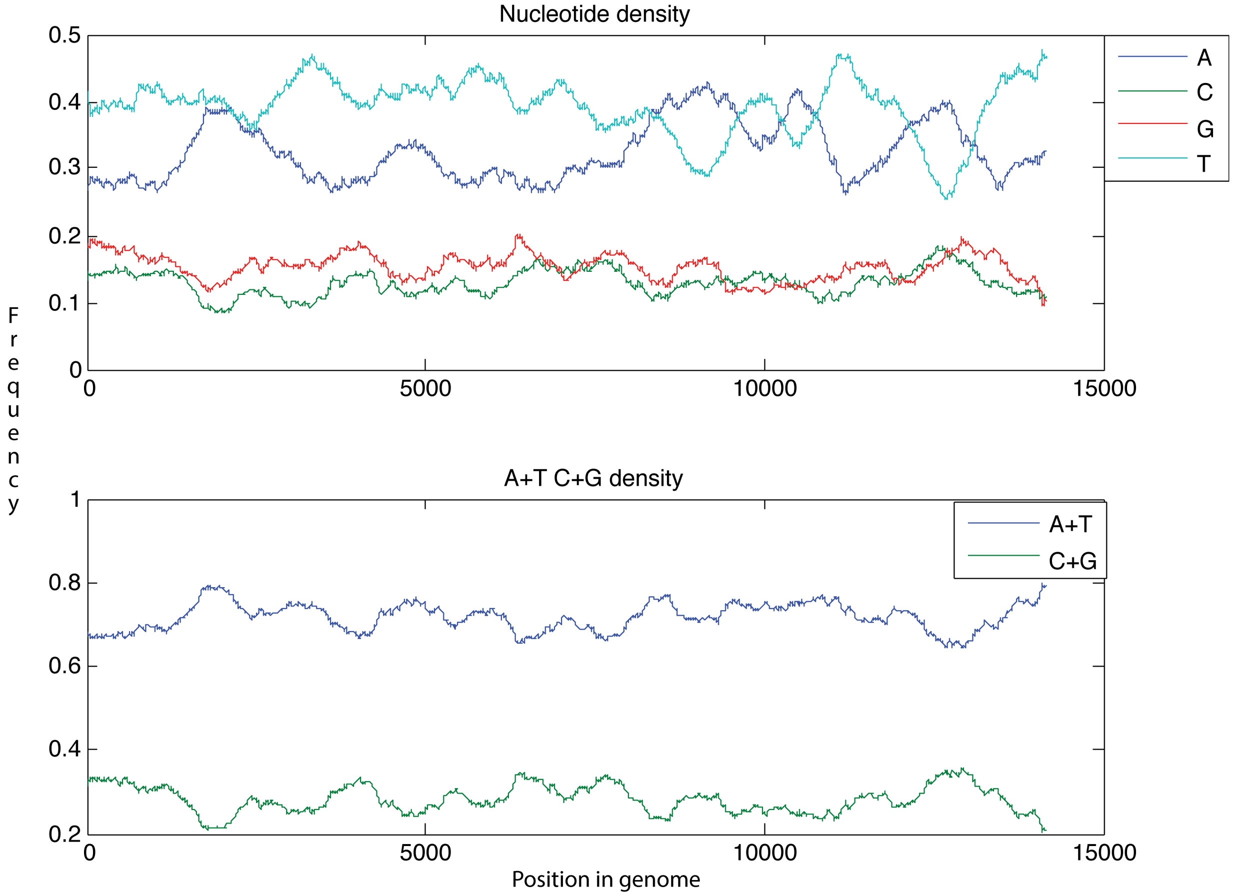

Figure 7: Nucleotide density plot for mt genome of *V. pusilla*. Matlab automatically determined the bin size. Position 1 is at the begining of *cox1*.

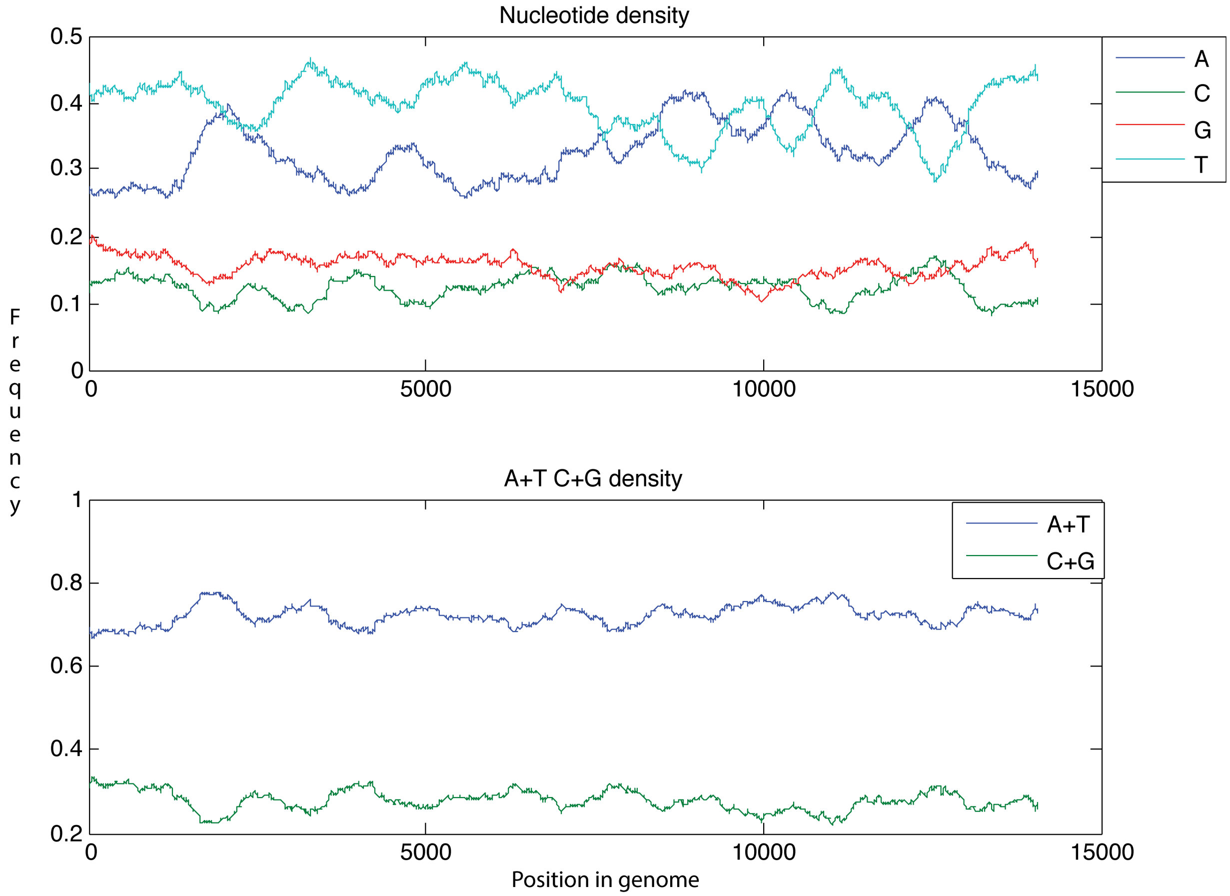

Figure 8: Predicted tRNA structures for *G. cristata.* non-standard tRNA are indicated with a black star. The number of missing bonds, missing arms and non-standard spacing determine which tRNA are considered non-standard. tRNA are in 5' -> 3', left to right and are based on DNA sequence.

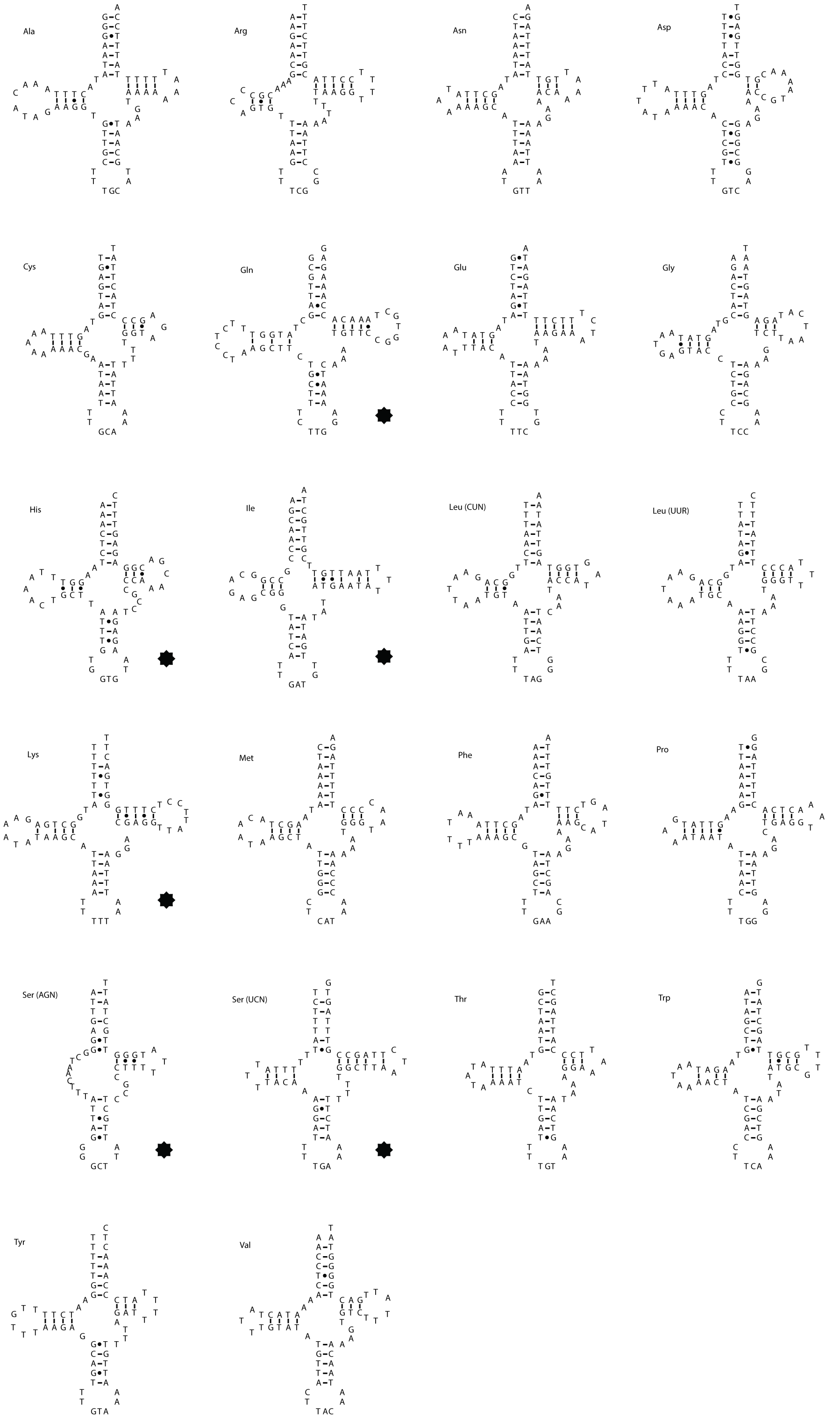

Figure 9: Predicted tRNA structures for *P. muscorum.* Non-standard tRNA are indicated with a black star. The number of missing bonds, missing arms and non-standard spacing determine which tRNA are considered non-standard. tRNA are in 5' -> 3' , left to right and are based on DNA sequence.

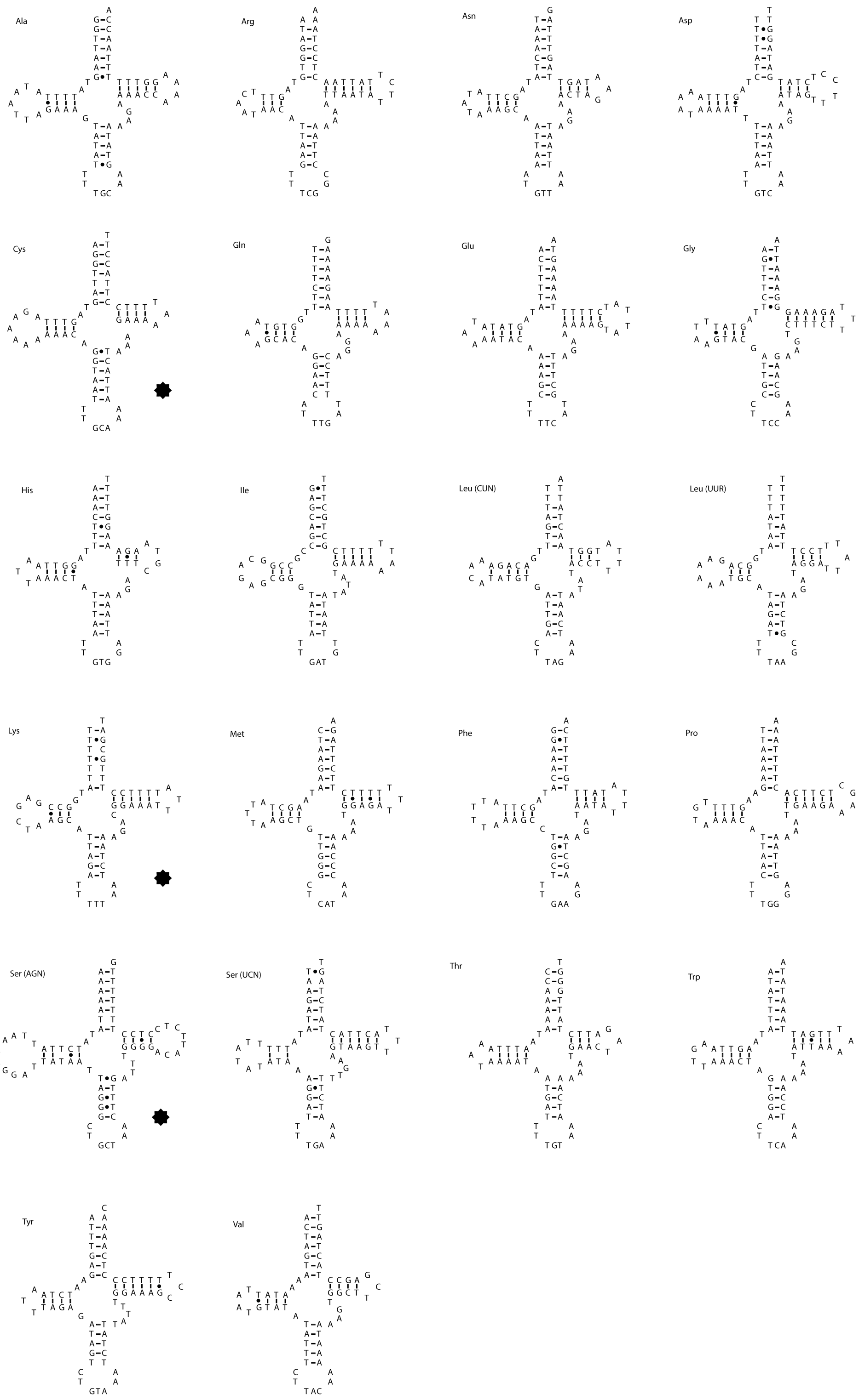

Figure 10: Predicted tRNA structures for *V. pusilla*. Non-standard tRNA are indicated with a black star. The number of missing bonds, missing arms and non-standard spacing determine which tRNA are considered non-standard. tRNA are in 5' -> 3' , left to right and are based on DNA sequence.

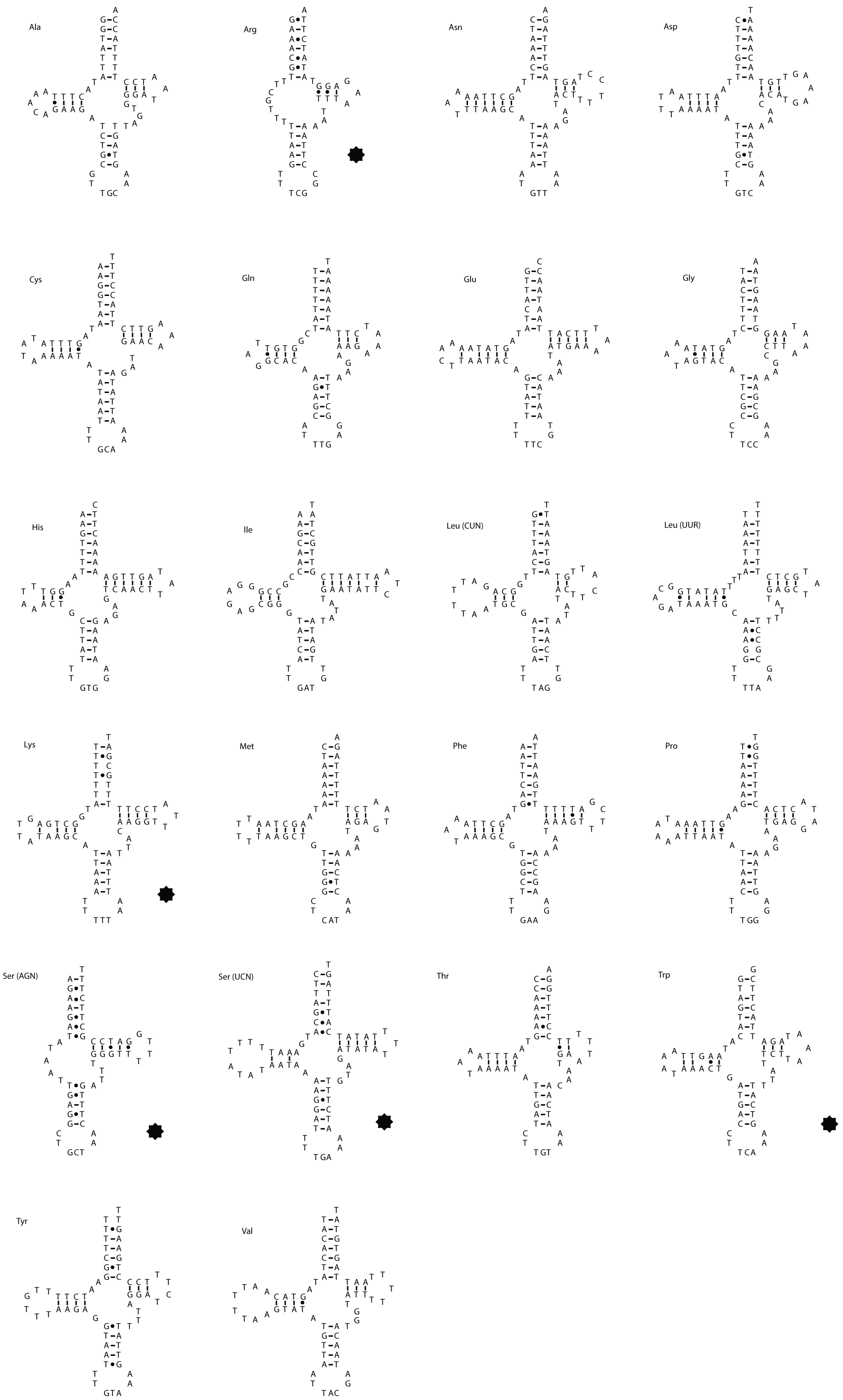

Figure 11: Nucleotide density plot for mt genome of *A. caerulea*. Matlab automatically determined the bin size. Position 1 is at the begining of *cox1*.

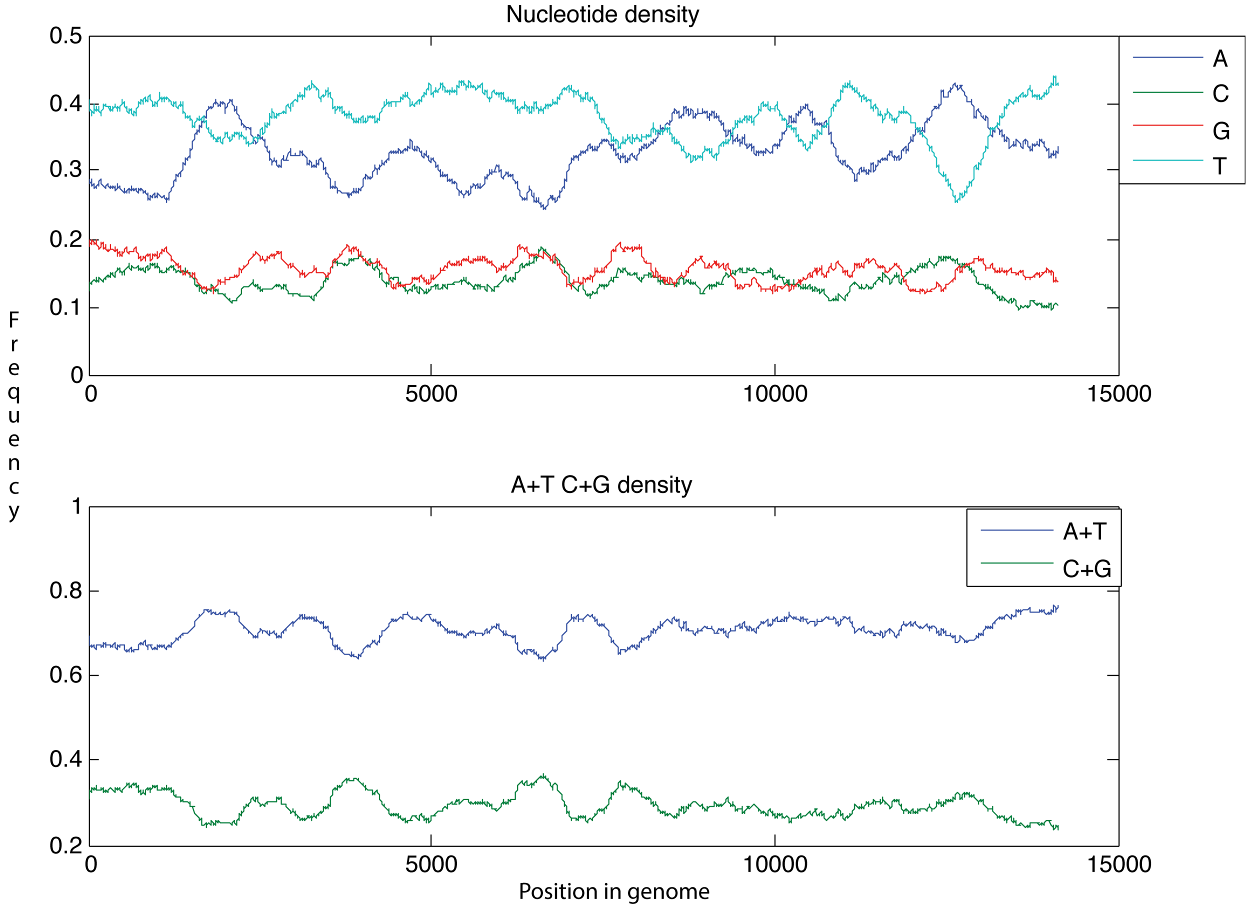

Figure 12: Nucleotide density plot for mt genome of *C. nemoralis*. Matlab automatically determined the bin size. Position 1 is at the begining of *cox1*.

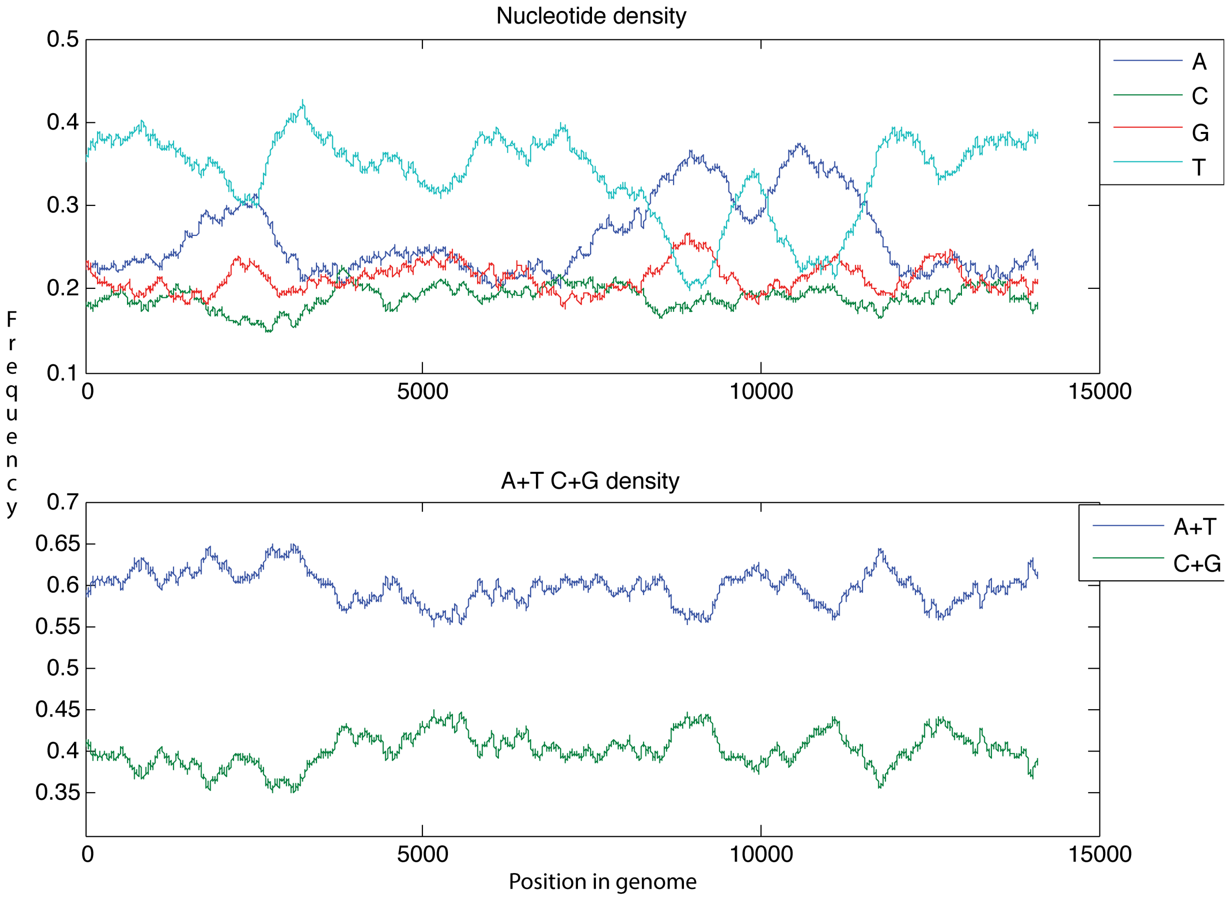

Figure 13: A+T and G+C percentages by mt genome.

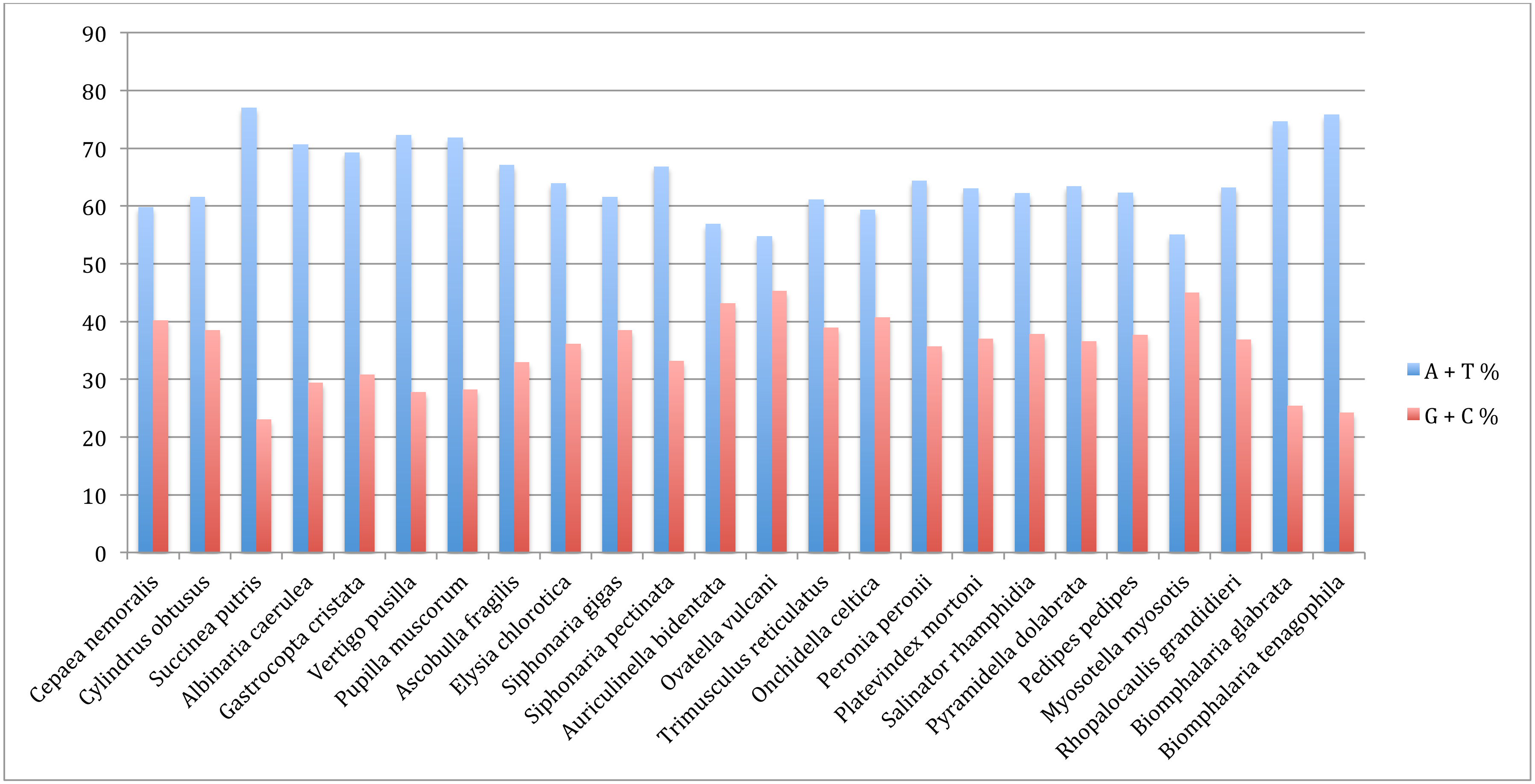

Figure 14: Gene order comparison across data set. Green boxes are tRNA, red boxes are rRNA and blue boxes are protein coding genes. Underlined genes are in the opposite orientation to non underlined genes.

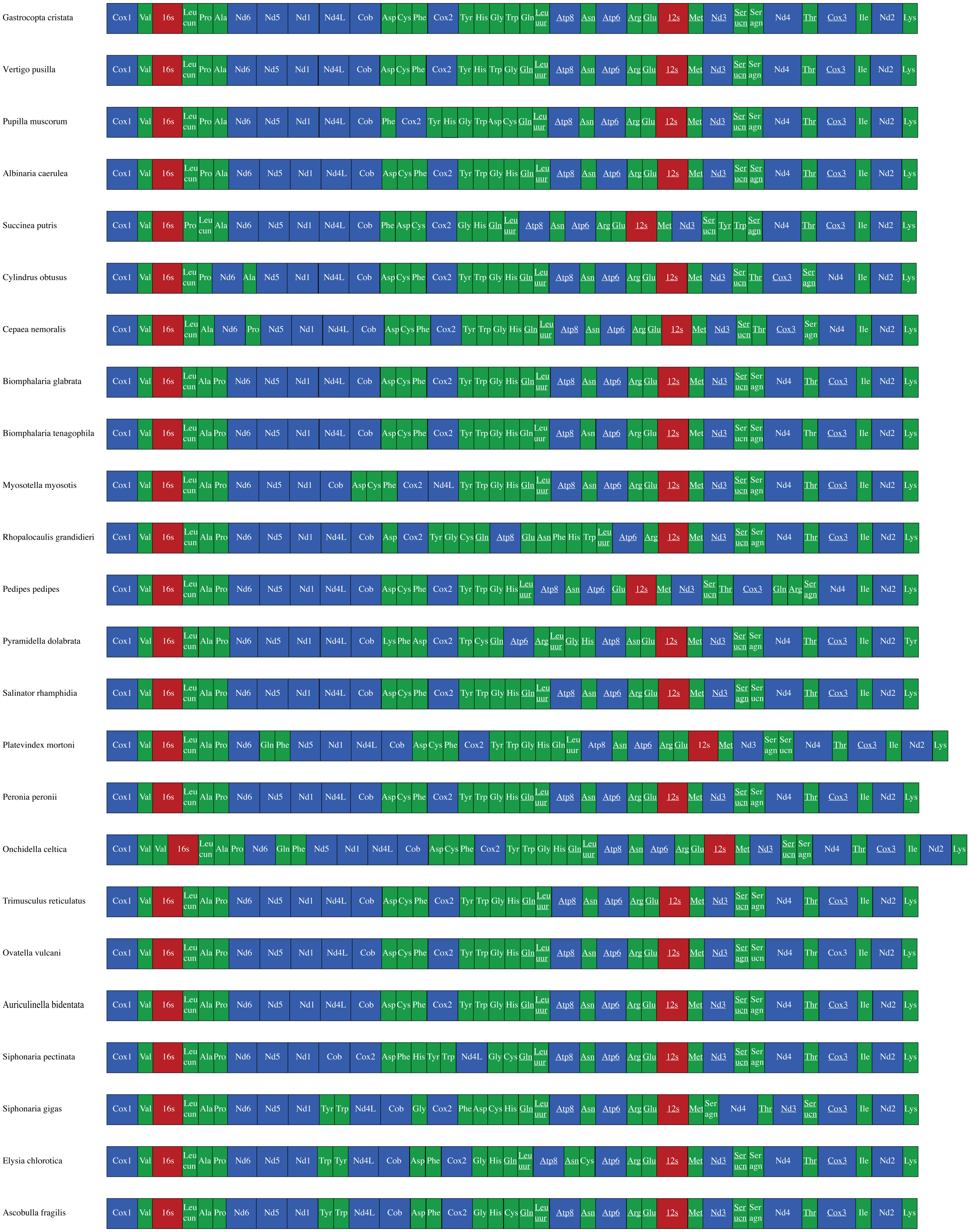

Figure 15: G/C skew comparison of *G. cristata*, *P. muscorum* and *V. pusilla*. Made using a coding sequence alignment. Upper half is ratio of G and lower half is ratio of C. Positive peaks mean G is more prevalent, negative peaks mean C is more prevalent. Bin size was 210 nucleotides, advancing 3 nucleotides at a time.

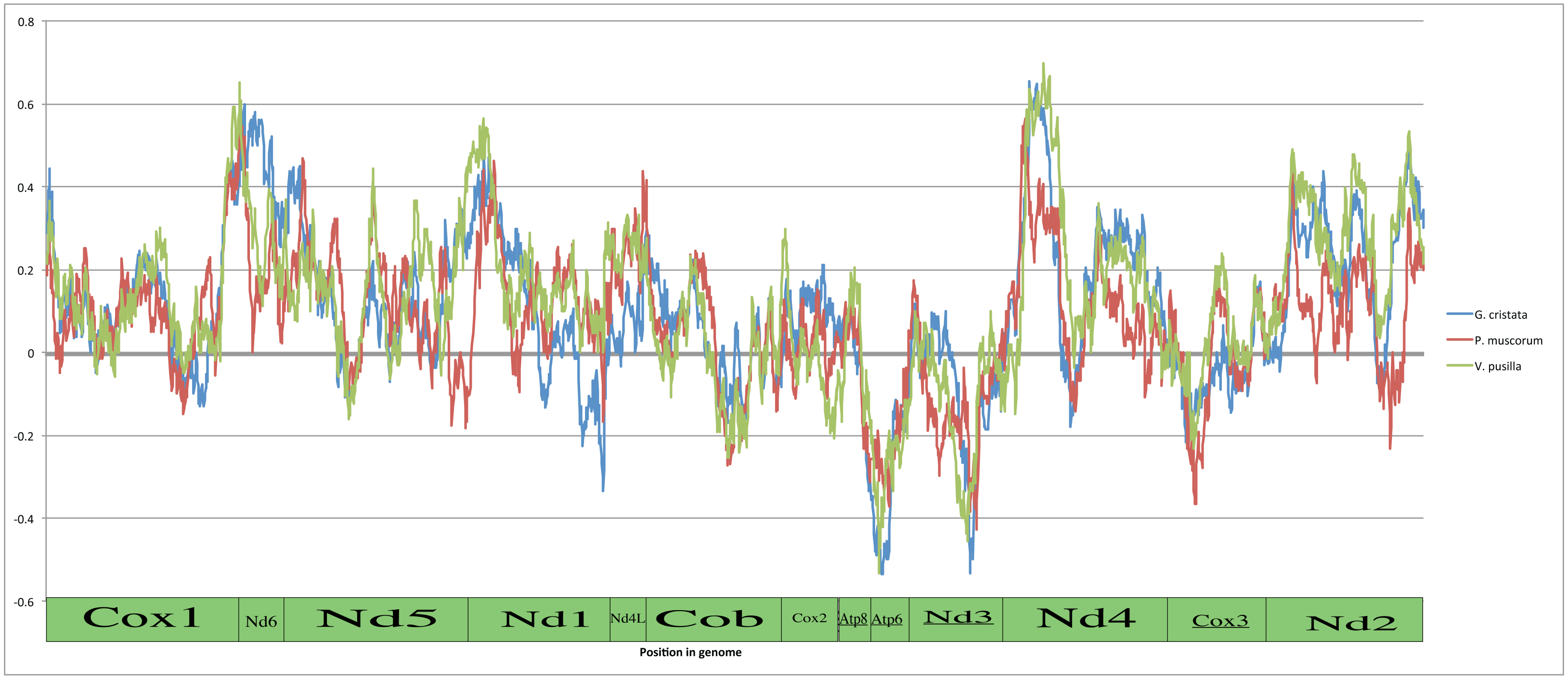

Figure 16: Codon usage pie chart for mt genome of *G. cristata* by amino acid. The two serine and two leucine tRNA's are grouped together under serine and leucine respectively.

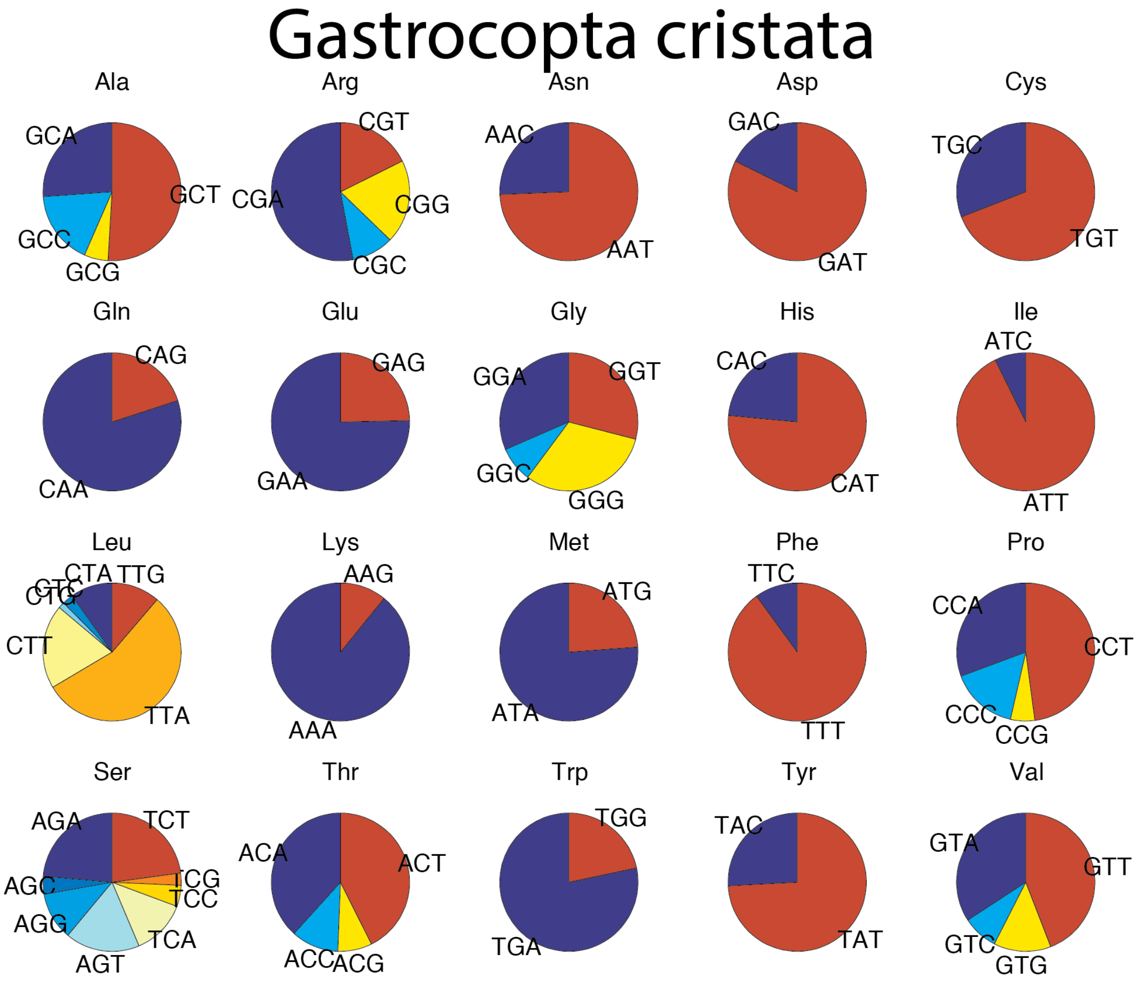

Figure 17: Codon usage pie chart for mt genome of *P. muscorum* by amino acid. The two serine and two leucine tRNA's are grouped together under serine and leucine respectively.

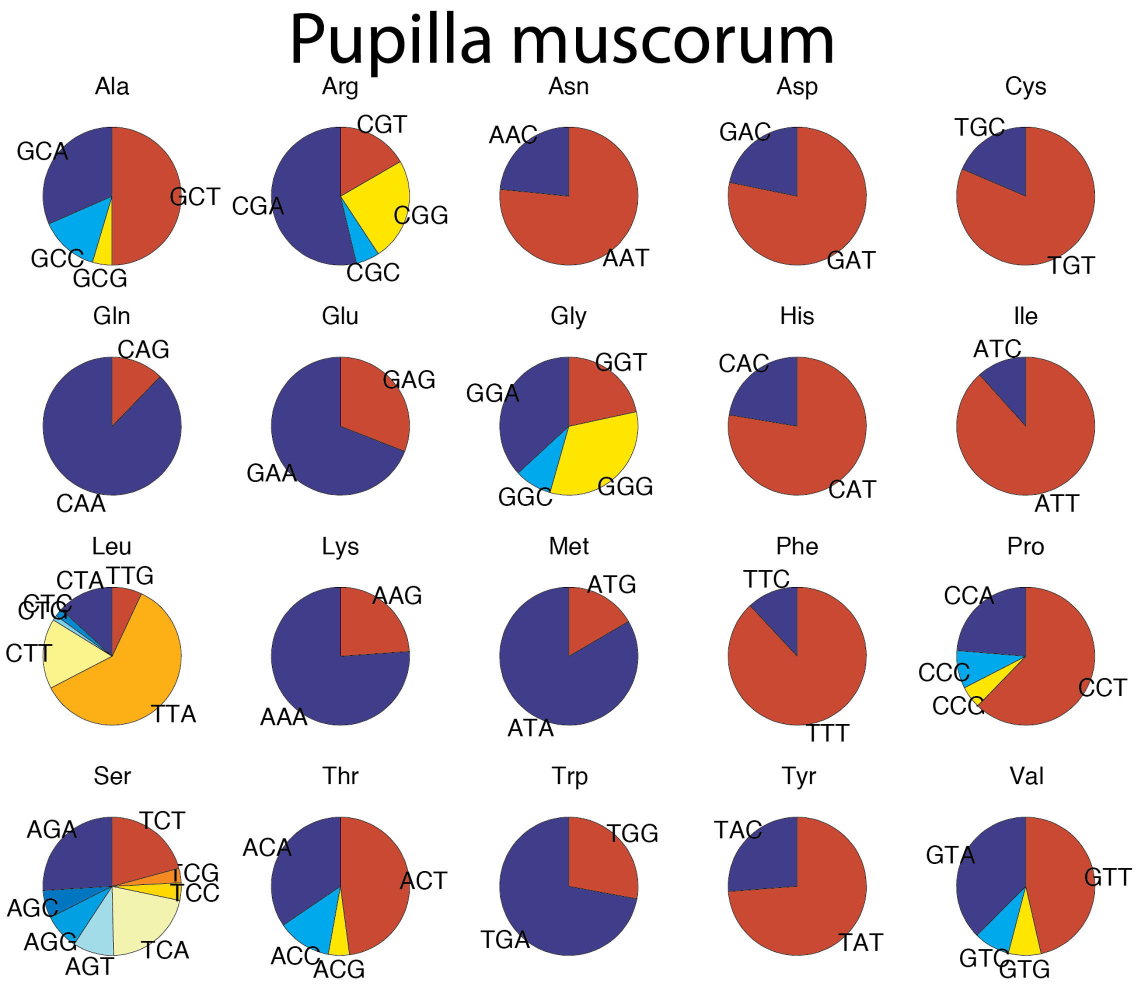

Figure 18: Codon usage pie chart for mt genome of *V. pusilla* by amino acid. The two serine and two leucine tRNA’s are grouped together under serine and leucine respectively.

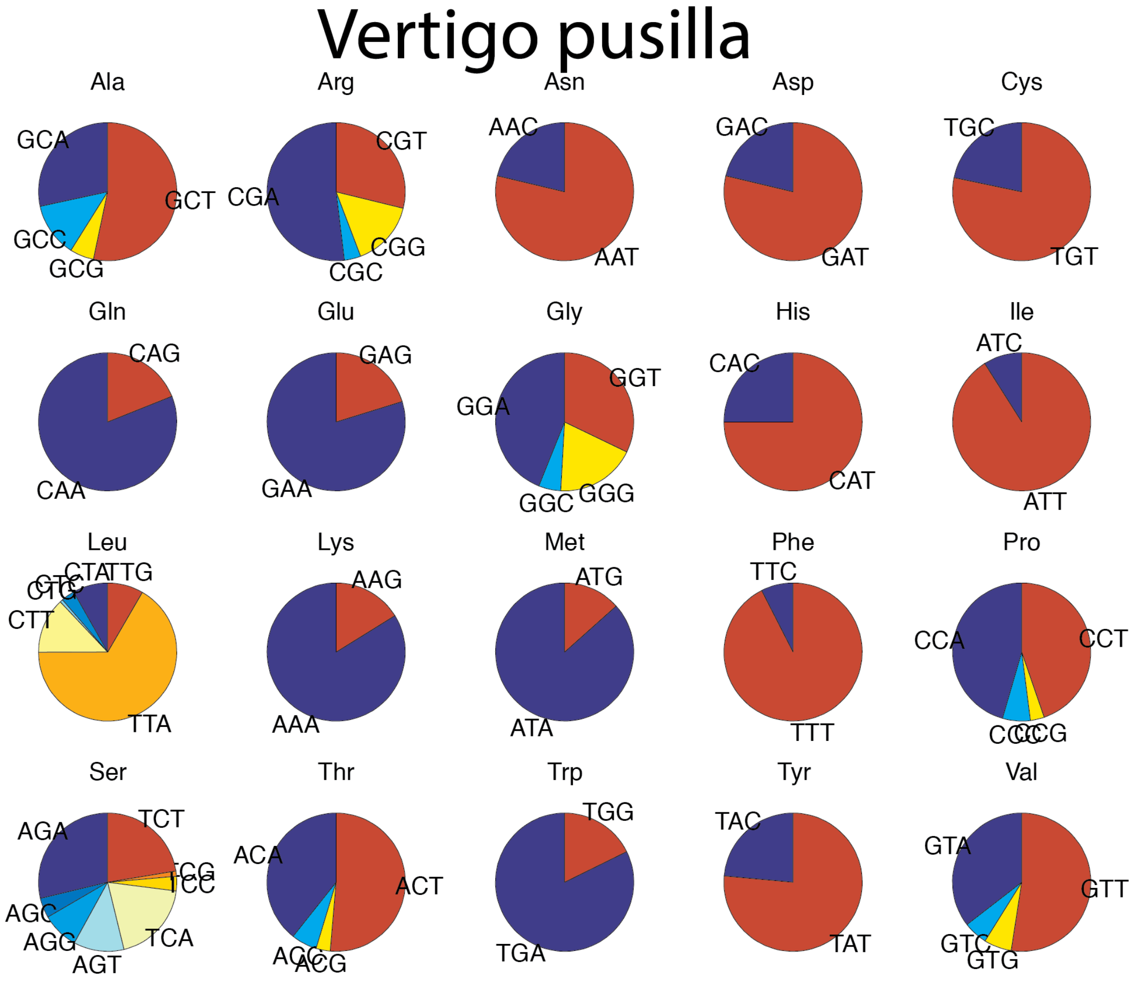

Figure 19: Codon usage pie chart for mt genome of *A. caerulea* by amino acid. The two serine and two leucine tRNA's are grouped together under serine and leucine respectively.

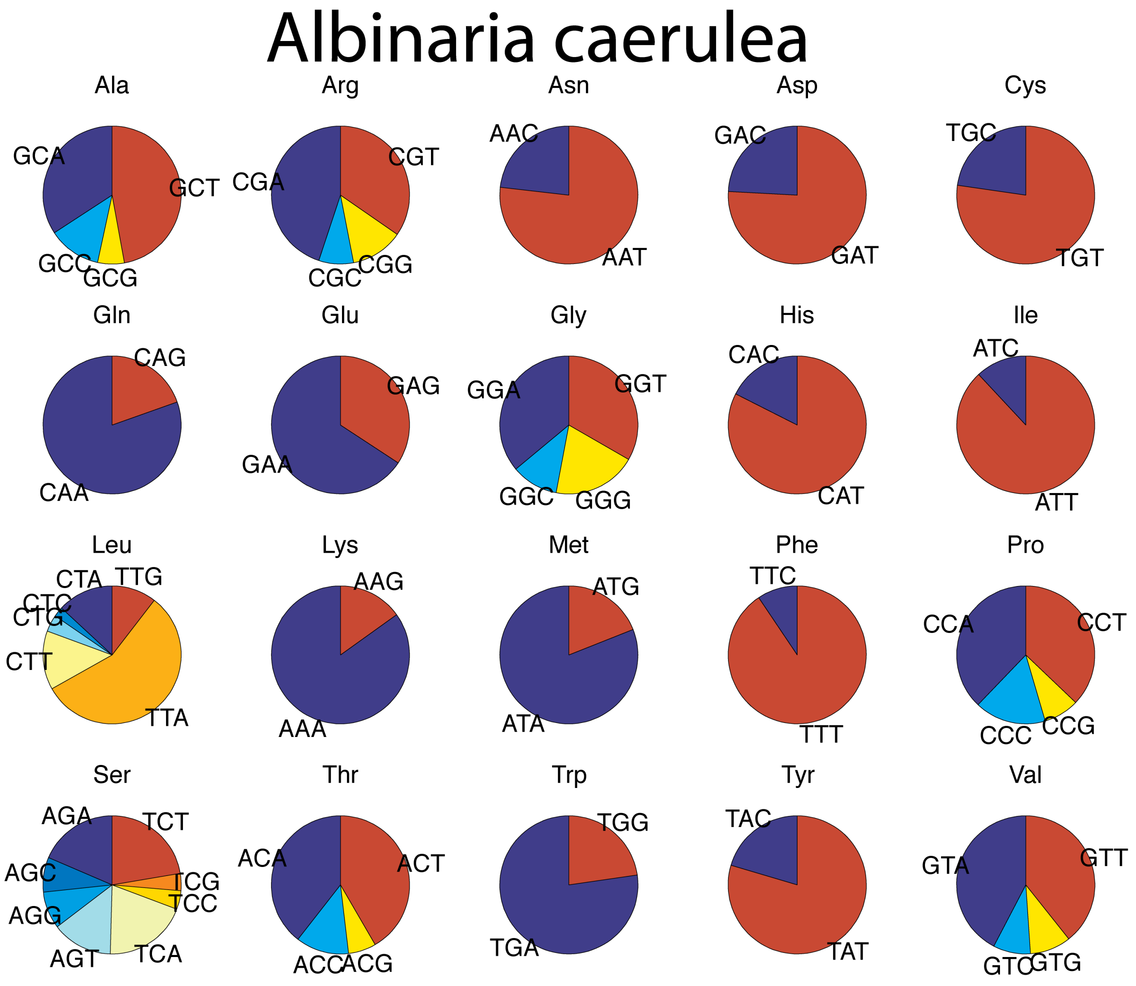

Figure 20: Codon usage pie chart for mt genome of *C. nemoralis* by amino acid. The two serine and two leucine tRNA's are grouped together under serine and leucine respectively.

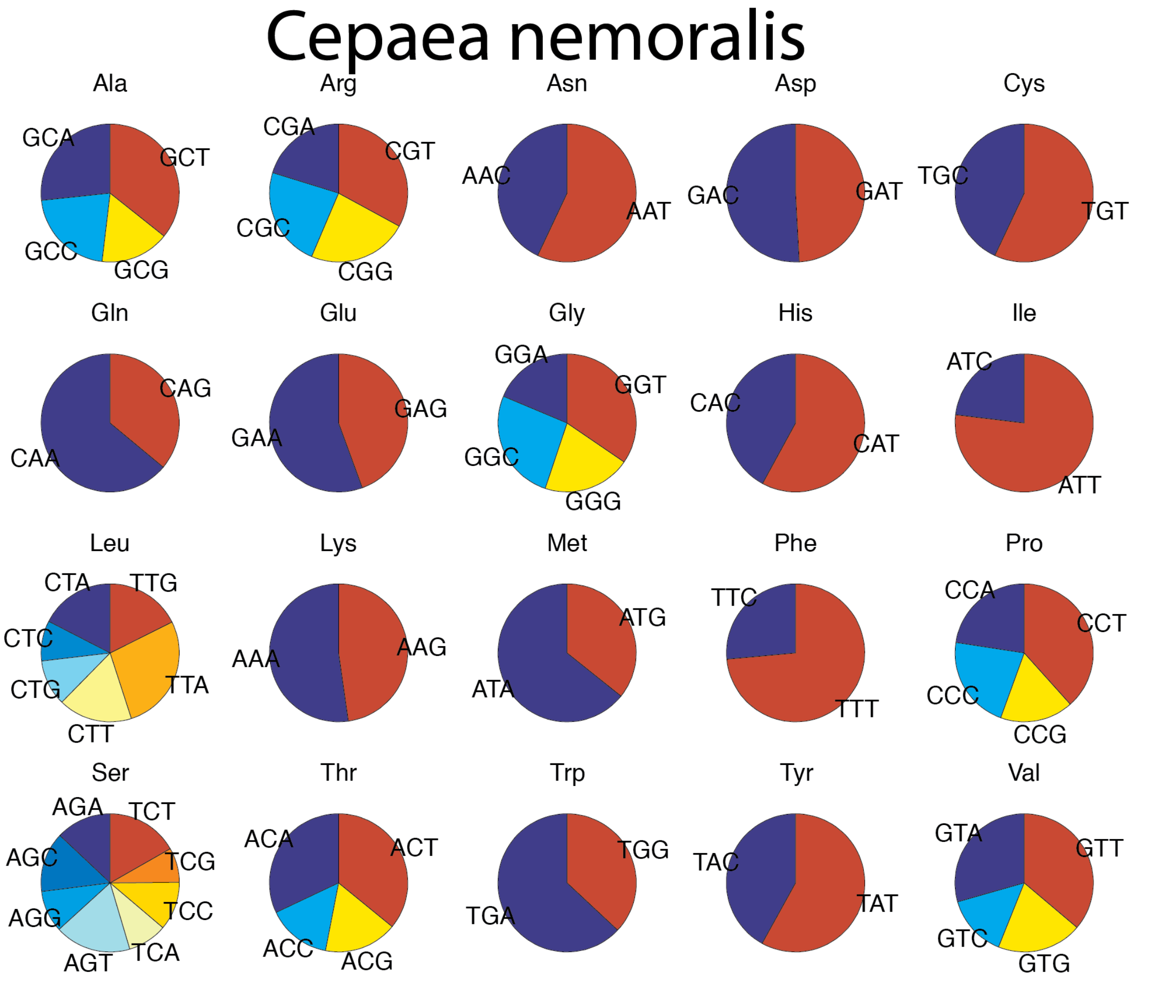

Figure 21: Bayesian phylogenetic tree for complete data set. Node labels are the posterior probabilities.

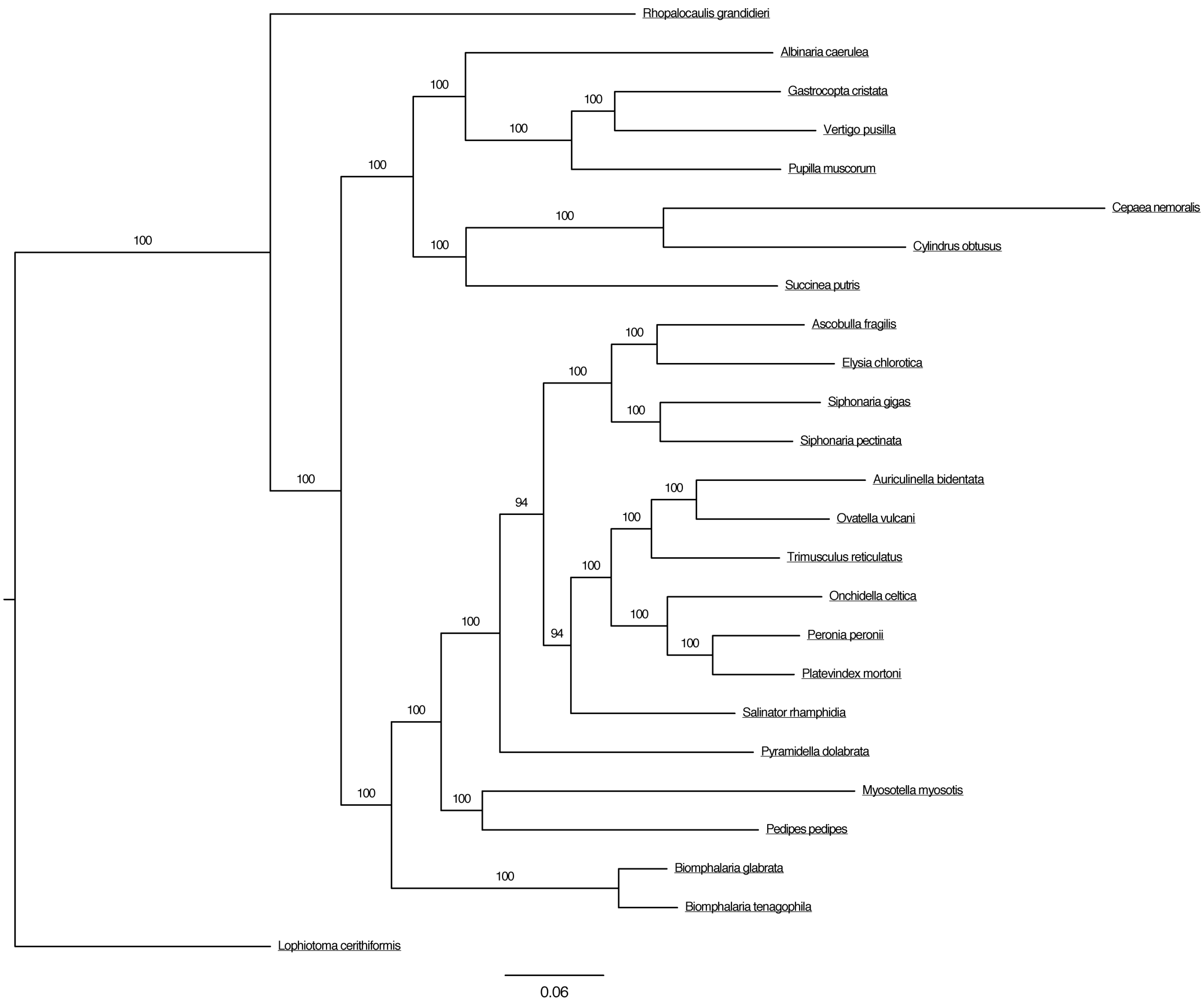

Figure 22: Maximum likelihood tree for complete data set. Node labels are bootstrap values.

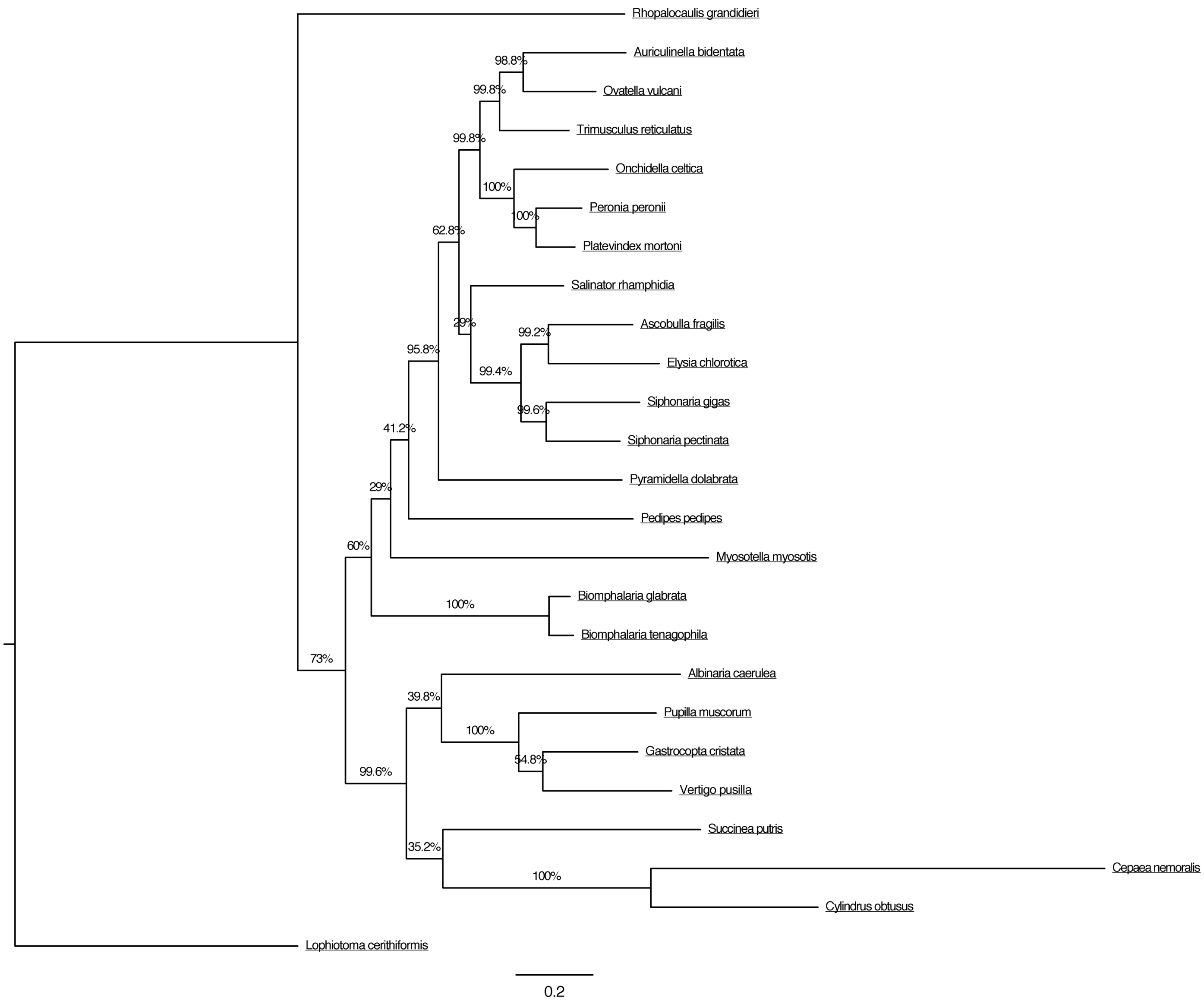

Figure 23: Maximum parsimony tree for complete data set. Node labels are bootstrap values.

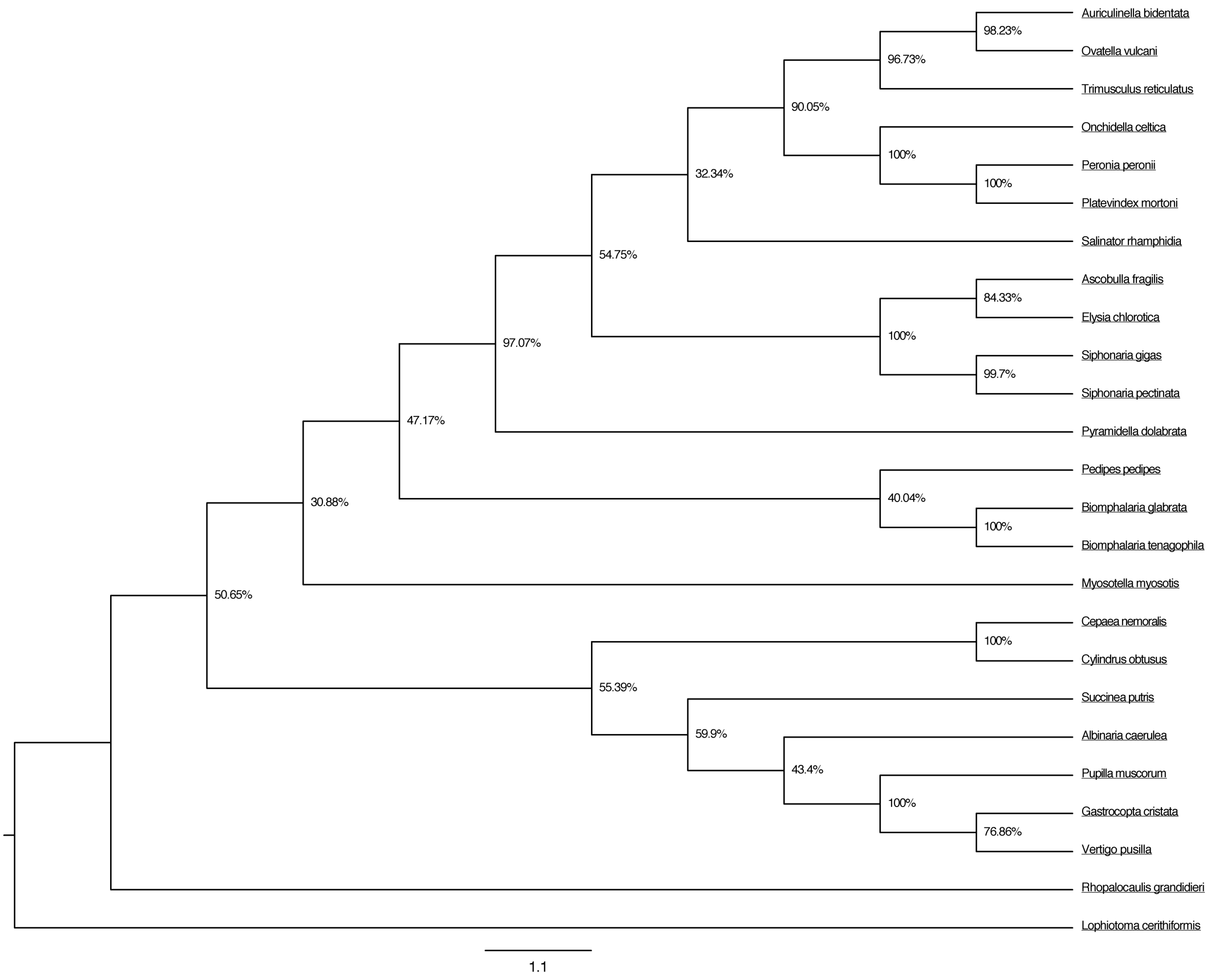

Figure 24: Nearest neighbor joining tree for complete data set. Node labels are bootstrap values.

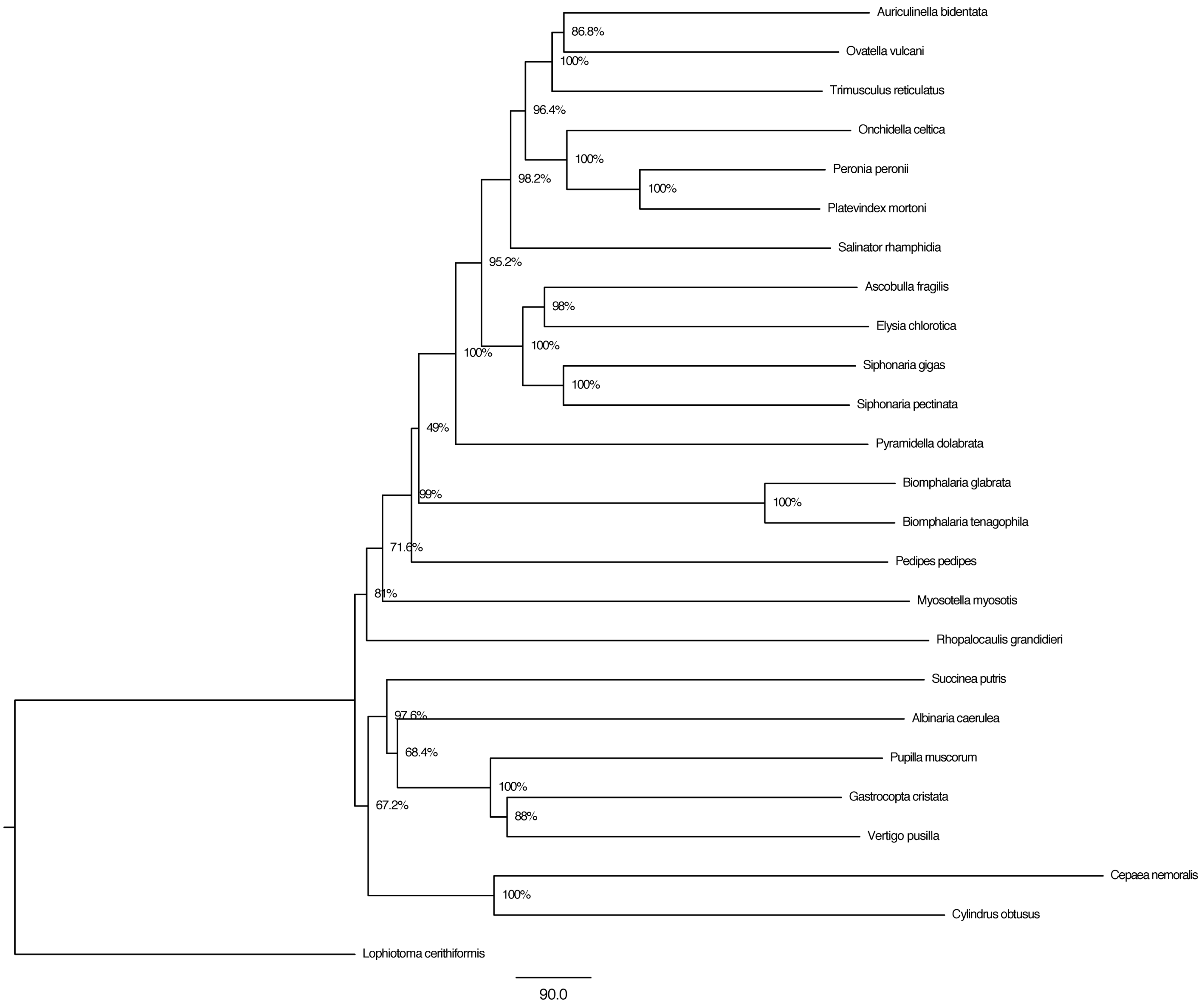

Table 1: List of species used for this project and their associated Genbank accession numbers.

Species name	Genbank acession number
Albinaria caerulea	NC_001761.1
Ascobulla fragilis	NC_012428.1
Auriculinella bidentata	NC_016168.1
Biomphalaria glabrata	NC_005439.1
Biomphalaria tenagophila	EF433576.1
Cepaea nemoralis	NC_001816.1
Cylindrus obtusus	NC_017872.1
Elysia chlorotica	EU599581.1
Gastrocopta cristata	KC185403
Myosotella myosotis	NC_012434.1
Onchidella celtica	AY345048.2
Ovatella vulcani	NC_016175.1
Pedipes pedipes	NC_016179.1
Peronia peronii	NC_016181.1
Platevindex mortoni	GU475132.1
Pupilla muscorum	KC185404
Pyramidella dolabrata	NC_012435.1
Rhopalocaulis grandidieri	NC_016183.1
Salinator rhamphidia	NC_016185.1
Siphonaria gigas	NC_016188.1
Siphonaria pectinata	NC_012383.1
Succinea putris	JN627206.1
Trimusculus reticulatus	NC_016193.1
Vertigo pusilla	KC185405
Lophiotoma cerithiformis	NC_008098.1

Table 2: List of all start codons, by gene, for *G. cristata, P. muscorum* and *V. pusilla.*

Yellow hilighted codons are alternative start codons.

	G. cristata	*P. muscorum*	*V. pusilla*
Atp8	ATG	ATG	ATG
Atp6	ATG	TTG	ATG
Cox1	TTG	TTG	TTG
Cox2	ATG	ATG	ATG
Cox3	ATG	ATG	ATG
Cytb	TTG	TTG	TTG
Nd1	ATG	ATG	GTG
Nd2	ATA	GTG	ATG
Nd3	GTG	TTG	TTG
Nd4	ATG	ATA	TTG
Nd4l	ATG	ATA	ATC
Nd5	ATA	ATG	ATA
Nd6	ATG	TTG	ATG

Table 3: Relative rate comparisons, using Tajima's relative rate test, between *G. cristata*, *P. muscorum V. pusilla* and *A. caerulea* with *S. putris* as the out group. The rate comprision was conducted using Mega 5.0 and coding only sequence with the divergent regions concatenated out.

Unique differences in Gastrocopta cristata	249
Unique differences in Albinaria caerulea	291
Unique differences in Succinea putris (outgroup)	373
p-value	0.07
value needed for significance using the sequential Bonferroni correction	0.0125

Unique differences in Pupilla muscorum	285
Unique differences in Albinaria caerulea	294
Unique differences in Succinea putris (outgroup)	358
p-value	0.7
value needed for significance using the sequential Bonferroni correction	0.05

Unique differences in Vertigo pusilla	261
Unique differences in Albinaria caerulea	275
Unique differences in Succinea putris (outgroup)	351
p-value	0.54
value needed for significance using the sequential Bonferroni correction	0.025

Unique differences in Gastrocopta cristata	187
Unique differences in Pupilla muscorum	237
Unique differences in Albinaria caerulea (outgroup)	501
p-value	0.015
value needed for significance using the sequential Bonferroni correction	0.01

Unique differences in Pupilla muscorum	224
Unique differences in Vertigo pusilla	209
Unique differences in Albinaria caerulea (outgroup)	469
p-value	0.47
value needed for significance using the sequential Bonferroni correction	0.0167

Unique differences in Gastrocopta cristata	190
Unique differences in Vertigo pusilla	244
Unique differences in Pupilla muscorum (outgroup)	308
p-value	0.009
value needed for significance using the sequential Bonferroni correction	0.0083

Table 4: D_n/D_s between *G. cristata*, *P. muscorum V. pusilla*. Calculated using a matlab script (Appendix A, sec. 2) and the coding sequence only with the divergent regions concatenated out.

	G. cristata vs. V. pusilla			G. cristata vs. P. muscorum			P. muscorum vs. V. pusilla		
	D_n	D_s	D_n/D_s	D_n	D_s	D_n/D_s	D_n	D_s	D_n/D_s
Atp6	0.221	1.370	0.161	0.316	2.185	0.144	0.322	1.446	0.223
Atp8	0.699	0.841	0.831	0.810	1.292	0.627	0.697	0.715	0.975
Cytb	0.170	1.477	0.115	0.194	2.172	0.089	0.192	1.247	0.154
Cox1	0.049	1.234	0.040	0.050	1.205	0.042	0.055	1.182	0.047
Cox2	0.125	1.287	0.097	0.141	1.285	0.109	0.160	2.161	0.074
Cox3	0.161	1.685	0.095	0.144	2.249	0.064	0.181	1.038	0.174
Nd1	0.249	1.017	0.245	0.272	1.600	0.170	0.288	1.500	0.192
Nd2	0.450	1.202	0.374	0.402	1.163	0.346	0.463	1.185	0.390
Nd3	0.360	2.110	0.170	0.318	0.840	0.378	0.384	0.990	0.388
Nd4	0.287	1.247	0.231	0.328	1.167	0.281	0.345	0.950	0.363
Nd4l	0.444	1.142	0.389	0.522	1.084	0.481	0.668	1.138	0.587
Nd5	0.359	1.201	0.299	0.353	1.566	0.225	0.369	1.247	0.296
Nd6	0.334	0.840	0.398	0.413	1.757	0.235	0.380	0.715	0.532

Appendix A: Section 1

```
% Codon bias pie graphs by Jason Marquardt

clc
clear all
format('short')

% genome to import
name='no crop protein alignment copy.fasta';

%how many entries
x=24;

for i=1:1:x

fastaentry=i;
%==========================
fasta1=fastaentry;
fasta2=fastaentry;
[header, genome]= fastaread(name,'Blockread', [fasta1
fasta2],'IgnoreGaps','True');
len=length(genome);
cb(i)= codonbias(genome,'pie',true,'GeneticCode',5);
h=uicontrol('units','normalized','style','text','position',[.2 .95 .6 .
05],'string',header,'fontsize',20);
set(h,'BackgroundColor',[1 1 1]);
saveas(gcf,[header,'.ai'],'ai')
end
```

Appendix A: Section 2

```
% Calculate Dn/Ds by Jason Marquardt

clc
clear all

%name of file to import
name='no crop protein alignment.aln';

%number of sequences
x=24;

%size of gene
win=1554

count=1;

[Headers, Sequences]=multialignread(name);

for i=1:1:x

    for j=1:1:x

[dn1(:,count) ds1(:,count) vardn1(:,count)
vards1(:,count)]=dnds(Sequences{i}, Sequences{j}, 'window',
win,'GeneticCode',5);

count= count+1;

    end

end
```

Appendix A: Section 3

```
% various whole genome nucleotide analysis by Jason Marquardt

clc
clear all
format('short')

% genome to import
name='no crop protein alignment copy.fasta';

%how many entries
x=24;

disp(sprintf('name                              len      A        T
G        C'));

for i=1:1:x

fastaentry=i;
%=========================
fasta1=fastaentry;
fasta2=fastaentry;
[header, genome]= fastaread(name,'Blockread', [fasta1
fasta2],'IgnoreGaps','True');
len=length(genome);


base=basecount(genome);
A=base.A;
T=base.T;
G=base.G;
C=base.C;
disp(sprintf('%s                %d   %d    %d   %d    %d',header,len,A,
T,G,C));


end
```

Appendix A: Section 4

```
% Nucleotide composition & base counts by Jason Marquardt


clear all
clc

name='vert complete genome copy.txt';
[header, genome]= fastaread(name);


base=basecount(genome);
length=length(genome)
A=base.A
T=base.T
G=base.G
C=base.C

figure
ntdensity(genome)
```

Appendix B: Section 1

```
LOCUS       Gastrocopta_cristata   14060 bp    DNA     circular     16-NOV-2012
DEFINITION  Gastrocopta cristata.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      mitochondrion Gastrocopta cristata
  ORGANISM  Gastrocopta cristata
            Unclassified.
REFERENCE   1  (bases 1 to 14060)
  AUTHORS   Marquardt,J.D., Adema,C.M., Nekola,J.C. and Bergthorsson,U.
  TITLE     Mitochondrial Genome Evolution in Pupillid Land Snails
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 14060)
  AUTHORS   Marquardt,J.D., Adema,C.M., Nekola,J.C. and Bergthorsson,U.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-NOV-2012) Department of Biology, University of New
            Mexico, 1 University of New Mexico, Albuquerque, New Mexico
            87131-0001, USA
COMMENT     ##Assembly-Data-START##
            Assembly Method       :: Sequencher v. 5.0
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..14060
                     /organism="Gastrocopta cristata"
                     /organelle="mitochondrion"
                     /mol_type="genomic DNA"
     tRNA            join(1..13,13999..14060)
                     /product="tRNA-Lys"
     gene            2..1531
                     /gene="COX1"
     CDS             2..1531
                     /gene="COX1"
                     /codon_start=1
                     /transl_table=5
                     /product="cytochrome c oxidase subunit I"
                     /translation="MRWLYSTNHKDIGTLYMIFGVWCGMVGTGLSLLIRLELGTSGVL
                     MDDHFFNVVVTAHAFVMIFFMVMPIMIGGFGNWMVPLLIGAPDMSFPRMNNMSFWLLP
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                     ITTIFNMRAPGMTFERLSLFVWSILVTVFLLLLSLPVLAGAITMLLTDRNFNTSFFDP
                     AGGGDPILYQHLFWFFGHPEVYILILPGFGIISHILGNASVKQPFGSLGMIYAMISIG
                     ILGFIVWAHHMFTVGMDVDTRAYFTAATMIIAVPTGIKVFSWLMTLYGMNSKFDASMY
                     WVLGFIFLFTLGGLTGIVLSNSSLDIVLHDTYYVVAHFHYVLSMGAVFAIFAGFVYWF
                     PVMTGLVLQERLAKAQFIIMFIAVNLTFFPQHFLGLSGMPRRYSDYPDSYFKWNQISS
                     FGSLMSIFAVLLFVLIVWEAFLSQRSVMFSSAAPYSREWTENNFPPDFHGNLIPSISV
                     L"
     tRNA            1537..1600
                     /product="tRNA-Val"
     rRNA            1601..2650
                     /product="16S ribosomal RNA"
     tRNA            2644..2706
                     /product="tRNA-Leu"
     tRNA            2707..2771
                     /product="tRNA-Pro"
     tRNA            2770..2836
                     /product="tRNA-Ala"
     gene            2864..3316
                     /gene="ND6"
     CDS             2864..3316
                     /gene="ND6"
                     /codon_start=1
                     /transl_table=5
                     /product="NADH dehydrogenase subunit 6"
                     /translation="MIVLFGLGMFLVFSFCFLRSPVSYGGALIALSTIGALLLSLGCS
                     TWYGYLLFLVYVGGLLVLFLYIIMLSSNFNLQVSFKLMGLIFLAFLVSKLYNFSYPKN
                     SLGVSLSECSEDFSLGLFLGLGGLLLLVFFAIVHIVFLKGQPVQVKND"
     gene            3318..4969
```

```
                     /gene="ND5"
     CDS               3318..4969
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                     /transl_table=5
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                     STLFIFDKISLSFGAVVTLISFCVFSFANTYMSEDNFQFRFILILAMFVLSMNILIFS
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                     SFNIFPIMSSYETIIFILAIAALTKSAQYPFSAWLPAAMAAPTPVSALVHSSTLVTAG
                     VYLIIRLSVNCSMSNSTASLLLFCGSVTCVLGGLSALYENDLKKIIAFSTLSQLGLMM
                     FSLGLKLPNLALLHLYAHAMFKALLFLSAGLILISGFGSQDLRLLGATLYTIPSVVVF
                     FNISSLCLMGIPFLSAFYSKHVIYEVVAMSETNAISFFLMLIGAILTTIYSIRTVKIL
                     SWNSISGLSVFSRLPLYTYVPLCILFFTSIISGKTFSMLDISYLTFMFTSSYYQLMLH
                     FLMALGVMIGLFLSGEKKSHMLSSMFFLWPSSNNLTKYFWPVLKSAKMLDYGWVEPVV
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     CDS               4961..5866
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                     SMCWFSALHTPPSSLLVFPLLAIWFASSLAETNRAPFDFAEGESELVSGFNVEYEGGL
                     FALLFLAEYTSIIFMSMATTVWFLSSWFTIDFVLITLMICIWFLFARGVYPRYRYDLL
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Appendix B: Section 2

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ACCESSION
VERSION
KEYWORDS    .
SOURCE      mitochondrion Pupilla muscorum
  ORGANISM  Pupilla muscorum
            Unclassified.
REFERENCE   1  (bases 1 to 14149)
  AUTHORS   Marquardt,J.D., Adema,C.M., Nekola,J.C. and Bergthorsson,U.
  TITLE     Mitochondrial Genome Evolution in Pupillid Land Snails
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 14149)
  AUTHORS   Marquardt,J.D., Adema,C.M., Nekola,J.C. and Bergthorsson,U.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-NOV-2012) Department of Biology, University of New
            Mexico, 1 University of New Mexico, Albuquerque, New Mexico
            87131-0001, USA
COMMENT     ##Assembly-Data-START##
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            Sequencing Technology :: Sanger dideoxy sequencing
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Appendix B: Section 3

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SOURCE      mitochondrion Vertigo pusilla
  ORGANISM  Vertigo pusilla
            Unclassified.
REFERENCE   1  (bases 1 to 14078)
  AUTHORS   Marquardt,J.D., Adema,C.M., Nekola,J.C. and Bergthorsson,U.
  TITLE     Mitochondrial Genome Evolution in Pupillid Land Snails
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 14078)
  AUTHORS   Marquardt,J.D., Adema,C.M., Nekola,J.C. and Bergthorsson,U.
  TITLE     Direct Submission
  JOURNAL   Submitted (16-NOV-2012) Department of Biology, University of New
            Mexico, 1 University of New Mexico, Albuquerque, New Mexico
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9061 taaaatagtt cctcagcaaa ttaaagcaag gcttctattt actcataatc tggttgtaga
9121 tctaaacact agaggcacta aacccaaaaa atttcttaaa ataataaata atattagaga
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9301 tataactaaa ggtattgctc aaattacaat agagtgatta ccgtctatag atgaaaataa
9361 gtctgttatc atttgataag aatgtaagat atacctctta aaattttaag gccgaaactt
9421 aaaaacgaaa cgtttcattc ttgggttatt aatgaaattt tcatatttgt attacgaaaa
9481 aatactgtat taagttttat actatagtaa catgaatttg tttttgcagt aaatgcaaag
9541 aagcaatttc cattacttct actttgttac gacttgtctc atttttaaac gagaatgacg
9601 ggcgatttgt gcactactaa aaaaatattt agattttatt ttttatttaa atcttacttt
9661 caagtccatc ttcaaaattt agtacttaaa tttatctgta ttaaaataat attgtaactc
9721 gccttaatta aaactataag ttgcaccttg atctgtctta ttgattactc ttaaaaaaat
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9841 tggttatcaa ttattaggta agttcccctg attattttta ttagccgcca taactcttga
9901 gtttatatat taaatatagt actactcatc aaataaggtt gaatatctag aataataggg
9961 tatctaatcc tagttttctt tctagcttta aaataaaaat atagaagcaa aaataaaaaa
10021 gaatatattt cactattatt caaagttttg tctataaaaa ttctatttga tttattgtaa
10081 ttagtttttt tataggtctg accgcggttg ctggcaccta ttaaaccaaa aaagaaataa
10141 tttactaagt taacatttct gttatttatt aaaatttata actggttctt acaaaatttc
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10321 aattaacact tcattctaaa gctcctatgg ctcactcata aattaaacct agtaaaaggg
10381 ctaaaagaaa aattaagagt gtcaaagtta aaaaagttga tgatattgca tttattaaca
10441 ttaagagagg gaagagcaaa actacttcaa cgtcaaaaac taaaaataaa attactaata
10501 taaaaaaacg gagtgaaaaa ggcttacgca tatttcttat tggatcaaaa ccacactcaa
10561 aaggcgatct tttttctcta tcttctgaaa agatagggct tcagtaagta ataaaaaaaa
10621 gtataattaa tgctacacta agaactcctg gaaaaattga gtagtatagc aatttagtga

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   10801 tctttttgat ttctacttta gttttttaa gtttagcaag aatttttatg tttaggtgag
   10861 atatagtttt attaagatgc tttataatag ttttaattag atttgttaat atacatataa
   10921 attttagatt aaatttagaa agaagagtat ttataattga atcatttaat aggtttttaa
   10981 ttttttaag ggtagttatt attttttgt cattaacaaa ttctttttga ttaaaaatta
   11041 ataggtttag gaaagtagta atatcattaa acttgtttct agttttagct tttagttgaa
   11101 aagatttatt agggttttat ttttttcg aatcttcttt aattccgaca ttaattttaa
   11161 ttatagtatg agggtaccag ccagagcgac tacaagcggg aacttatata atactttaca
   11221 cagtatttgc gtctttaccc ttactaatat taattctata ttttttaat aagaggagag
   11281 atttaagaat ctataatttt aagttattag gattagaaat aagtactttt atattaataa
   11341 tttttgtgat agctttttta gttaaattac ctatttttag agctcatttg tgacttccaa
   11401 aagcacatgt agaagctcct ttaagaggtt ctataatttt agctggagtt ttattaaaat
   11461 taggaggata tggtctttat ttaacaaacc aatgttttaa ttttagctct cttaatttaa
   11521 taagagtaag gattaggttt ttaagaatat gaggaggatt atttgcagct ttaatgtgcc
   11581 ttcaacagac agatataaaa gcaatagttg cttactcttc agtagctcat ataagtttag
   11641 ttattagtgg aatttttta aactctgtat gaggggaata ttgtgccaaa gttaccataa
   11701 ttgcccatgg ttttacttca tcagctttgt ttgttttagt aaatataaga tataaaaaat
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   11881 gagagttaat acttcttcca tctttatata tttactcagt aatgttagtt atcattatag
   11941 gtcttgtaat attttttaga gctacttata atatattttt atatactcag attaatcacg
   12001 ggatagctaa tcctttaatt aattctggaa gggcttatta ttcagcagat tatttagggc
   12061 tatttcttca tcttttaact tttattatat tattaaaatt aaatttattt tcaacagaaa
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   12301 atgatataag tatattcgta taagacatac tattaaaaat gttgctccaa cagcaacatg
   12361 gagtccgtga aaacctgttg caataaaaaa agttcttcca taaactctat cagcgataga
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   12481 agtaaaaatt aagctataaa ttgcggataa atattttctt tcttcaatag catgatgagc
   12541 tcatgttacc cttactcctg ataataaaag aactctagta tttaagaggg gaacttgaaa
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   12721 aaataagaaa actcctaatt ttaatccact aattacataa gatgtatgaa aaccttgata
   12781 tgttctttct cgaacaatat ctcgtcatca taaatatgca accagtgcag ttgcaataat
   12841 tccaaaaatt attaaattaa tcgagtgtaa tcgtaagtaa tatactaagc ctaaaggcat
   12901 acaagttaag agagttgatc ctaaaattgg ccatggtcta aattctacta aatgaaaagg
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   13261 attttttagt tcaagcttta gctagtttaa ttgttttaat tagtggtatt aacattttta
   13321 tatttaatag aaataggata ataattcttg gttttttttt aattggttta actttaaaat
   13381 taggtatttt tcctttacat ttttgagtga tcccagtttt aaagactagc ggttataatt
   13441 taataatatt gttactaggt cctataaaaa ttgtaccttt aaggttatta ataaaatttt
   13501 ttcaggtttt taacattaga aattttagag tactatgatt tgcggtcttt agtgcattaa
   13561 ttggggcaat tattggtaat aatgttagaa gaattcgagc agttttaggg gcttcttcta
   13621 tttctcattc tgggtgattt atagtaggag cttggagagg agggatgtgg tcttattttt
   13681 ttatttactt aattagccta tttttaatat tatgatattt agaagaatcc tttagaaaag
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   13801 aagtaaacat tttattgaga tttttaacag taaatgcctc tttgcttttc ttagtaattt
   13861 taattattag ggcagttctt aggctaaact tctacttgaa gttcagttat atttttttatt
   13921 taaaggaaaa aaaattagga tttacagggc gctgaaattg attatttttt ttaagtgctt
   13981 ggttagtggg agctttttta gttttgctat tttaaaaagt tttttatggc tgagttataa
   14041 gcattaaatt tttaatttaa ttacaaggtt tatccttt
//
```

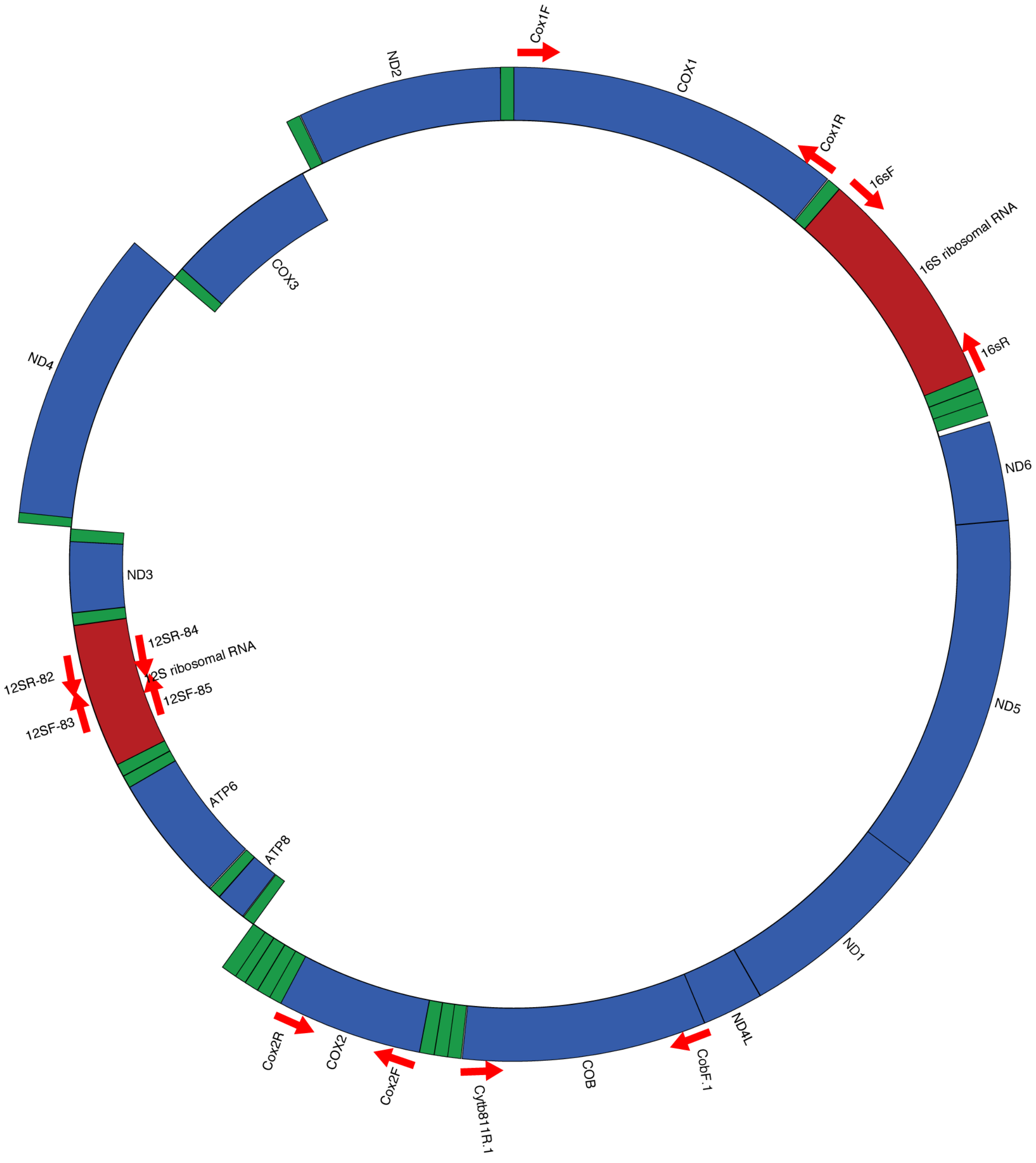
Appendix C: Section 1

12s universal primers designed in house from NCBI GenBank sequence alignment. *cytb* primers based on primers from vonProschwitz *et al.* 2009. *Cox1*, *cox2* and *16S* primers from the work of Gittenberger *et al.* 2004, Hugall *et al.* 2002, and Nekola *et al.* 2009. For relative locations and orientations see Appendix C: Section 2.

Primer name	Primer sequence
12sF-85	5-GACCGCGGTTGCTGGCACCTA-3
12sR-84	5-AGGTGCCAGCAACCGCGGTCA-3
12sF-83	5-AATTATATGGTAAGTTCCCCTAA-3
12sR-82	5-TYAAACTTAAAWRTTATGGCGG-3
CobF.1	5-CATTTTGAGGTGCAACAGTAATTAC-3
Cytb811R.1	5-GCRTAYAGAAAGTATCATTCWGG-3
Cox2F	5-AAATAATGCTATTTCATGAYCAYGC -3
Cox2R	5-GCTCCGCAAATCTCTGARCAYTG -3
Cox1F	5-GGTCAACAAATCATAAAGATATTGG-3
Cox1R	5-TAAACTTCAGGGTGACCAAAAAATCA-3
16sF	5-GCGCTGTTTATCAAAAACAT-3
16sR	5-GCCGGTCTGAACTCAGATCAT-3

Appendix C: Section 2

The relative locations and orientations of universal primers.



Appendix C: Section 3

A list of working amplicons by species and region. Use of custom primers is indicated with a (C). Universal primers were used to get initial sequence inside of the genes then that sequence was used to design the custom primers.

G. cristata
cox1F --> 16SR
cox2F --> cox1R
16SF --> cobR
cobF(C) --> cox2R(C)
cox2F(C) --> 12SR-82/12SR-84
cox1R(C) --> 12SF-85/12SF-83
cox1F --> cox1R
16SF --> 16SR
cox2F --> cox2R
cobF --> cobR

P. muscorum
cobF --> cox2R
cox1F --> 16SR(C)
16SF(C) --> cobR(C)
cox2F(C) --> cox1R(C)
cox1F --> cox1R
16SF --> 16SR
cox2F --> cox2R
cobF --> cobR
12sF --> 12SR (used only for sequencing)

V. pusilla
cox1R(C) --> cox2F(C)
cox1F(C) --> cox2R(C)
cox1F --> cox1R
16SF --> 16SR
cox2F --> cox2R
cobF --> cobR
12sF --> 12SR (used only for sequencing)

Appendix D: section 1

LDPCR thermo-cycler program for *G. cristata* (a), *P. muscorum* (b) and *V. pusilla* (c).

1. 93°C for infinity (hot-start, manually continue after reactions loaded)
2. 93°C for 3 minutes
3. 93°C for 15 seconds
4. TM for 30 seconds
    a. 55°C
    b. 55°C
    c. 50°C
5. 68°C for 10 minutes
6. Go to #3 - 9 times
7. 93°C for 15 seconds
8. TM for 30 seconds
    a. 55°C
    b. 55°C
    c. 50°C
9. 68°C for 10 min +20 seconds a cycle
10. Go to #7 – 10 times
11. 4°C for infinity

Appendix D: section 2

Sequencing thermo-cycler program for *G. cristata* (a), *P. muscorum* (b) and *V. pusilla* (c).

1. 96°C for 1 minute
2. 96°C for 10 seconds
3. TM °C for 5 seconds
    a. 55°C
    b. 51°C
    c. 51°C
4. 60°C for 1 minute and 15 seconds
5. Go to 2, 14 times
6. 96°C for 10 seconds
7. TM °C for 5 seconds
    a. 55°C
    b. 51°C
    c. 51°C
8. 60°C for 1 minute and 30 seconds
9. Go to 6, 4 times
10. 96°C for 10 seconds
11. TM °C for 5 seconds
    a. 55°C
    b. 51°C
    c. 51°C
12. 60°C for 2 minutes
13. Go to 10, 4 times
14. 4°C for infinity

Appendix D: section 3

Ethanol precipitation protocol for all BigDye sequencing reactions.

1. Add 10 µl of sodium acetate
2. Add 30 µl of ice cold 100% ETOH
3. Invert 4X
4. Set in dark 4°C space for 15 minutes
5. Spin down and transfer contents from PCR tube to 1.7 ml eppendorf tube
6. Centrifuge at 13,000 rpm, 4°C, for 30 minutes
7. Invert into paper towel and flick GENTLY
8. Add 35 µl of ice cold 70% ETOH
9. Centrifuge at 13,000 rpm, 4°C, for 15 minutes
10. Invert into paper towel and flick GENTLY
11. Vacufuge at 45°C for 10 minutes
12. Submit to MBF for sequencing

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